UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

EFFECT OF *BOOPHONE DISTICHA* ON THE BEHAVIOUR AND HIPPOCAMPAL NEUROANATOMY IN A BALB/c MOUSE MODEL

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Johannesburg, 2022

DECLARATION

I Nkosiphendule Khuthazelani Xhakaza declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

9 day of June 2023 in Pretoria

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DEDICATION

I dedicate this thesis to my parents, Mr Phillemon and Mrs Nelisiwe Xhakaza, who taught me and raised me in accordance with the principles of the Kingdom of God. While not educated, they sacrificed a lot to ensure that I get the education. They understood the value of an inheritance of education. They always stood in prayer and support for me. To my lovely wife Makhosi, my one and only son Nkazimulo, my two daughters, Ndumiso and Wandile, thank you so much for your prayers, love, support and enduring my unavailability during my long journey of the PhD studies. You have been my pillar of strength and joy in the midst of what has been a tough journey. You played a critical role in this achievement. To my entire family for always encouraging and believing in and praying for me.

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***Boophone disticha* attenuates effects of swimming stress on behaviour and neuroblast differentiation in Balb/c mouse hippocampus.**

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2. 49th Congress of the Anatomical Society of Southern Africa (ASSA Conference: from the 19th to the 21st of April 2022).

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ABSTRACT

Depression is one of the most common neuropsychiatric disorders and is associated with dysfunction of the neuroendocrine system and alterations in specific brain proteins. *Boophone disticha (BD)* is an indigenous psychoactive bulb that belongs to the *Amaryllidacae* family, which is widely used in Southern Africa to treat depression, with scientific evidence of potent antidepressant-like effects. The present study examined the antidepressant effects of *BD* and its mechanisms of action by measuring some behavioural parameters in the elevated plus maze, light dark box, open field forced swimming, brain content of corticosterone, brain derived neurotropic factor (BDNF), and neuroblast differentiation in the hippocampus of Balb/c mice exposed to the five-day repeated forced swim stress (5dRFSS) and 28 days chronic restraint stress. Male Balb/c mice were subjected to the 5dRFSS and 28 days chronic restraint protocols to induce depressive-like behaviour (decreased swimming, increased floating, decreased open arm entry, decreased time spent in the open arms and decreased head dips in the elevated plus maze test, increased time in dark box in the light dark box test, reduced frequency of rearing and increased time on the sides of the open field in the open field test), and treated with distilled water, fluoxetine and *BD*.

Three weeks *Boophone disticha* treatment (10mg/kg/p.o) significantly attenuated both the 5dRFSS and chronic restraint-induced behavioural abnormalities and the elevated brain tissue corticosterone levels observed in stressed mice. Additionally, 5dRFSS exposure significantly decreased the number of neuroblasts in the hippocampus and BDNF levels in the brain of Balb/c mice, while fluoxetine and *BD* treatment attenuated these changes. In the chronic restraint stressed mice, similar effects of *BD* treatment were observed after 21 days of treatment, however, the levels of corticosterone were not different in control and stressed animals, probably due to habituation to stress. In both 5dRFSS and chronic restraint stress, the antidepressant effects of *BD* were comparable to those of fluoxetine, but unlike fluoxetine, *BD*

did not show any anxiogenic effects, suggesting better pharmacological functions. It is important to note that in chronic restraint stress mice, it appeared that animals seemed to have habituated to stressful conditions, demonstrated in part by brain tissue levels of corticosterone that were not elevated in stressed animals treated with distilled water. However, BDNF levels remained significantly low in stressed animals treated with distilled water, suggesting that the effect of chronic stress in this parameter were not reversed when animals habituated.

In conclusion, our study shows that *BD* exerted antidepressant-like effects in both 5dRFSS and chronic restraint stress mice, mediated in part by normalizing brain corticosterone and BDNF levels. Due to some degree of habituation in chronic stress model, caution should be exercised when evaluation effects of treatment in different parameters to evaluate antistress effects of tested agents, particularly levels of corticosterone. Furthermore, the persistent low levels of BDNF suggest that habituation of animals to chronic stress is due to normalising levels of corticosterone but not BDNF. The above occurrence could suggest that recovery from chronic stress without antidepressant treatment could alleviate other behavioural symptoms but not cognitive impairment which is influenced in part by BDNF levels.

Key words: Depression, Antidepressants, Hippocampus, Stress, Behaviour

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LIST OF ABBREVIATIONS

ACC- Anterior cingulate cortex

ACTH- Adrenocorticotropic hormone

Aβ-Amyloid beta

ATP- Adenosine triphosphate

BDNF- Brain derived neurotropic factor

BD- *Boophone disticha*

CA1- Cornu Ammonis area 1

CA2- Cornu Ammonis area 2

CA3- Cornu Ammonis area 3

CORT- Corticosterone

COX-1- Cyclo-oxygenase 1

CRH- Corticotrophin-releasing hormone

CRH1- Corticotropin-releasing hormone receptor one

CRH2- Corticotropin-releasing hormone receptor two

CRP- C-reactive protein

DA- Dopamine

dACC- Dorsal anterior cingulate cortex

DAT- Dopamine Transporter

DCX-Doublecortin

D2- Dopamine receptor subtype 2

DG- Dentate gyrus

DIP- Direct intrahippocampal pathway

DLPFC-Dorsolateral prefrontal cortex

ELS-Early life stress

GC- Glucocorticoids

GFAP- Glial brillary acidic protein

GR- Glucocorticoid receptors

HPA- hypothalamic-pituitary-adrenal axis

5-HIAA- Serotonin primary metabolite

5-HT- Serotonin

5-HT1A to 5-HT7- Serotonin receptor subtypes

5-HTTLPR- Serotonin transporter gene

IL-1-Interleukin one

IL-1β-Interleukin beta

IL-6-Interleukin 6

IPCs- Intermediate progenitor cells

LC- Layer Chromatography

LPS- Lipopolysaccharide

LTP- Long-term potentiation

MR- Mineralocorticoid receptor

MDD- Major depressive disorder

MRIs- Magnetic resonance imaging

NAT- Noradrenaline Reuptake Transporters

NE- Norepinephrine

NF-κB- Necrosis factor caper beta

NGF -Nerve growth factor

NSC- Neuronal stem cells

NSF- Novelty suppressed feeding

NT-3- Neurotrophin-3

OFC-Orbitofrontal cortex

PFC- Prefrontal cortex

PIP-The polysynaptic intrahippocampal pathway

PVN- Paraventricular nucleus

RGL- Radial glial cells

SERT- Serotonin transporter

sgACC- Subgenual anterior cingulate cortex

SGZ- Subgranular zone

Sox2- Sex determining region Y-box 2

SSRI- Selective serotonin reuptake inhibitor

SVZ- Subventricular zone

TNF-Tumour necrosis factor

TNF-α- Tumour necrosis factor alpha

TrkB- Tyrosine kinase receptor

VMPFC-Ventromedial prefrontal cortex

WRAF- Wits Animal Research Facility

LIST OF SYMBOLS

α- Alpha

β-Beta

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CHAPTER ONE

1.0 INTRODUCTION

World Health Organization identifies depression as one of the leading causes of morbidity and mortality worldwide (Jogdand, 2014). Depression is a complex disease that can occur as a result of a multitude of different factors, including biological, emotional, and environmental influences (Zayka *et al*., 2015). Diagnosis of depression is mainly based on symptomatic behavioural criteria, such as depressed mood, low self-esteem, and recurrent thoughts of death and suicide. The heterogeneity of depression suggests that multiple different biological mechanisms may underlie its aetiology (Anacker, 2014). It is estimated that 40% risk of developing depression is genetic, though the specific genes involved are partially understood (Homung and Heim, 2014). The other 60% non-genetic risk remains poorly defined, with diverse theories implicating acute or chronic stress, childhood trauma, viral infections, and even random processes during brain development as possible causes (Kessler *el. al*, 2003).

In addition to structural, learning and memory changes observed in depression, chronic stress results in dendritic remodelling i.e dendritic atrophy and spine loss in the hippocampus (Sierakowiak *et al*., 2015). It has also been shown that chronic stress causes a decrease in cell proliferation and concomitant increase in granule cell death in the dentate gyrus of the adult hippocampus (Saaltink and Vreugdenhil, 2014). There is however, no evidence to date to show if stress reduces the total number of neurons in the hippocampus and if so, the cellular mechanisms by which this neuronal loss occurs (Kheirbek and Hen 2013).

Other reports suggested that dendritic restructuring, decreased hippocampal neurogenesis, and decreased neuronal cell survival associated with stress may provide a cellular basis for the impairments seen in the brains of patients with depression (Qiao *et al*., 2016). Several studies have also shown that stress and depression causes a reduction in hippocampal neurotrophin levels, including brain derived neurotropic factor (BDNF), leading to dysfunction of the

cholinergic system (Smith *et al*., 1995; Kaneko *et al*., 2006). The neurotropic factor family has been the focus of much of the work on stress and depression, and the most widely studied member of this family is BDNF (Duman, 2002). Brain derived neurotropic factor, nerve growth factor (NGF) and neurotrophin-3 (NT-3), influence the proliferation, differentiation, and growth of neurons during development, but are also expressed in the adult brain and play a critical role in the survival and function of mature neurons (McAllister, 2002). High levels of BDNF are expressed in limbic brain structures implicated in mood disorders, including the hippocampus, prefrontal cortex, and amygdala, and act through a transmembrane tyrosine kinase receptor referred to as TrkB (Tanis et al., 2007). Exposure to immobilization stress results in a dramatic reduction in levels of BDNF in the rodent hippocampus (Smith et.al, 1995) relative to the major subfields of the CA3 pyramidal cell layer and granule cell layer of the hippocampus whereas dendritic atrophy and decreased neurogenesis are observed respectively in response to stress (Smith et.al, 1995). Although there is no evidence suggesting that depression associated anatomical and chemical changes in the hippocampus involve a change in neuronal number, antidepressants may supposedly mediate their long-term therapeutic effects by triggering cellular mechanisms that counteracts the structural impairments.

Czen *et al*, (2001) further demonstrated that chronic, not acute, treatment with the atypical antidepressant tianeptine, reverses the stress-induced impairments such as changes in metabolite concentrations, decreases in neurogenesis and reduction in volume of the dentate gyrus in the hippocampus. A study by Pawluski *et.,al*, 2014 showed that minipump administration of fluoxetine in female rats increased cell proliferation in the hippocampal granular cell layer.

Most of the current antidepressant drugs have undesirable side effects and are beyond the reach of many South Africans, and Africans in general. As a result, the populace mainly relies on alternative and complimentary traditional medicines for antidepressant therapeutics. These

factors have led to an investigation into natural products and herbal medicines with potential anxiolytic and antidepressant properties including *Boophone disticha* (*BD*) as affordable and easily accessible alternatives to conventional antidepressants. Therefore, the present study evaluated the anxiolytic/ fluoxetine-like action of *BD* in search for a cheap, easily available and accessible alternative to modern conventional antidepressant drugs.

Boophone disticha (L) (family Amaryllidaceae; tumbleweed/sore-eye flower) is an indigenous psychoactive bulb that is widely used in Southern Africa. *Boophone disticha* belongs to the Amaryllidaceae family, *Boophone* genus, which can be found throughout Southern and Tropical Africa (Gadaga *et al*., 2011). It has been reported that South African traditional healers use the bulbs and leaves of *Boophone disticha* to treat anxiety (Sandager *et al*., 2005). The study done by Gadaga and colleagues (2011), described the neurotoxicological effects of the *Boophone* hydro-ethanolic extract ranging from mild tremors to limb paralysis and death at high doses. In the above study, the LD50 was estimated to be above 120 mg/kg, while 50 mg/kg dose showed little toxicity, and hence 50 mg/kg was recommended for therapeutic purposes. A dose of 10mg/kg has previously been shown to significantly reduce mean arterial pressure in mice (Pote et.al, 2014). In addition to the above, crude extracts of the leaves of *Boophone disticha* have been shown to have affinity for the selective serotonin reuptake inhibitor (SSRI) site of the serotonin transporter (Pedersen et al., 2008). Furthermore, two alkaloids isolated from *Boophone disticha* (buphanadrine and buphanamine), have been shown to be responsible for binding to the selective serotonin reuptake inhibitor (SSRI) site of the serotonin P transporter (Cheesman et al., 2012). These mechanisms may partially explain why *Boophone disticha* is used traditionally for the management of anxiety and depression. Thus, much needs to be done in current and future studies to test this hypothesis. The focus of the present study is to validate if a crude extract of *Boophone disticha* has any anxiolytic-like

and/or antidepressant-like activity and its effects on the chemical neuroanatomy using the established mouse models of depression.

Available literature showed no report on the effects of *Boophone disticha* treatment on disorders due to stress on behaviour and brain structure in animal models of depression, and the present study is designed against this backdrop to elucidate the neuro-therapeutic effects of the hydroethanolic extract of the bulbs of *Boophone disticha.* This study further, attempts to bridge the knowledge gap between the toxicity and beneficial effects of *Boophone disticha* in in-vivo treatment of anxiety disorders in an effort to further extend the frontiers of the search for suitable, affordable and available antidepressant drugs from traditional herbs.

Boophone disticha is expected to demonstrate antidepressant, anxiolytic and neuroprotective effects in the mouse models of depression.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of depression

Major depressive disorder is a devastating mental illness that has profound effects in the quality of life of the affected individuals. Symptoms of depression that mostly affect the individuals quality of life include low-self esteem, poor concentration, sleep disorders, loss of pleasure and interest, and sadness amongst other things (Depression, W.H.O., 2017). While the aetiology underlying this disorder is not clearly understood, psychosocial stress is one of the major factors implicated in this condition (Costello et at., 2002; Yang *et al*., 2015). Stress is described as the external or internal events or conditions that affect the organism (Goldberger and Breznitz, 2010). These adverse events challenge the equilibrium in an organism's physiological state, triggering a cascade of adaptive responses (Chrousos and Gold, 1992; Chrousos, 2009). It has been suggested that the ability of an individual to respond to stress is influenced by genetic factors, with the inheritability of 32 to 38% chances (Feder et al., 2009). The hypothalamic pituitary adrenal (HPA) axis is an essential component of the stress system, and its accurate functioning is crucial for the success of the adaptive response to stress (Tsigos, and Chrousos, 1994). The response to stress is meant to be temporal, to restore the physiological equilibrium and disappear (Tafet and Nemeroff, 2016). However, chronic stressful conditions result into maladaptive behaviours that lead to pathological conditions such as mood disorder including depression (Caspi et al., 2003; Heim et al.,2008; Nemeroff and Seligman, 2013).

2.2 Prevalence of depression

The report by the Institute of Health Metrics and Evaluation indicated that 280 million people, including 23 million children and adolescents, were living with depression in 2019 (Institute of Health Metrics and Evaluation, 2021). The burden of depression has been shown to be high

in both males and females across the entire life span in many locations around the world (GBD 2019 Mental Disorders Collaborators, 2022). However, studies have shown that the prevalence of depression is higher in females (5.1%) compared to males (3.6%) (Ferrari et al., 2013). There is also evidence that despite many majors put in place to reduce the impact of depressive disorders globally, no reduction in prevalence of depression is realized since 1990 (Patel et al.,2016). The African continent is no exception to the pandemic of depression representing about 10% of the global burden of mental (WHO, 2017). A review by Gbadamosi et al. 2022 noted that the epidemiological data in Sub-Saharan African countries is poor with only a few countries, including South Africa, represented in the published data. The disease was aggravated by the outbreak of SARS-COVID-19 pandemic which caused a 25% increase in prevalence of depression worldwide (COVID-19, WHO, 2022). One study estimated that 53.2 million new depression patients were added globally after the outbreak of COVID-19 throughout the year 2020 (Santomauro et al., 2021). In areas like the African continent where poverty and mental health issues already have a negative interaction in these vulnerable population, COVID-19 had a significant negative impact (Álvarez-Iglesias et al., 2021). The increase in prevalence of depression after COVI-19 outbreak has become one of the main driving factors for plant derived antidepressant agents that can be used with easy access and minimal negative effects (Fedorova et al., 2021).

2.3 Endocrine process in depression

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is among the most prevalent biological findings in major depression (Baumeister *et al*., 2016). Altered levels of cortisol is one of the important varieties of hormonal abnormalities, indicating the existence of endocrine disturbances, more specifically the dysfunctions in the hypothalamic-pituitary- adrenal (HPA) axis (Brigitta, 2002). The HPA axis consists of positive and negative feedback loops that involve the brain, pituitary, and adrenal glands, which regulates glucocorticoid production (Keller *et al*., 2017). When environmental stress is experienced, monoamines, serotonin, norepinephrine and dopamine are released from the amygdala, hippocampus, and other brain regions (Manke, 2019), which triggers the paraventricular nucleus (PVN) of the hypothalamus to synthesize corticotrophin-releasing hormone (CRH). This hormone binds to corticotropin-releasing hormone receptor one (CRH1) and two (CRH2) in the anterior pituitary. The binding of the CRH causes secretion of the adrenocorticotropic hormone (ACTH) into the circulation, which activates the production and release of glucocorticoids (GCs) in the adrenal glands (cortisol in humans, corticosterone in rodents). Homeostasis is restored by the negative feedback mechanisms initiated by binding of GCs to glucocorticoid receptors (GR) of the hippocampus, hypothalamic paraventricular nucleus (PNV) and the anterior pituitary gland, inhibiting further release of CRH (Figure 2.1) (de Kloet., 2005). However, in the major depressive disorder (MDD), the sensitivity of the GR is impaired leading to a reduced negative feedback mechanism resulting in hypersecretion of CRH and an elevated production of GCs (Holsboer, 2000). These elevated levels result into HPA shifting into high set point causing prolonged elevated HPA activity in patients with MDD (Raison and Miller, 2003, Young *et al*., 2003). In 2020, a Human Sciences Research Council conducted a study which showed that 33% of South Africans were depressed (Nguse and Wassernaar, 2021).

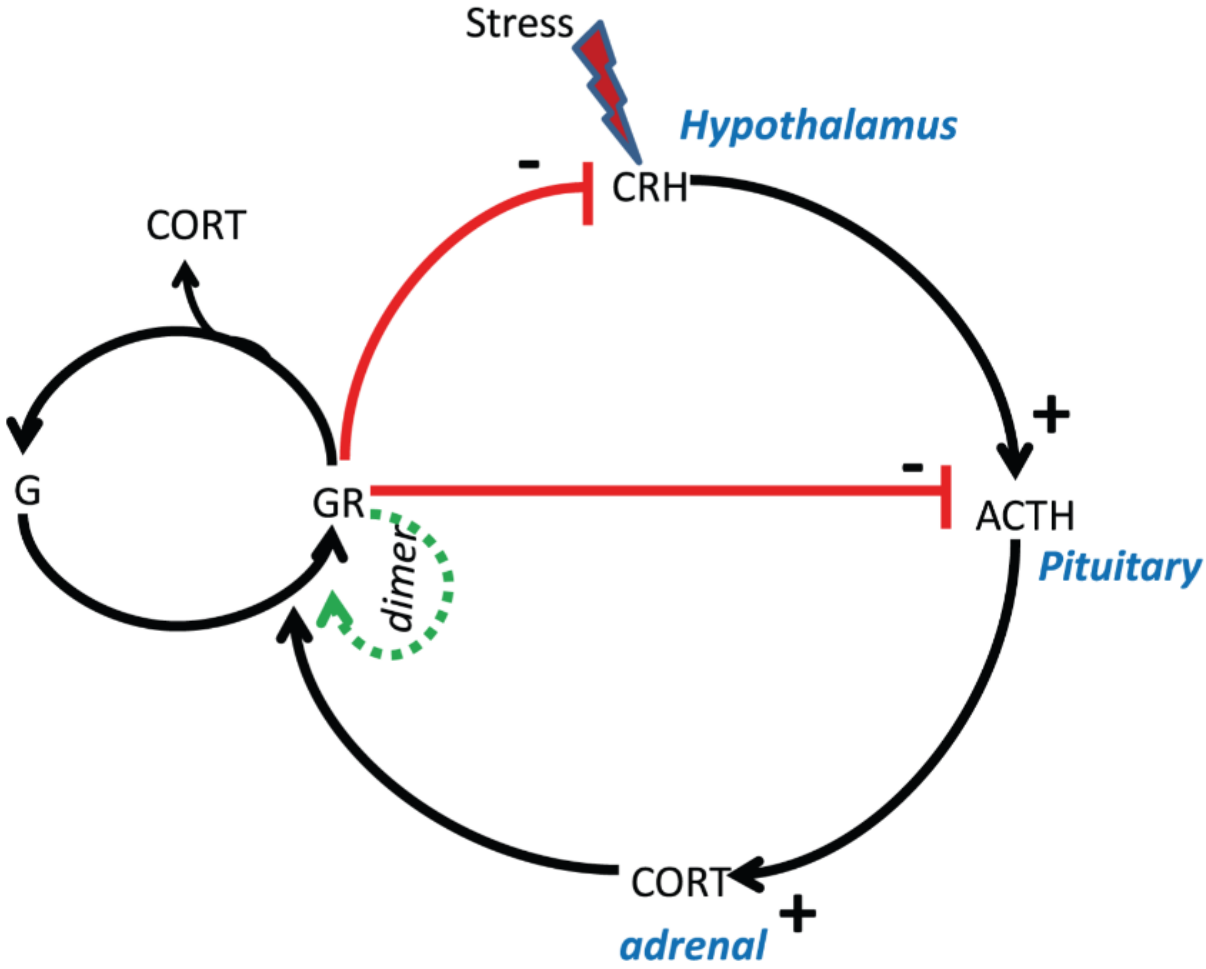

Figure 2.1: Regulation of corticosterone in HPA axis:
Exposure to stress stimulate the hypothalamus to secret corticotrophin releasing hormone (CRH). The CRH activates the adreno-corticotropic hormone (ACTH) in the pituitary gland. Aceto-corticotrophin hormone stimulates the adrenal gland to release corticosterone (CORT). Corticosterone will than bind to the glucocorticoid receptor and form GR complex which subsequently undergoes dimerization. The binding of the GR complex to CRH and ACTH makes a closed loop that results into a negative feedback (Sriram et al., 2012).

Furthermore, changes in serotonin receptors are some of the effects of hypercortisolism (Angst *et al*., 2007, de Kloete, 1998), which are thought to be the mechanism for specific symptoms observed in severe MDD (Leonard, 2005).

In humans, association between MDD and cortisol levels seems to be dependent on the stage and severity of the disease (Nandam *et al*., 2020). A study using dexamethasone suppression testing recorded no differences in salivary and serum cortisol levels between controls and patients who had chronic MDD for more than two years (Watson *et al*., 2002). On the contrary,

high cortisol levels were observed in remitted MDD patients compared to controls after exposure to a visual stress cue (Holsen *et al*., 2013). The above suggests that in remitted MDD, persistent hyperresponsiveness to dexamethasone can be used to predict relapse, while in chronic MDD, cortisol levels do not provide prognostic information (Zobel *et al*., 2001; Watson *et al*., 2002).

In addition, a number of studies have shown that severity of MDD is proportional to cortisol levels, with more severe subtypes, like melancholic and psychotic, consistently displaying elevated baseline cortisol levels than atypical MDD which is a less severe subtype (Zobel *et al*., 2001; Posener *et al*., 2000; Schatzberg *et al*., 2014; Keller *et al*.,2006; Karlović *et al*., 2013). Early life stress (ELS) is one of the factors that are responsible for programming responses to abnormal adult corticosterone during stressful events (Hunter *et al*., 2011). Changes to GR and mineralocorticoid receptor (MR), glucocorticoid resistance and increased central CRH activity are the potential mechanisms via which responses to corticosterone changes are programmed (Heim and Binder, 2012, Juruena, 2014). Impairment of HPA axis in adulthood has been recorded in a number of childhood trauma associated psychiatric disorders including MDD, suggesting persistent abnormal cortisol into adulthood (Heim *et al*., 2001).

2.4 Neurotransmitter systems in depression

Neurotransmitters are important connecting molecules in the transmission of neural signals with precision and intensity at either presynaptic or postsynaptic level (Branco and Staras, 2009). In the 1750's, effective antidepressant drugs were introduced stimulating the interest in the brains neurochemistry and neurotransmission system (Palazidou, 2012). Deficiency of mono-amine neurotransmitters together with the abnormal function of the neurotransmitter receptors is the biological etiology of the major depressive disorder (MDD) (Liu *et al*., 2020). Abnormalities in expression of dopamine (DA), serotonin (5-HT), and norepinephrine (NE)

monoamine neurotransmitter systems has been observed in several neuronal circuits in different regions of the brains of subjects with MDD (Castren, 2005; Hamon and Blier, 2013). The disruption of monoamine neurotransmitters has a potential to affect the function of their receptors.

Decreased levels of DA in the receptors causes over excitability of the amygdala due to failure of inhibition from the frontal cortex, resulting in fear and pathological anxiety (Liu *et al*., 2018). The D2 receptor subtype of DA has been specifically found to have lower density in patients with anxiety disorders compared to their heathy counter parts (Shin and Liberzon, 2010). Reports by previous researchers recorded evidence that decreased expression of DA receptors in the striatal pathways, with enhanced activity of the insula and adjacent operculum, play a role in mood alterations and related behaviours including eating disorders (Stice *et al*., 2009; Frank *et al*., 2012), suggesting that the regulation of mood by the dopaminergic system is through the insula.

Furthermore, it has long been hypothesized that decreased activity of 5-HT pathways is part of the mechanism in the pathophysiology of depression (Coppen A, 1967; Hogenelst *et al*., 2016). The administration of reserpine in humans has been shown to deplete brain stores of 5-HT and to interfere with its synaptic vesicles, resulting into depressive symptom (Shore *et al*., 1955). Further research noted that reserpine induced depressive symptoms could be reversed by monoamine precursors (Hirschfeld, 2000). In addition, patients with MDD have been shown to have lower 5-HT serum concentrations compared to healthy controls, suggesting 5-HT deficiency in patients with MDD (Bot *et al*., 2015; Phillips, 2017). A number of studies have recorded low levels of primary metabolite of 5-HT (5-HIAA) in patients with MDD, specifically those exhibiting suicidal behaviour (Kruesi *et al*., 1990; Asberg, 1997; Placidi *et al*., 2001). The low concentrations of 5-HT observed in MDD are probably due to low neuronal 5-HT synthesis and abnormal function of 5-HT receptors (Artigas, 2013). At least five of the

fourteen 5-HT receptor subtypes: 5-HT1A, 5-HT1B, 5-HT4, 5-HT6, and 5-HT7 presumably play a role in depression (Hamon *et al*., 1990; Beck *et al*., 1992; Riad *et al*., 2000; Richardson-Jones *et al*., 2010).

The subsequent development of selective serotonin reuptake inhibitors (SSRIs), that work by increasing availability of 5-HT in the receptors ameliorating depressive symptoms in patients, supported the theory that 5-HT systems are a biochemical basis for MDD (Cowen, 1990). A preclinical study in transgenic mice discovered that altering levels of raphe 5-HT1A autoreceptors is a determining factor to response and no response to antidepressant treatment (Richardson-Jones *et al*., 2010). The above study found mice with lower levels of 5-HT1A autoreceptors to be more resistant to stress, with better response to SSRI treatment than mice that had elevated levels of 5-HT1A autoreceptors. Furthermore, mice exhibiting decreased levels of 5-HT1A autoreceptors have also been shown to respond to acute treatment with SSRIs in novelty suppressed feeding (NSF), a behavioural paradigm that usually responds to chronic antidepressant treatment of at least 14 days (Richardson-Jones *et al*., 2010; Samuel and Hen, 2011) indicates that the negative feedback of raphe 5-HT1A autoreceptors has a temporal limiting effect in behavioural response to SSRI treatment (Yohn *et al*., 2017).

The involvement of NE in the pathophysiology of depression was proposed in 1979, whereby it was suggested that depressive symptoms are caused in part by the reduction of NE in the central nervous system (Zis and Goodwin, 1979). Further research has shown that inability to adequately regulate stress response is one of the key factors in the pathophysiological of depression (Carboni *et al*., 2010; Weger and Sandi, 2018). Several studies have shown that there is overstimulation of NE in MDD patients (Yehuda *et al*., 1998; Mausbach *et al*., 2005; Nutt *et al*., 2006). In addition, norepinephrinergic neuronal cell bodies have been recorded to have increased binding of agonist ligands at α2-adrenergic autoreceptors, indicating higher

activity of these NE autoreceptors, which is indicative of reduced noradrenergic neurotransmission in MDD (Hamon and Blier, 2013).

2.4.1 Inflammatory process in depression

Activated inflammatory pathways and increased proinflammatory cytokines have been observed in patients with major depression (Raison et al., 2006; Miller et al., 2009, Stetler and Miller, 2011). Furthermore, cytokines reach the brain and interact with neurotransmitter metabolism, neuroendocrine function, neural circuitry, and synaptic plasticity, all of which plays a role in pathophysiology of depression (Borden and Parkinson, 1998). In the brain, cytokine signals stimulate synthesis, release, and reuptake of dopamine, norepinephrine and serotonin, which are mood-relevant neurotransmitters (Felger et al., 2007; Anisman et al., 2008; Miller, 2009). Other effects of proinflammatory cytokines are on the HPA-axis where they cause increase in levels of CRH and ACTH, as well as cortisol in patients suffering from depression (Besedovsky and del Rey, 1996; Pariante and Miller, 2001).

Prolonged activation of cytokine networks in the brain systems can cause dysregulation of cognitive function and glial/neuronal circuits thereby contributing to the pathophysiology of depression (Miller et al., 2009). Increased serum and plasma concentrations of interleukin 6 (IL-6) and C-reactive protein (CRP) are frequently observed inflammatory markers in depressed patients (Howren et al., 2009). In addition, BDNF, an important requirement of antidepressant response which fosters neurogenesis has been shown to be reduced by increased levels of interleukin beta (IL-1β), tumour necrosis factor (TNF) and necrosis factor caper beta (NF-κB) in stress-induced animal models of depression (Goshen et al., 2008; Koo et al., 2010). Furthermore, inflammatory mediators have been associated with depressive symptoms, whereby both acute and chronic administration of cytokines/cytokine inducers like lipopolysaccharide (LPS) can cause behavioural symptoms similar to those seen in patients

with major depression (Reichenberg et al., 2001; Brydon et al., 2008). Elevated levels of hippocampal TNF-α and IL-1 and cognitive impairment have been observed after peripheral administration of LPS resulting from reduction in expression of hippocampal BDNF (Wu et al., 2007). Additionally, hippocampal dependent learning and memory, synaptic plasticity and neuronal survival are linked to BDNF and its signalling pathways, partially explaining the cognitive impairment seen with elevated levels of proinflammatory cytokines (Sairanen et al., 2005).

2.4.2 Genetic predisposition in depression

Epidemiological evidence suggests that heritability of bipolar disorders is about 80% based on the studies done on twins, adoption and family, where genetic factors were found to play a significant role in the aetiology of affective sicknesses (Berrettini, 1999). Furthermore, development of the MDD has been found to have a very strong dependence on gene-environmental interactions (Kendler et al., 1997; Klengel and Binder, 2015; Binder, 2017). Gene–environment interaction is a different effect of an environmental exposure on disease risk in persons with different genotypes or a different effect of a genotype on disease risk in persons with different environmental exposures (Ottman, 1996). The pioneering study on gene-environment interaction in depression was done in 2003, and demonstrated close interaction between a functional polymorphism in the serotonin transporter gene (5-HTTLPR) and stressors that predict depression (Caspi *et al*., 2003). Additionally, a study done in healthy male adults who were exposed to public speaking stress task showed significant effect of the BDNF Val66Met polymorphism on HPA-axis reactivity (Alexander *et al*., 2010).

2.5 Neuroanatomy of depression

The connection between the cingulate gyrus, hippocampus, the hypothalamus and the anterior thalamic nuclei, the structures collectively referred to as a limbic system, was first described by James Papez in 1937. He described the above circuit as a communication between these brain structures that plays a role in the storing of memory and also enabling the cortex to control the emotions. Further advances in technology including the introduction of neuroimaging techniques broadened the knowledge on the importance on 'neurocircuit of emotion' where the prefrontal cortex (PFC) was also found to play a role in the circuit (Pelazidou, 2012). This circuit has been shown to be responsible for maintaining emotional stability, and the destruction in the structures involved in it is believed to be central to the pathophysiology of depression.

2.5.1 The role of the prefrontal cortex in depression

The PFC is located anterior to the premotor and the primary motor area of the frontal cortex. It is divided into three main parts: (i) the dorsolateral, (ii) the paralimbic, and (iii) the anterior cingulate cortex (ACC). The ventromedial (VMPFC) and the dorsolateral (DLPFC) connect with each other via the cingulate gyrus and the hippocampus (Pelazidou, 2012).

Decreased activity of the PFC, including its connections to and from other depression linked limbic and subcortical structures, has been observed in depressed patients (Savitz and Drevets, 2009). Previous studies conducted on MRIs of MDD patients have shown large volumetric reduction in anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC) (Koolschijn *et al*., 2009; Gray *et al*., 2020). In addition, MMD patients with large grey matter volume in the right dorsal anterior cingulate cortex (dACC) and right inferior frontal gyrus have better clinical outcomes and fewer symptoms of depression (Serra-Blasco *et al*., 2016). Studies in rodents found the right ventromedial prefrontal cortex (vmPFC), an analog of the human subgenual

cingulate cortex (sgACC) is of critical importance in the regulation of HPA axis (Sullivan and Gratton, 1999).

Chronic stress that lasts for a number of days to weeks is known to induce behavioural depressive symptoms and brain structural changes (Ferenczi *et al*., 2016; Willner, 2017). The structural changes due to chronic stress are not only limited to the amygdala and hippocampus, but they also involve the PFC that includes dendritic spine density amongst other changes (McEwen and Morrison, 2013; McEwen *et al*., 2016).

2.5.2 The role of amygdala in depression

The amygdala is a brain structure connected to the superior aspect of the hippocampus. It plays an important role in processing emotions, affective state, conditioning of fear and social behaviours (Fox and Shackman, 2019; Hur *et al*., 2019; Janak and Tye, 2015; Lindquist *et al*., 2012; Putnam and Chang, 2021; Shackman *et al*., 2016). Studies indicate that the amygdala function acquires a sustained increase in the activity during acute phase of depression, which normalises after antidepressant treatment (Sheline *et al*., 2001; Fu *et al*., 2004; Siegle *et al*., 2007). The recorded increase in amygdala activity has been shown to be associated with severity of symptoms of depression (Drevets *et al*., 2002). Furthermore, depressed patients with first episode of MDD were shown to have increased volume of the amygdala while on depression treatment (Frodl *et al*., 2002; Lange and Irle, 2004; Weniger *et al*., 2006). On the other hand, patients who recovered from recurrent depression showed no structural or volumetric increase of the amygdala, suggesting that the volume normalised after antidepressant treatment (Mervaala *et al*., 2000; Caetano *et al*., 2004; Hastings *et al*., 2004). It has been suggested that the increase in the volume of amygdala is related to the increase in the generation of oligodendrocytes (Wennström *et al*., 2004).

2.5.3 Role of the hippocampus in depression

The hippocampus is a key hub of the limbic system known to be dysfunctional in the neuropathology of the MDD (Campbell and MacQueen, 2004). The hippocampus is an elevated tissue of gray matter that lies within the parahippocampal gyrus in the floor of the temporal horn of the lateral ventricle (Fogwe *et al*., 2018). It consists of three distinct zones: the dentate gyrus (DG), the hippocampus proper, and the subiculum, which is a transitional zone joining the DG and the hippocampus proper (Fogwe *et al*., 2018). Definitions may also include the presubiculum and parasubiculum, which are the anatomically adjacent areas, and the functionally connected areas which includes entorhinal, parahippocampal cortices, and white matter areas including the fimbriae and the fornix (Amaral *et al*., 2007). The hippocampus proper consist of the cornu ammonis subfields 1 to 4 (CA1–CA4) (Amaral *et al*., 2007). CA3 and CA2 form the borders of the hilum of the dentate gyrus on either side (Fogwe *et al*., 2018). CA3 forms the larger subfield of the hippocampus that receives fibers from the proximal dendrites of the granule cells of the DG (Daugherty *et al*.,2016).

Each subfield of the hippocampus is further organized into distinct layers. The main cellular layer is the pyramidal cell layer which is also referred to as stratum pyramidale which contains approximately 300,000 – 400,000 cells in rodents (Miettinen *et al*., 2012). The neurones of the above layer have been shown to connect to different cortical and subcortical structures with the exception of few cases where one neurone project into two different regions (Ishikawa and Nakamura, 2006). The above connections are believed to project between the ventral hippocampus to the medial nucleus of the amygdala, whereby they modulate bulbar cardiorespiratory control circuits (Ajayi et al., 2018).

Other layers include a noncellular stratum oriens which lies deep to the pyramidal cell layer, and the noncellular stratum lucidum, which lies above the pyramidal cell layer in the CA3 sub-field (Ajayi, 2019). The stratum lucidum is also present immediately superficial to the

pyramidal cell layer in CA1 and CA2 subfields, where it contains the apical dendrites of the cells in the pyramidal cell layer. In addition, mossy fibres projecting from the DG to the CA3 subfield mainly occupy the above layer (Ajayi, 2019). Lying superficial to the stratum lucidum is the stratum radiatum which constitutes intrinsic connections within the subfield, and lastly, the superficial area of the hippocampal regions is the stratum lacunosum-moleculare, which receives terminal projections from the thalamus, entorhinal cortex, and other cortical areas (Amaral, 1995). The DG is made up of three layers namely, the superficial stratum moleculare that has scattered neuronal cells, the middle stratum granulare with densely packed oval shaped granule cells, and the stratum multiforme which consists of polymorphic neuronal cells (EI Falougy *et al*., 2008).

The main hippocampal circuit functions within the hippocampal formation which includes subiculum inferiorly, the DG medially and the molecular layer (Schultz and Engelhardt, 2014). Autopsy and imaging studies have shown that the hippocampus, parahippocampal region, and the neocortical association are essential for memory processing (Fogwe *et al*., 2018). Bilateral damage to the above regions has been shown to cause impairment of short-term memory resulting into an inability to form new memories (Kizilirmak *et al*., 2019). Learning and memory circuit comprises two important pathways, the polysynaptic (Fig. 2.2) and direct (Fig. 2.3) intrahippocampal pathways (Anand and Dhikav, 2012). The polysynaptic intrahippocampal pathway (PIP) comprises entorhinal cortex, DG, CA and the subiculum (Prasad *et al*., 2019). Inputs to the PIP originate from the occipital cortex, temporal cortex, and posterior parietal cortex via the entorhinal cortex as summarised in Figure 2.2 (Prasad et al., 2019).

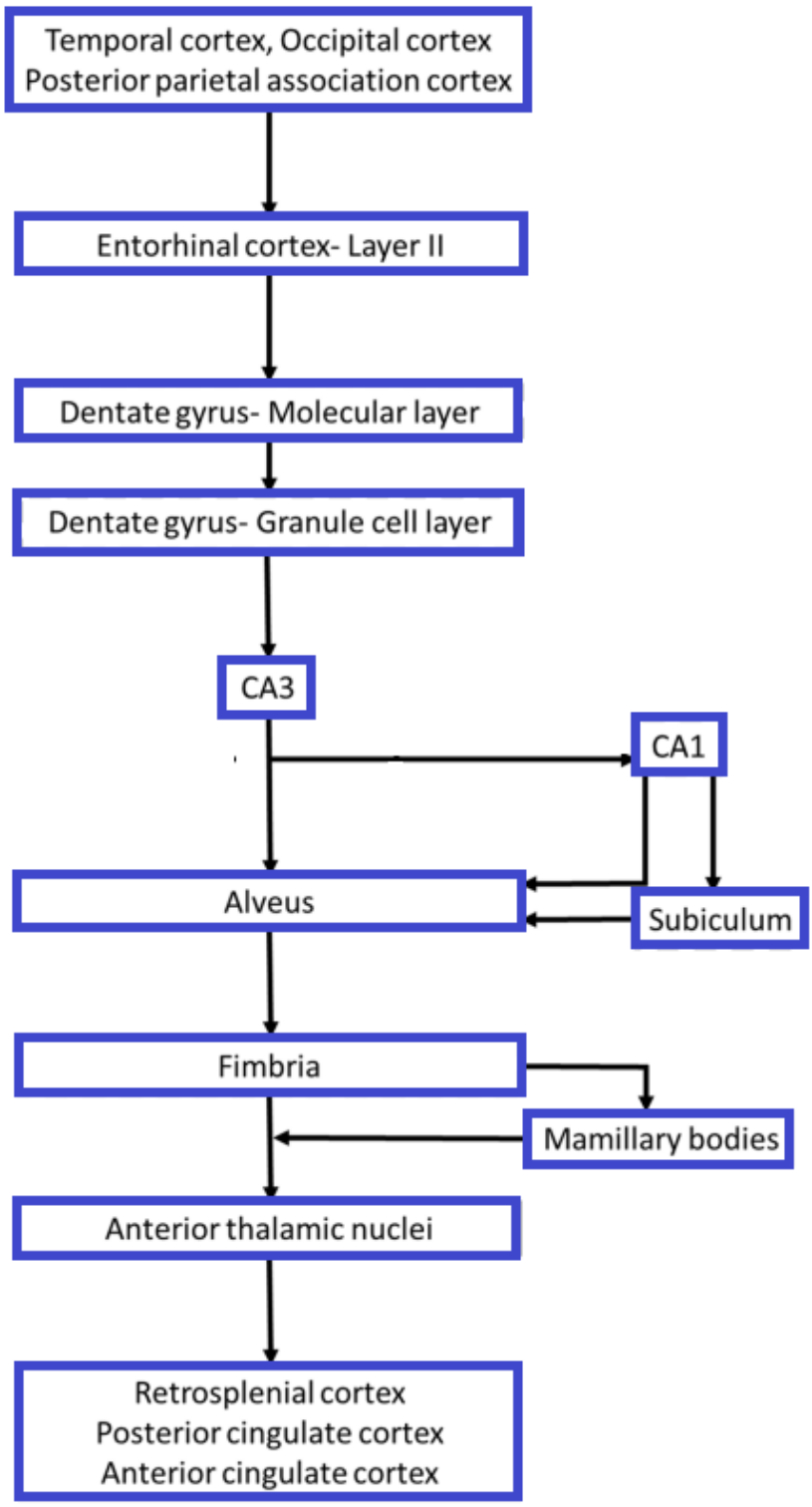

Figure 2.2: Schematic representation of the intrahippocampal polysynaptic pathway (Prasad *et al*., 2019).

The direct intrahippocampal pathway (DIP) receives input from the inferior temporal association cortex and connect directly with CA1 through layer III of the entorhinal cortex as illustrated in Figure 3 (Prasad *et al*., 2019).

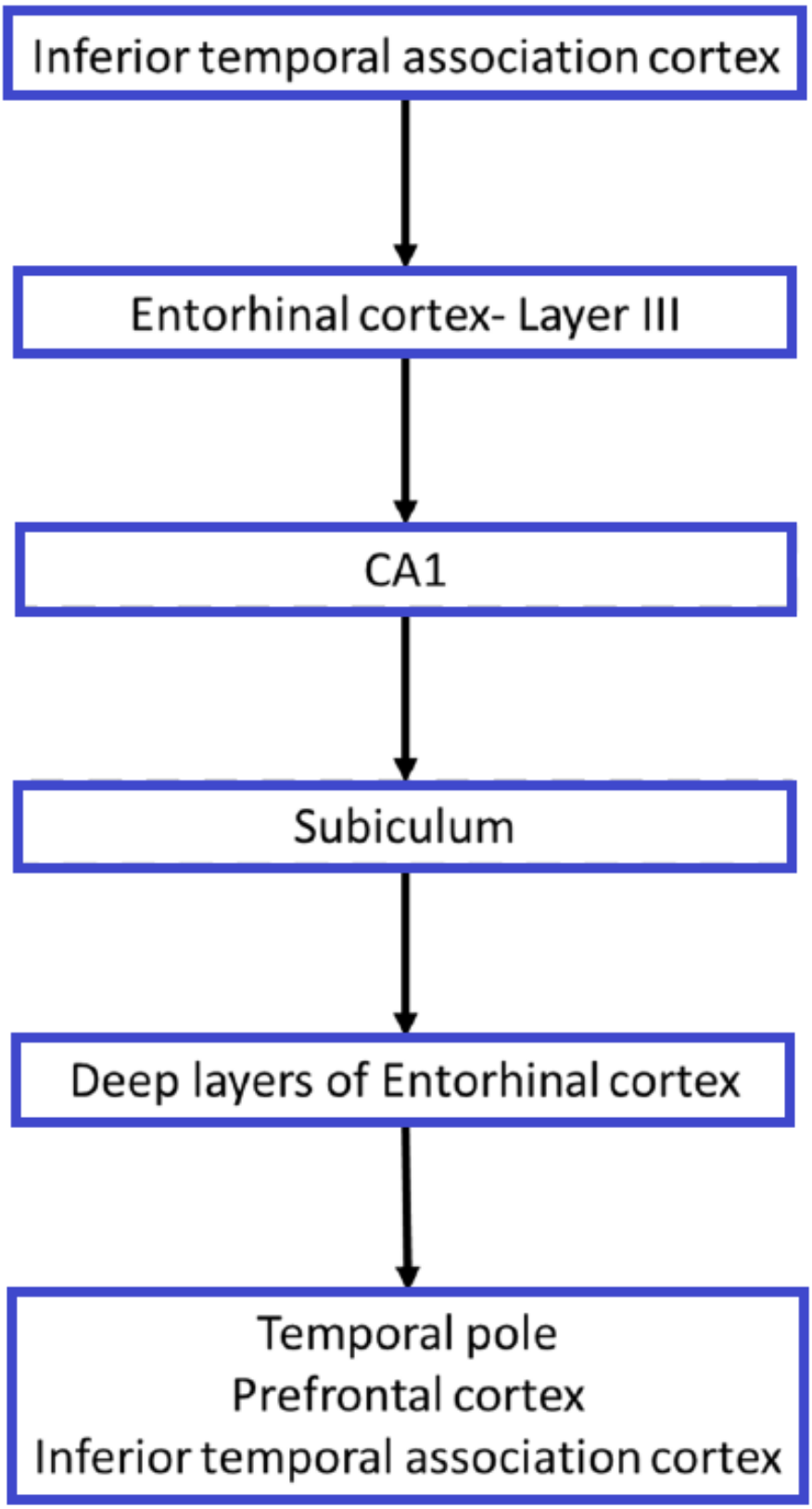

Figure 2.3: Schematic representation of the direct intrahippocampal pathway (Prasad et al., 2019)

The hippocampus is intricately sensitive to stress and the stress hormone class, glucocorticoids. The DG sub-region has a high density of GC receptors that respond to increased levels of circulating GCs resulting into hippocampal atrophy (De Kloet *et al.,* 1998). Hippocampal atrophy has been linked to a number of mechanisms (Dhikav and Anand, 2011). One of the important mechanisms resulting into hippocampal atrophy is stress resultant from increase in circulating glucocorticoids (Gilbert and Brushfield, 2009). Deposition of beta amyloids, suppression of hippocampal neurogenesis, impairment of long term potentiation, oxidative and metabolic stress are five mechanisms implicated in stress induced hippocampal atrophy (Hiramatsu *et al*., 1992; Hamaguchi *et al*., 2006; Howland and Wang, 2008; Srivareerat *et al*., 2009; Ondrejcak *et al*., 2010; Rothman, and Mattson, 2010; Tran *et al*., 2010; Yassa and Stark, 2011).

2.5.3.1 Beta amyloid deposition and hippocampal atrophy

Amyloid beta (Aβ) is a peptide that is secreted by neurones in an activity-modulated manner as observed in the pathophysiology of Alzheimer's disease whereby it removes dendritic spines, and sites of excitatory synaptic transmission (Wei *et al*., 2010). The effects of Aβ accumulation are profound, including loss and deterioration of synapse, inflammation and eventually, cell death (Cline *et al*., 2018). Previous studies have shown that large accumulation of Aβ accompanies reduction in the volume of hippocampus (Knopman, 2013; Jack *et al*., 2014; Gordon *et al*., 2016). Similarly, chronic stress has been implicated in dendritic atrophy and loss of dendritic spines in areas CA1, CA3, and loss of hippocampal volume probably due to the same Aβ mechanism (McEwen, 1999; Sousa *et al*., 2000; Pawlak *et al*., 2005).

2.5.3.2 Suppression of neurogenesis and hippocampal atrophy

A process of production of new neurons in the adult hippocampus, referred to as neurogenesis, is now believed to be taking place in several mammalian species (Peter et al., 1998; Lucassen et al., 2010). Neurogenesis is believed to play an important role in the pathophysiology of depression, however, there is still a lack of explanation as to how the newborn neurons play a role in mood and other symptoms of depression (Kempermann et al., 2008). A view that neurogenesis is part of the mechanism of action of the antidepressant drugs has been suggested (Santarelli et al., 2003). The above is because reduction in neurogenesis cannot produce depressive symptoms on its own even though the prolonged reduction in the formation of new granule cells in the dentate gyrus (DG) can impair the composition of the cell population in the DG and make it vulnerable to impaired functioning (Lucassen et al., 2014). It is for this reason that part of the success of antidepressant treatment should be the restoration of the normal rate of neurogenesis (Kempermann et al., 2008).

Most neuronal stem cells (NSC) in the adult brain are inactive, however, NSCs in the subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) slowly divide to generate new neurons, a process called neurogenesis (Kempermann and Gage, 1999; Encinas et al., 2011; Ming and Song, 2011). The belief in the existence of adult neurogenesis started in the 1960s when adult generated brain cells were identified in rodents (Altman, 1962). This philosophy of newly generated adult brain cells was met with great skepticism for three decades until confirmed in numerus mammals including humans and non-human primates (Cameron and Gould, 1994; Peter et al., 1998; Gould et al., 1999). Recent studies have also shown the persistence of neurogenesis in Balb/c mice and avians, noting that the proliferation and migration of newly formed cells decline with age (Nkomozepi et al., 2019a; Nkomozepi et al., 2019b). In animals, the newly formed cells migrate into the olfactory bulb and subsequently get integrated into the olfactory neuronal circuitry (Alvarez-Buylla and

Garcıa-Verdugo, 2002; Sun and Moon, 2010; Faiz et al., 2005). Five stages of developing granule cells have been described (Fig. 2.4) (von Bohlen Und Halbach, 2007). The first stage is the proliferation stage which is represented by neuronal stem cells or by type 1 radial glial cells (RGL) which can be labelled with glial brillary acidic protein (GFAP), Sex determining region Y-box 2 (SOX2) and Nestin or even other markers of stem cells. Stage 2 is marked by intermediate progenitor cells (IPCs) that arise from RGLs. The IPCs continue to divide and show expression of doublecortin (DCX). In stage 3, IPCs give rise to migrating neuroblasts which also express DCX and give rise to stage 4 mature dentate gyrus neurones. In stage 5, newly formed neurones are integrated into the hippocampal circuitry.

However, research has shown that neurogenesis is upregulated by a number of factors such as pregnancy, environmental enrichment, exercise, antidepressant treatments, while other factors like aging and stress downregulates neurogenesis (Fan et al., 2017; Trinchero et al., 2019; Wan et al., 2019). The reduction in neurogenesis has been shown to cause reduction in the volume of the dentate gyrus and CA3 in the hippocampus after four weeks of suppression of neurogenesis, and reduction of whole hippocampal volume after eight weeks (Schoenfeld *et al*., 2017). However, suppression of neurogenesis is not a main contributor of hippocampal volume loss compared to factors such as reduction in number of inhibitory interneurons and astrocytes seen in depression throughout the hippocampus (Czéh *et al*., 2015; Tata *et al*., 2006).

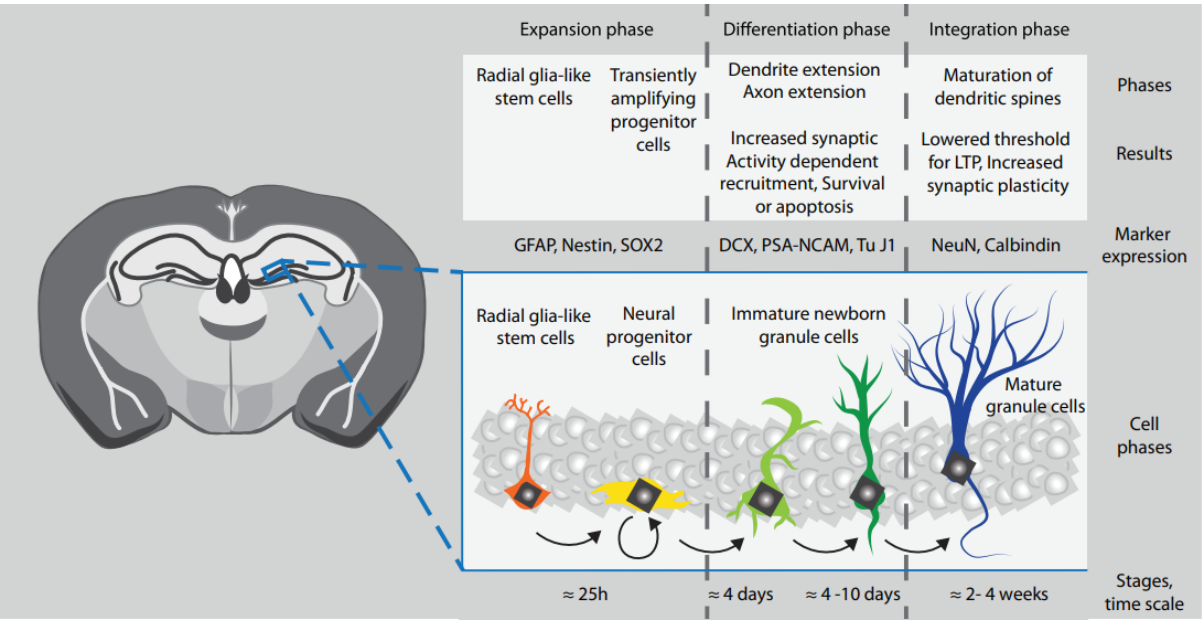

Figure 2.4: Cell stages in neurogenesis (Adapted from Samuels et al., 2016).

2.5.3.3 Long term potentiation and hippocampal atrophy

Long-term potentiation (LTP) is described as a persistent strengthening of synapses leading to long-lasting increase in signal transmission between neurons (Fu and J hamandas, 2020). Long-term potentiation can be disrupted by chronic stress and/or glucocorticoid administration which may lead to depression (Srivareerat *et al*., 2009). Deposition of Abeta, glucocorticoid administration, and chronic stress are some of the factors thought to be responsible for disruption of LTP in long term depression (Hamaguchi and Yamada, 2006; Howland and Wang, 2008; Adekar *et al*., 2010). It is believed that enhanced postsynaptic potentials following presynaptic stimulation serves as a substrate in the hippocampal neuronal circuit during learning and memory (Molnár, 2011).

2.5.3.4 Oxidative stress and hippocampal atrophy

Stress and Abeta (Alpha beta) is broadly known to increase production of free radicals in the brains of experimental animals (Howland and Wang, 2008; Srivareerat et al., 2009). A number of authors have shown that the pyramidal cells of CA3 and granule cells of the dentate gyrus

are prone to oxidative stress, with some authors suggesting that pyramidal cells of CA1 are the ones that are more susceptible to oxidative damage (Chang et al., 2012; Huang et al., 2012, 2013; Uysal et al., 2012).

2.6 Stress effects on plasticity and molecular structure

As mentioned in sections above, the most widely understood explanation of mental and brain illnesses is the imbalances in the monoaminegic neurochemical system. Modern research has shown that pathophysiology of stress involves abnormalities in the plasticity and the changes in the volume of limbic structures of the brain (Lucassen *et al*., 2014). The above changes include apoptosis, remodeling of dendrites, and changes in glial cells (Czéh and Lucassen, 2007; MacQueen and Frodl, 2011; Rajkowska and Stockmeier, 2013). These changes in limbic brain structures are thought to be coupled with behaviors observed in stressed individuals, even though it is uncertain whether the structural changes are reversible adaptations to stress or pathologic changes (Lucassen *et al*., 2014).

2.6.1 Changes in dendritic spines

The importance of the dendritic spines is their involvement in storing the information and formation of synapses, all of which are impaired by prolonged chronic stress (Tasker and Herman, 2011). Exposure to chronic stress and prolonged administration of high doses corticosterone is known to cause blunting of the apical spines of the dendrites of neurons in CA3 and CA1 of the hippocampus after a couple of weeks of stressful conditions (Woolley et al., 1990). Other effects of chronic stress include changes in vesicles and mitochondria at synapses, larger surface area of postsynaptic density and reduction of synapses of mossy fibers (Sandi et al., 2003; Tata et al., 2006).

Reduction in dendritic spine densities and atrophy which was reversible with training or recovery period has been recorded in stressed animals (Magariños *et al*., 1996; Sandi et al.,

2003; Stewart et al., 2005). The above effects have been observed specifically in the prefrontal cortex and pyramidal cells of the hippocampus (Goldwater et al., 2009; Radley and Morrison, 2005; Radley et al., 2005). In addition, the morphology of both pre- and post-synaptic neurons is markedly altered after prolonged exposure to stress and glucocorticoids (Liston et al., 2013).

The opposite of the above effects has been recorded in the nucleus accumbens and amygdala whereby exposure to chronic stress resulted in increased density and hypertrophy of dendritic spines (Mitra et al., 2005; Vyas et al., 2004).

2.6.2 Stress and apoptosis

Apoptosis, also called programmed cell death is a process whereby cell growth and division ceases resulting into the dead of cells that are subsequently removed from the system by the process of phagocytosis (D'Arcy, M.S., 2019). Prolonged exposure to glucocorticoids has also been shown to causes shrinkage and volume reduction of the hippocampus and cerebral cortex, probably due to increase in neuronal cell apoptosis (Swaab et al., 2005; Lucassen et al., 2006; Sapolsky et al., 2002; McKernan et al., 2009; Shelton et al., 2011). Contrary observations have been reported in patients and animal models of depression where no neuronal loss or hippocampal shrinkage was observed (Lucassen et al., 2001; Müller et al., 2001). In a previous study where the dentate gyrus of unmedicated patients showed fewer granule cells, it was suggested that the cellular changes in the hippocampus of depressed individuals could be related to the length of time of the disease (Boldrini et al., 2013). In rodent stress models, apoptosis has been observed in acute stress (Heine et al., 2004; Lucassen et al., 2006; Yu et al., 2011), while in chronic stress, apoptosis only happens in some hippocampal structures and normalises after treatment (Lucassen et al., 2004). The above is crucial in interpretation of the structural and neuronal changes observed in the experiments of the depression animal models to properly explain both the effects of depression and antidepressant treatment (Huang et al., 2013; Lucassen et al., 2001).

2.7 Treatment of depression

Given the known pathophysiology of depression, the medical sciences have developed antidepressant drugs that would counteract the symptoms of depression in order to maintain healthy living (Khushboo and Sharma, 2017). The principle of treatment is based on the foundation of neurotransmitter functionality of the pre and post synaptic transmission, which is highly regulated by enzymes and several mechanical steps (Bondy, 2012). The steps of neurotransmitter function include synthesis, storage, release, induction of cellular response and termination of the neurotransmitter release (Bondy, 2012) . Firstly, the synthesis step is the process where neurotransmitter amino acid precursors, go through facilitated transport from the blood to the brain. In the brain, the precursors will be converted by enzymes into a specific neurotransmitter (Bondy, 2012). Secondly, in the storage step, the synthesized neurotransmitter will get stored in synaptic vesicles which are found within the neurons in the brain (Bondy, 2012) . Thirdly, the release step is the process of regulated release of the neuro-transmitter into the synaptic cleft (Bondy, 2012). Fourthly, the induction of cellular response step is the process where the neurotransmitter molecules within the synaptic cleft induce a signal transduction cascade reaction (Bondy, 2012) . It is also important to note that the induction reaction will be triggered by binding of the neurotransmitter on the surface of the neurotransmitter receptors located on the post-synaptic membrane (Whalen, 2018). However, neurotransmitter molecules do not cross or go through the post-synaptic membrane (Whalen, 2018). Finally, the termination step is carried out by the enzymatic process (Bondy, 2012). The antidepressant drugs will act by intervening in one of the steps above as described below.

2.8 Antidepressants and their mode of action

Antidepressant drugs are divided into five categories which are: tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), serotonin noradrenaline reuptake inhibitors (SNRI), monoamine oxidase inhibitors, and atypical antidepressants (Ferguson, 2001).

Selective Serotonin Reuptake Inhibitors are a group of commonly used anti-depressant agents, mainly because of their high efficacy in alleviating depressive episodes (Whalen, 2018). According to Fasipe (2018) the mechanism of action of SSRIs is explained by their selective inhibition of the serotonin transporter (SERT). A more precise mechanism of SSRI is however its ability delaying the inhibition of serotonergic neurotransmission in about four pathways that occur after desensitization of 5-HT1A and 5-HT1B auto-receptors (Fasipe, 2018). However, SSRIs affect a wide range of postsynaptic receptor subtypes, which result in a wide range of side effects including nausea, headaches, dry mouth and sexual dysfunction (Ferguson, 2001). Thus, though effective, the resultant side effects limits its overall usefulness in the treatment of depression and necessitates the search of a cheap and better alternative treatment drug from natural plant sources.

2.9 Medicinal plants with antidepressant properties

There is a fair amount of literature documenting the use of medicinal plants to treat depression (Aderibigbe, 2018; Ahmadpoor et al., 2019; Jahani et al., 2019; Rabiei and Setorki, 2019; Ilkhanizadeh et al., 2021). The study done on the antidepressant effects of Albizia adianthifolia, one of the plants used by traditional healers in African countries to treat depression showed reduction in immobility time in both forced swimming tests and tail suspension test in Swiss mice (Aderibigbe, 2018). *Adiantum capillus-veneris* (Pteridaceae), a cosmopolitan plant that is widely distributed in parts of the world that have tropical climate and high humidity is one of the plants that has been shown to have a wide range of medicinal properties including analgesic, anti-inflammatory, and anti-oxidant properties (Trojan-Rodrigues et al., 2012; Rajurkar and Gaikwad, 2012; Gaikwad et al., 2013). In addition to the above medicinal properties, a study conducted in male Balb/c mice exposed to chronic stress showed that *Adiantum capillus-veneris* possesses antidepressant properties in elevated plus maze and forced swimming test where the frequency of entry in open arms was increase and the immobility time

reduced in forced swimming test and elevated plus maze respectively (Ahmadpoor et al., 2019). From the African perspective, the study of *Amaryllidaceae* alkaloids began in the late 1990s with the investigation of the alkaloidal content of *Crinum bulbispermum* (Elegorishi et al., 1999). Since ancient times, medicinal plants belonging to this family have been used to treat depression in different cultures (Elgorashi, 2019). The rationale behind the use of *Amaryllidaceae* in traditional medicine and their medicinal efficacy is attributed largely to the presence of a unique type of alkaloids. These alkaloids are present exclusively in this family and have been isolated from each member of *Amaryllidaceae* investigated so far. Few South African *Amaryllidaceae* species have been investigated for their affinity for the serotonin re-uptake transport protein (Nielsen et al, 2004). Extracts of the leaves and bulbs of *B. disticha* have exhibited strong affinity to the SSRI site, while leaf extracts of *Brunsvigia grandiflora* and root extracts of *G. ciliaris* have moderate to low affinity to SSRI site, respectively (Nielsen et al, 2004). In addition, ethanolic extracts of *BD* showed functional inhibition of serotonin transporter, noradrenalin transporter, and dopamine transporter upon screening in a functional inhibition assay using COS-7 cells (Pedersen et al, 2008). The efficacy of *Amaryllidaceae* alkaloids from *Boophone disticha* (*BD)* stimulated the screening of many *Amaryllidaceae* alkaloids isolated from a number of *Crinum* and *Cyrtanthus* species for their affinity to the SSRI site (Elegorishi et al., 1999). These activities were also confirmed *in vivo* using the tail suspension and the forced swim tests in mice and rat models (Viladomat et al, 1997, Pote et al, 2018, Gadaga et al., 2010). There is currently a paucity of information regarding the mechanism by which BD exerts its antidepressant effects on the brain. The current study used mice model to close this gap. Mice and rats possesses numerous advantages for research purposes, such as sharing 90% genome homology with humans, in addition to many genetic, physiological, and organ anatomical similarities (Tabassum et al, 2015).

2.10 Overview of *Boophone Disticha*

Boophone disticha (BD) belongs to a large family of bulbous flowering plants known as Amaryllidaceae (Fig. 2.5) (Nair and Van Staden, 2014). The genus "*Boophone*" consists of only two species, namely: *Boophone disticha* and *Boophone haemanthoides* (Nair and Van Staden, 2014).

Boophone disticha plant has been used for centuries by traditional healers within the Southern African regions (Neuwinger *et al*.,1997). Rock paintings and Khoi-San mummies that contained *Boophone disticha* scales, further substantiate the fact that this plant has been used by South African natives for centuries (Nair and Van Staden, 2014).

Figure 2.5: Representing the Amaryllidaceae, bulbous plant known as *Boophone Disticha* (SMGrowers.co.za).

The *Amaryllidaceae* plants have been extensively used for medicinal purposes by some of the most popular South African tribes namely: Sotho, Zulu and Xhosa (Nair and Van Staden, 2014). Different parts of the plants are used to treat different conditions. For example, dry bulbs are used for the feeling of weakness, fresh bulbs are taken to increase sexual potency, leaves

are taken for gastrointestinal wash and roots are burned and ground to powder in order to be applied to an area where one would experience paralysis (Gadaga et al. 2011).

2.10.1 Alkaloids of Boophone Disticha

The medicinal activity can be attributed to the alkaloids of the plant (Neuwinger, Leon-Rot and Mebs, 1997). There are eleven alkaloids that have been isolated in the BD plant, which are *buphanidrine, undulatine, buphanisine, buphanamine, nerbowdine, crinine, crinamidine,* and *lycorine, distichamine, acetylnerbowdine* and *buphacetine* (Fig. 2.6) (Neuwinger *et al*., 1997)

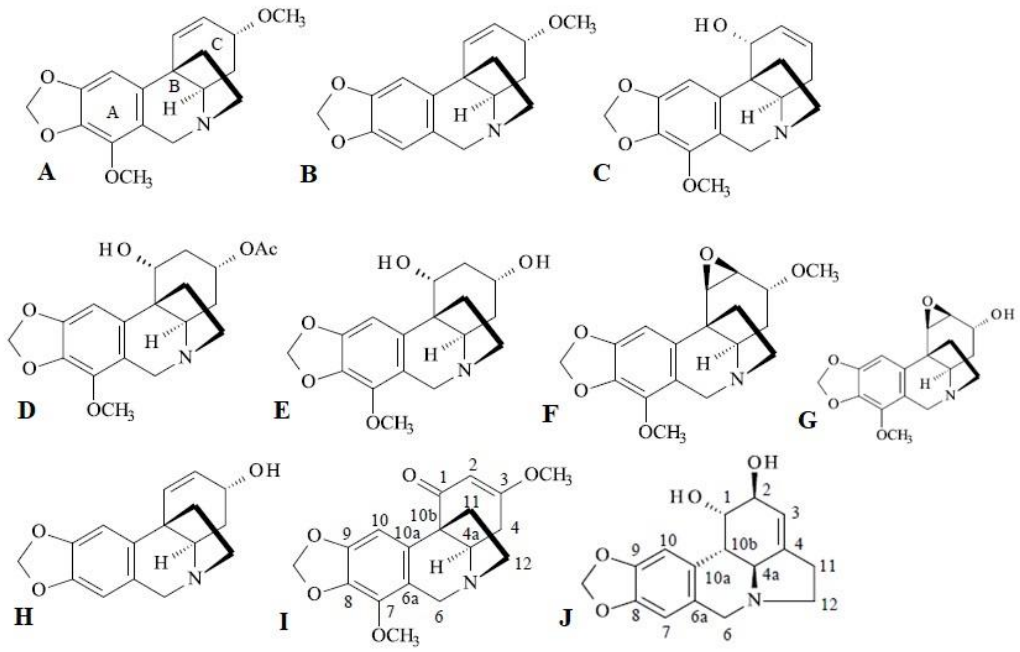

Figure 2.6: Representing the structures of the *Boophone Disticha* Alkaloids as follows:
(A) Buphanamine **(B)** Buphanisine **(C)** Buphanidrine **(D)** Acetylenerbowdine, **(E)** Nerbowdine**, (F)** Udantaline, **(G)** Crimanidine, **(H)** Crinine, **(I)** Distichamine and **(J)** Lycorine (Cheeseman et al. 2013).

A previous study on the inhibitory binding affinity of the alkaloids, of BD extract, on the Serotonin Reuptake Transporters (SERT) showed that *Buphanadrine*, *Buphanamine* and *Distichamine* have high binding affinity for the serotonin reuptake site (Neergaard *et al.*, 2009).

According to Gadaga *et al*. (2011), *buphanidrine* is the most abundant alkaloid found in BD, occurring at a percentage abundance of 19.4%. The plant is well known for its powerful analgesic and neurotoxicity effects. Furthermore *buphanidrine* has a high hepatotoxicity at profile at high doses (Neuwinger, Leon-Rot and Mebs, 1997). *Buphanidrine*, has an LD50 of 8.9 mg/kg for mice administered Subcutaneously. A dose of 10 mg/kg has previously been shown to significantly reduce mean arterial pressure in mice (Pote *et al.*, 2014).

2.10.2 Pharmacological Activity of *Boophone Disticha*

The alkaloids of the *BD* give the plant a source of its pharmacological activity (Nair and Van Staden, 2014). *Boophone disticha* exerts its anti-inflammatory effects by inducing the production of Adenosine triphosphate (ATP) on isolated human neutrophils while simultaneously inhibiting the release of superoxide from neutrophils (Gadaga *et al*. 2011). The above mechanism could account for the plant's traditional use to alleviate rheumatic pains, muscular sprains and other inflammatory conditions. Additionally, *BD* inhibit cyclo-oxygenase 1 (cox-1) enzyme (Gadaga *et al*. 2011). This enzyme is responsible for maintaining hemostasis by inducing platelet aggregation (Whalen 2018).

A previous study showed that *BD* displaces more than 50% of the transport protein bound citalopram at various concentrations (Neergaard *et al.*, 2009). A study on a serotonin binding assay showed inhibition of serotonin (Nair and Van Staden, 2014). A study done by Gadaga et al, (2011) showed that *BD* has a high affinity for binding to the receptors of the SSRIs, and inhibition of Serotonin Reuptake Transporters (SERT), Noradrenaline Reuptake Transporters (NAT) and affinity for Dopamine Transporter (DAT). The anticholinesterase effects (inhibition of cholinesterase enzyme) of *BD* have been demonstrated using thin Layer Chromatography (LC), explaining the use of this plant for memory enhancement and tretment Alzheimer's disease (Gadaga *et al*., 2011).

2.11 Rodent models of depression

Despite a tremendous effort dedicated to neuro-psychiatric pharmacological research, not much has been attained to counteract the onset and progression of affective disease, with most treatment strategies simply targeting the mono-aminagic neurotransmitter system (Hyman, 2014). This setback is due to the lack of detailed understanding of the molecular, cellular and neuronal circuits involved in the pathophysiology of the affective disorders, with some suggestions genetic and environmental interplay in the disease process (Caspi and Moffitt, 2006; Sullivan, 2015; Han and Nestler, 2017; Wray et al., 2018). To acquire a deeper understanding of the pathophysiology of affective disorders, rodent models have become increasingly relevant in the research of mental disorders (Italia et al., 2020). Mice are the most commonly used animals model in the research of mental disorders due to ease of handling and versatility in different behavioral tests (Hylander et al., 2022). Forced swimming test (FST) is one of the commonly used stress models in mice to demonstrate the effects of mild unpredictable stress (Becker et al., 2021). On the other hand, chronic restraint stress is useful for modeling chronic life psycho-emotional stressors which might have more longer lasting effects (Huang et al., 2015; Xu et al., 2017; Wu et al., 2018).

Based on the argument that FST is a test for copying mechanism and not necessarily a stressor, it is important to combine the FST with chronic stress in drug testing as chronic stress might be more resistant to treatment than the acute stress (Molendijk and de Kloet, 2022). For example, a previous study demonstrated that neuropeptide Y is more effective in reversing the effects of acute stress and less effective in reversing the effects of chronic stress (Andriushchenko et al., 2022).

2.12 Aims and objectives of the study

2.12.1 Aim of the study

To determine the effect of *Boophone disticha* on the behaviour and hippocampal neuroanatomy in male Balb/c mouse model of depression.

2.12.2 Objectives

(i) To determine the anxiolytic like and antidepressant-like activity of orally gavaged hydroethanolic extract of *Boophone disticha* and compare with similar effects of fluoxetine using open field test, elevated plus maze and light dark box in established mouse models of depression.

(ii) To compare the effects of orally gavaged hydroethanolic extract of *Boophone disticha* and fluoxetine on levels of brain derived neurotropic factor (BDNF) and corticosterone in the brains of mice subjected to different established mouse models of depression by ELISA technique.

(iii) To compare the effect of orally gavaged hydroethanolic extract of *Boophone disticha* to fluoxetine on the volume of the hippocampus and its dentate gyrus of the mice subjected to different established mouse models of depression on Nissl stained sections using Volumest plugin of image J.

(iv) To compare the effect of orally gavaged hydroethanolic extract of *Boophone disticha* and fluoxetine on the expression of doublecortin (DCX) in the dentate gyrus of the hippocampus using immunohistochemical techniques (immunostained brains sections) in mouse brain subjected to different established mouse models of depression.

2.12.3 Hypothesis

Buphoone disticha attenuate the effects of stress on the behaviour and neuroanatomy of Balb/c mouse model.

CHAPTER THREE

METHODOLOGY

3.1 Experimental animals

Seventy-two adult male Balb/c mice (Postnatal day 30) were used in the current study. Animals were purchased and housed in the Wits research animal facility (WRAF) and handled with the help of professional personnel. Mice were randomly allocated to one of the following two established models of depression, 1) five days repetitive forced swimming forced swimming model (5d-RFSS), 2) 28 days repetitive restraint stress model. The procedure for induction of the stress models is described below (section 3.4 - 3.5). Animals randomly assigned to each stress model were treated for 21 days post stress induction as shown in Appendix 1. Briefly, each stress model had four groups of animals (Control, distilled water, *BD* and fluoxetine groups) with 9 animals in each group (n=9), adding up to 36 animals per stress model. The Control group was the normal animals that were not exposed to stress, the distilled water group was the stressed animals that were orally gavaged with 0.2 ml of distilled water, the BD group was the group that was orally gavaged with 10 mg/kg of BD dissolved in 0.2 ml distilled water, the fluoxetine group was the positive control that was orally gavaged with 10mg/kg of fluoxetine. During days 7, 14 and 21 of the treatment period, the animals underwent the following behavioural tests: open field test, elevated plus maize and light dark box as described in section 3.6.1 below. For a forced swimming stress model, the forced swimming was used for both induction of stress and a behavioural test during the treatment period. The tests were done in a random order. Following behavioural studies, animals were anesthetized using isoflurane for a short period, followed by perfusion using normal saline. After this procedure, brains were harvested as described in section 3.7 below.

Male mice were chosen in the current study to eliminate the confounding hormonal factors as the female mice could be at different oestrous cycles. While it is not impossible to monitor the

individual for differences in the estrous cycles, the current study design did not cater for that procedure.

3.2 Plant material and extraction of phytochemicals

Bulbs of *BD* were purchased from Random Harvesters in Muldersdrift Johannesburg, South Africa and were authenticated at the Department of Botany, University of Johannesburg, South Africa. A table knife was used to chop the fresh bulbs of *BD* into small pieces and sun dried for seven days. The dried plant material was ground into a fine powder using a kitchen blender. The powder was then mixed with 70% ethanol in a ratio 5:1 (70% ethanol: *BD* powder) and placed on an automatic shaker for 24 hours at room temperature. After 24 hours, plant debris were filtered using Whatman No. 1 filter paper. The resultant extract was concentrated using a rotary evaporator at 70ºC. The final product was a dark brown solid paste which was air dried, crushed into powder and stored at room temperature. The administered dose of the powder was weighted in grams according to each animal's body weight and dissolved in 2ml distilled water and administered through orogastric gavage.

3.3 Identification of phytochemicals using LC-MS

A Thermoscientific ultimate 3000 Ultra High-Performance Liquid Chromatography (UHPLC) in conjunction with Bruker Compact Q-TOF high resolution mass spectrophotometer (HRMS) was used to analyse 20 ul of BD extract dissolved in methanol. Gradient elution techniques was applied to elute 20 ul of the sample that was injected into the UHPLC in a period of 14 minutes. Solvent A with a concentration ranging from 95%-5% of 0.1% formic acid in water (v/v) and a solvent B with a concentration ranging from 5%-95% of 0.1% formic acid in acetonitrile (v/v) was used through Raptor ARC C18 Column (2.7 um 2.1 x100 mm). Analyses of the sample was done using the UHPLC-HRMS analyses, while Bruker Daltonics analyses was used for mass spectrum analyses.

3.4 Five day repeated forced swimming stress model (5d RFSS)

Forced swimming stress model was first introduced by Porsolt *et al*, in 1977. The procedure for this model is to place the mouse for 6 minutes into a glass-polycarbonate cylinder (25 cm high×10 cm wide) filled to a depth of 10cm with water maintained at 24°C (Fig. 3.1). Immediately following the 6 minutes exposure into the water cylinder, the animals are removed from the cylinder and dried using a water absorbent pad. In the current study, we used a recently described 5 d repeated forced swim stress (5d-RFSS) protocol (Serkov et al., 2015), in which mice were forced to swim in an open cylindrical container (diameter, 10 cm; height, 25 cm) containing 10 cm deep water (25°C) for five consecutive days. The five days procedure constituted the induction phase of the stress model. This protocol had been shown to produce the depressive symptoms that last for at least four weeks, allowing for a time window to test and study therapeutic interventions (Serkov et al., 2015). After the five days induction period, animals were treated for 21 days vial oral gavage as described in section 3.1, while behavioural tests were conducted and video recorded at intervals on days 7, 14 and 21, after 5d RFSS. The period/duration of swimming and immobility during the 6 minutes test was measured from the video recordings and recorded using a timer. While the FST has received extensive criticism regarding its validity and reproducibility, a meta-analysis of 73 behavioral studies that used FST in mice demonstrated external validity and reproducibility of the FST where different types of antidepressant drugs reduced immobility in the test (Kara et al., 2018).

The issue of ethics regarding the use of the FST has been raised due to its perceived suffering to animals (PETA, 2020). However, there is currently no regulation against the use of the FST. The fact sheet published by British Association for Psychopharmacology in conjunction with, the Laboratory Animal Science Association and Understanding Animal Research advocated for the continued use of FST as a valuable behavioral screening tool (LASA, 2020). A recent

commentary suggested the use of none-behavioral neurochemical measures like BDNF as an alternative to the FST (Sewell et al., 2021).

Figures 3.1: Schematic representation of the forced swimming test. (Adapted from: Creative biolab. Accessed on 21 December 2022).

3.5 Repetitive restraint stress

Clinical observations suggest that stress can act as a precipitating factor in the onset of affective illnesses, especially major depression (Bidzinska, 1984). The pathophysiology of depression and the neurobiology of stress are linked by their shared association with the hypothalamic pituitary-adrenal (HPA) axis and in particular with serotonin and norepinephrine containing neuronal systems (Breslow *et.al.,* 1989). In animals, the behavioural deficits and the abnormalities in the nervous system and neuroendocrine system that are induced by exposure to uncontrollable and unpredictable chronic stress, such as hypo-activity in the open field, aberrations in the HPA system, and alterations in neuroplasticity and neurogenesis, can be

reversed by antidepressant treatments (Xu *et.al*., 2009). In this study, mice were restrained in acrylic cylinders (6.5 cm inner diameter, 20 cm long, with air ventilation at the sides and at the nasal end of the cylinder, Fig. 3.2) for 6 h (10:00–16:00 h) for 28 consecutive days (Makhathini *et al*.,2017). After the induction of chronic restraint, the 21 days treatment was conducted as described in section 3.1 with the behavioural tests in days 7, 14 and 21.

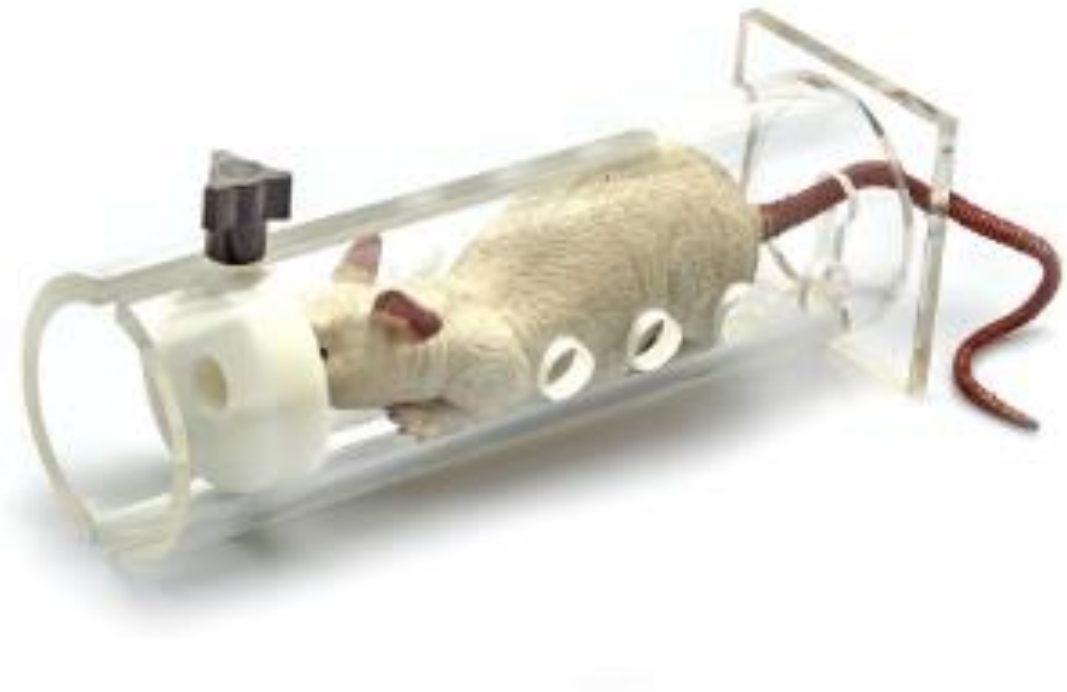

Figure 3.2: Picture demonstrating the restrain procedure during the chronic restraint stress induction.
(Adapted from: **https://midsci.com/item/ASRATREST/Rat-Restraints/**).

3.6 Behavioural tests

On the test day, mice were brought into the test room for at least one hour before the test. All testing was performed between 9:00 am and 15:00 pm by the experimenter. Behavioral tests were done randomly (Elevated plus maize, open field test, light dark box and forced swimming test). All behavioral activities were video recorded via an overhead video camera (Canon Legria, HF R806, Makro, South Africa) and later analyzed by the independent observer blinded to the experimental groups.

3.6.1 Elevated plus maze

The maze is plus-shaped with four identical 50 cm (length) X 10cm (width) arms, elevated 70 cm above the floor (Fig. 3.3Two opposite arms are surrounded on three sides by 30cm tall opaque walls, and the other two arms are open with no protecting walls on the sides. Each animal was introduced in the centre area (10 cm X 10 cm) facing an open arm and allowed to explore freely for 5min. The number of arm entries and time spent in each arm was scored. An arm entry was scored when all four paws of the animals entered an arm and time in arm was counted only if all four paws of the animal are within the arm (Porsolt et al., 1977). Behaviours in this task (i.e., activity in the open and closed arms) reflect a conflict between the rodent's preference for protected areas (e.g., closed arms) and their innate motivation to explore novel environments (e.g., open arms). Anti-anxiety behaviour (increased open arm time and/or open arm entries) can be determined simultaneously with a measure of spontaneous motor activity (total and/or closed arm entries (Nalloor et al., 2011; Walf and Frye, 2007).

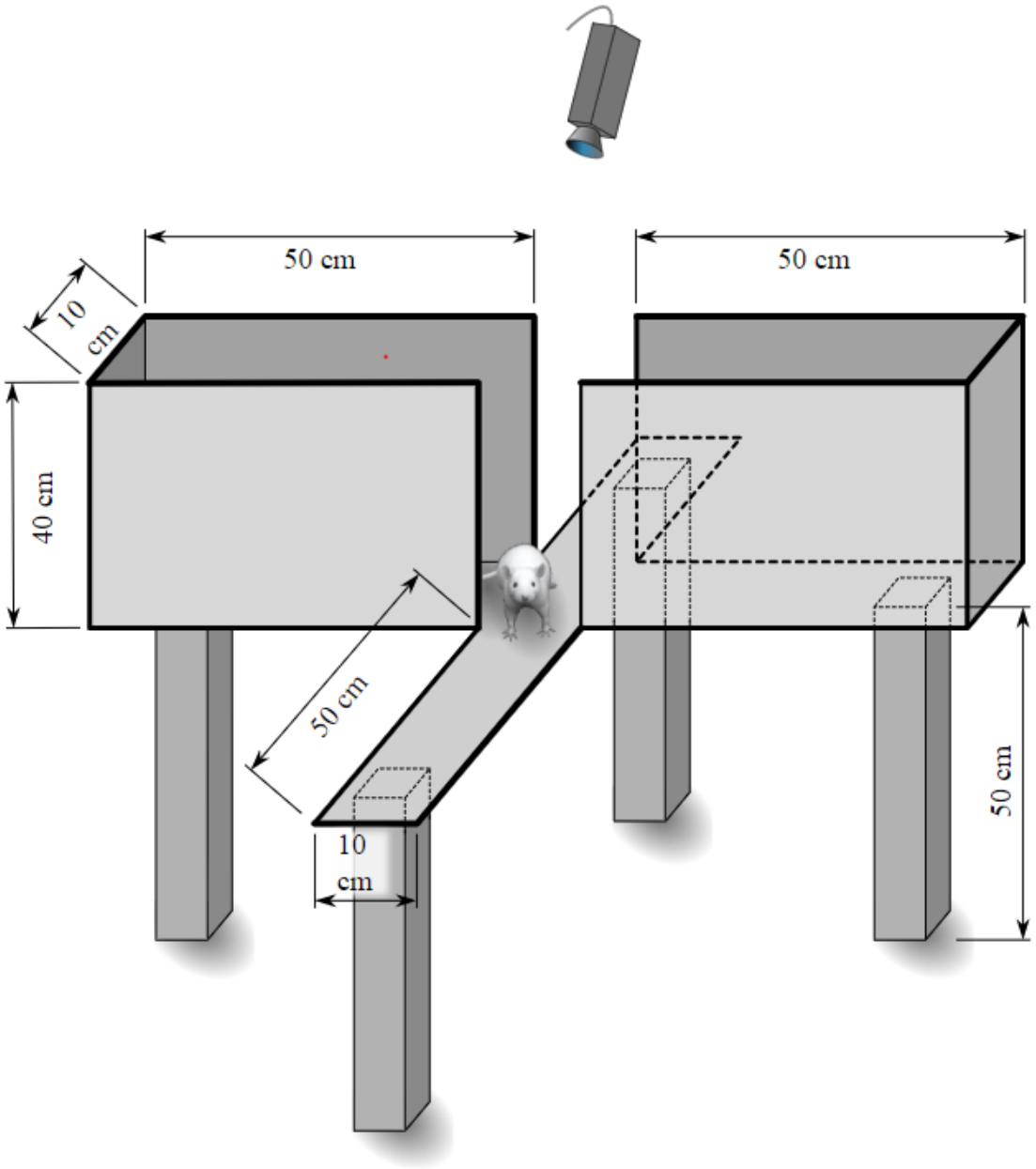

Figure 3.3: Schematic representation of the elevated plus maze (Adapted from: Media Wiki pedia: Accessed on 21 December 2022).

3.6.2 Open field test

The open field is an arena with walls to prevent escape. Commonly, the field is marked with a grid and square crossings (Fig. 3.4). The centre of the field is marked with a different colour to differentiate from the other squares. The Open Field Test provides simultaneous measures of locomotion, exploration and anxiety (Walsh & Cummins, 1976). The number of central square

entries and the duration of time spent in the central square are measures of exploratory behaviour and anxiety. A high frequency/duration of these behaviours indicates high exploratory behaviour and low anxiety levels. Stretch attend postures are “risk-assessment” behaviours which indicate that the animal is hesitant to move from its present location to a new position (Blanchard, Griebel & Blanchard, 2001) and thus a high frequency of these postures indicates a higher level of anxiety. A mouse was gently placed into the corner of the field, and allowed to explore the arena for 10 minutes. Movements was recorded by a video camera mounted above the arena and analysed manually. The number of entries into the centre, the time in the centre, the total distance number of grid line crossing during the test period was measured.

Figure 3.4: Schematic representation of the open filed test (Adapted from: Biomed.au.dk: Accessed on 22 December 2021).

3.6.3 Light/dark box

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light (Crawley and Goodwin, 1980). A natural conflict situation occurs when an animal is exposed to an unfamiliar environment or novel objects. The conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia) situations. The exploratory activity reflects the combined result of these tendencies in novel situations. Thus, in the light/dark test, drug-induced increase in behaviors in the white part of a two-compartment box, illuminated and a black compartment, darkened, is suggested as an index of anxiolytic activity. An increase in transitions without an increase in spontaneous locomotion is considered to reflect anxiolytic activity (Bourin, Martine Hascoet, 2003). The apparatus for this test consists of two equally sized compartments (h:24 X w:25 X l:33 cm) connected by an 8x8 cm opening (Fig. 3.5). One compartment was painted black and covered with a black lid (dark box). The other compartment was opaque and remained uncovered during the test (light box). An opaque black Plexiglas with a door separated the two boxes. Inside the light box was illuminated by a 30 lux light. Inside the dark box there was no appreciable illumination (0.2 lux). On test days, mice were moved to the test room 30 minutes prior to testing. To start the test, the mouse were placed in the center of the light box facing away from the door to the dark box and allowed to freely explore the apparatus for 5 min. The movements of the animals during the 5-minute test period was tracked by a video camera positioned above the center of the light dark box. The data of behavior analysis included the time spent in the light box and dark box with the entry in each chamber defined as at least half of the animal's body from one chamber to the next.

Figure 3.5: Schematic representation of the light dark box
(Adapted from: https://brainstuff.org/blog/what-does-the-light-dark-box-test-measure)

3.7 Terminal procedures

After behavioural tests, animals were euthanized by an overdose of 5% isoflurane at a flow of 2L/min, followed by intra cardiac perfusion with buffered saline. Following perfusion, brains were rapidly removed from the skull and separated into two hemispheres. The left hemisphere was immediately snap frozen in dry ice before being stored at -80º C for biochemical analyses. The Elabscience competitive ELISA kit (Biocom Africa, South Africa, catalogue No. E-EL-M0203, BDNF and E-OSEL-M0001, Corticosterone) for BDNF and corticosterone was used according to the product specifications. The detection range is 3.12-200 ng/mL, with no significant cross reactivity, a standard curve range of 0-1000 pg/ml, sensitivity of 1.93ng/mL and a repeatability: coefficient of variation is <10%. The brain stem and cerebellum was

removed from the brains, after which the tissues were weighed and then homogenized in ice cold PBS (0.01M, pH=7.4) in a volume ratio of 1:9 tissue to PBS with a kitchen blender in 1,5 ml PCR tubes dipped in ice. The homogenates were then centrifuged for 5-10 min at 5000×g at 2-8℃ to get the supernatant. 100 µL each dilution of standard, blank and sample was added into the appropriate plates. Plates were then covered and incubated for 90 min at 37℃. After decanting the solution, 100 µL of Biotinylated Detection Ab working solution was added to each well, followed by 1 hour incubation at 37°C. The solution was decanted and 350 µL of wash buffer was added to each well, and pat dried after 1 minute. Washes were repeated 3 times. 100 µL of HRP conjugate working solution was then added to each well followed by 30 min incubation at 37°C. Wells were washed 5 times in a buffered solution and then 90 µL of substrate Reagent was added to each well, followed by 15 minutes incubation at 37°C. 50 µL of stop Solution was added to each well and readings were done at 450 nm using a preheated microplate reader. The mean absorbance was calculated. The right hemisphere was immersion fixed in 4% buffered paraformaldehyde overnight before being transferred into 30% sucrose for Nissl staining and immunohistochemistry.

3.7.1 Sectioning of the brain tissues

The right cerebral hemispheres were sectioned in a coronal plane at 50 µm, using a Leica croyostat at -25℃. One in 5 serial sections were collected free-floating into 24 well culture plates with 0.1 M PB. The first set of serial sections were mounted in positively charged slides and air dried, followed Cresyl violet staining. These sections were used to orientate the sections, measure the volumes of the DG, granule cell layer (GC) and hippocampus proper. The second set of the serial sections were stained with DCX for migrating cells using immunohistochemical techniques. The remainder of the sections were stored for further analyses.

3.7.2 Nissl staining

For anatomical orientation, and brain tissue architecture, Cresyl violet or Nissl staining was used. Decreasing concentrations of 5 minutes alcohol baths (100%, 95%, 70%, and 50%) were used to rehydrate the slide mounted tissues, followed by 1 minute deionization in water. Slides were than immersed for 1 minute in 1% Cresyl violet staining solution, transferred into distilled water for 1 minute. Five minutes baths of increasing concentrations of alcohol (50%, to 100%) were used to dehydrate the slides. Dehydration was interrupted with a differentiation in 70% alcohol/acetic. Slides were dipped in the above solution until the desired intensity of the Cresyl violet stain was achieved. After differentiation, dehydration was continued, and slides were than cleared in xylene and cover slipped with DPX.

3.7.3 Immunohistochemistry

A protocol modified by Nkomozepi *et al*, (2019) was used for DCX immunohistochemistry stains. Brain tissue sections were incubated in endogenous peroxidase inhibitor solution containing 49.2% 0.1M PB, 49.2% methanol and 1.66% H_2O_2, and then the sections were washed in three ten minutes washes in 0.1 M PB. Washes were followed by a 2 hours incubation with gentle shaking at room temperature in a blocking buffer solution containing 0.25 % Triton X-100 in 0.1 M PB, 2% bovine serum albumin and 3% normal goat serum. The sections were then incubated with gentle shaking for 48 hours in primary antibody solution of rabbit anti-DCX [(5dRFSS: 1:2000, AB18723, Abcam, UK); (Chronic restraint stress:1:250, AB135349, mouse monoclonal, Abcam, UK)] in a blocking buffer solution, followed by three ten minutes washes in 0.1 M PB. Sections were than incubated under gentle shaking for 2 hours in 1,000 dilution of biotinylated anti-rabbit IgG] (5dRFSS: BA-5000; Vector Laboratories, USA or for Chronic restraint stress: 1:200: AS10 1427, Goat anti-Mouse IgG (H&L), HRP

conjugated, min, cross-reactivity to; vector laboratories, USA)] secondary antibody solution in blocking buffer solution, followed by three 10 minute washes in 0.1 M PB. Sections were then incubated for one hour in an avidin-biotin solution [1:125 A and 1:125 B (Vector Laboratories, USA) in 0.1 M PB] under gentle shaking, followed by three ten minutes washes of 0.1 M PB. The sections were then transferred into a solution containing 0.05% 3, 3-di-amino-benzidine tetrachloride (DAB) in 0.1 M PB for five minutes. 3.3 µL of 30% H_2O_2 was added to each 1 ml of the above solution, and the reaction was monitored by viewing the tissues under the stereomicroscope. To stop the reaction, sections were transferred to a 0.1 M PB, and then washed for 10 minutes in another fresh solution of 0.1 M PB. Stained sections were mounted on positively charged slides and air dried overnight, followed by dehydration in increasing concentrations of alcohol. Air dried slides were cleared in xylene and cover slipped with DPX. Random control sections were stained following the above procedure but omitting either the secondary or primary antibody to eliminate the confounding effects of none specific staining. The control sections did not show DCX stained cells.

3.7.4 Volumes of the hippocampus and its subfields

The volumes the DG, granule cell layer (GC), pyramidal cell layer and hippocampus proper for each animal were measured in all serial sections that were 250 μm part from each of the 72 brains (36 5dFSS and 36 chronic restraint stress) in each group on photomicrographs acquired using a Leica ICC50 HD digital camera. The volumes were measured using a free hand tool of ImageJ plugin, VOLUMEST (Schneider et al., 2012).

3.7.5 Doublecortin quantification

Six brain sections that were 250 μm apart were quantified for DCX in the SGZ of hippocampus in each animal. Cells were manually counted by means of a cell counter of ImageJ (Schneider et al., 2012) on photomicrographs taken by a Leica ICC50 HD microscope mounted camera. Adobe photoshop version 7 software (Adobe Systems, Mountain View, CA) was used to prepare the sample of micrographs of the DG of the hippocampus. The originality of the micrographs was not interfered with.

3.7.5 Statistical analysis

Statistical analysis was done using Graph pad prism (version 7.0). All tests were parametric, and $P \leq 0.05$ was considered significant. ANOVA followed by Bonferroni's *post-hoc* tests was used for comparisons of significant differences among the treatment groups. Comparisons of all scored behaviours were expressed as means ± standard error and compared across treatments groups. A paired t-test was used to compare the weights and food consumption changes between the normal and the restrained animals during the chronic restraint stress induction period.

CHAPTER FOUR

RESULTS

4.1.1 Identification of phytochemicals in the hydro-ethanolic extracts of *boophone disticha* using LC-MS

The spectrum view at 5.92 minutes had parental peaks as indicated by the black squares in Fig. 4.1 with the circle dots showing the fragments of the compounds. With the aid of a metabolite mass spectral database, the parental peaks in figure 7 were identified according to their specific m\z as follows: 246.1492 is *Tropococaine*; 274.1439 is *Galanthamine*; 286.1439 is *Narwedine*; 302.1388 is *Buphanamine*, and 316.1537 is *Buphanidrine*. All the phytochemicals obtain in this spectrum view are alkaloids and they are the most abundantly isolated phytochemicals, probably due to the use ethanol solvent.

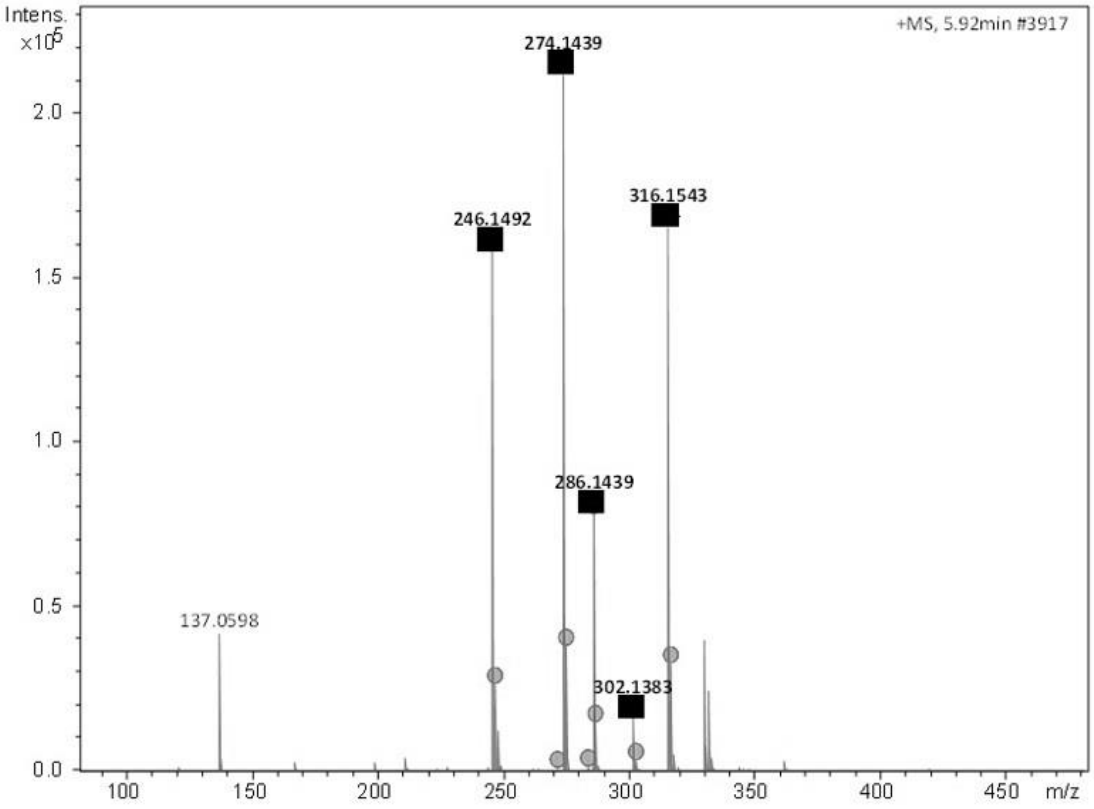

Figure 4.1: Spectrum view of LCMS chromatogram, acquired in the positive-ion mode, from an extract of the bulb of *boophone disticha*.

4.1.2 Phytochemical profile of BD based on LC-MS data

The table below indicates the alkaloids of BD detected in the current study (Table 4.1). In the table below, m/z is calculated as mass divided by charge number with the horizontal axis in a mass spectrum expressed in units of m/z. z is almost always equal to 1, hence the value of m/z is considered as mass.

Table 4.1: Phytochemical profile of BD based on LC-MS data

Retention time	m/z	Common name	Structural formulae
	104.1072	Choline	$C_5H_{14}NO$
0.71min	302.1388	Buphanamine	$C_{17}H_{19}NO_4$
	315.1537	Buphanidrine	$C_{18}H_{21}NO_4$
3.77 min	205.097	Vasicinol	$C_{11}H_{12}N_2O_2$
5.15 min	288.1241	Lycorine	$C_{16}H_{17}NO_4$
5.48 min	332.1493	Tazittine	$C_{18}H_{21}NO_5$
	272.1298	Crinine	$C_{16}H_{17}NO_3$
5.92 min	246.1492	Tropacocaine	$C_{15}H_{19}NO_2$
6.86 min	286.144	Narwedine\Nerbowdine	$C_{17}H_{19}NO_3$
7.77 min	330.1335	Distichamine	$C_{16}H_{17}NO_3$
	274.1439	Galanthamine	$C_{16}H_{19}NO_3$
14.92mm	275.1471	Carboxyprimaquine	$C_{15}H_{18}N_2O_3$
18.16 min	353.2691	Montalnol	$C_{21}H_{36}NO_4$
24.39 min	264.2411	Caranine	$C_{16}H_{17}NO_3$
26.02 min	556.4332	Chloroxathin/OH-Nerosporene	$C_{40}H_{60}O$

4.1.3 Animal weights

Table 4.2 shows the changes in body weights of the animals in this study. Initial body weights were taken at the start of the experiment, and then once every week. Terminal weights were also recorded and compared to initial weights as shown in table 4.2. There was no statistically significant difference in weight gain across the experimental groups (P=0.449). However, the percentage weight gain of the control group was significantly higher than the experimental groups (P=0.01).

Table 4.2: Body masses (grams)

Parameter	Treatments				
	Control	**Stress + Distilled water**	**Stress + BD**	**Stress + Fluoxetine**	**P value**
Initial body mass (g)	18.75	21. 625	22	22.33	0.029
Final body mass (g)	24.125	24.25	24	24.83	0.449
Body mass gain (g)	5.375	2.625	2	2.5	0.001
% change in body mass	28.66	12.14	9.09	11.19	0.01

4.1.4 Five days repetitive forced swimming stress

4.1.4.1 Induction Phase

During the induction phase, mice were forced to swim for 6 minutes daily for 5 days. On day one, the swimming and immobility times were 141.06±29.55 and 218.93±29.55 respectively. These two indices significantly changed to 76.37±29.77 and 283.62±29.77 respectively on day five (P=0.000). From day six, the animals were assigned to three different treatment groups (stress + distilled water; stress + BD; stress + fluoxetine) as described in the methods section and were retested again after days 7, 14 and 21 of the treatment period post 5dRFSS.

4.1.4.2 Changes in swimming times during the treatment period

There were significant differences in swimming times across all the groups after 21 days of treatment (F= 8.647, P=0.004; Fig. 4.2A). Bonferroni's *post hoc* analysis showed that the swimming time for both fluoxetine and *BD* treated groups was significantly higher than that of the distilled water treated group (P<0.05). A time dependant effect of treatments recorded in behavioural tests that were conducted in days 14 and 21 was performed. The *post hoc* analyses showed that by treatment day 14, the swimming time for the fluoxetine treated animals was significantly higher (P=0.0012) compared to the distilled water and *BD* treated animals, while *BD* treated animals only showed a significant increase in swimming time by day 21 (P=0.004;

Fig. 4.2B), at which time the fluoxetine treated group had reached a plateau of increase in swimming time.

4.1.4.3 Changes in immobility times

On day 14 of the treatment period, immobility times were significantly different across the treatment groups as demonstrated in ANOVA test (F=4.237, P=0.029). Furthermore, Bonferroni's p*ost hoc* analysis revealed that the immobility times were significant reduced in the fluoxetine treated group compared to both the distilled water and *BD* treated groups (P= 0.01). On the contrary, no significant differences were found between the distilled water and *BD* treated groups in *post hoc* test (P=0.185).

In day 21, ANOVA test detected significant differences in immobility times across the treatment groups (F=8.597, P=0.004, Fig. 4.2C). Bonferroni's *post hoc* analysis revealed that immobility times were significantly reduced in both the fluoxetine (0.001) and *BD* (0.004) groups compared to the distilled water treated group.

When compared to the control, distilled water treated group had significantly high immobility times by treatment day 21 (P=0.001). However, no differences were found between the control and both the fluoxetine (P=0.067) and *BD* (P=1.00) treated groups.

A time dependant steady reduction in immobility time from day 5 to day 21 was observed in the *BD* treated animals, whereas a steady reduction was observed in a fluoxetine treated group from day 5 to day 14 and plateaued between day 14 and 21. The immobility time for distilled water treated group remained high throughout the treatment period (P= 0.001, Fig. 4.2D).

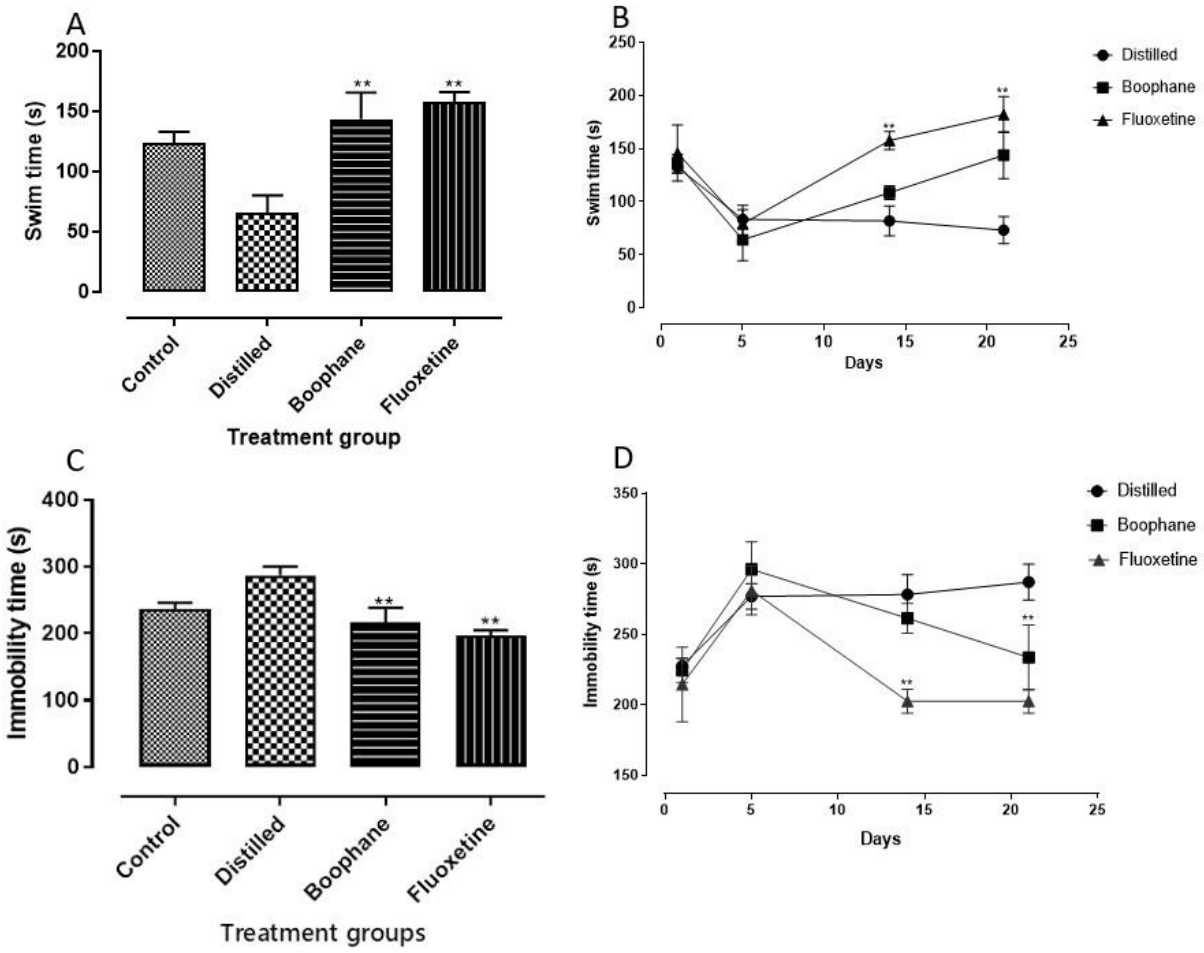

Figure 4.2: Graphic representation of changes in swimming times after 21 days of treatment. A) Significant differences in swimming time across different groups were observed in ANOVA test (P=0.04). B) The *post hoc* test showed that in day 14 of treatment, the swimming time for the fluoxetine treated animals was significantly higher (P=0.0012). C) In day 21, immobility times were significantly different across the treatment groups as demonstrated in ANOVA test (P=0.029). D) A significant time dependant steady reduction in immobility time from day 5 to day 21 was observed in the *BD* treated animals (P=0.004). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.1.5 Elevated plus maze

4.1.5.1 Time spent in open arms

Figure 14 shows the results of the elevated plus maze. The ANOVA test showed that the time spent in the open arms of the elevated plus maze was significantly different among the groups (F= 44.41, P= 0.0001, Fig. 4.3A). Bonferroni's *post hoc* test revealed that the time spent in the open arms was significantly higher in the control group compared to experimental groups (P<0.05). Furthermore, *BD* treated group recorded significantly high time in the open arms compared to both distilled water and fluoxetine treated groups (P<0.05). The time spent in the open arms for fluoxetine and distilled water treated groups was not significantly different (P>0.05).

4.1.5.2 Time spent in closed arms

The time spent in closed arms was not significantly different across the groups as shown in ANOVA test (F= 1.835, P= 0.195). The control group recorded the lowest time in closed arms, though non-significant, when compared with the treatment groups. However, both fluoxetine and *BD* recorded low time in the closed arms as compared to the distilled water group (Fig. 4.3B).

4.1.5.3 Number of head dips and frequency of open and closed arm entries

The number of head dips was significantly different across the groups as shown by ANOVA test (F= 6.122, P= 0.0105, Fig. 4.3C). Bonferroni's *post hoc* test showed that the control group had significantly high number of head dips compared to distilled water and fluoxetine treated groups, both of which scored the lowest number of head dips (P<0.05). Furthermore, Bonferroni's *post hoc* test showed that the head dips of the *BD* treated group were not significantly different from those of the control group (P>0.05). In addition, the post *hoc test* showed that both the control and *BD* treated groups recorded significantly high frequency of open arm entries compared to both distilled water and fluoxetine treated groups (P<0.05, Fig.

4.3D). Furthermore, *BD* treated group had significantly low frequency of closed arm entries compared to distilled water and fluoxetine treated groups (P<0.05; Fig. 4.3E).

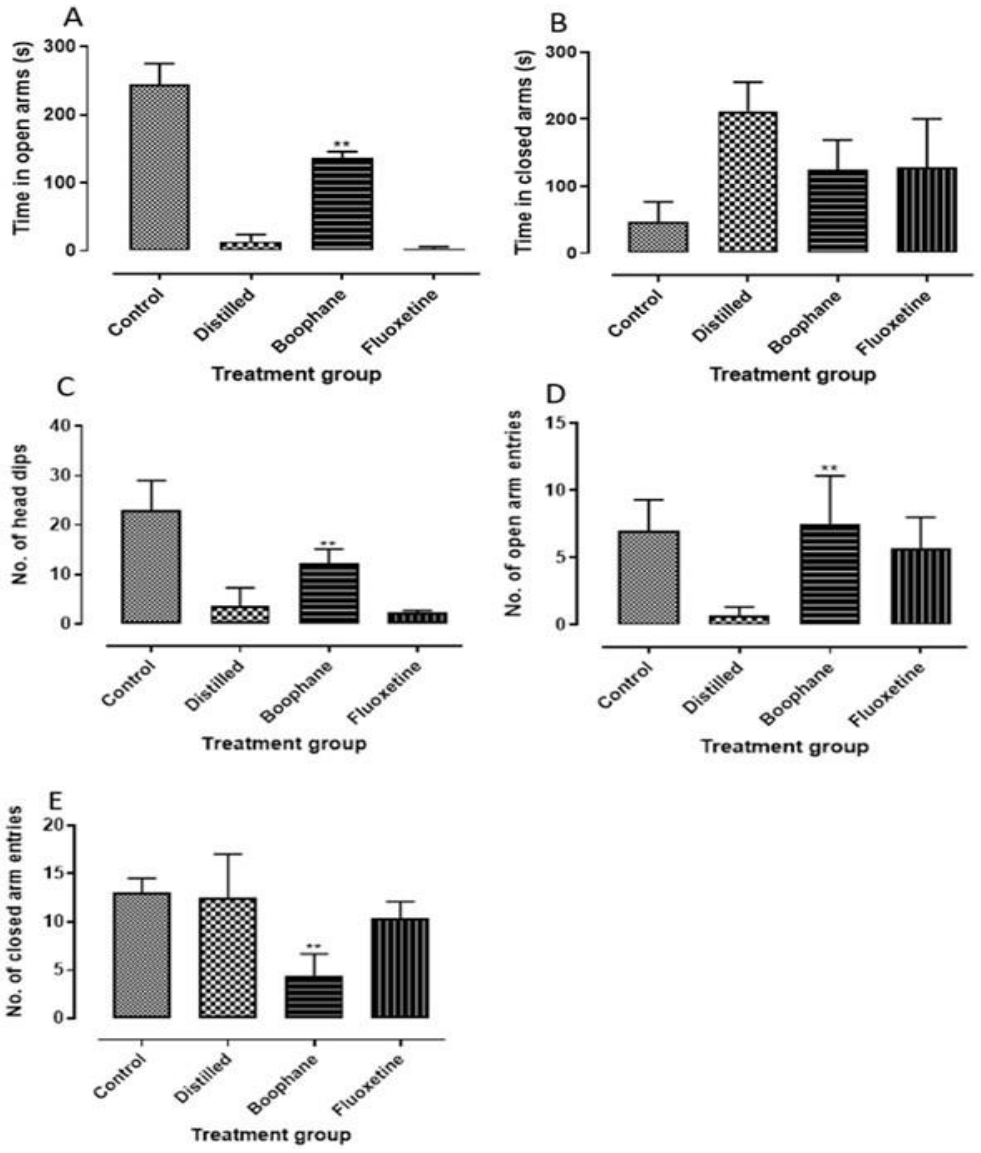

Figure 4.3: Graphic representation of the elevated plus maze results.
A) The time spent in the open arms was significantly different across the groups in ANOVA test (P=0.0001). B) The time spent in the closed arms was not significantly different across the groups (P=0.195). C) The number of head dips was significantly different across the groups as shown by ANOVA test (F= 6.122, P= 0.0105). D) Both the control and BD treated groups recorded high frequency of open arm entries. E) BD treated group had significantly low frequency of closed arm entries (P<0.05). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.1.6 Volume of hippocampus

The ANOVA test did not detect any significant differences in the volume of the dentate gyrus (F= 0.3083, P= 0.8190, Fig. 4.4A), whole hippocampus (F= 3.110, P= 0.1101, Fig. 4.4B), and granule cell layer of the dentate gyrus (F= 1.501, P= 0.2955, Fig. 4.4C) across all the groups. However, there was a slight reduction of the dentate gyrus volume in the experimental groups compared to the control group. Similarly, a slight reduction in the hippocampal volume of the experimental groups was observed compared to the control group. The same observation was also noted in the volume of the granule cell layer of the experimental groups compared to the control group.

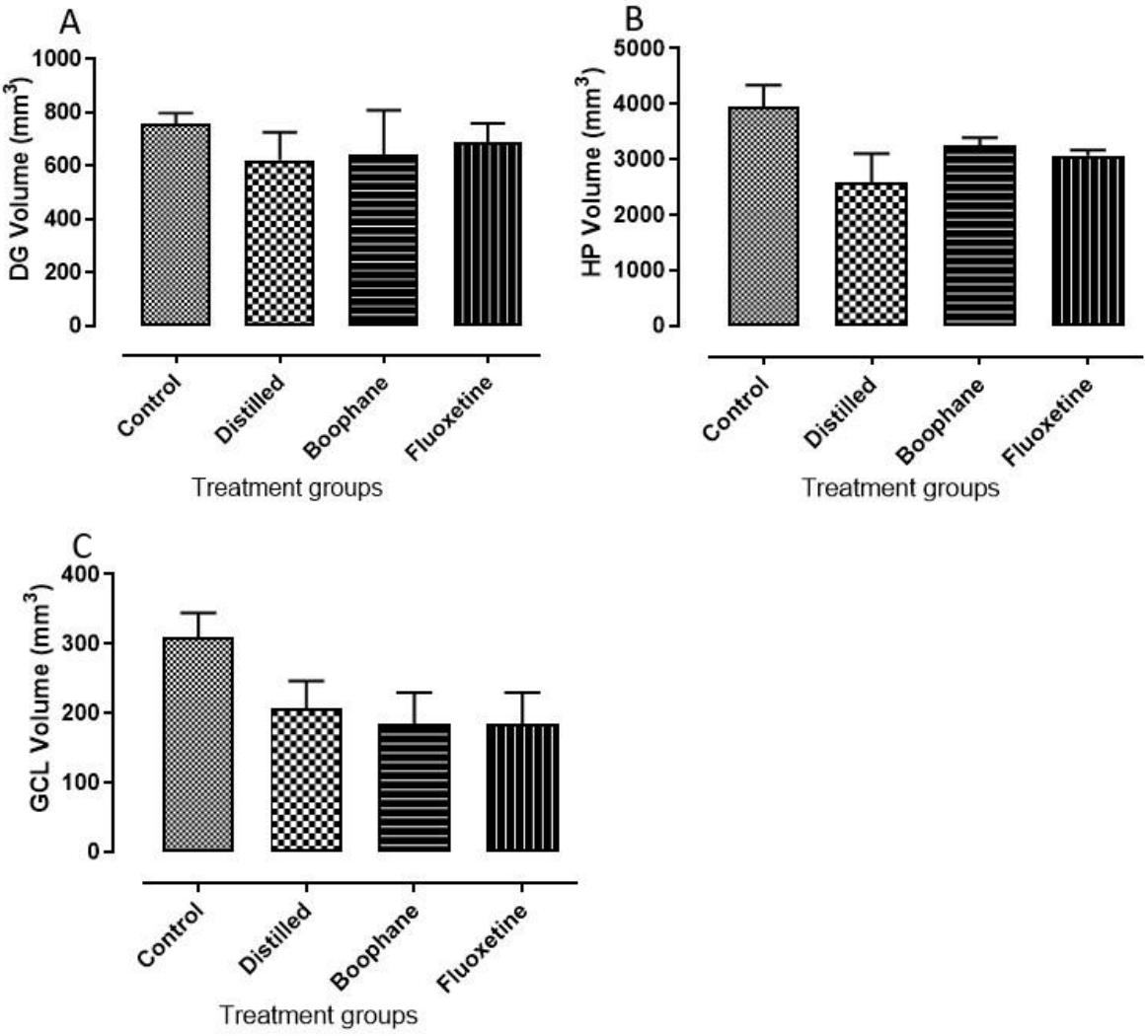

Figure 4.4: Graphic representation of the volumes of the dentate gyrus and hippocampus across the groups.

A) The ANOVA test did not detect any significant differences in the volume of the dentate gyrus (F= 0.3083, P= 0.8190); B) whole hippocampus (F= 3.110, P= 0.1101): and C) granule cell layer of the dentate gyrus (F= 1.501, P= 0.2955), Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.1.7 DCX Expression

There were statistically significant differences in the DCX expression across the groups as detected by the ANOVA test (F= 10.23, P=0.0005). Bonferroni's *post hoc* test showed that the DCX expression was significantly lower in the distilled water treated group compared to both fluoxetine and *BD* treated groups (P<0.05, Fig. 4.5 and 4.6A). The DCX expression in fluoxetine and *BD* treated animals was similar and comparable to the control group. Furthermore, the DCX immunoreactive cells of the control, *BD* and fluoxetine treated groups had well developed dendritic spines, while those of the distilled water treated group had poorly developed dendritic spines (Fig. 4.6).

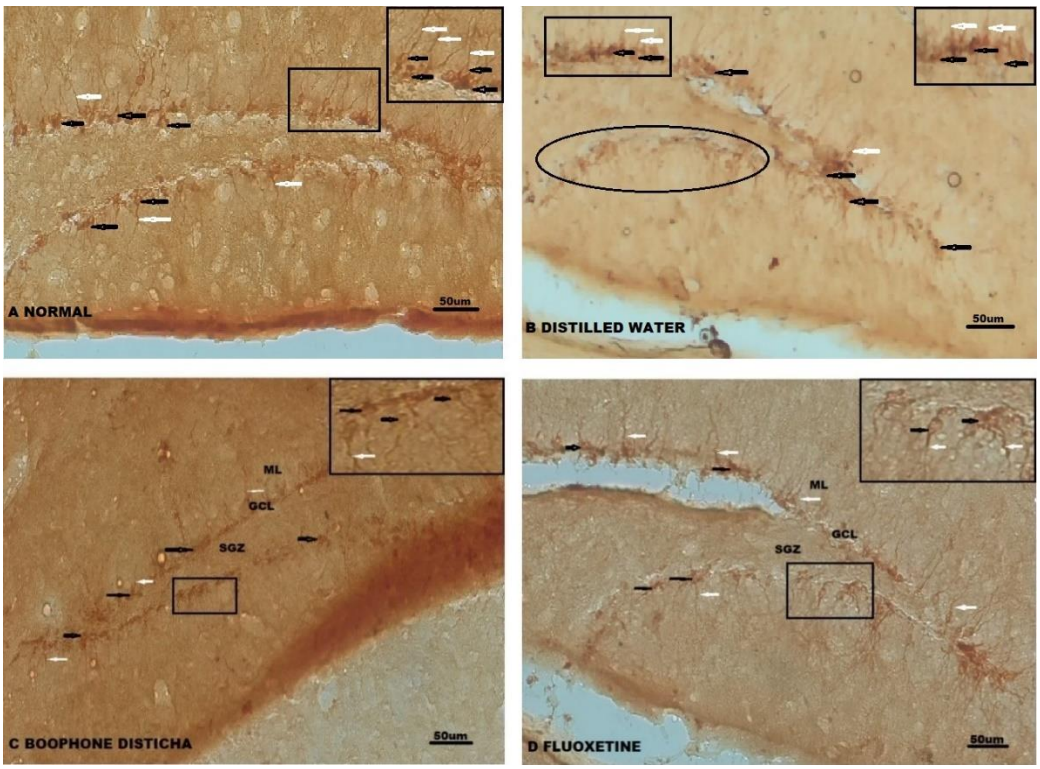

Figure 4.5: Photomicrographs showing DCX immunoreactive cells in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus of the normal (control) (A) and treated (B to D) groups.
The black arrows are showing the cell bodies of the neuroblasts and the white arrows are showing the dendritic spines. Poorly developed dendritic spines can be seen in the distilled water treated group B. The oval circle in group B shows one of the patches in the lower limb of the dentate gyrus where DXC immunoreactive cells were absent leading to low cell count in this group. The magnified areas on the top right of each micrograph represent the areas indicated by the black rectangles.

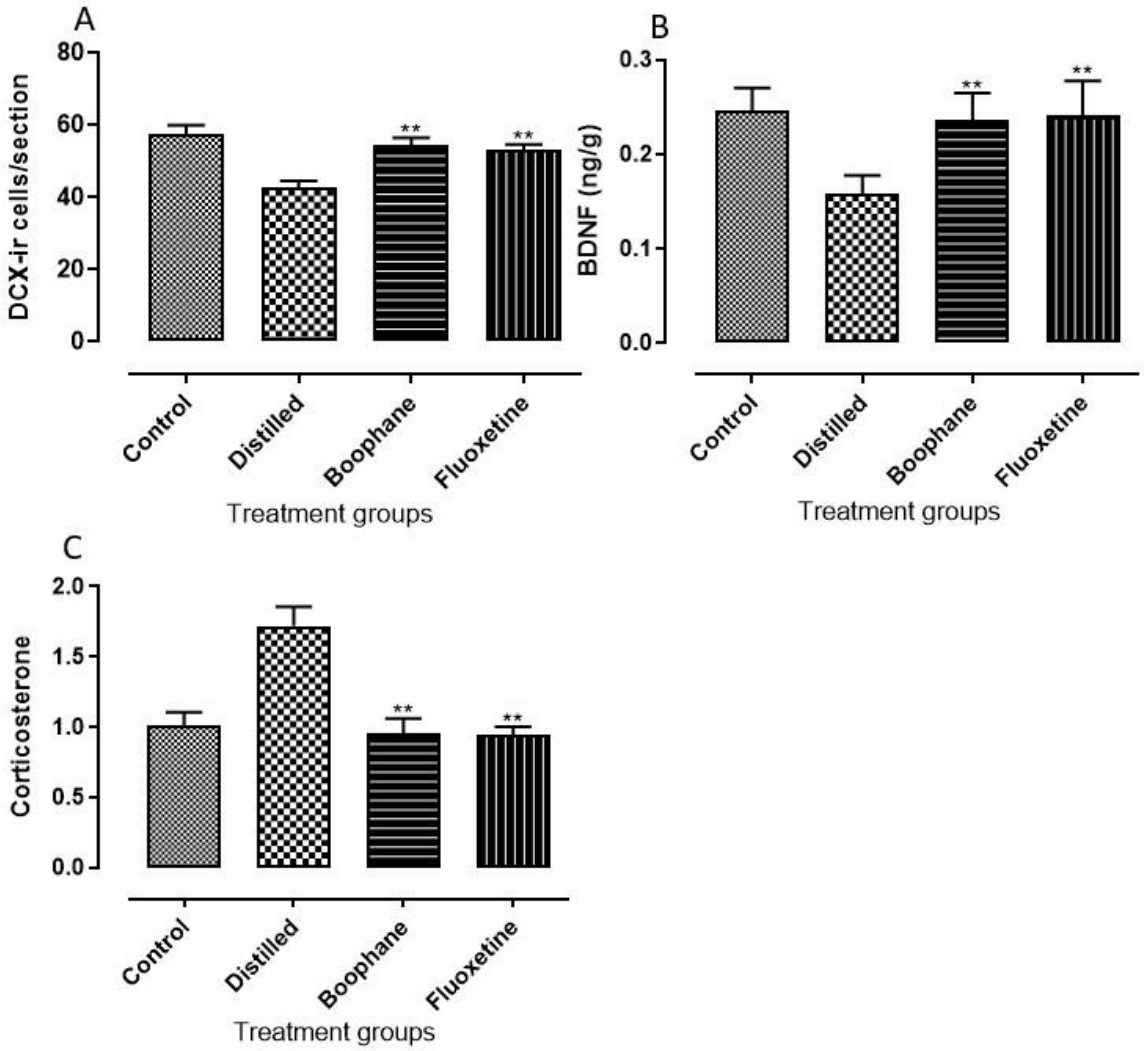

Figure 4.6: Graphic representation of the expression of DCX immunoreactive cells (DCX-ir) and biochemical analyses of brain derived neurotropic factor (BDNF) and corticosterone.
A) The expression of DCX-ir was significantly different across the groups (P=0.0005). **B)** The expression of BDNF was significantly different across the groups (P =0.0026). **C)** The expression of corticosterone was significantly different across the groups (P= 0.001). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.1.8 Brain derived neurotropic factor (BDNF)

The expression of BDNF was significantly different across the groups in ANOVA test ($F= 9.051$, $P =0.0026$, Fig. 4.6B). The expression of BDNF was significantly lower in the distilled water group compared to the control, fluoxetine and *BD* treated groups in the *post hoc* test ($P<0.05$). Furthermore, the *post hoc* test showed that fluoxetine and *BD* treatment significantly increased the expression of BDNF compared to the distilled water group ($P<0.05$). There were no significant differences when comparing the control group to both fluoxetine and *BD* treated groups ($P>0.05$).

4.1.9 Corticosterone

The tissue levels of corticosterone were significantly different across the groups ($F= 53.55$, $P= 0.001$, Fig. 4.6C). The *post hoc* test showed that corticosterone levels remained significantly high in the distilled water treated group when compared to the control group ($P<0.05$). On the contrary, treatment with either fluoxetine or *BD* significantly reduced corticosterone levels ($P<0.05$). The levels of corticosterone in the fluoxetine and *BD* treated groups were not significantly different from that of the control group ($P>0.05$).

4.2 CHRONIC RESTRAINT

4.2.1Chronic stress induction phase

4.2.1.1 Food consumption and weight changes during chronic restraint

During the induction phase of chronic restraint stress model, the experimental animals were restrained for 6 hours as described in section 3.5. The control animals were not restrained and had access to food and water *ad- libitum*. The paired sample T test was used to compare the weight and food consumption changes between the control and the restrained animals during the days 7, 14, 21 and 28 of the chronic restraint stress period to determine the effect of the restraint in these two parameters. The t-test showed that the weight gain of the chronic restraint animals was not significantly different from that of the control animals by day 7 of chronic restraint (P=0.196). However, the weight gain was significantly lower in the chronic restraint animals after days 14 (P=0.007), 21 (P=0.017) and 28 (P=0.004; Table 4.3).

Table 4.3: Showing weight changes during the 28 days chronic restraint induction period

Experimental group	Parameter	Chronic restraint day 7 weight gain	Chronic restraint day 14 weight gain	Chronic restraint day 21weight gain	Chronic restraint day 28 weight gain
Control group	**Initial mass (g)**	20±0.6324			
	Mass change (g)	21.75±0.0680	23±1.1175	23.875±1.2578	24.5±1.3524
	Body mass gain (g)	1.75	3	3.875	4.5
	% Change in body mass	8.75	15	19.35	22.5
Chronic stress group	**Initial mass (g)**	20.35±0.6324			
	Mass change (g)	21.64±1.5667	21.38±4.26711	21.85±1.9752	22.02±1.9069
	Body mass gain (g)	1.29	1.03	1.5	1.67
	% Change in body mass	7.5	3.69	5.53	6.16
P value: % change in initial body mass		0.196	0.007	0.017	0.004

The average food consumption during the restraint period is shown in Fig. 4.7 and Table 4.4 below as recorded in days 7, 14, 21 and 28. The paired t-test was also conducted to compare the changes in average food consumption between the normal and the restrained animals during the 28 days of the chronic restraint period. The t-test showed that the average food consumption

was significantly reduced in stressed animals compared to the control animals in day 7 of the restraint period (P=0.033). On the contrary, the food consumption was not significantly different between the control and stressed animals in days 14 (P=0.459) and 21 (P=0.433) of the restraint period. By day 28, the food consumption was significantly reduced in stressed animals compared to the control animals (P=0.041).

Table 4.4: Showing changes in food consumption during the induction of the chronic restraint stress model.

Experimental group	Food consumption (g) day 7	Food consumption (g) day 14	Food consumption (g) day 21	Food consumption (g) day 28
Control group	19.5±1.8518	31±3.7729	28.7±2.7439	25.3±3.1245
Chronic stress group	18.2±1.5667	29.5±4.2711	29.2±1.9752	20.7±1.9069
P value	0.033	0.459	0.433	0.041

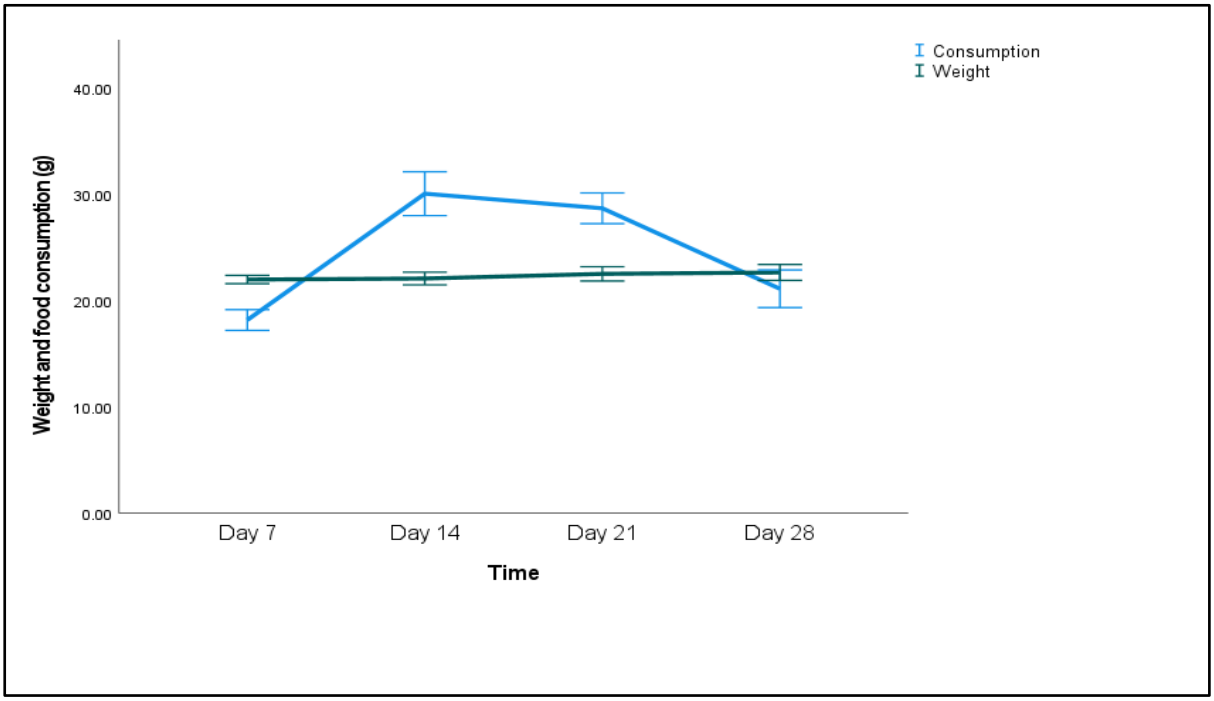

Figure 4.7: Summary of changes in food consumption and weigh changes in the intervals of days 7, 14, 21, and 28 during chronic restraint Stress induction period.

The t-test showed that the average food consumption was significantly reduced in stressed animals compared to the control animals (P=0.033). On the contrary, in days 14 (P=0.459) and 21 (P=0.433), the food consumption was not significantly different between the control and stressed animals. By day 28, the food consumption was significantly reduced in stressed animals compared to control animals (P=0.041).

4.2.2 Animal weight changes after the treatment period

Table 4.5: Body masses (g)

Parameter	Treatments				
	Control	**Stress + Distilled water**	**Stress + BD**	**Stress + Fluoxetine**	**P value**
Initial body mass (g)	20±0.00	20±0.00	21±0.00	21±0.8165	0.003
Final body mass (g)	25.37±1.2500	23±0.9128	23.62±0.5000	24.12±1.2500	0.051
Body mass gain (g)	5.37±1.2583	3±0.9574	2.62±0.8539	3.12±1.2500	0.036
% Change in body mass	26.85	15	12.47	14.85	0.015

4.2.3 Elevated plus maze

4.2.3.1 Elevated plus maze day 7 test during the treatment period

After the chronic restraint test, animals underwent 21 days of treatment. During the treatment period, behavioural tests were conducted in days 7, 14 and 21 using elevated plus maze, light dark box, and open field test.

The time spent in closed arms of the elevated plus maze was significantly different across the groups in the ANOVA test (F= 3.775; P=0.041; Fig. 4.8). However, the Bonferroni's post hoc test showed a p value of 0.075 when comparing the time spent in closed arms for distilled water and fluoxetine, with fluoxetine recording low time in closed arms (Fig. 4.8A). The time spent in the closed arms by BD treated and control animals was not significantly different (P=1.00).

The time spent in the centre was significantly different across the groups (F=3.559; P=0.055). Bonferroni's post hoc test showed that the fluoxetine group had significantly higher time in the centre when compared to the distilled water treated animals (P=0.055; Fig. 4.8B), while the BD treated, and normal animals had comparable time spent in the centre (P=1.00). The time spent in the open arms was not significantly different across the groups (F=1.910; P=0.182; Fig. 4.8C). Similarly, no significant differences were detected across the groups in the number

of open arm entries (F= 1.910; P=0.182; Fig 4.8D), number of closed arm entries (F=1.529; P=0.257; Fig 4.8E), and the number of head dips (F= 3.257; P=0.060; Fig 4.8F).

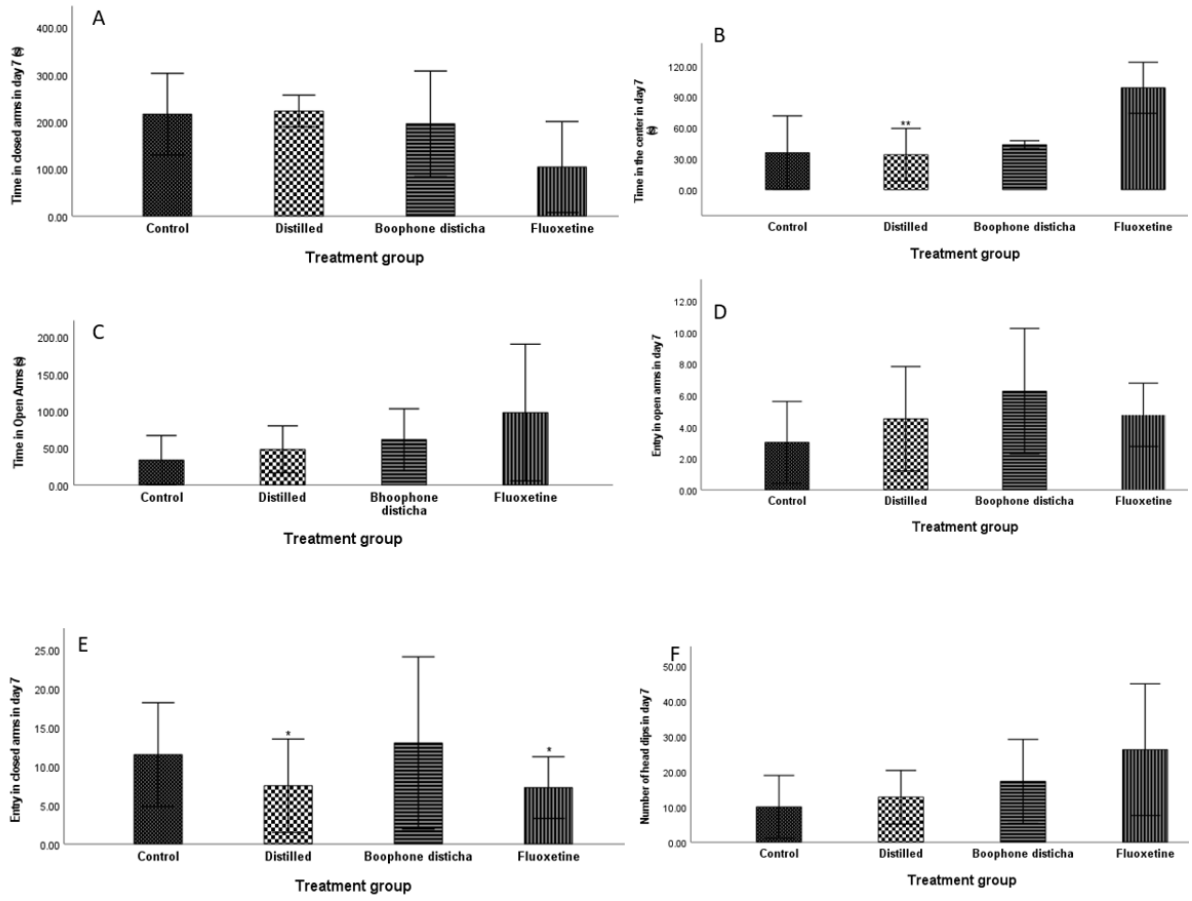

Figure 4.8: Graphic representation of the elevated plus maze results in day seven of the treatment period.

A) Fluoxetine had marginally low time in closed arms. B) Fluoxetine group had significantly higher time in the centre. C) The time spent in the open arms was not significantly different across the groups (P=0.182). D) No significant differences were detected across the groups in the number of open arm entries (P=0.182), E) number of closed arm entries (P=0.257), and F) the number of head dips (P=0.060). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.3.2 Elevated plus maze day 14

There were no statistically significant differences in the time spent in the centre (P= 0.517; Fig. 4.9 A), closed arms (P= 0.185; Fig. 4.9B) and the number of closed arm entries (P=1.00; Fig. 4.9 C).

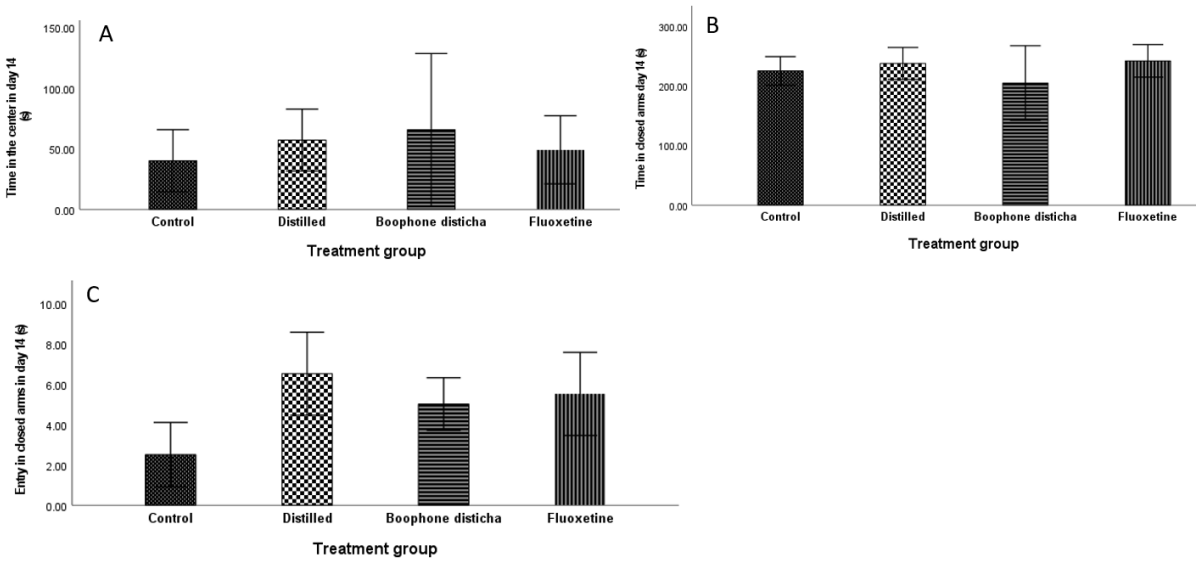

Figure 4.9: Graphic representation of the none-significant elevated plus maze results in the 14th day.
A) No statistically significant differences in the time spent in the centre (P= 0.517), B) closed arms (P= 0.185), C) number of closed arm entries (P=1.00). Error bars correspond to the SEM.

There were statistically significant differences in the time spent in the open arms across the groups (F= 58.814; P= 0.0001; Fig. 4.10A). The Bonferroni's post hoc test showed that the distilled water group had significantly low time in open arms when compared to the control (P= 0.0001), BD (P= 0.0001), and fluoxetine (P= 0.003) treated groups. Furthermore, the time spent in the open arms for control and BD treated animals was not significantly different (P= 0.313). Additionally, the time spent in open arms was significantly lower in fluoxetine treated group compared to BD treated (P=0.001), and control groups (P=0.0001).

The number of closed arm entries was significantly different across the groups (F= 9.267; P=0.002; Fig. 4.10B). Bonferroni's post hoc test showed that the number of closed arm entries were significantly higher in the distilled (P= 0.002), BD treated (P=0.049), and fluoxetine (P=0.015) treated animals when compared to the control group.

The number of head dips was also significantly different across the groups in the ANOVA test (F= 35.952; P= 0.0001; Fig 4.10C). The Bonferroni's post hoc test showed that the number of

head dips was significantly lower in distilled water, BD and fluoxetine groups when compared to the control group, all with a P value of 0.0001.

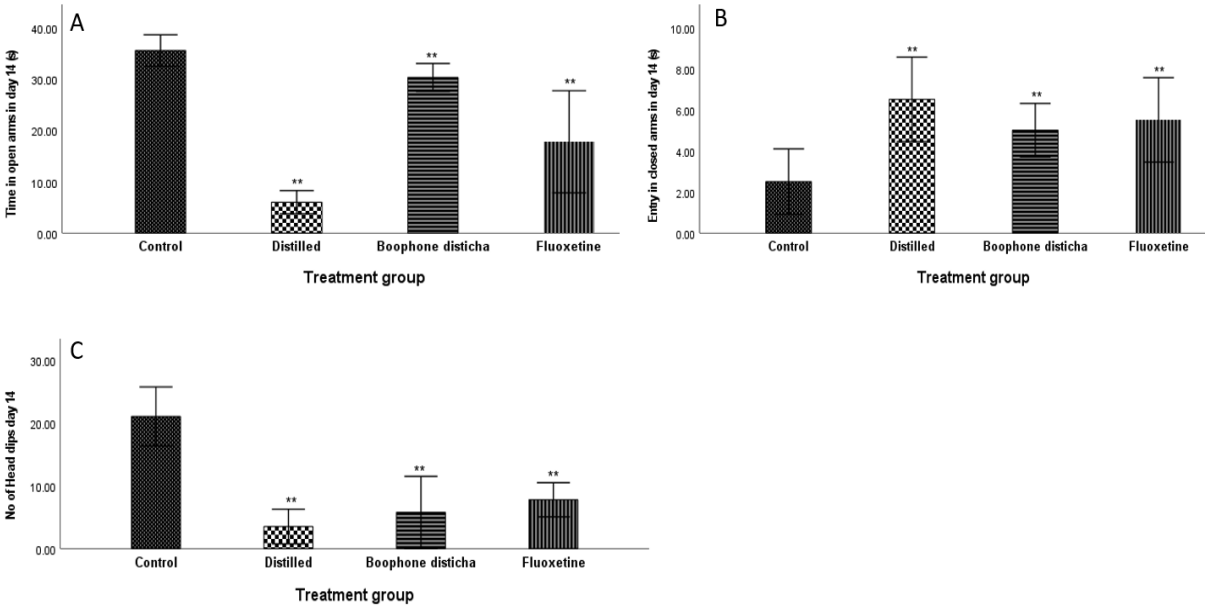

Figure 4.10: Graphic representation of the significant differences in the elevated plus maze results in the 14th day of treatment.
A) Significantly low time in open arms when compared to the control (P= 0.0001), BD (P= 0.0001), and fluoxetine (P= 0.003) treated groups. B) The number of closed arm entries was significantly higher in the distilled (P= 0.002), BD (P=0.049), and fluoxetine (P=0.015) treated animals. C) The number of head dips was significantly lower in distilled water, BD and fluoxetine groups when compared to the control group, all with a P value of 0.0001. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4. 2.3.3 Elevated plus maze day 21

There were no statistically significant differences in the time spent in the center of the maize across the groups (F=4.437; P=0.057; Fig. 4.11A). The time spent in open arms of the maize was significantly different across the groups in the ANOVA test (F= 4.311; P= 0.028; Fig. 4.11B). The Bonferroni's post hoc test showed that the time spent in open arms of the maze was significantly lower in the fluoxetine group (P=0.051). On the contrary, the time spent in the open arms of the maze was not significantly different when comparing distilled water (P= 0.139) and BD (P=1.00) groups to the control group.

There were statistically significant differences in the time spent in closed arms across the groups in the ANOVA test (F= 16.726; P=0.0001; Fig. 4.11C). The Bonferroni's post hoc test showed that the time spent in closed arms was higher in the distilled water group compared to the control group (P=0.019). Furthermore, the time spent in the closed arms was significantly lower in the BD treated group compared to the control group (P=0.046). The time in the open arms for the fluoxetine group was not significantly different from that of the control group (P=0.922). In addition, the time spent in closed arms was significantly higher in the fluoxetine treated group compared to the BD treated group (P=0.003).

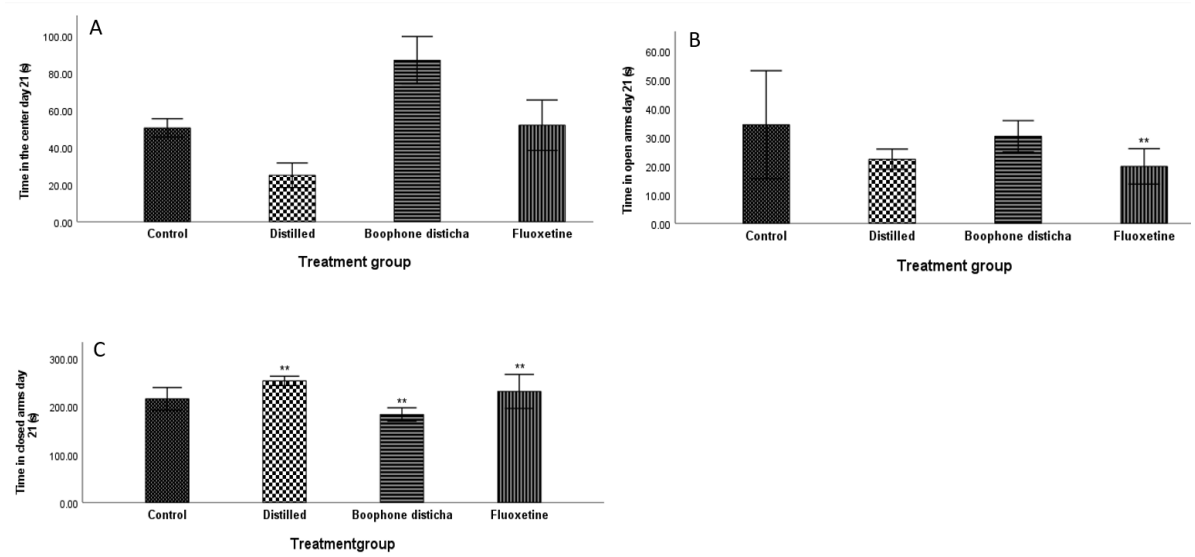

Figure 4.11: Graphic representation of the significant differences in the elevated plus maze results in the 21st day of treatment.
A) No statistically significant differences in the time spent in the centre of the maize across the groups (F=4.437; P=0.057). B) The Bonferroni's post hoc test showed that the time spent in open arms of the maze was significantly lower in the fluoxetine group (P=0.051). C) The time spent in closed arms was higher in the distilled water group compared to the control group (P=0.019). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

The number of entries in open arms were significantly different across the groups in the ANOVA test (F=8.800; P=0.002; Fig. 4.12A). The Bonferroni's post hoc test showed that the number of open arm entries was significantly higher in the distilled water group compared to the control group (P=0.013). The post hoc comparisons of the number of open arm entries for fluoxetine, and BD to the control and the treatment groups themselves did not reveal any significant differences (P=1.000).

There were no statistically significant differences in the number of entries in closed arms across the groups (F=1.448; P= 0.278; Fig. 4.12B). However, the number of closed arm entries was slightly lower in the *BD* treated group compared to distilled and fluoxetine treated group. This slightly low number of closed arm entries of the *BD* group was comparable to that of the control group.

The number of head dips was significantly different across the groups in the ANOVA test (F=21.504; P= 0.0001). Bonferroni's post hoc test showed that the number of head dips was significantly lower in distilled water (P= 0.004), BD (P= 0.001), and fluoxetine (P= 0.001) treated groups compared to the control group (Fig. 4.12C). Further analyses of the Bonferroni's post hoc test revealed no differences amongst the distilled water, *BD* and fluoxetine treatment groups.

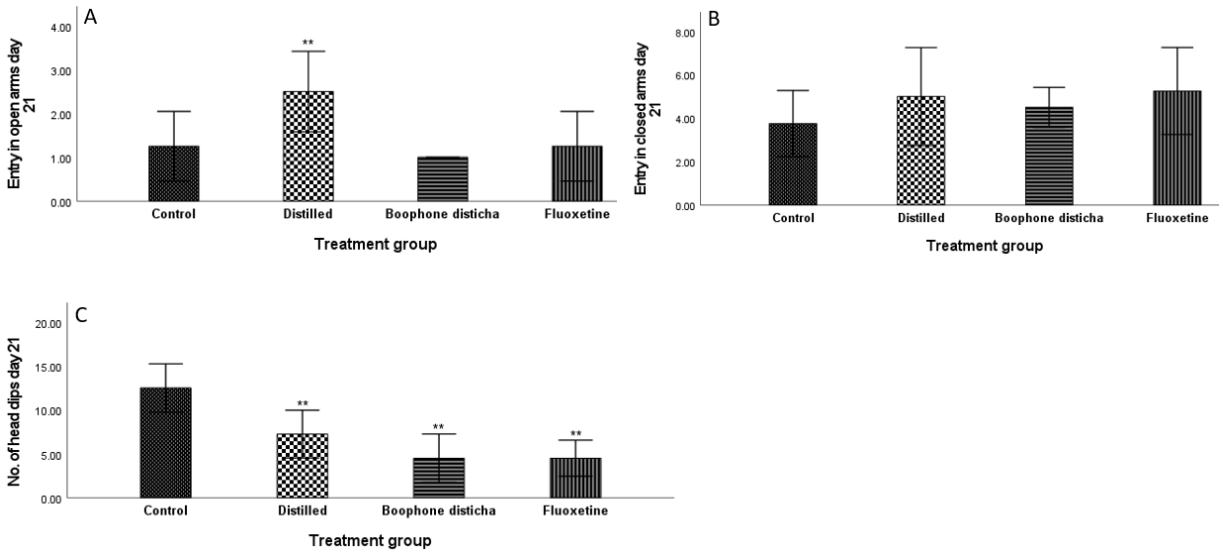

Figure 4.12: Graphic representation of the significant differences in the elevated plus maze results in the 21st day of treatment.
A) The Bonferroni's post hoc test showed that the number of open arm entries was significantly higher in the distilled water group compared to the control group (P=0.013). B) No statistically significant differences in the number of entries in closed arms across the groups (F=1.448; P= 0.278). C) The number of head dips was significantly lower in distilled water (P= 0.004), BD (P= 0.001), and fluoxetine (P= 0.001) treated groups compared to the control group. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.4 Light dark box

4.2.4.1 Time spent in light and dark box during treatment period

There were statistically significant differences across the groups in the time spent in the light box as demonstrated by the ANOVA test (P= 0.0001). Interestingly, the Bonferroni post hoc test showed that the control animals had significantly low time in the white box compared to all treatment groups, all with a P value of 0.0001 (Fig. 4.13A). Furthermore, the time spent in the white box for BD treated group was not significantly different from that of the distilled water treated group (P=0.158), while the time spent in the light box was significantly higher in the fluoxetine treated group when compared to all other groups, all with a P value of 0.0001. As the time spent in the dark box was measured in the same time period as the time in the white

box, the results of the time spent in the dark box are a direct opposite of the time spent in the white box (Fig.4.13B).

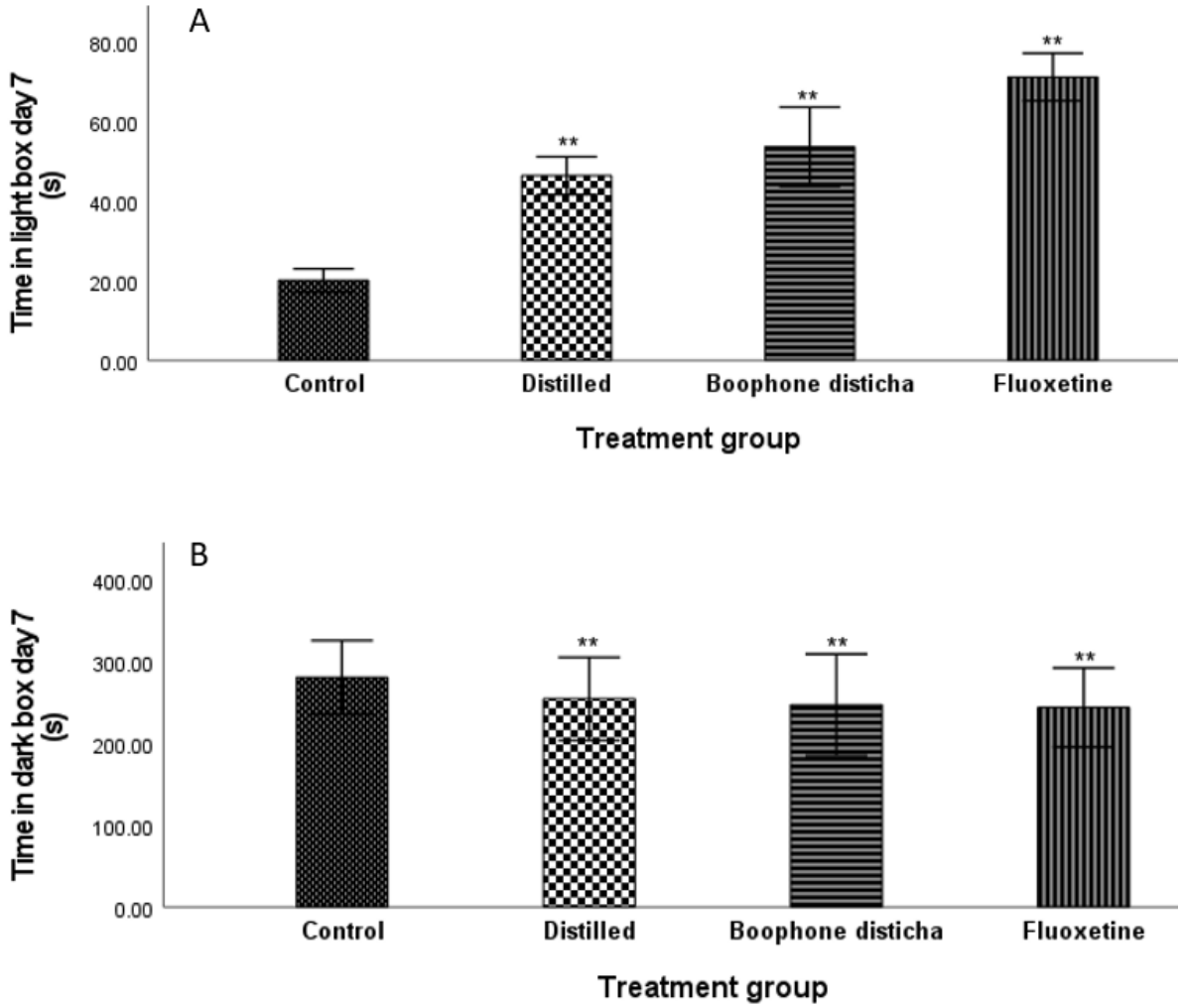

Figure 4.13: Graphic representation of the time spent in light and dark box in day 7 of the behavioral tests.
A) Bonferroni post hoc test showed that the control animals had significantly low time in the white box compared to all treatment groups, all with a P value of 0.0001. B) As the time spent in the dark box was measured in the same time period as the time in the white box, the results of the time spent in the dark box are a direct opposite of the time spent in the white box. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

In day 14 of the treatment period, there were statistically significant differences in the time spent in the light box across the groups in the ANOVA test (P= 0.0001). The Bonferroni's post hoc test showed that both the distilled water and BD treated groups had significantly low time

in the light box compared to the control group, both with the p value of 0.0001 (Fig. 4.13A). On the contrary, the fluoxetine treated group had significantly high time in the light box compared to the control, distilled water and BD treated groups, all with a p value of 0.0001. The time spent in the light box for BD and distilled water groups were not significantly different (P= 1.00). The time in the dark box was a direct contrast of the time in the light box as animals were either in the light or dark box (Fig. 4.13B).

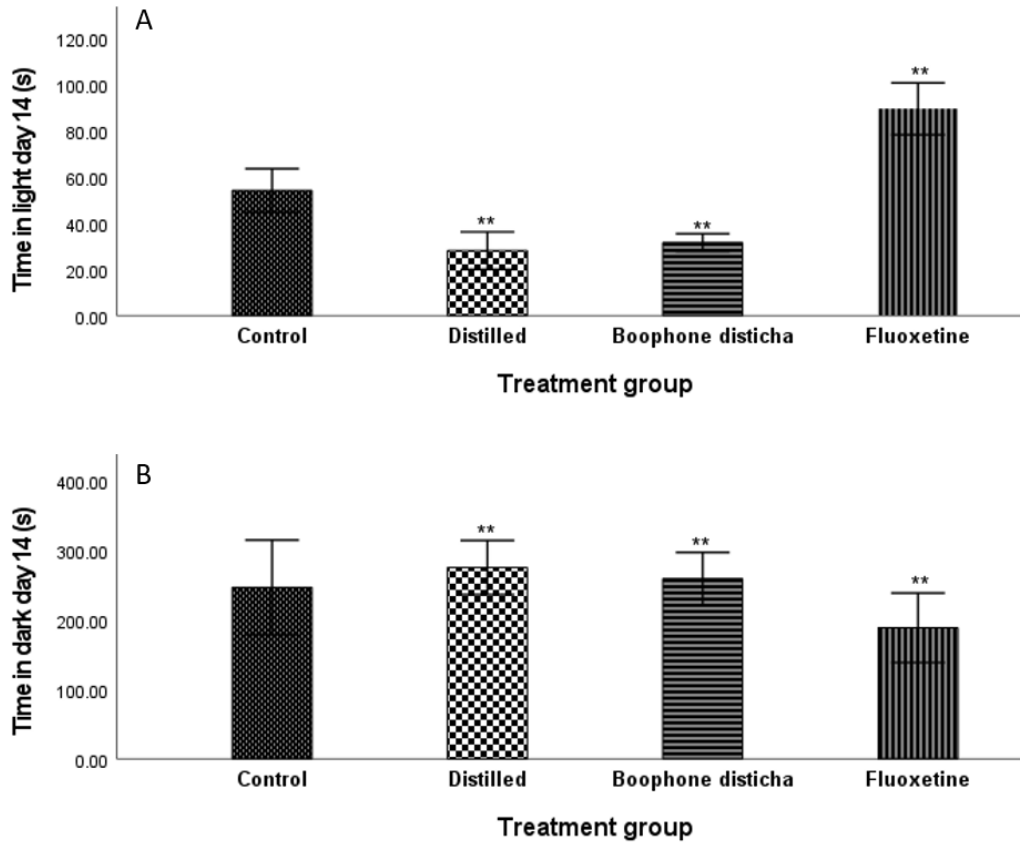

Figure 4.13: Graphic representation of the time spent in light and dark box in day 14 of the behavioral tests.
A) The Bonferroni's post hoc test showed that both the distilled water and BD treated groups had significantly low time in the light box compared to the control group, both with the p value of 0.0001. B) The time in the dark box was a direct contrast of the time in the light box as animals were either in the light or dark box. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

In day 21 of the treatment period, there were statistically significant differences in the time spent in the light box across the groups (F=7.020; P=0.006; Fig. 4.14A). The Bonferroni's post hoc test showed that the time spent in the light box was significantly lower in the fluoxetine treated group compared to the control group (P=0.032). Additionally, the time spent in the light box was significantly higher in the BD treated group compared to the fluoxetine treated group (P=0.006). On the contrary, the time spent in the light box for distilled water and BD treated groups was not significantly different from the control group, both with a p value of 1.00. The time spent in the dark box was a direct contrast of the time spent in the light box (Fig. 4.14B).

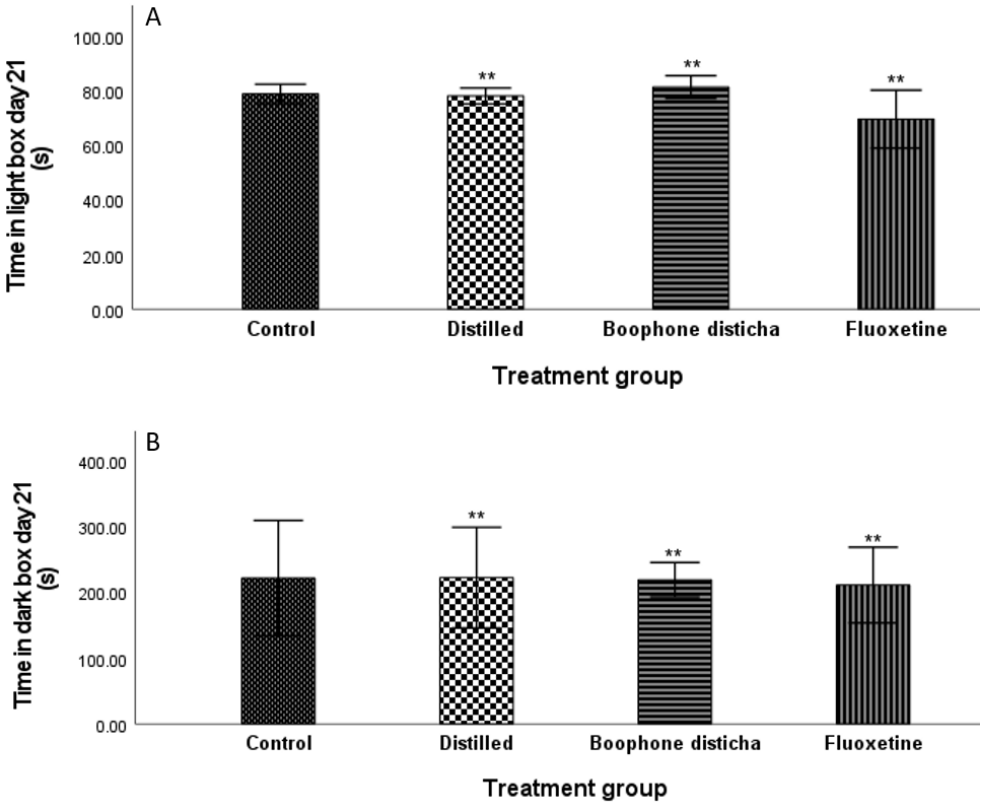

Figure 14: Graphic representation of the time spent in light and dark box in day 21 of the behavioral tests.
A) The Bonferroni's post hoc test showed that the time spent in the light box was significantly lower in the fluoxetine treated group compared to the control group (P=0.032). B) The time spent in the dark box was a direct contrast of the time spent in the light box as the animals were either in the light or a dark box. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.5 Open field test

4.2.5.1 Time spent in the center of the field

There were statistically significant differences in the time spent in the center of the field across the groups in day 7 of the treatment period as shown by the ANOVA test (F=3.577; P=0.047). However, the Bonferroni's post hoc test showed a p value of 0.074 when comparing the time spent in the center of the field for the distilled water and BD treated groups, suggesting a marginal difference, whereby the BD treated group had higher time spent in the center of the field than the distilled water treated group. The comparison of distilled water (P= 1.00), BD (P=0.115) and fluoxetine (P=1.00) groups to the control group showed no statistically significant differences (Fig. 4.15A).

Similarly, in day 14 there were statistically significant differences in the time spent in the center of the field across the groups as detected by the ANOVA test (F=6.476; P= 0.01). Contrary to day 7 however, the Bonferroni's post hoc test showed that the time spent in the center of the field was significantly higher in the BD treated compared to the control (P=0.028) and fluoxetine treated (P=0.033) groups. The time spent in the center of the field was not significantly different when comparing the distilled water (P=0.291) and fluoxetine (P=1.000) treated groups to the control group (Fig. 4.15B).

Furthermore, in day 21 of the treatment period, the ANOVA test did not detect any significant differences in the time spent in the center of the filed across the groups (F=2.094; P=0.165; Fig. 4.15C).

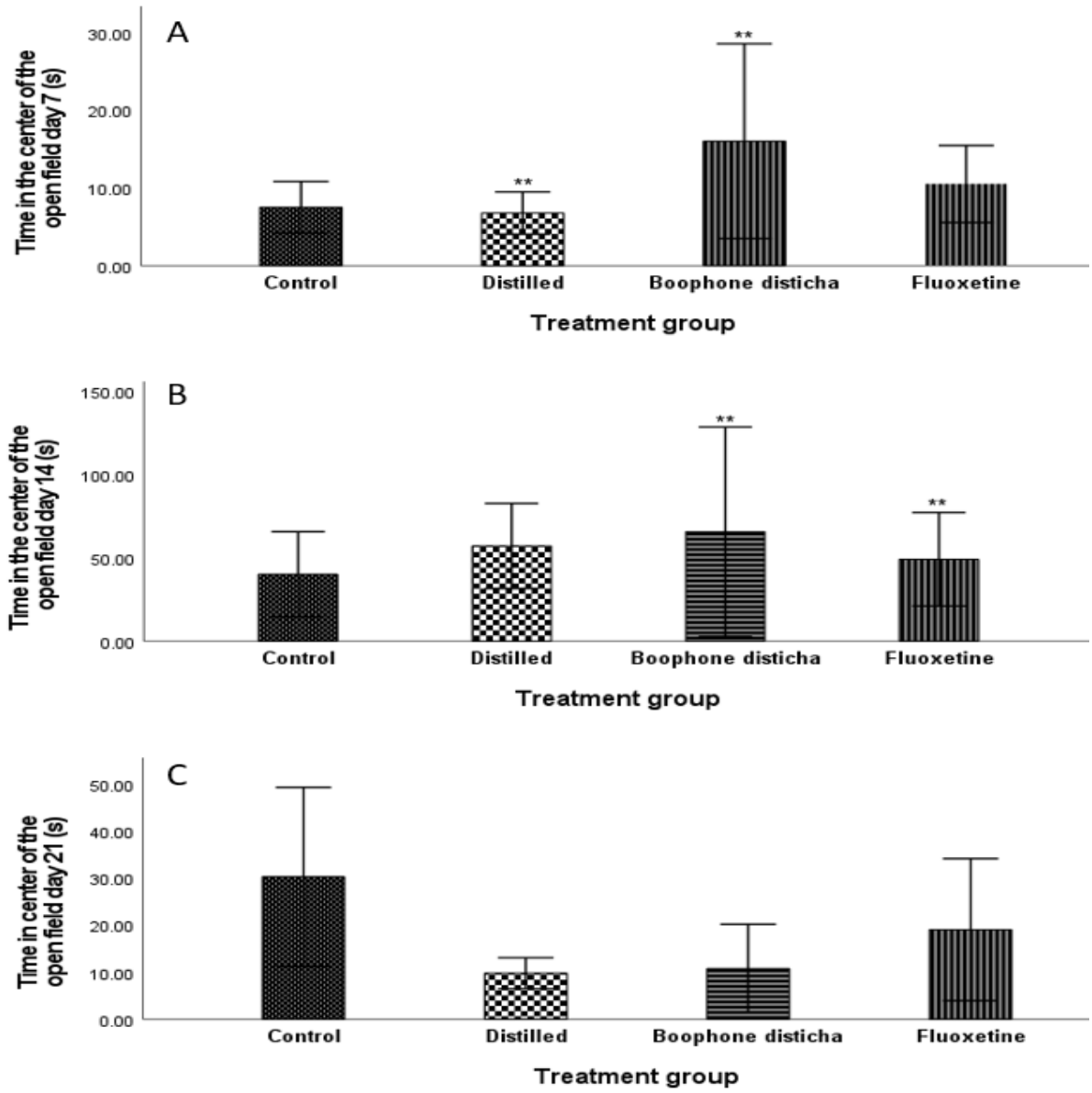

Figure 4.15: Graphic representation of the time spent in the center of the open field during behavioral tests.

A) There were statistically significant differences in the time spent in the centre of the field across the groups in day 7 of the treatment period as shown by the ANOVA test (F=3.577; P=0.047). B) The Bonferroni's post hoc test showed that the time spent in the centre of the field was significantly higher in the BD treated compared to the control (P=0.028) and fluoxetine treated (P=0.033) groups. C) In day 21 of the treatment period, the ANOVA test did not detect any significant differences in the time spent in the centre of the filed across the groups (F=2.094; P=0.165). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.5.2 Time spent on the sides of the open field

The time spent on the sides of the open field was significantly different across the groups in day 7 of the treatment period as detected by the ANOVA test (F=3.577; P=0.047). However, for a comparison between the time spent on the sides of the open field for the distilled water and the BD groups, the Bonferroni's post hoc test showed a p value of 0.074 suggesting that thought the ANOVA test showed significant differences, the significance was marginal, whereby the time spent on the sides for a distilled water group was higher than that of the BD group. Bonferroni's post hoc test further showed there were no significant differences in the time spent on the sides of the open field when comparing between the control and distilled water (P= 1.00), BD (P=0.115) and fluoxetine (P=1.00) groups (Fig. 4.16A).

Similarly, in day 14 of the treatment period, the time spent in the center of the filed was significantly different across the groups in the ANOVA test (F=3.728; P=0.045). However, the Bonferroni's post hoc test showed a p value of 0.063 when comparing the time spent on the sides of the open field by the control and BD treated groups indicating a marginal significance of high time on the sides for a BD group. The comparison of the time spent on the sides of the open field for distilled water (P=0.498) and fluoxetine (P=1.000) to the control group did not show significant differences (Fig. 4.16B).

In day 21 of the treatment period, the ANOVA test demonstrated that there were no significant differences in the time spent on the sides of the open field across the groups (F= 1.058; P=0.406; Fig. 4.16C).

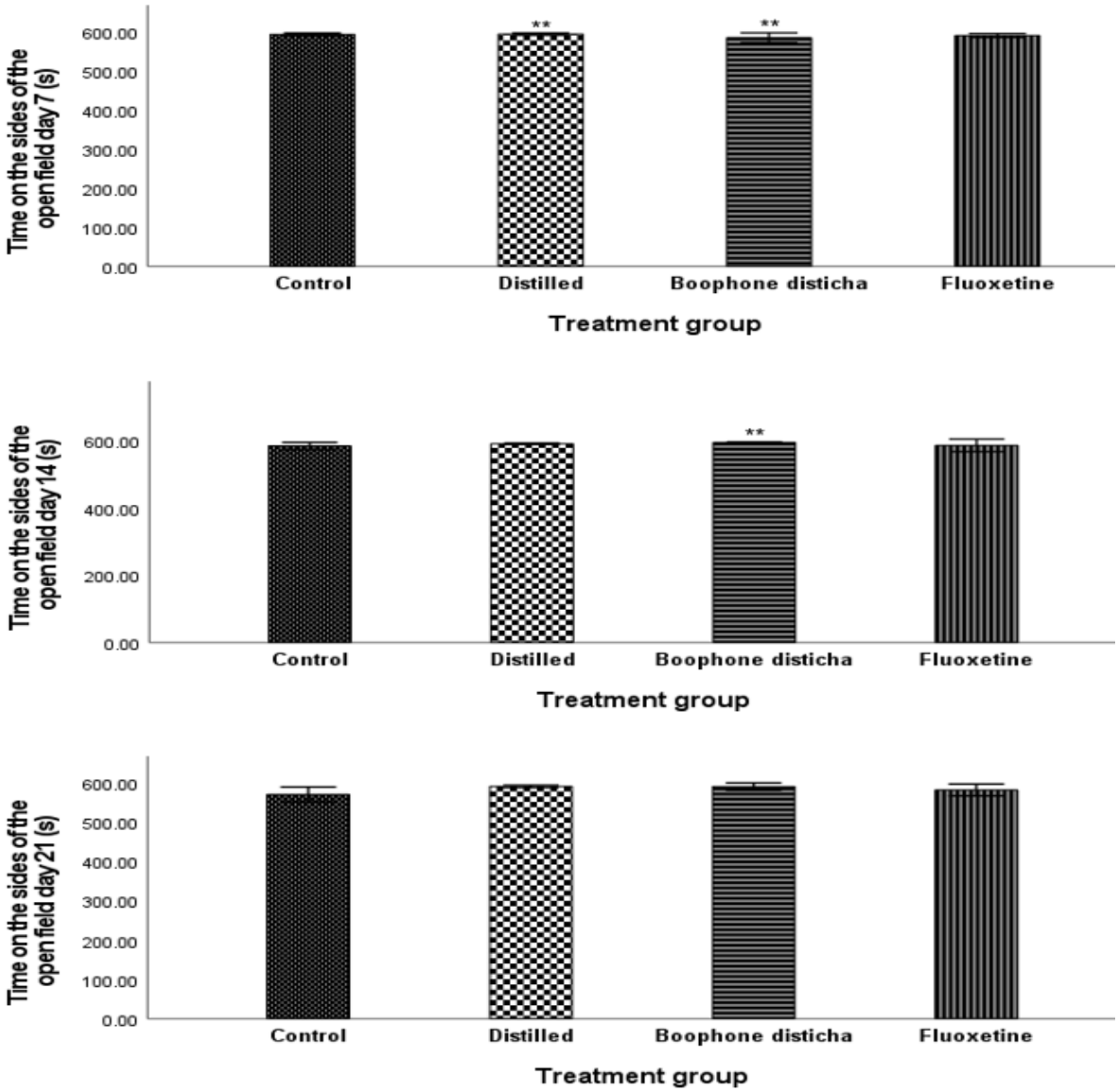

Figure 4.16: Graphic representation of the time spent on the sides of the open field during behavioral tests.

A) Bonferroni's post hoc test showed a p value of 0.074 suggesting that thought the ANOVA test showed significant differences, the significance was marginal. B) In day 14 of the treatment period, the time spent in the centre of the filed was significantly different across the groups in the ANOVA test (F=3.728; P=0.045). C) In day 21 of the treatment period, the ANOVA test demonstrated that there were no significant differences in the time spent on the sides of the open field across the groups (F= 1.058; P=0.406). Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.5.3 Frequency of rearing

The frequency of rearing was not significantly different across the groups in days 7, 14 and 21 of the treatment period. The results are detailed in Fig. 4.17 below.

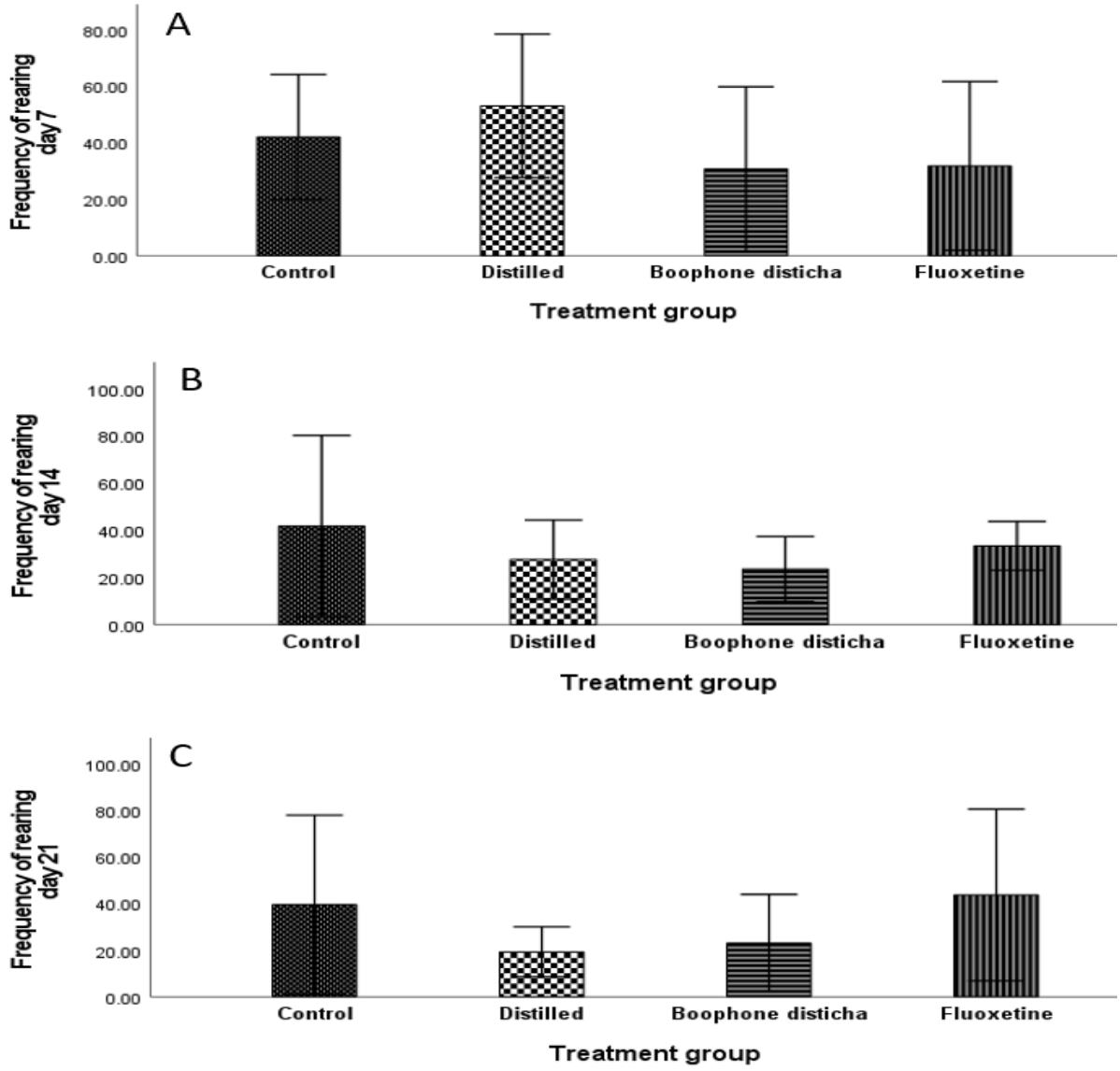

Figure 4.17: Graphic representation of the frequency of rearing in the open field during behavioral tests.

A) BD and fluoxetine treated groups recorded similar low frequency of rearing when compared to both distilled water and control groups, even though the differences were not significant. B) The ANOVA test did not detect any significant differences in the frequency of rearing across the groups in day 14 (F=0.893; P=0.478). C) Day 21, the frequency of rearing was not significantly different across the groups (F= 2.323; P=0.136). Error bars correspond to the SEM.

4.2.5.4 Frequency of line crossing

The frequency of line crossing was not statistically significant in treatment days 7, 14 and 21 as detailed in Fig. 4.18 below.

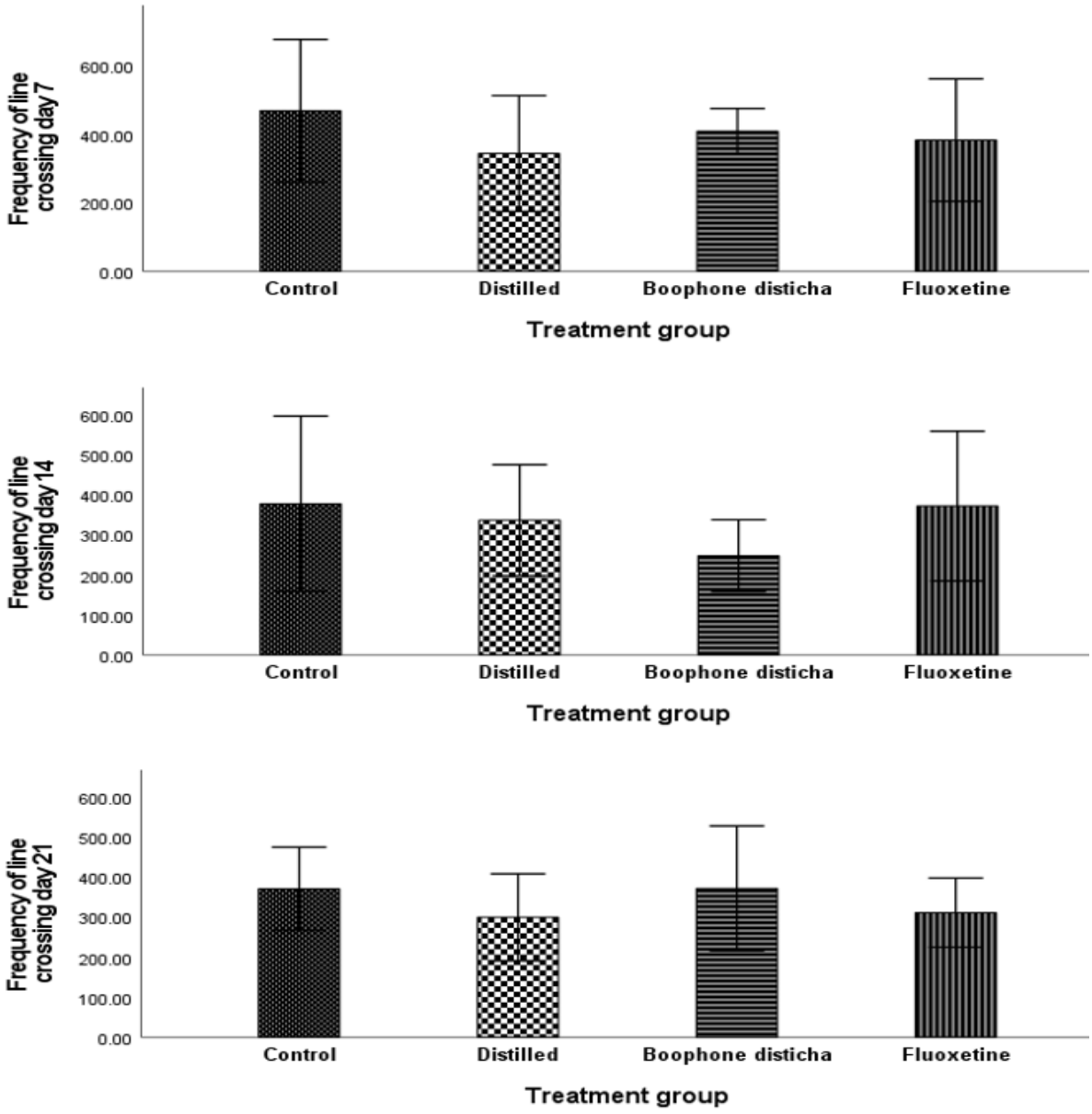

Figure 4.18: Graphic representation of the frequency of line crossing in the open field during behavioral tests.

A) The number of line crossings in day 7 of the treatment period was not significantly different across the groups as detected by the ANOVA test (F=1.029; P= 0.414). B) Similarly, the ANOVA test did not detect significant differences in line crossing in day 14 of the treatment period (F= 1.516; P=0.265). C) The same trend was also observed in day 21 of the treatment period with nonsignificant differences in the frequency of line crossing across the groups (F=0.676; P=0.585). Error bars correspond to the SEM.

4.2.6 Volume of the hippocampus

There were no statistically significant differences in the volume of the hippocampus proper (F= 1.288; P=0.323; Fig. 4.19A and B), dentate gurus (F=0.962; P=0.462; Fig. 4.20 A and B) and the granule cell layer (F=0.675; P=0.594; Fig. 4.21 A and B) across the groups. Even though the differences were not significant, a slight volumetric reduction of the hippocampus proper, was observed across the treatment groups compared to the control group (Fig. 4.19B). Interestingly, the volume of the dentate gyrus in the distilled water treated group was slightly higher than that of the BD and fluoxetine treated animals (Fig. 4.20B). Volumetric analyses of the granule cell layer of the dentate gurus showed a different trend whereby the BD treated group recorded slightly lower volume compared to control, distilled water treated and fluoxetine groups (Fig. 4.21B).

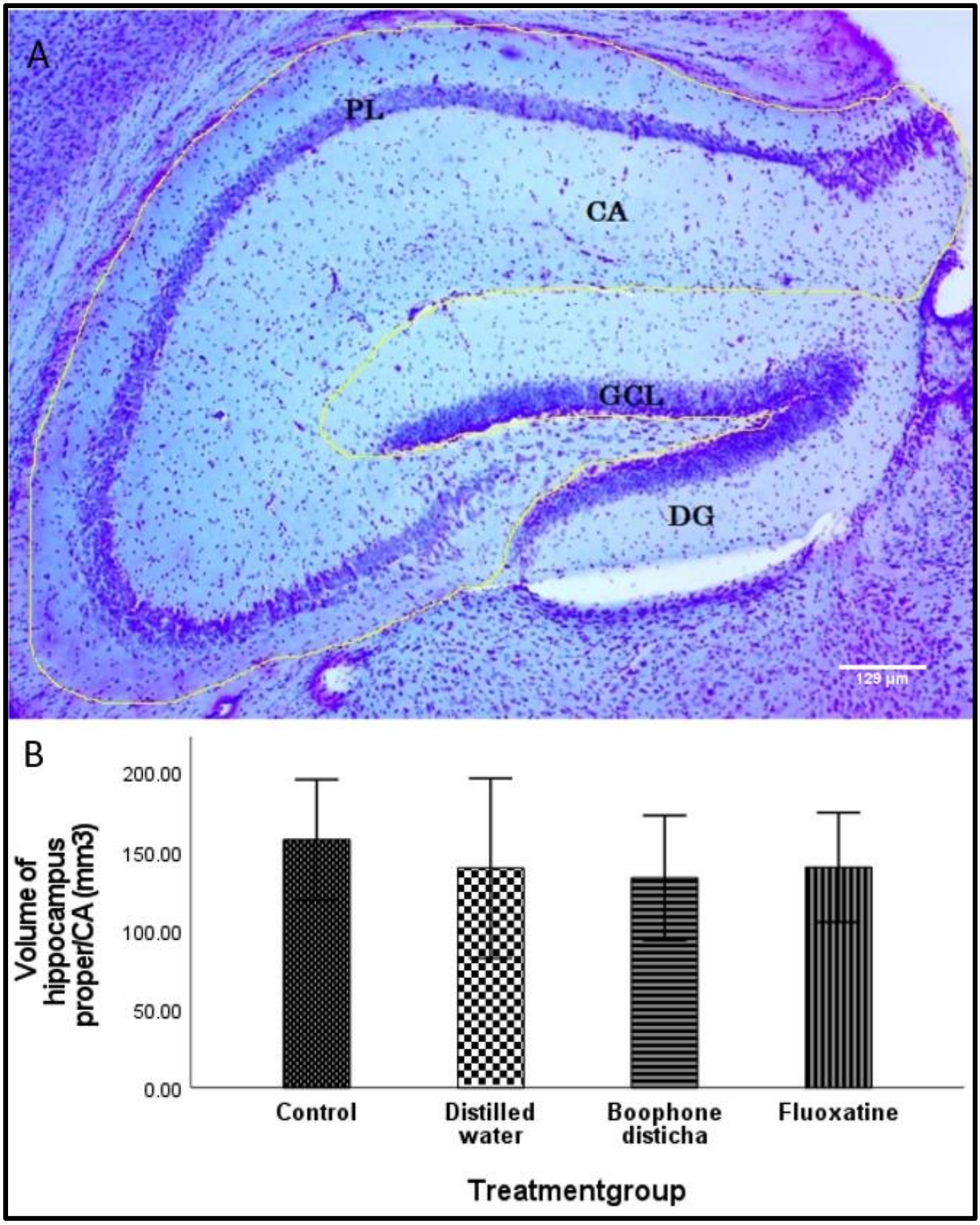

Figure 4.19: Graphic representation of the volume of the hippocampus proper/CA.
A) Photomicrograph showing Image J hand tool highlighted hippocampus proper for computation of the volume. B) Bar graph indication nonsignificant differences in the volumes across the groups (F= 1.288; P=0.323). Error bars correspond to the SEM. CA: Cornu Amonis, PL: Pyramidal cell layer, DG: Dentate gyrus, GCL: Granule cell layer. Error bars correspond to the SEM.

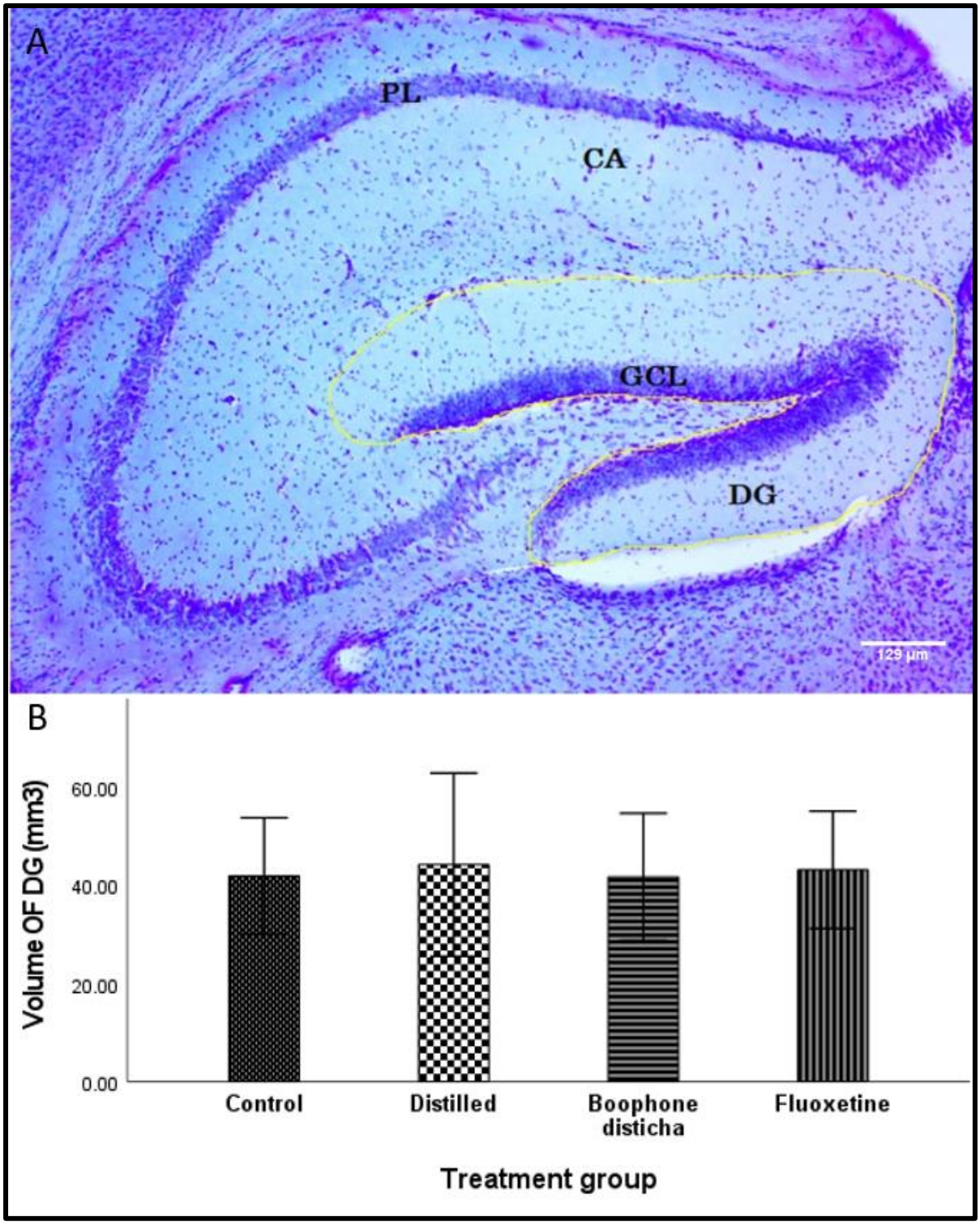

Figure 4.20: Graphic representation of the volume of the dentate gyrus of the hippocampus. A) Photomicrograph showing Image J hand tool highlighted dentate gyrus computation of the volume. B) Bar graph indicating nonsignificant differences in the volumes of the pyramidal layer across the groups (F=0.962; P=0.462). Error bars correspond to the SEM. **CA**: Cornu Amonis, **PL**: Pyramidal cell layer, **DG**: Dentate gyrus, **GCL**: Granule cell layer. Error bars correspond to the SEM.

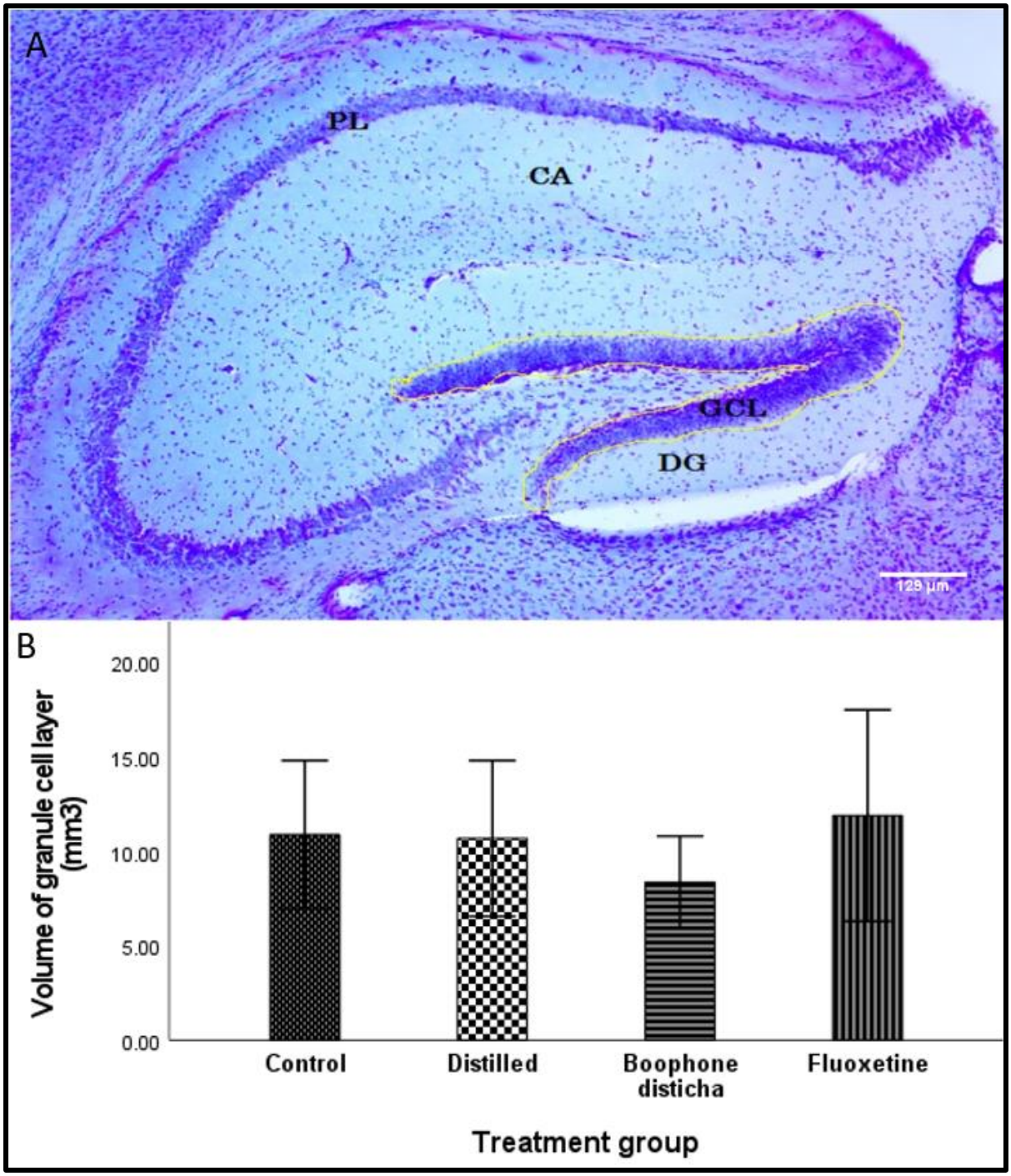

Figure 4.21: Graphic representation of the volume of the granule cell layer of the hippocampus.
A) Photomicrograph showing Image J hand tool highlighted dentate gyrus computation of the volume. B) Bar graph indicating nonsignificant differences in the volumes of the pyramidal layer across the groups (F=0.675; P=0.594). Error bars correspond to the SEM. **CA**: Cornu Amonis, **PL**: Pyramidal cell layer, **DG**: Dentate gyrus, **GCL**: Granule cell layer.

4.2.7 BDNF and Corticosterone

In the current study, tissue levels of BDNF and corticosterone were analyzed in homogenized brain tissue using the ELSA kit. There were statistically significant differences in the brain tissue levels of BDNF as shown by ANOVA test (F= 4.962; P=0.0182). The Bonferroni's post hos test showed that the distilled water treated animals had significantly low levels of BDNF compared to the control group (P= 0.017). The comparison of BD (P=0.245) and Fluoxetine (p=0.130) indicated that there were no significant differences between these groups (Fig. 4.22A). Interestingly, the ANOVA test did not detect any significant differences in corticosterone levels across the groups (F=0.966; P=0.433). However, both distilled water and fluoxetine showed a slightly high levels of corticosterone compared to both the control and BD groups (Fig 4.22B).

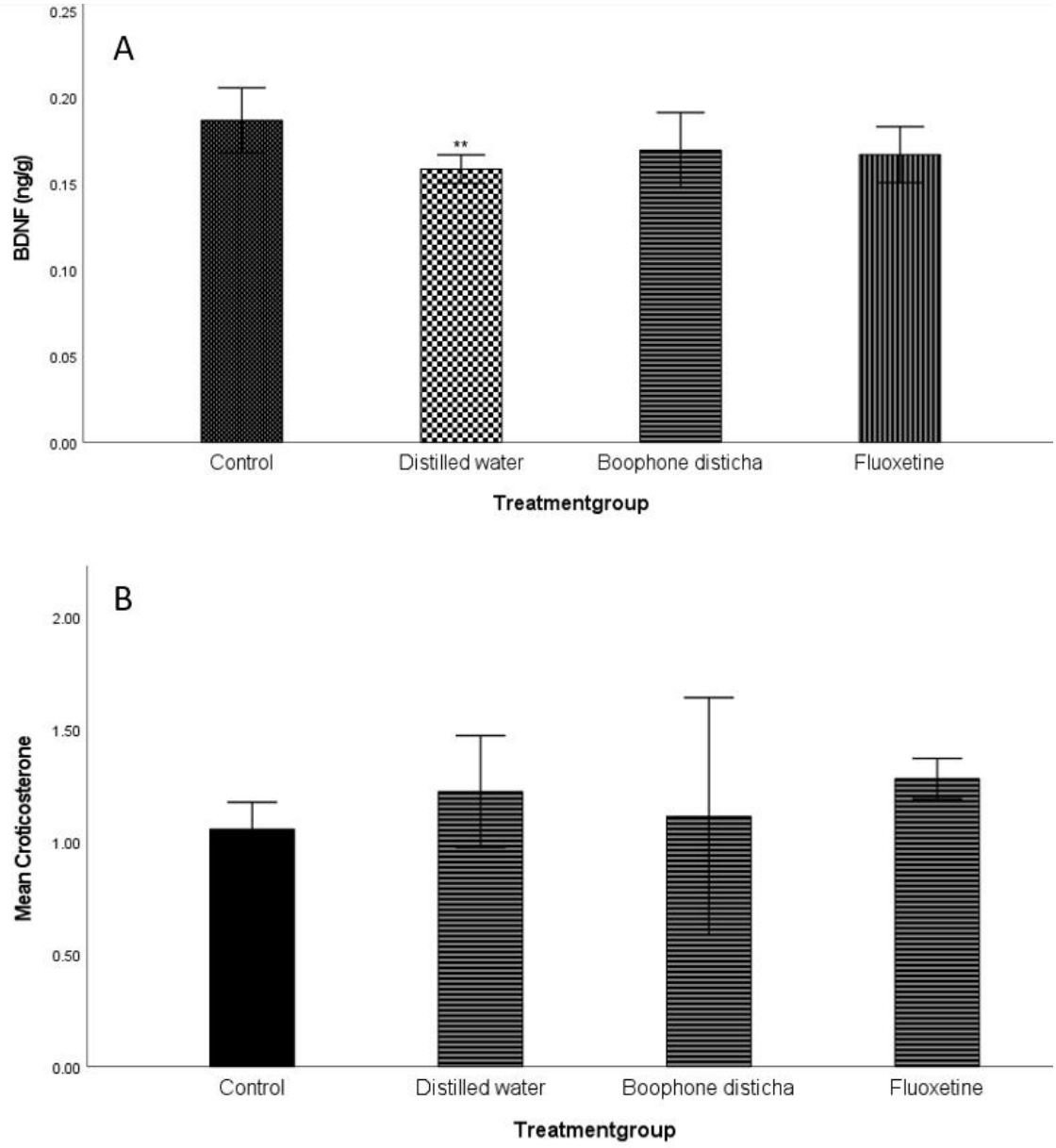

Figure 4.22: Graphic representation of the brain levels of BDNF and corticosterone.
A) Bar graph showing significantly low levels of BDNF in distilled water treated group. B) Bar graph show comparable levels of brain corticosterone across all the groups. Error bars correspond to the SEM, ** indicates significant changes due to treatments.

4.2.8 DCX expression

DCXir cells could not be analysed in the chronic restraint stress model. The supplier of the antibodies terminated the production/supplier of the rabbit polyclonal antibodies (AB18723, Abcam, UK) which were successfully used in the forced swimming stress model. Several attempts to stain with the monoclonal antibodies (AB135349, mouse monoclonal, Abcam, UK) could not yield positive results. Due to time frames of the current study, eventually a decision was made to addendum this aspect of the study. It was considered that this decision will not negatively affect the outcome of the study as the trend was established in the forced swimming stress model and inferences could be made based on the results of the biochemical analyses of BDNF and corticosterone. Below we present the pictures of the nonspecific stain obtained from the failed antibodies for illustrative purposes (Fig. 4.23).

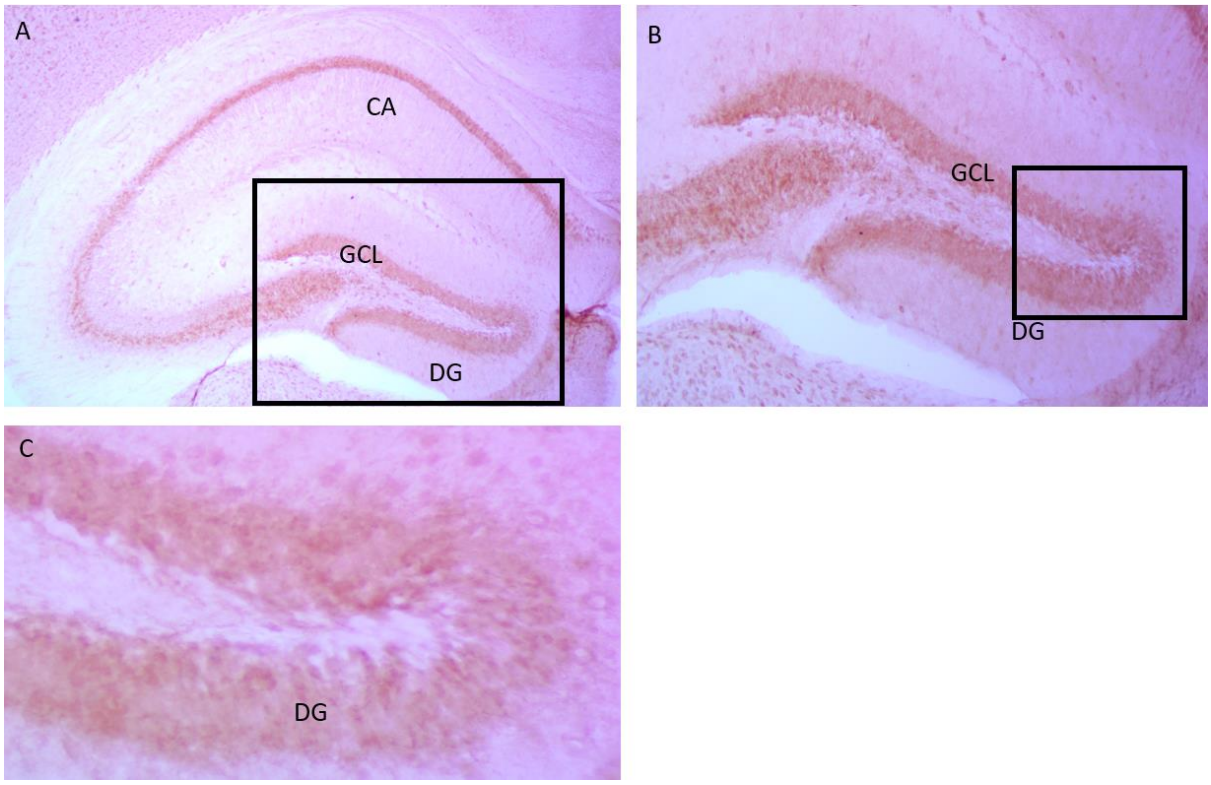

Figure 4.23: Demonstration of nonspecific failed DCX stain:
A) Low magnification (4X) of the hippocampus. B) Medium magnification (10X) of the dentate gyrus (DG), (square rectangle in A). C) Hi magnification (40X) of the granule cell layer (GCL), (square rectangle in B), showing the nonspecific staining of the granule cells.

CHAPTER FIVE
DISCUSSION

5.1 INTRODUCTION

In the current study, two stress models were used, FST and chronic restraint stress. The idea of the two stress models was based on the fact that no one stress model is able to replicate stressful condition as seen in humans (Campos et al., 2013). It has been established that the effective animals stress models must meet three basic criteria reflected by human beings in stressful conditions viz: face validity, correlating the physiological and behavioral changes associated with specific emotional states, construct validity, the etiology of diseases, and predictive validity, responses to pharmacological treatments (Becker et al., 2021). While both the FST and the chronic restraint stress reflect the same characteristic in terms of the behavior of animals, pathophysiology of the disease and response to treatment, they have different strengths in reflecting the above three basic criteria. The weakness of the FST is that the response of animals to this stressor is inconsistent due to genetic factors that are not yet explained (Henn and Vollmayr, 2005). In the chronic restraint stress, animals tend to adapt or habituate to the stressful conditions resulting in short lived depressive symptoms (Christiansen et al., 2011; Grizzell et al., 2014; Park et al., 2018). In the current study, while the behavior of animals did not show inconsistencies in the FST, signs of habituation were notable in the chronic restraint stress animals as reflected in the results section and specified in the sections below. Overall, the results of the current study were a good reflections of the three basic criteria specified to extrapolate the results to human conditions in that the behavior of animals in the tests, elevated levels of corticosterone, reduction of BDNF levels and response to treatment was similar to that observed in humans (Nakagawa et al., 2019; Russell and Lightman, 2019; Dong et al., 2022). Contrary to volumetric reduction seen in human hippocampi of stressed

patients (Belleau et al., 2019), the current study did not observe significant changes in the volume of hippocampus.

The LCMS of BD was conducted in the current study to detect the alkaloid constituent. Of the alkaloids detected in the current study (Table4.1), *buphanamine, lycorine, nerbowdine, crinine* and *distichamine* have also been detected in a previous study conducted by Neergaard *et al*, 2009. The above study also detected *undulatine, acetylnerbowdine, crinamidine, buphacetine* and *buphanisine* which were not detected in the current study. Of importance in the results of the current study, is the detection of *buphanidrine* and *buphanamine,* the two alkaloids which are known to be responsible for binding to the serotonin transporter site (Table 4.1).

5.2 Forced swimming test

5.2.1 Changes in immobility time

In the current study, we investigated the therapeutic efficacy of the hydroethanolic extract of *BD* in attenuating the effects of five days forced swimming stress in male Balb/c mice. In the induction phase of the stress model, mice were forced to swim for 6 minutes, each day, in a glass cylinder for five consecutive days followed by 21 days treatment interventions mixed with behavioral tests in-between. The results indicate that the five days forced swimming significantly increased the immobility time in day five compared to day one, thus confirming a successful stress induction. The immobility time was high in control group compared to either fluoxetine or *BD* treated groups throughout the 21 days treatment period. The persistent high immobility time observed in distilled water treated group corroborates with previous reports indicating that the 5 days forced swimming induced stress persists for a period of four weeks (Mul et al., 2016; Serchov et al., 2016). Treatment with fluoxetine caused significant reduction in immobility time by day 14. By day 21, immobility time for fluoxetine treated group remained

similar to day 14 but *BD* treated group showed significant decline in immobility time compared distilled water group.

The absence of change in immobility time in fluoxetine treated group between day 14 and 21 suggest fast onset and peak of action by fluoxetine, followed by plateaued effect. Previous studies reported similar reduction in immobility time following 10mg/kg fluoxetine treatment for 24 days in Balb/c mice but did not include the weekly intervals of behavioural test assessments as in the present study (Dulawa et al., 2004). However, the result is consistent with other previous reports of continuous reduction in immobility time between week one and week two of fluoxetine treatment (Mezadri et al., 2011). Therefore, the distinction between the present study results and the previous report, (Mezadri et al., 2011), is the third week behavioural test assessment which showed a plateauing in the action of fluoxetine.

The present study has therefore revealed lack of prolonged fluoxetine action relative to prolonged continuous potency of *BD* against the effects of the stress (Dulawa et al., 2004).

The time dependent increase in the efficacy of *BD* with the same dose could be an advantage in maintaining depression patients without increasing the dosage of the drug. A previous study showed that two alkaloids of BD, *buphanidrine* and *distichamine* have the affinity for the SERT, a property which is believed to be responsible for the antidepressant effects of *BD* (Sandager et al., 2005; Neergaard et al., 2009). Overall, the effects of both fluoxetine and *BD* on the swimming behavior of animals are in agreement with the known principle that serotonergic compounds like fluoxetine are sensitive to swimming behavior while tricyclic antidepressants are sensitive to climbing behavior of animals [Detke et al.,995; Page et al., 1999).

5.2.2 Elevated plus maze

Fluoxetine treated group performed poorly in the elevated plus maze with regards to the number of head dips and time spent in the open arms when compared *BD* treated group. The lower fluoxetine scores in the open arms and head dips is in agreement with the reports by the previous authors who recorded that acute administration of selective serotonin reuptake inhibiters paradoxically increase symptoms of anxiety for some individuals whereby anxiolytic effects only become observable after chronic treatment (Bagdy et al., 2001; Nutt, 2005). A sharp increase in serotonergic function of SSRIs provoke an anxiogenic effects in animal models (Birkett et al., 2011). The same effect has been observed in humans where anxiety is exacerbated at the initiation of SSRIs treatment (Handley and McBlane, 1993). It is possible that the slow action of *BD* shown in this study gives an advantage of a slow increase in serotonergic function as opposed to a sharp increase caused by SSRIs which has been suspected to cause anxiogenic symptoms in patients taking these drugs. The number of head dips, time spent in open arms and the frequency of open arm entries in the *BD* treated group was comparable to that of the control group, suggesting that *BD* attenuated the effects of stress. These findings are in agreement with a previous study where 10, 25 and 40mg/kg doses of *BD* produced high scores of head dips and frequency of unprotected arm entries (Pote et al., 2018). We hypothesize that the observed time dependant increase in the efficacy of *BD* is the reason for anxiolytic effects of *BD* treated animals as demonstrated in the elevated plus maze test.

5.2.3 Volume of the hippocampus

Not much has been reported about how acute stress affect the volumes of the hippocampus. Previous studies have reported a 3% reduction in hippocampal volume in rats after exposure to chronic stress (Rocher et al., 2004; Lee et al., 2009; Malhi et al., 2019; Moica et al.,2016;

Rahman et al., 2016; Zimmerman et al., 2016). The neuronal loss caused by stress in the hippocampus could be one of the explanations for the reduction in the volume of the hippocampus (Kara et al., 2003; Mirescu and Gould, 2006). A previous study done do demonstrate the effects of prenatal stress on the volume of the hippocampus found that the reduction in volume specifically affected DG, subiculum, molecular layer, all subfields of the cornu armonis and GC (Marečková et al., 2018). The above hippocampal subfields are identified as sensitive to stress and glucocorticoids (Marečková et al., 2018). Furthermore, animal studies have shown that changes in hippocampal volume are interlinked to changes in cell proliferation, dendritic structure and neurogenesis (Maguire et al., 2006; Leuner and Gould, 2010).

5.2.4 Doublecortin (DCX) expression

In the current study, the chronic stress significantly reduced the expression of DCX immunoreactive cells. This reduction in expression of DCX immunireactive cells was attenuated by both fluoxetine and *BD* treatments. The above results are consistent with the previous findings that provided evidence that acceleration of neurogenesis is one of the cellular mechanisms underpinning SSRIs antidepressant efficacy (Kobayashi et al., 2010; Ohira and Miyakawa, 2011; Miller and Hen, 2015; Schoenfeld and Cameron, 2015 Ohira et al., 2019; Shuto et al., 2020). Chronic administration of SSRIs including fluoxetine has long been shown to play a role in promoting proliferation of progenitor cell early phase maturation and cell survival stages of neurogenesis (Pawluski et al., 2014; Encinas et al., 2006; Wang et al., 2003). In human patients, it has also been shown that the number of mature granule cells in the dentate gyrus of major depressive disorder patients treated with SSRIs is higher than in untreated patients (Boldrini et al., 2013). On the hand, a previous study in 5-HT1A receptor knockout mice showed that proliferation and early maturation of cells in the DG was lacking after

treatment with fluoxetine (Samuels et al., 2016). The above results suggest that the effects of SSRIs on granule cell layer proliferation is mediated through 5-HT1A receptors (Turcotte-Cardin et al., 2019). The involvement of 5-HT1A receptors in SSRIs mediated neurogenesis is also confirmed by the studies that used a 5-HT1A receptor agonist, 8-OH-DPAT, where the cell proliferation in the DG was seen to increase after short-term administration in rodents (Klempin et al., 2010; Arnold and Hagg, 2012). In addition, 5-HT4 receptor activation has also been shown to facilitate proliferation and maturation of new-born neurones (Mendez-David et al., 2014). In the current study, *BD* treated animals showed results comparable to fluoxetine in terms of expression of DCX immunoreactive cells. Previous studies have demonstrated that two alkaloids of *BD*, *buphanidrine* and *buphanamine* have the affinity for the 5HT (1A) receptors (Ramaekers, 2003). The above is thought to be the explanation for the antidepressant effects of BD. While previous studies did not look at the effect of *BD* on the expression of the DCX immunoreactive cells of the dentate gyrus, the observations of the current study suggest that *BD* causes antidepressant effects with the mechanism similar to that of fluoxetine in rectifying the decline of DCX imminoreactive cell expression.

5.2.5 Corticosterone and BDNF levels

A significant increase in the brain corticosterone was observed in stressed control animals of the current study, in agreement with previous authors (Browne et al., 2014; Habr et al., 2014; Gong et al., 2015; Wasinski et al., 2016; Sousa et al., 2019; Safari et al., 2020). In the current study, treatment with either fluoxetine or *BD* normalised the levels of brain corticosterone. Similarly, other studies have recorded reduction in corticorsterone levels in depressed human patients and animals after treatment with SSRIs (Aihara et al., 2007; Knorr et al., 2012; Hernandez et al., 2013; Ruhé et al., 2015). Furthermore, our observations confirm the findings by previous research that SSRIs are able to downregulate the HPA axis activity in both rats and

human subjects (Inder et al., 2001; Paile-Hyvärinen et al., 2003). The reduction of corticosterone levels seen after SSRIs treatment has been associated with improvement in depressive symptoms including memory impairment (Hinkelmann et al., 2012).

We recorded significantly reduced levels of BDNF in the distilled water group of the current study. These findings are in agreement with previous authors who reported low levels of BDNF in animals exposed to stress and in post mortem brains of depressed patients (Aydemir et al., 2005; Castrén and Rantamäki, 2010; Nagahara and Tuszynski, 2011; Guilloux et al., 2012; Tripp et al., 2012; Wu et al., 2012; Phillips, 2017). Evidence has been previously provided that the reduction in BDNF levels are linked to depressive symptoms in mice (Monteggia et al., 2007; Lindholm and Castrén, 2014). Similar to corticosterone, treatment with either fluoxetine or BD restored the levels of BDNF to the levels comparable to that of the control group in the current study. Early studies established that part of the mechanism of action of antidepressant drugs is enhancement of BDNF and TrkB mRNA expression in the hippocampus and cortical regions (Nibuya et al., 1995; Nibuya et al., 1996). Fluoxetine in particular has been shown to promote BDNF expression in the visual cortex and hippocampus through BDNF signalling (Vetencourt et al., 2008). Furthermore, the increased levels of BNDF coupled with increase in DCX immunoreactive cells recorded in the current study further support the theory that the activity of the antidepressant drugs is related to neuronal plasticity (Castrén, 2014; Castrén and Kojima, 2017). It is therefore possible that *BD* exerts its antidepressant effects on the BDNF levels through the same mechanism described for fluoxetine and SSRIs in general.

5.3 Chronic restraint stress

5.3.1 Weight and food consumption changes

The results of the current study showed that the chronic restraint did not affects the weight gain of the animals in the first seven days compared to normal animals. The significant weight reduction was only observed from days 14 to day 28. Interestingly, the significant food consumption changes were observed in the first (day 7) and the last week (day 28) of chronic restraint. It is not clear why the reduction in food consumption did not effect the weight of the animals in day 7 as the significant weight reduction was only observed in day 14. Our results are contrary to the study conducted by Jeong *et al* (2013). The above authors observed significant decline in both weight and food consumption in the five day of a 2 hours daily chronic restraint that was applied for 15 days in mice. Similar to the current study, the above authors also observed a significant recovery in food consumption after the first five days of the chronic restraint. This recovery could be due to stress induced increase in corticosterone levels which is known to activate the stimulation of the appetite in the hypothalamus (Dallman et al., 2004). However, the current study observed that the prolonged stress caused a relapse is reduction of food intake in the fourth week. This is in line with the weight reduction that was consistently lower in stressed animals compared to control animals. Glucocorticoids stimulate the activity of the lipoprotein lipase in the adipose tissues of the organs, which in turn elevates the fat storage around the organs, which would promote weigh gain in stressed individuals (Bjorntorp, 2001). However, glucocorticoids also cause the release of the protein lipase which supresses the appetite (Jequier, 2002). The above mechanism could explain the observed weight reduction of the stressed animals in the current study. Furthermore, it has also been suggested that chronic restraint increase energy metabolism due to heat loss that happens as result of sympathetic nervous system stimulation which cause vasodilation (Rybkin et al., 1997). The terminal percentage weight gains after the treatment period was significantly lower

in all treatment groups of animals compared to the control group in the current study. This is consistent with the previous studies which indicated that the weight gain in stressed animals never reach full restoration even after the recovery or treatment period (Bjorntorp, 2001; Jequier, 2002; Jeong *et al*., 2013; Zareian et al., 2015).

5.3.2 Anxiolytic effects of BD using elevated plus maze

To evaluate the anxiolytic effects of BD, after 28 days of chronic restraint, stressed animals in the current study were divided into distilled water, BD and fluoxetine groups. Distilled water was used as a negative control while fluoxetine was a positive control. Animals were treated for 21 days, and behavioural tests conducted in days 7, 14, and 21 of the treatment period. Below, we will start by consolidating the results of the elevated plus maze test in days 7, 14 and 21 followed by discussion.

In day 7, the only significantly different parameters were the time spent in open arms and time spent in the centre of the elevated plus maze, both of which were observed in the fluoxetine treated animals.

In day 14, the significant differences across the groups were observed in the tome spent in open arms, number of closed arm entries, and the number of head dips. When comparing BD and fluoxetine treated animals, a shift was observed in day 14 whereby the time spent in open arms was significantly higher in BD treated compared to fluoxetine treated animals. In fact, the time spent in open arms for BD treated animals was comparable to the control group. In addition, BD, fluoxetine and distilled water treated animals recorded significantly high number of closed arm entries when compared to the control group. The above was also observed with the number of head dips.

In Day 21, the significantly different parameters were the time spent in both closed and open arms, number of open arm entries, and the number of head dips. The trend of the significantly low time spent in the open arms for fluoxetine treated animals continued in day 21. Interestingly, the distilled water and the BD treated groups scored similarly in the time spent in open arms, and they were comparable to the control group.

It is also interesting to note that the BD treated group scored significantly low time in closed arms compared to the control group. The comparison of the time spent in closed arms between the fluoxetine and BD treated animals showed that BD performed better than fluoxetine whereby BD treated animals had significantly low time in closed arms compared to fluoxetine.

With the number of head dips, all treatment groups scored significantly low compared to the control group, with no significant differences when comparing the treatment groups amongst themselves.

The time spent in open arms for fluoxetine treated animals was significantly low only in treatment day 7 in the current study. These results are central to the popular believe that acute administration of fluoxetine produces anxiety like or anxiogenic effects in rodent models due to sharp increase of serotonin (Silva and Brandao, 2000; Drapier *et al*., 2007; Robert *et al*., 2011; Abuhamdah *et al*., 2015). The above was also emphasised in a review of 18 rodent studies where all studies reviewed noted the does dependant anxiogenic effects of chronic fluoxetine administration (Kryst et al., 2022). On the contrary, while BD treated animals had low time in the open arms in day 7, the subsequent tests showed significant increase in time spent in open arms in the in this group of animals compared to fluoxetine. These results confirm the finding observed in the five days forced swimming stress model as documented in section 5.1 above in our published data (Xhakaza *et al*., 2022). The said results indicated that BD is a slow acting agent which only produce anxiolytic effects after 14 days of treatment. It is evident

that these anxiolytic effects of BD are the same for both chronic and acute stress, making this plant extract an effective antidepressant agent in both these conditions.

5.3.3 Anxiolytic effects of BD using light dark box

The light dark box test was also performed in days 7, 14 and 21 as detailed for the elevated plus maize in section 5.2.2 above.

Our results showed that the time spent in the light box was significantly lower in the control, BD and distilled water treated groups compared to fluoxetine which recorded significantly high time in the light box in day 7.

In day 14, fluoxetine treated animals continued to perform better than control, BD and distilled water treated animals. However, the performance of the control animals approved when compared to BD and distilled water.

It is interesting to note that the anxiogenic effects of fluoxetine only started to show in day 21 in the light dark box test where the time spent in the light box became significantly lower than all other groups. The control animals also showed the expected performance whereby the time spent in the light box was not exceeded by the treatment groups even though it was similar to that of the distilled water and BD treated animal.

The observed poor performance of the control animals in days 7 and 14 could due to lack of habituation to handling as these animals were handle less due them not undergoing chronic restraint stress. It has been suggested that handling experience reduces anxious behaviour in rodents during behavioural tests (Abuhamdah *et al*., 2015). It does seem this in the current study, the time spent in the light box was a matter of habituation more than the effects of treatment for the distilled water and BD treated groups. This is due to the fact that by day 2, both the distilled water and the BD treated animals had similar time in light box which was also comparable to that of the control animals. Our results are contrary to the previous studies

where normal animals had significantly high time in the light box compared to the dark box (Ardayfio and Kim, 2006; Ibironke and Olley, 2014; Orellana et el., 2015; Anandan et al., 2020 Ghosh et al., 2021). It is not clear why this is the case as studies do not give details regarding animal handling before the light dark box test. Further investigate is necessary for accurate interpretation of the results. We however hypothesise that the effects of the repetitive handling during the chronic stress induction is a major factor of the difference in the results obtained in the current study. The repetitive handling been shown to reduce the anxiety behaviour in experimental animals (Ueno *et al*., 2020).

5.3.4 Anxiolytic effects of BD using open field test

In the open field test, the analyses of the time spent in the centre and the sides in days 7, 14 and 21 showed significant differences in day 14 where BD treated animal had significantly high time spent in the centre of the field. The lack of the effects of the fluoxetine and BD in day 21 is agreement with the notion that SSRIs are no not effective in the centre time in the open filed test (Prut and Belzung, 2003).

The frequency of rearing and line crossing also did not show significant differences across the groups. The open filed test is important for exploring motor activity in addition to anxiolytic effects of the tested agents. The line crossings in the current study were used a measure of distance the differences in the distance travelled. It is important to note while BD did not show anxiolytic effects in all parameters of the open field test, the absence of significant differences in line crossing is a good indicator that BD does not have any negative effects on the mobility or motor activity in the animals. The distance travelled, measured by frequency of line crossing in the current study is of great importance in the open field test as the reduction in the mobility of the animal can confound other parameters that are dependant on the mobility of the animals (Seibenhener and Wooten, 2015). Due to manual analyses of the behaviour of animals in the

open field test of the current study, other factors like free, and immobility could not be accounted for. It has been suggested that the above parameters could affect the time spent on the centre and sides of the open field (Rodgers, 2007).

5.3.5 Volume of the hippocampus

The volume of the hippocampus did not show any significant changes in all parts of the hippocampus measured in the current study. The current study had the same findings for the volume of the hippocampus in the forced swimming stress. Our finding are contrary to those of several previous authors who reported that chronic stress causes reduction of the volume of the hippocampus (Rocher et al., 2004; Lee et al., 2009; Malhi et al., 2019; Moica et al.,2016; Rahman et al., 2016; Zimmerman et al., 2016). However, all the said previous studies were done on MRIs and not on histological sections as is the case with the current study, a possible reason for the contradiction in the findings.

5.3.6 BDNF and corticosterone

The current study recorded significantly low levels of BDNF in the distilled water treated animals, while those animals treated with both BD and fluoxetine had BDNF levels comparable to those of the control animals. These finding are in agreement with the previous studies which suggested that stress reduces the levels of BDNF (Cunha et al., 2006; Palomino; Guilloux et al., 2012). A previous study showed that the mice that were exposed to chronic stress with had poor memory compared to the control mice in a habituation open filed test (Gamaro *et al*., 1999). While distilled water treated animals in the chronic restraint stress model showed signs of habituation, in behavioural tests, it is possible that the effects of low BDNF levels might have persistent effects on memory, even though the current study had no interest in memory deficits.

The corticosterone levels were not significantly different across the groups. The above findings could support the idea that during the chronic restraint stress experiment, animals undergo some degree of habituation and resistance to stress (Benini *et al*., 2019; Ueno *et al*., 2020). However, the persistent low levels of BDNF indicate that the habituation could be related to normalising levels of corticosterone but not BDNF. This finding is of great clinical significance as it may explain the persistence of memory deficits observed in adult depression patients with a history of childhood trauma (Bücker et al., 2013; Burri et al., 2013; Petkus et al., 2017; Nakanyama et al., 2020). In the above studies, it was noted that treatment regiment of children exposed to trauma should carefully consider the long impact the trauma may have in these patients at a later stage. The findings of the current study maybe therefore suggest that antidepressant treatment should be part of the treatment regimen in childhood trauma.

6. CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

We hypothesize that the hydroethanolic extract of *BD* exerts its antidepressant effects with a mechanism similar to that of fluoxetine. Both the above treatments caused a reduction of corticosterone and an increase in BDNF levels. The increase in BDNF levels is believed to be responsible for the improvement in the count of DCX immunoreactice cell counts in the treated animals when compared to the control group. Meanwhile both BD and fluoxetine demonstrated anxiolytic effects in the forced swimming test, animals treated with fluoxetine showed anxiogenic like behaviour in the open field test where the animals spent more time in the protected arms and had fewer count of head dips, while animals treated with *BD* showed good anxiolytic activities where the above parameters were significantly higher. In the current study, we have also shown that the potency of *BD* has a time dependant increase in the forced swimming test. Fluoxetine treatment reached its maximum potency by day 14 of the treatment period as shown by the swimming time of the animals, while in *BD* treated animals swimming time continued to increase steadily, and by day 21 it was comparable to that of fluoxetine treated animals. On the other hand, in chronic restraint stress animals, the therapeutic effects BD were more delayed, only becoming significant in treatment day 21, at which point fluoxetine began showing anxiogenic effects. These results suggest that *BD* has a potential for better outcomes as an antidepressant medication when compared to the current conventional medication.

In BD treated animals, the lack of anxiogenic effects observed that is observed after chronic fluoxetine treatment suggest the long term use of BD could have a better therapeutic effects compared to fluoxetine.

Overall, the results of the current study suggest that BD attenuates the effects of stress on the behaviour and neuroanatomy of mouse model of depression.

In the current study, behavioural tests were manually analysed due to lack of access to the relevant softwares and equipment for analysing and recording the animal behaviours. This was one of the limitations of the current study as the softwares give more accurate results. Furthermore, the supplier's termination of the polyclonal antibodies in the middle of the experiment resulted into an immerse struggle to get the antibody compatible with the mouse brain tissue. The above resulted into exclusion of the DCX analyses in the chronic restraint stress. This also contributed to the limitations of this study. The use of male mice might be a limitation as response to stress is known to have sex differences. The results obtained in the current study could therefore be applicable to males and probably not to females.

It is recommended that future studies of this kind be conducted using the software to analyse the behaviours of animals for accuracy and ease of comparisons with other studies. It is also recommend that the chronic restraint stress to conducted with the inclusion of DCX analyses. Analysis of the volume of the hippocampus using MRIs is also recommended for similar studies in future as the protocol of the current study differed from the previous ones that used MRIs to analyse the volumes of the hippocampus. Further recommendation is made that future studies should include western blot for expression of corticosterone and BDNF receptors since in the brain, physiological effects of biomolecules are closely linked to receptor expression.

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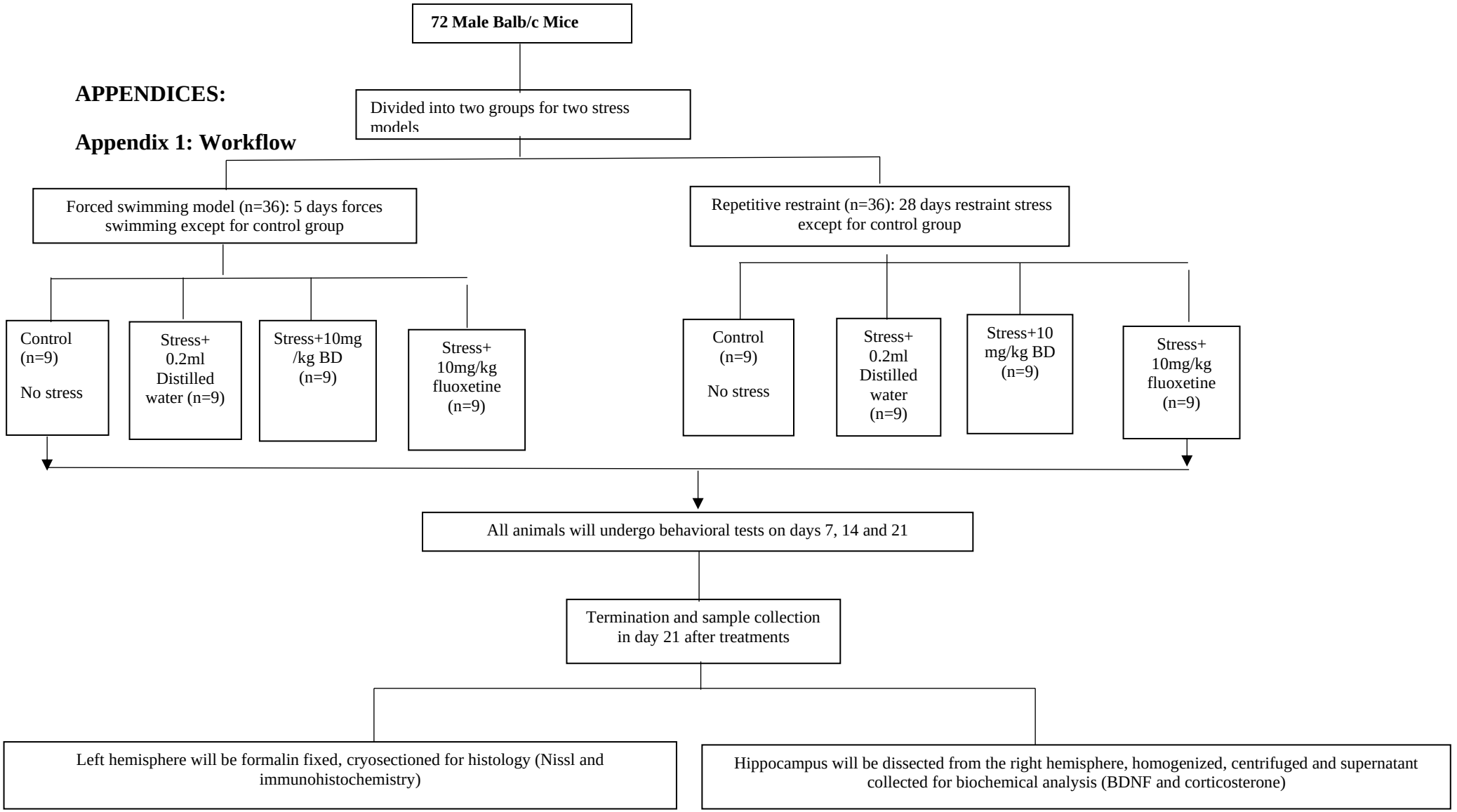
APPENDICES:

Appendix 1: Workflow



Appendix 2: Ethics certificate 1 (Original)

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/011/78/D

APPLICANT: Ms N Xhakaza

SCHOOL: Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: Effect of boophone disticha on the behaviour and hippocampal neuroanatomy in a BALB/c mouse

Number and Species

70X 6 weeks male Balb/c mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/11/28. This approval remains valid until 2020/01/09.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 12 January 2018

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 10 January 2018

cc: Supervisor: Professor E Mbajiorgu
Director: CAS

Works 2000\lain0915\AESCCertLwps

Appendix 3: Ethics certificate 2 (Repeat experiment)

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2021/02/01/D

APPLICANT: Mr N Xhakaza
School: School of Anatomical Sciences; Department: N/A; Location: WRAF

PROJECT TITLE: Effect of boophone disticha on the behaviour and hippocampal neuroanatomy in a BALB/c mouse model of depression
Category: D; Species and Numbers involved: *21X 6months old, male Balb/c Mice*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 23 Feb 2021. This approval remains valid until 9 May 2023 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: ____10/05/2021___

(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: _____ _____ Date: _11 May 2021_____

(Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

Appendix 4: Plagiarism Declaration

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Nkosiphendule Khuthazelani Xhakaza (Student number: 1509905) am a student registered for the degree of Doctor of Philosophy in the academic year 2022.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 27/12/2022

Appendix 5: Turn it in report

EFFECT OF BOOPHONE DISTICHA ON THE BEHAVIOUR AND HIPPOCAMPAL NEUROANATOMY IN A BALB/c MOUSE

ORIGINALITY REPORT

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APPENDIX 6:

Original Article
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Anatomy & Cell Biology acb

Boophone disticha attenuates five day repeated forced swim-induced stress and adult hippocampal neurogenesis impairment in male Balb/c mice

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Abstract: Depression is one of the most common neuropsychiatric disorders and is associated with dysfunction of the neuroendocrine system and alterations in specific brain proteins. *Boophone disticha* (BD) is an indigenous psychoactive bulb that belongs to the *Amaryllidacae* family, which is widely used in Southern Africa to treat depression, with scientific evidence of potent antidepressant-like effects. The present study examined the antidepressant effects of BD and its mechanisms of action by measuring some behavioural parameters in the elevated plus maze, brain content of corticosterone, brain derived neurotropic factor (BDNF), and neuroblast differentiation in the hippocampus of Balb/c mice exposed to the five day repeated forced swim stress (5d-RFSS). Male Balb/c mice were subjected to the 5d-RFSS protocol to induce depressive-like behaviour (decreased swimming, increased floating, decreased open arm entry, decreased time spent in the open arms and decreased head dips in the elevated plus maze test) and treated with distilled water, fluoxetine and BD. BD treatment (10 mg/kg/p.o for 3 weeks) significantly attenuated the 5d-RFSS-induced behavioural abnormalities and the elevated serum corticosterone levels observed in stressed mice. Additionally, 5d-RFSS exposure significantly decreased the number of neuroblasts in the hippocampus and BDNF levels in the brain of Balb/c mice, while fluoxetine and BD treatment attenuated these changes. The antidepressant effects of BD were comparable to those of fluoxetine, but unlike fluoxetine, BD did not show any anxiogenic effects, suggesting better pharmacological functions. In conclusion, our study shows that BD exerted antidepressant-like effects in 5d-RFSS mice, mediated in part by normalizing brain corticosterone and BDNF levels.

Key words: Depression, Antidepressant agents, Hippocampus, Corticosterone, Fluoxetine

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Introduction

World Health Organization identifies depression as one of the leading causes of morbidity and mortality worldwide [1]. Depression is a complex disease that can occur because of multitude of factors, including biological, emotional, and environmental influences [2]. Diagnosis of depression is mainly based on symptomatic behavioural criteria such as depressed mood, low self-esteem and recurrent thoughts of death and

suicide. The heterogeneity of depression suggests that multiple biological mechanisms may underlie its etiology [3]. It is estimated that 40% risk of developing depression is genetic, though the specific genes involved are partially understood [4]. The other 60% non-genetic risk remains poorly defined, with diverse theories implicating acute or chronic stress, childhood trauma, viral infections, and even random processes during brain development as possible causes [5].

The most prescribed antidepressant drugs include tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) [6]. Fluoxetine in particular has been shown not only to have antidepressant properties but to also have antioxidant activities [7]. However, these antidepressant drugs have undesirable side effects. For instance, acute administration of SSRIs such as fluoxetine has been reported to paradoxically increase symptoms of anxiety for some individuals thereby complicating the treatment objective [8, 9]. This increase in anxiety is related to increased serotonergic function of SSRIs, which is believed to provoke an anxiogenic effect in animal models [10]. Co-administration of benzodiazepines and SSRIs is believed to cause faster onset of anxiolytic effects of the SSRI [9]. However, research has shown that non-sedating anti-depressants in combination with benzodiazepines could result in driving impairment [11]. Furthermore, the conventional antidepressant drugs such as fluoxetine are highly expensive and beyond the reach of the poor and the needy. Consequently, the need for alternative antidepressant drugs becomes a great necessity and have resulted in the search for affordable, cost effective and easily accessible natural products and herbal medicines with potential anxiolytic and antidepressant properties such as *Boophone disticha* (BD).

BD (tumbleweed/sore-eye flower) is an indigenous bulb that is widely used by traditional healers in Southern Africa for the treatment of a variety of ailments including anxiety [12]. BD belongs to the *Amaryllidaceae* family, *Boophone* genus, found throughout Southern and Tropical Africa [13], and has been reported to reduce mean arterial pressure of maternally separated mice [14] and in normal non-stressed mice [15].

Furthermore, crude leaf extracts of BD have also been shown to have a high affinity for the serotonin transporter (SERT) [16], with its two alkaloids *buphanadrine* and *buphanamine*, being responsible for binding to the SSRI site of the serotonin P transporter [17]. These mechanisms may partially explain why BD is used traditionally for the management of anxiety. One of the proposed mechanisms of action of many antidepressants is through regulation of adult hippocampal neurogenesis (AHN). AHN is a complex multistep process involving precursor cell proliferation in the subgranular zone (SGZ) of the hippocampal dentate gyrus, differentiation into neuroblasts, migration and subsequent integration into the granule cell layer [18]. SSRIs have been shown to enhance AHN, which might contribute to their antidepressant properties [19]. Conversely, stress and major depression have been shown to reduce volume, cell proliferation and neurogenesis in the adult mammalian hippocampus [20].

Given that BD possibly acts through a similar mechanism as SSRIs, we hypothesized that it would have a regulatory effect on AHN. Therefore, the aim of this study was to evaluate the potential of a crude hydroethanolic extract of BD to attenuate the five day repeated forced swim (5d-RFS) induced stress, associated effects and possible AHN impairment in male Balb/c mice. The 5d-RFS has been shown to result in progressive increases in immobility time in rodents usually persisting for at least 4 weeks and often considered the onset and persistence of a depression-like state [21]. Additionally, the 5d-RFS has been reported to decrease sucrose preference for at least 4 weeks after the last swimming session, suggesting anhedonia, which is also observed in human depression [21, 22]. The progressive increase in immobility time and persistence of anhedonia has been shown to last for at least 4 weeks, giving a window of opportunity to evaluate therapeutic interventions [21].

Materials and Methods

Animals

Twenty (n=20) 6 week old male Balb/c mice were used in the current study. Animals were purchased and housed at Wits Research Animal Facility (WRAF) and were handled by professional personnel of the facility. Before the start of the experiment, animals were acclimatized for one week. Consequently, at the start of the experimental procedure, the animals were seven weeks old. Animals were individually housed in perspex cages with wood shavings as a bedding. A 12 hours light dark cycle was maintained throughout the experimental period. Food and water was provided *ad libitum*. Initial body weights were taken at the start of the experiment, and then once every week. Terminal weights were also recorded and compared to initial weights. All animal

experiments were carried out according to the protocols approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC number: 2017/011/78/D).

Plant material and extraction of phytochemicals

Bulbs of BD were purchased from Random Harvesters in Muldersdrift Johannesburg, South Africa and were authenticated at the Department of Botany, University of Johannesburg, South Africa.

A table knife was used to chop the fresh bulbs of BD into small pieces and sun dried for seven days. The dried plant material was ground into a fine powder using a blender. The powder was then mixed with 70% ethanol in a ratio 5:1 (70% ethanol: BD powder) and placed on an automatic shaker for 24 hours at room temperature. After 24 hours, plant debris were filtered using Whatman No. 1 filter paper. The resultant extract was concentrated using a rotary evaporator at 70°C. The final product was a dark brown solid paste which was air dried, crushed into powder and stored at room temperature. The administered dose of the powder was weighted in grams according to each animal's body weight and dissolved in 2 ml distilled water and administered through orogastric gavage.

Identification of phytochemicals using liquid chromatography–mass spectrometry

A Thermoscientific ultimate 3000 Ultra High-Performance Liquid Chromatography (UHPLC) in conjunction with Bruker Compact Q-TOF high resolution mass spectrophotometer (HRMS) was used to analyse 20 µl of BD extract dissolved in methanol. Gradient elusion techniques was applied to elute 20 µl of the sample that was injected into the UHPLC in a period of 14 minutes. Solvent A with a concentration ranging from 95%–5% of 0.1% formic acid in water (v/v) and a solvent B with a concentration ranging from 5%–95% of 0.1% formic acid in acetonitrile (v/v) was used through Raptor ARC C18 Column (2.7 µm 2.1×100 mm). Analyses of the sample was done using the UHPLC-HRMS analyses, while Bruker Daltonics analyses was used for mass spectrum analyses.

Experimental design

The twenty mice were randomly divided into two main groups: A (treatment group: n=15) and B (control: n=5). Group A was exposed to 5d-RFSS and was further divided into three treatment subgroups (Fluoxetine, BD and Distilled water groups) with 5 mice each. Fluoxetine group received a dose of 10 mg/kg body weight of fluoxetine [23, 24] via oral gavage for 21 days, BD group received 10 mg/kg body weight of BD extract via oral gavage for 21 days [14], and Distilled water group received 0.2 ml distilled water, the vehicle for the treatment drugs for 21 days. Group B (Control) consisted of five normal mice that were not exposed to 5d-RFSS and received distilled water for 21 days.

Five day repeated forced swimming stress model

Forced swimming stress model was first introduced by Porsolt et al. [25], in 1977. The procedure for this model is to place the mouse for 6 minutes into a glass-polycarbonate cylinder (25 cm high×10 cm wide) filled to a depth of 10cm with water maintained at 24°C. Immediately following the 6 minutes exposure into the water cylinder, the animals are removed from the cylinder and dried using a water absorbent pad. In the current study, we used a recently described five day repeated forced swim stress (5d-RFSS) protocol [22], in which mice were forced to swim in an open cylindrical container (diameter, 10 cm; height, 25 cm) containing 10 cm deep water (25°C) for five consecutive days. The five days procedure constituted the induction phase of the stress model. This protocol had been shown to produce the depressive symptoms that last for at least four weeks, allowing for a time window to test and study therapeutic interventions [22]. After the five days induction period, animals were treated for 21 days, while five minutes behavioural tests were conducted and video recorded at intervals on days 7, 14 and 21, after 5d-RFSS. The period/duration of swimming and immobility during the 6 minutes test was measured from the video recordings and recorded using a timer. While the FST has received extensive criticism regarding its validity and reproducibility, a metanalysis of 73 behavioral studies that used FST in mice demonstrated external validity and reproducibility of the FST where different types of antidepressant drugs reduced immobility in the test [26].

The issue of ethics regarding the use of the FST has been raised due to its perceived suffering to animals [27]. However, there is currently no regulation against the use of the FST. The fact sheet published by British Association for Psychopharmacology in conjunction with, the Laboratory Animal Science Association and Understanding Animal Research advocated for the continued use of FST as a valuable behavioral screening tool [28]. A recent commentary suggested the

use of none-behavioral neurochemical measures like brain derived neurotropic factor (BDNF) as an alternative to the FST [29]. The current study included the testing of the BDNF levels in the brain and showed that the changes in the BDNF are in solidarity with the reduction in immobility time in the FST paving a way for a shift away from the FST.

Behavioural tests

On the test day, mice were brought into the test room for at least one hour before the test. All testing was performed between 9:00 am and 15:00 pm by the experimenter. Behavioral tests were done randomly (Elevated plus maize and forced swimming test). All behavioral activities were video recorded via an overhead video camera (Canon Legria, HF R806, China) and later analyzed by the independent observer blinded to the experimental groups.

Elevated plus maze

The maze is plus-shaped with four identical 50 cm (length)×10 cm (width) arms, elevated 70 cm above the floor. Two opposite arms are surrounded on three sides by 30 cm tall opaque walls, and the other two arms are open with no protecting walls on the sides. Each animal was introduced in the centre area (10×10 cm) facing an open arm and allowed to explore freely for 5 minutes. The number of arm entries and time spent in each arm was scored. An arm entry was scored when all four paws of the animals entered an arm and time in arm was counted only if all four paws of the animal are within the arm [25]. Behaviours in this task (*i.e.*, activity in the open and closed arms) reflect a conflict between the rodent's preference for protected areas (*e.g.*, closed arms) and their innate motivation to explore novel environments (*e.g.*, open arms). Anti-anxiety behaviour (increased open arm time and/or open arm entries) can be determined simultaneously with a measure of spontaneous motor activity (total and/or closed arm entries [30, 31].

Terminal procedures

After behavioural tests, animals were euthanized by an overdose of 5% isoflurane at a flow of 2 l/min, followed by intra cardiac perfusion with buffered saline. Following perfusion, brains were rapidly removed from the skull and separated into two hemispheres. The left hemisphere was immediately snap frozen in dry ice before being stored at –80°C for biochemical analyses. The Elabscience ELISA kit (Biocom Africa, South Africa, catalogue No. E-EL-M0203, BDNF and E-OSEL-M0001, Corticosterone) for BDNF and corticosterone was used according to the product specifications. The brain stem and cerebellum was removed from the brains, after which the tissues were weighed and then homogenized in ice cold phosphatebuffered saline (PBS; 0.01 M, pH=7.4) in a volume ratio of 1:9 tissue to PBS with a kitchen blender in 1.5 ml PCR tubes dipped in ice. The homogenates were then centrifuged for 5–10 minutes at 5,000×g at 2°C–8°C to get the supernatant. A 100 µl each dilution of standard, blank and sample was added into the appropriate plates. Plates were then covered and incubated for 90 minutes at 37°C. After decanting the solution, 100 µl of Biotinylated Detection Ab working solution was added to each well, followed by 1 hour incubation at 37°C. The solution was decanted and 350 µl of wash buffer was added to each well, and pat dried after 1 minute. Washes were repeated 3 times. 100 µl of HRP conjugate working solution was then added to each well followed by 30 minutes incubation at 37°C. Wells were washed 5 times in a buffered solution and then 90 µl of substrate Reagent was added to each well, followed by 15 minutes incubation at 37°C. A 50 µl of stop Solution was added to each well and readings were done at 450 nm using a preheated microplate reader. The mean absorbance was calculated. The right hemisphere was immersion fixed in 4% buffered paraformaldehyde overnight before being transferred into 30% sucrose for Nissl staining and immunohistochemistry.

Sectioning of the brain tissues

The right cerebral hemispheres were sectioned in a coronal plane at 50 µm, using a Leica croyostat at –25°C. One in 5 serial sections were collected free-floating into 24 well culture plates with 0.1 M PB. The first set of serial sections were mounted in positively charged slides and air dried, followed Cresyl violet staining. These sections were used to orientate the sections, measure the volumes of the DG, granule cell (GC) layer and hippocampus proper. The second set of the serial sections were stained with doublecortin (DCX) for migrating cells using immunohistochemical techniques. The remainder of the sections were stored for further analyses.

Nissl staining

For anatomical orientation, and brain tissue architecture, Cresyl violet or Nissl staining was used. Decreasing concentrations of 5 minutes alcohol baths (100%, 95%, 70%, and 50%) were used to rehydrate the slide mounted tissues, followed by 1 minute deionization in water. Slides were than

immersed for 1 minute in 1% Cresyl violet staining solution, transferred into distilled water for 1 minute. Five minutes baths of increasing concentrations of alcohol (50% to 100%) were used to dehydrate the slides. Dehydration was interrupted with a differentiation in 70% alcohol/acetic. Slides were dipped in the above solution until the desired intensity of the Cresyl violet stain was achieved. After differentiation, dehydration was continued, and slides were than cleared in xylene and cover slipped with DPX.

Immunohistochemistry

A protocol modified by Nkomozepi et al. [18], was used for DCX immunohistochemistry stains. Brain tissue sections were incubated in endogenous peroxidase inhibitor solution containing 49.2% 0.1 M PB, 49.2% methanol and 1.66% H_2O_2, and then the sections were washed in three ten minutes washes in 0.1 M PB. Washes were followed by a 2 hours incubation with gentle shaking at room temperature in a blocking buffer solution containing 0.25 % Triton X-100 in 0.1 M PB, 2% bovine serum albumin and 3% normal goat serum. The sections were then incubated with gentle shaking for 48 hours in primary antibody solution of rabbit anti-DCX (1:2,000, AB18723; Abcam, Cambridge, UK) in a blocking buffer solution, followed by three ten minutes washes in 0.1 M PB. Sections were than incubated under gentle shaking for 2 hours in 1,000 dilution of biotinylated anti-rabbit IgG (BA-5000; Vector Laboratories, Burlingame, CA, USA) secondary antibody solution in blocking buffer solution, followed by three 10 minute washes in 0.1 M PB. Sections were then incubated for one hour in an avidin-biotin solution [1:125 A and 1:125 B (Vector Laboratories) in 0.1 M PB] under gentle shaking, followed by three ten minutes washes of 0.1 M PB. The sections were then transferred into a solution containing 0.05% 3, 3-di-amino-benzidine tetrachloride in 0.1 M PB for five minutes. 3.3 μl of 30% H_2O_2 was added to each 1 ml of the above solution, and the reaction was monitored by viewing the tissues under the stereomicroscope. To stop the reaction, sections were transferred to a 0.1 M PB, and then washed for 10 minutes in another fresh solution of 0.1 M PB. Stained sections were mounted on positively charged slides and air dried overnight, followed by dehydration in increasing concentrations of alcohol. Air dried slides were cleared in xylene and cover slipped with DPX. Random control sections were stained following the above procedure but omitting either the secondary or primary antibody to eliminate the confounding effects of none specific staining. The control sections did not show DCX stained cells.

Volumes of the hippocampus

The volumes the DG, GC layer and hippocampus proper for each animal was measured in 6 serial sections on photomicrographs acquired using a Leica ICC50 HD digital camera using a free hand tool of ImageJ plugin, VOLUMEST [32, 33].

Doublecortin quantification

Six brain sections that were 250 μm apart were quantified for DCX in the SGZ of hippocampus in each animal. Cells were manually counted by means of a cell counter of ImageJ [32] on photo micrographs taken by a Leica ICC50 HD microscope mounted camera. Adobe photoshop version 7 software (Adobe Systems, Mountain View, CA, USA) was used to prepare the sample of micrographs of the DG of the hippocampus. The originality of the micrographs was not interfered with.

Statistical analysis

Statistical analysis was done using GraphPad Prism 7.0 (GraphPad Prism Software Inc., San Diego, CA, USA). All tests were parametric, and $P \leq 0.05$ was considered significant. ANOVA followed by Bonferroni's *post-hoc* tests was used for comparisons of significant differences among the treatment groups. Comparisons of all scored behaviours were expressed as means±standard error and compared across treatments groups.

Results

Identification of phytochemicals in the hydro-ethanolic extracts of BD using LC-MS

The spectrum view at 5.92 minutes had parental peaks as indicated by the black squares in Fig. 1 with the circle dots showing the fragments of the compounds. With the aid of a metabolite mass spectral database, the parental peaks in Fig. 1 were identified according to their specific m/z as follows: 246.1492 is *Tropococaine*; 274.1439 is *Galanthamine*; 286.1439 is *Narwedine*; 302.1388 is *Buphanamine*, and 316.1537 is *Buphanidrine*. All the phytochemicals obtain in this spectrum view are alkaloids and they are the most abundantly isolated phytochemicals, probably due to the use ethanol solvent.

Phytochemical profile of BD based on LC-MS data

Of the alkaloids detected in the current study (Table 1), *buphanamine, lycorine, nerbowdine, crinine* and *distichamine* have also been detected in a previous study conducted by Neergaard et al. [34], 2009. The above study also detected *undulatine, acetylnerbowdine, crinamidine, buphacetine* and *buphanisine* which were not detected in the current study. Of importance in the results of the current study, is the detection of *buphanidrine* and *buphanamine*, the two alkaloids which are known to be responsible for binding to the SERT site (Table 1).

Animal weights

Table 2 shows the changes in body weights of the animals in this study. Initial body weights were taken at the start of the experiment, and then once every week. Terminal weights were also recorded and compared to initial weights as shown in Table 2. There was no statistically significant difference in weight gain across the experimental groups (P=0.449). However, the percentage weight gain of the control group was significantly higher than the experimental groups (P=0.01). It is possible that the significant weight gain noted above is due to the significantly low (P=0.029) average initial weight of the control group as all groups ended with a similar weight.

Forced swimming test

Induction phase

During the induction phase, mice were forced to swim for 6 minutes daily for 5 days. On day one, the swimming and immobility times were 141.06±29.55 and 218.93±29.55 respectively. These two indices significantly changed to 76.37±29.77 and 283.62±29.77 respectively on day five (P=0.000). From day six, the animals were assigned to three different treatment groups as described in the methods above and were retested again after days 7, 14 and 21 of the treatment period post 5d-RFSS.

Changes in swimming times during the treatment period

There were significant differences in swimming times across all the groups after 21 days of treatment (F=8.647, P=0.004; Fig. 2A). Bonferroni's *post-hoc* analyses showed that the swimming time for both fluoxetine and BD treated groups was significantly higher than that of the distilled wa-

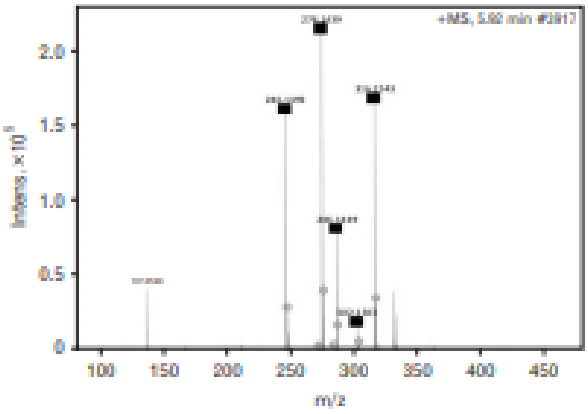

Fig. 1. Spectrum view of LC-MS chromatogram, acquired in the positive-ion mode, from an extract of the bulb of BD. LC-MS, liquid chromatography-mass spectrometry; BD, *boophone disticha*.

Table 1. Phytochemical profile of BD based on LC-MS data

Retention time	m/z	Common name	Structural formulae
0.71 min	104.1072	Choline	$C_5H_{13}NO$
	302.1588	Buphanamine	$C_{17}H_{23}NO_4$
3.77 min	315.1537	Buphanidrine	$C_{17}H_{21}NO_4$
	205.097	Vasicinol	$C_{11}H_{11}N_2O_2$
5.15 min	288.1241	Lycorine	$C_{16}H_{17}NO_4$
5.48 min	332.1493	Tazettine	$C_{18}H_{21}NO_5$
5.92 min	272.1298	Crinine	$C_{16}H_{17}NO_3$
	246.1492	Tropacocaine	$C_{15}H_{19}NO_2$
6.86 min	286.144	Narwedine/Nerbowdine	$C_{17}H_{19}NO_3$
7.77 min	330.1335	Distichamine	$C_{18}H_{19}NO_5$
14.92 mm	274.1439	Galanthamine	$C_{17}H_{19}NO_3$
	275.1471	Carboxyprimaquine	$C_{14}H_{18}N_2O_3$
18.16 min	353.2891	Montalnol	$C_{21}H_{39}NO_3$
24.39 min	264.2411	Caranine	$C_{17}H_{29}NO_2$
26.02 min	556.4332	Chloroxathin/OH-Neosporene	$C_{40}H_{52}O$

BD, *boophone disticha*; LC-MS, liquid chromatography-mass spectrometry.

Table 2. Body masses (g)

Parameter	Treatments				
	Control	Stress+distilled water	Stress+BD	Stress+fluoxetine	P-value
Initial body mass (g)	18.75	21. 625	22	22.33	0.029
Final body mass (g)	24.125	24.25	24	24.83	0.449
Body mass gain (g)	5.375	2.625	2	2.5	0.001
% change in body mass	28.66	12.14	9.09	11.19	0.01

BD, *boophone disticha*.

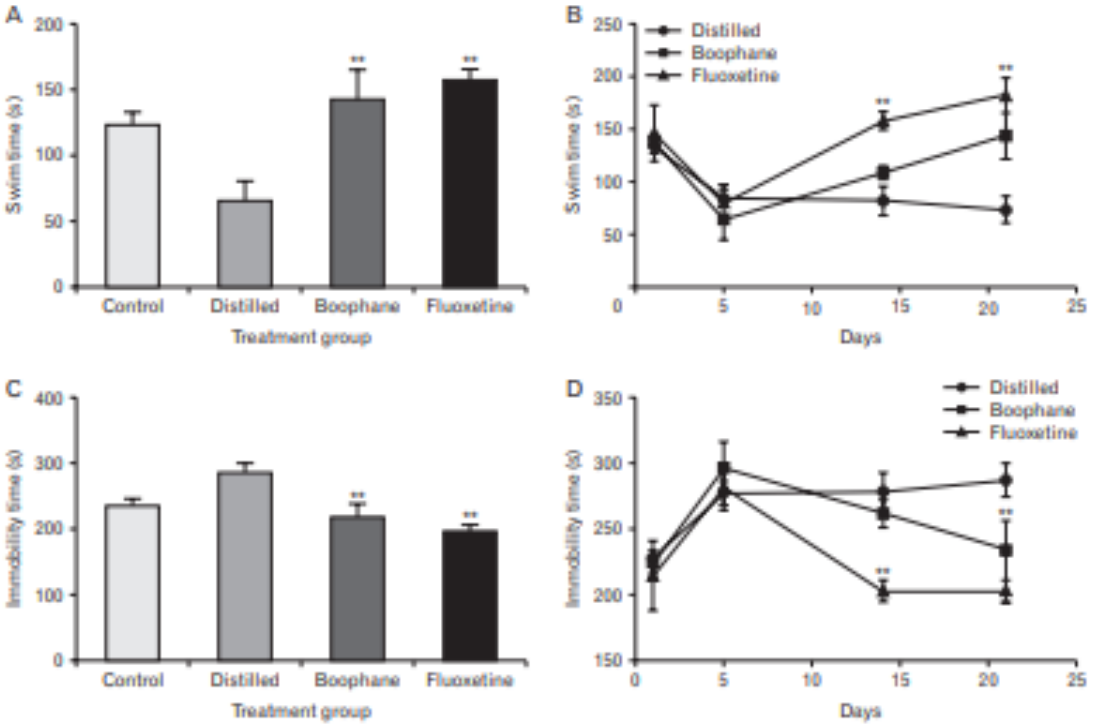

Fig. 2. Graphic representation of changes in swimming times after 21 days of treatment. (A) Significant differences in swimming time across different groups were observed in ANOVA test (P=0.04). (B) The *post-hoc* test showed that in day 14 of treatment, the swimming time for the fluoxetine treated animals was significantly higher (P=0.0012) compared to the distilled water and BD treated animals, while BD treated animals only showed a significant increase by day 21, whereby the fluoxetine group had reached a plateau of increase in swimming time. (C) In day 21, immobility times were significantly different across the treatment groups as demonstrated in ANOVA test (P=0.029). (D) A significant time dependant steady reduction in immobility time from day 5 to day 21 was observed in the BD treated animals (P=0.004), whereas a steady reduction was observed in fluoxetine treated group from day 5 to day 14 and plateaued between day 14 to day 21. The immobility time for distilled water treated group remained high throughout the treatment period (P=0.001). Error bars correspond to the standard error of the mean, **indicates significant changes due to treatments. BD, *boophone disticha*.

ter treated group (P<0.05). A time dependant effect of treatments recorded in behavioural tests that were conducted in days 14 and 21 was performed. The *post-hoc* analyses showed that by treatment day 14, the swimming time for the fluoxetine treated animals was significantly higher (P=0.0012) compared to the distilled water and BD treated animals, while BD treated animals only showed a significant increase in swimming time by day 21 (P=0.004; Fig. 2B), whereby the fluoxetine treated group had reached a plateau of increase in swimming time.

Changes in immobility times

On day 14 of the treatment period, immobility times were significantly different across the treatment groups as demonstrated in ANOVA test (F=4.237, P=0.029). Furthermore, Bonferroni's *post-hoc* analysis revealed that the immobility times were significant reduced in the fluoxetine treated group compared to both the distilled water and BD treated groups (P= 0.01). On the contrary, no significant differences were found between the distilled water and BD treated groups in *post-hoc* test (P=0.185).

In day 21, ANOVA test detected significant differences in immobility times across the treatment groups (F=8.597, P=0.004, Fig. 2C). Bonferroni's *post-hoc* analysis revealed that immobility times were significantly reduced in both the fluoxetine (0.001) and BD (0.004) groups compared to the distilled water treated group.

When compared to the control, distilled water treated group had significantly high immobility times by treatment day 21 (P=0.001). However, no differences were found be-

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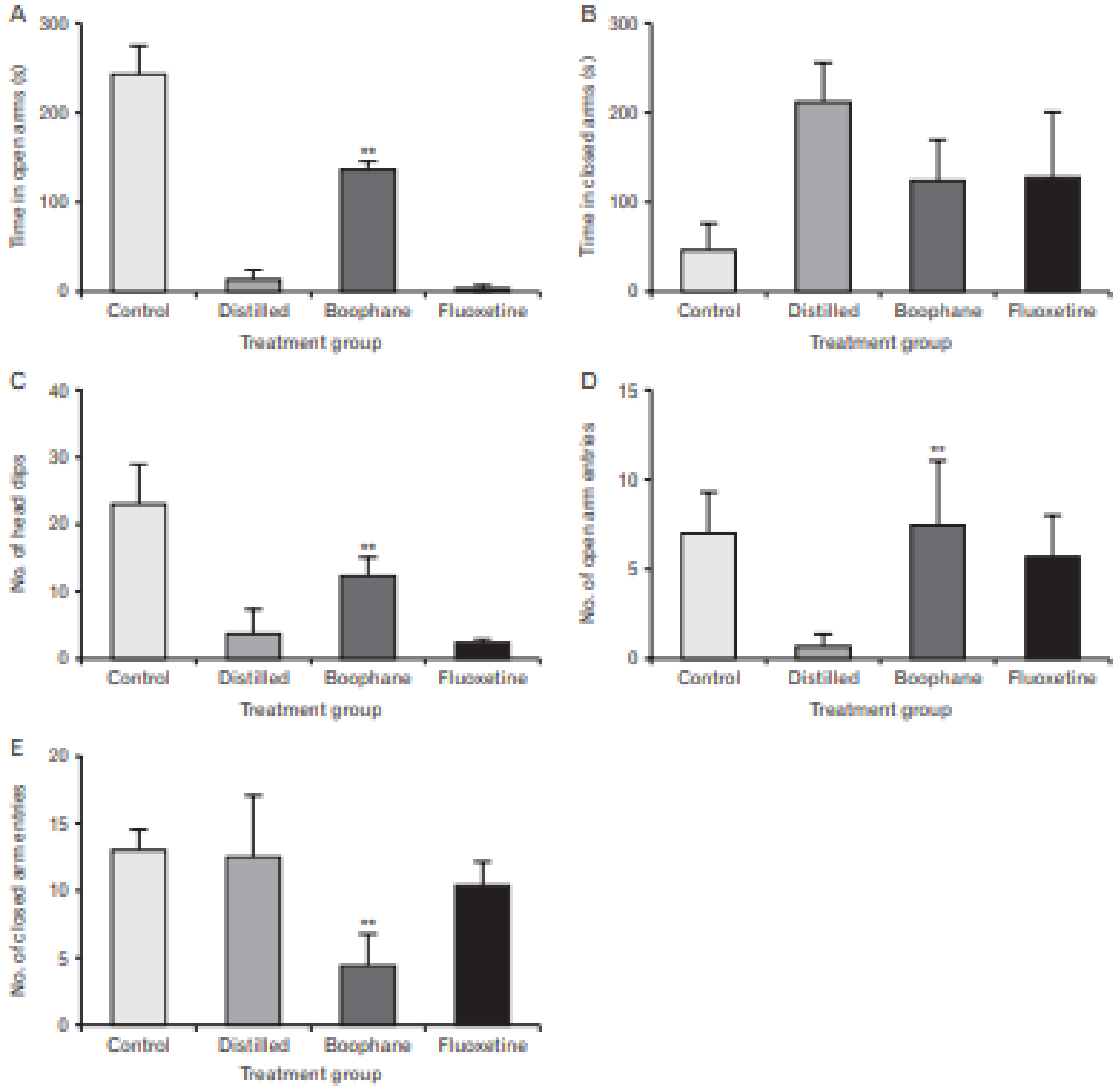

Fig. 3. Graphic representation of the elevated plus maze results. (A) The time spent in the open arms was significantly different across the groups in ANOVA test (P=0.0001). Bonferroni's *post-hoc* test revealed that the time spent in the open arms was significantly higher in the control group compared to experimental groups (P<0.05). BD treated group recorded significantly high time in the open arms compared to both distilled water and fluoxetine groups (P<0.05). The time spent in the open arms for fluoxetine and distilled water group was not significantly different (P>0.05). (B) The time spent in the closed arms was not significantly different across the groups (P=0.195). The control group recorded the lowest time in closed arms though non-significant in the ANOVA test when compared with the treatment groups. Both fluoxetine and BD recorded low time in the closed arms as compared to the distilled water group. (C) The number of head dips was significantly different across the groups as shown by ANOVA test (F=6.122, P=0.0105). Bonferroni's *post-hoc* test showed that the BD group had significantly high number of head dips compared to distilled water and fluoxetine treated groups both of which scored the lowest number of head dips (P<0.05). Both the control and BD treated groups recorded high frequency of open arm entries compared to both distilled water and fluoxetine treated groups (P<0.05). (D) Both the control and BD treated groups recorded high frequency of open arm entries when compared to both distilled water and fluoxetine treated groups (P<0.05). (E) BD treated group had significantly low frequency of closed arm entries compared to distilled water and fluoxetine treated groups (P<0.05). Error bars correspond to the standard error of the mean, **indicates significant changes due to treatments. BD, *boophane disticha*.

tween the control and both the fluoxetine (P=0.067) and BD (P=1.00) treated groups.

A time dependant steady reduction in immobility time from day 5 to day 21 was observed in the BD treated animals, whereas a steady reduction was observed in a fluoxetine treated group from day 5 to day 14 and plateaued between day 14 and 21. The immobility time for distilled water treated group remained high throughout the treatment period (P=0.001, Fig 2D).

Elevated plus maze

Time spent in open arms

Fig. 3 shows the results of the elevated plus maze. The ANOVA test showed that the time spent in the open arms of the elevated plus maze was significantly different among the groups (F=44.41, P=0.0001, Fig. 3A). Bonferroni's *post-hoc* test revealed that the time spent in the open arms was significantly higher in the control group compared to experimental groups (P<0.05). Furthermore, BD treated group recorded significantly high time in the open arms compared to both distilled water and fluoxetine treated groups (P<0.05). The time spent in the open arms for fluoxetine and distilled water treated groups was not significantly different (P>0.05).

Time spent in closed arms

The time spent in closed arms was not significantly different across the groups as shown in ANOVA test (F=1.835, P=0.195). The control group recorded the lowest time in closed arms, though non-significant, when compared with the treatment groups. However, both fluoxetine and BD recorded low time in the closed arms as compared to the distilled water group (Fig. 3B).

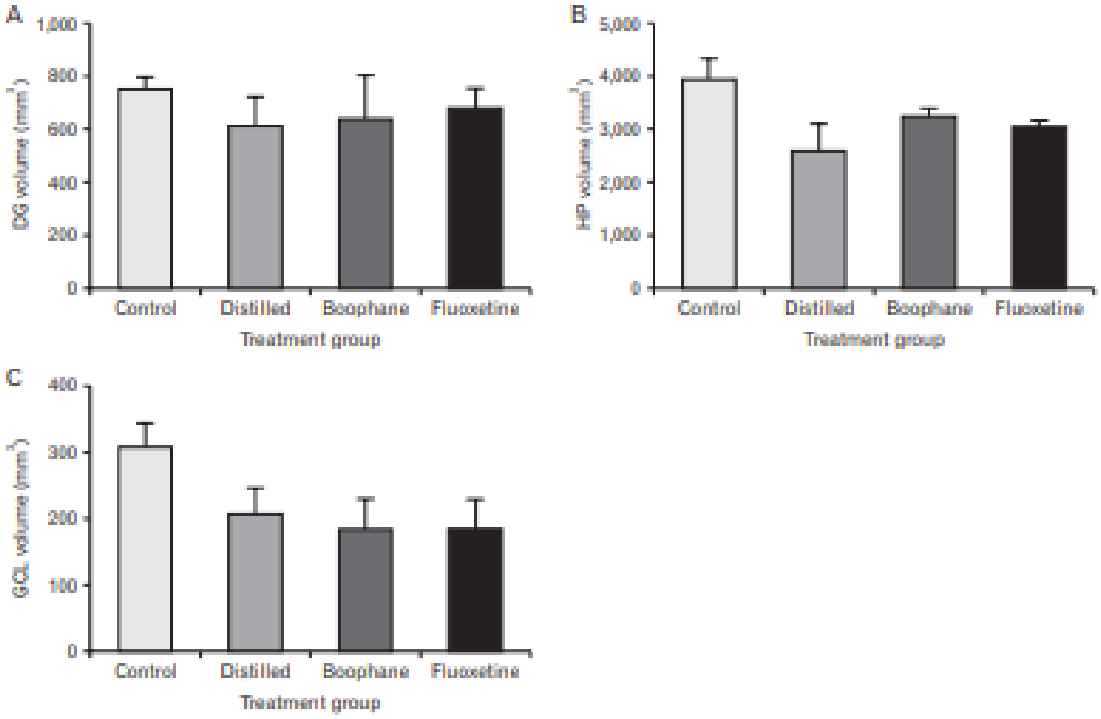

Fig. 4. Graphic representation of the volumes of the dentate gyrus and hippocampus across the groups. (A) The ANOVA test did not detect any significant differences in the volume of the dentate gyrus (F=0.3083, P=0.8190), (B) whole hippocampus (F=3.110, P=0.1101), and (C) granule cell layer of the dentate gyrus (F=1.501, P=0.2955) across all the groups. There was a slight reduction of the dentate gyrus volume in the experimental groups compared to the control group. A slight reduction in the hippocampal volume of the experimental groups as compared to the control group was also observed. The same observation was also noted in the volume of the granule cell layer of the experimental groups compared to the control group. Error bars correspond to the standard error of the mean, **indicates significant changes due to treatments.

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Number of head dips and frequency of open and closed arm entries

The number of head dips was significantly different across the groups as shown by ANOVA test ($F=6.122$, $P=0.0105$, Fig. 3C). Bonferroni's *post-hoc* test showed that the control group had significantly high number of head dips compared to distilled water and fluoxetine treated groups, both of which scored the lowest number of head dips ($P<0.05$). Furthermore, Bonferroni's *post-hoc* test showed that the head dips of the BD treated group were not significantly different from those of the control group ($P>0.05$). In addition, the *post-hoc* test showed that both the control and BD treated groups recorded significantly high frequency of open arm entries compared to both distilled water and fluoxetine treated groups ($P<0.05$, Fig. 3D). Furthermore, BD treated group had significantly low frequency of closed arm entries compared to distilled water and fluoxetine treated groups ($P<0.05$, Fig. 3E).

Volume of hippocampus

The ANOVA test did not detect any significant differences in the volume of the dentate gyrus ($F=0.3083$, $P=0.8190$, Fig. 4A), whole hippocampus ($F=3.110$, $P=0.1101$, Fig. 4B), and granule cell layer of the dentate gyrus ($F=1.501$, $P=0.2955$, Fig. 4C) across all the groups. However, there was a slight reduction of the dentate gyrus volume in the experimental groups compared to the control group. Similarly, a slight reduction in the hippocampal volume of the experimental groups was observed compared to the control group. The same observation was also noted in the volume of the granule cell layer of the experimental groups compared to the control group.

Doublecortin expression

There were statistically significant differences in the DCX expression across the groups as detected by the ANOVA test ($F=10.23$, $P=0.0005$). Bonferroni's *post-hoc* test showed that the DCX expression was significantly lower in the distilled water treated group compared to both fluoxetine and BD treated groups ($P<0.05$, Figs. 5, 6A). The DCX expression in fluoxetine and BD treated animals was similar and comparable to the control group. Furthermore, the DCX immunoreactive cells of the control, BD and fluoxetine treated groups had well developed dendritic spines, while those of the distilled water treated group had poorly developed dendritic spines (Fig. 5).

Brain derived neurotropic factor

The expression of BDNF was significantly different across the groups in ANOVA test ($F=9.051$, $P=0.0026$, Fig. 6B). The expression of BDNF was significantly lower in the distilled water group compared to the control, fluoxetine and BD

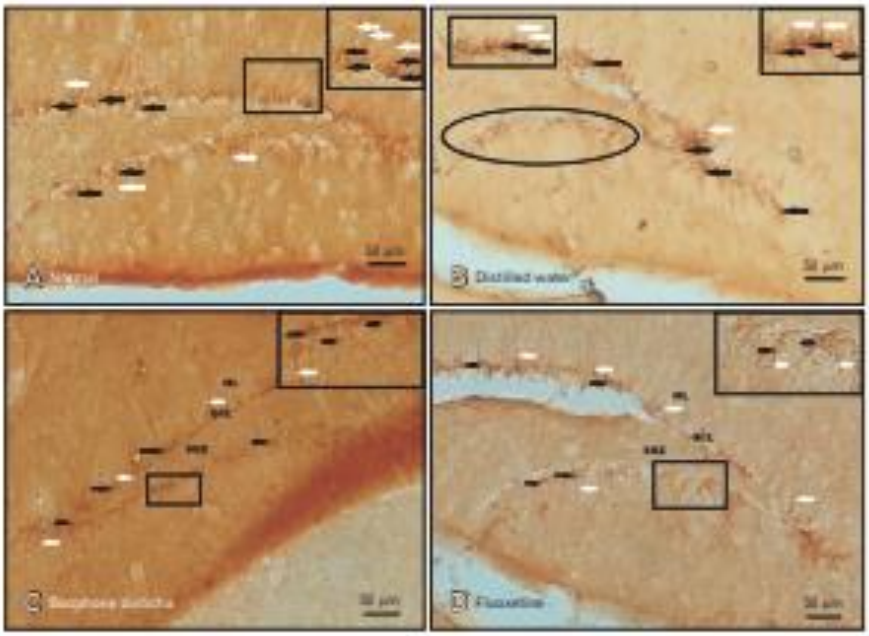

Fig. 5. Photomicrographs showing DCX immunoreactive cells in the SGZ of the dentate gyrus of the hippocampus of the normal (control) (A) and treated (B–D) groups. The black arrows are showing the cell bodies of the neuroblasts and the white arrows are showing the dendritic spines. Poorly developed dendritic spines can be seen in the distilled water treated group B. The oval circle in group B shows one of the patches in the lower limb of the dentate gyrus where DXC immunoreactive cells were absent leading to low cell count in this group. The magnified areas on the top right of each micrograph represent the areas indicated by the black rectangles. DCX, doublecortin; SGZ, subgranular zone; GCL, granule cell layer; ML, molecular layer.

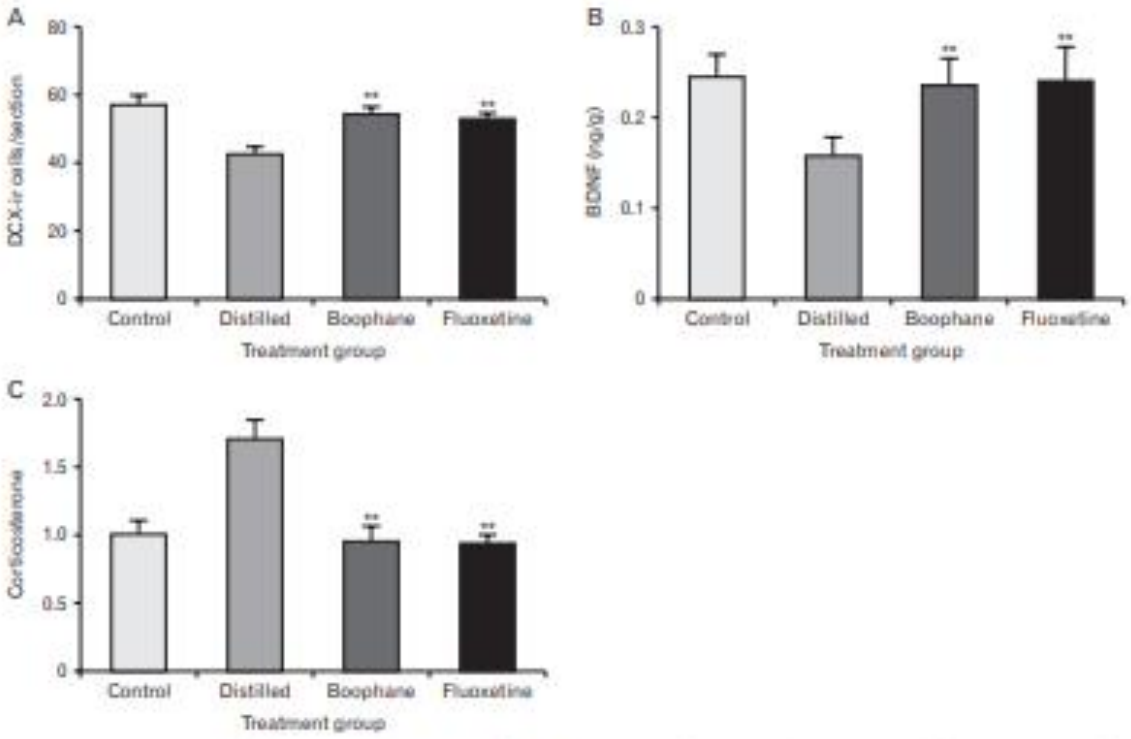

Fig. 6. Graphic representation of the expression of DCX-ir and biochemical analyses of BDNF and corticosterone. (A) The expression of DCX-ir was significantly different across the groups (P=0.0005). DCX-ir expression was significantly lower in the distilled water treated group when compared to both fluoxetine and BD treated groups as shown by the *post-hoc* test (P<0.05). *Post-hoc* analyses further showed that the counts of DCX-ir cells in both fluoxetine and BD groups were significantly higher than the distilled water group (P>0.05). (B) The expression of BDNF was significantly different across the groups (P=0.0026). BDNF expression was significantly lower in the distilled water treated group when compared to the control, fluoxetine and BD treated groups in the *post-hoc* test (P<0.05). The *post-hoc* test further showed that fluoxetine and BD treatment significantly increased the expression of BDNF when compared to the distilled water group (P<0.05). (C) The expression of corticosterone was significantly different across the groups (P=0.001). The *post-hoc* test showed that corticosterone levels remained significantly high in the distilled water treated group when compared to the control group (P<0.05). On the contrary, treatment with either fluoxetine or BD significantly reduced corticosterone levels (P<0.05). The levels of corticosterone in the fluoxetine and BD treated groups were not significantly different from that of the control group (P>0.05). Error bars correspond to the standard error of the mean, **indicates significant changes due to treatments. BDNF, brain derived neurotropic factor; DCX-ir, doublecortin immunoreactive cells; BD, *boophone disticha*.

treated groups in the *post-hoc* test (P<0.05). Furthermore, the *post-hoc* test showed that fluoxetine and BD treatment significantly increased the expression of BDNF compared to the distilled water group (P<0.05). There were no significant differences when comparing the control group to both fluoxetine and BD treated groups (P>0.05).

Corticosterone

The expression of corticosterone was significantly different across the groups (F=53.55, P=0.001, Fig. 6C). The *post-hoc* test showed that corticosterone levels remained significantly high in the distilled water treated group when compared to the control group (P<0.05). On the contrary, treatment with either fluoxetine or BD significantly reduced corticosterone levels (P<0.05). The levels of corticosterone in the fluoxetine and BD treated groups were not significantly different from that of the control group (P>0.05).

Discussion

Changes in immobility time

In the current study, we investigated the therapeutic efficacy of the hydroethanolic extract of BD in attenuating the effects of five days forced swimming stress in male Balb/c mice. In the induction phase of the stress model, mice were forced to swim for 6 minutes, each day, in a glass cylinder

for five consecutive days followed by 21 days treatment interventions mixed with behavioral tests in-between. The results indicate that the five days forced swimming significantly increased the immobility time in day five compared to day one, thus confirming a successful stress induction. The immobility time was high in control group compared to either fluoxetine or BD treated groups throughout the 21 days treatment period. The persistent high immobility time observed in distilled water treated group corroborates with previous reports indicating that the 5 days forced swimming induced stress persists for a period of four weeks [21, 22]. Treatment with fluoxetine caused significant reduction in immobility time by day 14. By day 21, immobility time for fluoxetine treated group remained similar to day 14 but BD treated group showed significant decline in immobility time compared distilled water group.

The absence of change in immobility time in fluoxetine treated group between day 14 and 21 suggest fast onset and peak of action by fluoxetine, followed by plateaued effect. Previous studies reported similar reduction in immobility time following 10 mg/kg fluoxetine treatment for 24 days in Balb/c mice but did not include the weekly intervals of behavioural test assessments as in the present study [35]. However, the result is consistent with other previous reports of continuous reduction in immobility time between week one and week two of fluoxetine treatment [36]. Therefore, the distinction between the present study results and the previous report [36] is the third week behavioural test assessment which showed a plateauing in the action of fluoxetine.

The present study has therefore revealed lack of prolonged fluoxetine action relative to prolonged continuous potency of BD against the effects of the stress [35].

The time dependent increase in the efficacy of BD with the same dose could be an advantage in maintaining depression patients without increasing the dosage of the drug. A previous study showed that two alkaloids of BD, *buphanidrine* and *distichamine* have the affinity for the SERT, a property which is believed to be responsible for the antidepressant effects of BD [12, 34]. Overall, the effects of both fluoxetine and BD on the swimming behavior of animals are in agreement with the known principle that serotonergic compounds like fluoxetine are sensitive to swimming behavior while TCAs are sensitive to climbing behavior of animals [37, 38].

Elevated plus maze

Fluoxetine treated group performed poorly in the elevated plus maze with regards to the number of head dips and time spent in the open arms when compared BD treated group. The lower fluoxetine scores in the open arms and head dips is in agreement with the reports by the previous authors who recorded that acute administration of selective serotonin reuptake inhibiters paradoxically increase symptoms of anxiety for some individuals whereby anxiolytic effects only become observable after chronic treatment [8, 9]. A sharp increase in serotonergic function of SSRIs provoke an anxiogenic effects in animal models [10]. The same effect has been observed in humans where anxiety is exacerbated at the initiation of SSRIs treatment [39]. It is possible that the slow action of BD shown in this study gives an advantage of a slow increase in serotonergic function as opposed to a sharp increase caused by SSRIs which has been suspected to cause anxiogenic symptoms in patients taking these drugs. The number of head dips, time spent in open arms and the frequency of open arm entries in the BD treated group was comparable to that of the control group, suggesting that BD attenuated the effects of stress. These findings are in agreement with a previous study where 10, 25, and 40 mg/kg doses of BD produced high scores of head dips and frequency of unprotected arm entries [15]. We hypothesize that the observed time dependant increase in the efficacy of BD is the reason for anxiolytic effects of BD treated animals as demonstrated in the elevated plus maze test.

Volume of the hippocampus

Not much has been reported about how acute stress affect the volumes of the hippocampus. Previous studies have reported a 3% reduction in hippocampal volume in rats after exposure to chronic stress [40–45]. The neuronal loss caused by stress in the hippocampus could be one of the explanations for the reduction in the volume of the hippocampus [46, 47]. A previous study done do demonstrate the effects of prenatal stress on the volume of the hippocampus found that the reduction in volume specifically affected DG, subiculum, molecular layer, all subfields of the cornu armonis and GC [48]. The above hippocampal subfields are identified as sensitive to stress and glucocorticoids [48]. Furthermore, animal studies have shown that changes in hippocampal volume are interlinked to changes in cell proliferation, dendritic structure and neurogenesis [49, 50].

Doublecortin expression

In the current study, the chronic stress significantly re-

duced the expression of DCX immunoreactive cells. This reduction in expression of DCX immunireactive cells was attenuated by both fluoxetine and BD treatments. The above results are consistent with the previous findings that provided evidence that acceleration of neurogenesis is one of the cellular mechanisms underpinning SSRIs antidepressant efficacy [51-54]. Chronic administration of SSRIs including fluoxetine has long been shown to play a role in promoting proliferation of progenitor cell early phase maturation and cell survival stages of neurogenesis [24, 55, 56]. In human patients, it has also been shown that the number of mature granule cells in the dentate gyrus of major depressive disorder patients treated with SSRIs is higher than in untreated patients [57]. On the hand, a previous study in 5-HT1A receptor knockout mice showed that proliferation and early maturation of cells in the DG was lacking after treatment with fluoxetine [58]. The above results suggest that the effects of SSRIs on granule cell layer proliferation is mediated through 5-HT1A receptors [59]. The involvement of 5-HT1A receptors in SSRIs mediated neurogenesis is also confirmed by the studies that used a 5-HT1A receptor agonist, 8-OH-DPAT, where the cell proliferation in the DG was seen to increase after short-term administration in rodents [60, 61]. In addition, 5-HT4 receptor activation has also been shown to facilitate proliferation and maturation of new-born neurones [62]. In the current study, BD treated animals showed results comparable to fluoxetine in terms of expression of DCX immunoreactive cells. Previous studies have demonstrated that two alkaloids of BD, *buphanidrine* and *buphanamine* have the affinity for the 5-HT1A receptors [11]. The above is thought to be the explanation for the antidepressant effects of BD. While previous studies did not look at the effect of BD on the expression of the DCX immunoreactive cells of the dentate gyrus, the observations of the current study suggest that BD causes antidepressant effects with the mechanism similar to that of fluoxetine in rectifying the decline of DCX imminoreactive cell expression.

Corticosterone and BDNF levels

A significant increase in the brain corticosterone was observed in stressed control animals of the current study, in agreement with previous authors [63-68]. In the current study, treatment with either fluoxetine or BD normalised the levels of brain corticosterone. Similarly, other studies have recorded reduction in corticorsterone levels in depressed human patients and animals after treatment with SSRIs [69-72]. Furthermore, our observations confirm the findings by previous research that SSRIs are able to downregulate the HPA axis activity in both rats and human subjects [73, 74]. The reduction of corticosterone levels seen after SSRIs treatment has been associated with improvement in depressive symptoms including memory impairment [75].

We recorded significantly reduced levels of BDNF in the control group of the current study. These findings are in agreement with previous authors who reported low levels of BDNF in animals exposed to stress and in post mortem brains of depressed patients [76-82]. Evidence has been previously provided that the reduction in BDNF levels are linked to depressive symptoms in mice [83, 84]. Similar to corticosterone, treatment with either fluoxetine or BD restored the levels of BDNF to the levels comparable to that of the control group in the current study. Early studies established that part of the mechanism of action of antidepressant drugs is enhancement of BDNF and TrkB mRNA expression in the hippocampus and cortical regions [85, 86]. Fluoxetine in particular has been shown to promote BDNF expression in the visual cortex and hippocampus through BDNF signalling [87]. Furthermore, the increased levels of BNDF coupled with increase in DCX immunoreactive cells recorded in the current study further support the theory that the activity of the antidepressant drugs is related to neuronal plasticity [88, 89]. It is therefore possible that BD exerts its antidepressant effects on the BDNF levels through the same mechanism described for fluoxetine and SSRIs in general.

In conclusion, we hypothesize that the hydroethanolic extract of BD exerts its antidepressant effects with a mechanism similar to that of fluoxetine. Both the above treatments caused a reduction of corticosterone and an increase in BDNF levels. The Increase in BDNF levels is believed to be responsible for the improvement in the count of DCX immunoreactice cell counts in the treated animals when compared to the control group. Mean while both BD and fluoxetine demonstrated anxiolytic effects in the forced swimming test, animals treated with fluoxetine showed anxiogenic like behaviour in the open field test where the animals spent more time in the protected arms and had fewer count of head dips, while animals treated with BD showed good anxiolytic activities where the above parameters were significantly higher. In the current study, we have also shown that the potency of BD has a time dependant increase in the forced swimming test. Fluoxetine treatment reached its maximum potency by day 14 of the treatment period as shown by the swimming

time of the animals, while in BD treated animals swimming time continued to increase steadily, and by day 21 it was comparable to that of fluoxetine treated animals. These results suggest that BD has a potential for better outcomes as an antidepressant medication when compared to the current conventional medication.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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