Antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats

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B. PHARM (Honours)

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ABSTRACT

Introduction: People with major depressive disorder and comorbid anxiety disorders tend to have poor response to treatment compared to patient with major depressive disorder alone. The standardized extract of *Sceletium tortuosum* (Zembrin®) is a multi-model serotonin reuptake transporter (SERT) and phosphodiesterase (PDE) 4B inhibitor that may be a promising novel therapy for patients with depression, either as monotherapy or as an augmentation strategy in poor responders. Therefore, investigation into its therapeutic potential alone following sub-chronic treatment and in combination with a known standard antidepressant is required.

Aim of the study: To assess the dose-related antidepressant- and anxiolytic-like effects of Zembrin® (ZEM) in the Flinders Sensitive Line (FSL) rats, a genetic model of depression, alone and as an adjunctive treatment with low-dose desipramine (DMI), and correlation with altered regional brain monoamines and phosphodiesterase 4B expression.

Material and methods: For confirmation of the model, 12 male Flinders Resistant Line (FRL) rats and 12 male FSL rats (control group) were treated with saline for 14 days via oral gavage. Seven groups (*n* =12) of male FSL rats were treated with a 3-tier dose of ZEM (10, 25 & 30 mg/kg/day), and a 2-tier dose of DMI (15 & 30 mg/kg/day) for 14 days via oral gavage to establish predictive validity. To assess augmentation potential, ZEM (10 & 30 mg/kg/day), was combined with a low dose of DMI (15 mg/kg/day) over 14 days. Following treatment, depressive- like behaviour was assessed in the Forced Swim Test (FST), and anxiety-like behaviour was assessed in the Open Field Test (OFT) and Elevated Plus Maze (EPM). The behavioural assessment was followed by analyses of cortical and hippocampal monoamines and PDE4B levels.

Results: Depressive-like behaviour was significantly increased in FSL rats versus Flinders Resistant Line (FRL) control rats. DMI (15 & 30 mg/kg/day) significantly decreased immobility and increased struggling behaviour in the FST. ZEM (10, 25 & 30 mg/kg/day) showed no antidepressant-like properties in the FST versus SAL (saline) treated FSL rats. Similarly, combinations of ZEM+DMI showed no antidepressant-like properties in FST versus FSL saline treated rats. ZEM-10+DMI-15 and ZEM-30+DMI-15 showed no antidepressant-like effects in FSL rats versus DMI-15 treated rats. ZEM-

ABSTRACT

10+DMI-15 and ZEM-30+DMI-15 significantly reduced immobility in the FST in FSL rats versus ZEM- (10 & 30 mg/kg/day) monotherapies. FSL rats did not demonstrate anxiety in either the OFT or EPM versus FRL rats. There was significantly increased cortical norepinephrine (NE) levels in FRL rats versus FSL saline control, nevertheless, the hippocampal serotonin (5-HT) levels were reduced in the FRL rats versus FSL saline control. In the DMI-30 treated rats, hippocampal and cortical NE levels were reduced versus FSL saline rats, whereas the hippocampal NE levels were reduced in DMI-15 versus FSL saline rats. ZEM-30 significantly increased frontal cortical NE and 5-HT levels versus the FSL saline control group. Neither DMI (15 mg/kg/day) nor ZEM (10 & 30 mg/kg/day) monotherapies reduced cortical and hippocampal PDE4B levels in FSL rats versus SAL FSL control group. However, ZEM-10+DMI-15 and ZEM-30+DMI-15 combination therapies significantly reduced hippocampal PDE4B levels versus FSL SAL treated rats. ZEM-30+DMI-15 significantly increased cortical PDE4B levels versus FSL SAL treated rats.

Conclusions: FSL rats showed distinct depressive- like characteristics versus FRL controls. DMI alone reverse depressive-like behavioural characteristics in FSL rats. We were unable to confirm the antidepressant-like effects of ZEM in this study, either alone or in combination with DMI. These results may be model-related. Nevertheless, studies on hippocampal PDE4 levels do lend some support for the augmentation potential of ZEM as an add-on therapy for patients responding poorly to standard antidepressants, especially where specific actions on the hippocampal PDE4B are required. Combination of ZEM with antidepressants i.e., NRIs may be depressogenic displaying increased cortical PDE4B levels. However, further work is needed.

Keywords: **Zembrin® PDE4B, Major depressive disorder, Flinders Sensitive Line rat**, **Forced swim test, monoamines**

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LIST OF ABBREVIATIONS

↑	Increase
↓	Decrease
μ-opioid	Mu- opioid receptor
5-HMT	5-hydroxy-Nω-methyptryptamine oxalate
5-HT	5- Hydroxytryptamine (serotonin)
5-HT'ergic	Serotonergic
5-HT_{1A}	Serotonin-1A receptor
5-HT_{1B}	Serotonin- 1B receptor
5-HT_{2A}	Serotonin-2A receptor
$5\text{-HTR}_{1A/1D/7}$	Serotonin receptors
5-HIAA	5- hydroxyindoleacetic acid
α1	Alpha-1 adrenoceptor
α1/2 AR	Alpha 1 or 2-adrenergic receptors
α1AR	Alpha-1 adrenoceptor
β1	Beta-1 adrenoceptor
β2	Beta -2 adrenoceptor
βAR	Beta adrenergic receptors
β-hydroxylase	Beta-hydroxylase enzyme
Bγ	Beta-gamma- subunits
AAALAC	American Association for Accreditation of Laboratory

LIST OF ABBREVIATIONS

	Animal Care
AADC	Aromatic amino acid decarboxylase
AC	Adenylyl cyclase
Ads	Anxiety disorders
Ach	Acetyl choline
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AMPA	Alpha-Amino-3-Hydroxy-5-methyl-4-Isoxazole Propionic– Acid
APA	American Psychiatric Association
ANOVA	Analysis of variance
ARRIVE	Animal Research Reporting of in vivo Experiment's guidelines
BDNF	Brain derived neurotrophic factor
$Ca2^{+}$	Calcium
CAM	Complementary and alternative medicines
Camp	Cyclic adenosine monophosphate
CBT	Cognitive behavioural therapy
Cm	centimetres
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
COVID-19	Coronavirus disease of 2019

LIST OF ABBREVIATIONS

CREB	cAMP- respond element binding protein
CRF	Corticotrophin releasing factor
CUS	Chronic unpredictable stress
CYP 2C19	Cytochrome P450 iso-enzyme 2C19
CYP 450	Cytochrome P450
CYP1A2	Cytochrome P450 iso-enzyme 1A2
CYP2D6	Cytochrome P450 iso-enzyme 2D6
$D_{1/2}$	Dopamine-like 1 or 2 receptor
DA	Dopamine
DAT	Dopamine transporter
DHPG	3,4-Dihydroxyphenylglycol
DFP	Diisopropyl fluorophosphate DFP
DMI	Desipramine
DNB	Dorsal noradrenergic bundle
DCC	Dopa decarboxylase
DOPA	Dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders -5
DST	Department of Science and Technology
ECG	Electrocardiogram
EEG	Electroencephalogram
ECT	Electroconvulsive therapy

LIST OF ABBREVIATIONS

EPM	Elevated plus maze
FDA	Food Drug Administration
fMRI	Functional magnetic resonance imaging
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
FST	Forced swim test
GABA	Gamma- amino- butyric- acid
$GABA_{A/B}$	Gamma-amino-butyric-acid-A/B receptor
GAD	Generalized anxiety disorders
GDNF	Glial cell derived factor
$G_{I/O}$ protein	Inhibitory G-coupled protein
Glu	Glutamate
GLP	Good Laboratory Practice
H_1-receptors	Histamine-1 receptor
HAM-D	Hamilton depression rating scale
HAM-A	Hamilton anxiety rating scale
HPA-axis	Hypothalamic-pituitary-adrenal axis
HPLC-ECD	High-performance liquid chromatography-electrochemical detection
I.P	Intraperitoneal route
I.V	Intravenous route
IDO	Indolamine 2, 3-dioxygenase

LIST OF ABBREVIATIONS

IFN -γ	Interferon-gamma
IFN-α	Interferon-alpha
IL-6,10,12, 17	Interleukin-6,10,12,17
IP3	Inositol 1, 4, 5- triphosphate
IPT	Interpersonal psychotherapy
IVC	Individually ventilated cage
Kg	Kilograms
LAT	Laboratory animal technician
LC	Locus coeruleus
LCSPT	Limbic-cortico-striato-pallidal-thalamic circuit
L-tryptophan	Levo-tryptophan
L-tyrosine	Levo-tyrosine
MAO	Monoamine-oxidase
MAO-A/B	Monoamine-oxidase-A or B enzyme
MAOIs	Monoamine-oxidase inhibitors
MDD	Major depressive disorder
Mg	milligrams
MHGP	3-methoxy-4-hydroxyphenylglycol
$MT_{1/2}$	Melatonin-type 1 and 2 receptor
Mrna	Messenger Ribonucleic acid
NAC	N-acetyl cysteine
NA	Noradrenaline

LIST OF ABBREVIATIONS

NA'ergic	Noradrenergic
NE	Norepinephrine
NET	Norepinephrine transporter
NMDA	N-methyl-D-aspartate
NRIs	Noradrenaline reuptake inhibitors
NWU	North-West University
NWU-AnimCareREC	North-West University Animal Care Research Ethics Committee
OCD	Obsessive compulsive disorder
OFT	Open field test
PD	Panic disorder
PDE4	Phosphodiesterase4
PDE4A/B/C/D	Phosphodiesterase- isoforms A, B,C &D
PET	Positron Emission Tomography
Pharmacen	Centre of excellence for Pharmaceutical Sciences
PKA	Protein kinase A
PTSD	Post-Traumatic Stress Disorder
SA	South Africa
SAD	Social anxiety disorders
SADAG	South African Depression and Anxiety Group
SAL	Normal Saline
SANAS	South African National Accreditation System

LIST OF ABBREVIATIONS

SANS	South African National Standards
SAVC	South African Veterinary Council
SD	Sprague Dawley rats
SERT	Serotonin reuptake transporter
SNRIs	Serotonin and noradrenaline reuptake inhibitors
SSRIs	Selective serotonin reuptake inhibitors
ST	*Sceletium tortuosum*
TCAs	Tricyclic antidepressants
TNF-α	Tissue necrosis factor-alpha
UPLC-MS	Ultra performance liquid chromatography-tandem mass spectrometry
VEGF	Vascular endothelial growth factor
VMAT-2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WHO	World Health Organization
ZEM	Zembrin®

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CHAPTER 1
INTRODUCTION

1.1. BACKGROUND

Most of patients with major depressive disorder (MDD) often present with co-morbid anxiety (Kessler *et al.,*2015), experience a slower response to existing treatment (Goddard *et al.,*2010). Long-term maintenance treatment in these patients is often hampered by premature discontinuation leading to higher risk of relapse (Harvey, 2006). Furthermore, these individuals often show decreased functioning, and increased risk of suicide ideation (Altin *et al.,*2014).

Major depressive disorder remains a serious mood disorder that is difficult to treat, mainly due to a relatively poor understanding of its aetiology and pathogenesis (Jin *et al.,*2018). MDD is characterized by debilitating symptoms that negatively affect daily life activities, including fatigue, hopelessness, impaired memory and cognition, impaired concentration, anhedonia, suicidal thoughts, disturbed sleep, increased or decreased appetite, anxiety, psychomotor retardation, and decreased sex drive (APA, 2013). Progress in neuroscience has identified that although monoaminergic pathways such as noradrenergic, serotonergic, and dopaminergic systems play a key role in the pathophysiology of MDD and for many years have contributed to the development of new antidepressants (Boku *et al.,*2018; Moret and Briley, 2011), the illness is known to involve several of other confirmed and/or suspected biological causes (Brand *et al.,*2015).These include hypothalamic-pituitary- adrenal axis (HPA-axis) abnormalities, dysregulation of the pro-inflammatory and anti-inflammatory cytokines (neuroinflammation), mitochondrial dysfunction, oxidative stress, disturbed circadian rhythm, glutamatergic dysfunction, decreased neurogenesis and neuroplasticity (Dean and Keshvan, 2017). Since It is known that MDD is a multifactorial mental disorder, the current treatment for MDD should involves integration of a multimodal approach based on the combination of the psychosocial, electroconvulsive therapy and pharmacotherapy (McIntyre, *et al.,*2017). Under pharmacotherapy, majority of the current available standard antidepressants including the monoamine-oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs) and atypical antidepressants (Lierberman and Massey, 2009; McIntyre, *et al.,*2017). Unfortunately, only a limited number of patients respond to these

and other standard treatments (Brand *et al.,*2015; Harvey and Slabbert, 2014), highlighting the need for improved antidepressant treatments that target these newly identified pathways.

As noted above, the current standard antidepressants mainly target one or two of the causal mechanisms of MDD leaving most of the patients inadequately treated with less than 50% of patients eventually achieving complete remission (Brand *et al.,*2015; Harvey and Slabbert, 2014). This problem could be due to delayed onset of action (Boku *et al.,*2018), side effects, and non-compliance, but mostly due to inappropriate target engagement (Harvey and Slabbert, 2014; Nahas and Sheikh, 2011). In addition to the aforementioned problems, the treatment of MDD is further complicated by the pathological presentation in any given patient that is often unique, with treatment that assumes a "one size fits all" approach. Thus, the contribution of additional biological processes, for instance mitochondrial dysfunction, oxidative stress, neuroinflammation, disturbed circadian rhythm etc, are not immediately addressed which compromises successful therapeutic outcomes. Over and above this, patient preference, cost of treatment, symptoms profile, clinical factors, drug-drug interaction, safety, and tolerability profile also need to be considered (Haller *et al.,*2019).

Accumulating evidence has since confirmed that antidepressants regulating multiple targets relative to a single target offer a better opportunity to treat MDD more effectively and thus achieve better and faster remission (Liu *et al.,*2018; Jainer *et al.,*2013; Blier *et al.,*2010). This approach has for instance led to the development and marketing of first-in-class agents like ketamine/S-ketamine, agomelatine and vortioxetine that target monoamines in more specific and/or indirect ways but with additional biological targets included, e.g., glutamate and melatonin receptors amongst others (Brand *et al.,*2015). Alternatively, the current practice could comprise of two traditional agents but from different mechanistic classifications (Harvey *et al.,*2011), or combining a traditional agent with complementary and herbal therapies (Haller *et al.,*2019). In fact, the WHO has introduced new incentives to drive the exploration and development of traditional medicines for the treatment of psychiatric illness (WHO, 2013). This is often done to limit or prevent the side effects and costs of traditional anti-depressants, as well as to improve a delayed onset of action and overall clinical outcome (Pilkington *et al.,*2006). However, combining antidepressant drugs with a dual mechanism of action or that combine multiple metabolic pathways especially that involves monoamines (i.e. serotonin and

norepinephrine) may lead to adverse events such as serotonin syndrome and hypertensive crisis (Osuch and Marais, 2017; Pilgrim *et al.,*2011; Thase, 2011). Furthermore, drugs with narrow therapeutic indices or that present with competitive metabolism with the monoamines may also lead to serotonin syndrome due to excessive synaptic stimulation of 5-HT_{1A} or 5-HT_{2A} receptor (Franscescangeli *et al.,*2019; Scotton *et al.,*2019). This is especially an issue with traditional medicines where the mode of action is either unknown or unclear.

Considering serotonin syndrome, this potentially life-threatening toxicity occurs when combining two powerful serotonergic agents, and manifests as hypomania, confusion, agitation, tremor, diarrhoea, fever, hyperreflexia, diaphoresis, shivering, incoordination, tachycardia, etc. (Franscescangeli *et al.,*2019; Boyer and Shannon, 2005). Indeed, this potential drug-herbal or drug-dietary supplement interaction is frequently overlooked as a possible cause of serotonin syndrome (Patel and Marzella, 2017).

1.2 PROBLEM STATEMENT

Earlier study in our laboratory demonstrated that a combination of the SSRI escitalopram with the standardized extract of *Sceletium tortuosum* (ST) (Zembrin®), a herbal product known to exhibit serotonergic actions, has pronounced serotonergic actions that may lead to the risk of a 5-HT syndrome (Gericke, 2019). The risk of this interaction occurring in the clinical context is high, given that traditional agents may inadvertently be combined with over the counter complementary and herbal therapies as treatment.

There is therefore a need for seeking more rapidly acting, safe, and effective monotherapy or combination antidepressant regimens with a broad activity to cover various biological mechanisms of MDD (Dale *et al.,*2015). In this regard, the use of phosphodiesterase-4 (PDE4) inhibitors such as rolipram has been widely considered as potential antidepressant agents by their ability to potentiate the duration of cyclic adenosine monophosphate (cAMP, a second messenger) -mediated signaling (Murthy and Mangot, 2015), and hence to improve antidepressant response. However, rolipram presents with dose-limiting side effects, mostly nausea, emesis, headache, diarrhoea, fatigue, dyspepsia, nasopharyngitis, and gastroenteritis, which severely restricts its therapeutic utility (Kumar *et al.,*2013; O' Donnell and Zhang, 2004; Montana and Dyke, 2002). Currently, there are efforts to improve the safety and efficacy profile of PDE4 inhibitors, such as the new generation PDE4 inhibitor, apremilast, although studies have

not yet been conducted in MDD (Heckaman *et al.,*2018; Kumar *et al.,*2013). Given these setbacks, complementary and alternative medicines (CAM) presenting with PDE4 inhibitory actions are attractive alternatives (Yeung *et al.,*2018).

Interestingly, *Sceletium tortuosum* (ST) holds great promise for future development in the treatment of anxiety and MDD (Gericke and Viljoen, 2008), both as a monotherapy and as adjunctive therapy (Olatunji *et al.,*2021). ST has been used for centuries by the Khoisan hunter-gatherers of South Africa to promote a sense of calm, improve health, as stress relief, reduce anxiety, improve mood, and enhance concentration (Olatunji *et al.,*2021). A recent systematic review (Olatunji *et al.,*2021), previous preclinical (Murbach *et al.,*2014) and clinical (Nell *et al.,*2013) studies revealed that Zembrin® is safe and tolerable.

Multiple targets versus single target engagement offers a better opportunity to more effectively treat MDD (Liu *et al.,*2018). A standardised extract of ST (Zembrin®) has been documented to work by various mechanisms of action under *in vitro* conditions, many of which are antidepressant and anxiolytic in their own right (Harvey *et al.,* 2011). These include serotonin transporter (SERT), DA transporter (DAT), μ-opioid, cholecystokinin-1 , PDE4 (Harvey *et al.,*2011), MAO-A inhibition, upregulation of vesicular monoamine transporter 2 (VMAT-2) (Coetzee *et al.,*2016), and activity at $GABA_{A/B}$ receptors (Olatunji *et al.,*2021). Zembrin® contains a standardized ratio and number of alkaloids, including mesembranol, mesembrine, mesembrenone, and mesembrenol (Gericke *et al.,*2022) of which two main pharmacologically active alkaloids (mesembrine and mesembrenone) have shown to inhibit both SERT and PDE4 activity (Harvey *et al.,*2011). The SSRI-like properties of Zembrin® have also been demonstrated *in vivo* (Gericke *et al.,*2022). The inhibitory actions on SERT have afforded it with viable psychopharmacological potential to treat MDD and anxiety (Gericke and Viljoen, 2008), while its actions on PDE4 presents therapeutic potential in inflammation and specifically neuro-inflammatory conditions (Smith, 2011; Olatunji *et al.,*2021). Moreover, its ability to modulate inflammatory cytokines like IL-1 -and IL-10 (Smith, 2011) reaffirm possible application in treating neuro-inflammation, of which MDD is one such condition of interest (Dean and Keshavan, 2017). Although pro-inflammatory and anti-inflammatory cytokines are released to mediate immune system in response to infections (Leonard and Song, 2002), it is well documented that infections, chronic stress and increased PDE4B activity lead to the dysregulation of cytokine release from microglia and astrocytes resulting in the over-production of the pro-

inflammatory cytokines (i.e., IL-6) while also decreasing anti-inflammatory cytokines (i.e., IL-10) (Wang *et al.,*2017). The balance between IL-6 and IL-10 can induce MDD via various mechanisms, such as excessive glutamatergic neurotransmission, activation of the kynurenine pathway, HPA axis hyperactivity, as well as decreased neurogenesis and neuroplasticity (Kalkman,2019; Brand *et al.,*2015). Therefore, evidence of the PDE4 inhibitory actions of Zembrin® from *in vitro* studies have hinted at the possibility of using it for treatment of MDD and anxiety related to neuro-inflammation (Harvey *et al.,* 2011).

Given the evidence that Zembrin® boosts the serotonergic actions of an SSRI described above, and its associated risk of 5-HT syndrome, it is necessary to re-evaluate this scenario by exploring whether Zembrin® preferentially benefits a non-serotonergic antidepressant such as desipramine (DMI) during adjunctive treatment.

The Flinders Sensitive Line (FSL) rat model of depression has been widely used as a translational model of MDD (Overstreet and Wegener, 2013; Overstreet *et al.,*2005) and for evaluating the antidepressant activity of standard (Mokoena *et al.,*2015; Steyn *et al.,*2018; Brand and Harvey, 2017), as well as herbal products i.e., Zembrin® (Gericke *et al.,*2022) and *Garcinia mangostana Linn* (Oberholzer *et al.,*2018). The model presents with various bio-behavioural symptoms akin to human MDD such as elevated rapid eye movement, reduced appetite and psychomotor function (Overstreet and Wegener, 2013; Overstreet *et al.,*2005), as well as responds preferentially to chronic treatment with a standard antidepressant (Mokoena *et al.,*2015; Overstreet and Wegener, 2013).

The purpose of the current study was to confirm the therapeutic dose and the minimum effective dose of desipramine (DMI) that may be bolstered by Zembrin®. Phase 1 involved a behavioural validation of the FSL rat model with respect to MDD versus control, Flinders Resistant Line (FRL) rats. Phase 2 evaluated the predictive validity of Zembrin® using a sub-chronic 3-tier dose response (10, 25, and 30mg/kg/day), and a 2-tier dose of DMI (30 mg/kg/day-high dose) (15 mg/kg/day-low dose) versus saline. Sub-chronic treatment with ZEM-10 or ZEM-30 could produce divergent results although speculative, therefore, it was important to assess these two (low and high) doses for augmentation potential to low dose DMI. Finally, Phase 3 investigated the antidepressant activity of the combination of low dose DMI (15 mg/kg/day) with 2-tier doses of Zembrin® (10 and 30 mg/kg/day). In all instances, locomotor activity, depressive- and anxiety-like behaviour were measured to assess the anxiolytic-like and antidepressant-like effects of the aforementioned drugs and

combinations. Finally, hippocampal, and frontal cortex monoamines together with the PDE4B levels were also determined.

1.3 AIM OF THE STUDY

The study aimed to assess the dose related antidepressant and anxiolytic -like effects of Zembrin® extract in the FSL rat alone and against a reference standard antidepressant, DMI, as well as an adjunctive treatment together with a low dose of DMI.

1.3.1 Objectives

1. To validate the FSL rat model vs FRL rats with respect to locomotor, depressive- and anxiety-like symptoms using the OFT, FST, and EPM, respectively, while simultaneously assessing hippocampal and cortical monoamines, as well as hippocampal and cortical PDE4B levels.
2. To compare the dose-related efficacy of monotherapy with Zembrin® (10, 25 and 30) mg/kg/day and DMI (15 and 30 mg/kg/day) versus saline control in FSL rats after 14 days' sub-chronic treatment on the above-mentioned bio-behavioural markers.
3. To assess whether a high and low dose of Zembrin® (identified in 2 above) can augment the response to a low dose of DMI (15 mg/kg/day) in FSL rats with respect to the above-mentioned bio-behavioural markers.

1.4 STUDY LAYOUT

In summary, this sub-chronic treatment study used 96 male FSL and 12 male control FRL rats. The study was divided into three (phases). In Phase I, 24 rats (12 FSL and 12 FRL) were used for validation of the FSL rat model for MDD and anxiety-related bio-behavioural manifestations. Both groups of FRL and FSL rats were treated with saline at a constant dosing volume of 10ml/kg bodyweight (bw). given via oral gavage over 14 days. Twelve (12) FSL rats from phase I were used in Phase 2 as control group. To evaluate the dose-dependent antidepressant-like and anxiolytic-like effects of Zembrin® and/or DMI, sixty (60) FSL rats were randomly divided into five groups, as follows; Group *(III)* treated with Zembrin® low dose (10 mg/kg/day) given via oral gavage. Group *(IV)* treated with Zembrin® medium dose (25 mg/kg/day) given via oral gavage. Group *(V)* treated with Zembrin® high dose (30 mg/kg/day) given via oral gavage. Group *(VI*) treated with low dose of DMI (15 mg/kg/day) given via oral gavage, and Group *(VII)* treated with high dose of DMI (30 mg/kg/day) via oral gavage.

Behavioural analysis across the groups was assessed with the OFT, FST, and EPM. The monoamine analyses (measured with HPLC-ECD) for ZEM treatment groups (10, 25 and 30 mg/kg/day) were done for selection of two doses to be used in augmentation study. To evaluate the antidepressant-like and anxiolytic-like effects of a combination of Zembrin® low dose (10 mg/kg/day) and low dose of DMI (15 mg/kg/day) or a combination of Zembrin® high dose (30 mg/kg/day) and low dose of DMI (15 mg/kg/day). Twenty-four (24) FSL rats were randomly divided into two groups as follows; Group *(VIII)* treated with the combination of Zembrin® low dose (10 mg/kg/day) and low dose of DMI (15 mg/kg/day) via oral gavage, and Group *(IX)* treated with a combination of Zembrin® high dose (30 mg/kg/day) and low dose of DMI (15 mg/kg/day) via oral gavage. Behavioural analysis across the groups was assessed with the OFT, FST, and EPM. Later on, the monoamine analyses were done for remaining treatment groups.

24 hours after the last treatment, the animals were decapitated, the hippocampus and frontal cortex collected and stored at -80°C until the day of bioanalysis of regional monoamines and PDE4B. Immediately after decapitation, trunk blood samples were also collected in 10ml EDTA tubes and centrifuged at 5500 rotations per minute (rpm) for 10 minutes at 4°C and stored at -80°C for analysis of plasma cytokines (IL-6 and IL-10).

1.5 HYPOTHESIS

In this sub-chronic pharmacotherapy study, we propose that FSL rats will display hypo-locomotor and depressive- and anxiety-like behaviours as measured in the OFT (decreased locomotor activity), and FST (decreased climbing/struggling and swimming, and increased immobility) and EPM (decreased exploration of open arms). Neurobiologically, we propose that decreased hippocampal and frontal cortical monoamines, increased hippocampal and frontal cortical PDE4B levels will be observed in FSL rats, which will correlate with depressive- and anxiety-like behaviour. All these bio-behavioural changes will show dose-dependent reversal by monotherapy with low (15 mg/kg/day) and high dose (30 mg/kg/day) of DMI and Zembrin® (10, 25 and 30 mg/kg/day). Sub-chronic adjunctive treatment with low dose (10 mg/kg/day) or high dose (30 mg/kg/day) of Zembrin® and low dose of DMI (15 mg/kg/day) will show equal or better antidepressant-and anxiolytic-like effects versus either treatment alone.

1.6 EXPECTED OUTCOMES

We expect FSL rats to display significantly decreased locomotor activity in OFT, less active coping (climbing and swimming), increased immobility in FST, and decreased exploration of the open arms in the EPM, versus their FRL controls. These are indicative of depressive- and anxiety-like behaviour. From the neurochemical analysis we expect saline treated FSL rats to show reduced hippocampal and cortical monoamines, and increased hippocampal and cortical PDE4B levels

Thereafter we expect Zembrin® to show dose-dependent antidepressant and anxiolytic-like effects in FSL rats by reversing the above-mentioned bio-behavioural changes following 14 days treatment.

Regarding DMI treatment alone, we expect to see dose-dependent reverse of most if not all these bio-behavioural changes in FSL rats, although the effects will not be better than with Zembrin®.

We expect Zembrin® to dose-dependently reverse most if not all the bio-behavioural changes in FSL rats similar and/or better than DMI alone, while the combination of Zembrin® plus low dose of DMI will show greatest benefit comparable to ZEM and/or low dose DMI alone. This will differ because multiple pharmacological mechanisms of action increase remission rates by synergizing with other neurotransmitter systems (Sarris *et al.,*2021).

Finally, a combination of Zembrin® and DMI will produce similar if not better effects than either monotherapy in reversing said bio-behavioural changes following repeated administration over 14 days.

Overall, we expect to see increased PDE4B levels in FSL versus FRL rats in both brain regions (frontal cortex and hippocampus), while this will be associated with reduced cortico-hippocampal monoamines as well as co-presenting symptoms of anxiety and depression. Zembrin® in turn will dose-dependently reverse most if not all these bio-behavioural changes as earlier study in our laboratory showed dose-dependent antidepressant effect of Zembrin® following acute treatment (Gericke *et al.,*2022), while combined low dose Zembrin® plus DMI will show the greatest benefit, especially in augmenting low dose DMI.

1.7 ETHICAL CONSIDERATIONS

All experimental procedures described in this study were approved by the North-West University Animal Care Research Ethics Committee (NWU-AnimCareREC) (NHREC reg. number AREC-130913-015) **(ethics approval number: NWU-00520-20-A5).** Animals were bred, supplied, and housed at the vivarium (SAVC registration number FR15/13458; SANAS GLP compliance number G0019, AAALAC accredited file # 1717) of the Pre-Clinical Drug Development platform of NWU, established by the National Department of Science and Innovation (DSI). The animals were housed in groups of 2 to 3 rats per cage in polypropylene individually ventilated cages (IVC) using a standard vivarium-approved nesting material. Food and water were supplied *ad libitum*. The animals were maintained under environmental control with a constant ambient temperature of 25±1°C and relative humidity of 55% ± 10%, with a full spectrum of light in a 12h/12 h light-dark cycle (lights switched on at 06:00 and off at 18:00) and a positive air-pressure and air exchange rate of 18/hr. All procedures were performed following the code of ethics in research, training, and testing of drugs in South Africa. The animals were handled from PND-21 during weaning and daily during weighing to monitor the weight and to habituate them to human handling prior to drug administration. The wellbeing of the animals was monitored daily and regularly after behavioural testing using a general animal monitoring sheet (see Addendum G). The overall experimental procedures and data reporting in this study were compliant with the national legislation and guidelines, including the South African National Standard (SANS): The care and use of animals for scientific purposes (SANS10386; 2008) and Animal Research Reporting of in vivo Experiments (ARRIVE) guidelines were followed throughout the study to ensure transparent reporting and reproducible data from this study. The 4Rs concept was applied to ensure that animals were handled well, and research conducted humanely, as follows:

Replace: The FSL rat model and its control FRL rat have been widely used for behavioural research on MDD and for determining the therapeutic properties of novel antidepressants (Overstreet and Wegener, 2013). Different non-animal alternatives such as computer models, tissue cell culture, and micro-dosing were considered to replace the use of *in vivo* animal models (Doke and Dhawale, 2015; Balls, 2002), although such methods have distinct translational limitations and can only complement, rather than replace the use of a well-validated *in vivo* animal model. The FSL rat model was therefore

selected as an ideal model to achieve the objectives of this study and cannot be replaced with non-animal alternatives.

Refine: All the experiments and procedures in this study were performed as reviewed and approved by the NWU-AnimCareREC. All techniques and methodologies have been refined in that only validated methods and techniques, as well as ethics-approved standard operating procedures (SOPs), were applied to minimize distress and pain to the animals.

Reduce: Statistical power analysis was used prior to beginning the study to determine the sample size required in this study and only those animals required were used for obtaining statistically significant results.

Responsible: All researchers have undergone the required ethics training with AnimCareREC as well as the SAVC, with the student responsible for monitoring the animal's health and welfare completing the general animal welfare monitoring sheets daily. The animals were handled under the supervision of a veterinarian and laboratory animal technicians (LAT).

References

Altin, M., Harada, E., Schacht, A., Berggren, L., Walker, D., Duenas, H. 2014. Does early improvement in anxiety symptoms in patients with major depressive disorder affect remission rates? A post-hoc analysis of pooled duloxetine clinical trials. *Open Journal of Depression*, 3(3):112-123

American Psychiatric Association. 2013. Diagnostic and Statistical Manual of Disorders 5th Edition (DSM-5). Washington, D.C. https://www.psychiatry.org/psychiatrists/practice/dsm Date of access: 05. May.2020

Balls, M. 2002. Future improvements; replacement in in vitro methods. *Institute for Laboratory Animal Research*,43: 69-72

Blier, P., Ward, H.E., Tremblay, P., Larberge, L., Hebert, C., Bergeron, R. 2010. Combination of antidepressant medication treatment initiation for major depressive disorder: A double- blind randomized study. *American Journal of Psychiatry*,167(3): 281-288

Boku, S., Nakagawa, S., Toda, H., Hishimoto, A. 2018. Neural basis of major depressive disorder; beyond monoamine hypothesis. *Psychiatric and Clinical Neurosciences*,72: 3-12.

Brand, S.J., Harvey, B.H. 2017a. Exploring a Post-traumatic stress disorder paradigm in Flinders Sensitive Line rats to model treatment resistant depression I; bio-behavioural validation and response to imipramine. *Acta Neuropsychiatrica*, 29(4):193-206

Brand, S.J., Moller, M., Harvey, B.H. 2015. A review of biomarkers in mood and Psychotic of disorders: A dissection of clinical vs pre-correlates. *Current Neuropharmacology*, 13(3): 324-368

Coetzee, D.D., Lopez, V.V., Smith, C. 2016. High –Mesembrine Sceletium extract (Trimesemine™) is a monoamine releasing agent, rather than only a selective serotonin reuptake inhibitor. *Journal of Ethnopharmacology*, 177: 111-116

Dale, E., Bang-Anderson, B., Sanchez, C. 2015. Emerging mechanism and treatment for depression beyond SSRIs and SNRIs. *Biochemical Pharmacology*, 95: 81-97

Dean, J., Keshavan, M. 2017. The neurobiology of depression; an integrated view. *Asian Journal of Psychiatry*, 27: 101-111

Doke, S.K., Dhawale, S.C. 2015. Alternatives to animal testing; a review. *Saudi Pharmaceutical Journal*, 23:223-229

Franscescangeli, J., Karamchandani, K., Powell, M., Bonavia, A. 2019. The serotonin syndrome; Molecular mechanism for clinical practice. *International Journal of Molecular Science*, 20(9): 2288

Gericke, J. 2019. Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, A.M., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:114550

Goddard, A.W., Ball, S.G., Martinez, J., Robinson, M.J., Yang, C.R., Russel, J.M., Shekhar, A. 2010. Current perspectives of the roles of central norepinephrine system in anxiety and depression. *Anxiety and Depression,* 27(4):339-350.

Haller, H., Anheyer, D., Cramer, H., Dobos, G. 2019. Complementary medicine for clinical depression; an overview of systematic reviews. *British Medical Journal Open*, 9: 1-9

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P. 2011. Pharmacological actions of South African medicinal and functional plant *Sceletium tortuosum* and its principle alkaloids. *Journal of Ethnopharmacology*,137: 1124-1129.

Harvey, B.H. 2006. Adaptive plasticity during stress and depression and the role of glutamate-nitric oxide pathways. *South African Psychiatry Review*, 9: 132-139

Harvey, B.H., Slabbert, F.N. 2014. New insight on the antidepressant discontinuation syndrome. *Human Psychopharmacology: Clinical and Experimental*, 299(6): 503-516

Jainer, A.K., Kamatchi, R., Martzanski, M., Somashekar, B. 2013. Current Advances in the treatment of Major Depression: Shift towards receptor specific drugs. Mental Disorder- Theoretical and Empirical Perspectives.*Intech,* 12:270-288

Jin, Y., Sun, L.H., Yang, W., Cui, R.J., Xu, S.B. 2019. The role of BDNF in the neuroimmune axis regulation of mood disorders. *Frontiers in Neurology*,10 (515): 1-19

Kalkman, H. O. 2019. Novel Treatment Targets Based on insights in the etiology of depression; Role of IL-6 trans-signaling and stress-induced elevation of glutamate and ATP. *Pharmaceuticals*, 12(3): 113

Kessler, R.C., Sampson, N.A., Berglund, P., Gruber, M.J., AL-Hamzawi, A., Andrada, L., Bunting, B., Demyttenaere, K., Florescu, S., De Girolamo, G., Gureje, O., He, Y., Hu, C., Huang, Y., Karam, E., Kovess-Masfety, V., Lee, S., Levinsom, D., Medina Mora, M.E., Moskalewicz, J., Nakamura, Y., Navarro-Mateu, F., Browne, M.A.O., Piazza, M., Posada-Villa, J., Slade, T., Ten Have, M., Torres, Y., Vilagut, G., Xavier,M., Zarkov, Z., Shahly, V., Wilcox, M.A.2015. Anxious and non-anxious major depressive disorder in the World Health Organization World Mental Surveys. *Epidemiology and Psychiatric Sciences*, 24:210-226

Kumar, N., Goldminz, M.A., K, N., Gottlieb, A. B. 2013. Phosphodiesterase 4-targeted treatments for autoimmune diseases. *BMC Medicine*, 96 (11): 1-8

Leonard, B.E., Song, C. 2002. Changes in the immune system in rodents' model of depression. *International Journal of Neuropsychopharmacology*, 5: 345-356

Li, H., Zuo, J.,Tang, W. 2018. Phosphodiesterase4 inhibitor for treatment of inflammatory diseases. *Frontiers in Pharmacology*, 1048(9):1-21

Lieberman, D.Z., Massey, S.H. 2009. Desvenlafaxine in major depressive disorder: an evidence-based review of its place in therapy. *Core Evidence Dovepress*, 4: 67-82

Mclntyre, R.S., Suppes, T., Tandon, R., Ostacher, M. 2017. Florida Best Practice psychotherapeutic medication guideline for adults with major depressive disorder. *The Journal of Clinical Psychiatry*, 78(6):703-713

Mokoena, M.L., Harvey, B.H., Viljoen, F., Ellis, S.M., Brink, C.B. 2015.Ozone exposure of Flinders Sensitive Li ne rats is a rodent translational model of neurobiological

oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232(1): 2921-2938.

Montana, J.G., Dyke, H.J. 2002. Update on the therapeutic potential of PDE-4 inhibitors. *Expert Opinion on Investigational Drug*, 11(1): 1-13

Moret, C., Briley, M. 2011. The importance of noradrenaline in depression. *Neuropsychiatric Disease and Treatment*, 7(1):9-13.

Murbach, T.S, Hirka, G., Szakonyine, I.., Gericke, N., Endres, J.R. 2014. A toxicological safety assessment of a standardized extract of *Sceletium tortuosum* (Zembrin ®) in rats. *Food and Chemical Toxicology*, 74:190-199

Murthy, V.S., Mangot, A.G. 2015. Psychiatric aspect of phosphodiesterase's. *Indian Journal of Pharmacology*, 47(6): 594-599

Nahas, R., Sheikh, O. 2011. Complementary and alternative for the treatment of major depressive disorder; clinical review. *Canadian Family Physician*, 5: 659-663

Nell, H., Siebert, M., Chellan, P., Gericke, N. 2013. A randomized, double-blind parallel-group, Placebo-controlled trial of extract *Sceletium tortuosum* (Zembrin®) in healthy adults. *Journal of Alternative and Complementary Medicine*,19(11):898-904

O' Donnell, J.R., Zhang, H.T. 2004. Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE). *Trends in Pharmacological Sciences*, 25(3): 158-163

Oberholzer, I., Moller, M., Holland, B., Dean, O.M., Berk, M., Harvey, B.H. 2018. Garcinia of depression Linn displays antidepressant-like and pro-cognitive effects in a genetic animal model of depression; a bio-behavioural study in the Flinders Sensitive Line rat. *Metabolic Disease*, 33(2): 467-480

Olatunji, T.L., Siebert, F., Adetunji, A., E., Harvey, B.H., Gericke, J., Hamman, J.H., Van der Kooy, F. 2021. *Sceletium tortuosum*; A review on its phytochemistry, pharmacokinetics, biological and clinical activities. *Journal of Ethnopharmacology*, 280:114476

Osuch, E., Marais, A. 2017. The pharmacology management of depression. *South African Pharmaceutical Journal*, 84 (2): 36-45

Overstreet, D.H., Wegener, G. 2013. The Flinders Sensitive Line rat model of depression -25 years and still producing. *Pharmacology Review,* 65(1): 143-155

Overstreet, D.H., Friedman, E., Mathe, A.A., Yadid, G. 2005. The Flinders Sensitive Line rat; A selectively bred putative animal model of depression. *Neuroscience and Bio-behavioural Reviews*, 29: 739-759

Patel, Y.A., Mazella, N. 2017. Dietary supplement drug interaction induced serotonin syndrome progressing to acute compartment syndrome. *American Journal of Case Reports*, 18: 926-930

Pilgrim, J.L., Gevostamoulos, D., Drummer, O.H. 2011. Pharmacogenetics aspects of the effects of cytochrome P450 polymorphism on serotonergic drug metabolism response, interaction, and adverse effects. *Forensic Science Medicine and Pathology,* 7(2):162-184

Pilkington, K., Rampes, H., Richardson, J. 2006. Complementary medicines for depression. *Expert Review of Neurotherapeutics*, 6(11):1741-1751

Sarris, J., Marx, W., Ashton, M.M., Ng, C.H., Psych, M., Galvao-coelho, N., Ayati, Z., Zhang, Z., Kasper, S., Ravindra, A., Harvey, B.H. 2021. Plant-based medicines (Phytoceuticals) in the treatment of psychiatric disorders; a meta-review of meta-analyses of randomized controlled trials. *The Canadian Journal of Psychiatry*, 66(10):849-862

Scotton, W., Hill, L., Williams, A.C., Barnes, N, M. 2019. Serotonin syndrome, pathophysiology, clinical features, management, and potential future directions. *International Journal of Tryptophan Research*, 12: 1-14

Smith, C. 2011. The effects of *Sceletium tortuosum* in an *in vivo* model of psychological stress. *Journal of Ethnopharmacology*, 133(1): 31-36.

SANS (South African National Standards).2008. The care and use of animals for scientific purposes. Pretoria: SABS standards Division. (SANS10386):180-187

Steyn, S.F., Harvey, B.H., Brink, C.B. 2018. Immediate and long-term antidepressive-like effects of pre-pubertal escitalopram and omega-3 supplementation combination in young adult stress-sensitive rats. *Behavioural Brain Research*, 351:49-62.

Thase, M.E. 2011. Antidepressant combinations widely used, but far from empirically validated. *Canadian Journal of Psychiatry*, 56 (6):317-323

Wang, C., Wang, Z., Li, M., Li, C., Yu, H., Zhou, D., Chen, Z. 2017, 'Reducing Neuroinflammation in Psychiatric Disorders: Novel Target of Phosphodiesterase 4 (PDE4) and Developing of the PDE4 Inhibitors', in G. E. A. Abreu (ed.), Mechanisms of Neuroinflammation, *IntechOpen*, London. 10.5772/intechopen.69154. http://www.intechopen.com/books/mechanisms-ofneuroinflammation Date of access: 06. Jun.2021

World Health Organisation (WHO)., 2013. WHO traditional medicine strategy 2014-2023. https://www.who.int/publications-detail-redirect/9789241506096 Date of access: 19. Nov.2021

World Health Organization (WHO). 2017. Depression and other common mental disorders: Global Health Estimates. Geneva. https://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2 Date of access: 05. May.2020

Wu, T., Xiaoqian, J., Huifeng, S., Jieqion, N., Yin, J. X., Wang, X. 2021. Prevalence of mental health problems during the COVID-19 pandemic; A systematic review and metal analysis. *Journal of Affective Disorders*, 281:91-98

Yeung, K.S., Hernandez, M., Mao, J.J., Haviland, I., Gubili, J. 2018. Herbal medicines for depression and anxiety; systematic review assessment of potential psycho-oncologic relevance. *Phytotherapy Research*, 32 (5): 865-891

CHAPTER 2

Literature review

This chapter provides an overview of the literature on matters relating to the comorbidity of anxiety disorders (ADs) and major depressive disorder (MDD) in adults and will cover aspects such as epidemiology, signs and symptoms, diagnostic criteria, and current pharmacotherapy. In addition, this entails a brief overview of the biological theories underlying the pathophysiology of MDD, but with more intensive discussion on the role of the noradrenergic system, as well as relevant groupings of clinically used antidepressants, but with deeper discussion on agents relevant to this study, viz. noradrenaline reuptake inhibitors (NRIs) and phosphodiesterase 4 inhibitors (PDE4 inhibitors). Finally, a brief discussion of animal models of MDD was provided.

2.1. EPIDEMIOLOGY AND PREVALENCE OF MAJOR DEPRESSIVE DISORDER (MDD)

Major depressive disorder (MDD) is a common mood disorder widely distributed in the population and is among the leading causes of disease burden globally and in South Africa (Nglazi *et al.,*2016). It is predicted to be the major component of disease burden by 2030 (Albert, 2015). The proportion of the population with MDD globally is 10-20% a figure reported prior COVID-19 pandemic (Kessler *et al.,*2007). While MDD can occur at any age, its prevalence rate peaks in older adult patients aged between 55 and 74 years (WHO, 2017). Studies reveal that MDD is the leading cause of disability and suicide attempts worldwide, especially in patient's aged between 15 and 29 years (WHO, 2017; Bilsen, 2018). Suicide is the second leading cause of death among people aged 15-29 years old, with over 800 000 estimated mortalities globally (Thornton *et al.,* 2019;WHO, 2017). The MDD affects over 300 million people world-wide (WHO, 2017), and while it can occur at any age, its prevalence rate peaks mostly in older adult patients aged between 55 and 74 years (WHO, 2017). The South African Depression and Anxiety Group (SADAG) considers MDD as one of the most prevalent of mental disorders in South Africa (SADAG, 2020), with approximately 20% of South Africans expected to experience a major depressive disorder once in their lifetime (SADAG, 2020).

Recently the detrimental impact of the Covid-19 pandemic and associated lockdown restrictions has seen the global prevalence of MDD increase to 31.4%, this due to

financial stress, anxiety, and social isolation (Wu *et al.,*2021), with over 177 million total confirmed cases and 3.84 million deaths reported to date worldwide (18th June 2021) (www.worldometers.info).

In South Africa, review studies showed that an estimate of 9.8% of the adult population had experience MDD in their lifetimes (Cuadros *et al.,*2019; Mungai and Bayat, 2019) before the COVID-19 pandemic (Ngelazi *et al.,*2016), while a recent review study describes the prevalence of MDD at 33%, this as a result of lockdown and other measures to combat the spread of COVID-19 (Nguse and Wassenaar, 2021). MDD is a major national health concern that contributes 7.2% of health loss or disease burden (WHO, 2017). The COVID-19 pandemic is ceaselessly affecting the health and economy of South Africa (Nguse and Wassenaar, 2021). To this date (18th June 2021) there has been a total of over 1.79 million confirmed cases with over 58,000 deaths in South Africa (www.worldometers.info).

2.2. SIGNS, SYMPTOMS AND DIAGNOSIS OF MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) is a mood disorder characterised by persistently depressed mood or loss of interest in activities ultimately leading to significant impairment in daily life (Otte *et al.,*2016) that can negatively affect the feelings, behaviour, and emotions of an individual and lead to a variety of emotional and physical problems (APA, 2013). Major depressive disorder is characterized by anhedonia (loss of interest in daily activities), depressed mood, impaired concentration or decision making, changes in sleep patterns (hypersomnia or insomnia), changes in appetite (increased or decreased), weight gain or loss (a change of > 5%), fatigue, psychomotor retardation, feeling of hopelessness or worthless and suicidal ideation or thoughts (Oscu and Marais, 2017; APA, 2013). Importantly, additional symptoms such as anxiety, headache, muscle aches, pain, and irritability may also diminish the patient's quality of life (Alev *et al.,*2013).

The diagnosis of MDD is based primarily on the criteria of the American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criteria for MDD (APA, 2013). According to these criteria (Table 2.1), at least 5 of the 9 symptoms of MDD must be present and sustained for at least two weeks, and such symptoms must significantly diminish the functioning and quality of life, or impair patient social, occupational, or family relationships (APA, 2013).

Table 2.1. Diagnostic criteria for MDD as outlined in DSM-V (APA, 2013)

Core criteria (A)	Additional criteria
1. Depressed mood- most time of the day, nearly every day characterised by feeling of sad, hopelessness or tearful. 2. Loss of interest or pleasure in almost all the activities in most time of the day or nearly every day	3. Significant weight loss (when not in diet) or gain (a change > 5%) or changes in appetite 4. Changes in sleep nearly every day (hypersomnia or insomnia) 5. Psychomotor retardation nearly every day 6. Fatigue or loss of energy nearly every day 7. feeling of worthlessness or guilt nearly every day 8. Impaired concentration nearly every day 9. Suicidal ideation or thoughts with or without a specific plan to committing suicide
B. the above clinical symptoms must diminish the patient role functioning and quality of life or cause impairment in social and occupational functions	
C. The episode must not be associated with any medical condition or physiological effects of any substance such as alcohol or substance of abuse.	

2.3 THE PATHOPHYSIOLOGY OF MAJOR DEPRESSIVE DISODER (MDD)

Different pathophysiological changes during MDD in different brain regions and neurochemical processes has promulgated various hypotheses in antidepressant research (Kharade *et al.,* 2010), which are presented in summarized form in *Table 2.2.*

While some of these pathological processes may present subsequent pathways set in motion during the development of the disorder, others may occur concurrently.

2.3.1 The neuroanatomy of Major depressive disorder (MDD)

Different brain regions are responsible for different symptoms of MDD such as anhedonia (ventral striatum), cognitive impairment (hippocampus), difficulty in concentration and poor decision making (prefrontal cortex, anterior cingulate, orbitofrontal cortex and sub-genual cingulate), fatigue, sleep disturbance, changes in appetite (hypothalamus and pituitary), fear and anxiety (amygdala) (Marije *et al.,*2009). A review study showed that the limbic-cortico-striato-pallidal-thalamic circuit (LCSPT) play important role in neuropathophysiology of MDD (Helm *et al.,*2018). These connections consist of the orbital medial prefrontal cortex, hippocampus, amygdala, striatum, thalamus and ventral pallidum (Helm *et al.,*2018). Previous studies showed changes in grey matter volume and neurophysiological activity on the brain regions making these network in patients with MDD (Drevets *et al.,*2008; Zhang *et al.,*2021)..

Major depressive disorder is associated with volume reduction in the grey matter in the anterior cingulate and orbitofrontal cortex (Ballmaier *et al.,*2004; Treadway *et al.,*2015) as well as in the subgenual cingulate (Marije *et al.,*2009; Botteron *et al.,*2002). Steingard and co-workers (2002) reported reduced volume of the white matter in frontal cortex coupled to increased volume of the gray matter specifically in left-sided brain region (Bremner *et al.,*2002). These abnormalities may cause mood, emotion, impulse and social dysregulation (Brand *et al.,*2015; Bremner *et al.,*2002) and poor clinical outcomes with the standard antidepressants (Zhang *et al.,*2018).

Furthermore, impaired hippocampal function, due to a reduction in the dendritic neurogenesis (Sheline, 2003) and hippocampal atrophy (Nestler *et al.,*2002), has been correlated with decreased hippocampal volume (Liu *et al.,*2017). These structural changes contribute to the memory problems which manifest as memory impairments (Anacker and Hern, 2017). Previous studies showed that chronic stress may also contribute to the structural changes leading to MDD mainly through activation of the hypothalamic-pituitary-adrenal axis (HPA-axis), leading to release of cortisol (Marije *et al.,*2009).

Chronic stress may activate (HPA-axis) and increase the release of corticotropin releasing factor and stress hormone (cortisol) which may lead to structural changes in the hippocampus (i.e., reduced neurogenesis and hippocampal atrophy producing abnormal negative feed-back to the HPA -axis (Feltes *et al.,*2017). Cortisol stimulates glucocorticoids receptors on the prefrontal cortex, hippocampus and amygdala and attenuates the negative feedback which results in MDD (Mikulska *et al.,*2021). Earlier review studies showed inconsistency in the data regarding abnormalities of the amygdala in MDD (Marije *et al.,*2009; Frodl *et al.,*2003). Marije *et al.* (2009) reported reduced volume and increased amygdala activity in MDD, whereas Frodl and co-workers (2003) observed increased amygdala volumes in patients with first episode of MDD. In fact, the amygdala stimulates the HPA -axis via noradrenergic and serotonergic transmission, and attenuates the negative feedback exerted by cortisol leading to hyperactivity of the HPA -axis seen leading to MDD (Mikulska *et al.,*2021).

Previous studies showed that ventral striatum relates to multiple brain regions in the limbic system (i.e., hippocampus and amygdala) (Marije *et al.,*2009) and cortex (i.e., anterior cingulate cortex and prefrontal cortex) (Ng *et al.,*2019), such that its functional abnormalities lead to dysfunction in the reward circuit (Ng *et al.,*2019). Meta-analysis study reported inconsistencies in literature which identify the role of ventral striatum in pathophysiology of MDD (Ng *et al.,*2019). However, convergent evidence showed that anhedonia is associated with reduced dopaminergic function in the ventral striatum (Arrondo *et al.,*2015).

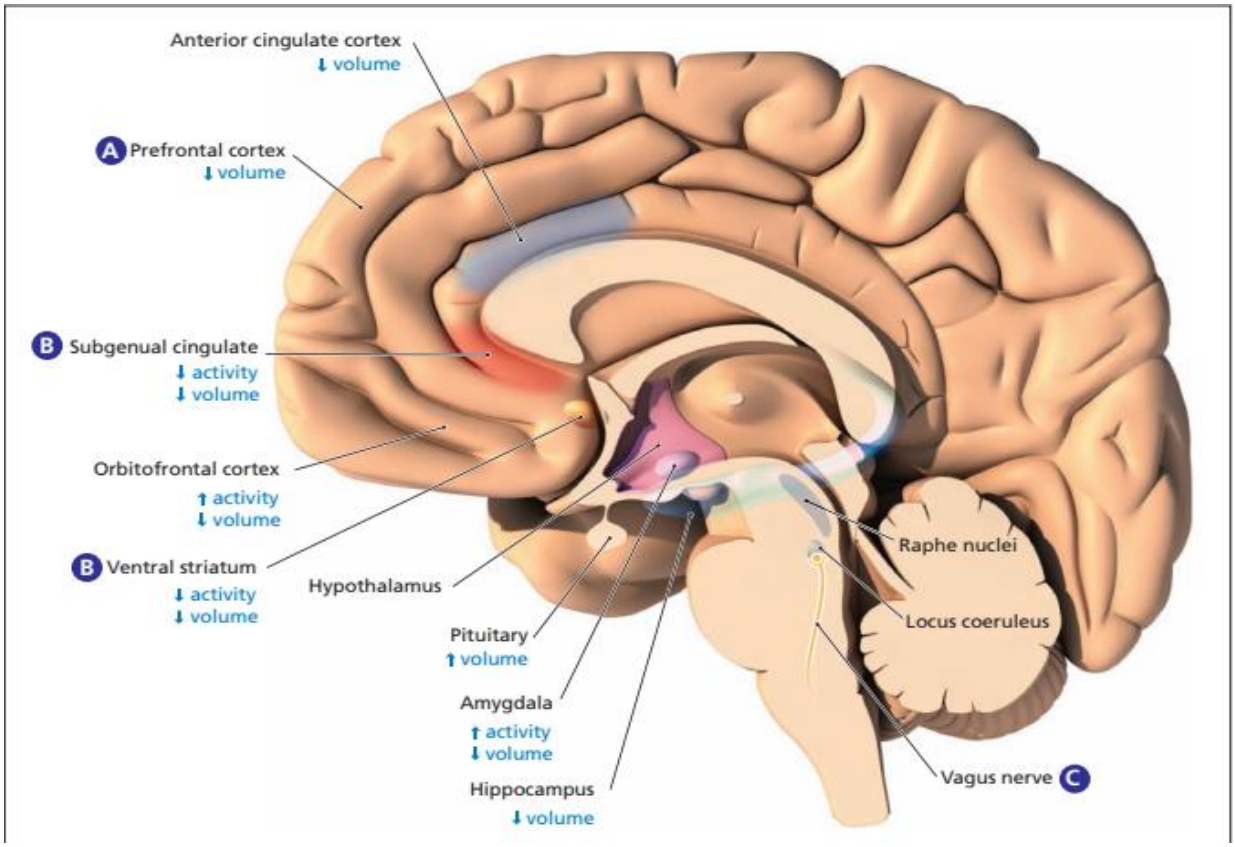

Fig. 2.1. Major brain regions associated with major depressive disorder (Adapted from Marije *et al.,*2009).

Overall, all the above abnormalities and structural changes contribute to decreased synaptic concentration of monoamine neurotransmitters, a mainstay of the monoamine hypothesis (Anecker and Hen, 2017; Belujon and Grace, 2017; Marije *et al.,*2009).

Table. 2.2.

Summary of hypotheses of major depressive disorder, preclinical and biological evidence supporting the hypotheses

Reference	**Hypotheses**	**Preclinical evidence**	**Biological evidence in humans**
Goddard *et al.,*2010 Jainer *et al.,*2013	**Monoamine** The pathophysiology of MDD is associated with depletion of NE, 5-HT, and DA in the synapse	$5\text{-}HT_{1A}$,$5\text{-}HT_{2A/C}$ are ↑ α_{A2} adrenoceptors are ↑ D_2 autoreceptors are ↑	Standard antidepressants elevate monoamines
Feltes *et al.,*2017	**Hypothalamic-pituitary-adrenal-axis (HPA-axis/stress)** MDD is associated with hyperactivity of HPA-axis due to chronic stress	↑ CRF and cortisol in animals CRF antagonist reverse elevated CRF	↑ CRF and cortisol in MDD patients and respond to CRF antagonist
Wang *et al.,*2017	**Neuro-inflammation** Increased pro-inflammatory cytokines and activation of kynurenine pathway	↑ IL-6 and ↓ tryptophan and serotonin	Antidepressants ↓ IL-6 and ↑ serotonin in depressed patients

Yang *et al.,*2020	**Neuroplasticity and neurogenesis** Impaired neuroplasticity and neurogenesis	↓neuroplasticity and neurogenesis ↓ BDNF ↓ hippocampal volume	Antidepressants ↑ neuroplasticity, neurogenesis, BDNF and reverse hippocampal atrophy
Dale *et al.,*2015	**Cholinergic/adrenergic** MDD is associated with hyperactivation of cholinergic system and decreased activity of noradrenergic system	Muscarinic-3 (M_3) receptors are ↑	Muscarinic antagonists (scopolamine) and TCAs reduce MDD symptoms
Wang *et al.,*2017; O'Donnell and Zhang, 2004	**Phosphodiesterase 4 (PDE-4) activity** MDD is associated with overexpression of PDE-4	↑ PDE-4 over expression ↓ cAMP ↑ IL-6 coupled with ↓-IL 10	PDE4 inhibitors ↓ PDE-4 expression, reverse IL-6 and -IL 10 balance in MDD patients
Dale *et al.,*2015	**GABA** MDD is associated with reduced GABA neurotransmission in the brain	$GABA_A$ (inhibition)	Antidepressants may indirectly activate $GABA_A$ receptors and restores reduced GABA neurotransmission in depressed patients
Brand *et al.,*2015	**Glutamate** MDD is associated with prolonged glutamate excitation on the N-methyl-D- Aspartate (NMDA) receptors		MDD patients respond to ketamine

2.3.2 Synthesis, storage, release, and metabolism of the biogenic monoamines

The monoamine hypothesis is critical to our understanding of the neurobiology and treatment of MDD. The synthesis, storage, release, and metabolism of the biogenic monoamines, i.e., catecholamines (DA, NE, and 5-HT), is central.

Dopamine (DA): According to Olguin *et al.* (2016), dopamine as a neurotransmitter is synthesized via distinct enzymatic steps (Fig. 2.2). Briefly, tyrosine hydroxylase (TH) converts the amino acid tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine), which is metabolised by dopa decarboxylase (DCC) to DA. Dopamine is transported via vesicle monoamine transporter-2 (VMAT-2) where it is packaged and stored in the vesicles. Dopamine release into the synapse is negatively regulated by the D_{2A} auto receptor located on the presynaptic neuron. According to Gilsbach and Hein (2012), auto receptors are receptors located on the pre-synaptic neurons which are responsible for regulating the release of the same neurotransmitters. On the other hand, heteroreceptors are pre- or post-synaptic receptors responsible for regulation of the release of other neurotransmitters (Gilsbach and Hein, 2012) and located on another neuron (i.e., 5-$HT1_A$ on a dopaminergic, noradrenergic, and glutamatergic neuron) (Harvey and Slabbert, 2014). The fate of DA is either to bind to pre- and post-synaptic receptors or to be terminated through reuptake into the neuron via the DA transporter (DAT). DA is metabolized by intracellular monoamine oxidase (MAO) to DOPAC (3,4-Dihydroxyphenylacetic acid), whereas metabolism by extracellular catechol-O methyl transferase (COMT) produces homovanillic acid (HVA) (Olguin *et al.,*2016). Importantly, DA is a precursor for biosynthesis of NE via catalytic action of DA β-hydroxylase (Olguin *et al.,*2016).

Norepinephrine (NE): NE is mainly synthesized from the precursor amino acid, L-tyrosine, through various intermediate steps (Fig. 2.2). Tyrosine is converted to L-DOPA by tyrosine hydroxylase, the rate limiting enzyme in NE synthesis. L-DOPA is metabolized by aromatic amino acid decarboxylase (AADC) to DA which is transported via vesicular monoamine transporters (VMAT-2) for storage in vesicles of the presynaptic neuron (Montoya *et al.,*2016). Its release occurs via calcium-dependent exocytosis mediated α_{2A} and α_{2c} adrenoceptors located on the presynaptic noradrenergic neuron (Maletic *et*

*al.,*2017; Chandley and Ordway, 2012). NE released from the pre-synaptic neuron can stimulate different adrenoceptors located on the pre- and post-synaptic neuron or be terminated through reuptake into the pre-synaptic neuron via the norepinephrine transporter (NET) (Chandley and Ordway, 2012). Finally, NE can be metabolized by either intracellular MAO to inactive metabolites, MHGP (3-methoxy-4-hydroxyphenylglycol) or extracellular enzyme (COMT) to NM (normetanephrine) (Montoya *et al.,*2016).

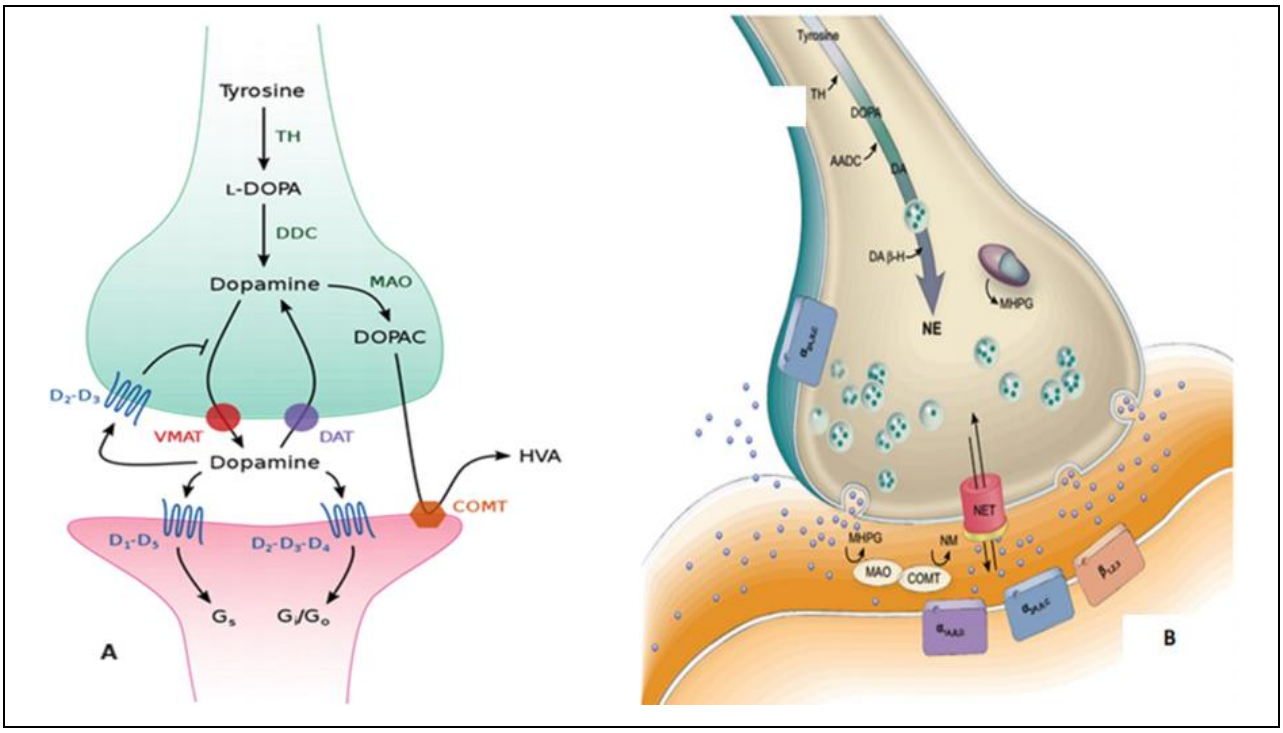

Fig. 2.2. Key steps in the synthesis, storage, release and metabolism of DA and NE. **A.** DA synthesis, storage, release, and metabolism pathway. **B.** NE synthesis, storage, release, and metabolism pathway. (Adapted from Olguin *et al.,*2016 and Montoya *et al.,*2016).

Serotonin (5-HT); 5-HT is synthesized via hydroxylation of the amino acid, L- tryptophan to 5-hydroxytryptophan (5-THP) in a process catalyzed by tryptophan hydroxylase (Fig. 2.3), followed by decarboxylation of 5-THP to serotonin (5-HT) in a process catalyzed by AADC (Pourhamzeh *et al.,*2021). Serotonin is transported through vesicular monoamine transporter-2 (VMAT-2) to be stored in vesicles, whereupon it can be released via Ca^{2+} dependent exocytosis into the synaptic cleft to activates various subtypes of 5-HT receptors on the pre- and post-synaptic neuron (Pourhamzeh *et al.,*2021). The release of 5-HT is negatively regulated by $5\text{-HT}_{1A/B}$ auto receptors, while its action is terminated

either by removal from the synapse through the serotonin reuptake transporter (SERT) embedded on the pre-synaptic neuron or deactivated by MAO to 5-hydroxyindoleacetic acid (5-HIAA) (Pourhamzeh *et al.,*2021).

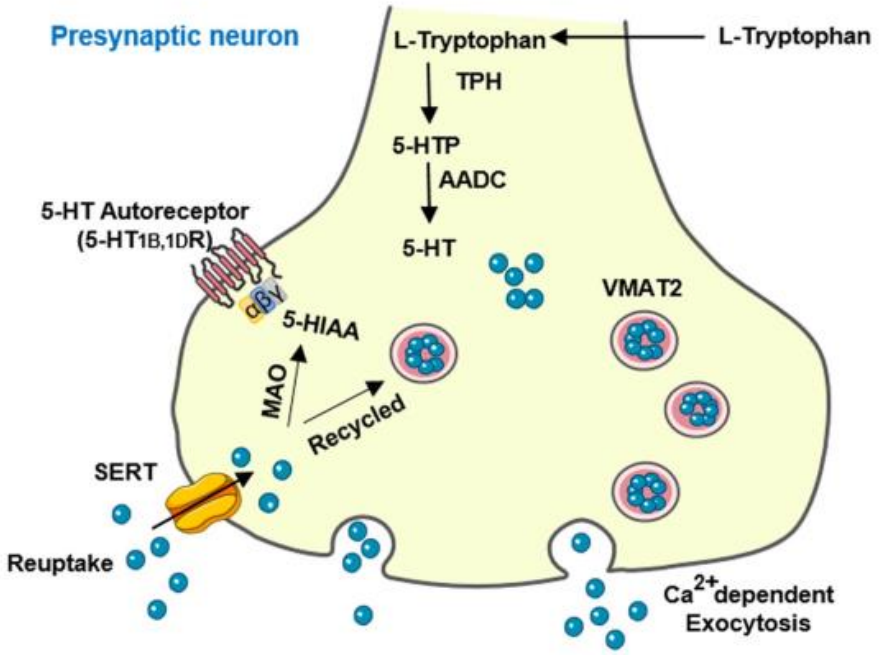

Fig. 2.3. Key steps in the synthesis, storage, release, and metabolism of serotonin in a pre-synaptic neuron. (Adapted from Pourhamzeh *et al.,*2021).

2.3.3 The biogenic amine hypothesis of major depressive disorder

The monoamine hypothesis proposes that MDD is caused by depletion of the monoamine neurotransmitters NE, 5-HT, and DA, in the central nervous system (Mulinari, 2012; Boku *et al.,*2018). Considering the origin of dopaminergic, serotonergic and noradrenergic neurons in the midbrain and brain stems nuclei, and their projection to the frontal cortex, hippocampus, amygdala, ventral striatum, nucleus accumbens and hypothalamus which are brain regions implicated in MDD (Marije *et al.,*2009) , it is not surprising that the monoaminergic system is responsible for regulating many components of the mood continuum, including attention, reward, memory, sleep, sexual function, appetite, and cognition (Hasler,2008; Bondy, 2002). This has further been supported by earlier work describing the presentation of depression-like symptoms following the use of reserpine to treat for instance hypertension (Robinson, 2018). Reserpine acts to deplete brain monoamine stores thereby contributing to the development of the chemical imbalance theories of depression, such as the monoamine hypothesis (Robinson, 2018; Bondy,

2002). Moreover, this hypothesis was strengthened by the discovery that iproniazid, a medicine used to treat tuberculosis, block behavioural effects of reserpine (Robinson, 2018; Bondy, 2002), through actions that bolstered synaptic monoamine levels through MAO inhibition (Di Giovanni *et al.,*2016). This fortuitous discovery prompted the development of imipramine, the first clinically effective antidepressant, which engenders the same effect on synaptic monoamine levels by blocking the reuptake of serotonin and norepinephrine (Brand and Harvey, 2017).

Previous studies have shown that depletion of monoamines is associated with several causative factors, for example, increased metabolism by MAO and COMT (Huotari *et al.,*2002), relative deficiency in the availability of the monoamine precursors, mainly 5-hydroxytryptophan and L-3,4-dihydroxyphenylalanine (Hinz *et al.,*2011). This has prompted intensive research to uncover how monoamine neurotransmitters contribute separately to the pathophysiology of MDD. This in turn has promulgated the development of SSRI's, SNRI's and various atypical agents for treating the disorder.

Role of Dopamine (DA) in Major depressive disorder

Dopamine is produced in the substantia nigra (SN) and ventral tegmental area (VTA) of the brain (Fig. 2.4) regions that plays a vital role in reward and movement regulation in the brain (Olguin *et al.,*2016). Neurons projecting from the SN innervate the dorsal striatum (nigrostriatal system), with a small percentage innervating the cortex, nucleus accumbens, and globus pallidus (GP) (Fig. 2.4), while VTA neurons primarily innervate the nucleus accumbens, cortical areas (mesolimbic and mesocortical systems respectively), amygdala, hippocampus and hypothalamus, with a small percentage innervating the dorsal striatum (Brichta *et al.,*2013). Dopamine is involved in regulating cognition, motivation, reward, sleep, working memory, and learning (Belujon and Grace, 2017; Olguin *et al.,*2016; Moret and Briley, 2011). Based on multiple studies, the role of diminished dopaminergic neurotransmission in MDD has been ascribed to depletion of DA in the synaptic cleft, impaired DA-mediated signal transduction, or due to changes in receptor quantity and function or altered intracellular signalling (Amidfar, 2018; Belujon and Grace, 2017; Cannon *et al.,*2009; Dunlop and Nemeroff, 2007). Dopamine receptors are classified into two main groups (Fig. 2.4) with distinct expression in the brain regions (Brichta *et al.,*2013). Previous studies revealed that D_1-like receptors are exclusively located on the post-synaptic neuron where they are coupled to G-proteins to stimulate

adenylyl cyclase (AC) and increase second messenger i.e., cAMP (Belujon and Grace, 2017; Olguin *et al.,*2016) to regulate reward, motivation, attention and working memory (Santos *et al.,*2013). Reduced D_1-like receptors in the mesocortical areas has been associated with DA dysfunction in MDD (Cannon *et al.,*2009). Previous studies have demonstrated that DA could play an important role in the pathophysiology of MDD mainly through D_1- like receptors in the hippocampus and cortical areas (Cannon *et al.,*2009). In fact, animal studies revealed that chronic treatment with antidepressants (i.e., fluoxetine) induce the expression of D_1-like receptor in hippocampus which may contribute to efficacy of the antidepressants (Belujon and Grace, 2017). Whereas the D_1-like receptors are exclusively hetero-receptors (Belujon and Grace, 2017), D_2-like receptors are located both in the pre-synaptic (autoreceptors) and post-synaptic neurons coupled to $G_{i/o}$ proteins, thereby decreasing production of cAMP (Belujon and Grace, 2017). Major depressive disorder has been associated with upregulation of the D_{2A}-autoreceptors in the hippocampus and cortex (Dunlop and Nemeroff, 2007). The potential of D_{2A}-autoreceptors to regulate the release of DA from the frontal cortex and hippocampus offer a potential target for drugs such as antidepressants (Amidfar, 2018) that block DAT or desensitize D_{2A}-autoreceptors (Amidfar, 2018; Liu *et al.,*2018). Moreover, previous review studies demonstrated that atypical antipsychotics with fewer side effects (i.e., olanzapine, clozapine, risperidone, aripiprazole and quetiapine) that selectively inhibit D_2-like receptors and increase DA in prefrontal cortex have the potential to elicit antidepressant-like effects (Belujon and Grace, 2017; Amidfar, 2018; Liu *et al.,*2018).

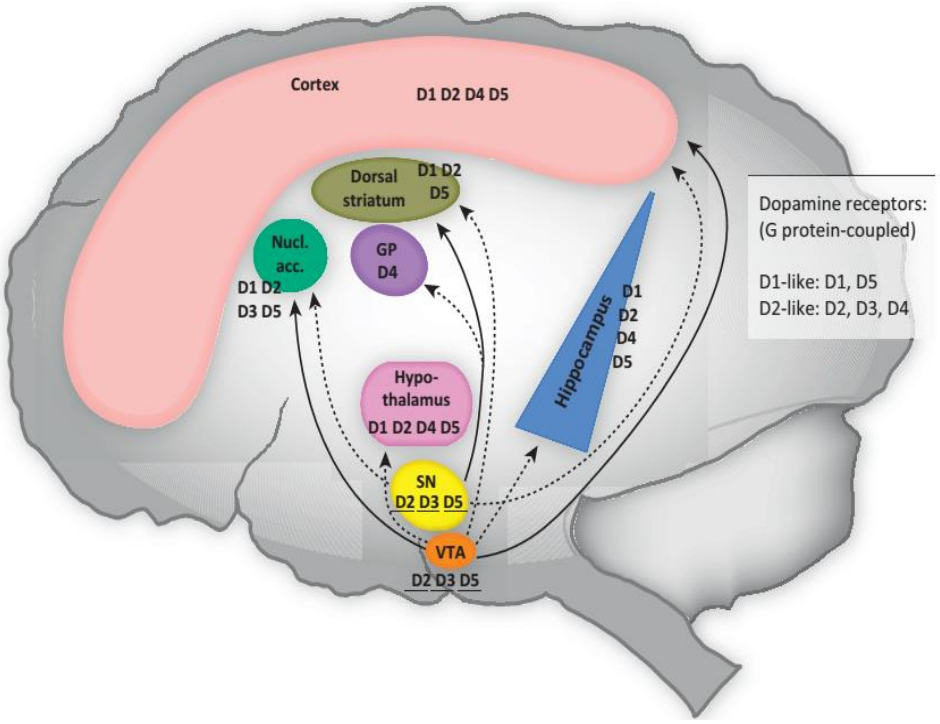

Fig. 2.4. Dopaminergic projection pattern and expression of DA receptors in the brain. (Adapted from Brichta *et al.,*2013).

Role of Serotonin (5-HT) in Major depressive disorder pathophysiology

Serotonergic neurons emerge exclusively in the raphe nuclei (RN) clustered in the midline of the brain stem and innervate diverse regions of the brain, including the hippocampus, frontal cortex, striatum, nucleus accumbens (Brichta *et al.,*2013) (Fig. 2.5). This brain distribution allows 5-HT to modulate a diverse array of behavioural and neuropsychological functions, including mood, perception, reward, anger, aggression, appetite, sexual function, attention, cognition, sleep awake cycle, thermoregulation, locomotion, and memory (Charnay and Leger, 2010; Berger *et al.,*2009). Dysfunction of the serotonergic system is widely recognised to be associated with the pathophysiology of MDD (Charnay and Leger, 2010; Aghanian and Liu, 2009). Nutt and colleagues suggest that depleted 5-HT levels in MDD is due to alteration in synthesis, release, transport, and/or reuptake, which in turn drives symptoms such as anxiety, obsessions, decreased libido, changes in appetite and changes in sleep patterns (Nutt *et al.,*2008).

Converging evidence has shown that serotonin receptors are not only involved in modulation of depression, but also response to antidepressant medication (Carr and

Lucki, 2011; Aghajanian and Liu, 2009). Serotonergic neurons emanate from the dorsal and median raphe nuclei (see Fig. 2.5 below) to innervate serotonin post-synaptic receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_4, 5-HT_{5A}, 5-HT_6, 5-HT_7) and pre-synaptic receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}) in the hippocampus and frontal cortex where they differently modulate depression-related manifestations and response to treatment (Carr and Lucki, 2011; Aghajanian and Liu, 2009). In this regard, 5-HT_{1A} and 5-HT_{2A} receptors mediate mood, emotion, cognition, learning and memory (Charnay and Leger,2010), 5-HT_{2C} receptors mediate agitation and anxiety (Harvey and Slabbert, 2014), 5-HT_{3A} receptors mediate nausea (Charnay and Leger,2010), 5-HT_4 receptors mediate reward cognition (Charnay and Leger,2010), 5-HT_{5A} receptors mediate circadian rhythm and sleep (Charnay and Leger,2010), 5-HT_6 receptors mediate learning and memory (Charnay and Leger,2010) 5-HT_7 receptors mediate mood, sleep and cognition (Charnay and Leger, 2010), 5-HT_{1A}, receptors are implicated in regulating the onset of antidepressant action, amongst other aspects (Celada *et al.,*2004).

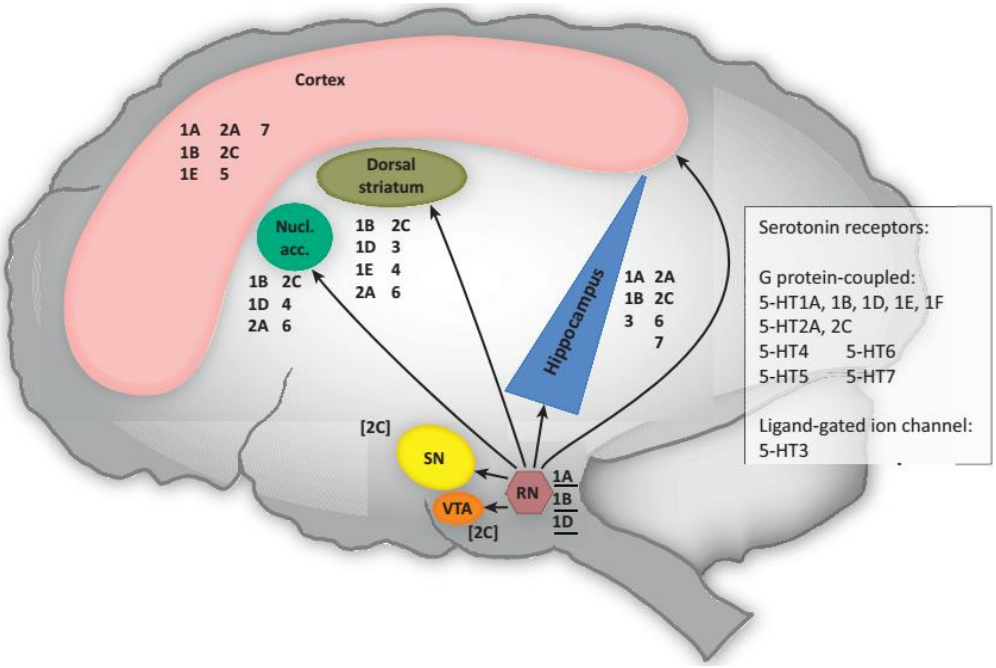

Fig. 2.5. Serotonergic projections and expression of the 5-HT receptors in the brain. (Adapted from Brichta *et al.,*2013).

However, given the number and diversity of 5-HT receptors, for the purpose of this dissertation only the autoreceptors (5-HT_{1A}, 5-HT_{1B}) and post-synaptic receptors (5-HT_{1A} and 5-HT_{2A}) will be discussed, regarding their modulation of MDD and response to

antidepressants. Both 5-HT_{1A} and 5-HT_{1B} are $G_{i/o}$-coupled receptor that exist either as pre-synaptic autoreceptors or post-synaptic heteroreceptors which reduce serotonergic firing mainly through inhibition of adenylyl cyclase or through hyperpolarization of the neurons in the corticolimbic system (Vahid-Ansari *et al.,*2017). Infact, previous review studies showed that blockade of 5-HT auto-receptors or stimulation of the 5-$HT1_A$ receptors produce antidepressant-like effects (Charney and Leger, 2010).

Review studies have demonstrated that hyperactivation of 5-HT_{1A} autoreceptors coupled with its overexpression can result in depression-like behaviours due to decreased 5-HT levels and neuronal activity in the dorsal raphe nuclei (Vahid-Ansari *et al.,*2017; Carr and Lucki, 2011; Celada *et al.,*2004). Somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei and axonal 5-HT_{1B} receptors are responsible for regulation of 5-HT neurotransmission and response to SSRIs through negative feedback inhibition (Quentin *et al.,*2018; Charney and Leger, 2010; Celada *et al.,*2004). Chronic administration of SSRIs leads to increase in potassium conductance, thus hyperpolarization and reduction of expression/desensitization of the somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei, probably through the negative feedback-associated reduction in the firing of serotonergic neurons in the hippocampus and cortex (Carr and Lucki, 2011; Celada *et al.,*2004). These signaling pathways have been proposed to explain the clinical delay of SSRIs in reducing symptoms of MDD (Charnay and Leger, 2010; Celada *et al.,*2004). More importantly, MDD is associated with increased expression of 5-$HT1_{A/B}$ receptors (Brand *et al.,*2015). Thus, hyperactivation of these autoreceptors in the dorsal raphe nuclei (5-HT_{1A}) and at the axon terminal (5-HT_{1B}) leads to uncontrolled negative feedback inhibition and drastically reduces 5-HT release and activation of post-synaptic receptors (Quentin *et al.,*2018; Aghajanian and Liu, 2009). On the other hand, stimulation of post-synaptic *5*-HT_{1A} and 5-HT_{1B} in the hippocampus, amygdala and cingular cortex inhibits adenylyl cyclase and results in antidepressant-like effect (Charnay and Leger, 2010).

5-HT_{2A} in MDD- Serotonin 5-HT_{2A} receptors located at the terminal axon of the neocortex are G-protein coupled receptors which via $G_{q/11}$ proteins activate downward signalling such as protein kinase C and increase inositol triphosphate (IP3) or intracellular calcium (Charnay and Leger, 2010). These receptors may also play a vital role in modulation of response to SSRIs via desensitization or down regulation following a repeated treatment with SSRIs (Charnay and Leger, 2010; Celada *et al.,*2004).

Role of norepinephrine (NE) in Major depressive disorder

The dorsal noradrenergic bundle (DNB) originates from the locus coeruleus in the dorsal pons and innervate multiple brain regions (Fig. 2.6 below) including the cortex, hippocampus, hypothalamus, striatum, cerebellum, and the dopaminergic cell bodies in the substantia nigra (SN) (Brichta *et al.,*2013). Previous review studies demonstrated that abnormalities in most of these regions (i.e., prefrontal cortex, hippocampus, amygdala, and hypothalamus have been associated with pathophysiology of MDD (Goddard *et al.,*2010; Mandela and Ordway, 2006). There is clear evidence for the contribution of the LC/NE system in the pathogenesis of MDD through its actions at α_{1A}, α_{2A}, $\beta_{1,}$ and β_2 adrenergic receptor subtypes (Doze *et al.,*2009). Review studies demonstrated that α_{1A}, $\alpha_{1B,}$ α_{1D} and $\beta_{1\text{-}3}$ adrenoceptors act as stimulators (G_s) of adenylyl cyclase (AC), thereby increasing cAMP or phospholipase C (PLC) respectively (Doze *et al.,*2009; Maletic *et al.,*2017; Montoya *et al.,*2016). A resultant increase in NE stimulation of α_1 adrenergic receptors improves prefrontal cortical related functions such as attention, behavioural flexibility, and learning (Seki *et al.,*2018), On the other hand, β-adrenergic receptors regulate hippocampal functions such as working memory and retrieval (Borodovitsyna *et al.,*2017) and in this way ameliorate symptoms of MDD such as reduced concentration, fatigue, disrupted sleep/wakefulness and circadian rhythm (Maletic *et al.,*2017).

Most importantly, Gilsbach and Hein (2012) showed that α_{2A}, α_{2B} and α_{2C} adrenoceptor subtypes exist as autoreceptors or heteroreceptors. Whereas the α_1 and β adrenoceptors are coupled to G_s proteins (Maletic *et al.,*2017; Montoya *et al.,*2016), the aforementioned α_2 adrenoceptor subtypes are coupled to $G_{i/o}$ proteins (Gilsbach and Hein, 2012) and play an important role in neurobiology of MDD (Maletic *et al.,*2017). These receptors also form one the major targets in the treatment of MDD (Uys *et al.,*2017; Cottingham and Wang, 2015). Previous studies showed that stimulation of the α_2 pre-synaptic (autoreceptors) receptors inhibit the release of NE in the synaptic cleft and may lead to MDD (Maletic *et al.,*2017; Cottingham and Wang, 2015), whereas stimulation of α_{2A}, α_{2B} and α_{2C} heteroreceptors on the dopaminergic, serotonergic, and glutamatergic neurons (Harvey and Slabbert, 2014) decrease activity of adenylyl cyclase thereby decreasing cAMP (Maletic *et al.,*2017).

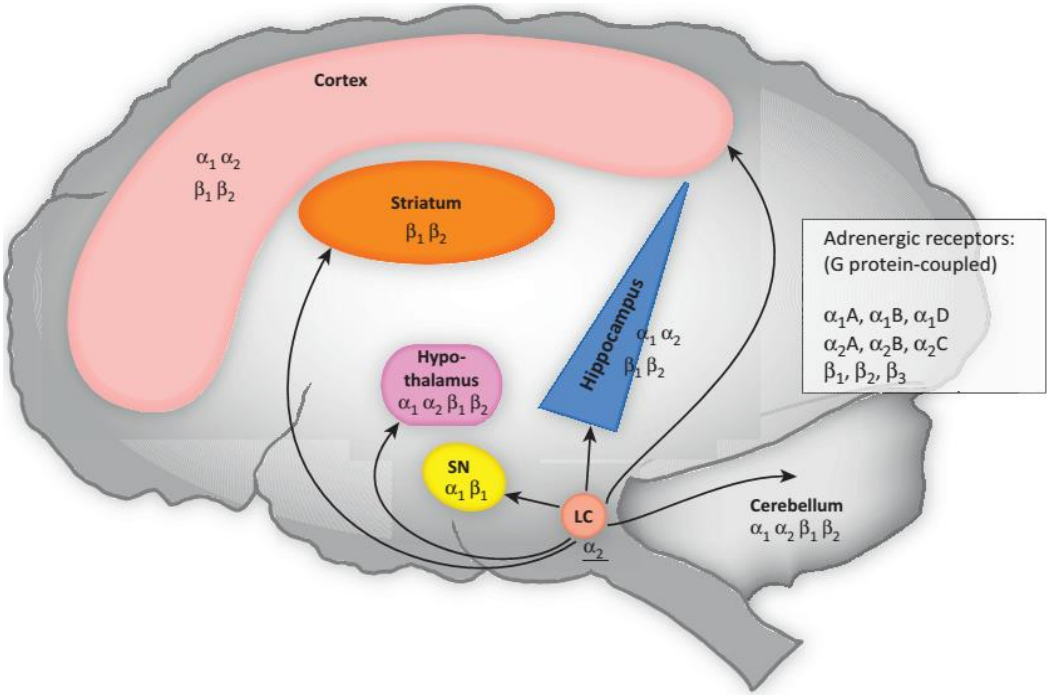

Fig. 2.6. Noradrenergic projections and expression of the norepinephrine (NE) receptors in the brain (Adapted from Brichta *et al.,*2013).

Due to its ability to crosstalk with many neurotransmitter systems, especially 5HT, DA, Glu (Harvey and Slabbert 2014), NE is considered one of the key neurotransmitters in the pathophysiology of MDD, being especially important in regulating motor activity, appetite, arousal, and cognition (Dell'Osso *et al.,*2011). In MDD, the noradrenergic system is important in the regulation of anxiety, arousal, and stress response (Kravets *et al.,*2015). Review studies showed that symptoms i.e., drowsiness or fatigue, impaired cognition, and executive functions in MDD patients have been associated with reduced levels of prefrontal cortical NE (Montoya *et al.,*2016; Maletic *et al.,*2017). Psychosocial stress is a major predisposing factor in the aetiology of MDD due to the activation of the central noradrenergic system (Seki *et al.,*2018; Chandley and Ordway, 2012). Converging evidence has demonstrated that continuous stress leads to long-term dysregulation within the NE system leading to pathogenesis of MDD (Goddard *et al.,*2010). Importantly, low NE levels in the presynaptic cleft are associated with decreased locomotion, deficits in reward behaviour and decreased cognitive capacity (Nutt *et al.,*2008; Mulinari, 2012).

The norepinephrine transporter (NET) is a 12- transmembrane protein known to be a target for a variety of drugs used clinically for the treatment of mood and behavioural disorders such as MDD (Mandela and Ordway, 2006; Zhou, 2004). Specifically, drugs that can inhibit NE reuptake via blockade of NET can produce clinically relevant anxiolytic-like and antidepressant-like effects which further strengthens the hypothesis that dysfunction of NET might contribute to Major depressive disorder and anxiety disorders (Perona *et al.,*2008). In the absence of NRIs greater amounts of NE are taken back into the pre-synaptic nerve terminal by NET and metabolised by monoamine oxidase to one of NE's major metabolites, 3,4-dihydroxyphenylgycol (DHPG) leaving small amounts (<20%) active in the synaptic cleft (Shannon *et al.,*2000) (Fig. 2.7). Therefore, chronic administration of NA-ergic selective antidepressants such as desipramine (Zhao *et al.,*2008), or dual 5-HT/NA'ergic antidepressants like venlafaxine (Arakawa *et al.,*2019) and duloxetine (Moruguchi *et al.,*2017) alter the function and expression of the NET *m*RNA protein by either acting as inhibitors or by competing as a substrate for uptake (Mandela and Ordway, 2006). Indeed, pre-clinical studies have demonstrated that reduced NET expression due to chronic administration of desipramine leads to increased synaptic concentrations of NE which underlie its antidepressant-like effects on behaviour (Zhao *et al.,*2008) as illustrated in Fig 2.7.

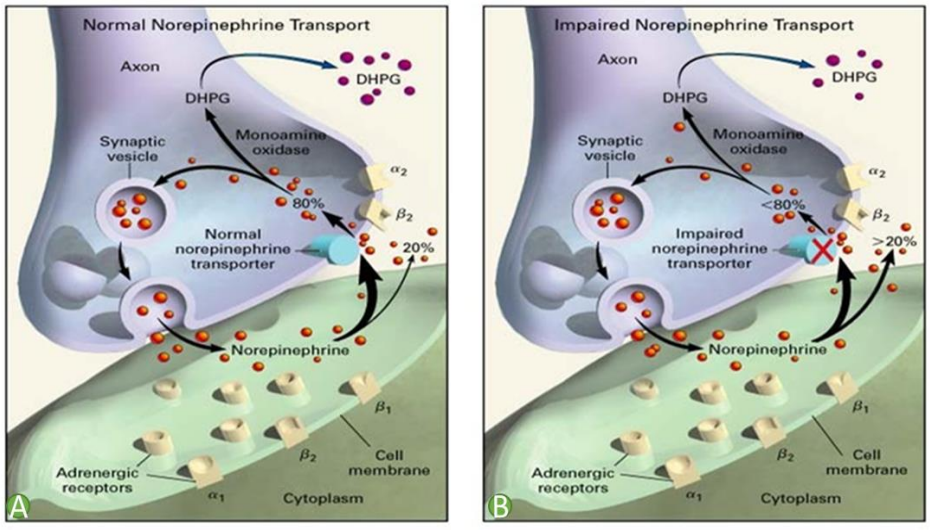

Fig. 2.7. NE action on the pre-synaptic and post-synaptic adrenoceptors before and after chronic treatment with desipramine; **A)** Small amounts of NE (<20%) active in the synaptic cleft. **B)**. NET is blocked and greater concentrations of NE exert prolonged effects on the noradrenergic post-synaptic receptors (Adapted from Shannon *et al.,*2000).

2.3.4 Monoaminergic systems potential interaction and relevance in Major depressive disorder pathophysiology, and relevance for antidepressant action

Multiple findings have shown that the monoaminergic neurotransmitter systems (serotonergic, noradrenergic, and dopaminergic) do not function in isolation mainly due to crosstalk between multiple receptors subtypes which may produce excitation or inhibition on the post-synaptic neuron and results in many behavioural overlaps in MDD symptoms (Slamlo and Fazlali, 2020; Dean and Keshvan, 2017; Guard *et al.,*2008; Blier, 2001). Indeed, review studies showed 5-HT projections that may stimulate the 5-HT_{1A} auto receptors on the noradrenergic pre-synaptic neurons may decrease the levels of NE in the synapse (Blier, 2001; Koeze *et al.,*2012), whereas 5-HT projection to the striatum may result in increased DA in the synapse (Koeze *et al.,*2012). Furthermore, NE projections to the serotonergic pre-synaptic neuron can stimulate α_{2A}/α_{2C} auto- and hetero-receptors and inhibit release of 5-HT (Blier, 2001), as well as NE, DA and Glu (Fig. 2.8). More importantly, stimulation of the α_2 adrenoceptor in the dorsal hippocampus and in the frontal cortex by NE can reduce DA concentration in the synaptic cleft (Guard *et al.,*2008). Therefore, crosstalk between the monoamine systems has been shown to have multiple effects that engender positive benefits on the treatment of MDD when appropriately targeted by an antidepressant (Blier, 2001). This is especially useful for patients presenting with comorbid major depressive disorder and anxiety disorders where antidepressant-like venlafaxine, fluoxetine (Outhoff, 2016), clomipramine (Zohar and Westenberg, 2000), agomelatine (Harvey and Slabbert, 2014), vortioxetine (Ostuzzi *et al.,*2020), mirtazapine (Croom *et al.,*2009) and bupropion (Dhillon *et al.,*2008) can produce a broader range of therapeutic effects.

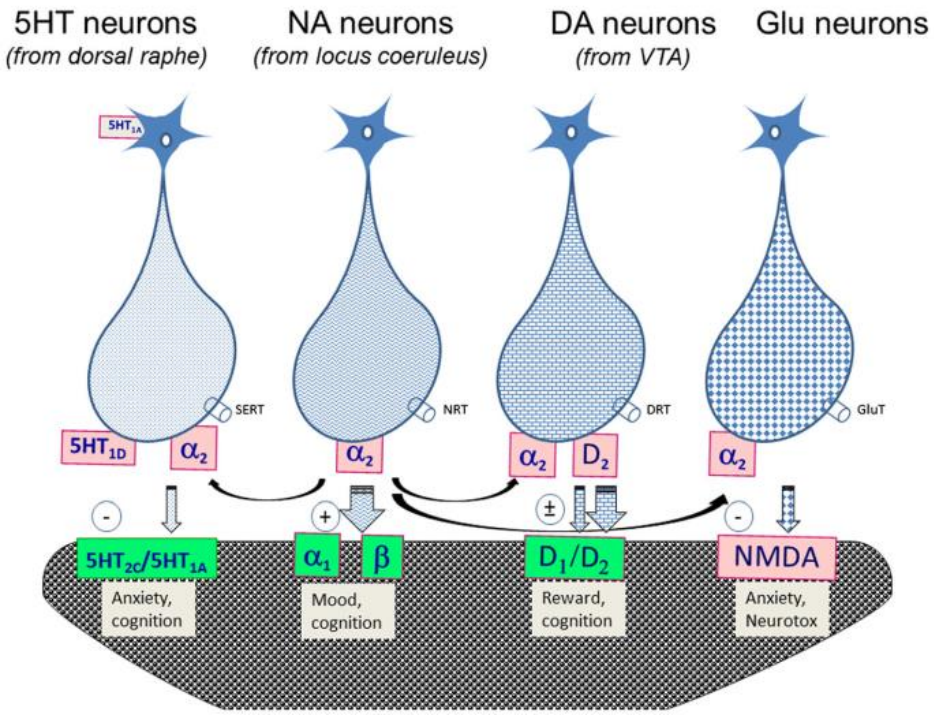

Fig. 2.8. Illustration of crosstalk between multiple receptors subtypes which may produce excitation or inhibition on the post-synaptic neuron (Harvey and Slabbert, 2014)

2.3.5 The role of neuro-inflammation in the pathophysiology of MDD

There is accumulating evidence for inflammation in the pathophysiology of MDD (Dantzer, 2017; Wang *et al.,*2017; Cowen, 2016; Brand *et al.,*2015). Systemic inflammation results due to various mechanisms, such as chronic stress or infection leading to an inflammatory response accompanied by sickness behaviour, often presenting with symptoms of MDD such as fatigue, anhedonia, anorexia and impaired concentration (Cowen; 2016). Chronic stress underpins over-activation of the innate immune cells, such as microglia, astrocytes, and macrophages, resulting in the production of pro-inflammatory cytokines (i.e., IL-1β, IL-6, IL-12, IL-17, IL-22, IL-23, TNF-α and IFN- α) (Feltes *et al.,*2017; Jin *et al.,*2019; Roohi *et al.,*2021) (Fig. 2.9). These activate the indoleamine-2,3-deoxygenase (IDO) enzyme that metabolizes tryptophan to kynurenic acid and quinolinic acid, thereby decreasing 5-HT levels (Feltes *et al.,*2017). Further, pro-inflammatory cytokines might activate the hypothalamic-pituitary –adrenal axis (HPA-axis) which produce corticotrophin-releasing hormone (CRH) that mediates release of adrenocorticotropic hormone (ACTH) ultimately increasing production of cortisol from adrenal glands (Feltes *et al.,*2017).

Recent review studies have revealed that elevated levels of IL-6 are reliable and consistent biomarkers in MDD patients (Jin *et al.,*2019; Roohi *et al.,*2021), concluding that systematic inflammation via IL-6 could induce MDD via various mechanisms not limited to excessive activation of inflammatory pathways (Dantzer, 2017; Wang *et al.,*2017; Cowen, 2016; Brand *et al.,*2015). In fact, numerous review studies have revealed that decreased neurogenesis and neuroplasticity due to IL-6 overexpression may reduce the volume of the anterior cingulate cortex, and prefrontal cortex, neocortical grey matter and hippocampus leading to MDD (Feltes *et al.,*2017; Roohi *et al.,*2021).

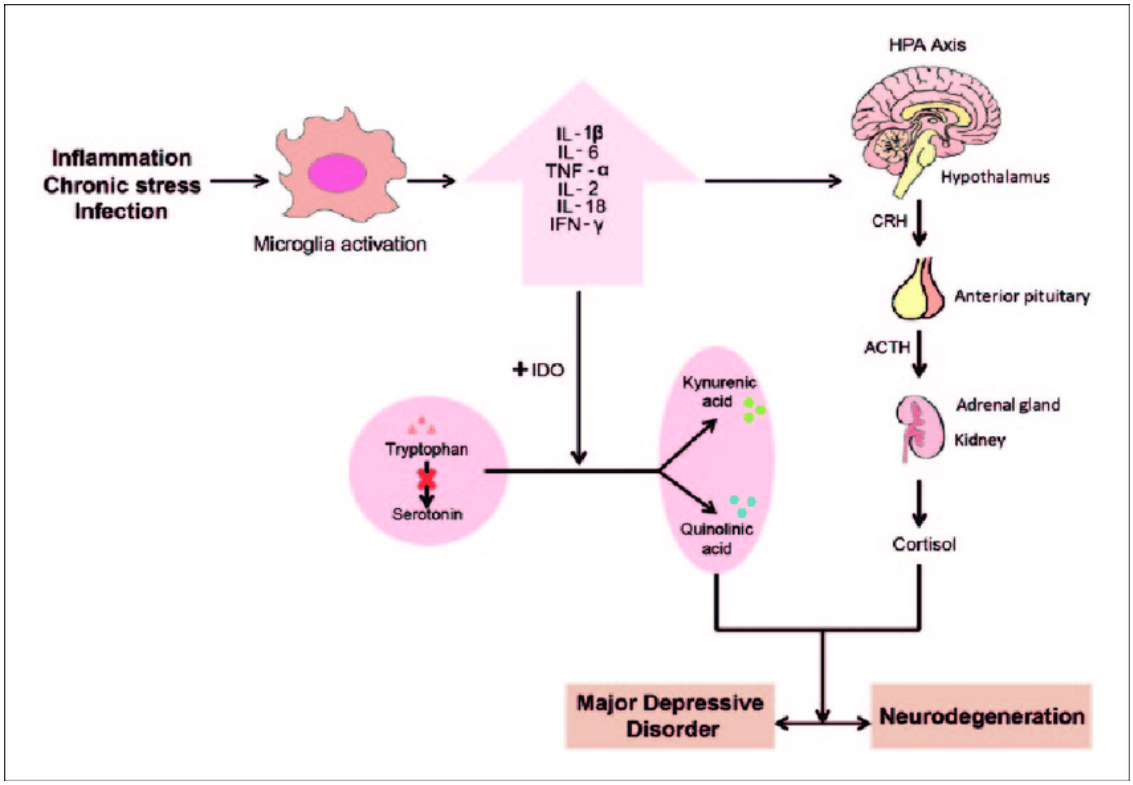

Fig. 2.9. Inflammation cascade leading to major depressive disorder pathophysiology and neurodegeneration. (Adapted from Feltes *et al.,*2017).

Overall, chronic stress or infection may lead to overproduction of IL-6 from the microglia and astrocytes to cause hyper activation of the HPA-axis which increase cortisol release. Co-activation of IDO increase the metabolism of tryptophan (5-HT precursor) thereby depleting 5-HT levels in the synapse (Dantzer, 2017; Feltes *et al.,*2017; Roohi *et al.,*2021) (Fig. 2.9). Both elevated cortisol and reduced 5-HT are hallmark traits of MDD (Brand *et al.,*2015).

2.3.6 The Role of Phosphodiesterase 4 (PDE4) in the pathophysiology of MDD

The cyclic nucleotide phosphodiesterases (PDEs) are a family of ubiquitous enzymes that selectively hydrolyse cyclic adenosine 3'5' monophosphate (cAMP), ubiquitous second messengers involved in intracellular signalling cascades (Murthy and Mangots, 2015; Cashman *et al.,*2008). Based on the sequence homology, substrate, and genetic expression, PDEs are classified into 11 families with the PDE4 subgroup of particular importance in neuromodulation (Wang *et al.,*2017; Kumar *et al.,*2013; Cashman *et al.,*2008) where it acts specifically to hydrolyse cAMP. Cyclic AMP is the prerequisite messenger for the activation of the protein kinase A (PKA)--cAMP-dependent response element-binding (CREB) protein cascade. Through these actions, PDE4 is implicated in learning, long term memory, anxiety, and mood regulation (Li *et al.,*2009; Wang *et al.,*2017).

The PDE4 gene encodes four subgroups of enzymes, i.e., PDE4A, PDE4B, PDE4C and PDE4, all of which are present in the brain (Wang *et al.,*2017; Kumar *et al.,*2013; Cashman *et al.,*2008; Itoh *et al.,*2004). Converging evidence from animal and clinical studies have demonstrated that PDE4B and PDE4D are involved in the pathogenesis of MDD (Wang *et al.,*2017; Li *et al.,*2009; Itoh *et al.,*2004), as well as mediating the antidepressant-like effects of PDE4 inhibitors (Dlaboga *et al.,*2006).

Increased PDE4B and PDE4D activity in the hippocampus and prefrontal cortex has been associated with decreased BDNF expression coupled with decreased cAMP concentration and/or reduced expression of CREB, leading to atrophy and structural changes in these brain regions and associated with impaired learning and memory (Miranda *et al.,*2019; Jin *et al.,*2019; Wang *et al.,*2017) (Fig. 2.10). Findings from pre-clinical studies have shown that the above-mentioned alterations in cAMP-PDE4-CREB signalling can be restored with chronic administration of PDE4 inhibitors such as rolipram (Wang *et al.,*2017; Li *et al.,*2009). Furthermore, selective PDE4 inhibitors like rolipram have antidepressant-like effects in animal models of MDD (Dlaboga *et al.,*2006; Li *et al.,*2009) and in clinical studies of MDD (Fujita *et al.,* 2012).

More importantly, PDE4 plays a key role in neuro-inflammation contributing to the pathophysiology of MDD (Wang *et al.,*2017). Many studies have revealed that PDE4 overexpression can lead to neuro-inflammation via over-activation of microglia cells and the subsequent production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-

6, IL-12, IL-17, IL-22, IL-23, TNF-α, IFN-γ, and decreased production of anti-inflammatory cytokines such as IL-10 and IL-4 (Jin *et al.,*2019; Zou et al.,2018; Wang *et al.,*2017) (Fig. 2.10). Moreover, chronic treatment with PDE4 inhibitors such as rolipram can restore the imbalance in the release of pro- and anti-inflammatory cytokines in microglia (Wang *et al.,*2017). Numerous studies have demonstrated that pro-inflammatory cytokines exert neurotoxic effects via oxidative stress that is damaging to neuronal cells in the prefrontal cortex and hippocampus, thus reducing neurogenesis and neuroplasticity ultimately leading to depressive-like behaviour (Jin *et al.,*2019; Wang *et al.,*2017). The reverse is also true, that anti-inflammatory agents like celicoxib (Hesham *et al.,*2021) and/or antioxidants like N-acetyl cysteine (NAC) (Fan *et al.,*2020) also have antidepressant-like effects, at least in animal models.

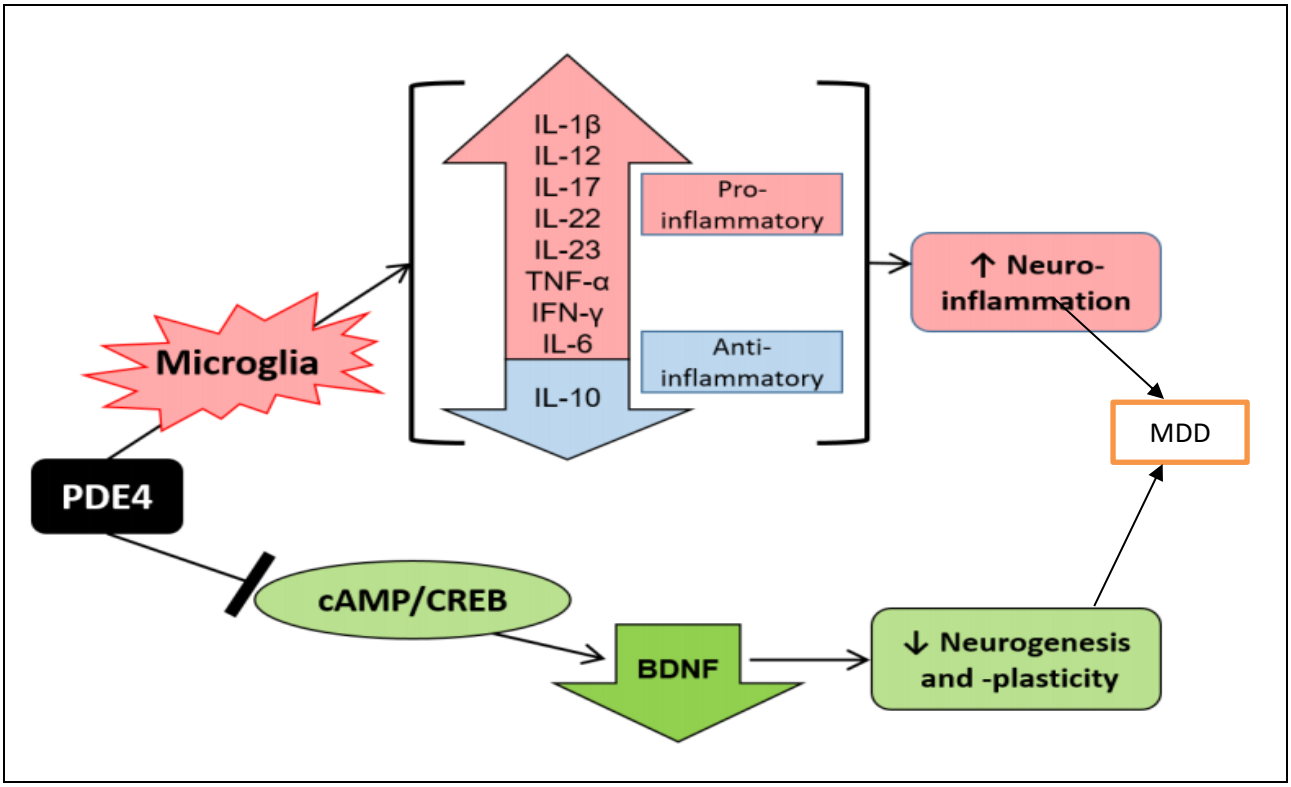

Fig. 2.10. Role of PDE4 in major depressive disorder pathophysiology**,** namely increasing neuro-inflammation, and decreasing neurogenesis and neuroplasticity (Adapted from Gericke, 2019).

Overall, findings have demonstrated that inhibition of PDE4 activity in the hippocampus and prefrontal cortex not only restores the imbalance in the release of pro- and anti-inflammatory cytokines in the brain, but also increases BDNF and so produce antidepressant-like and cognition-enhancing effects via bolstering actions on the cAMP-CREB cascade. These actions ultimately regulate learning, memory, attention, and

mood, and hence offer the potential to treat MDD and other psychiatric disorders (Wang *et al.,*2017; Jin *et al.,*2019).

2.3.7 The contribution of the noradrenergic system and phosphodiesterase 4 enzymes co-interaction in the pathophysiology of MDD

The monoaminergic system in the brain is a critical regulator of cAMP signalling and PDE4 activity through NE-mediated stimulation of postsynaptic β-adrenergic receptors (Lourenco *et al.,*2006). Previous studies have shown that PDE4 overexpression and reduced cAMP-CREB signalling may ultimately lead to increased pro-inflammatory cytokines such as IL -1α, lL-6, and TNF- α which in turn activate and lead to overstimulation of the HPA-axis, ultimately increasing the release of CRF and cortisol (Feltes *et al.,*2017). This leads to decreased BDNF expression and a further decrease in noradrenergic and serotonergic functions, hence connecting with the monoamine hypothesis of MDD (Dantzer *et al.,*2008). This series of events has prompted further consideration of the co-interaction between the noradrenergic system and PDE4 in the pathophysiology of MDD (Fig. 2.11.). Briefly, decreased synaptic NE concentration reduces β-adrenoceptor mediated activation of adenylyl cyclase leading to disturbances in NE-cAMP-PDE4 signalling (Liu *et al.,*2009). Cyclic adenosine monophosphate (cAMP) is produced by AC following β-NE'ergic receptor stimulation (Wang *et al.,*2015). Therefore, decreased AC activation and a decrease in the intracellular cAMP can be exacerbated by overexpression of PDE4 that inactivates cAMP (Zhu *et al.,* 2001). The latter is accompanied by either downregulation of cAMP-CREB signalling which result in decreased expression of BDNF, overexpression of pro-inflammatory cytokines (i.e., IL-6) and downregulation of anti-inflammatory cytokines (i.e., IL-10), ultimately leading to decreased neurogenesis, hippocampal atrophy, and prefrontal cortex hypoactivity (Zhu *et al.,* 2001). In addition to the disturbances in the cAMP-CREB signalling cascade, chronic stress and IL-6 associated neuro-inflammation leads to hyper-activation of the HPA-axis (Feltes *et al.,*2017) and altered NE'ergic firing. Indeed, dysfunction of the PDE4-cAMP-CREB signalling and HPA-axis hyper-activation have been associated with a number of presumed biomarker changes of MDD, including decreased BDNF production, hippocampal atrophy and decreased neurogenesis, depletion of monoamine stores (i.e., NE) accompanied by upregulation of pre-synaptic transporters and autoreceptors (Fig. 2.11)

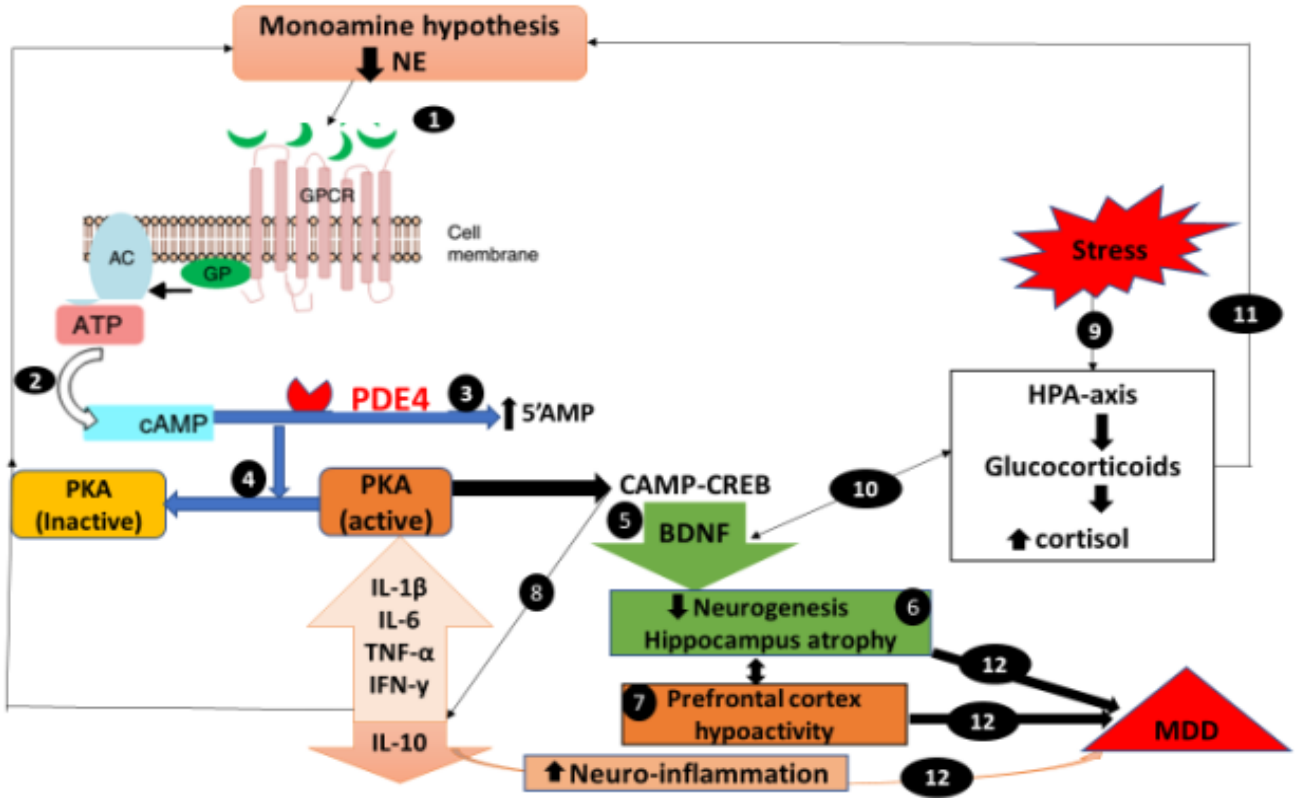

Fig. 2.11. The neurobiological association between altered noradrenergic transmission, PDE4 overexpression and cAMP-CREB signalling in Major depressive disorder pathophysiology (Wang *et al.,*2017; Li *et al.,*2009; Zhu *et al.,*2001).

Murthy and Mangot (2015) reviewed the role of PDE as a possible drug target in treating MDD and anxiety, especially involving the PDE4 subtype. Given that the inhibition of PDE4 will promote cAMP-dependent sub-cellular signal transduction, this action is theorized to have beneficial psychopharmacological effects by modulating the same subcellular NA'ergic processes activated by SNRI and/or TCA antidepressants, but doing so directly i.e., independent of an extra-cellular target (Harvey,1997). Stimulation of G-protein coupled postsynaptic membrane receptors, in particular β-adrenoceptors coupled to AC, increases intracellular cAMP which as noted earlier will activate PKA and the transcription of CREB gene. CREB then modulates the expression of BDNF responsible for neurogenesis and neuroplasticity (Li *et al.,*2017; O'Donell and Zhang, 2004). Previous pre-clinical studies have shown that stimulation of the β-adrenergic receptors enhances the activity of PDE4 inhibitors (mainly rolipram) by upregulating the PDE4 receptor expression (Lourenco *et al.,*2006).

Importantly, chronic treatment with desipramine, a noradrenergic TCA, significantly increases PDE4 protein affinity through stimulation of PDE4 protein expression and/or by

phosphorylation (Lourenco *et al.,*2006). Moreover, similar results are evident following chronic administration of the PDE4 inhibitor, rolipram, viz. increasing intracellular concentration of cAMP (Li *et al.,*2009), thus illustrating their common end-point actions despite alternate biological targets. These actions, as alluded to earlier, reduce the release of pro-inflammatory cytokines while also promoting transcription of anti-inflammatory cytokines (Jin *et al.,*2019; Wang *et al.,*2017). It is through this action that some newer antidepressants with dual or multiple pharmacological mechanisms of action increase remission rates by synergizing with other neurotransmitter systems and in so doing may be more effective than unimodal strategies (Sarris *et al.,*2021). Such a strategy not only benefits outcome for MDD alone, but also offers an effective treatment for MDD presenting with various comorbidities, such as anxiety.

2.4 THE COMORBIDITY OF ANXIETY DISORDERS AND MAJOR DEPRESSIVE DISORDER AND ITS COMPLICATIONS

Anxiety disorders (ADs) such as general anxiety disorders (GAD), post-traumatic stress disorder (PTSD), obsessive-compulsive disorder (OCD), panic disorder, social anxiety disorder and phobia are the major psychiatric disorders co-existing with Major depressive disorder (MDD) (APA, 2013; SADAG, 2020). If left untreated these anxiety disorders often decrease response to standard antidepressants, resulting in reduced patient quality of life (Altin *et al.,*2014; Coplan *et al.,*2015) and increased risk of suicide (Altin *et al.,*2014; SADAG, 2020). Indeed, numerous studies demonstrate poor treatment outcomes in MDD patients co-presenting with an accompanying anxiety disorder (Adams *et al.,*2020; Kessler *et al.,*2015). These findings have been further supported by preclinical results from studies utilizing dual-hit anxiety-depression models that show the development of treatment resistance to a standard antidepressant like imipramine (Harvey and Brand, 2017).

The global prevalence of comorbid ADs and MDD has been reported to be between 47-67% (Kessler *et al.,*2015). Furthermore, comorbid anxiety and MDD may lead to increased risk for earlier onset of MDD, more prolonged depressive episodes, and high risk of suicide compared to patients with MDD (Kessler *et al.,*2015) and increase the risk of treatment resistance (Coplan *et al.,*2015; Matteo *et al.,*2021).

Anxiety disorders are characterized by excessive fear, anxiety, agitation, sleep disturbance, irritability, and psychomotor retardation (APA, 2013). Numerous review studies have demonstrated that anxiety disorders are associated with hyperactivation of the limbic (i.e., amygdala, prefrontal cortex, anterior cingulate cortex, and hippocampus) and HPA-axis which results in excessive release of cortisol and NE mostly linked to symptoms of anxiety disorders (i.e., agitation, hyperarousal, insomnia) (Outhoff, 2016; Liu *et al.,*2018). Although anxiety may be an appropriate response to a threat or stressful event (Outhoff, 2016), severity of the symptom of anxiety disorders are so serious that quality of life is significantly compromised (Outhoff, 2016), or disease burden is increased (Garakani *et al.,*2020).

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2.5. TREATMENT OPTIONS FOR MAJOR DEPRESSIVE DISORDER WITH/WITHOUT COMORBID ANXIETY DISORDERS

The current treatment for major depressive disorder (MDD) and anxiety disorders involves the integration of a multimodal approach based on the combination of non-pharmacological treatment, including interventions stimulus (i.e., electroconvulsive therapy (ECT)), vagus nerve stimulation (VNS) psychotherapy (i.e., cognitive behavioural therapy (CBT), interpersonal therapy (IPT) (McIntyre, *et al.,*2017) and pharmacotherapy (Adams *et al.,*2008). Psychological therapies such as behavioural therapy and cognitive behavioural therapy are employed in the treatment of anxiety disorders such as GAD (SADAG,2020). Although there are several pharmacological and non-pharmacological treatment options for MDD and anxiety disorders, except for the rapid antidepressant effects associated with ketamine especially in patients with TRD (Bratsos and Saleh, 2019), the current armamentarium of antidepressants continues to be limited with regard to delayed onset of action (Boku *et al.,*2018; Liu *et al.,*2018). The majority of the MDD patients do not adequately respond to currently available treatment, with less than 50% of patients achieving remission after antidepressant treatment (Brand *et al.,*2015; Jainer *et al.,*2013). However, co-morbid anxiety presents with a number of complicating factors in the diagnosis and effective management of MDD (Kessler *et al.,*2015). The current pharmacological treatment options for MDD and MDD with anxiety as well as anxiety disorders are briefly discussed below

2.5.1 Pharmacotherapy of major depressive disorder

The Food Drug Administration (FDA) has approved different classes of drugs that have been proven suitable and effective for the treatment of MDD (Lierberman and Massey, 2009; McIntyre, *et al.,*2017). Clinically available antidepressants are grouped into five major classification approaches; namely, monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), and atypical antidepressants (such as agomelatine, vortioxetine, mirtazapine, bupropion, PDE4 inhibitors, amongst others) (Khushboo, 2017; McIntyre, *et al.,*2017). The first successful pharmacological classes of antidepressants acted primarily to increase synaptic concentration of the monoamine transmitters, i.e., 5-HT, DA and NE, and includes the MAOIs, TCA's, SSRIs, and SNRI's.

Monoamine oxidase inhibitors (MAOIs)

The first successful pharmacological treatment of MDD became the MAO inhibitors, of which the non-selective irreversible agents among the group led to safety concerns such as hypertensive crises and ultimately led to their removal from the market (Hillhouse and Porter, 2015). These drugs prevent breakdown of the monoamine neurotransmitters (norepinephrine, serotonin, and dopamine) and increase their availability in stored vesicles by reversibly or irreversibly inhibiting the activity of the monoamine oxidase enzyme that significantly metabolizes the monoamines (Dell'Osso *et al.,*2011). In an attempt to improve the safety of non-selective MAO inhibitors, reversible selective MAO-A inhibitors such as moclobemide was developed and studies revealed that the aforementioned drug was effective as SSRIs, non-selective MAO inhibitors and TCAs for reducing symptoms of MDD (Papakostas and Fava, 2006; Avasthi *et al.,*2005). However, the MAO inhibitors are reserved as third-line or fourth-line treatment of MDD due to concerns over safety and tolerability (Culpepper, 2013).

Tricyclic antidepressants (TCAs)

Accumulating evidence has shown that TCAs have similar clinical efficacy to other antidepressants and are still relevant for the treatment of MDD (Fasipe, 2018). Interestingly, while no longer considered as first line treatment due to a poor tolerability profile (Furukawa et al.,2016), some studies have noted an improved response rate with TCA's (Alvano and Zieher, 2020; Dell'Osso *et al.,*2011), with some TCA's such as clomipramine being advocated in TRD (Tundo *et al.,*2015). Despite tolerability concerns TCAs, most patients with MDD are treated in primary health care, where compliance has been found to be much higher with lower versus higher doses of TCAs (Furukawa *et al.,*2002).

TCAs approved by FDA include amitriptyline, imipramine, nortriptyline, desipramine and clomipramine (Adams *et al.,*2008; Lierberman and Massey, 2009). Although the FDA has only approved clomipramine for treating OCD (Wilson and Tripp, 2021), it is listed under FDA approved drugs for MDD (Lierberman and Massey, 2009). Interestingly, evidence for a faster onset of action for clomipramine in treating TRD is due to a stronger affinity for SERT than other TCAs (Tundo *et al.,*2015; Wilson and Tripp, 2021). Amitriptyline and imipramine exert their effect by variably blocking both NET and SERT (Fasipe, 2018), with nortriptyline and desipramine targeting NET more selectively (Ordway *et al.,*2003).

Tertiary amine TCA's (i.e., amitriptyline and imipramine) not only act on their recognised molecular targets, i.e., NET, SERT, but are more potent inhibitors of H1, acetylcholine muscarinic and α_{1-} adrenergic receptors (Alvano and Zieher, 2020). While these actions introduce possible side effects such as constipation, sedation, dry mouth, blurred vision, urinary retention, and orthostatic hypotension (Alvano and Zieher, 2020), some of these properties especially antimuscarinic actions, may have a distinct role on treating MDD. In fact, the lesser-known cholinergic theory of depression proposes increased cholinergic activity in certain types of MDD (Dulawa and Janowsky, 2019). In fact, the centrally acting anti-muscarinic drug scopolamine has shown efficacy in TRD (Ellis *et al.,*2014; Furey *et al.,*2010).

Recently considerable interest has been shown for secondary amine TCAs such as desipramine and nortriptyline, metabolites of imipramine and amitriptyline, respectively. Both show a high affinity and selectivity for NE transporter while having lower affinity for histamine, muscarinic and adrenergic receptors (Adams *et al.,*2008). This has afforded them as a viable second choice treatment for both MDD and anxiety (Alvano and Zieher, 2020; Dell'Osso *et al.,*2011; Gillman, 2007). In this regard, the FDA has recently approved desipramine for treatment of MDD (Maan *et al.,*2021).

Selective serotonin reuptake inhibitors (SSRIs)

Although SSRIs have developed as the first-line treatment of MDD (Ansari *et al.,*2019) with a low side effects profile (Harvey, 1997), they have some adverse effects. Thus, citalopram, paroxetine and sertraline may produce muscarinic (i.e., dry mouth, constipation) and antihistaminic side effects (i.e., sedation, dizziness, weight gain, nausea), and sexual dysfunction (Carvalho *et al.,*2016) akin to those of TCAs (Harvey, 1997). Their efficacy rate is not better than TCA's, with full remission varying between 50-60% (Kovich and Dejong, 2015), while a delayed onset of action remains problematic (Ansari *et al.,*2019). The SSRIs are variably metabolized in the liver by cytochrome P450 iso-enzymes and are variably associated with drug-drug interactions and unexpected adverse drug events (Gillman, 2007). Finally, premature discontinuation has been linked to withdrawal symptoms as well as possible worsening long-term outcome (Harvey *et al.,*2003; Harvey and Slabbert, 2014).

Serotonin-Noradrenaline reuptake inhibitors (SNRIs)

Previous studies have shown that SNRIs such as venlafaxine and duloxetine offer better antidepressant efficacy than SSRIs (Altin *et al.,*2014; Alev *et al.,*2013; Dell'Osso *et al.,*2011). These drugs block the SERT, and NET sodium-chloride transmembrane protein embedded in the presynaptic to inhibit reuptake of serotonin and norepinephrine, thereby increasing the concentration of serotonin and norepinephrine in the synaptic cleft (Alev *et al.,*2013). Indeed, venlafaxine has a 60% efficacy rate (Debonnel *et al.,*2007) versus 50% for escitalopram (Jayatissa *et al.,*2006). In this regard, venlafaxine is widely regarded as the gold standard antidepressant (Mansuy, 2010) and is often used in TRD where SSRIs have failed (Kivrak *et al.,*2014). Nevertheless, these drugs still do not lead to complete remission in some patients (Dell'Osso *et al.,*2011) and, like all currently used antidepressants with the exception of ketamine (Bratsos and Saleh, 2019), is hampered by a delayed onset of action (Boku *et al.,*2018; Liu *et al.,*2018; Hillhouse and Porter,2015). For the most, SNRI's have a similar side effect profile as SSRI's, especially serotonergic, although increased noradrenergic activity such a hypertension, increased heart rate, increased sweating, tremor, and dizziness is a problem with venlafaxine, especially at higher doses (Adams ,2008; Kivrak *et al.,*2014).

Atypical antidepressants

In addition to four approved major groups of antidepressants (MAIOs, TCAs, SNRIs and SSRIs) (Lieberman and Massey, 2009), the FDA has approved another category of antidepressants (atypical antidepressants) such as vortioxetine (Ostuzzi *et al.,*2020), mirtazapine (Croom *et al.,*2009), bupropion (Dhillon *et al.,*2008), agomelatine (Kennedy and Rizvi, 2010), ketamine and/or esketamine (Matteo *et al.,*2021) not classified under any of the aforementioned class due to multiple mechanisms of action (Lieberman and Massey, 2009). Although, the mechanisms of action of the aforementioned atypical antidepressants are not fully understood, converging evidence from previous studies showed enhanced serotonergic effects with vortioxetine (Ostuzzi *et al.,*2020). Vortioxetine is a partial 5-$HT_{1A/B}$ receptor agonist, which also blocks the 5-HT_3 and 5-HT_7 receptors in addition to its potential SERT inhibition (Ostuzzi *et al.,*2020). Due to multiple mechanisms of action, clinical (Cao *et al.,*2019) and review studies (Sanchez *et al.,*2015; Ostuzzi *et al.,*2020) showed improved anhedonia in patient treated with vortioxetine (Sanchez *et al.,*2015; Cao *et al.,*2019; Ostuzzi *et al.,*2020).

Mirtazapine has both noradrenergic and serotonergic effects not through NET or SERT inhibition, rather the drug blocks central α_2-adrenergic autoreceptors and heteroreceptors, and antagonizes the effects of 5-HT on the post-synaptic 5-HT_2 and 5-HT_3 receptors (Croom *et al.,*2009). Bupropion has both noradrenergic and dopaminergic actions, via inhibition of dopamine and norepinephrine reuptake transporters (Dhillon *et al.,*2008).

On the other hand, novel drugs such as agomelatine gain a popularity in treatment of MDD due to their non-monoaminergic mechanism which may account for favourable side effects compared to monoaminergic antidepressants (Harvey and Slabbert, 2014). Since agomelatine acts as a melatonergic MT_1 and MT_2 receptors agonist with potential to antagonize the effects of 5-HT on the 5-HT_{2C} receptors (Kennedy and Rizvi, 2010), this offers advantage for treatment of both MDD and anxiety disorders over standard antidepressants including SSRIs (Harvey and Slabbert, 2014).

Most importantly, the FDA has approved ketamine and esketamine for treatment of resistant depression (TRD) (Bratsos and Saleh, 2019; Matteo *et al.,*2021). According to Matteo *et al.* (2021) TRD occurs when patients fail to respond to two or more standard antidepressants given at maximal dose. Both ketamine and esketamine acts as N-methyl-D-aspartate receptor antagonists which blocks the action of glutamate and produce rapid onset of action (Matteo *et al.,*2021; Bratsos and Saleh, 2019).

2.5.2 Complementary and Alternative Medicines (CAMs) for MDD

Use of complementary therapies and herbal medicines (i.e., *Agapanthus campanulatus, Boophone distica, Mondia whitei, Xysmalobium undulatum, Hypericum perforatum*) to alleviate depression-like symptoms has been well documented (Lee and Bae, 2017; Pedersen *et al.,*2008; Pilkington *et al.,*2006). Their activity has been associated with the chemical complexity thought to provide advantages in terms of synergy of pharmacological activity, and/or to enhance solubility or bioavailability (Pilkington *et al.,*2006). *In vitro* (Nielsen *et al.,*2004) and *In vivo* (Sandager *et al.,*2005) studies demonstrated that *Agapanthus campanulatus, Boophone distica* and *Xysmalobium undulatum* exhibit selective serotonin reuptake inhibition. Pedersen *et al. (*2008) further showed that the afore-mentioned plants also have affinity for the norepinephrine transporter (NET) and dopamine transporter (DAT). *Hypericum perforatum* (St John's Wort) has been extensively studied for its ability to reduce anxiety and depression (Haller

*et al.,*2019; Lee and Bae, 2017), nevertheless the plant displayed a serious drug-herb interaction which limits its use (Nahas and Sheikh, 2011). Due to its promising modes of action, *Sceletium tortuosum*, a South African plant, heralds promise for future development for the treatment for anxiety and MDD both as a monotherapy and as adjunctive therapy (Olantuji *et al.,*2021; Brendler *et al.,*2021).

2.5.3 Pharmacotherapy of anxiety disorders

Pharmacologically, the benzodiazepines are considered the treatment choice for acute anxiety states because of their rapid anxiolytic effects although are not considered the first line treatment for chronic anxiety disorders (Garakani *et al.,*2020). Their anxiolytic effects are mediated via activation of $GABA_A$ inotropic receptor subtype thereby bolstering GABA-mediated hyperpolarization leading to reduced anxiety (Katzung *et al.,*2018). The commonly used benzodiazepines include diazepam, lorazepam, and midazolam (Katzung *et al.,*2018). Despite their rapid onset of action, side effects include psychomotor impairment, cognitive dysfunction, risk of dependence and dose dependent amnestic effects which may interfere with patient's quality of life (Katzung *et al.,*2018). Additionally, these drugs are contraindicated in patients who may need to perform duties demanding mental alertness i.e., driving (Katzung *et al.,*2018). Importantly, because they are less effective in the treatment of long-term anxiety disorders i.e., GAD, PTSD, Panic disorders and OCD (Katzung *et al.,*2018), benzodiazepines are not prescribed chronically for MDD but as required to manage severe bouts of anxiety.

Alternatives to benzodiazepines include older sedative TCAs with calming effects, starting at lower doses, and gradually increasing to full dose (Katzung *et al.,*2018). Nevertheless, slow onset of action, failure of the patients to tolerate antimuscarinic and antihistaminic side effects limit their use (Alvano and Zieher, 2020). However, desipramine, a secondary amine TCA has less antimuscarinic and antihistaminic side effects (Alvano and Zieher, 2020; Dell'Osso *et al.,*2011; Gillman, 2007). The Food Drug Administration (FDA) has approved the use of new generation SSRIs and SNRIs as the first line treatment for anxiety disorders including GAD, certain PD, PTSD and OCD (Katzung *et al.,* 2018).

2.5.4 Pharmacological treatment of major depressive disorder with comorbid anxiety

Due to the overlap of anxiety and major depressive disorder (MDD) symptoms, patient with MDD may respond poorly to standard antidepressants (Coplan *et al.,*2015). Previous studies showed that SSRIs such as escitalopram at dose between 10-40 mg/kg/day (Coplan *et al.,*2015) are recommended first-line choice for treatment of MDD with generalised anxiety disorders (Coplan *et al.,*2015). With poor outcome from the SSRIs, antidepressants with dual mechanism of action (i.e., SNRIs) may offer a better clinical outcome (Alev *et al.,*2013). In fact, previous studies showed that SNRIs such as duloxetine at dose of 60-120 mg/kg/day (Altin *et al.,*2014; Coplan *et al.,*2015) or venlafaxine 150 mg/kg/day may be more effective than SSRIs in the treatment of comorbid of generalised anxiety disorders with MDD accompanied by functional pain syndromes (Alev *et al.,*2013; Coplan *et al.,*2015). With poor outcome from the aforementioned legitimate first line drugs, combination of SSRIs and/or atypical antidepressants with partial 5-HT_{1A} binding may improve treatment outcomes compared to the monotherapy (Coplan *et al.,*2015). In fact, Reed, and co-workers (2012) showed that vilazodone, an SSRIs with partial 5-HT_{1A} affinity has anxiolytic potential at lower doses (10-40 mg/kg/day) and can be used as an alternative treatment of MDD with comorbid anxiety disorders. Despite tolerability concerns, TCAs (i.e., amitriptyline, clomipramine) are still effective for treatment of MDD with anxiety disorders accompanied by functional pain syndromes (Fibromyalgia) (Copla *et al.,*2015).

2.6 Drugs relevant to this study

2.6.1 Desipramine in the treatment of Major depressive disorder

Desipramine (DMI) selectively blocks NET (Ordway *et al.,*2003) in the limbic brain regions (Lapiz *et al.,*2007), ultimately enhancing NE function at postsynaptic α_1 and $\beta_{1/2}$ adrenoceptors (Lapiz *et al.,*2007; Ordway *et al.,*2003). Animal studies have shown that chronic administration of DMI reduces the density (downregulate) and activity of the α_{2A} pre-synaptic receptors (autoreceptors) in the limbic regions (Cottingham and Wang, 2012). It is this process of adaptive neuroplasticity that explains its delayed onset of action, albeit typical of all drugs in this class (Ordway *et al.,*2003).

The most pharmacologically desirable activity of DMI is as norepinephrine reuptake inhibitor (NRI), as well as its lower risk for drug-drug interactions when co-administered with SSRIs (i.e., fluoxetine) and atypical agents like the PDE4 inhibitor, rolipram (Palaniyappan *et al.,*2009; Gillman, 2007; Lourenco *et al.,*2006). In this regard, a combination of DMI with rolipram was found to enhance noradrenergic functions by increasing PDE4 protein affinity through stimulation of PDE4 protein expression and/or by phosphorylation (Lourenco *et al.,*2006), illustrating its potential as an augmentation agent.

Pharmacokinetics of desipramine

DMI is rapidly and completely absorbed from the gastrointestinal tract (GIT) with an oral bioavailability of between 50-70%, undergoes first-pass metabolism in the liver by CYP2D6, with approximately 70% of an orally administered dose excreted in the urine as metabolites (Lemke *et al.,*2012). The lipophilic nature of DMI offers an advantage for the drug to accumulate in the membrane and exert maximum efficacy on the NE transporters embedded in the membrane (Zhao *et al.,*2008). Interestingly, DMI is one of the NRIs with the least drug interaction compared to the SSRIs as it weakly inhibits CYP 450, 2C19, and 1A2 (Gillman, 2007).

Desipramine dose selection

Clinically, desipramine (DMI) is administered orally at the initial dosage of 25 mg/kg/day up to 50 mg/kg/day for treatment of MDD (Maan *et al.,*2021). The dose is titrated up gradually based on therapeutic effects and tolerability, but not exceeding the maximum dose of 300 mg/kg/day (Maan *et al.,*2021). Animal studies provide robust support for classification of DMI as a selective norepinephrine inhibitor (NRI) (Mokoena *et al.,*2015; Overtreet *et al.,*2010). DMI is commonly used as a positive control in studies employed in animal models of MDD to assess antidepressant activity (Mokoena *et al.,*2015; Overstreet *et al.,*2005), as well as when the comparative effects of an SSRI versus an NRI are sought (Korff *et al.,*2008). Other sub-acute dose-response studies in rats have confirmed these findings (Simpson *et al.,*2012; Furgama *et al.,*2011). Furthermore, sub-chronic studies showed that low- (7.5 mg/kg/day), 10 and 15 mg/kg/day (Shah and Frazer, 2014) and full therapeutic dose (15 mg/kg/day) of DMI (Mokoena *et al.,*2015), given via intraperitoneal route, are significantly antidepressive in the FST. In conclusion 7.5 and 15 mg/kg/day i.p. were selected and convert to 15 and 30 mg/kg/day for oral

treatment. According to Lemke *et al.* (2012), DMI is rapidly and completely absorbed from the gastrointestinal tract (GIT) with the oral bioavailability between 50-70%. Therefore, higher than normal doses of DMI for oral gavage were calculated based on the oral bioavailability of DMI, which ranges between 50-70% (Lemke *et al.,*2012).

2.6.2 PDE4 inhibitors in the treatment of Major depressive disorder

A dysregulation of PDE4 activity is believed to have a significant role in the pathophysiology and treatment of MDD, so much so that the use of PDE4 inhibitors has been widely considered as potential antidepressant agents due to their ability to potentiate the intensity and duration of cAMP-mediated signalling, critical in mood (Murthy and Mangot, 2015). Despite clinical and pre-clinical evidence to support rolipram's antidepressant effects, its side-effect profile, notably emetic and sedative actions, limited its clinical utility in MDD (O' Donnell and Zhang, 2004), and eventually ended its further development as an antidepressant (Montana and Dyke, 2002). Other adverse effects associated with early PDE4 inhibitors include headache, diarrhoea, fatigue, dyspepsia, nasopharyngitis, and gastroenteritis (Kumar *et al.,*2013). New generation PDE4 inhibitors, such as apremilast, has been found to be well tolerated with few side effects in phase I and phase II studies although that study did not focus on its antidepressant-like effects (Man *et al.,*2009).

Despite the above setbacks with early PDE4 inhibitors, historical data for herbal plant medicines with putative PDE4 inhibitory properties have shown better safety and tolerability profiles (Manosroi *et al.,*2017; Anand *et al.,*2021). One such example is a standardized extract of *Sceletium tortuosum* (ST) (Zembrin®) (Olantuji *et al.,*2021; Brendler *et al.,*2021). Zembrin® has shown dual pharmacological activity as a SERT and PDE4 inhibitor in *in vitro* studies (Harvey *et al.,*2011), while studies from our laboratory have demonstrated dose-dependent antidepressant effects (Gericke *et al.,*2022). There is thus promising potential to develop this extract as a new treatment for mood and anxiety disorders that targets both SERT and PDE4.

2.7. *Sceletium tortuosum (*ST) in the treatment of major depressive disorder

Traditional use of ST: The South African plant, ST, is part of the subfamily of Mesembryanthemaceae which has been used for centuries by the Khoisan hunter-gatherers of South Africa to promote a sense of calm, improve health, as a masticatory, relief of hunger, thirst, fatigue, restorative, sedative, hypnotic, analgesic, antispasmodic, as stress relief, reduce anxiety, improve mood and to enhance concentration (Olantuji *et al.,*2021; Brendler *et al.,*2021). The whole plant parts are used as fresh plant material, baked plant material, dried plant, and dried "fermented" plant material or the stem as well as the roots are chewed (Gericke and Viljoen, 2008). This succulent plant is distributed in the South-West Cape province of South Africa ranging from Namaqualand to Montagu and is usually found growing under the shrub in an arid environment (Gericke and Viljoen, 2008). The plant is commonly recognised by the white to light yellow or pink petals (about 4 cm in length) with prominent dry leaf veins that resemble the skeleton-like structure of the dry, withered, or old leaves (Fig 2.12).

Fig. 2.12. *Sceletium tortuosum* (ST) plant. A). The full blooming plant; B) the skeletonised appearance of withered dry leaves, and C) ST region of distribution in South Africa (Gericke, 2019; Gericke and Viljoen, 2008).

Pre-clinical and clinical studies with ST and its products: *In vivo* analysis of the alkaloid mixture of ST were compared to paroxetine (an SSRI) in an acute rat model of MDD, where the authors noted that the high mesembrine containing alkaloid preparation (Trimesemine ^{TM}) produced antidepressant-like effects like paroxetine (Schell, 2014).

Various ST products (i.e., powders, tablets, capsules) and crude extracts (i.e., *Sceletium* Extract M55 capsules high Mesembrine 60 x15 mg) are available, with the raw plant extract shown to present several alkaloids that vary in concentration in the products

(Patnala, 2007; Dimpfel *et al.,*2016). Some of the marketed products of ST include *Sceletium tortuosum* tablets Big Tree®, *Sceletium tortuosum* herbal care capsules®, *Sceletium tortuosum* capsules and *Sceletium tortuosum* alcoholic tincture® manufactured by Essential source® (Partnala, 2007). However, the variability in constituents in these preparations introduces inconsistency in bio-assay results, which propagated the development of a standardized extract of ST, such as Zembrin® and Elev8® (Africanbotanicals.com).

2.7.1 The Zembrin® as antidepressant

Zembrin® is a standardized hydroethanolic extract of ST referred to as a chemotype, containing 0.35-0.45 total alkaloids, and comprises the following mesembrine alkaloids: mesembrenone and mesembrenol ≥ 60%, mesembrine ≤ 20%, and mesembranol ≥ 5% (Fig. 2.13). A typical fingerprint is provided demonstrates the composition of the main four alkaloids in Zembrin®; mesembrenone (1.84 µg/mg), mesembrenol (1.23 µg/mg), mesembrine (0.51 µg/mg) and mesembranol (0.26 µg/mg) (Gericke *et al.,*2022).

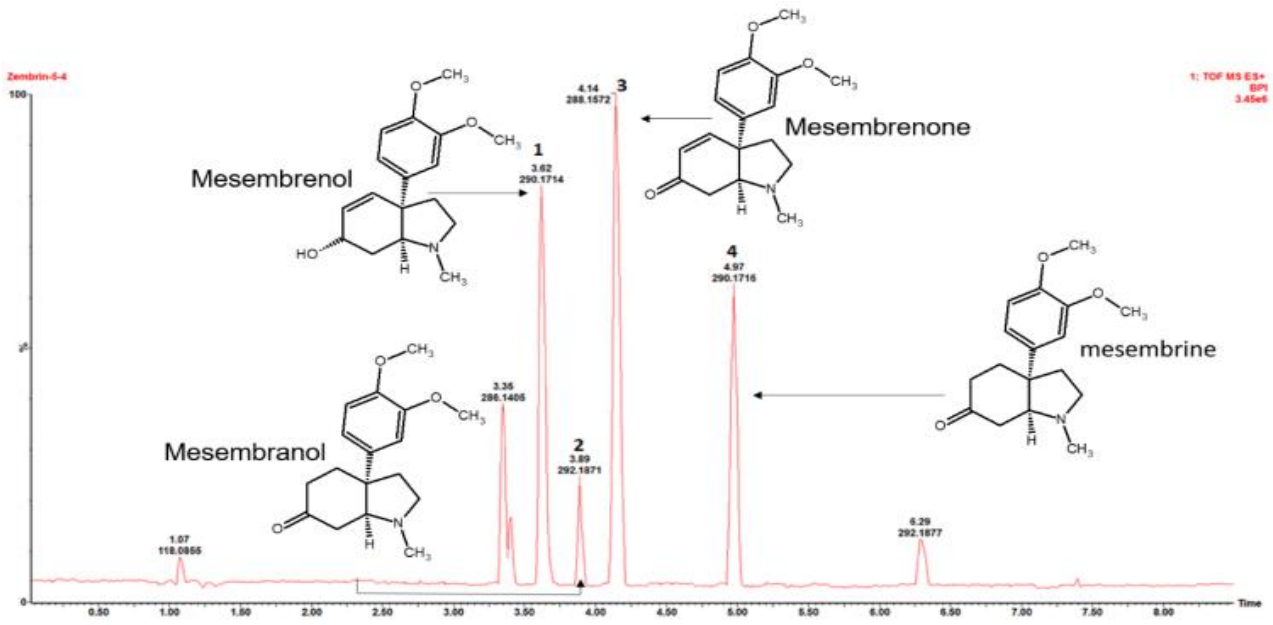

Fig. 2.13. Ultra-performance liquid chromatography tandem mass spectrometry-chromatogram of Zembrin® with four main peaks at 3.35 (mesembranol), 3.62 (mesembrenol),4.14 (mesembrenone) and 4.97 (mesembrine) minutes, confirming the Zembrin® chemotype (3.84 µg/mg) (Adapted from Gericke *et al.,*2022).

Pharmacological mechanism of action: Zembrin® shows significant inhibition of SERT and PDE4 activity, and at high concentrations activates μ-opioid, cholecystokinin-1 and exert non-selective actions on the $GABA_{A/B}$ receptors (Harvey *et al.,*2011). Mesembrine and mesembrenone show significant affinity for SERT and PDE4 comparable to fluoxetine and rolipram, respectively (Harvey *et al.,*2011), while a recent study from our laboratory showed that the combination of Zembrin® 50 mg/kg/day (given via oral gavage) and escitalopram (5 mg/kg/day) significantly increased both 5-HT and NE in the hippocampus (Gericke, 2019), supporting earlier referenced actions on SERT and its pro-serotonergic actions (although here a high-mesembrine ST extract not Zembrin® was used (Coetzee *et al.,*2016).

The above-described pharmacological actions for Zembrin® illustrate the potential to be developed as an antidepressant and anxiolytic agent, which is based on the capability of the two main pharmacologically active alkaloids (mesembrine and mesembrenone) to inhibit SERT and PDE4 activity (Harvey *et al.,*2011). Moreover, actions on PDE4 hint at possible anti-inflammatory actions, which have been described elsewhere (Smith, 2011).

Pre-clinical and clinical studies with Zembrin®: Recently, study from our laboratory showed that the combination of Zembrin® 50 mg/kg/day (given via oral gavage) and escitalopram (5 mg/kg/day) significantly increased both 5-HT and NE in the hippocampus (Gericke, 2019), whereas clinical studies on Zembrin® have also confirmed the safety and tolerability of Zembrin® (25 mg/kg/day) (Nell *et al.,*2013; Chiu *et al.,*2014; Murbach *et al.,*2014) with subsequent efficacy studies performed in healthy individuals showing benefit (Zembrin® 25 mg/kg or 50 mg/kg/day) to improve mood, cognitive performance and anxiety (Dimpfel *et al.,*2016b). Magnetic resonance imaging studies in healthy humans have demonstrated functional activity for Zembrin® in the amygdala, the major fear-regulating centre of the brain (Terburg *et al.,*2013), further hinting at its potential to relieve anxiety and stress-related conditions. A single dose of Zembrin® (25 mg/kg/day or 50 mg/kg/day) was found to impact electrical circuitry of the brain resulting in reduction of psychological stress and enhancement of concentration during treatment (Dimpfel *et al.,*2017).

Currently, clinical studies have been limited to healthy volunteers, looking at cognitive function and anxiety (Dimpfel *et al.,*2017). In these studies, no biomarkers were measured except behavioural tests such as cognitive tests (i.e., memory test, calculation

performance test) and anxiety (Hamilton Anxiety Rating Scale (HAM-A)) (Dimpfel *et al.,*2016b). Although recent studies showed that Zembrin® has potential for treatment of MDD (Olantuji *et al.,*2021; Brendler *et al.,*2021), it is surprising that Hamilton Rating Scale for Depression ($HAM\text{-}D_{17}$) was not used in the earlier study conducted to measure anxiety (HAM-A) (Dimpfel *et al.,*2017). Fourier magnetic resonance imaging (FMRI) was used to evaluate anxiolytic-like effects in healthy individuals (Terburg *et al.,*2013). Additionally, changes in electropharmacogram (EEG), which correlates with neurotransmitter activity (i.e., dopaminergic, noradrenergic, and cholinergic systems), showed that Zembrin® (25 mg/kg/day or 50 mg/kg/day) improve cognitive function and anxiety in healthy adults (Dimpfel *et al.,*2017). While these are not depressed populations, the studies provided promising data in support of Zembrin® to improve mood, relieve stress and reduce anxiety (Murbach *et al.,*2014). Clearly clinical studies are required, but until then it is imperative to test the extract in more robust animal models with greater validity for the human condition.

2.8 RAT MODELS OF DEPRESSION

Animal models that display various neurobiological features of MDD (Planchez *et al.,*2019) provide an appropriate means for examining the neuronal circuit, signal transduction, and neurobiology that may contribute to MDD pathophysiology (Becker *et al.,*2021; Wang *et al.,*2017). Such models are designed to provoke neurobiological and behavioural changes in animals that are akin to human MDD, termed construct and face validity, and allow researchers to explore new biological targets as well as new antidepressants (Wang *et al.,*2017; Vollmayr *et al.,*2007). An animal model as a non-human organism produces human pathophysiological characteristics with some degree of predictive validity (Vollmayr *et al.,*2007). Predictive validity implies that the bio-behavioural manifestations noted in the animal model responds to chronic treatment with clinical antidepressants or electroconvulsive therapy (ECT) (Abeleira *et al.,*2013). Rodent depression models include learned helplessness (LH), chronic unpredictable mild stress (CUMS) (Willner,2016), early-life stress (maternal deprivation, post-weaning social isolation (SIR), olfactory bulbectomy (OBX), glucocorticoid exposure (chronic ACTH), and genetic animal models such as the Flinders Sensitive Line (FSL) (Overstreet and Wegener, 2013) and Wistar-Kyoto (WK) rats (Albelaira *et al.,*2013; Wang *et al.,*2017).

2.8.1 Flinders Sensitive Line (FSL) rat model

Flinders Sensitive Line rat has been widely used for behavioural research in MDD; for investigating novel biological markers and drug targets, and for determining the therapeutic properties of novel antidepressants (Overstreet and Wegener, 2013). FSL rats are selectively bred from Sprague-Dawley strain rats that present with the typical behavioural attributes of MDD (Overstreet and Wegener, 2013; Wang *et al.,*2017). However, the FSL rats were bred to show high sensitivity to the anticholinesterase diisopropyl fluorophosphate (DFP) for the purpose of creating rats highly sensitive to the effects of muscarinic receptor agonists. Its primary purpose has since developed to emulate the biological and behavioural mechanisms that parallel the symptoms of MDD in humans (Overstreet *et al.,*2005). Following initial behavioural characterization, the FSL and their normal control, the Flinders Resistant Line (FRL) rat, were proposed as a rat model of human MDD (Overstreet and Wegener, 2013). The model has subsequently been found to present with important characteristics that align with MDD, according to three distinct validation criteria as outlined below:

*Face validity***:** FSL rats display several symptoms that resemble depressive-like behaviour in humans, such as increased rapid eye movement (REM) sleep, reduced appetite, reduced activity, anhedonia, less active coping in a stressful environment (climbing and swimming), and increased despair (immobility) (Overstreet and Griebel, 2005; Overstreet and Wegener, 2013).

*Construct validity***:** FSL rats display various neurobiological features of MDD (Overstreet and Wegener, 2013), such as reduced levels of 5-HT in the hippocampus (Gericke, 2019), abnormalities in the HPA axis (Overstreet and Wegener, 2013), reduced synaptic plasticity (Eriksson *et al.,*2012), decreased BDNF in the hippocampus (Elfving *et al.,*2010), altered alpha 2 AR expression (Lillethorup *et al.,*2015), oxidative stress (Mokoena *et al.,*2015), mitochondrial dysfunction (Chen *et al.,*2013), metabolic dysfunction (Abildgaard *et al.,*2011), and altered glutamate-nitric oxide signalling (Wegener *et al.,*2010). Its original feature of hypersensitivity to cholinergic agonists is also in line with the cholinergic hypothesis of MDD (Overstreet and Janowsky,1991).

Predictive validity: As is typical in treatment response in MDD, the bio-behavioural manifestations akin to MDD noted in FSL rats respond preferentially to chronic treatment with clinical antidepressants, such as TCAs (e.g. imipramine) (Oberholzer *et al.,*2018)

and desipramine (Mokoena *et al.,*2015), SSRIs (e.g. escitalopram) (Gericke, 2019), SNRI's (e.g., venlafaxine) (Steyn, 2011), sildenafil (Saayman *et al.,*2021), ketamine (Liebenberg *et al.,*2015) and a large variety of experimental drugs with potential antidepressant activity in human MDD (Nish *et al.,*2009; Overstreet and Wegener, 2013; Uys *et al.,*2017), including plant extracts (Oberholzer *et al.,*2018; Gericke *et al.,*2022).

2.9 BEHAVIOURAL TESTS IN RODENTS

For neuropharmacology and neurobiology research, behavioural assessments have long been used to assess animal emotional behaviour to explore new treatment strategies for either anxiety or MDD (Belovicova *et al.,* 2017).

2.9.1 Anxiety

Due to high prevalence of anxious depression and overlaps in the symptoms (Altin *et al.,*2014), two widely used anxiety assessments in rodents including the open field test (OFT) and the elevated plus-maze (EPM), were used in this study. Both the OFT and EPM are designed to measure animal emotional activity, especially anxiety, when subjected to certain aversive stimuli, in this instance bright light and open spaces where natural risk aversion to predation is increased (Belovicova *et al.,* 2017). The EPM is generally considered a more robust test than the OFT (Regenass *et al.,*2018; Steyn *et al.,*2018).

2.9.1.1. Open Field Test (OFT)

The OFT is a paradigm commonly employed to measure general locomotor activity (Harvey and Brand,2017). The apparatus may be constructed from various materials such as Plexiglass, aluminium, painted (black or white) plywood, while the shape may be square, rectangular, or circular and surrounded by high walls to prevent escape (Gould *et al.,*2009; Stanford, 2007). The animal is placed in the center of the arena, allowed to explore for 5 min under red light (80lx) (Steyn *et al.,*2020) and the total distance covered during the specified time and the time spent in the central zone are recorded and analysed (Oberholzer *et al.,*2018; Steyn *et al.,*2020). Thigmotaxis, which refers to the animal staying close to the wall, is an indicator of anxiety in rodents which correlates with fear behaviour in humans (Walz *et al.,*2016). Animals that spend more time in the central zones of the box are regarded as less anxious, while more anxious animals stay near to the walls of the arena (Oberholzer *et al.,*2018; Saayman, 2019).

Importantly, the OFT can be performed before the Forced Swim Test (FST) to control for locomotor activity (Brand and Harvey; 2017).

2.9.1.2. Elevated Plus Maze (EPM)

The EPM is a paradigm used to evaluate anxiety-like behaviour in rodents and has been validated to assess the anxiolytic effects of various drugs (Schneider *et al.,*2011; Walf and Frye, 2007). The EPM consists of two open arms and two closed arms which together form a plus ("+") (Regenass *et al.,*2018) and illumination of the closed arms under red light (80lx), as described previously (Steyn, 2011). The EPM apparatus and its features may differ among laboratories, with the apparatus made from plexiglass, polyvinylchloride (PVC), aluminium, etc., and is usually elevated 0.5 m above the ground (Regenass *et al.,*2018; Hogman *et al.,*2014). These characteristics are necessary in to measure innate fear of rodents for open arms and elevated spaces (Mokoena *et al.,*2015). In the text, anxious rats tend to spend more time in the closed arms (perceived as more protective) whereas less anxious rats tend to explore the open arms more frequently (Steyn, 2011). The animal is placed at the junction of the plus with the nose facing an open arm. The exploration times (the entries or time spent in closed versus open arm) are recorded and analysed (Regenass *et al.,*2018; Steyn, 2011). Reduced open arm exploration and increased closed arm exploration are scored as measures of increased anxiety, with the opposite an indication of reduced anxiety (Steyn, 2011).

Accumulating evidence has demonstrated that various antidepressant drugs such as desipramine (Tanyeri *et al.,*2018; Mokoena *et al.,*2015), imipramine, venlafaxine, and bupropion (Tanyeri *et al.,*2018) increase open arm exploration as well as increase the number of open arms entries when compared to non-treated or vehicle-treated animals (Tanyeri *et al.,*2018), thereby supportive of the anxiolytic effects of these treatments.

2.9.2. Depression

2.9.2.1. Forced Swim Test (FST)

The FST is a widely used behavioural test to evaluate depression-like behaviour in rats (Porsolt *et al.,*1978). Further, the test is used to determine the depressogenic effects of various stressful procedures and to determine the antidepressant-like properties of drugs (Brand and Harvey, 2017). The test primarily assesses learned helplessness and despair, both of which are typical behavioural manifestations of MDD. The modified FST enables

investigators to further subdivide behaviours into various coping strategies, specifically climbing and swimming. Moreover, the aforementioned coping behaviours are based on NA'ergic (climbing) and 5-HT'ergic (swimming) mediated responses, thus informing on the possible monoaminergic actions of the tested antidepressant (Cryan *et al.,*2005). In the modified FST, the animals are placed individually into the cylinders (20 cm x 60 cm) filled with water to a 30cm depth and allowed to swim at ambient temperature of 25±1°C. Thereafter, the duration of immobility, swimming, and climbing behaviours are scored using video-recordings (Cryan *et al.,*2002). The FSL rat has its foundation in the ability to display depressive-like behaviour (i.e., increased immobility in FST, decreased struggling and swimming) without requiring pre-condition swim 24 hr before conducting the FST test typically used when studying other rodent strains (Overstreet *et al.,*2005).

The ability of the FST to allow classification of antidepressants according to their neurochemical underpinnings, i.e., serotonergic (swimming) or noradrenergic (climbing and struggling), is a particularly useful attribute. Thus, antidepressants that increase swimming duration include SSRIs such as escitalopram (Gericke, 2019), whereas those that increase struggling/ climbing include NRIs such as desipramine (Mokoena *et al.,*2015). The FST has seen increasing use to screen novel compounds for potential antidepressants activity, such as plant extracts (Gericke *et al.,*2022; Oberholzer *et al.,*2018) as well as novel treatments (Delport *et al.,*2014; Delport *et al.,*2018; Uys *et al.,*2017). The FST has good face validity for MDD, viz. immobility reflecting learned hopelessness and despair, while also assessing stress sensitivity in rodents (Gericke, 2019; Brand and Harvey, 2017; Bogdanova *et al.,*2013). As for construct validity, the assessment of coping behaviours speaks to the possible neurobiological underpinnings of MDD, especially when combined with regional brain monoamine analysis (Uys *et al.,*2017; Brand and Harvey, 2017), while robust predictive validity is confirmed by the test's ability to respond to NRIs, SSRIs, SNRIs, as well as miscellaneous compounds (Gericke *et al.,*2022; Oberholzer *et al.,*2018; Delport *et al.,*2014; 2018; Uys *et al.,*2017).

Despite the popular use of the FST to predict compounds with potential antidepressant activity, the test has some limitations and shortcomings that should not be ignored or overlooked (Bogdanova *et al.,*2013; de Kloet and Molendijk, 2015). In their review study, Bogdanova *et al*. (2013) outlined different factors that may affect the credibility of behavioural despair as assessed in the FST (Table 2.3).

Table. 2.3. Summary of factors that can affects interpretation of the behaviours in Forced Swim Test (FST) (Bogdanova *et al.,*2013)

Biological factors	**Preconditioning and treatment**	**Test design**	**Environmental conditions**
Animal strain- FSL rat has ↑ immobility	Handling-long term handling (2 months prior FST) ↑ immobility	Test equipment and settings	Light
Age and body weight-old animals have ↑ immobility	Housing-enriched environment↓ immobility	Time effects	Noise
Gender	Diet- high fat diet ↑ immobility	Duration of the procedure	Odour
Individual animal variability-variation in immobility	Dosing schedule, drug type and dose-	Scoring and statistical analysis	
	Stressors	Combination with other behavioural tests	

The above-mentioned factors may lead to either false-positive results, like non-antidepressant drugs such as psychostimulants, benzodiazepines, and barbiturates, or false-negative results seen with antidepressants that may fail to reduce immobility yet prove to be effective antidepressants, e.g., SSRI's (de Kloet and Molendijk, 2015; Bogdanova *et al.,*2013; Slattery and Cryan, 2012).

However, many of the above detractions to the FST can be addressed by applying the modified FST technique. This involves increasing the depth of the water from 15-18cm to 30cm, excluding the first and the last minutes in a 7 min recording time to exclude the confounding effect of trapped air pockets in the fur of the animal that may provide buoyancy and thus give a false positive (Bogdanova *et al.,*2013; Slattery and Cryan, 2012; Cryan, 2002). More importantly, the inclusion of an additional test such as the OFT immediately before the FST can be used to control whether locomotor activity contributes

in any way to the psychomotor effects of the tested compound (Gericke *et al.,*2022; Brand and Harvey, 2017; Mokoena *et al.,*2015), and thus also introduce a false positive risk.

2.10 Synopsis

Major depressive disorder remains a seriously debilitating condition with clinical symptoms that diminish the patient's functioning and quality of life, or cause impairment in social and occupational functions (APA, 2013). However, the aetiology of the condition remains unknown. Different pathophysiological changes in brain regions such as the hippocampus and prefrontal cortex has promulgated various hypotheses in antidepressant research, including hypotheses that emphasize disturbances in monoamine, HPA-axis, neuro-inflammation, neuroplasticity, cholinergic super-sensitivity, glutamatergic, and gene-environment (Jianer *et al.,*2013; Feltes *et al.,*2017; Wang *et al.,*2017; Dale *et al.,*2017; Brand *et al.,*2015; Klenge and Binder, 2015). Another factor that further complicates the diagnosis of MDD is the absence of diagnostic biomarkers (Hacimusalar and Esel, 2018). Additionally, MDD is often co-morbid with anxiety, so that co-presenting anxiety conditions complicate diagnosis, treatment as well as the long-term outcome of MDD.

The FDA has approved different classes of antidepressants currently used for the treatment of MDD (Liebarman and Massey, 2009). However, these drugs are only effective in approximately 50% of patients (Brand *et al.,*2015; Harvey and Slattery, 2014). Importantly, many of these conventional antidepressants, with exception of atypical newer agents like vortioxetine, typically target one, perhaps two of the recognized pathological mechanisms of MDD. This accounts for loss of efficacy, while they are also associated with enduring side effects that compromise patient compliance, hence contributing to poor remission rates (Boku *et al.,*2018; Harvey and Slabbert, 2014). That said, the main problem with currently available antidepressants, bar ketamine, is a delayed onset of action which poses further challenges regarding non-compliance and poor remission (Boku *et al.,*2018; Liu *et al.,*2018).

Multi-target antidepressants, such as vortioxetine (Ostuzzi *et al.,*2020) offer a better potential to treat symptoms of MDD more effectively (Liu *et al.,*2018). Research has noted that PDE4B plays an important role in the pathophysiology and antidepressant and anxiolytic like effects of various drugs, including PDE4 inhibitors (Liu *et al.,*2009; Zhang *et al.,*2008). Studies have also suggested the value of multi-target PDE4 inhibitors

(Cashman *et al.,*2009). With combined SERT and PDE4 inhibitory properties, Zembrin® heralds promise for future development for the treatment of anxiety and MDD, both as a monotherapy and as adjunctive therapy (Gericke, 2019) as it works by various mechanisms that have potential antidepressant and anxiolytic actions. The latter aspects have been explored in various pre-clinical and clinical studies (Smith, 2011; Terburg *et al.,*2013).

References

Abelaira, H.M., Reus, G.Z., Quevedo, J. 2013. Animal models as a tool to study the Pathophysiology of depression. *Brazilian Journal of Psychiatry*, 35(2):112-120.

Abildgaard, A., Solskov, L., Volke, V., Harvey, B.H., Lund, S., Wegener, G. 2011. A high-fat diet exacerbates depressive-like behaviour in the Flinders Sensitive Line (FSL) rat, a genetic model of depression. *Psychoneuropharmacology*, 36(5): 623-633

Adams, G.C., Balbuena, L., Meng, X., Asmundson, G.J.G. 2016. When social anxiety and depression go together, A population study of comorbidity and associated consequences. *Journal of Affective Disorders*, 206:48-54

Adams, S.M., Miller,K.E., Zylstra,R.G. 2008. Pharmacological management of adult depression. *American Family Physician*, 77(6):786-791

Aghajanian, G., Liu, R.J. 2009. Serotonin (5-Hydroxytryptamine-5-HT); CNS pathways and neurophysiology. *Encyclopaedia of Neuroscience*, 715-722

Albert, R.P. 2015. Why depression more prevalent in women? *Journal of Psychiatry Neuroscience*, 40(4):219-221

Alev, L., Smith, A.L., Altin, M., Duenas, H., *et al.* 2013. A review of serotonin norepinephrine reuptake inhibitors; pharmacologic aspects and clinical implications for treatment of major depressive disorder and associated painful physical symptoms. *Open Journal of Depression*, 4(2): 54-63

Altin, M., Harada, E., Schacht, A., Berggren, L., Walker, D., and Duenas, H. 2014. Does early improvement in anxiety symptoms in patients with major depressive disorder affect remission rates? A post-hoc analysis of pooled duloxetine clinical trials. *Open Journal of Depression*, 3(3):112-123

Alvano, A.S., Zieher, L.M. 2020. An updated classification of antidepressants; A proposal to simplify treatment. *Personalized Medicine in Psychiatry*, 100042:19-20

American Psychiatric Association. 2013. Diagnostic and Statistical Manual of Disorders 5th Edition (DSM-5). Washington, D.C. https://www.psychiatry.org/psychiatrists/practice/dsm Date of access: 05. May.2020

Anacker, C., Hen, R. 2017. Adult hippocampal neurogenesis and cognitive flexibility-linking memory and mood. *Nature Review Neuroscience*, 18(6):335-346

Arakawa, R., Stenkrona, P., Takano, A., *et al.* 2019. Venlafaxine ER blocks the norepinephrine transporter in the brian of patient with major depressive disorder; a PET study using [^{18}F] FMeNER-D_2. *International Journal of Neuropsychopharmacology*, 22(4):278-285

Arrondo, G., Segarra, N., Metastasio,A., Ziauddeen H., Spencer, J., Reinders ,N.R., Dudas, R.B., Robbins, T.W., Fletcher, P.C., Murray, G.K. 2015. Reduction in ventral striatal activity when anticipating a reward in depression and schizophrenia; a replicated cross-diagnostic finding. *Frontier in Psychology*, 6(1280):1-12

Avasthi, A., Kulhara, P., Singh, G., Sharma, R., Kamir, R. 2005. Comparison of the efficacy and safety of moclobemide and imipramine in the treatment of depression in Indian patients. *Indian Journal of Psychiatry*, 47(2): 84-88

Ballmaier, M., Toga, A., Blanton, R.E., Sowell, E.R., Lavretsky, H., Peterson, J., Pham, D., Kumar, A. 2004. Anterior cingulate, gyrus rectus and orbitofrontal abnormalities in elderly depressed patients; An MRI based parcellation of the prefrontal cortex. *American Journal of Psychiatry*, 161(1):99-108

Becker, M., Pinhasov, A., Ornoy, A. 2021. Animal Models of Depression: What Can They Teach Us about the Human Disease? *Diagnostics*, 11(1):123

Belovicova, K., Bogi, E., Dubovicky, M. 2017. Animal testing for anxiety-like and depression-like behavior in rats. *Interdisciplinary Toxicology*, 10(1):40-43.

Belujon, P., Grace, A.A. 2014. Restoring mood balance in depression; ketamine reverses deficit in dopamine-dependent synaptic plasticity. *Biological Psychiatry*, 76(12): 927-936

Berger, M., Gray, J.A., Roth, B.L. 2009. The expanded biology of serotonin. Annual *Review of Medicine*, 60: 355-366

Bilsen, J., 2018. Suicide and youth; risk factors. *Frontiers in Psychiatry*. 540 (9): 1-5

Blier, P. 2001. Crosstalk between the norepinephrine and serotonin systems and its role in the antidepressant response. *Journal of Psychiatry and Neuroscience*, 26 (suppl): S3-10

Bogdanova, O. V, Kanekar, S., Renshaw, P. F. 2013. Factors influencing behavior in the forced swim test. *Physiology and Behavior*, 118: 227–239

Boku, S., Nakagawa, S., Toda, H. and Hishimoto, A. 2018. Neural basis of major depressive disorder; beyond monoamine hypothesis. *Psychiatric and Clinical Neurosciences*, 72(1):3-12

Bondy, B. 2002. Pathophysiology of depression and mechanism of treatment. *Dialogues in Clinical Neuroscience*, 4(1):7-20

Botteron, K.N., Raichle, M.E., Drevets, W.C., Heath, A.C., Todd, R.D. 2002. Volumetric reduction in left subgenual prefrontal cortex in early onset of depression. *Society of Biological Psychiatry*, 51(4): 342-344

Brand, S.J., Harvey, B.H. 2017a. Exploring a Post-traumatic stress disorder paradigm in Flinders Sensitive Line rats to model treatment resistant depression I; bio-behavioural validation and response to imipramine. *Acta Neuropsychiatrica*, 29(4):193-206

Brand, S.J., Moller, M., and Harvey, B.H. 2015. A review of biomarkers in mood and Psychotic of disorders: A dissection of clinical vs pre-correlates. *Current Neuropharmacology*, 13(3): 324-368

Bratsos., S., Saleh, S.N. 2017. Clinical efficacy of ketamine for treatment-resistant depression. *Cureus*,11(7):2-5

Bremner, J.D., Vythilingam, M., Vermittin, E., *et al.* 2002. Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry*, 51 (4): 273-279

Brendler, T., Brinckmann, J.A., Feiter, U., Gericke, N., Lang, L., Pozharitskaya, O.N., Shikov, A.N., Smith, M., Van Wyk, B-E. 2021. Sceletium for managing anxiety, depression, and cognitive impairment; A traditional herbal medicine in modern -day regulatory systems. *Current Neuropharmacology*,19 (9):1394-1400

Brichta, L., Greengard, P., Flajolet. M. 2013. Advances in the pharmacological treatment of Parkinson's disease; targeting neurotransmitter systems. *Trends in Neurosciences*, 36(9):543-554 52.

Cannon, D.M, Klaver, J.M., Peck, S.A., Rallisvoak, D., Erickson,K., Drevets, W.C. 2009. Dopamine type-1 receptor binding in major depressive disorder assessed using positron

emission tomography and [11C] NNC-112. *Neuropsychopharmacology*, 34(5): 1277-1287

Cao, B., Park, C., Subramaniapillai, M., Lee, Y., Iacobucci, M., Mansur, R.B., Zuckerman, H., Phan, L., McIntyre, R.S. 2019. The efficacy of vortioxetine on anhedonia in patients with major depressive disorder. *Frontiers in Psychiatry,* 10(17) :1–8.

Carr, G.V., Lucki, I. 2011. The role of serotonin receptor sub-types in treating depression; a review of animal studies. *Psychopharmacology*, 213(2-3):265-287

Carvalho, A.F., Sharma, M.S., Brunoni, A.R., Vieta, E. 2016. The safety, tolerability and risks associated with the use of new generation antidepressant drugs: A critical review of the literature*. Psychotherapy and Psychosomatics*, 85:270-28

Cashman, J.R., Voelker, T., Zhang, H.T., O' Donnell, J.M. 2009. Dual inhibitors of phosphodiesterase-4 and serotonin reuptake. *Journal of Medicinal Chemistry*, 52(6):1530-1539

Celada, P., Puig, V., Bosc, M.A., Adell, A., Artiga, F. 2004. The therapeutic role of 5-HT_{1A} and 5-HT_{2A} receptors in depression. *Journal of Psychiatry and Neuroscience*, 29 (4): 252-65

Chandley, M.J., Ordway, G.A. 2012. Noradrenergic dysfunction in depression and suicide. *The neurobiological basis of suicides Frontiers in Neuroscience,* 3:1-67

Charnay, Y., Leger, L. 2010. Brain serotonergic circuitries. *Dialogue in Clinical Neuroscience*, 4(12): 471-487

Charney, D.S. 2002. Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry*, 51(4): 273-279

Chen, F., Wegener, G., Madsen, T.M., Nyengaard, J.R., 2013. Mitochondrial Plasticity of the Hippocampus in a Genetic Rat Model of Depression After Antidepressant Treatment.*Synapse*.67:127–134

Chiu, S., Gericke, N., Woodbury, M.F., *et al.* 2014. Proof-of-concept randomized controlled study of cognition effects of the proprietary extract Sceletium tortuosum (Zembrin®) targeting phosphodiesterases-4 in cognitively healthy subjects, implication for Alzheimer's Dementia. *Evidence-Based Complementary and Alternative Medicine*. https://doi.org/10.1155/2014/682014 Date of access: 08. Aug.2021

Coetzee, D.D., Lopez, V.V., Smith, C. 2016. High –Mesembrine Sceletium extract (Trimesemine^{TM}) is a monoamine releasing agent, rather than only a selective serotonin reuptake inhibitor. *Journal of Ethnopharmacology*, 177: 111-116

Coplan, J.D., Aaronson, C.J., Panthangi, V., Kim, Y. 2019. Treating comorbid anxiety and depression: Psychosocial and pharmacological approaches. *World Journal of Psychiatry*, 5(4):366-378

Cottingham, C., Ferryman, C.J., and Wang, Q. 2015. A2-adrenergic receptor trafficking as a therapeutic target in antidepressant drug action. *Progress in Molecular Biology and Translational Science*, 132: 207-225

Cottingham, C., Wang, Q. 2012. α_2-adrenergic receptor dysregulation in depressive disorders, implication for neurobiology of depression and antidepressant therapy. *Neuroscience and Bio-behavioural Review*, 36: 2214-2225

Cowen, P. 2016. Neuroendocrine and neurochemical process in depression. *Journal of Experimental Psychopathology*, 3(1): 315

Croom, K.F., Perry, C.M., Plosker, G., L. 2009. Mirtazapine. *CNS Drugs*, 23(5) :427-452

Cryan, J. F., Page, M. E., Lucki, I. 2002. Assessing antidepressant activity in rodents: recent development and future needs. *Trends in Pharmacological Sciences*, 25(5):238-245.

Cryan, J. F., Valentino, R. J., Lucki, I. 2005. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neuroscience and Bio-behavioral Reviews*, 29 (4–5): 547–569.

Cuadros, D.F., Tomita, A., Vandormael, A., Slotow, R., Burns J.K., Tanser, F. 2019. Spatial structure of depression in South Africa; A longitudinal panel survey of a national representative sample of household. *Open Scientific Reports*, 9(978):1-10

Culpepper, L. 2013. Reducing the burden of difficult to treat major depressive disorder, revisiting monoamine oxidase therapy. *The Primary Care Companion for CNS Disorders*, 15 (5). PCC.13r01515. https://doi.org/10.4088/PCC.13r01515 Date of access: 08. Aug.2020

Dale, E., Bang-Anderson, B., Sanchez, C. 2015. Emerging mechanism and treatment for depression beyond SSRIs and SNRIs. *Biochemical pharmacology*. 95:81-97

Dantzer, R., 2017. The role of kynurenine metabolism pathway in inflammation-induced depression- preclinical approaches. *Current Topics in Behavioural Neuroscience*, 31: 117-138

Dantzer, R., O' Connor, J.C., Freund, G.G., Johnson, R.W., and Kelley, K.W. 2008. From inflammation to sickness and depression when the immune system subjugates the brain. *Nature Review Neuroscience*, 9(1): 45-46

De Kloet, E.R., Molendijk, M.L. 2016. Coping with the forced swim stressor; towards understanding an adaptive mechanism. *Neural Plasticity*. https://doi.org/10.1155/2016/6503162 Date of access:10. Jul. 2020

Dean, J., Keshavan, M. 2017. The neurobiology of depression; an integrated view. *Asian Journal of Psychiatry*, 27: 101-111

Debonnel, G., Saint-Andre, Elise, Hebert, C., de Montigny, C., Lavoie, N., Blier, P. 2007. Differential physiological effects of a low dose and high dose of venlafaxine in major depression. *International Journal of Neuropsychopharmacology*, 10:51-61

Dell'Osso, B., Palazzo, M.C., Oldani, L., Altamura, A.C. 2011. The noradrenergic action in antidepressant treatments. Pharmacological and clinical aspects. *CNS Neuroscience and Therapeutics*, 17: 723-732

Delport, A, Harvey, B.H., Petzer, A., Petzer, J.P. 2014. Azure B and a synthetic structural analogue of methylene blue, ethylthioninium chloride, present with antidepressant-like properties. *Life Sciences*, 117(2):56-66

Delport, A., Harvey, B.H., Petzer, A., Petzer, J.P. 2018. Methylene Blue Analogues with Marginal Monoamine Oxidase Inhibition Retain Antidepressant-like Activity. *ACS Chemical Neuroscience*, 9(12):2917-2928

Dhillon, S., Yang, L., Curran, M.P. 2008. Bupropion; A review of its use in the management of major depressive disorder. *Drug*, 68(5):663-689

Di Giovanni, G., Strac, D.S., Sole, M., Unzeta, M., Tipton, K. F., Muck-Seler. M., Bolea, I., et al. 2018. Monoaminergic and histaminergic strategies and treatments in Brain diseases. *Frontiers in Neuroscience*, 10(541):1-28.

Dimpfel W., Gericke, N., Suliman, S., Gwladys, N. and Chiegoua, D. 2017. Psychophysiological effects of Zembrin® using quantitative EEG source density in combination with eye-tracking in 60 healthy subjects. A double-blind randomized, placebo- controlled, 3-armed study with parallel design. *Journal of Neuroscience and Medicine*, 7(1): 114-132.

Dlaboga, D., Hasshussein, H., O' Donnell, J.M. 2006. Regulation of phosphodiesterase4 expression in mouse brain by repeated antidepressant treatment; comparison with Rolipram. *Brain Research*, 1096:104-112

Doze, V. A., Handel, E.M., Jensen, K.A., Luger E.J., Haselton, J.R., Talbot, .J.N., Rorabaugh, B.R. 2009. $\alpha_{1\text{-}A}$ and α_{1B} -adrenergic receptor differentially modulate antidepressant like behaviour I the mouse. *Brain Research*, 128: 148-157

Drevets, W. C., Price, J. L., Furey, M. L. 2008. Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure & Function*, 213(1-2): 93–118

Dulawa, S.C., Janowsky,D.S. 2019. Cholinergic regulation of mood: From basic and clinical studies to emerging therapeutics. *Molecular Psychiatry*, 24(5):694-709

Ellis., J.S., Zarate, C.A., Luckenbaugh, D.A., Furey, M.L. 2014. Antidepressant treatment history as a predictor of response to scopolamine: clinical implications. *Journal of Affective Disorders*, 162:39-42

Eriksson, T.M., Delagrange, P., Spedding, M., Popoli, M., Mathe, A.A., Ogren, S.O. and Svenningsson, P. 2012. Emotional memory impairments in a genetic rat model of depression 5-HT/MEK/Arc signaling in restoration. *Molecular Psychiatry,* 17: 173-184

Fan, C., Long, Y., Wang, L., Liu, X., Liu, Z., Lan, T., Li., Y., Yu., S. 2020. N-Acetylcystein rescues hippocampal oxidative stress-induced neuronal injury via suppression of p38/JNK signalling in depressed rats*. Fronteirs in Cellular Science*,14(554613):1-11

Fasipe, O.J. 2018. Neuropharmacological classification of antidepressant agents based on their mechanism of action. *Archives of Medicine and Health Sciences*, 6:81-94.

Feltes, P.K., Doorduin, J., Klein, H.C., Orozco,L.E.J., Dierckx, R.,A., Jeckel, C.M.M, Vries, E.F. 2017. Anti-inflammatory treatment for major depressive disorder; Implication for patients with an elevated immune profile and non-responders to standard antidepressant therapy. *Journal of Psychopharmacology*, 31(9):1149-1165

Frodl, T., Meisenzahl, E.M., Zetzsche, T., Born, C., Jager, M., Groll, C., Bottlender, R., Leinsinger, G., Moller, H-J. 2003. Large amygdala volume in first depressive episode as compared to recurrent major depression and health control subjects. *Biological Psychiatry*, 53(4):338-344

Fujita, M., Hines, C.S., Zoghbi, S.S., Mallinger, A.G., *et al.* 2012. Down regulation of brain phosphodiesterase type IV measured with C-®- Rolipram positron Emission Tomography in major depressive disorder. *Journal of Biological Psychiatry*, 72(7):548-554

Furey, M.L., Khanna, A., Hoffman, E.M., Drevets, W. 2010. Scopolamine produces large antidepressant and antianxiety effects in women than in men. *Neuropsychopharmacology*, 35:2479-2488

Furmaga, H., Shah, A., Frazer, A. 2011. Serotonergic and noradrenergic pathways are required for the anxiolytic-like and antidepressant–like behavioral effects of repeated vagal nerve stimulation in rats. *Biological Psychiatry*, 70:937-945

Furukawa, T.A., McGuire, H., Barbui, C. 2002. Meta-analysis of effects and side effects of low dosage tricyclic antidepressants in depression: systematic review. *British Medical Journal*, 325:991-995

Furukawa, T.A., Salanti, G., Atkinso, L., Leucht, S., *et al.* 2015. Comparative efficacy and acceptability of first generation and second-generation antidepressants in the acute treatment of major depression: Protocol for a network meta-analysis. *British Medical Journal*, 6(7). https://doi.org/10.1136/bmjopen-2015-010919e010919 Date of access: 08. Dec. 2021

Garakani, A., Murrough, J.M., Freire, R.C., Thom, R.P., Larkin, K., Buono, F. D., Losifescu, D. V. 2020. Pharmacotherapy of anxiety disorders: current and emerging treatment options. *Frontiers in Psychiatry*, 11: 595584

Gericke, J. 2019. Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, A.M., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:114550

Gericke, N., Viljoen, A.M. 2008.*Sceletium*– A review update. *Journal of Ethnopharmacology*,119(3): 653-663

Gillman, P.K. 2007. Tricyclic antidepressant pharmacology and therapeutic drug interaction updated; review. *British Journal of Pharmacology*, 151: 737-748

Gilsbach, R., Hein, L. 2012. Are the pharmacology and physiology of α_2-heteroreceptors and autoreceptors respectively?. *British Journal of Pharmacology*,165:90-102

Goddard, A.W., Ball, S.G., Martinez, J., Robinson, M.J., Yang, C.R., Russel, J.M., Shekhar, A. 2010. Current perspectives of the roles of central norepinephrine system in anxiety and depression. *Review of Anxiety-Depression and Norepinephrine,* 27:339-350.

Gould, T.D., Dao, D.T., Kovacsics, C.C. 2009. Mood and Anxiety related Phenotypes in Mice; Characterization using behavioral tests. *Humana Press.*1-20.

Guiard, B.P., El monsai, M., Blier, P. 2008. Cross talk between the dopaminergic and noradrenergic systems in the rat ventral tegmental area, locus coeruleus, and dorsal hippocampus. *The American Society for Pharmacology and Experimental Therapeutics*, 74 (5): 1463-1475

Hacimusalar, Y., Esel, E. 2018. Suggested biomarkers for major depressive disorder. *Archives of Neuropsychiatry*, 55 (3): 280-290

Haller, H., Anheyer, D., Cramer, H., Dobos, G. 2019. Complementary medicine for clinical depression; an overview of systematic reviews. *British Medical Journal Open*, 9(8): 1-9

Halser, G. 2010. Pathophysiology of depression; do we have any solid interst to clinicians?. *World Psychiatry*, 9(3): 155-161

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P. 2011. Pharmacological actions of South African Medicinal and Functional plant Sceletium tortuosum and its principle alkaloids. *Journal of Ethnopharmacology*,137(3): 1124-1129

Harvey, B.H. 1997. The neurobiology and pharmacology of depression. A comparative overview of serotonin selective antidepressants. *South Africa Medical Journal*, 87(4):540-552

Harvey, B.H., Naciti, C., Brand, L., Stein, D.J. 2003. Endocrine, cognitive and hippocampal/cortical 5HT1A/2A receptor changes evoked by a time-dependent sensitisation (TDS) stress model in rats. *Brain Research*, 983:97-107

Harvey, B.H., Slabbert, F.N. 2014. New insight on the antidepressant discontinuation syndrome. *Human Psychopharmacology*, 29(6): 503-516

Helm, K., Viol, K., Weiger, T. M., Tass, P. A., Grefkes, C., Del Monte, D., Schiepek, G. 2018. Neuronal connectivity in major depressive disorder: a systematic review. *Neuropsychiatric Disease and Treatment*, 14:2715–2737

Hesham, E.Y., Anthony, S.P., David., B., S. 2021.Effects of celecoxib augmentation of antidepressant or anxiolytic treatment on affective symptoms and inflammatory markers in patients with anxiety disorders; explorative study. *International Clinical Psychopharmacology*, 36(3):126-132

Hillhouse, T.M., Porter, J.H. 2015. A brief history of the development of antidepressant drugs; from monoamine to glutamate. *Experimental and Clinical Psychopharmacology*, 23(1): 1-21

Hinz, M., Stein, A., Uncini, T. 2011. Monoamine depletion by reuptake inhibitors. *Drug, Health and Patient Safety*, 3: 69-77

Hogman, C. 2014. Explorative stratergies in the open field (OFT), elevated plus maze (EPM) and multivariate concentric square field (MCSF) in adolescent wistar rats. Uppsala University (MSc. dissertation)

Huotari, M., Gogos, J.A., Karayiorgou, M., *et al.* 2002. Brain catecholamine metabolism in catechol-O-methyl transferase (COMT) deficient mice. *European Journal of Neuroscience*, 15(2): 246-256

Itoh, T., Tokumura, M., Abe, K. 2004. Effects of Rolipram, a phosphodiesterase-4 inhibitor in combination with imipramine on depressive behaviour (CRE-binding activity and BDNF level) in learned helpless rats. *European Journal of Pharmacology*, 498 (1-3):135-142

Jainer, A.K., Kamatchi, R., Martzanski, M., Somashekar, B. 2013. Current Advances in the treatment of Major Depression: Shift towards receptor specific drugs. *Mental Disorder- Theoretical and Empirical Perspectives*, 12:270-288

Jayatissa, M., Bisgaard, C., Tingstrom, A., Papp, M., Wiborg, O. 2006. Hippocampal cytogenesis correlates to escitalopram-mediated recovery in a chronic mild stress rat model of depression. *Neuropsychopharmacology*, 31:2395-2404

Jin, Y., Sun, L.H., Yang, W., Cui, R.J., and Xu, S.B. 2019. The role of BDNF in the neuroimmune axis regulation of mood disorders. *Frontiers in Neurology*, 10 (515): 1-19

Katzung, B.G. 2018. Drugs that act on the Central Nervous System. *Basic and Clinical Pharmacology*. Page: 370-380: Mc Graw hill education

Kennedy, S.H., Rizvi, S.J. 2010. Agomelatine in the treatment of major depressive disorder, *CNS Drugs*.24(6):479-499

Kessler, R.C., Angermeyer, M., Anthony, J.C., De Graaf, R.O.N., Demyttenaere, K., Gasquet, I., De Girolamo, G., Gluzman, S., Gureje, O.Y.E., Haro, J.M. and Kawakami, N. 2007. Lifetime prevalence and age-of-onset distributions of mental disorders in the World Health Organization's World Mental Health Survey Initiative. *World Psychiatry*, 6(3):168

Kessler, R.C., Sampson, N.A., Berglund, P., Gruber, M.J., *et al*. 2015. Anxious and non-anxious major depressive disorder in the World Health Organization World Mental Surveys. *Epidemiology and Psychiatric Sciences*, 24(3):210-226

Kharade, S.M., Gumate, D.J. and Naikwade, N.S. 2010.A review: hypothesis of depression and roles of antidepressant drug. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2(4):3-6

Khushboo, S.B. 2017. Antidepressants: mechanism of action, toxicity and possible amelioration. *Journal of Applied Biotechnology and Bioengineering*, 3(5): 437-448.

Kivrak, Y., Guvenc, T.S., Akbulut, N., Yagci, I., Cigsar,G., Gunduz, S., Balci, B. 2014. Accelerated hypertension after Venlafaxine usage. *Case Reports in Psychiatry*, 659715. https://doi.org/10.1155/2014/659715 Date of access: 27.Nov. 2021

Klenge, T., Binder, E.B. 2015. Epigenetics of stress related psychiatric disorders and Gene X Environment interactive. *Neuron Review*, 86 (6):1343-57

Korff, S., Stein, D.J., Harvey, B.H. 2008. Stereotypic behaviour in the deer mouse: pharmacological validation and relevance for obsessive compulsive disorder. *Progress in Neuropsychopharmacology and Biological Psychiatry*, 32(2):348-355

Kovich, H., Dejong, A. 2015. Common questions about the pharmacologic management of depression in adults. *American Academy of Family Physicians*, 92(2):94-100

Kravets, J.L., Reyes, B.A., Unterwald, E.M, van Bocksaele, E.J. 2015. Direct target peptidergic amygdala neurons by noradrenergic afferents; linking stress –integrative circuitry. *Brain Structure and Function*, 220 (1): 541-558

Kroeze, Y., Zhou, H., Homberg,J.R. 2012. The genetics of selective serotonin inhibitors. *Pharmacology and Therapeutics*, 136:375-400

Kumar, N., Goldminz, M.A., K, N., Gottlieb, A. B. 2013. Phosphodiesterase 4-targeted treatments for autoimmune diseases. *BMC Medicine*, 96 (11): 1-8

Lapiz, S.M., Bondi, C.O., Morilak, D.A. 2007. Chronic treatment with desipramine improves cognitive performance of rats in an attention set-shifting test. *Neuropsychopharmacology*, 32; 1000-1010

Lee, G., Bae, H. 2017. Therapeutic Effects of phytochemicals and medicinal herb on depression. *Biomedical Research International*, 6596241:1-11

Lemke, T.L., Williams, D.A., Roches, F., Zito, W. 2012. Foye's principles of medicinal chemistry. 7th edition. Wolters Kluwer Health Adis/ Lippincott Williams & Wilkins

Li, Y., Huang, Y., Amsdell, S.L., Xiao, L., O'Donnell, J.M., *et al.* 2009. Antidepressant- and anxiolytic-like effects of the phosphodiesterase- 4 (PDE4) inhibitor Rolipram on behaviour depend on cyclic AMP-response element binding protein (CREB)-mediated neurogenesis in the hippocampus. *Neuropsychopharmacology*, 34(11): 2404–2419

Liebenberg, N., Wegener, G., Joca, S. 2015. Nitric oxide involvement in the antidepressant-like effect of ketamine in the Flinders Sensitive Line rat model of depression. *Acta Neuropsychiatrica*, 27(2):90-96

Lieberman, D.Z., Massey, S.H. 2009. Desvenlafaxine in major depressive disorder: an evidence-based review of its place in therapy. *Core Evidence Dovepress*, 4: 67-82

Lillethorup, T.P., Iversen, P., Wegener, G., Doudet, D.J., Landau, A.M. 2015. Adrenoceptors binding in Flinders Sensitive Line compared with Flinders Resistance Line and Sprague Dawley rats. *ACTA Neuropsychiatrica*, 27(6): 345-352

Liu, W., Ge, T., Leng, Y., Pan, Z., Fan, J., Yang, W., and Cui, R. 2017. The role of neural plasticity in depression from hippocampus to prefrontal cortex. *Neural Plasticity*,6871089. https://doi.org/10.1155/2017/6871089 Date of access: 18. Sep. 2020

Liu, Y., Zhao, J., Guo, W. 2018. Emotional roles of Mono-Aminergic neurotransmitters in major depressive disorders and anxiety disorders. *Frontiers in Psychology*, 9:2201

Lourenco, C.M., Kenk, M., Beanlands, R.S., Da Silva, J.N. 2006. Increasing synaptic noradrenaline, serotonin and histamine enhances in vivo binding of phosphodiesterase-4 inhibitor rolipram in rat brain, liver and heart. *Life Sciences*, 79(4): 356-364

Maan, J.S, Rosani, A., Saadabadi, A. 2021. Desipramine. In: StatPearls [Internet updated on Sep 14]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–. PMID: 29262158

Maletic, V., Eramo, A., Gwin, K., Offord, S.J and Duffy, R.A. 2017. The role of norepinephrine and its adrenergic receptors in the pathophysiology and treatment of major depressive disorders and schizophrenia. A systematic review. *Frontiers in Psychiatry*, 8 (42): 1-12

Man, H.W, Schafer, P., Wong, L.M., Gorral, L.G., Raymon, H., Blease, K., Leisten, J.,Shirley, M.A., Tang, Y., Babusis, D.M., Chen, R., Stirling, D., and Muller, G.W. 2009. Discovery of (s)-n-{2-[1-(3-Ethoxy-4-methoxyphenyl)-2-mehtanesulfonylethyl]-1,3-dioxa-2,3-dihydroxy-1H-isoindol-4-yl} acetamide (Apremilast), a potent and orally Active phosphodiesterase4 and Tumor Necrosis factor-α-inhibitor. *Journal of Medicinal Chemistry*, 52(6): 1522-1524

Mandela, P., Ordway, G.A. 2006. The norepinephrine transporter and its regulation. *Journal of Neurochemistry*, 97: 310-333.

Manosroi, A., Tangjai, T., Chankhampan, C., Manosroi, W., Najarut, Y., Kitdamrongtham, W., Manosroi, J. 2017. Potent Phosphodiesterase Inhibition and Nitric Oxide Release Stimulation of Anti-Impotence Thai Medicinal Plants from "mANOSROI III" Database. *Evidence-based Complement. Alternative Medicines*, 9806976. https://doi.org/10.1155/2017/9806976 Date of acess: 27.Oct.2021

Mansuy, L. 2010. Antidepressants therapy with milnacipran and venlafaxine. *Neuropsychiatric Disease and Treatment*, 6(1):17-22

Marije, R.T., Sanjay, J., Mathew, M.D., Dennis, S and Charney, M.A. 2009. Neurobiological mechanism in major depressive disorder. *Canadian Medical Association Journal*,180(3):305-313

Matteo, M., Cristian, P., Laura, M., Federico, M., *et al.* 2021. The use of esketamine in comorbid treatment resistant depression and obsessive-compulsive disorder following extensive pharmacogenomic testing; a case report: *Annals of General Psychiatry*, 20(43):1-7

Mclntyre, R.S., Suppes, T., Tandon, R., Ostacher, M. 2017. Florida Best Practice psychotherapeutic medication guideline for adults with major depressive disorder. *The Journal of Clinical Psychiatry*, 78(6):703-713

Mikulska, J., Juszczyk, G., Gawrońska-Grzywacz, M., Herbet, M. 2021. HPA Axis in the Pathomechanism of Depression and Schizophrenia: New Therapeutic Strategies Based on Its Participation. *Brain Sciences*, 11(10): 1298

Miranda, M., Morici, J.F., Zanoni, M.B., Bekinschtein, P. 2019. Brain-derived neurotrophic factor; Akey molecular for memory in the Healthy and pathological brain. *Frontiers in Cellular Neuroscience*, 363(13): 1-25

Mokoena, M.L., Harvey, B.H., Viljoen, F., Ellis, S.M., Brink, C.B. 2015.Ozone exposure of Flinders Sensitive Line rats is a rodent translational model of neurobiological oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232(1):2921-2938.

Montana, J.G., Dyke, H.J. 2002. Update on the therapeutic potential of PDE-4 inhibitors. *Expert Opinion on Investigational Drug*, 11(1): 1-13

Montoya, A., Bruins, R., Katzman, M.A., Blier, P. 2016. The noradrenergic paradox; implications in the management of depression and anxiety. *Neuropsychiatric Disease and Treatment*, 12: 541-557

Moret, C., Briley, M. 2011. The importance of noradrenaline in depression. *Neuropsychiatric Disease and Treatment*, 7(1):9-13.

Moruguchi, S. Yamada, M., Takano, H., *et al.* 2017. Norepinephrine transporter in major depressive disorders; A PET study. *American Journal of Psychiatry*,174(1): 36-41

Mulinari, S. 2012. Monoamine theories of depression; Historical impact on biomedical research. *Journal of the History of the Neuroscience*, 21(4): 366-92

Mungai, K., Bayat, A. 2019. An overview of trends in depressive symptoms in South Africa. *South African Journal of Psychology*, 49(4): 1-18.

Murbach, T.S, Hirka, G., Szakonyine, I.., Gericke, N., Endres, J.R. 2014. A toxicological safety assessment of a standardized extract of *Sceletium tortuosum* (Zembrin ®) in rats. *Food and Chemical Toxicology*, 74:190-199

Murthy, V.S., Mangot, A.G. 2015. Psychiatric aspect of phosphodiesterase's. *Indian Journal of Pharmacology*, 47(6):594-599

Nahas, R., Sheikh, O. 2011. Complementary and alternative for the treatment of major depressive disorder; clinical review. *Canadian Family Physician*, 57(6): 659-663

Nell, H., Siebert, M., Chellan, P., Gericke, N. 2013. A randomized, double-blind parallel-group, Placebo-controlled trial of extract *Sceletium tortuosum* (Zembrin®) in healthy adults. *Journal of Alternative and Complementary medicine*,19(11):898-904

Nestler, E.J., Barrot, M., DiLeone, R.J., Eisch, A.T., Gold, S.T., Monteggia, L.M. 2002. Neurobiology of depression. *Neuron*, 34(1): 13-25

Ng, T.H., Alloy, L.B., Smith, D.V. 2019. Metanalysis of reward processing in major depressive disorder reveals distinct abnormalities within the reward circuit. *Translational Neuropsychiatry*, 9(293):1-10

Nglazi, M., Joubert, J.D., Stein, D.J., Lund, C., Wiysonge, C.S., Vos, T., Wyk, V.P., Roomaney, R.A., Muhwava, L.S., Bradshaw, D. 2016. Epidemiology of major depressive disorder in South Africa (1997-2015): A systematic review protocol. *British Medical Journal*, *6*(7), e011749. https://doi.org/10.1136/bmjopen-2016-011749 Date of acess: 26. Nov. 2021

Nguse, S., Wassenaar, D. 2021. Mental health and COVID-19 in South Africa. *South African Journal of Psychology*, 51(2): 304-313

Nielsen, N.D., Sandager, M., Stafford, G.I., van Staden, V.J., Jager, A.K. 2004. Screening of indigenous plants from South Africa for affinity to the serotonin reuptake transporter protein. *Journal of Ethnopharmacology*, 94(1):159-163

Nishi, K., Kanemaru, K., Hasegawa, S., Watanabe, A., Diksic, M. 2009. Both acute and Chronic Buspirone treatments have different effects on regional 5-HT synthesis in Flinders Sensitive Line rats (a rat model of depression) than in control rats. *Neurochemistry International,* 54(3):205-214

Nutt, D. 2008. Relation of neurotransmitters to the symptoms of major depressive disorder. *The Journal of Clinical Psychiatry*, 69(1):4-7

O' Donnell, J.R., and Zhang, H.T. 2004. Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE). *Trends in pharmacological sciences*, 25(3): 158-163

Oberholzer, I., Moller, M., Holland, B., Dean, O.M., Berk, M., Harvey, B.H. 2018. Garcinia of depression Linn displays antidepressant-like and pro-cognitive effects in a genetic animal model of depression; a bio-behavioural study in the Flinders Sensitive Line rat. *Metabolic Disease*, 33(2): 467-480.

Olatunji, T.L., Siebert, F., Adetunji, A., E., Harvey, B.H., Gericke, J., Hamman, J.H., Van der Kooy, F. 2021. Sceletium tortuosum; A review on its phytochemistry, pharmacokinetics, biological and clinical activities. *Journal of Ethnopharmacology*. 280:114476

Olguin, H.J., Guzman, D.C, Garcia, E.H., Mejia, G.B. 2016. The role of dopamine and its dysfunction as a consequence of oxidative stress. *Oxidative Medicine and Cellular Longevity*, 9730467. https://doi.org/10.1155/2016/9730467 Date of access: 24.Sep. 2020

Ordway, G.A., Schenk, J., Stoclmeier, C.A., May, W., and Klimek, V. 2003. Elevated agonist binding to α_2 adrenoceptors in the locus coeruleus in major depression. *Society of Biological Psychiatry*, 53(4): 315-323

Ostuzzi, G., Gastaldon, C., Barbato, A., D'Avanzo, B., *et al.* 2020. Tolerability and efficacy of vortioxetine versus SSRIs in elderly with major depression. Study protocol of the VESPA study; a pragmatic, multicentre, open lable, parallel-group, superiority, randomized trial. *Trial*, 21(695):1-14

Osuch, E., Marais, A. 2017. The pharmacology management of depression. *South African Pharmaceutical Journal*, 84(2): 36-45

Otte, C., Gold, S.M., Penninx, B.W., Pariante, C.M., Etkin, A., Fava, M., Mohr, D,C., Schatzberg, A,F. 2016. Major depressive disorder. *Nature Reviews, Disease Primers*. https://doi:org/10.1038/nrdp.2016.65 Date of acess: 07. Mar. 2022

Outhoff, K. 2016. An update on the pharmacological treatment of anxiety and related disorders. *South African Family Practice*, 58(5):50-56

Overstreet, D.H., Friedman, E., Mathe, A.A., Yadid, G. 2005. The Flinders Sensitive Line rat; A selectively bred putative animal model of depression. *Neuroscience and Bio-behavioural Reviews*, 29(4-5):739-759

Overstreet, D.H., Griebel, G. 2005. Antidepressant-like effects of the vasopressin Vlb receptor antagonist SSR149415 in the Flinders Sensitive Line rat. *Pharmacology, Biochemistry and Behavior*, 82 (1):223-227

Overstreet, D.H., Janowsky, D.S.1991. A Cholinergic Supersensitivity Model of Depression. In: Boulton A.A., Baker G.B., Martin-Iverson M.T. (eds) *Animal Models in Psychiatry*, II. Neuromethods, vol 19. Humana Press, Totowa, NJ. https://doi.org/10.1385/0-89603-177-2:81 Date of acess: 27.Nov.2021

Overstreet, D.H., Naimoli, N.M., Griebel, G. 2010.Saredutant, an NK2 receptor antagonist, has both antidepressant-like effects and synergizes with desipramine in an animal model of depression. *Pharmacology, Biochemistry and Behaviour*, 96(2):206-210

Overstreet, D.H., Wegener, G. 2013. The Flinders Sensitive Line rat model of depression -25 years and still producing. *Pharmacology Review,* 65(1):143-155.

Palaniyappan, L., Insole, L., Ferrier, N. 2009. Combining antidepressants; a review of evidence. *Advance Psychiatric Treatment*, 15(2):90-99

Papakostas, G.I., and Fava, M. 2006. A meta-analysis of clinical trials comparing moclobemide with selective serotonin reupateke inhibitors for the treatment of major depressive disorder. *Canadian Journal of Psychiatry*, 51 (12):783-790.

Patnala., S. 2007. Pharmaceutical analysis and quality of complementary medicines: Sceletium and associates products. Rhodes University, Grahamstown, South Africa (PhD. thesis)

Pedersen, M.E., Szewczyk, B., Stachowicz, K., Wieronska, j., Andersen, J., Stafford, G.I., van Staden, j., Pilc, A., Jager, A.K. 2008. Effects of South African traditional medicines in animal model for depression. *Journal of Ethnopharmacology,* 119(3) 542-548

Perona, M.T., Waters, S., Hall, F.S., Sora, I., Lesch, K-P., Murphy, D.L., Caron, M., Uhl., G.R. 2008. Animal models of depression in dopamine, serotonin and norepinephrine transporters knockout mice; prominent effects of dopamine transporters deletions. *Behavioural Pharmacology*, 19 (5-6): 566-574

Pilkington, K., Rampes, H., Richardson, J. 2006. Complementary medicines for depression. *Expert Review of Neurotherapeutics*, 6(11):1741-51

Planchez, B., Surget, A., Belzung, C. 2019. Animal models of major depression: drawbacks and challenges. *Journal of Neural Transmission*,126(11):1383-1408

Porsolt, R.D., Bertin, A., Jalfre, M.1978. “Behavioural despair” in rats and mice: strain differences and the effects of imipramine. *European Journal of Pharmacology*, 51(3):291–294

Pourhamzeh, M., Moravej, F.G., Arabi, M., Shahriari, E., Mehrabi, S., Ward, R., Ahadi, R., Joghataei, M.T. 2021. The role of serotonin in Neuropsychiatric disorders. *Cellular and Molecular Neurobiology*. https://doi.org/10.1007/s10571-021-01064-9 Date of access: 27. Nov. 2021

Quentin, E., Belmer, A., Maroteus, L. 2018. Somato-dentritic regulation of raphe serotonin neurons; a key to antidepressant action. *Frontiers in Neuroscience*, 12 (982): 1-11

Reed, C.R., Kajdasz, D.K., Whalen, H., Athanasiou, M.C., Gallipoli, S., Thase, M.E. 2012. The efficacy profile of vilazodone, a novel antidepressant for the treatment of major depressive disorder. *Current Medical Research and Opinion*, 28(1):27-39

Regenass, W., Möller, M., Harvey, B. H. 2018. Studies into the anxiolytic actions of agomelatine in social isolation reared rats: Role of corticosterone and sex. *Journal of Psychopharmacology*, 32(2):134–145.

Robinson, E. 2018. Psychopharmacology; from serendipitous discoveries to rational design, but what next?. *Brain and Neuroscience Advances*, 2:1-11

Roohi, E., Jaafari, N., Hashemian, F. 2021. On inflammatory hypothesis of depression; what is the role of IL-6 in the middle of the chaos?. *Journal of Neuroinflammation*, 18(45):1-15

Saayman L.J.B. 2019. Long lasting bio-behavioural effects of early life sildenafil administration in stress sensitive versus healthy control rats. North-West University (South Africa), Potchefstroom campus (MSc dissertation)

Saayman, L.B.J., Steyn, S.F., Brink, C. B. 2021. The long-term bio-behavioural effects of juvenile sildenafil treatment in Sprague-Dawley versus Flinders Sensitive Line rats. *Acta Neuropsychiatrica,* 33(4):200-205

Sanchez, C., Asin, K. E., Artigas, F. 2015. Vortioxetine, a novel antidepressant with multimodality activity; Review of preclinical and clinical data. *Pharmacology and Therapeutics*,145:43-57

Sandager, M., Nielsen, N.D., Stafford, G.I., van Staden, T., Jager, A.K. 2005. Alkaloids from *Bophane distica* with affinity to the serotonin transporter in rat brain. *Journal of Ethnopharmacology*, 98:367-370

Sarris, J., Marx, W., Ashton, M.M., Ng, C.H., Psych, M., Galvao-coelho, N., Ayati, Z., Zhang, Z., Kasper, S., Ravindra, A., Harvey, B.H. 2021. Plant-based medicines (Phytoceuticals) in the treatment of psychiatric disorders; a meta-review of meta-analyses of randomized controlled trials. *The Canadian Journal of Psychiatry*, 66(10):1-14

Schell, R. 2014. Sceletium tortuosum and mesembrine; A potential Alternative Treatment for Depression (Claremont Colleges Scripps Senior Dissertation)

Schneider, P., Ho., Y.J Spanagel, R., Pawlas C.R. 2011. A novel elevated plus maze procedure to avoid one trial tolerance problem. *Frontiers in Behavioural Neuroscience*, 5 (43):1-8

Seki, K., Yoshida, S., and Jaiswal, M.K. 2018. Molecular mechanism of noradrenaline during stress-induced major depressive disorders. *Neural Regeneration Research*, 13 (7):1159-1169

Shah, A., Frazer, A. 2014. Influence of acute or chronic administration of ovarian hormone on the effects of desipramine in forced swim test in female rats. *Psychopharmacology*, 231(18):3685-3694

Shannon, J.R., Flattem, N.L., Jordan, J., Jacob, G., Black, B.K., Biaggioni, I., Blakely, R.D., Robertson, D. 2000. Clues to the origin of orthostatic intolerance, a genetic defect in the cocaine-and antidepressant sensitive norepinephrine transport. *New The New England Journal Medicine*, 342:541-549

Sheline, Y.I., Godo, M.H., Kraemer, H.C. 2002. Untreated depression and hippocampal volume loss. *The American Journal of Psychiatry*, 160(8):1516-8.

Simpson, J. Ryan, C., Curley, A., Mulcaire, J., Kelly, J.P. 2012. Sex differences in baseline and drug-induced behavioural responses in classical behavioural tests. *Progress in Neuropsychopharmacology Biological Psychiatry*, 37(2):227-236

Slamloo, Y.R., Fazlali, Z. 2020. Dopamine and noradrenaline in the brain; overlapping or dissociate function?. *Frontiers in Molecular Neuroscience*, 12(334):1-8

Slattery, D. A., Cryan, J. F. 2012. Using the rat forced swim test to assess antidepressant-like activity in rodents. *Nature Protocols*, 7(6):1009–1014.

Smith, C. 2011. The effects of Sceletium tortuosum in an *In vivo* model of psychological stress. *Journal of Ethnopharmacology*, 133(1): 31-36.

South African Depression and Anxiety Group (SADAG). 2020. Depression, treatment, and referral guide. https://www.sadag.org/. Date of access: 24. Jan.2021

Stanford, C. 2007. The open field testing; reinventing the wheel. *Journal of Psychopharmacology*, 21 (2):134-135

Steyn, S.F. 2011. The effects of early-life exposure stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood. North-West University (South Africa), Potchefstroom Campus (M.Sc. dissertation)

Steyn, S.F., Harvey, B.H., Brink, C.B. 2020. Pre-pubertal, low-intensity exercise does not require concomitant venlafaxine to induce robust, late-life antidepressant effects in Flinders sensitive line rats. *European Journal of Neuroscience*, 00:1-16

Steyn, S.F., Harvey, H.B., and Brink, C.B. 2018. Immediate and long-term antidepressive-like effects of pre-pubertal escitalopram and omega-3 supplementation combination in young adult stress-sensitive rats. *Behavioural Brain Research*, 351:49-62.

Tanyeri, M.H., Buyukokuroglu, M.C., Tanyeri, P., Mutlu,O., Ulaka, G., Akar, F.Y., Erden, B.F. 2018.Eavuation of the effects of antidepressants on depression and anxiety in mice forced swim test and elevated plus maze test. *Sakarya Medical Journal*, 84 (4):820-821

Terburg, D., Syal, S., Rosenberger, L.A., Heany, S., Phillips, N., Gericke, N., Stein, D.J., van Honk, J. 2013. Acute effects of Sceletium tortuosum (Zembrin®) A dual 5-HT reuptake and PDE-4 inhibitor in the human amygdala and its connection to the hippocampus. *Neuropsychopharmacology*, 38(13):2708-2716

Thornton, V.J., Asanbe, C.B., Denton, E.ge D.2019.Clinical risk factors among youth at high risk for suicide in South Africa and Guyana. *Anxiety and Depression Association of America*, 36:423-432

Treadway, M.T., Michael, L.W., Dillon, D.G, *et al.* 2015. Illness progression, recent stress and morphometry of hippocampal subfield and medial prefrontal cortex in major depression. *Society of Biological Psychiatry*, 77(3):285-294

Tundo, A., de Filippis, Proietti, L. 2015. Pharmacological approach to treatment resistant depression evidence and personal experience: *World Journal of Psychiatry*, 5(3):330-341

Uys, M.M, Shahid, M., Sallinen, J., Harvey, B.H. 2017. The α2C-adrenoceptor antagonist, ORM-10921, exerts antidepressant-like effects in the Flinders Sensitive Line rat. *Behavioural Pharmacology*, 28(1):9-18

Vahid-Ansari, F., Zhang, M., Zahrai, A, Albert, P.R. 2019. Overcoming resistance to selective serotonin reuptake inhibitors; targeting serotonin-1A receptors and adult neuroplasticity. *Frontiers in Neuroscience*,15 (404):1-16

Vollmayr, B., Mahlstedt, M. M., Henn, F. A. 2007. Neurogenesis and depression: what animal models tell us about the link. *European Archives of Psychiatry & Clinical Neuroscience*, *257*(5): 300–303

Walf, A.A., Frye, C.A. 2007. The use of the elevated plus maze as an assay of anxiety-related behaviour in rodents. *Nature Protocols,* 2(2):322‑328

Walz, N., Muhlberger, A., Pauli, P. 2016. A human open filed test reveal thigmotaxis related to agoraphobic fear. *The Society of Biological Psychiatry*, 80:390-397

Wang, C., Wang, Z., Li, M., Li, C., Yu, H., Zhou, D., Chen, Z. 2017, 'Reducing Neuroinflammation in Psychiatric Disorders: Novel Target of Phosphodiesterase 4 (PDE4) and Developing of the PDE4 Inhibitors', in G. E. A. Abreu (ed.), Mechanisms of Neuroinflammation, *IntechOpen*, London. 10.5772/intechopen.69154. http://www.intechopen.com/books/mechanisms-ofneuroinflammation. Date of access: 06. Jun.2021

Wang, Q., Timberlake, M.A., Prall, K., Dwivedi, Y. 2017. The recent progress in animal models of depression. *Progress in Neuropsychopharmacology and Biological Psychiatry,* 77: 99–109

Wang, Z.Z, Yang, W.X., Zhang, Y., *et al.* 2015. Phosphodiesterase4D knock down in the prefrontal cortex alleviate chronic unpredictable stress induced depressive behaviour and memory deficit in mice. *Scientific Report*, 5 (112332): 1-14

Wegener, G., Harvey, B.H., Bonefeld, B., Müller, H.K., Volke, V., Overstreet, D.H., Elfving, B. 2010. Increased stress-evoked nitric oxide signalling in the Flinders sensitive line (FSL) rat: A genetic animal model of depression. *International Journal of Neuropsychopharmacology*,13(4):461-473

Willner, P. 2016. The chronic mild stress (CMS) model of depression: History, evaluation and usage. *Neurobiology of Stress*, 6:78–93

Wilson, M., Tripp, J. 2021. Clomipramine. In *StatPearls*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK541006 Date of access: 06. Dec. 2021

World Health Organization (WHO). 2017. Depression and other common mental disorders: Global Health Estimates. Geneva. https://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2 Date of access: 05. May.2020

Wu, T., Xiaoqian, J., Huifeng, S., Jieqion, N., Yin, J. X., and Wang, X. 2021. Prevalence of mental health problems during the COVID-19 pandemic; A systematic review and metal analysis. *Journal of Affective Disorders*, 281:91-98

https://www.worldometers.info/coronavirus. Date of access: 18. Jun. 2020

Yang, T., Nie, Z., Shu, H., Kuang, Y., Chen, X., Cheng, J., Yu, S., Liu, H. 2020. The Role of BDNF on Neural Plasticity in Depression. *Frontiers in Cellular Neuroscience*, 14 (82):1-12

Zhang, F.F., Peng, W., Sweneey, J.A., Jia, Z.Y., and Gong, Q.Y. 2018. Brain structure alteration in depression; Psychoradiological evidence. *CNS Neuroscience and Therapeutics*, 24(11):994-1003

Zhao, Z., Baros, A. M., Zhang, H. T., Lapiz, M. D., Bondi, C. O., Morilak, D. A., O'Donnell, J. M.2008. Norepinephrine transporter regulation mediates the long-term behavioral effects of the antidepressant desipramine. *Neuropsychopharmacology*, *33*(13): 3190–3200

Zhang, Y., Yang, Y., Zhu, L., Zhu, Q., Jia, Y., Zhang, L., Peng, Q., Wang, J., Liu, J., Fan, W., Wang, J., 2021. Volumetric deficit within the frontal-limbic-striatal circuit in first episode drug naïve patients within major depression disorder. *Frontiers in Psychiatry*. 11:600583

Zhu., J., Mix., E., Winblad., B. 2001. The antidepressant and anti-inflammatory effects of Rolipram in the central nervous drug review.*CNS Drugs Review*, 7(4):387-398

Zmudzka, E., Salaciak, K., Sapa, J., Karolina, P. 2018. Serotonin receptors in depression and anxiety; Insights from animal studies. *Life Sciences*, 210:106-124

Zohar, J., Westenberg, H.M. 2000. Anxiety disorders; a review of tricyclic antidepressants and selective reuptake inhibitors. *Acta Psychiatrica Scandinavica,* 101(403):39-49

Zou, W., Feng, R., Yang, Y. 2018. Changes in the serum levels of inflammatory cytokines in antidepressant drug-naïve patients with major depression. *PloS one*, *13*(6), e0197267. https://doi.org/10.1371/journal.pone.0197267 Date of access: 06. Aug. 2021

CHAPTER 3

MANUSCRIPT

This chapter presents the findings from this study wherein the results are prepared as a concept manuscript for publication in a peer review journal. The manuscript article titled: ''*Evaluating the antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats: A bio-behavioural study*.'' has thus been prepared in accordance with guidance and instructions applicable to the journal **Behavioural Brain Research.** The complete guidelines can be found on the journal website: https://www.journals.elsevier.com/behavioural-brain guide for authors. Additional behavioural and neurochemical data not included in the manuscript are presented in an addendum **(Addendum B)** and submitted as part of the thesis.

Evaluating the antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats: A bio-behavioural study.

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ABSRACT

Introduction: Comorbid anxiety disorders in patients with major depressive disorder often reduce response to standard antidepressants. The standardized extract of *Sceletium tortuosum* (Zembrin®) is a multi-model serotonin reuptake transporter (SERT) and phosphodiesterase (PDE) 4B inhibitor that may be a promising novel therapy for patients with depression, either as monotherapy or as an augmentation strategy in poor responders. Therefore, investigation into its therapeutic potential alone in combination following sub-chronic treatment and in combination with a known standard antidepressant is required.

Aim of the study: To assess the dose-related antidepressant- like effects of Zembrin® (ZEM) in the Flinders Sensitive Line (FSL) rats, a genetic model of depression, alone and as an adjunctive treatment with low-dose desipramine (DMI), and correlation with altered regional brain monoamines and phosphodiesterase 4B expression.

Material and methods: For confirmation of the model, 12 male FRL rats and 12 male FSL rats (control group) were treated with saline for 14 days via oral gavage. Seven groups (*n* =12) of male FSL rats were treated with a 3-tier dose of ZEM (10, 25 & 30 mg/kg/day), and a 2-tier dose of DMI (15 & 30 mg/kg/day) for 14 days via oral gavage to establish predictive validity. To assess augmentation potential, ZEM (10 & 30 mg/kg/day), was combined with a low dose of DMI (15 mg/kg/day) over 14 days. Following treatment, depressive-like behaviour was assessed in the Forced Swim Test (FST), followed by analyses of cortical and hippocampal monoamines and PDE4B levels.

Results: Depressive-like behaviour was significantly increased in FSL rats versus Flinders Resistant Line (FRL) control rats. DMI (15 & 30 mg/kg/day) significantly decreased immobility and increased struggling behaviour in the FST. Both combinations significantly reduced immobility versus ZEM- (10 & 30 mg/kg/day) monotherapies. ZEM-30 significantly increased frontal cortical NE and 5-HT levels versus the FSL saline control group. While both combination therapies significantly reduced hippocampal PDE4B levels versus ZEM- (10 & 30 mg/kg/day) monotherapies, however, they increased cortical PDE4B levels compared to ZEM- (30 mg/kg/day) alone.

Conclusions: FSL rats showed distinct depressive- like characteristics versus FRL controls. DMI alone reversed depressive-like behavioural characteristics in FSL rats. We were unable to confirm the antidepressant-like effects of ZEM in this study, either alone

or in combination with DMI. These results may be model-related. That said, studies on hippocampal PDE4 levels do lend some support for the augmentation potential of ZEM as an add-on therapy for patients responding poorly to standard antidepressants, especially where specific actions on the hippocampal PDE4B are required. Combination of ZEM with antidepressants i.e., NRIs may be depressogenic displaying increased cortical PDE4B levels. However, further work is needed.

Keywords: **Zembrin® PDE4B, Major depressive disorder, Flinders Sensitive line rat, Forced Swim Test, monoamines**

1. Introduction

Comorbid anxiety disorders in patients with major depressive disorders (MDD) often reduce patient quality of life, increase risk of suicidality, decrease response to standard antidepressants and results in poor treatment outcomes compared to MDD alone (Altin *et al.,*2014). The global prevalence of comorbid anxiety disorders and MDD has been reported to be between 47-67% (Kim, 2020). MDD remains a serious mood disorder with more than 300 million people affected worldwide (WHO, 2017), but not effectively treated due to a relatively poor understanding of its aetiology and pathogenesis (Jin *et al.,*2018) and incomplete target engagement by current treatments (Simon *et al.,*2013; Nahas and Sheikh, 2011). The Covid-19 pandemic and national lockdowns have had a detrimental impact on global health and economy (Onyeaka *et al.,*2021), with the prevalence of MDD increasing from 10-20% (Kessler, 2007) to 31.4% during COVID-19 (Wu *et al.,*2021). This is due to financial stress, anxiety, and social isolation (Rajkumar, 2020; Onyeaka *et al.,*2021) as well as via Covid-19 associated neuro-inflammation that may precipitate or worsen existing MDD (Jansen van Vuren *et al.,*2021). Most of the current standard antidepressants target one or two of the recognized mechanisms of MDD leaving the majority of MDD patients inadequately treated. In fact, less than 50% of patients eventually achieve complete remission (Brand *et al.,*2015; Harvey and Slabbert, 2014). This problem could be due to delayed onset of action (Boku *et al.,*2018; Liu *et al.,*2018), side effects, and non-compliance, but mostly due to inappropriate target engagement (Harvey and Slabbert, 2014; Nahas and Sheikh, 2011). Novel biological targets and treatment strategies are therefore urgently required.

Converging evidence has revealed phosphodiesterase (PDE)-4B as a promising novel antidepressant target as its inhibition is linked with upregulation of hippocampal brain-derived neurotrophic factor (BDNF), promotion of neurogenesis and suppression of neuroinflammation (Li *et al.,*2009). Increased PDE4B activity in the hippocampus and prefrontal cortex has been associated with decreased BDNF expression coupled with decreased cAMP (cyclic adenosine monophosphate) concentration and/or reduced expression of cAMP-response element binding protein (CREB), leading to atrophy and structural changes in these brain regions and associated impaired learning and memory (Miranda *et al.,*2019; Wang *et al.,*2017). Converging evidence from animal and clinical studies have demonstrated that PDE4B is involved in the pathogenesis of MDD (Li *et al.,*2009; Itoh et al. 2004) and anxiety disorders (Zhu *et al.,*2001; Zhang *et al.,*2008) as

well as mediating the antidepressant- and anxiolytic- like effects of PDE4 inhibitors (Dlaboga *et al.,*2006; Li *et al.,*2009). Since inhibition of PDE4 promotes cAMP-dependent sub-cellular signal transduction, this action is theorized to have beneficial psychopharmacological effects by modulating the same subcellular NA'ergic and 5-HT'ergic processes activated by SSRI, SNRI and TCA but doing so directly i.e., independent of an extra-cellular target (Harvey,1997). Therefore, combined treatment that comprise two traditional agents but from different mechanistic classifications or combining the traditional agent with complementary and alternative medicines (CAMs), may offer a better treatment strategy (Pilkington *et al.,*2006). This is often done to limit or prevent the side effects and costs of current antidepressants, and not just to improve treatment response, e.g., delayed onset of action (Pilkington *et al.,*2006). According to WHO (2018), CAMs refers to a broad set of health care practices, including traditional medicines that are not part of conventional treatment.

Plant-based medicines, or "phytoceuticals", have had a long-standing history of use in psychiatric disorders. A recent study of available clinical trials of plant-based medicines has provided a "meta-review" of this top-tier evidence (Sarris *et al.,*2021). The standardized extract of *Sceletium tortuosum* (Zembrin®) heralds promise for future development for the treatment for anxiety and MDD both as a monotherapy and/or as adjunctive therapy (Olatunji *et al.,*2021). *Sceletium tortuosum* has been used for centuries by the Khoisan hunter-gatherers of South Africa to promote a sense of calm, improve health, as stress relief, reduce anxiety, improve mood and to enhance concentration (Olatunji *et al.,*2021). Importantly, the safety and tolerability of Zembrin® has been documented in preclinical and clinical studies (Olatunji *et al.,*2021). Mechanistically, it has been shown to induce robust PDE4B and serotonin reuptake transporter (SERT) inhibition *in vitro,* known biological targets for antidepressant action (Harvey *et al.,*2011). In the latter study, two pharmacologically active alkaloids in *Sceletium totuosum* (ST), viz. mesembrine and mesembrenone, were found to inhibit SERT and PDE4 activity, respectively (Harvey *et al.,*2011).

Recently, dose-dependent antidepressant-like effects of Zembrin® versus an SSRI (i.e., escitalopram) following acute treatment using the Flinders Sensitive Line rat genetic model of depression was demonstrated (Gericke *et al.,*2022). Follow-up sub-chronic treatment studies showed that the combination of Zembrin® with escitalopram significantly elevated hippocampal BDNF, 5-HT and NE levels, although produced a

significant worsening of depression-like symptoms (Gericke, 2019). Although unexpected, these results were indicative of the potential of Zembrin® to boost the serotonergic actions of an SSRI, which the authors have suggested may underlie this apparent paradoxical response (Gericke, 2019).

Given the findings, it is necessary to re-evaluate whether Zembrin® may preferentially benefit a non-serotonergic antidepressant such as desipramine (DMI) during adjunctive treatment, as well as more comprehensively explore target engagement for Zembrin® following sub-chronic dosing. DMI is a secondary amine TCA approved for the treatment of MDD (Maan *et al.,*2021). DMI demonstrates few interactions when co-administered with antidepressants such as SSRIs (i.e., fluoxetine) and the PDE4 inhibitor, rolipram (Palaniyappan *et al.,*2009; Gillman, 2007; Lourenco *et al.,*2006).

The aim of the current study was to assess the dose-related antidepressant-like effects of Zembrin® (ZEM) in the Flinders Sensitive Line (FSL) rat model of MDD, alone and as an adjunctive treatment with low-dose DMI following 14-days treatment. To obtain a broader read-out of pharmacological activity, depressive-like behaviour was correlated with the analysis of cortical and hippocampal monoamines (NE, 5-HT, and DA) and PDE4B levels.

2. Materials and methods

2.1 Test subject and treatment strategies

2.1.1 Animals

The FSL rat has been widely used and extensively validated for behavioural research on MDD and for determining the therapeutic properties of novel antidepressants (Overstreet and Wegener, 2013). This sub-chronic treatment study used 96 male FSL and 12 male control Flinders Resistant Line (FRL) rats from PND-50 to PND-64. Animals were bred, supplied, and housed at the vivarium (SAVC registration number FR15/13458) of the Pre-Clinical Drug Development platform of North-West University (NWU), established by the National Department of Science and Innovation (DSI). The animals were housed in groups of 2 to 3 rats per cage in polypropylene individually ventilated cages (IVC) under a standard vivarium nesting material. Food and water were supplied *ad libitum*. The animals were maintained under complete environmental control with a constant ambient temperature of 22± 2°C and relative humidity of 50% ± 10%, with a full spectrum of light

in a 12h/12h light-dark cycle (lights switched on at 06:00 and off at 18:00), a positive air-pressure and air exchange rate of 18/hr.

All experimental procedures were approved by the North-West University Animal Care Research Ethics Committe approval number: **NWU-00520-20-A5** and performed in accordance with code of ethics in research, training and testing of drugs in South Africa. Furthermore, the animals were handled from PND-21, monitored daily, and regularly after the behavioural tests, using an approved vivarium animal monitoring sheet. The pups were weaned at PND-21 and randomly divided into groups of 12 rats per group. The overall experimental procedures and data reporting in this study followed the national legislations and guidelines (i.e., ARRIVE) with the 4Rs concept applied throughout the study to ensure that the smallest number of animals were used in the study and to minimize pain, suffering or discomfort.

The study was divided into three phases (Fig 1). All animals were orally dosed by oral gavage over 14 days, with treatment initiated on PND 50. Phase 1: twenty-four rats (12 FSL and 12 FRL) were divided into two groups as follows: Group *(I)* FRL rats and Group *(II)* FSL rats (was also used in phase 2 as control group), both treated with saline given at a constant dosing volume of 10 ml/kg body weight (bw) (maximum volume) (Murbach *et al.,*2014). FRL rats were used for validation of the model.

Phase 2: comprised monotherapy treatments, where in sixty (60) FSL rats were randomly divided into five groups and treated via oral gavage as follows; Group *(III-V)* was treated with 3 doses of ZEM (10, 25 and 30 mg/kg/day) respectively. Groups *(VI and VII*) were treated with low dose (15 mg/kg/day) and high dose DMI (30mg/kg/day), respectively.

Phase 3: comprised augmentation of DMI by selected ZEM doses. Twenty-four (24) FSL rats were randomly divided into two groups as follows; Group *(VIII)* was treated with the combination of low dose ZEM (10 mg/kg/day) and low dose of DMI (15 mg/kg/day) while Group *(IX)* with a combination of high dose ZEM (30 mg/kg/day) and low dose of DMI (15 mg/kg/day).

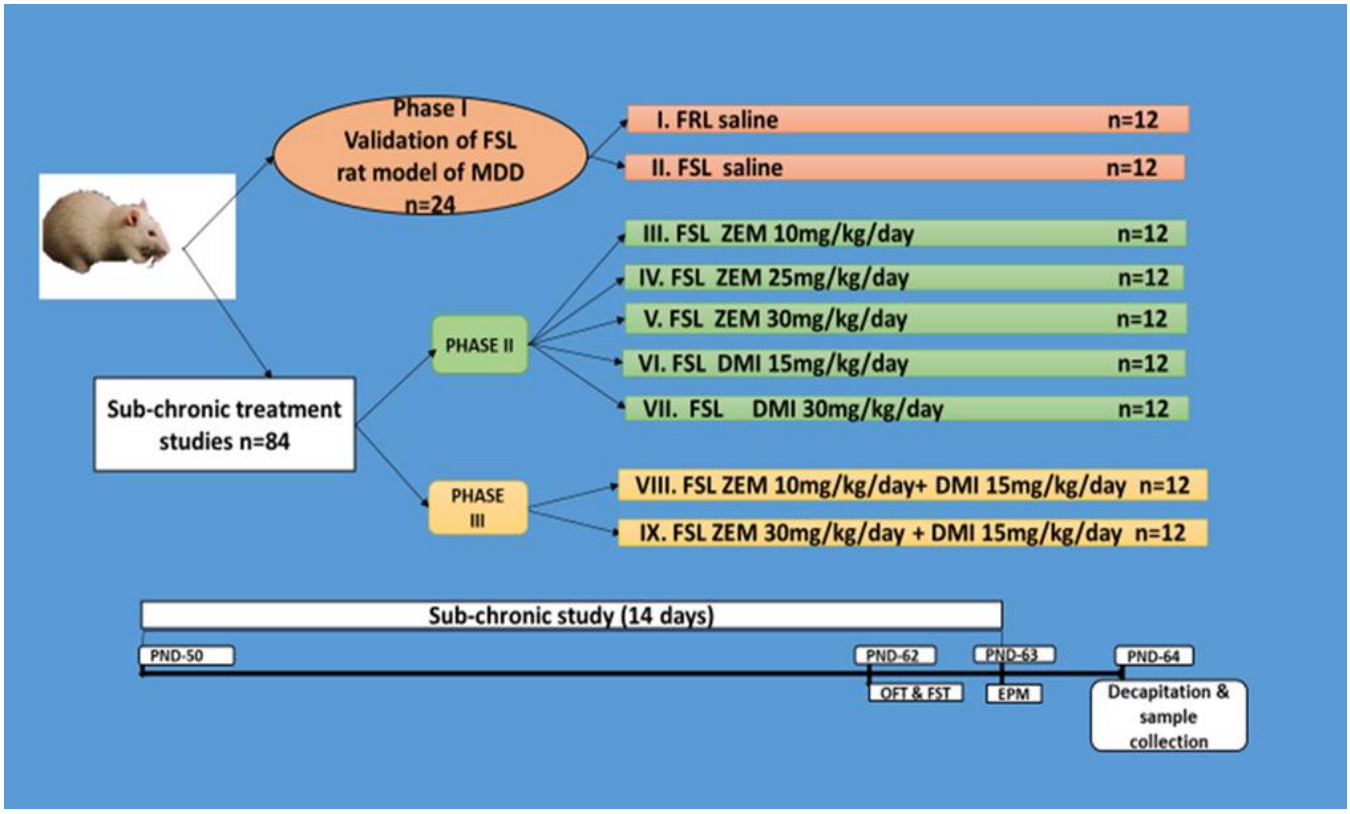


Fig. 1. Sub-chronic treatment study divided into three phases; Phase I validation of FSL model; Phase II-monotherapy studies; Phase III- augmentation study.

2.1.2 Drug preparation and administration

2.1.2.1 Zembrin®

Zembrin® (a standardised hydroethanolic extract of ST) was a donation from HG and H Pharmaceuticals (Pty) Ltd (Bryanston, South Africa). All the doses used in this study were chosen based on previous behavioural studies (Gericke, 2019; Gericke *et al.,*2022; Smith, 2011; unpublished data; HG&H Pharma, Johannesburg, SA. communicated by Dr Ralph Tettey-Amlalo). Zembrin® 25 mg/kg/day or 50mg/kg/day is effective as an antidepressant following acute treatment versus ESC (Gericke *et al.,*2022). However, antidepressants show clinical response after long-term treatment (Bennabi *et al.,*2019), so a sub-chronic design has a better predictive validity and is the logical step to take following the recently published acute dose ranging study (Gericke *et al.,*2022). A sub-chronic 3-tier dose-response study of Zembrin® (10, 25 and 30 mg/kg/day) was done to establish predictive validity while also exploring at least two doses of ZEM that could bolster the response to low-dose DMI. Zembrin® was dissolved in physiologic saline (0.9% *w/v*) and freshly prepared daily based on body weights to determine the dosage.

2.1.2.2 Desipramine

Desipramine (DMI), a noradrenaline reuptake inhibitor (NRI), has been extensively studied as an antidepressant under sub-chronic treatment in rats (Mokoena *et al.,*2015; Shah and Frazer, 2014; Furmaga *et al.,*2011; Overstreet *et al.,*2010; Lapiz *et al.,*2007). However, in these studies DMI was administered intraperitoneally (i.p.). Oral administration has a more predictable bioavailability compared to the intraperitoneal route (Lemke *et al.,*2012). Higher than normal doses of DMI for oral gavage were calculated based on the oral bioavailability of DMI, which ranges between 50-70% (Lemke *et al.,*2012). This choice was supported by previous studies employing oral administration of DMI in rats (Zhou *et al.,*2012; Singewald *et al.,*2004: Nava *et al.,*2015; Kalshetti *et al.,*2015). Based on previous studies (Shah and Frazer, 2014: Mokoena *et al.,*2015), we selected a low dose (7.5mg/kg/day) (Shah and Frazer, 2014) and full therapeutic dose (15 mg/kg/day) of DMI (Mokoena *et al.,*2015), culminating in doses of 15 and 30 mg/kg/day used in this study. Desipramine hydrochloride was purchased from Sigma-Aldrich, Johannesburg, South Africa and dissolved in saline (0.9% *w/v*) prior to oral gavage.

2.2 Behavioural analysis

On day 13 (PND-62) of treatment, the animals were subjected to behavioural testing starting with the less stressful Open Field Test (OFT) followed by the more stressful Forced Swim Test (FST), with the Elevated Plus Maze test (EPM) (anxiety submitted in addendum B) performed on day 14 (PND-63) of treatment. All behavioural tests were conducted during the rat's dark cycle, with each test separated by an acclimatization period of 30 min to minimize stress caused by movement of home cages to the behavioural assessment room.

2.2.1 Open Field Test (OFT)

The OFT is a well-described assay to assess locomotor activity behaviour in rodents (Brand and Harvey,2017). Here the test was performed according to previously described methods (Gericke, 2019; Oberholzer *et al.,*2018). Briefly, the apparatus consisted of an open black square arena (1 m^2) surrounded by high walls (45 cm) to prevent the animals from escaping. Animals were allowed to freely explore the arena, while behavioural parameters were recorded by cameras mounted above the arena. All tests were performed under red light (80 lx). On PND-63, animals were moved in their home cages

to the experimental room and allowed to acclimatize for 30 min (Gericke, 2019; Oberholzer *et al.,*2018). The rats were placed individually in the centre of the open field arena and allowed to explore for 6 min, with the last 5 min recorded and used for statistical analyses. The arena was cleaned with 10% (*v/v*) alcohol after each test to minimize olfactory cues present in the apparatus left by a previous animal (Hershey *et al.,*2018). Behavioural parameters were scored with EthoVision© XT 14 software (Noldus Information Technology, Wageningen, NLD) and included total distance covered (cm) during the specified time (Gericke *et al.,*2022).

2.2.2 Forced Swim Test (FST)

The FST is a well-described assay to assess depressive-like behaviour in rodents (Porsolt *et al.,*1978). The FST was conducted according to previously described methods (Brand and Harvey, 2017; Gericke *et al.,*2022). The apparatus consisted of four Perspex® cylinders (diameter 20 cm, height 60 cm) positioned next to each other. On PND-63, the cylinders were filled with water up to the height 30 cm and the water temperature maintained at 25°C ± 1°C. The animals were allowed to acclimatize for 30 min; thereafter, they were individually placed in the cylinders and allowed to swim for 7 min. The first and the last minute were excluded with the remaining 5 min behaviour scored with manual FST scoreboard 2.0 software; Academic Support services: Information Technology in Education, NWU, RSA as previously described (Steyn *et al.,*2020). The behavioural parameters were coded as immobility (when rats only make necessary movement to keep their heads above water), struggling/climbing (upward-directed movement of the forepaws along the side of the cylinder) and swimming (horizontal movement throughout the swim cylinder plus crossing to another quadrant) (Mokoena *et al.,*2015; Cryan *et al.,*2002). These behaviours also inform on noradrenergic (struggle/climbing) versus serotonergic (swimming)–mediated behaviours in the FST (Slattery and Cryan, 2012). All the cylinders were thoroughly rinsed with water between sessions.

2.2 Neurobiological analysis

2.3.1 Brain dissection and sample collection

The hippocampus and frontal cortex were selected for analysis as these regions are intimately associated with the pathophysiology of MDD (Brand *et al.,*2015), especially monoamine and other neurochemical changes. The animals were decapitated 24 hours after their last treatment. The brain was dissected on an ice-cooled dissection slab, with

the frontal cortex and hippocampus removed according to previous methods (Moller *et al.,*2013). The hippocampus and frontal cortex were split into aliquots according to the left or right brain tissues, fixed in liquid nitrogen, stored in 1.5 ml Eppendorf tubes and preserved at -80°C until the day of the analysis. The rest of the brain was collected and stored for use in future studies.

2.3.2 Quantification of the hippocampal and cortical monoamines

Quantification of hippocampal and cortical monoamines (NE, 5-HT and DA) were conducted with High Performance Liquid Chromatography (HPLC) coupled with electrochemical detection (HPLC-ECD) using a validated method as previously described (Viljoen *et al.,*2018). Briefly, the hippocampal and frontal tissues were thawed on ice weighed and 1 ml of solution A was added to the Eppendorf tubes. Brain tissue was sonicated and placed on ice for 20 min to allow for completion of perchlorate precipitation of proteins and extraction of monoamines. The samples were centrifuged at 4°C for 25 min at 14, 000 rpm. The supernatant was collected for each sample and transferred to 2 ml Eppendorf tubes. Thereafter pH was adjusted with one drop of 10 M Potassium acetate and the solution vortexed. Then, 20 µl of 5-HMT (internal standard) was added to 200 µl of tissue supernatant. The final samples were vortexed and centrifuged at 4°C. for 5 min at 14, 000 rpm, with 200 µl of the supernatant transferred to HPLC vials and analysed. The instrumentation used consisted of an Ultimate 3000 Ultra- High Performance Liquid Chromatography (UHPLC) system equipped with an ISO-3100SD isocratic pump and WPS-3000TSL analytical autosampler, coupled to an ECD-3000RS rapid separation electrochemical detector with 2-Channel 6011RS ultra Coulometric Analytical Cell and Chromeleon® chromatography management system version 7.2 (purchased from Thermo Fisher Scientific, Waltham, MA USA). The concentrations of the monoamine analysed were expressed as ng/g weight of tissue analysed.

2.3.3 Regional brain phosphodiesterase-4B (PDE4B) analysis

The hippocampus and frontal cortex samples were prepared and analysed in duplicate using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cloud-Clone Corp.) for PDE4B, cAMP specific (PDE4B) SEF642Ra for tissue homogenates according to procedures described by the manufacturer. Briefly, the hippocampal and frontal tissues were thawed on ice, rinsed with ice-cold phosphate buffer saline (PBS-0.01mol/L, pH 7.4), weighed and homogenised in 9 ml to disrupt the cells. The homogenates were

sonicated and centrifuged at 10, 000 x g for 5 min. The supernatant was collected and stored at -80°C. On the day of the analysis, the reagents and samples were prepared, washed, and added to the appropriate 96-T wells. The absorbance of each concentration of the samples was measured with a Molecular device Spectra max® Spectrophotometer (paradigm multi-mode detection platform Austria Lagerhausstrasse, 45, 5021 wals) at 450 nm wavelength. The results were obtained from a typical standard curve plotted and expressed as concentration (ng/ml).

2.4 Statistical analysis

The Grubbs' test was used to identify any outlier from the given data sets, which was consequently removed for data analysis. The Shapiro-Wilk test was used to test for normality of distribution. The Mann-Whitney *U* or unpaired *t*-test with Welch's correction was used to compare behaviour between two data sets, based on distribution of data. A Brown-Forsythe one-way ANOVA or Kruskal-Wallis test, followed by a Dunnett's T3 or Dunn's multiple comparison post-hoc test, to compare bio-behavioural data between the monotherapy's treatment groups. The Tukey's multiple comparison post-hoc test was used to compare bio-behavioural data between the monotherapies and combination treatment groups for the data that showed normal distribution. Data are expressed as mean ± standard error of the mean (S.E.M), with statistical significance accepted as $p < 0.05$. In addition, the effect size indicators (applied only where statistically significance was narrowly missed, and/or strong trends were obvious) between the specific groups were calculated and presented as the unbiased Cohen's *d* (d_{unb}) value (with a 95% CI of the effect size) to indicate the magnitude of the response to treatment (Cumming, 2013; Grissom and Kim, 2012) (large when $d_{unb} \geq 0.8$ (Lakens, 2013)). The unbiased Cohen's *d* (d_{unb}) value is calculated from the Cohen's *d*- values to provide best estimates of effect sizes for the entire population. The graphs were generated with GraphPad Prism (GraphPad software, version 8, San Diego, USA).

3.Results

3.1. Phase-1 Validation of the FSL rat

3.1.1 Confirmation of face validity of the FSL/FRL Model for depressive-like behaviour (Table 1)

All the data sets were normally distributed. At the end of the intervention period, SAL FSL rats weighed significantly more than their FRL counterparts ($t_{13.18} = 3.26$; $p = 0.0061$),

despite having similar baseline body weights prior to treatment initiation (197.8 ± 7.44 *vs.* 190.3 ± 5.55; $t_{12.63} = 1.34$; $p = 0.2038$).

Following the intervention period, SAL FSL rats were significantly more immobile in the FST ($t_{21.52} = 8.75$; $p \leq 0.0001$), despite covering a significantly greater distance in the OFT ($t_{21.91} = 3.61$; $p = 0.0016$) in relation to FRL controls. Furthermore, SAL FSL rats also displayed significantly less time engaged in struggling ($t_{19.96} = 2.47$; $p = 0.0228$) and swimming behaviours ($t_{21.55} = 7.24$; $p \leq 0.0001$), compared to their FRL counterparts.

Table 1: Face validation of the FSL/FRL model

In all instances, animals received SAL treatment. Data is presented as mean ± S.E.M.

Parameters measured	Behavioural test	FRL	FSL	p-values
Body weight (g)	-	249.0±5.56	267.2±18.15	p = 0.0061
Distance moved (cm)	OFT	1771.76 ± 608.94	2649.60 ± 650.44	p = 0.0016
Immobility (sec)	FST	147.59 ± 16.96	204.10 ± 14.60	p ≤ 0.0001
Struggling (sec)	FST	79.49 ± 24.55	57.96 ± 17.62	p = 0.0228
Swimming (sec)	FST	72.94 ± 13.16	36.58 ± 11.38	p ≤ 0.0001

3.2. Phase-2 ZEM and DMI Monotherapy dose response studies

The data in this section illustrates the effects of saline, escalating doses of ZEM (10, 25 and 30 mg/kg/day) and DMI (15 and 30 mg/kg/day) on the mean weight gain, locomotor activity (measured in OFT) and depressive-like behaviour (measured in FST) in FSL rats following monotherapy treatment for 14 days.

3.2.1. Monotherapy study; body weights

In Fig. 2, all data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00, 55.31} = 28.19$; $p \leq 0.0001$). Compared to the SAL FSL, groups receiving DMI-15 ($p \leq 0.0001$); DMI-30 ($p \leq 0.0001$), ZEM-10 ($p =$

0.009) and ZEM-30 (p = 0.0045), gained significantly less weight during the 14-day treatment period.

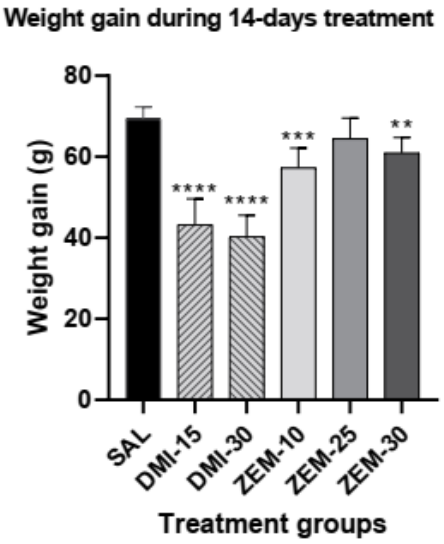


Fig.2. *Monotherapy study. Effects of saline, escalating doses of ZEM (10, 25 and 30 mg/kg/day) and DMI (15 and 30 mg/kg/day) on mean weight gain over 14-days treatment. Data expressed as means ± S.E.M. with **p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0001 vs. SAL.*

3.2.2 Monotherapy study: Locomotor activity and depressive- like behaviour (FST & OFT)

Locomotor activity (*Fig. 3A*)*:* All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00,\ 59.87}$ = 3.25; p = 0.0116). Compared to SAL FSL rats, the Dunnett's T3 multiple comparison test revealed significantly reduced locomotor activity in DMI-30 (p = 0.0310) and ZEM-10 (p = 0.0229) rats.

Immobility (Fig. 3B): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00,\ 40.13}$ = 11.87; $p \leq 0.0001$). Both DMI-15 (p = 0.0009) and DMI-30 (p = 0.0219) significantly reduced time spent immobile in the FST compared to SAL FSL control.

Struggling (Fig. 3C): Not all data sets were normally distributed. There were statistically significant differences between the treatment groups (x^2 = 42.21; p ≤ 0.0001). The Dunn's post-hoc test revealed that DMI-15 (p = 0.0282) and DMI-30 (p = 0.0249) significantly increased struggling behaviour in FST compared to SAL FSL control.

Swimming (Fig. 3D): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00, 47.57} = 40.31$; $p \leq 0.0001$). DMI-15 ($p = 0.0004$) and DMI-30 ($p = 0.0004$) treated rats displayed significantly less time engaged in swimming behaviour in FST, compared to SAL FSL rats.

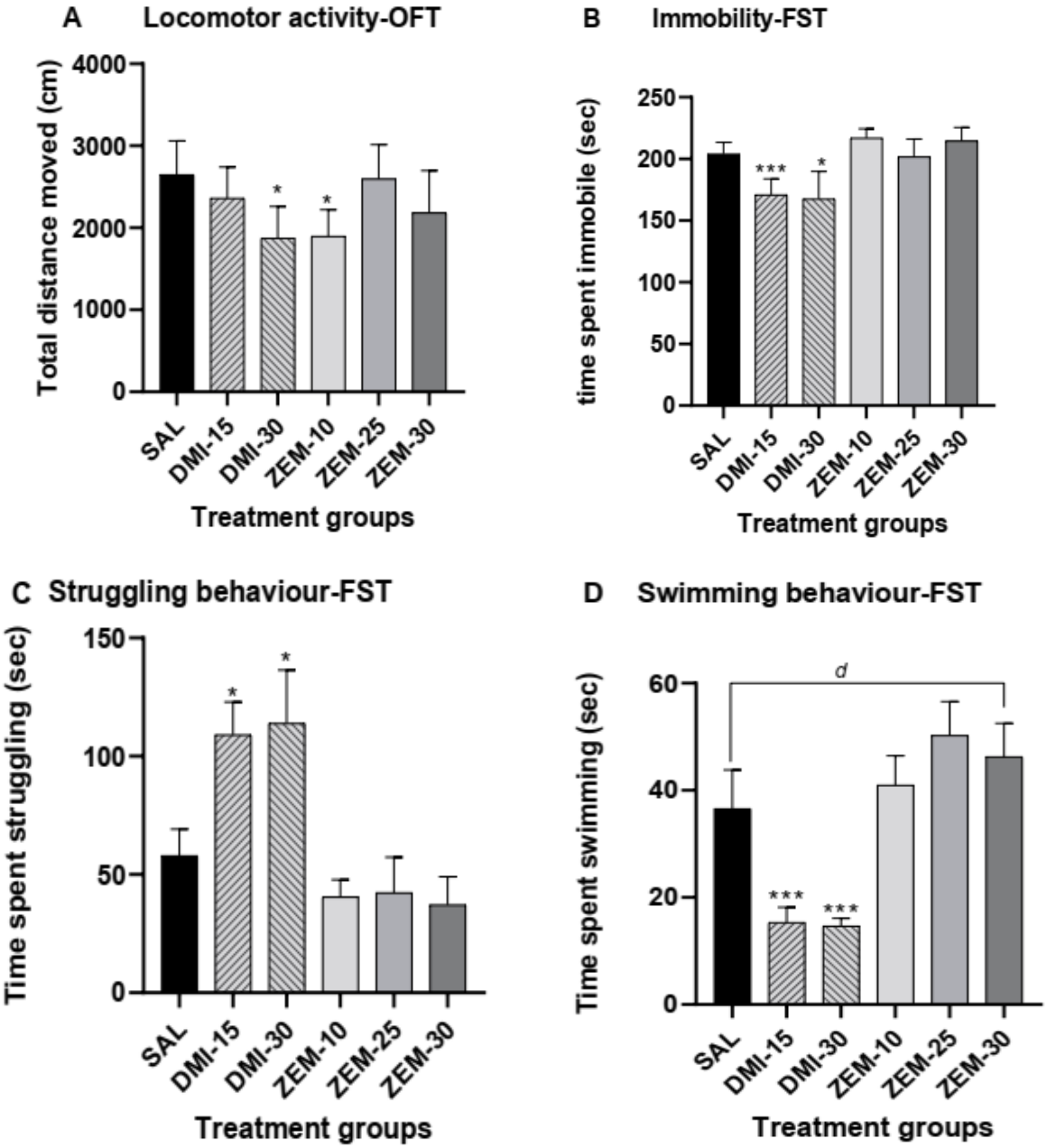


Fig.3. *Monotherapy study. Effects of saline, escalating doses of ZEM (10, 25 & 30 mg/kg/day), low dose DMI (15 mg/kg/day) and DMI (30 mg/kg/day) on locomotor activity and depressive-like behaviour versus FSL saline treated rats following 14 days treatment.* ***(A)*** *Total distance moved in the OFT (outlier identified and removed from the ZEM-25 group).* ***(B)*** *Time spent immobile (outlier identified and removed from the DMI-15 group),* ***(C)*** *struggling (outlier identified and removed from the DMI-15 group), and* ***(D)*** *swimming in the FST. Data expressed as means ± S.E.M. with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs. SAL*

3.3 Selection of DMI and/or ZEM doses for the augmentation study

Conclusion on DMI dose selection for augmentation study

The behavioural results from the FST above (Fig. 3) revealed that DMI is effective at both doses (15 and 30 mg/kg/day) to reverse depressive-like behaviour in FSL rats, which supports the predictive validity of the FSL rat model using DMI. Despite both DMI doses being statistically significant, DMI-15 had the bigger effect size value (i.e., more robust effect) (Fig. 3B) and had no potential to alter psychomotor activity, whereas DMI-30 reduced locomotor activity in FSL rats (Fig. 3A). Therefore, DMI-15 was selected for use in the augmentation study. Although the effects of DMI (15 and 30 mg/kg/day) on the monoamines were determined, and because of the behavioural data was clear that DMI-15 had a better therapeutic outcome in terms of efficacy and safety (see Fig. 3A & B) the monoamine results were not required for selecting a dose for DMI for augmentation study.

Since none of the doses of ZEM were significantly effective to reverse depressive-like behaviour in the FST (Fig 3), we consulted a previous acute dose-ranging study in our laboratory (25 mg/kg/day and 50 mg/kg/day) (Gericke *et al.,*2022) to provide direction, as well as considered the effects of Zembrin® on hippocampal and cortical NE and 5-HT, to select an appropriate dose for the sub-chronic augmentation study.

Effects of saline, Zembrin® and desipramine on hippocampal and frontal cortical NE levels

Figure 4 below represent effects of saline and escalating doses of ZEM (10, 25 and 30 mg/kg/day) and DMI (15 and 30 mg/kg/day) on hippocampal and frontal cortical NE levels in FSL rats following 14 days treatment.

Hippocampal NE (Fig. 4A): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00,\ 32.53}$ = 14.73; $p \leq$ 0.0001). Compared to SAL FSL rats, the Dunnett multiple comparison post-hoc test indicated significantly reduced hippocampal NE levels in DMI-15 (p = 0.0042) and DMI-30 (p = 0.0028) treated rats. The Dunnett multiple comparison post-hoc test showed no significant difference in hippocampal NE levels in FSL rats treated with ZEM-10 (p = 0.0813); ZEM-25 (p = 0.8961) and ZEM-30 (p = 0.9922) versus SAL FSL rats.

Cortical NE (Fig. 4B): Not all data sets were normally distributed. There were statistically significant differences among the treatment groups (X^2 = 38.60; $p \leq$ 0.0001). Compared to

SAL FSL rats, the Dunn's multiple comparison post-hoc test showed no significant differences in frontal cortical NE levels in FSL rats treated with DMI-15 (p = 0.8549) versus FSL SAL control rats. The Dunn's multiple comparison post-hoc test showed significantly reduced cortical NE levels in FSL rats treated with DMI-30 (p = 0.0296) compared to the SAL FSL rats. Of note, ZEM-30 significantly increased frontal cortical NE levels in FSL rats compared to the SAL FSL rats (p = 0.0297). The Dunn's multiple comparison post-hoc test showed no significant differences in frontal cortical NE levels in FSL rats treated with ZEM-10 (p = 0.9999) and ZEM-25 (p = 0.9999) compared to the SAL FSL rats.

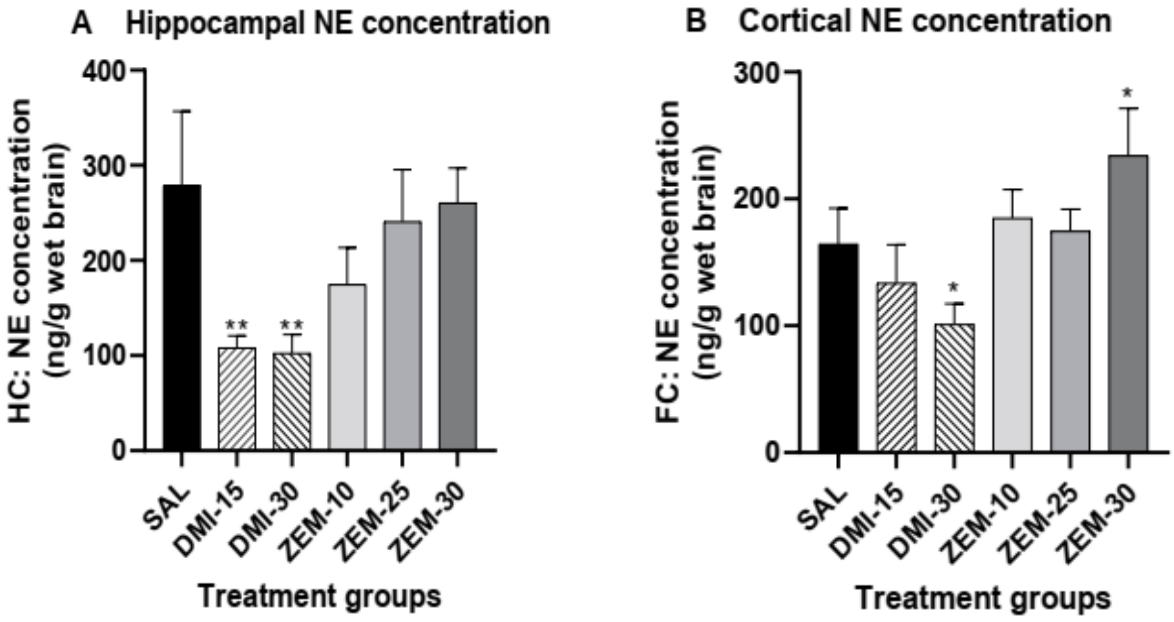


Fig. 4*. Effects of saline and escalating doses of ZEM (10, 25 & 30 mg/kg/day) and DMI (15 & 30 mg/kg/day) on tissue brain NE levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal NE concentration (ng/g) (two outliers identified and removed from the FSL-SAL group). (B). Cortical NE concentration (ng/g) (outlier identified and removed from the FSL-SAL group). Data expressed as means ± S.E.M. with *p ≤ 0.05, **p ≤ 0.01vs. SAL*

Effects of saline, Zembrin® and desipramine on hippocampal and frontal cortical 5-HT levels

Figure 5 represent effects of saline and escalating doses of ZEM (10, 25 and 30 mg/kg/day) and DMI (15 and 30 mg/kg/day) on hippocampal and frontal cortical 5-HT levels in FSL rats following 14 days treatment.

Hippocampal 5-HT (Fig. 5A): Not all data sets were normally distributed. In DMI-15 treated rats hippocampal 5-HT levels were below the limit of detection (BLOD). There were statistically significant differences among the treatment groups (x^2 = 18.28; p = 0.0011). Although the Dunn's multiple comparison post-hoc test showed significantly reduced hippocampal 5-HT levels in FSL rats treated with in DMI-30 compared to the SAL FSL rats (p = 0.0082), hippocampal NE levels were detected in five (5) samples only. Compared to SAL FSL rats, the Dunn's multiple comparison post-hoc test showed significantly reduced hippocampal 5-HT levels in FSL rats treated with ZEM-10 (p = 0.0402). The Dunn's multiple comparison post-hoc test showed no significant differences in hippocampal levels in FSL rats treated with ZEM-25 (p = 0.9999) and ZEM-30 (0.9999) versus the FSL SAL rats.

Cortical 5-HT (Fig. 5B): All data sets were normally distributed. In DMI-15 treated rats the hippocampal 5-HT level was detected in three (3) samples only. There were statistically significant differences among the treatment groups ($F_{5.00,38.20}$= 14.12; $p \leq 0.0001$). The Dunnett multiple comparison post-hoc test showed no significant differences in cortical levels in FSL rats treated with DMI-30 (p = 0.1139); ZEM-10 (p = 0.3606) and ZEM-25 (p = 0.8120) compared to the SAL FSL rats. Of note, ZEM-30 induced a significant increase in frontal cortical 5-HT levels compared to SAL FSL rats (p = 0.0027).

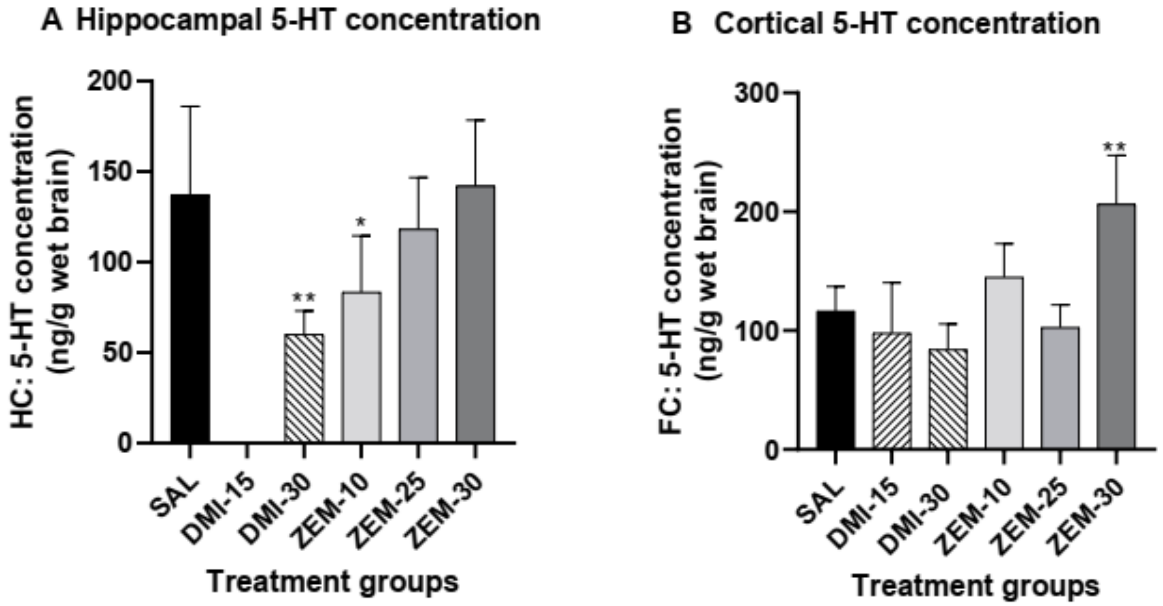


Fig. 5. *Effects of saline and escalating doses of ZEM (10, 25 & 30 mg/kg/day) and DMI (15 & 30 mg/kg/day) on tissue brain 5-HT levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal 5-HT concentration (ng/g) (FSL-SAL group (n=7), DMI-15 group (n = 0), DMI-30 group (n = 5), ZEM-10 (one outlier identified and removed reducing sample size n = 9), ZEM-25 (n = 12) and ZEM-*

*30 (n = 12)). (B). Cortical 5-HT concentration (ng/g) (FSL-SAL group (n = 12), DMI-15 group (n = 3), DMI-30 group (n = 9), ZEM-10 (n = 12), ZEM-25 (n = 12) and ZEM-30 (n = 12)). Data expressed as means ± S.E.M. with*p ≤ 0.05, **p ≤ 0.01 vs. SAL.*

Conclusion on ZEM dose selection for augmentation study

The FST behavioural results above showed no antidepressant-like effects with escalating doses of ZEM (Fig. 3) in the FST following 14-days treatment. However, a significant effect on regional brain monoamines was noted (Fig. 4 & 5). Only ZEM-30 significantly increased cortical NE and 5-HT levels that could support its antidepressant potential. This dose also correlates well with the effective doses for ZEM described by Gericke et al in their acute dose-ranging study in FSL rats (2021). Therefore, based on the neurochemical results, the decision was made to choose ZEM-30 over ZEM-25 as the higher dose in this study. ZEM-10 in turn did not affect immobility (Fig. 3B) or any of the monoamine biomarkers, excepting to reduce hippocampal 5-HT (Fig. 5A). It was thus chosen as the (low) sub-effective dose of ZEM (possible reduced risk of side effects in augmentation study) for combining with low dose DMI in the adjunctive treatment study. This will allow for investigating the augmentation potential of low DMI with ZEM in a dose dependent manner.

3. 4 Phase 3 Combination therapy of DMI and escalating doses of ZEM

Results below represent the effects of saline, escalating doses of ZEM (10 and 30 mg/kg/day), low dose of DMI (15 mg/kg/day) and the combination therapies (ZEM-10+DMI-15 or ZEM-30+DMI-15 mg/kg/day) on the mean weight gain, locomotor activity (measured in OFT) and depressive-like behaviour (measured in FST) and PDE4B levels (hippocampal and frontal cortical) in FSL rats following 14 days treatment.

3.4.1. Augmentation study: body weights

In *Fig. 6,* all data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5, 66} = 43.03$; $p \leq 0.0001$). The Tukey's multiple comparison post-hoc test revealed that DMI-15 ($p \leq 0.0001$); ZEM-10 ($p = 0.0010$); ZEM-30 ($p = 0.0500$); ZEM-10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 ($p \leq 0.0001$) treated rats gained significantly less weight compared to the SAL FSL rats during the treatment period. The Tukey's multiple comparison post-hoc test revealed that DMI-15 significantly reduced weight gain in FSL rats compared to ZEM-10 ($p \leq 0.0001$) and ZEM-30 ($p \leq$

0.0001) rats during the treatment period. The Tukey's multiple comparison post-hoc test revealed that ZEM-10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 ($p \leq 0.0001$) treated rats gained significantly less weight compared to both ZEM- (10 and 30) monotherapies during the treatment period.

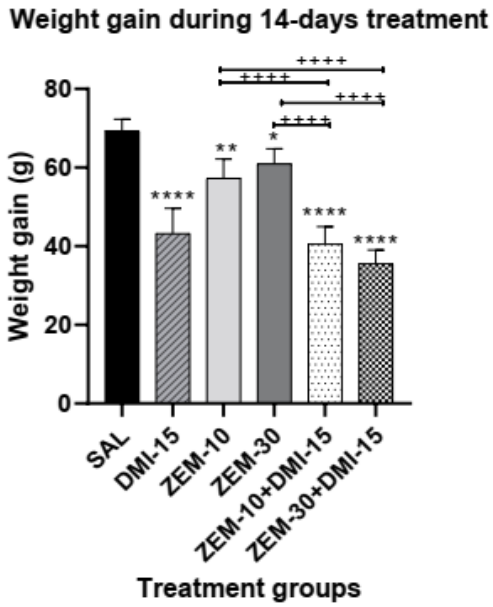


***Fig. 6**. Augmentation study. Effects of saline, escalating doses of ZEM (10 & 30 mg/kg/day), low dose of DMI (15 mg/kg/day) and the combination therapies (ZEM-10+DMI-15 or ZEM-30+DMI-15 mg/kg/day) on mean weight gain during 14-days treatment. Data expressed as means ± S.E.M. with *p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001 vs. SAL, ++++ p ≤ 0.0001 vs. ZEM*

3.4.2. Augmentation study: Locomotor activity and depressive- like behaviour

Locomotor activity: In *Fig. 7A,* all data sets were normally distributed. There were statistically significant differences between the treatment groups ($F_{5, 66} = 3.15$; $p = 0.0130$). Compared to the SAL FSL rats, the Tukey's multiple comparison post-hoc test revealed significantly reduced locomotor activity only in ZEM-10+DMI-15 ($p = 0.0326$) treated rats. Compared to the DMI-15 treated rats, the Tukey's multiple comparison post-hoc test showed no statistically significant differences in ZEM-10 ($p = 0.4655$); ZEM-30 ($p = 0.9818$); ZEM-10+DMI-15 ($p = 0.3645$) and ZEM-30+DMI-15 ($p = 0.9948$) regarding locomotor activity in OFT. Compared to ZEM-10 treated rats, the Tukey's multiple comparison post-hoc test showed no statistically significant differences in ZEM-10+DMI-15 ($p = 0.9999$) and ZEM-30+DMI-15 ($p = 0.1925$) regarding locomotor activity in OFT. Likewise, the Tukey's multiple comparison post-hoc test showed no statistically

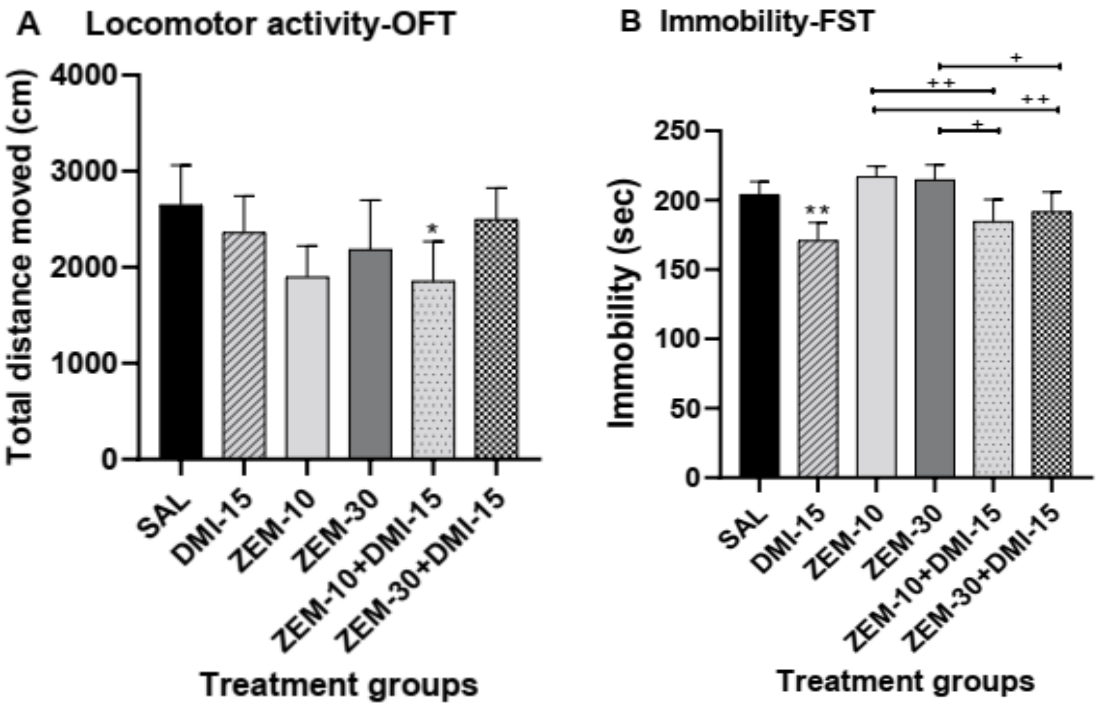
significant differences in ZEM-10+DMI-15 (p = 0.7926) and ZEM-30+DMI-15 (p = 0.8226) compared to ZEM-30 treated rats regarding locomotor activity in OFT.

Immobility: In *Fig. 7B*, all data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5, 65}$ = 10.58; $p \leq 0.0001$). DMI-15 significantly reduced time spent immobile in the FST compared to SAL FSL rats (p = 0.0011); ZEM-10 ($p \leq 0.0001$) and ZEM-30 ($p \leq 0.0001$) treated rats. Compared to SAL FSL rats, the Tukey's multiple comparison post-hoc test showed no statistically significant differences in ZEM-10 (p = 0.5518); ZEM-30 (p = 0.7368); ZEM-10+DMI-15 (p = 0.1313) and ZEM-30+DMI-15 (p = 0.5944) regarding time spent immobile in the FST. The Tukey's multiple comparison post-hoc test showed no statistically significant differences in ZEM-10+DMI-15 (p = 0.5109) and ZEM-30+DMI-15 (p = 0.1031) compared to the DMI-15 rats regarding time spent immobile in the FST. Of note, the Tukey's multiple comparison post-hoc test revealed significantly reduced time spent immobile in the FST in ZEM-10+DMI-15 (p = 0.0011) and ZEM-30+DMI-15 (p = 0.0194) compared to ZEM-10 treated rats. Likewise, the Tukey's multiple comparison post-hoc test revealed significantly reduced time spent immobile in the FST in ZEM-10+DMI-15 (p = 0.0029) and ZEM-30+DMI-15 (p = 0.0431) compared to ZEM-30 treated rats.

Struggling: In *Fig. 7C,* not all data sets were normally distributed. There were statistically significant differences among the treatment groups (x^2 = 37.39; $p \leq 0.0001$). DMI-15 treated rats displayed significantly more time engaged in struggling behaviour, compared to ZEM-30+DMI-15 (p = 0.0496) treated rats. Compared to the SAL FSL rats, the Dunn's multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 (p = 0.999) and ZEM-30+DMI-15 (p = 0.9999) regarding the time engaged in struggling behaviour in FST. The Dunn's multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 (p = 0.0793) and ZEM-30+DMI-15 (p = 0.4011) compared to the ZEM-10 treated rats regarding the time engaged in struggling behaviour in FST. Likewise, compared to the ZEM-30 treated rats, the Dunn's multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 (p = 0.0546) and ZEM-30+DMI-15 (p = 0.2939) treated rats regarding the time engaged in struggling behaviour in FST

Swimming. In *Fig. 7D,* all data sets were normally distributed. There were statistically significant differences between the treatment groups ($F_{5, 66}$ = 22.10; $p \leq 0.0001$). DMI-15 treated rats displayed significantly less time engaged in swimming compared to ZEM-

10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 ($p \leq 0.0001$) treated rats. Compared to the SAL FSL rats, the Tukey's multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 ($p = 0.7506$) and ZEM-30+DMI-15 ($p = 0.3370$) treated rats regarding the time engaged in swimming behaviour in the FST. The multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 ($p = 0.9999$) and ZEM-30+DMI-15 ($p = 0.9775$) treated rats compared to ZEM-10 treated rats, regarding the time engaged in swimming behaviour in the FST. Likewise, compared to ZEM-30 treated rats, the multiple comparison post-hoc test showed no significant differences in ZEM-10+DMI-15 ($p = 0.6632$) and ZEM-30+DMI-15 ($p = 0.9602$) treated rats regarding the time engaged in swimming behaviour in the FST.



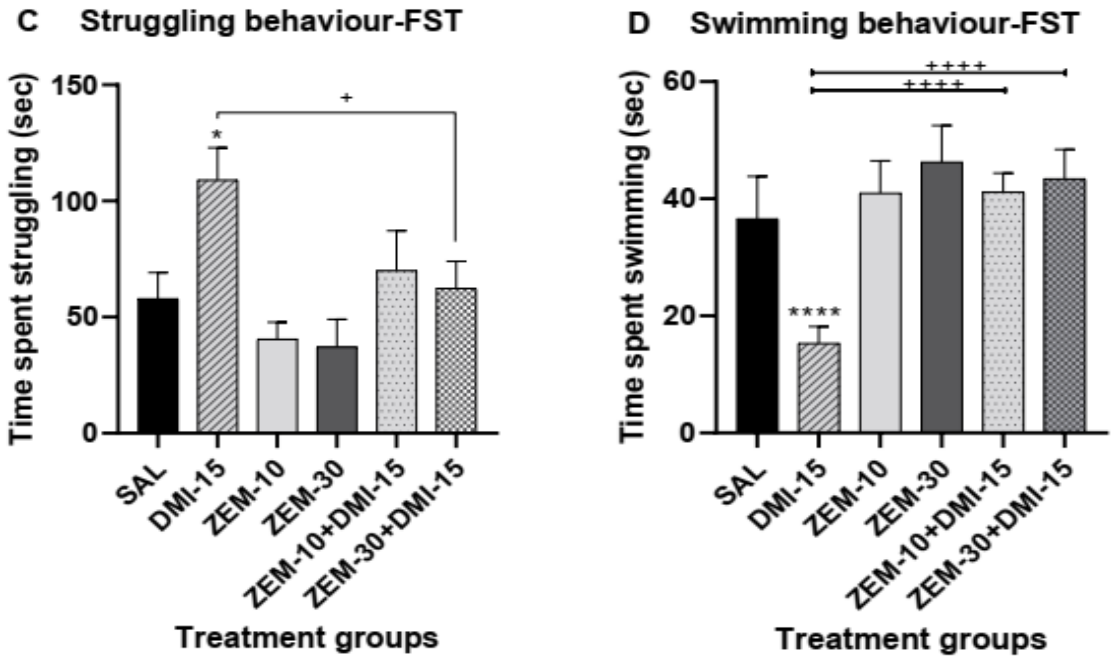


Fig.7. *Augmentation study. Effects of saline, escalating doses of ZEM (10 & 30 mg/kg/day), low dose DMI (15 mg/kg/day), ZEM-10+DMI-15 (mg/kg/day) and ZEM-30+DMI-15 (mg/kg/day) on locomotor activity and depressive-like behaviour versus FSL saline treated rats following 14 days treatment.* ***(A)*** *Total distance moved (cm) in the OFT* ***(B)*** *Time spent immobile (outlier identified and removed from the DMI-15 group),* ***(C)*** *struggling (outlier identified and removed from the DMI-15 group) and* ***(D)*** *swimming in the FST. Data expressed as means ± S.E.M. with *p ≤ 0.05, **p ≤ 0.01, **** p ≤ 0.0001 and d ≥ 0.8 vs. SAL. $^{+}$p ≤ 0.05 vs. DMI/ZEM. $^{++}$p ≤ 0.01, $^{++++}$p ≤ 0.0001 vs. ZEM.*

3.5. Augmentation study: Phosphodiesterase4 (PDE4B)

3.5.1. Effects of Zembrin®, DMI and/or combination therapies

Hippocampal PDE4B levels (ng/ml). In *Fig. 8A*. Not all data sets were normally distributed. There were statistically significant differences among the treatment groups (X^2 = 51.91; $p \leq 0.0001$). Compared to SAL FSL rats, ZEM-10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 ($p \leq 0.0001$) significantly decreased hippocampal PDE4B levels in FSL rats. Compared to the DMI-15 treated rats, the Dunn's multiple comparison post-hoc test revealed no statistically significant differences in hippocampal PDE4B levels in ZEM-10+DMI-15 ($p = 0.0637$) and ZEM-30+DMI-15 ($p = 0.1463$) treated rats. Of note, the Dunn's multiple comparison post-hoc test revealed that ZEM-10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 ($p \leq 0.0001$) significantly decreased hippocampal PDE4B levels compared to ZEM-10. Likewise, compared to ZEM-30, ZEM-10+DMI-15 ($p = 0.0011$) and ZEM-30+DMI-15 ($p = 0.0013$) significantly decreased hippocampal PDE4B levels in FSL rats.

Cortical PDE4B (ng/ml) levels. In *Fig. 8B.* Not all data sets were normally distributed. There were statistically significant differences among the treatment groups (x^2 = 47.17; $p \leq 0.0001$). Compared to the SAL FSL rats, the Dunn's multiple comparison post-hoc test revealed no statistically significant differences in ZEM-10+DMI-15 ($p = 0.9683$) treated rats regarding the effects on the frontal cortical PDE4B levels. ZEM-30+DMI-15 ($p = 0.0121$) significantly increased frontal cortical PDE4B levels in FSL rats compared SAL FSL rats. Of note, ZEM-10+DMI-15 ($p = 0.0067$) and ZEM-30+DMI-15 ($p \leq 0.0001$) significantly increased frontal cortical PDE4B levels in FSL rats compared to the DMI-15 treated rats. Surprisingly, the Dunn's multiple comparison post-hoc test revealed that ZEM-30 ($p = 0.0073$) significantly decreased PDE4B levels in FSL rats compared to ZEM-10 treated rats. Of note, ZEM-10+DMI-15 ($p = 0.0003$) and ZEM-30+DMI-15 ($p \leq 0.0001$) significantly increased frontal cortical PDE4B levels in FSL rats compared to the ZEM-30 treated rats. Compared to the ZEM-10 treated rats, the Dunn's multiple comparison post-hoc test showed no statistically significant differences in ZEM-10+DMI-15 ($p = 0.9999$) and ZEM-30+DMI-15 ($p = 0.3413$) treated rats regarding the effects on the frontal cortical PDE4B levels.

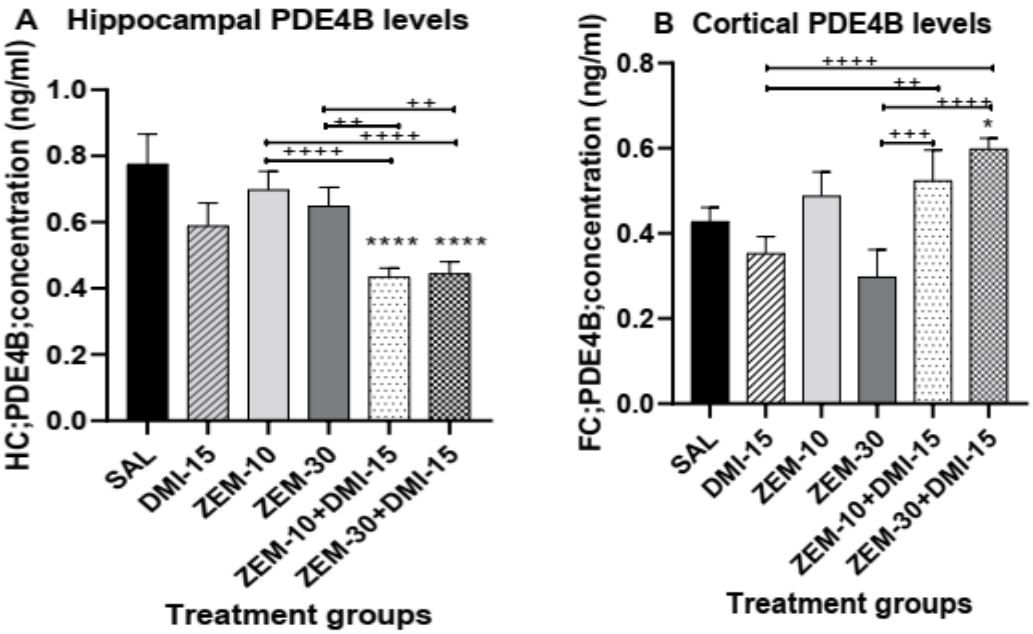


Fig.8. *Effects of saline, escalating doses of ZEM (10 & 30 mg/kg/day), low dose DMI (15 mg/kg/day), ZEM-10+DMI-15 (mg/kg/day) and ZEM-30+DMI-15 (mg/kg/day) on the hippocampal and cortical PDE4B (ng/ml) levels versus FSL saline treated rats following 14 days treatment.* ***(A).*** *Hippocampal PDE4B levels (ng/ml) (FSL-SAL group-(n=9)).* ***(B)*** *Cortical PDE4B (ng/ml) levels Data expressed as means ± S.E.M. with *p ≤ 0.05, **** p ≤ 0.0001 vs. SAL. $^{++}$p ≤ 0.01, $^{+++}$p ≤ 0.001, $^{++++}$p ≤ 0.0001 vs. ZEM/DMI*

4. Discussion

The FSL rat model is regarded as a robust and valid model of depression. Regarding face validity, it presents with reduced appetite, lower body weight, psychomotor retardation, reduced locomotor activity and increased despair/reduced coping versus control FRL rats (Overstreet and Wegener, 2013; Overstreet *et al.,*2005). According to DSM-V, increased appetite and weight gain are defining features of MDD in certain individuals (APA, 2013). The FSL model of MDD has subsequently been found to present with reduced appetite and lower body weight compared to FRL rats (Overstreet and Wegener, 2013; Overstreet *et al.,*2005), so that body weight and appetite are important parameters to consider (Saayman, 2019; Tillmann *et al.,*2019; Abildgaard *et al.,*2011). Although we did not measure daily food intake, our data show equivalent baseline body weight between treated naïve FSL and FRL rats *(Table 1)***.** After the intervention period, FSL rats weighed significantly more than FRL rats *(Table 1)* somewhat unexpected given earlier finding (Tillmann *et al.,*2019; Overstreet and Wegener,2013; Husum *et al.,*2003). Still, other studies do support our findings (Abildgaard *et al.,*2011). Although not common, increased appetite and weight gain may present in MDD (APA, 2013), while both clinical and review studies have described variable changes in appetite and weight in depressed individuals (Cosgrove *et al.,*2020; Simmons *et al.,*2018).

Turning to depressive- like behaviour, the findings in the present study show FSL rats to exhibit significantly elevated immobility (despair) and reduced struggling and swimming (coping) in the FST, also hallmark traits of MDD, as well as significantly higher locomotor activity in the OFT (total distance moved in cm) versus FRL rats *(Table1).* Although the latter is contrary to previous studies (Overstreet and Wegener, 2013; Overstreet *et al.,*2005), this is in line with findings from our laboratory (Gericke *et al.,*2022; Liebenberg *et al.,*2012) and others (Tillmann *et al.,*2019; Jefsen *et al.,*2019). However, in agreement with previous studies (Gericke *et al.,*2022; Tillmann *et al.,*2019; Mokoena *et al.,*2015; Liebenberg *et al.,*2012), drugs screened for antidepressant-like effects did not show psychostimulant activity despite the same or elevated locomotor activity in the OFT, thereby confirming a psychomotor basis to the observed locomotor changes (Gericke *et al.,*2022; Tillmann *et al.,*2019; Mokoena *et al.,*2015; Liebenberg *et al.,*2012). Importantly, our data confirm the depressive-like phenotype of the FSL rat (i.e., increased immobility, decreased coping behaviours in the FST) *(Table1), and* are in line with previous studies

that also showed increased immobility and decreased coping behaviours in FST (Gericke *et al.,*2022; Wegener *et al.,*2012).

In considering the response to monotherapy, we first considered body weight in FSL rats treated with escalating doses of ZEM (10, 25 and 30mg/kg/day) and/or DMI (15 and 30 mg/kg/day). Both ZEM and DMI significantly decreased weight gain in FSL rats compared to SAL-FSL rats (Fig. 2), with DMI showing the greatest effect. ZEM has been noted to suppress appetite (Brendler *et al.,*2021), while DMI might engender the same via its small but probably sufficient antihistaminic effects on the histamine H_1 receptors on the hypothalamus which is the main centre for appetite control (Kang *et al.,*2012).

The OFT showed significant differences in locomotor activity following sub-chronic treatment with either DMI-30 or ZEM-10 alone (Fig. 3A.). These findings differ from previous studies that showed no change in locomotor activity with ZEM (Gericke *et al.,*2022) and DMI (Mokoena *et al.,*2015; Overstreet *et al.,*2010). Previous studies have shown that DMI not only blocks the NE transporter (NET) but has dose-related antihistaminic and antimuscarinic side effects (i.e., sedation) (Maan *et al.,*2021), which may corroborate with reduced locomotor activity in DMI-30 treated rats (Fig. 3A). Previous studies have shown that lower doses of SSRIs may lead to upregulation of the 5-HT $_{1A}$ autoreceptors on the dorsal raphae nuclei due to lower 5-HT synaptic levels produced from partial inhibition of SERT (Yohn *et al.,*2017; Gericke et al.,2022). In fact, partial 5-HT_{1A} agonists reduce synaptic level of 5-HT (Liu *et al.,*2017). Therefore, we could suggest that ZEM-10, by virtue of SERT inhibition (Harvey *et al.,*2011), may produce partial inhibition of SERT at lower doses leading to upregulation of 5-HT_{1A} autoreceptors and a further inhibition of 5-HT release (see later; Fig. 5A). This may correlate with reduced locomotor activity in ZEM-10 treated rats (Fig.3A).

Considering the effects of escalating doses of ZEM (10, 25 and 30mg/kg/day) and/or DMI (15 and 30 mg/kg/day) on depressive-like behaviour in the FST, both DMI-15 and DMI-30 (Fig. 3B) showed significant antidepressant-like responses with the former showing a more superior effect size reduction in immobility (Fig. 3B). These findings firstly confirm the predictive validity of the FSL model using DMI and are in line with previous studies using low (5 and 10 mg/kg/day) (Overstreet *et al.,*2010; 2005) and high doses (15 mg/kg/ day) (Mokoena *et al.,*2015) of DMI in FSL rats. However, these earlier studies administered DMI via the intraperitoneal route. Interestingly, Simpson *et al. (*2012)

reported significant antidepressant-like effect with low (5 mg/kg/day) but not high doses of DMI (10 mg/kg/day). This dose discrepancy may explain the reduced antidepressant-like effects with high doses of DMI in this study (Fig. 3B). Alternatively, high dose DMI-30 may introduce other factors that could influence behaviour in the FST (Bogdanova *et al.,*2013; Simpson *et al.,*2012), such as sedative effects, due to stronger antimuscarinic and antihistaminic actions (Maan *et al.,*2021). Post-synaptic α_2-adrenoceptors activation (Ma *et al.,*2005) may also correlate with reduced locomotor activity in DMI-30 rats (Fig. 3A). Our data showed significantly decreased swimming behaviour in DMI-15 and DMI-30 treated rats (Fig. 3D), in agreement with previous studies, showing increased climbing behaviour and decreased swimming behaviour in rats treated with DMI (Mokoena *et al.,*2015; Overstreet *et al.,*2005). In fact, DMI reduces presynaptic NET expression (Zhao *et al.,*2008) and downregulates α_2-presynaptic receptors (Cottingham and Wang, 2012), which could underlie its antidepressant-like effects in the FST (Overstreet *et al.,*2005).

In the current study, ZEM (10, 25 and 30 mg/kg/day) showed no antidepressant-like effect in the FST following sub-chronic treatment *(*Fig. 3B*),* albeit in line with a recent sub-chronic study in our laboratory using ZEM (50 mg/kg/day) (Gericke, 2019). A recent acute treatment study showed dose-dependent antidepressant-like effects with ZEM (Gericke *et al.,*2022). Interestingly, Schell (2014) describes the antidepressant-like effects of an acid extract of mesembrine following acute treatment in BALB/c mice, a mouse model of MDD. Since antidepressant response across all classes, except ketamine, invariably requires chronic treatment, the antidepressant-like effect of ZEM in the FST following sub- or chronic treatment needs further study*.* Furthermore, differences in response may also be model-dependent, so that studies in other translational models may be revealing. Aspects of this are discussed later under monoaminergic effects. A large effect size increase in swimming behaviour in ZEM-30 rats (Fig. 3D) concurs with enhanced serotonergic activity (Fig. 5B) and hence re-affirms the purported serotonergic actions of ZEM and is in accord with its potential to inhibit SERT (Harvey *et al.,*2011). Considering this, a dose-dependent increase in swimming behaviour in ZEM- treated rats was expected but did not occur (Fig. 3D). However, these findings correlate with a previous sub-chronic study showing no significant differences in climbing and swimming behaviour in the FST after treatment with ZEM (50 mg/kg/day) (Gericke, 2019).

While CAMS may have clinical value as monotherapy, it is in adjunctive treatment that it sees its most common use, with both increased risk of improved response but also

adverse drug-herb interactions (Haller *et al.,*2019; Nahas and Sheikh, 2011). In considering the augmentation study data, both ZEM and DMI alone reduced weight gain in FSL rats (Fig. 2) with a similar trend evident in the combination treatments (Fig. 6). This might be related to the combined appetite suppressant effects of ZEM (Brendler *et al.,*2021) and the antimuscarinic and histaminergic actions of DMI (Maan *et al.,*2021). Although low dose (15 mg/kg/day) DMI does not alter psychomotor activity (Fig. 3A), it significantly reduced weight gain in FSL rats versus SAL and ZEM (10 and 30 mg/kg/day) alone. Being a TCA, DMI possess very mild anticholinergic properties (Maan *et al.,*2021) which even at low doses, may exert antimuscarinic and histaminergic actions leading to reduced weight gain in FSL rats. In fact, Kozisek *et al.* (2007) observed significant weight reduction coupled to regional increased serum concentration of DMI and its active metabolite, desmethyladesipramine (especially in the prefrontal cortex) in male Sprague-Dawley(SD) rats following treatment with DMI 10 mg/kg/day given via i.p.

Although DMI- 15 did not reduce weight gain in FSL rats compared to both ZEM-10+DMI-15 and ZEM-30+DMI-15 treatment (Fig. 6), both ZEM-10+DMI-15 and ZEM-30+DMI-15 reduced weight gain in FSL rats compared to ZEM- (10 and 30 mg/kg/day) alone (Fig.6), this may be related to the combined effects of ZEM and DMI which significantly reduced weight gain in FSL rats.

As with ZEM monotherapy challenges, we observed no antidepressant-like effects in the FST (immobility) following sub-chronic treatment with either ZEM-10+DMI-15 or ZEM-30+DMI-15, compared to SAL FSL rats and more importantly compared to DMI-15 alone. Results with DMI are incongruent with previous studies, where a synergistic effect has been reported with sub-effective doses of DMI (i.e., 2.5 mg/kg/day) combined with other compounds, although the drugs were administered via the i.p. route (Overstreet *et al.,* 2010). In fact, various rodent studies have shown reduced immobility in the FST with DMI alone and when combined with other compounds, via the i.p. (Lapiz *et al.,*2007; Simpson *et al.,*2012; Overstreet *et al.,*2010;Mokoena *et al.,*2015) or oral route (Singewald *et al.,*2004; Nava *et al.,*2015).

While we found no statistically significant differences in immobility in ZEM-10+DMI-15 and ZEM-30+DMI-15 compared to the DMI-15 treated rats, we observed significantly reduced immobility in ZEM-10+DMI-15 and ZEM-30+DMI-15 compared to ZEM-10 treated rats. Likewise, we noted significantly reduced time spent immobile in ZEM-

10+DMI-15 and ZEM-30+DMI-15 compared to ZEM-30 treated rats. Taken together, these results suggest that ZEM+DMI fails to augment the antidepressant action of DMI, but does augment the action of ZEM. Our earlier data indicates that DMI is a significantly better antidepressant than ZEM (Fig. 7B), so while the combination is not fully able to improve on the already significant antidepressant response of DMI, adding DMI to ZEM results in improving the response of ZEM. Our results differ with previous findings that showed increased immobility following sub-chronic treatment with ZEM (50 mg/kg/day) and escitalopram (5 mg/kg/day) (Gericke, 2019), possibly due to the combination of an SSRI (escitalopram) and ZEM exacerbating an already heightened serotonergic response in these animals (see below)(Overstreet *et al.,*1998).

To our knowledge, this is the first study to evaluate the antidepressant-like effects of DMI combined with ZEM- given via oral gavage. This approach is based on previous successful studies with DMI administered via the drinking water (Singewald *et al.,*2004; Nava *et al.,*2015) or via i.p. (Lapiz *et al.,*2007; Simpson *et al.,*2012; Overstreet *et al.,*2010; Mokoena *et al.,*2015). While DMI was an effective antidepressant alone (Figures 3B and 7B), combining it with ZEM seems to abrogate this response. Indeed, we also observed significantly increased frontal cortical PDE4B levels in the ZEM-30+DMI group compared to SAL FSL rats, as well as in both ZEM-10+DMI-15 and ZEM-30+DMI-15 treated FSL rats versus DMI-15 and ZEM-30 alone treatment groups which may underlie a paradoxical worsening of depressive-like behaviour (Fig. 8B). Moreover, an earlier study showed that ZEM + escitalopram seems to *worsen* depressive-like behaviour, also in the FSL rat (Gericke, 2019).

Importantly, the authors noted significantly increased hippocampal 5-HT and NE levels (Gericke, 2019). Since the FSL rat presents with 5-HT_{1A} receptor hypersensitivity (Overstreet *et al.,*1998), and that ZEM bolsters 5-HT release via SERT inhibition, the ensuing hyper-serotonergic state has been known to worsen MDD or induce treatment resistance (Andrews *et al.,*2015). That the FSL rat also presents with increased cortico-hippocampal α_2 -adrenoceptor density (Lillethorup *et al.,*2015), while ZEM also significantly increases NE release (Gericke, 2019), may also have implications for abrogating an antidepressant response, for e.g. by increasing PDE4B expression (Fig. 10).

To introduce greater relevance to the behavioural data, regional brain monoamine changes (NE & 5-HT) (sub-chronic monotherapy study) used in ZEM dose selection for augmentation study are now considered. However, the full monoamines result, and discussions are presented in Chapter 4. A decrease in hippocampal and cortical monoamine levels has been associated with MDD (Treadway *et al.,*2015). The locus coeruleus releases NE, which modulates cognitive functions and working memory via innervation of the hippocampus-prefrontal cortical (hippo-PFC) loop (Lim *et al.,*2010). We observed significantly reduced hippocampal and cortical NE in DMI- (15 and 30 mg/kg/day) treated rats versus SAL FSL rats (Fig. 4A & B). These results are not in line with previous studies which reported significantly increased struggling and reduced immobility in FSL rats following sub-chronic treatment with DMI, which corroborate with significant increased hippocampal and cortical NE levels in FSL rats (Mokoena *et al.,*2015, Overstreet *et al.,*2010). We were expecting DMI to elevate hippocampal and cortical NE levels as the drug blocks reuptake of NE and enhances NA'ergic effects (Mokoena *et al.,*2015; Overstreet *et al.,*2010). Therefore, part of the monoamines results is obscure and failed to support the behavioural data (Fig. 3B &C). We observed significantly reduced hippocampal 5-HT levels (Fig. 5A) in ZEM-10 compared to SAL FSL rats. This may be related to partial inhibition of SERT by a lower dose of ZEM, as previous studies have shown that lower doses of SSRIs (ZEM) upregulate and activate of 5-HT_{1A} auto receptors (Yohn *et al.,*2017), or indirectly stimulate the pre- and post-synaptic 5-HT_{1A} and 5-$HT_{2A/C}$ receptors (Gericke *et al.,*2022) leading to negative feedback inhibition of 5-HT release (Yohn *et al.,*2017; Gericke *et al.,*2022). Most importantly, our data showed increased frontal cortical NE (Fig. 4B) but no change in hippocampal NE (Fig. 4A) concentration in ZEM-30 compared to SAL FSL rats. Although we have previously shown no such change in either brain region with ZEM (50 mg/kg/day) alone (Gericke, 2019), ZEM does present with some noradrenergic effects via NET and MAO-A inhibition, and/or upregulation of VMAT-2 (Coetzee *et al.,*2016). Of note is that standardised extracts of herbal plants (i.e., St. John's wort) have shown dose-dependent reversing of bio-behavioural abnormalities in rodents at higher doses (Bukhari and Dar, 2013), possibly due to cumulative effects after repeated dosing (Paulke *et al.,*2008). Although speculative, sub-chronic treatment with ZEM-10 or ZEM-30 could produce divergent results via this explanation.

Enhanced serotonergic activity correlates with increased swimming behaviour in the FST (Cryan *et al.,*2005). Increased frontal cortical 5-HT (Fig. 4B) following treatment with

ZEM-30 in FSL rats versus SAL FSL rats correlate with a large effect size increase in the swimming behaviour in the FST (Fig. 3D), thus confirming the SERT inhibitory effects of ZEM (Harvey *et al.,*2011). Due to SERT inhibition playing a profound role in antidepressant action (Quentin *et al.,*2018; Celada *et al.,*2004), the lack of an antidepressant-like effects for ZEM is of particular interest and may be related to testing in the FSL model.

PDE4B has been associated with the pathogenesis of MDD and anxiety disorders (Li *et al.,*2009). In this regard PDE4 inhibitors like rolipram have antidepressant effects in animal models (Dlaboga *et al.,*2006; Li *et al.,*2009) as well as in humans (Fujita *et al.,* 2012). Itoh and co-workers, (2004) reported significantly increased PDE4 isoenzyme in the hippocampus and frontal cortex in learned helpless animal model of depression. Neither DMI-15 nor ZEM-30 monotherapies affected hippocampal PDE4B levels (Fig. 8A). In fact, an earlier study showed no significant effects of DMI or rolipram on PDE4B (Dlaboga *et al.,*2006). To our knowledge this is the first study to evaluate the effects of DMI and ZEM as a previous study only evaluated the effect of the combination of rolipram and DMI (Fujimaki *et al.,*2000) or rolipram and imipramine (Itoh *et al.,*2004). However, and more importantly, both ZEM-10+DMI-15 and ZEM-30+DMI-15 significantly reduced hippocampal PDE4B levels compared to SAL FSL control and versus ZEM- (10 and 30 mg/kg/day) monotherapies (Fig. 8A). Both ZEM-10+DMI-15 and ZEM-30+DMI-15 showed no statistically significant effects in hippocampal PDE4B levels versus DMI-15 alone, despite a trend towards decreased hippocampal PDE4B levels in FSL rats treated with both combinations versus DMI-15 alone (Fig. 8A). These results are in agreement with a previous study in which DMI alone did not affect hippocampal PDE4B levels (Fujimaki *et al.,*2000). That hippocampal PDE4B levels were decreased by a combination of DMI and rolipram (Fujimaki *et al.,*2000) is important, as with our findings presented here, and may represent a regional specific response given that ZEM30+DMI significantly increased cortical PDE4B levels compared to SAL FSL rats, whereas both ZEM-10+DMI-15 and ZEM-30+DMI-15 significantly increased cortical PDE4B levels in FSL rats compared to the DMI-15 and ZEM-30 monotherapies. This paradoxical response requires further study.

The central monoaminergic system is a critical regulator of cAMP signalling and PDE4 activity through NA-mediated stimulation of postsynaptic β-adrenergic receptors (Lourenco *et al.,*2006). Inhibition of PDE4 and bolstering of cAMP-dependent sub-cellular

signalling is theorized to have beneficial psychopharmacological effects by modulating the same subcellular NA'ergic and 5-HT'ergic processes activated by SSRI, SNRI and TCA (Harvey,1997). In fact, previous early studies showed that chronic treatment with DMI significantly downregulates NET (Zhao *et al.,*2008) and desensitizes α_2-adrenergic receptors (Cottingham and Wang, 2012). This would increase hippocampal NE concentrations, stimulate G-protein coupled β-adrenoceptors to increase CREB signalling leading to hippocampal neurogenesis and neuroplastic changes, ultimately to initiate an antidepressant response (Li *et al.,*2017).

Both DMI and ZEM monotherapies did not affect frontal cortical PDE4B levels in FSL rats versus FSL saline control (Fig. 8B). Although DMI did not affect frontal cortical PDE4B levels in FSL rats versus both ZEM doses alone, ZEM- (30 mg/kg/day) significantly reduced frontal cortical PDE4B levels in FSL rats versus low dose of ZEM- (10 mg/kg/day) (Fig. 8B). This may be related to the NA'ergic effects of ZEM- (30 mg/kg/day) which also increased frontal cortical NE levels in FSL rats (Fig. 4B). As mentioned above, ZEM has noradrenergic effects, which in turn may stimulate the post-synaptic β-adrenoceptor-mediated cAMP signalling (Lourenco *et al.,*2006) and this have beneficial psychopharmacological effects. ZEM-30+DMI-15 combination significantly increased cortical PDE4B concentration compared to SAL FSL rats (Fig. 8B). Unexpectedly, both ZEM-10+DMI-15 and ZEM-30+DMI-15 combinations significantly increased frontal cortical PDE4B in FSL rats versus either DMI-15 and ZEM-30 monotherapies (Fig. 8B). Considering PDE4 inhibitors are antidepressant, this appears paradoxical (see above). However chronic antidepressants such as DMI (O'Donnell and Zhang, 2004; Takahashi *et al.,*1999) are known to elevate PDE4B expression in the brain (Cashman *et al.,*2009) and is considered a compensatory response to antidepressant treatment (Takahashi *et al.,*1999). In this regard, DMI has shown a regionally specific down-regulation of the β-adrenergic receptors and upregulation of PDE4A versus an SSRI (i.e., fluoxetine) and MAOI, which appears to be a compensatory mechanism for increased noradrenergic activity (Cashman *et al.,*2009). Indeed, Dlaboga and co-workers reported increased cortical PDE4B expression in mice following chronic treatment with different antidepressants, including DMI (Dlaboga *et al.,*2006). These effects mainly occurred due to increased cortical synaptic NE levels, which as noted earlier upregulates PDE4B via G protein-coupled –B-adrenoceptor cAMP signalling (Zhu *et al.,*2001). Indeed, ZEM-30 significantly increased cortical NE concentration in FSL rats versus SAL FSL control (Fig.

4B), where ZEM-mediated upregulation of VMAT-2 (Coetzee *et al.,*2016) would mechanistically support its ability to increase cortical NE concentrations (Fig. 4B).

In conclusion, we failed to observe any significant antidepressant-like actions for Zembrin®, either alone or in combination with DMI. These findings agree with an earlier sub-chronic treatment study (Gericke, 2019), but contradicts that noted in acute-treatment study designs using various preparations of *Sceletium tortuosum* (Schell, 2014; Gericke *et al.,*2022). We posit that these differences may be model-related. On this note, the FSL rat model presents with hypersensitive α_{2A} and 5-HT_{1A} receptors that may underlie these unexpected findings. However, evidence for reduced hippocampal (but not cortical) PDE4 levels does lend some support for the augmentation potential of Zembrin® as an add-on therapy with DMI, especially where specific actions on the hippocampal PDE4B are required. Although multiple target engagement for Zembrin® arguably contribute to its antidepressant-like effects, including its NA'ergic effects, the latter may also have a paradoxical effect by upregulating cortical PDE4B expression (Takahashi *et al.,*1999) and hence counter an antidepressant response, especially when combined with another noradrenergic drug like DMI. Again, this may vary according to the translational model used. Further studies are needed on the cortical PDE4B to gain a better understanding of the potential of Zembrin® as add-on therapy in the treatment of MDD and anxiety disorders.

Limitations include absence of the FRL PDE4B results in the current study to establish the baseline PDE4B results. Therefore, recommendations for this study include basal analysis of PDE4B levels in FRL versus FSL rats to better observe the presence and nature of PDE4B changes versus a reference control, further supporting the argument that PDE4B inhibition will ultimately produce antidepressant-like effects. Such data would also enlighten on PDE4B findings in the cortex. Somewhat disappointingly, the monoamine results were obscure and could not support the behavioural data and PDE4B expression due to variability in time analysis between the treatment groups. Future studies should explore different mechanism of actions of Zembrin®, perhaps investigating different alkaloids separately versus the whole extract.

7.0 Conflict of interest and funding

Prof. BH Harvey has acted as a scientific advisor to HG&H. HG&H (manufacturers of Zembrin®) provided Zembrin® but has no other role in this study. Dr M. Lekhooa received

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8.0 Author's contribution

Rasemoko P. Polile- wrote up the proposal for the study, prepared it for ethics approval, undertook the behavioural tests, collected the tissues, performed the statistical analysis of the study and wrote-up the first draft of the manuscript.

Prof. Brian H. Harvey- co-supervised the study, contributed, and advised on the overall design of the study, proof-read, and contributed to the final preparation of the manuscript.

Dr Stephanus F. Steyn- assisted with the statistical analysis, data interpretation, behavioural tests and proof-read the final manuscript.

Dr 'Makhotso Lekhooa- contributed to planning of the study, supervised the study, and obtained ethical approval; contributed to and advised on the write-up of the manuscript.

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References

Abildgaard, A., Solskov, L., Volke, V., Harvey, B.H., Lund, S., Wegener, G. 2011. A high-fat diet exacerbates depressive-like behaviour in the Flinders Sensitive Line (FSL) rat, a genetic model of depression. *Psychoneuropharmacology*, 36(5): 623-633

Altin, M., Harada, E., Schacht, A., Berggren, L., Walker, D., and Duenas, H. 2014. Does early improvement in anxiety symptoms in patients with major depressive disorder affect remission rates? A post-hoc analysis of pooled duloxetine clinical trials. *Open Journal of Depression*, 3(3):112-123

American Psychiatric Association. 2013. Diagnostic and Statistical Manual of Disorders 5th Edition (DSM-5). Washington, D.C. https://www.psychiatry.org/psychiatrists/practice/dsm Date of access: 05. May.2020

Andrews, P.W., Bharwani, A., Lee, K.R., Fox, M. Thomson Jr, J.A. 2015. Is serotonin an upper or downer? The evolution of serotonergic system and its role in depression and the antidepressant response. *Neuroscience and Biobehavioural Reviews*, 51:164-188

Bay-richter, C., Peterson, E., Liebenberg, N., Elfving, B., Wegener, G. 2019. Latent toxoplasmosis aggravates anxiety- and depressive-like behaviour and suggest a role of gene environment interactions in the behavioural response to the parasite. *Behavioural Brain Research*, 364(9425):133-139

Bennabi, D., Yrondi, A., Charpeaud, T., Genty, J.-B., Destouches, S., Lancrenon, S., Allaili, N., Bellivier, F., Bougeroi, T., Camus, V., Doumy, O., Dorey, J.-M., Haesebaert, F., Holtzmann, J., Lancon, C., Lefebvre, M., Moliere, F., Nieto, I., Rabu, C., Richieri, R., Schmitt, L., Stephan, F., Valva, G., Walter, M., Leboyer, M., El-Hage, W., Aouizerate, B., Haffen, E., Llorca, P.-M., Courtet, P. 2019. Clinical guidelines for management of depression with specific comorbid psychiatric conditions French recommendations from expert (the French Association for Biological Psychiatry and Neuropsychopharmacology and the foundation Fonda Mental). *BMC Psychiatry*, 19:50

Bogdanova, O. V, Kanekar, S., Renshaw, P. F. 2013. Factors influencing behavior in the forced swim test. *Physiology and Behavior*, 118: 227–239

Boku, S., Nakagawa, S., Toda, H. and Hishimoto, A. 2018. Neural basis of major depressive disorder; beyond monoamine hypothesis. *Psychiatric and Clinical Neurosciences*, 72(1):3-12

Brand, S.J., Harvey, B.H. 2017a. Exploring a Post-traumatic stress disorder paradigm in Flinders Sensitive Line rats to model treatment resistant depression I; bio-behavioural validation and response to imipramine. *Acta Neuropsychiatrica*, 29(4):193-206

Brand, S.J., Moller, M., Harvey, B.H. 2015. A review of biomarkers in mood and Psychotic of disorders: A dissection of clinical vs pre-correlates. *Current Neuropharmacology*, 13(3): 324-368

Brendler, T., Brinckmann, J.A., Feiter, U., Gericke, N., Lang, L., Pozharitskaya, O.N., Shikov, A.N., Smith, M., Van Wyk, B-E. 2021. Sceletium for managing anxiety, depression and cognitive impairment; A traditional herbal medicine in modern -day regulatory systems. *Current Neuropharmacology*,19 (9): 1394-1400

Bukhari, I.A., Dar, A. 2013. Behavioral profile of Hypericum perfiratum (St. John's Wort) extract. A comparison with standard antidepressants in animals' model of depression. *European review for Medical and Pharmacology Sciences*, 17(8): 1082-1090

Cashman, J.R., Voelker, T., Zhang, H.T., O' Donnell, J.M. 2009. Dual inhibitors of phosphodiesterase-4 and serotonin reuptake. *Journal of Medicinal Chemistry*, 52(6):1530-1539

Celada, P., Puig, V., Bosc, M.A., Adell, A., and Artiga, F. 2004. The therapeutic role of 5-HT_{1A} and 5-HT_{2A} receptors in depression. *Journal of Psychiatry and Neuroscience*, 29(4): 252-65

Coetzee, D.D., Lopez, V.V., Smith, C. 2016. High –Mesembrine Sceletium extract (Trimesemine^{TM}) is a monoamine releasing agent, rather than only a selective serotonin reuptake inhibitor. *Journal of Ethnopharmacology*, 177: 111-116

Cosgrove, K.T., Burrows, K., Avery, J.A., Kerr, K.L., DeVille, D.C., Aupperte, R.L., Teague, T.K., Drevets, W. C. 2020. Appetite change profile in depression exhibit differential relationship between systemic inflammation and activity in reward and interoceptive neurocircuitry. *Brain, Behavior, and Immunity*, 83: 163-171

Cottingham, C., Wang, Q. 2012. α_2-adrenergic receptor dysregulation in depressive disorders, implication for neurobiology of depression and antidepressant therapy. *Neuroscience and Bio-behavioural Review*, 36: 2214-2225

Cryan, J.F., Markou, A., Lucki, I. 2002. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends in Pharmacological Sciences,* 23(5):238-245

Cryan, J.F., Valentino, R.J., Lucki, I. 2005. Assessing substrates underlying the behavioural effects of antidepressants using the modified rat forced swimming test. *Neuroscience and Bio-behavioural Reviews*, 29(4-5): 547-569

Cumming, G. 2013. The new statistics; why and how. *Association for Psychological Science*, 25(1):7-29

Dietze, S., Lees, K.R., Fink, H., Brosda, J., Voigt, J.-P. 2016. Food deprivation, body weight loss and anxiety-related behavior in rats. *Animals,* 6(4):2-14

Dlaboga, D., Hasshussein, H., O' Donnell, J.M. 2006. Regulation of phosphodiesterase4 expression in mouse brain by repeated antidepressant treatment; comparison with Rolipram. *Brain Research*, 1096:104-112

Fujimaki, K., Morinobu, S., Duman, R.S. 2000. Administration of a cAMP phosphodiesterase 4 inhibitor enhances antidepressant-induction of BDNF mRNA in rat hippocampus.*Neuropsychopharmacology*, 22 (1):2-10

Furmaga, H., Shah, A., Frazer, A. 2011. Serotonergic and noradrenergic pathways are required for the anxiolytic-like and antidepressant –like behavioral effects of repeated vagal nerve stimulation in rats. *Biological Psychiatry*, 70:937-945

Gericke, J. 2019. Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, A.M., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:114550

Gillman, P.K. 2007. Tricyclic antidepressant pharmacology and therapeutic drug interaction updated; review. *British Journal of Pharmacology*, 151: 737-748

Grissom, R.J., Kim, J.J. 2012. Effects sizes for research; univariate and multivariate applications (2nd edition). New York. NY; Routledge

Haller, H., Anheyer, D., Cramer, H., Dobos, G. 2019. Complementary medicine for clinical depression; an overview of systematic reviews. *British Medical Journal Open*, 9(8):1-9

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P. 2011. Pharmacological actions of South African medicinal and functional plant *Sceletium tortuosum* and its principle alkaloids. *Journal of Ethnopharmacology*,137(3):1124-1129

Harvey, B.H. 1997. The neurobiology and pharmacology of depression. A comparative overview of serotonin selective antidepressants. *South Africa Medical Journal*, 87(4):540-552

Harvey, B.H., Reganass, W., Dryer, W., Moller, M. 2019. Social isolation rearing-induced anxiety and response to agomelatine in male and female rats: role of corticosterone, oxytocin, and vasopressin. *Journal of Psychopharmacology*, 33(5):640-46

Harvey, B.H., Slabbert, F.N. 2014. New insight on the antidepressant discontinuation syndrome. *Human Psychopharmacology; Clinical and Experimental*, 299(6):503-516

Hershey, J.D., Gifford, J.J., Zizza, L.J., Pavlenko, D.A., Wagner, G., Miller, S. 2018. Effects of various cleaning agent on the performance of mice in behavioural assays of anxiety. *Journal of the American Association for Laboratory Animal Sciences*, 57(4):335-339

Hogman, C. 2014. Explorative stratergies in the open field (OFT), elevated plus maze (EPM) and multivariate concentric square field (MCSF) in adolescent wistar rats, Uppsala University (MSc. dissertation)

Husum, H., van Kammen, D., Termeer, E., Bolwig, T.G., Mathe, A.A. 2003. Topiramate normalizes hippocampal NPY-Li in Flinders Sensitive Line depressed rats and upregulates NPY, galanin, and CRH-LI in the hypothalamus; implications for mood-stabilizing and weight loss- inducing effects. *Neuropsychopharmacology*, 28: 1292-1299

Itoh, T., Tokumura, M., Abe, K. 2004. Effects of Rolipram, a phosphodiesterase-4 inhibitor in combination with imipramine on depressive behaviour (CRE-binding activity and BDNF level) in learned helpless rats. *European Journal of Pharmacology*, 498 (1-3):135-142

Jansen van Vuren, E., Steyn, S.F., Brink, C.B., Moller, M., Viljoen, F.P., Harvey, B.H. 2021. The neuropsychiatric manifestation of COVID-19: Interactions with psychiatric illness and pharmacological treatment. *Biomedicine and Pharmacotherapy*, 135(111200):1-20

Jefsen, O., Hojgaard, K., Christiansen, S.L., Elfving, B., Nutt, D.J., Wegener, G., and Muller, H.K. 2019. Psilocybin lacks antidepressant-like effect in Flinders Sensitive Line rat. *Acta Neuropsychiatrica*, 31(4): 213-219

Jin, Y., Sun, L.H., Yang, W., Cui, R.J., Xu, S.B. 2019. The role of BDNF in the neuroimmune axis regulation of mood disorders. *Frontiers in Neurology*, 10 (515): 1-19

Kalshetti, P.B., Alluri, R., Thakurdesai, P. A. 2015. Antidepressant effect of standardized extract of Commiphora mukul Engl. In Olfactory bulbectomized rats. *Brazilian Archies of Biology and Technology*, 58(1): 41-48

Kang, J-A, Lee, K., Lee, K.M., Cho, S., Seo, J., Hur, E-M., *et al.* 2012. Desipramine Inhibits Histamine H1 Receptor-Induced Ca^{2+} Signaling in Rat Hypothalamic Cells. *PLoS ONE*. 7(4): e36185. https://doi.org/10.1371/journal.pone.0036185 Date of access: 25 Feb. 2022

Kessler, R.C., Angermeyer, M., Anthony, J.C., De Graaf, R.O.N., Demyttenaere, K., Gasquet, I., De Girolamo, G., Gluzman, S., Gureje, O.Y.E., Haro, J.M. and Kawakami, N. 2007. Lifetime prevalence and age-of-onset distributions of mental disorders in the World Health Organization's World Mental Health Survey Initiative. *World Psychiatry*, 6(3):168

Kim, Y. 2020. Anxiety disorders; rethinking and understanding recent discoveries. *Advances in Experimental Medicine and Biology*, 1191:219-247

Kozisek, M.E., Deupree, J.D. Burke, W.J., Bylund, D.B. 2007. Appropriate dosing regimens for treating juvenile rats with desipramine for neuropharmacological and behavioral studies. *Journal of Neuroscience Methods*, 163(1):83-91

Lakens, D. 2013. Calculating and reporting effect sizes to facilitate cumulative science; A practical primer for t-tests and ANOVAs. *Frontiers in Psychology*, 4 (863):2-39

Lapiz, S.M., Bondi, C.O., Morilak, D.A. 2007. Chronic treatment with desipramine improves cognitive performance of rats in an attention set-shifting test. *Neuropsychopharmacology*, 32:1000-1010

Lemke, T.L., Williams, D.A., Roches, F., Zito, W. 2012. Foye's principles of medicinal chemistry. 7th edition. Wolters Kluwer Health Adis/ Lippincott Williams & Wilkins

Li, H., Zuo, J., Tang, W. 2018. Phosphodiesterase4 inhibitor for treatment of inflammatory diseases. *Frontiers in Pharmacology*, 1048(9):1-21

Li, Y., Huang, Y., Amsdell, S.L., Xiao, L., O'Donnell, J.M., *et al.* 2009. Antidepressant- and anxiolytic-like effects of the phosphodiesterase- 4 (PDE4) inhibitor Rolipram on behaviour depend on cyclic AMP-response element binding protein (CREB)-mediated neurogenesis in the hippocampus. *Neuropsychopharmacology*, 34(11): 2404–2419

Liebenberg, N., Harvey, B.H., Brand, L., Wegener, G., Brink, C.B. 2012. Chronic treatment with the phosphodiesterase type 5 inhibitors sildenafil and tadalafil display anxiolytic effects in Flinders Sensitive Line rats. *Metabolic Brain Disease*, 27(3):337-340

Lillethorup, T.P., Iversen, P., Wegener, G., Doudet, D.J., Landau, A.M. 2015. Adrenoceptors binding in Flinders Sensitive Line compared with Flinders Resistance Line and Sprague Dawley rats. *ACTA Neuropsychiatrica*, 27(6):345-352

Lim, E.P., Tan, C.H., Jay, T.M., Dawe, G.S. 2010. Locus coeruleus stimulation and noradrenergic modulation of hippocampo-prefrontal cortex long-term potentiation. *International Journal of Neuropsychopharmacology*,13:1219-1231

Liu, W., Ge, T., Leng, Y., Pan, Z., Fan, J., Yang, W., Cui, R. 2017. The role of neural plasticity in depression from hippocampus to prefrontal cortex. *Neural Plasticity*, 6871089: 1-11:

Lourenco, C.M., Kenk, M., Beanlands, R.S., Da Silva, J.N. 2006. Increasing synaptic noradrenaline, serotonin and histamine enhances in vivo binding of phosphodiesterase-4 inhibitor rolipram in rat brain, liver and heart. *Life Sciences*, 79(4): 356-364

Ma, D., Rajakumaraswamy, N., Maze, M. 2005. α_2- Adrenoceptor agonists; shedding light on neuroprotection? *British Medical Bulletin*, 71:77-92

Maan, J.S, Rosani, A., Saadabadi, A. 2021. Desipramine. In: StatPearls [Internet updated on Sep 14]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–. PMID: 29262158

Miranda, M., Morici, J.F., Zanoni, M.B., Bekinschtein, P. 2019. Brain-derived neurotrophic factor; Akey molecular for memory in the Healthy and pathological brain. *Frontiers in Cellular Neuroscience*, 363 (13):1-25

Mokoena, M.L., Harvey, B.H., Viljoen, F., Ellis, S.M., Brink, C.B. 2015.Ozone exposure of Flinders Sensitive Li ne rats is a rodent translational model of neurobiological oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232(1):2921-2938.

Moller, M., Du Preez, J.L., Viljoen, F.P., Berk, M.M., Emsley, R., Harvey, B.H. 2013. Social isolation rearing induces mitochondrial, Immunological, neurochemical and behavioural deficits in rats and is reversed by clozapine or N-acetyl cysteine. *Brain, Behaviour and Immunity*, 30:156-167

Murbach, T.S, Hirka, G., Szakonyine, I.., Gericke, N., Endres, J.R. 2014. A toxicological safety assessment of a standardized extract of *Sceletium tortuosum* (Zembrin ®) in rats. *Food and Chemical Toxicology*, 74:190-199

Nahas, R., Sheikh, O. 2011. Complementary and alternative for the treatment of major depressive disorder; clinical review. *Canadian Family Physician*, 5:659-663

Nava, N., Treccani, G., Liebenberg, N., Chen, F., Popoli, M., Wegener, G., Nyengaard, J.R. 2015. Chronic desipramine prevent acute stress induced reorganization of medial prefrontal cortex architecture by blocking glutamate vesicles, accumulation and excitatory synapse increase. *International Journal of Neuropsychopharmacology*, 18(3):1-11

Neumann, I.D., Wegener, G., Homberg, J.R., Cohen, H., Slattery, D.A., Zohar, J., Olivier, J.D., Mathe, A.A. 2011. Animal models of depression and anxiety; what they tell us about human conditions. *Progress in Neuropsychopharmacology and Biological Psychiatry, 35:1357-1375*

O' Donnell, J.R., and Zhang, H.T. 2004. Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE). *Trends in Pharmacological Sciences*, 25(3):158-163

Oberholzer, I., Moller, M., Holland, B., Dean, O.M., Berk, M., Harvey, B.H. 2018. Garcinia of depression Linn displays antidepressant-like and pro-cognitive effects in a genetic animal model of depression; a bio-behavioural study in the Flinders Sensitive Line rat. *Metabolic Disease,* 33(2): 467-480

Olatunji, T.L., Siebert, F., Adetunji, A., E., Harvey, B.H., Gericke, J., Hamman, J.H., Van der Kooy, F. 2021. *Sceletium tortuosum*; A review on its phytochemistry, pharmacokinetics, biological and clinical activities. *Journal of Ethnopharmacology*, 280(114476):1-15

Onyeaka, H., Anumudu, C.K., Al-Sharify, Z., Egele-Godswill, E., Mbaegbu, P. 2021. COVID-19 pandemic; A review of the global lockdown and its far-reaching effects. *Science Progress*, 104(2):1-18

Overstreet, D.H., Daws, L.C., Schiller, G.D., Orbach, J., Janowsky, D.S.1998. Cholinergic/serotonergic interactions in hypothermian, implications for rat models of depression. *Pharmacology, Biochemistry and Behavior*, 59(4):777-785

Overstreet, D.H., Friedman, E., Mathe, A.A., Yadid, G. 2005. The Flinders Sensitive Line rat; A selectively bred putative animal model of depression. *Neuroscience and Bio-behavioural reviews*, 29(4-5):739-759

Overstreet, D.H., Naimoli, N.M., Griebel, G. 2010.Saredutant, an NK2 receptor antagonist, has both antidepressant-like effects and synergizes with desipramine in an animal model of depression. *Pharmacology, Biochemistry and Behaviour*, 96(2): 206-210

Overstreet, D.H., Wegener, G. 2013. The Flinders Sensitive Line rat model of depression -25 years and still producing. *Pharmacology Review,* 65(1): 143-155

Palaniyappan, L., Insole, L., Ferrier, N. 2009. Combining antidepressants; a review of evidence. *Advance Psychiatric Treatment*, 15(2): 90-99

Paulke, A., Noldner, M., Schubert-Zsilavecz, M., Wurglics, M. 2008. St. John's wort flavonoids and their metabolites show antidepressant activity and accumulate in brain after multiple oral doses. *Article in Pharmazie*, 63(4): 296-302

Pilkington, K., Rampes, H., Richardson, J. 2006. Complementary medicines for depression. *Expert Review of Neurotherapeutics*, 6(11):1741-51

Porsolt RD, Bertin A, Jalfre M. 1978. Behavioural despair in rats and mice: strain differences and the effects of imipramine. *European Journal of Pharmacology*, 51(3):291–294

Quentin, E., Belmer, A., Maroteus, L. 2018. Somato-dentritic regulation of raphe serotonin neurons; a key to antidepressant action. *Frontiers in Neuroscience*, 12 (982): 1-11

Rajkumar, R.P. 2020. COVID-19 and Mental health; A review of the existing literature. *Asian Journal of Psychiatry*, 52.102066

Saayman L.J.B. 2019. Long lasting bio-behavioural effects of early life sildenafil administration in stress sensitive versus healthy control rats. North West University (South Africa), Potchefstroom campus (MSc dissertation)

Sarris, J., Marx, W., Ashton, M.M., Ng, C.H., Psych, M., Galvao-coelho, N., Ayati, Z., Zhang, Z., Kasper, S., Ravindra, A., Harvey, B.H. 2021. Plant-based medicines (Phytoceuticals) in the treatment of psychiatric disorders; a meta-review of meta-analyses of randomized controlled trials. *The Canadian Journal of Psychiatry*, 66(10):1-14

Schell, R. 2014. Sceletium tortuosum and mesembrine; A potential Alternative Treatment for Depression, (Claremont Colleges Scripps Senior Dissertation)

Shah, A., Frazer, A. 2014. Influence of acute or chronic administration of ovarian hormone on the effects of desipramine in forced swim test in female rats. *Psychopharmacology*, 231(18): 3685-3694

Simmons, W.K., Burrows, K., Avery, J.A., Kerr, K.L., Taylor, A., Bodurka, J., Drevets, W.C. 2018. Appetite changes reveal depression subgroups with distinct endocrine, metabolic, and immune states. *Molecular Psyschiatry*, 25(7):1457-1468

Simon, G.M., Niphakis, M.J., Cravatt, B.F. 2013. Determining target engagement in living systems. *Nature Chemical Biology*, 9(4): 200-205

Simpson, J. Ryan, C., Curley, A., Mulcaire, J., Kelly, J.P. 2012. Sex differences in baseline and drug-induced behavioural responses in classical behavioural tests. *Progress in Neuropsychopharmacology Biological Psychiatry*, 37(2):227-236

Singewald, N., Sinner, C., Hetzenauer, A., Sartori, S.B., Murck, H. 2004. Magnesium-deficient diet alters depression and anxiety–related behaviour in mice- influence of desipramine and *Hypericum perforatum* extract. *Neuropharmacology*, 47(8):1189-1197

Slattery, D.A., Cryan, J.F. 2012. Using the forced swim test to assess the antidepressant like activity in rodents. *Nature Protocols*, 7(6):1009-1014

Smith, C. 2011. The effects of Sceletium tortuosum in an *In vivo* model of psychological stress. *Journal of Ethnopharmacology*, 133(1):31-36.

Steyn, S.F. 2011. The effects of early-life exposure stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood. North-West University (South Africa), Potchefstroom Campus (M.Sc. dissertation)

Steyn, S.F., Harvey, B.H., Brink, C.B. 2020. Pre-pubertal, low-intensity exercise does not require concomitant venlafaxine to induce robust, late-life antidepressant effects in Flinders sensitive line rats. *European Journal of Neuroscience*, 00:1-16

Takahashi, M., Terwilliger, R., Lane, C., Mezes, P.S., Conti, M., Duman, S. 1999. Chronic antidepressant administration increases the expression of cAMP –Specific phosphodiesterase 4A and 4B isoforms. *The Journal of Neusroscience*,19(2): 610-618

Tillmann, S., Abildgaard, A., Winther, G., Wegener, G. 2019. Altered fecal microbiota composition in Flinders Sensitive Line rat model of depression. *Psychopharmacology*, 236(5):1445-1457

Treadway, M.T., Waskom, M.L., Dillon, D.G, Holmes, A.J., Park, M.T.M., Chakravarty, M.M., Dutra. S.J., Polli, F.E., Losifescu, D.V., Fava, M., Gabrieli, J.D.E., Pizzagalli, D.A. 2015. Illness progression, recent stress and morphometry of hippocampal subfield and medial prefrontal cortex in major depression. *Society of Biological Psychiatry,* 77(3): 285-294

Viljoen, F.P., du Preez, J.L., Wessels, J.C., Aucamp, M.E. 2018. HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples. *An International Journal of Pharmaceutical Sciences*, 73(10) 563–569

Wang, C., Wang, Z., Li, M., Li, C., Yu, H., Zhou, D., Chen, Z. 2017, 'Reducing Neuroinflammation in Psychiatric Disorders: Novel Target of Phosphodiesterase 4 (PDE4) and Developing of the PDE4 Inhibitors', in G. E. A. Abreu (ed.), Mechanisms of Neuroinflammation, *IntechOpen*, London. 10.5772/intechopen.69154. http://www.intechopen.com/books/mechanisms-ofneuroinflammation. Date of access: 06. Jun.2021

Wegener, G., Finger, B.C., Elfving, B., Keller, K., Lienbenberg, N., Fischer, C.W., Singewald, N., Slattery, D.A., Neumann, I.D., Mathe, A.A. 2012. Neuropeptide S-alters anxiety, but not depression-like behaviour in Flinders Sensitive Line rats; A genetic animal model of depression. *International Journal of Neuropsychopharmacology*, 15:375-387

World Health Organization (WHO). 2017. Depression and other common mental disorders: Global Health Estimates. Geneva. https://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2 Date of access: 05. May.2020

World Health Organization (WHO). 2018. Traditional and complementary medicines in primary health care. https://apps.who.int/iris/rest/bitstreams/1241145/retrieve Date of access: 02 Dec. 2021

Wu, T., Xiaoqian, J., Huifeng, S., Jieqion, N., Yin, J. X., Wang, X. 2021. Prevalence of mental health problems during the COVID-19 pandemic; A systematic review and metal analysis. *Journal of Affective Disorders*, 281:91-98

Yohn, C.N., Gergues, M.M., Samuels, B.A. 2017. The role of 5-HT receptors in depression. *Molecular Brain*, 10 (28): 2-12

Zhang, H.T., Huang, Y., Masood, A., Stolinski, L.R., Li, Y., Zhang, L., Dlaboga, D., Jin, C., Conti, M., O'Donnell, J.M. 2008. Anxiogenic-like behavioural phenotype of mice deficient in phosphodiesterase 4B (PDE4B). *Neuropsychopharmacology*, 33 (7):1611-1623.

Zhou, X.J., Liu, M., Yan, J. J., Cao, Y., Liu, P. 2012. Antidepressant-like effects of the extracted of the Kai Xin San, a traditional Chinese herbal prescription is explained by modulation of the central monoaminergic neurotransmitter system in the mouse. *Journal of Ethnopharmacology*, 139(2):422-428

Zhu., J., Mix., E., Winblad., B. 2001. The antidepressant and anti-inflammatory effects of Rolipram in the central nervous drug review.*CNS Drugs Review*, 7(4):387-398

CHAPTER 4

SUMMARY OF THE RESULTS, CONCLUSION, LIMITATIONS, AND RECOMMENDATIONS

4.1. Summary of the results

This chapter provides a summary of the results as presented in chapter 3 (manuscript), as well as the various addenda, as compared to the expected outcomes. The purpose of this chapter is to evaluate if the primary objectives of the current study have been accomplished and to formulate a general conclusion for the study. However, it is also necessary to identify certain constraints experienced during the study and in so doing to formulate recommendations for future studies.

Primarily, the present study was aimed at assessing the dose-related antidepressant-like effects of Zembrin® alone and in combination with DMI in a genetic rodent model of MDD, the FSL rat, following 14 days treatment, as well as the potential of either Zembrin® or combination therapy with DMI to reverse abnormalities in selected behavioural and neurobiochemical markers (i.e., monoamines, PDE4B activity) associated with MDD. In the end, we hope to provide a better understanding of the mode of action of Zembrin® as it relates to the neurobiology of MDD, as summarised in table 4.1

Key findings in the current study (*Table 4.2 below*)

- FSL rats displayed depressive-like behaviour in the FST (increased immobility, decreased swimming and struggling) compared FRL control rats
- DMI was effective as an antidepressant in the FST, significantly reversing immobility and struggling in FSL rats versus FSL saline treated rats
- Point 1 and 2 together confirm the face and predictive validity of the FSL rat model for use in this study
- Monotherapy treatment with 3-tier doses of Zembrin® (10, 25 and 30 mg/kg/day) failed to reverse depressive-like behaviour in the FST
- ZEM-30 induced a large effect size increase in cortical NE and 5-HT
- ZEM-10 and 30 were identified as the low and high doses of Zembrin®, respectively, and selected for the augmentation study with DMI-15

- Neither ZEM-10+DMI-15 (mg/kg/day) nor ZEM-30+DMI-15 (mg/kg/day) combination was effective to reverse depressive-like behaviour in the FST compared to FSL saline control or DMI-15 monotherapy
- Both the above combinations significantly reduced immobility in the FST compared to ZEM-(10 & 30) monotherapies, although neither ZEM-10+DMI-15 nor ZEM-30+DMI-15 augments response to low dose DMI (15 mg/kg/day
- Monotherapy treatment with either DMI-15 or 2-tier doses of ZEM (10 and 30 mg/kg/day) did not affect hippocampal and cortical PDE4B levels
- ZEM-10+DMI-15 (mg/kg/day) or ZEM-30+DMI-15 (mg/kg/day) combination therapies significantly reduced hippocampal PDE4B levels versus FSL saline rats
- ZEM-30+DMI-15 combination significantly increased cortical PDE4B levels versus FSL saline control
- ZEM-10+DMI-15 or ZEM-30+DMI-15 combination significantly increased cortical PDE4B levels versus DMI- 15 alone versus ZEM-30 alone

4.2 Discussion and conclusion

Although the aim of the current study was to assess antidepressant- and anxiolytic-like effects of ZEM and DMI in FSL rats, the FSL rats did not present with anxiety-related manifestations versus FRL rats. Therefore, any conclusions regarding the possible anti-anxiety-like effects of any of the treatments is not possible or relevant, hence anxiety-like behaviour data was not included in chapter 3 and in table 4.2.

Regarding the monoamine results, the findings were obscure as in most of the samples, the monoamines were not detected despite the antidepressant-like effect in the FST and their significant effects on the PDE4B levels. Therefore, the monoamines data presented under chapter 3 were used to support ZEM- dose selection. There may be a need to re-analyse the monoamines to observe if time factor does contribute to such results.

To conclude, FSL rats showed distinct depressive- like characteristics versus FRL controls, with the depressive-like behaviours evident in FSL rats reversed by DMI. However, no dose of Zembrin® tested in this study, either alone or in combination with DMI, was effective to reverse these behaviours. ZEM has potential effect on the monoamines possible elevating monoamines (NE & 5-HT) in specific brain region not in all brain regions. Since elevated PDE4 has been linked to MDD, reduced hippocampal PDE4 expression in ZEM-30+DMI-15 and ZEM-30+-DMI-15 groups vs ZEM (10 & 30)

monotherapies lends some support for the augmentation potential of Zembrin® as an add-on therapy, especially where specific actions on hippocampal PDE4B are required. Increased cortical PDE4 expression in the ZEM-10+DMI-15 or ZEM-30+DMI-15 group versus ZEM-30 (mg/kg/day) monotherapy seems paradoxical. Although, multiple target engagement for Zembrin® may have contributed to its antidepressant-like effects, its NA'ergic effects may also upregulate cortical PDE4B expression, especially when combined with drugs that can elevate synaptic concentration of NE - i.e., DMI.

Finally, regarding the augmentation study, our results suggest that ZEM+DMI fails to augment the antidepressant action of DMI but does augment the action of ZEM as compared to ZEM- (10 and 30 mg/kg/day) monotherapies. Overall, DMI is a significantly better antidepressant than ZEM, at least in this study.

TABLE 4.1: The study objectives (as outlined in chapter 1), the expected outcomes achieved or not achieved, and conclusion based on the observations. Abbreviations: FSL-Flinders Sensitive Line; FRL- Flinders Resistant Line; SAL-Saline; ZEM- Zembrin®; DMI-desipramine; OFT-Open Filed Test; FST-Forced Swim Test; EPM- Elevated Plus Maze; NE-Norepinephrine; 5-HT-Serotonin; &- AND, PDE4B-Phosphodiesterase -4B

Phase	Objectives	Expected outcomes	Results	Conclusions
FRL- SAL vs. FSL-SAL (**1**)	Confirmation of FSL rat model	FSL rats will display a depressive- (increased immobility, reduced struggling & swimming) & anxiety-like behaviour (OFT & EPM) versus FRL rats FSL will display increased locomotor activity vs FRL FSL will show reduced brain regions monoamines (NE, 5-HT & DA) & increased PDE4B levels vs. FRL	FSL show depressive-like behaviour in FST vs. FRL FSL fail to show anxiety-like behaviour vs. FRL FSL rats showed increased locomotor activity vs. FRL	FSL rat model is pure model of depression
Sub-chronic monotherapy study **(2)**	To compare the dose-related efficacy of	ZEM will dose-dependently reverse depressive- and anxiety-like behaviour in FSL rat's, elevate monoamines (5-	ZEM showed no antidepressant-like effect FSL rats in the FST vs. FRL. ZEM-30 significantly elevate cortical NE & 5-HT. ZEM-10 & ZEM-30 had not affected PDE4B levels alone vs FSL rats.	ZEM has no antidepressant-like and anxiolytic-like effects in

	monotherapy vs. FSL SAL to select two ZEM doses for augmentation study	HT & NE) and reduce PDE4B levels in FSL vs FSL SAL DMI will dose-dependently reverse depressive- and anxiety-like behaviour in FSL rat's vs FSL SAL Will be able to select two ZEM doses for augmentation study	DMI reverse depressive-like behaviour in FSL rats vs. FSL SAL, with DMI-15 being safe Monoamine's data was not supportive of the behavioural data in the FST.	FSL rat following sub-chronic treatment Desipramine has antidepressant-like effect in FSL rats, but has no anxiolytic-like effect in FSL rats following sub-chronic treatment
Augmentation study **(3)**	To assess if ZEM-10 or ZEM-30 can bolster low dose DMI versus monotherapies	ZEM+DMI will dose-dependently reverse depressive-and anxiety-like behaviours vs. monotherapies	ZEM-10+DMI-15 & ZEM-30+DMI-15 fail to reverse depressive-like behaviour in FSL rats vs. either FSL SAL or DMI-15 ZEM-10+DMI-15 & ZEM-30+DMI-15 reversed depressive-like behaviour vs. ZEM- (10 &30 monotherapies ZEM-10 +DMI-15 &ZEM-30+DMI-15 reduced hippocampal PDE4B vs. ZEM (10 &30) possible augmentation on ZEM	Combination of ZEM+DMI has no antidepressant-like effect in FSL rats, despite a potential for reducing hippocampal PDE4B expression

TABLE 4.2: Summary of results showing the expected outcomes and the findings as presented in chapter 3 (excluding the body weight, anxiety-like and monoamine data for augmentation study results). Abbreviations: FC-Frontal cortex; HC-Hippocampus; E-Expected outcome; F-Findings; ↑-increase; ↓-decrease; ↔-no difference; *-statistical significance, -_Below limit of detection/ not assessed

Phase	**Treatment groups**	**Immobility (depressive-like behaviour**		**Struggling**		**Swimming**		**Locomotion**		**NE**		**5-HT**		**PDE4-B**	
		E	**F**	**E**	**F**	**E**	**F**	**E**	**F**	**FC**	**HC**	**FC**	**HC**	**FC**	**HC**
Confirmation of FSL rat model **(1)**	FRL- SAL vs. FSL-SAL	↑	↑ ****	↓	↓ *	↓	↓ ****	↓	↑ **					-	-
Monotherapy study **(2)**	ZEM-10mg/kg/day	↓	↔	↑	↔	↑	↔	↔	↓	↔	↔	↔	↓ *	↔	↔
	ZEM-25mg/kg/day	↓	↔	↑	↔	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔
	ZEM-30mg/kg/day	↓	↔	↑	↔	↑	↔	↔	↔	↑ **	↔	↑ **	↔	↔	↔
	DMI-15mg/kg/day	↓	↓ **	↑	↑	↓	↓ ****	↔	↔	↔	↓ **	-	-	↔	↔
	DMI-30mg/kg/day	↓	↔	↑	↑	↓	↓ ****	↔	↓	↓ *	↓ **	-	-	-	-
Augmentation study **(3)**	ZEM-10 +DMI-15 (mg/kg/day)	↓	↔	↑	↔	↑	↔	↔	↓					↔	↓ ****
	ZEM-30 +DMI-15 (mg/kg/day)	↓	↔	↑	↔	↑	↔	↔	↔					↑ *	↓ ****

4.3. Limitation during the study

A few limitations to the study can be noted, as outlined below.

- Due to cost, baseline PDE4B levels for FRL and FSL control were not analysed, which could have provided a better observation of the presence and nature of PDE4B changes versus a reference control to support the argument that PDE4B inhibition will ultimately produce antidepressant-like effects. The baseline levels will be done before the publication to observe the presence and nature of PDE4B changes versus control rats.
- Quantification of monoamines were performed at two separate times during the study. This was done to select an appropriate dose for the sub-chronic augmentation study following failure of ZEM to reverse depressive-like behaviour in FSL in the FST. This compromised the monoamine data for the augmentation study, such that comparison of DMI- alone groups and combination therapies to FSL control group were not possible, therefore, the monoamines data does not correlates with the behavioural data. Most samples, the monoamines were below limit of detection.
- BDNF and pro-inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines were not analysed in this study (due to cost and time).
- The study used ZEM- extract and did not include isolated alkaloids of the extract (due to cost and time constraints). It would be relevant to compare the effects of isolated alkaloids with the crude extract in a similar study design
- While the FSL model has overall good validity for MDD, certain underlying biological anomalies with this model, such as α_{2A} and 5-HT_{1A} receptors hypersensitivity (Gericke *et al.,*2022; Lillethorup *et al.,*2015), could have resulted in some of the unexpected data presented, especially antidepressant-like effects of Zembrin® alone and in combination with desipramine.
- Use of only male rats in the study

4.4. Recommendations for future studies

- Brain tissue for the FRL and FSL rats have been fixed and should be used for later analysis of PDE4B levels, so to establish a baseline reference, and for correlating with behavioural data
- For the futures studies, the quantification of monoamines (NE,5-HT, and DA) should be conducted at the same time and analysed concurrently to avoid time variation between sample analysis.
- Plasma and brain tissue are available for analysis of other neurochemistry markers, such as pro-inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines and BDNF (i.e., for neuroplasticity). This will establish whether ZEM or combination therapies modulate neuro-inflammation and enhanced neuroplasticity processes considered beneficial in treatment of MDD.
- The above-mentioned biomarkers will provide a better understanding of the interaction of PDE4B with down-stream CAMP/CREB signalling, and whether these mechanisms underpin the antidepressant effects of ZEM.
- Biological processes, such as mitochondrial dysfunction, oxidative stress, neuroinflammation, disturbed circadian rhythm etc should be investigated in future studies
- Future studies should compare the effects of isolated alkaloids of ZEM and the extract, such as mesembrine and mesembrenone. Indeed, the latter are known to exert different pharmacodynamics and pharmacokinetics profiles, and which may account for differences in overall efficacy compared to the extract. Such work would be imperative to evaluate the individual effects of the different constituents on PDE4B versus that of the extract.
- The future studies should consider the use of another model of depression i.e., application of chronic mild stress in SD rats, to determine if indeed, the results are model related.
- The future studies should also include the female rats in the study

ADDENDUM A
ADDITIONAL METHODOLOGY

This addendum provides the background of the material and methods and additional information not included in chapter 2 and 3 together with the relevant references used in this addendum.

A. 1. Animals

As already elaborated in chapter 3 (*section 2.1.1*), this study employed a genetic model of MDD, Flinders Sensitive Line (FSL) rats and their normal control rats, Flinders Resistant Line (FRL) rats to assess the depressive-like and anxiety-like behaviours.

A. 1.1. The FSL rat as a genetic animal model of depression

The Flinders Sensitive Line rats (FSL) rats were developed in Australia at Flinders University and proposed as a genetic animal model of depression (Overstreet and Wegener,2013). The FSL rat has been widely used for behavioural research on MDD and for determining the therapeutic properties of novel antidepressants (Overstreet and Wegener, 2013). FSL rats are selectively bred Sprague-Dawley strain rats that present with the typical behavioural attributes of MDD (Overstreet *et al.,*2005; Overstreet and Wegener, 2013). Over the years reports from multiple laboratories across the globe including our laboratory further support validation of the FSL rat line as a genetic animal model of depression (Gericke *et al.,*2022; Saayman *et al.,*2021; Steyn *et al.,*2020) as the model has subsequently been found to present with three distinct characteristics (face, construct, and predictive validity) that align with MDD (Overstreet and Wegener, 2013) (see Chapter 1 and 3 for more elaboration).

A. 1.2 Limiting the study to males FSL and FRL rats only

Although the prevalence of MDD is higher in males than females (Kessler *et al.,*2007), this study was limited to include only male's rats. Converging line of evidence demonstrated a robust sex difference in animal model of MDD which may affect behavioural indices, depressive-like behaviours, neurobiological biomarkers, and response to treatment (Krokras and Dalla, 2014; Steyn, 2011; Autry *et al.,*2009; Becker *et al.,*2004). Previous studies revealed that hormonal cycles of female rats mainly oestrus cycle affects depressive-like behaviour and treatment complicating the interpretation of data from behavioural studies (Autry *et al.,*2009; Becker *et al.,*2004) and female's rats should be included in studies to examine whether gender differences can determine

vulnerability to depressive-like behaviour and treatment (Krokras and Dalla, 2014). Therefore, it was imperative to exclude the females' rats in this study so that the data obtain were proportionate and interpreted without complications.

A. 2. Background and methods for the behavioural tests

The animals were subjected to some behavioural tests to explore anxiety-like behaviour, measure locomotor activity and depression-like behaviour, starting with the least stressful to most stressful nevertheless, the Elevated Plus Maze (EPM) tests was performed after the FST to benefit from the bolstering action of the Forced Swim Test (FST) (Bay-Richter *et al.,*2019) (as discussed in chapter 3, *section 2.2*). All the tests were conducted according to previously described methods in our laboratories (Mokoena *et al.,*2015; Brand and Harvey, 2017; Oberholzer *et al.,*2018; Gericke., 2019) as approved by the NWU- Animal Care Research ethics committee. All the behavioural tests were conducted during the rat's dark cycle and the behavioural tests were separated by acclimatization period of 30 min to minimize stress caused during movement of home cages to behavioural room.

A. 2.1. Open Field Test (OFT)

The Open field test (OFT) was developed in 1932 by Calvin S. Hall to assess the general locomotor activity, anxiety, and explorative behaviours in rats (Hall and Ballachey, 1932). Originally, Hall assessed behaviour in open field arena in rats (Hall, 1932). However, with rising critics, different laboratories considered different paradigm and validated the methods to suit their laboratory conditions (Gould *et al.,*2009; Stanford *et al.,*2007). In this study, the OFT was conducted according to previously described method for our laboratory (Oberholzer *et al.,*2018; Gericke, 2019; Saayman, 2019). The apparatus consists of an empty black square open field arena (1 m^2) (*see Fig. A.1. below*) surrounded by high walls (45 cm) to prevent animals escape, with lamps mounted on the ceiling (red-light 80lx) to allow rats to explore the surrounding and video cameras mounted above the apparatus to record the behavioural parameters. The rats were placed individually in the centre of the open field arena and allowed to explore the box for 6 min, whereof 5 min behavior of the rats during OFT was recorded. The arena was cleaned with 10%v/v of alcohol each time before a rat is placed to minimize the cues present in the apparatus from previous animals which may alter the behaviours of the next subject (Hershey *et al.,*2018).

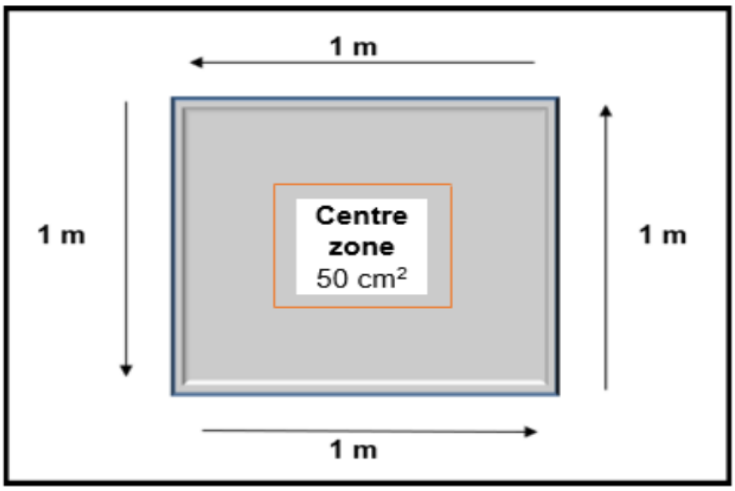


Fig A.1. Illustration of the OFT apparatus as implemented in this study (adapted from Saayman, 2019)

The behavioural parameters were scored using EthoVision© XT 14 software (Noldus Information Technology, Wageningen, NLD). The behavioural parameters that were measured during the OFT, the total distance covered during the specified time and the time spent in the central zone in this study correlates with general locomotor activity (Oberholzer *et al.,*2018; Gericke, 2019) and anxiety-like behaviour (Saayman, 2019) from previous studies (Oberholzer *et al.,*2018; Gericke, 2019). After the test, rats were return to their home cages and left undisturbed for 30 min after which they were exposed to the FST.

A. 2.2. Forced Swim Test (FST)

In 1977, Porsolt laboratory developed the forced swim test (FST) in mice which was later adapted to assess depressogenic–like behaviour in rats and screen for potential antidepressant in 1978 (Porsolt *et al.,*1977;1978). With recent ongoing research studies (Cryan *et al.,*2002; 2005) the paradigm was modified later to differentiate between serotonergic and noradrenergic antidepressants with the modified version commonly employed in recent depressive-like behaviour studies (Slattery and Cryan, 2012). The FST is primarily based on the observations that the rats will adopt a characteristic immobile from of mind when forced to swim from the cylinders where they cannot escape and such posture can be reduced by various antidepressants (Porsolt *et al.,*1978).

Although the FST has been considered as a robust and well validated measure of depressive-like behaviour and standard paradigm to screen the antidepressant drugs (Steyn, 2011), this paradigm is mostly affected by numerous factors including

environmental conditions (light, noise), biological factors (age, gender, strain, weight) and test design set up (equipment's) (Bogdanova *et al.,*2013). In this study, the FST was conducted according to previously described method for our laboratories (Brand and Harvey, 2017; Gericke, 2019).

The apparatus consists of four Perspex® cylinders (diameter 20cm, height 60cm) positioned next to each other (Gericke, 2019). In the day of the test, the cylinders were filled with water up to the height 30cm and the water temperature maintained at 25°C ± 1°C (Gericke, 2019) . On the day 13 of the treatment (at PND-62), 30 min after the OFT, the animals were individually placed in the cylinders and allowed to swim for 7 min, with all the cylinders thoroughly rinsed with water between the sessions to ensures that each rat swim in its own clean water, and behaviour despair was recorded with video cameras (*Fig. A.2*).

During the time of the test, the rats were monitored careful through the glass window to intervene in case the rats could drawn.

After the test, the animals were returned to their home cages and monitored according to the Vivarium animal monitoring sheet one hour after the swim as well as in the morning.

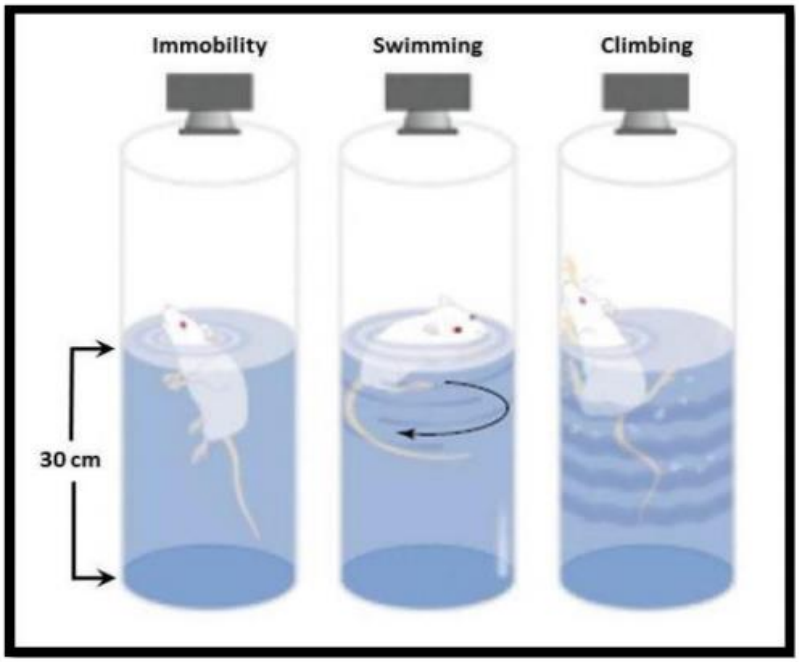


Fig. A. 2. Illustration of rat's behaviour in forced swim test apparatus. Parameters measured include immobility, swimming and struggling as adapted from Cryan *et al.,*2002.

The first and the last minute were excluded with the remaining 5 min behaviour scored with manual FST scoreboard 2.0 software; Academic Support services: Information Technology in Education, NWU, RSA as previously described (Steyn *et al.,*2020). The behaviour parameters were coded as immobility (when rats only make necessary movement to keep their heads above water), struggling/climbing (upward-directed movement of the forepaws along the side of the cylinder) and swimming (horizontal movement throughout the swim cylinder plus crossing to another quadrant) (Cryan *et al.,*2002; Mokoena *et al.,*2015).

A. 2.3. Elevated Plus Maze (EPM)

The elevated plus maze (EPM) was developed in 1984 by Handley and Mithani (Handley and Mithani, 1984), still considered as a robust, reliable, gold standard test used in rats to study anxiety-like behaviour and to assess the anxiolytic effects of various drugs (Hogman, 2014). Interestingly, the test was developed to study the involvement of the noradrenergic system in anxiety (Handly and Mithani, 1984), and further validated with various anxiogenic and anxiolytic drugs such as benzodiazepines, stimulants, neuroleptics, antidepressants and yohimbe (Pellow *et al.,*1985). Currently, the EPM is one of the widely used test to assess anxiety with numerous published studies (Carobrez *et al.,*2010). The standard paradigm consists of "+ plus –shaped maze with two open arms and two closed and elevated above the ground (Steyn, 2011; Regenass *et al.,*2018) as illustrated in *Fig.A-3.*

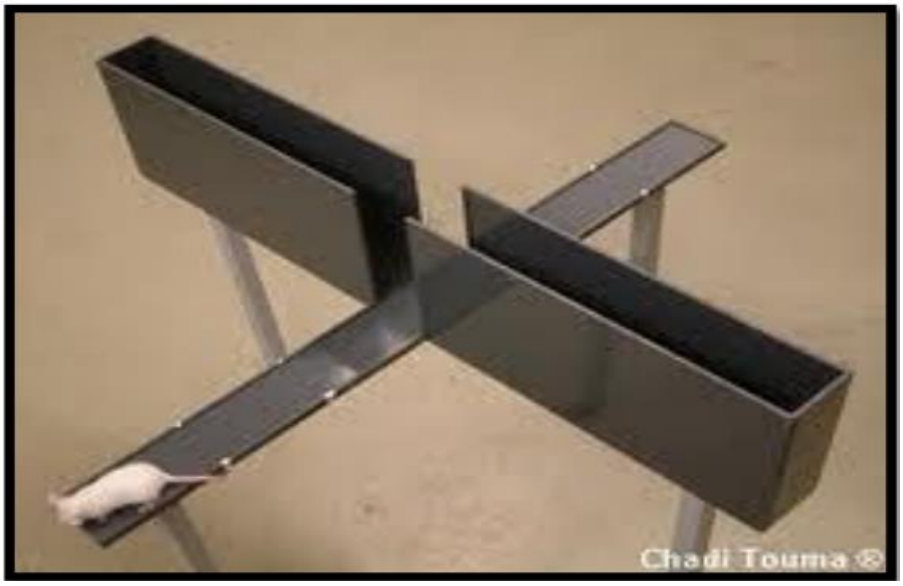


Fig A.3. Elevated plus maze with two opens arms two closed arms, elevated above the ground. (Adapted from Steyn, 2011)

Although EPM is one of the widely gold standard tests of anxiety-like behaviour (Hogman,2014) and a well validated paradigm (Pellow *et al.,*1985). Carobrez and Bertoglio (2005) revealed that several factors including animal strain, gender, age, housing condition and apparatus construction have influenced the behavioural results observed in EPM leading to intra-laboratory variability in the results (Carobrez and Bertoglio, 2005). Therefore, in this study, we conducted the EPM according to previously described methods for our laboratory (Steyn, 2011; Regenass *et al.,*2018). As mentioned in chapter 3 (*section 2.2*) that the FSL do not generally exhibit anxiety unless being exposed to a prior mild stress (Neumann *et al.,*2011; Wegener *et al.,*2012), the FST test its own is a stressor and can be conducted to induce anxiety-like behaviour (Yahav *et al.,*2015), Therefore, the EPM was performed at the day 14 of the treatment, a day after the FST to benefit from the bolstering ability of FST.

During the day of the test (at PND-63), the animals were moved in their home cages to the experimental room and allowed to acclimatize for 30 minutes. After cleaning the maze with 10% v/v alcohol, the rats were placed individually at the centre of the maze facing the other open arm opposite to the researcher and allowed to explore the EPM for five minutes under red light (80 lx) in which the time spent in open arms and closed arms were scored and analysed with EthoVision© XT 14 software (Noldus Information Technology, Wageningen, NLD).

References

Autry, A. E., Adachi, M., Cheng, P., Monteggia, L. M. 2009. Gender-specific impact of brain-derived neurotrophic factor signaling on stress-induced depression-like behavior. *Biological Psychiatry*, *66*(1): 84–90

Bay-richter, C., Peterson, E., Liebenberg, N., Elfving, B., Wegener, G. 2019. Latent toxoplasmosis aggravates anxiety- and depressive-like behaviour and suggest a role of gene environment interactions in the behavioural response to the parasite. *Behavioural Brain Research*, 364(9425):133-139

Becker, J. B., Arnold, A. P., Berkley, K. J., Blaustein, J. D., Eckel, L. A., Hampson, E., Herman, J. P., Marts, S., Sadee, W., Steiner, M., Taylor, J., & Young, E. 2005. Strategies and methods for research on sex differences in brain and behavior. *Endocrinology*, *146*(4): 1650–1673

Bogdanova, O.V., Kanekar, S., D'Anci, K.E., Renshaw, P.F. 2013. Factors influencing behaviour in the forced swim test. *Physiology and Behaviour*, 118 :227-239

Brand, S.J., Harvey, B.H. 2017a. Exploring a Post-traumatic stress disorder paradigm in Flinders Sensitive Line rats to model treatment resistant depression I; bio-behavioural validation and response to imipramine. *Acta Neuropsychiatrica*, 29(4):193-206

Carobrez, A.P., Bertoglio, L.J. 2005. Ethological and temporal analyses of anxiety-like behaviour; the elevated plus-Maze model 20 years. *Neuroscience and Bio-behavioural Reviews, 29(8)*: 1193-1205

Carobrez, A.P., Kinchenski. G.C., Bertoglio, L. 2010. Elevated plus maze. *Encyclopedia of Psychopharmacology*, 55:474-476

Cryan, J.F., Markou, A., Lucki, I. 2002. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends in Pharmacological Sciences,* 23(5):238-245

Cryan, J.F., Valentino, R.J., Lucki, I. 2005. Assessing substrates underlying the behavioural effects of antidepressants using the modified rat forced swimming test. *Neuroscience and Bio-behavioural Reviews*, 29(4-5): 547-569

Gericke, J. 2019. Evaluating the antidepressant-like properties of sceletium tortuosum, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, AM., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:114550

Gould, T.D., Dao, D.T., Kovacsics, C.C. 2009. Mood and Anxiety related Phenotypes in Mice: Characterization using behavioral tests. (vol.2):1-20. New York: Humana Press

Hall, C., Ballachey, E.L. 1932. A study of the rats behavior in a field. A contribution to method in comparative psychology. *Psychology*, 6:1-12

Handley, S. L., Mithani, S. 1984. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 327(1): 1–5.

Hershey, J.D., Gifford, J.J., Zizza, L.J., Pavlenko, D.A., Wagner, G., Miller, S. 2018. Effects of various cleaning agent on the performance of mice in behavioural assays of anxiety. *Journal of the American Association for Laboratory Animal Sciences*, 57(4):335-339

Hogman, C., 2014. Explorative stratergies in the open field (OFT), elevated plus maze (EPM) and multivariate concentric square field (MCSF) in adolescent wistar rats. Uppsala University (MSc. dissertation)

Kessler, R.C., Angermeyer, M., Anthony, J.C., De Graaf, R.O.N., Demyttenaere, K., Gasquet, I., De Girolamo, G., Gluzman, S., Gureje, O.Y.E., Haro, J.M. and Kawakami, N. 2007. Lifetime prevalence and age-of-onset distributions of mental disorders in the World Health Organization's World Mental Health Survey Initiative. *World Psychiatry*, 6(3):168

Kokras, N., Dalla, C. 2014. Sex differences in animal models of psychiatric disorders. *British Journal of Pharmacology*, *171*(20): 4595–4619

Mokoena, M. L., Harvey, B. H., Viljoen, F., Ellis, S. M., Brink, C. B. 2015. Ozone exposure of Flinders Sensitive Line rats is a rodent translational model of neurobiological oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232(16): 2921–2938.

Neumann, I.D., Wegener, G., Homberg, J.R., Cohen, H., Slattery, D.A., Zohar, J., Olivier, J.D., Mathe, A.A. 2011. Animal models of depression and anxiety; what they tell us about human conditions. *Progress in Neuropsychopharmacology and Biological Psychiatry*, 35:1357-1375

Oberholzer, I., Möller, M., Holland, B., Dean, O. M., Berk, M., Harvey, B. H. 2018. Garcinia mangostana Linn displays antidepressant-like and pro-cognitive effects in a genetic animal model of depression: a bio-behavioral study in the Flinders Sensitive Line rat. *Metabolic Brain Disease*, 33(2): 467–480.

Overstreet, D. H., Friedman, E., Mathé, A. A., Yadid, G. 2005. The Flinders Sensitive Line rat: A selectively bred putative animal model of depression. *Neuroscience and Biobehavioral Reviews*, 29(4–5): 739–759.

Overstreet, D. H., Wegener, G. 2013. The flinders sensitive line rat model of depression-25 years and still producing. *Pharmacological Reviews*, 65(1): 143–155.

Pellow, S., Chopin, P., File, S.E., Briley, M. 1985. Validity of open; closed arm entries in an elevated plus- maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*,14(3): 149-167

Porsolt, R.D., Bertin, A., Jalfre, M .1978. “Behavioural despair” in rats and mice: strain differences and the effects of imipramine. *European Journal of Pharmacology*, 51(3): 291–294

Porsolt, R.D, Bertin, A., Jalfre, M.1977. Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie*, 229 (2): 327–336.

Regenass, W., Möller, M., Harvey, B. H. 2018. Studies into the anxiolytic actions of agomelatine in social isolation reared rats: Role of corticosterone and sex. *Journal of Psychopharmacology*, 32(2): 134–145.

Saayman L.B.J., 2019. Long lasting bio-behavioural effects of early life sildenafil administration in stress sensitive versus healthy control rats. North West University (South Africa), Potchefstroom campus (MSc dissertation)

Saayman, L.B.J., Steyn, S.F., Brink, C. B. 2021. The long-term bio-behavioural effects of juvenile sildenafil treatment in Sprague-Dawley versus Flinders Sensitive Line rats. *Acta Neuropsychiatrica*,4(298): 200-205

Slattery, D. A. Cryan, J. F. 2012. Using the rat forced swim test to assess antidepressant-like activity in rodents. *Nature Protocols*, 7(6): 1009–1014

Stanford, C. 2007. The open field testing; reinventing the wheel. *Journal of Psychopharmacology*, 21 (2): 134-135

Steyn, S.F. 2011. The effects of early-life exposure stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood. North-West University (South Africa), Potchefstroom Campus (MSc dissertation).

Steyn, S.F., Harvey, B. H., Brink, C. B. 2020. Pre-pubertal, low-intensity exercise does not require concomitant venlafaxine to induce robust, late-life antidepressant effects in Flinders sensitive line rats. *European Journal of Neuroscience*, 52(8): 3979–3994.

Wegener, G., Finger, B. C., Elfving, B., Keller, K., Liebenberg, N., Fischer, C. W., Singewald, N., Slattery, D. A., Neumann, I. D., Mathé, A. A. 2012. Neuropeptide S alters anxiety, but not depression-like behaviour in Flinders Sensitive Line rats: A genetic animal model of depression. *International Journal of Neuropsychopharmacology*, 15(3): 375–387.

Yankelevitch-Yahav, R., Franko, M., Huly, A., Doron, R. 2015. The forced swim test as a model of depressive-like behavior. *Journal of Visualized Experiments*, 52587 (97): 1–7

ADDENDUM B

ANXIETY-LIKE BEHAVIOUR (OFT & EPM RESULTS)

This addendum contains anxiety-like behaviour as measured in the OFT and EPM that were not presented in the manuscript (Chapter 3). On critical review, these behavioural data failed to demonstrate any noteworthy differences between FSL and FRL rats. This shortcoming means we could not provide a basis for making valid conclusions as to the face validity of the FSL model in as far as anxiety is concerned, and hence the putative anxiolytic activity of the reference (DMI), the test drug (Zembrin®) and the combinations thereof. Consequently, face (behavioural) and predictive (response to DMI) validity was based on behavioural responses measured in the FST, as described in chapter 3. These data are then presented in completion of the aims and objectives of this study.

B.1. Introduction

The comorbidity of depression and anxiety has previously been documented (Goddard *et al.,*2010; Altin *et al.,*2014). This additional behaviour connected with depression has been explored using open field test (OFT) (Saayman, 2019; Steyn *et al.,*2018) and elevated plus maze (EPM) test (Mokoena *et al.,*2015; Steyn, 2011).

B. 2 Behavioural analysis of anxiety-like behaviour

On day 13 (PND-62) of treatment, the animals were subjected to Open Field Test (OFT) with the Elevated Plus Maze test (EPM) performed on day 14 (PND-63) of treatment. An earlier study demonstrated that performing the FST prior to EPM can bolster anxiety-like behaviour in FSL rats (Richter *et al.,*2016) which may be imperative as the FSL rats do not generally exhibit anxiety-like behaviour unless being exposed to a prior mild stressor (Neumann *et al.,*2011; Wegener *et al.,*2012). All behavioural tests were conducted during the rat's dark cycle, with each test separated by an acclimatization period of 30 min to minimize stress caused by movement of home cages to the behavioural assessment room.

B. 2.1 Open Field Test (OFT)

The OFT is a well-described assay to assess anxiety-like behaviour in rodents (Saayman, 2019). Here the test was performed according to previously described methods (Saayman, 2019; Steyn *et al.,*2018). Briefly, the apparatus consisted of an open black

square arena (1 m^2) surrounded by high walls (45 cm) to prevent the animals from escaping. Animals were allowed to freely explore the arena, while behavioural parameters were recorded by cameras mounted above the arena. All tests were performed under red light (80 lx). On PND-62, animals were moved in their home cages to the experimental room and allowed to acclimatize for 30 min (Gericke, 2019; Steyn *et al.,*2018). The rats were placed individually in the centre of the open field arena and allowed to explore for 6 min, with the last 5 min recorded and used for statistical analyses. The arena was cleaned with 10% (*v/v*) alcohol after each test to minimize olfactory cues present in the apparatus left by a previous animal (Hershey *et al.,*2018). Behavioural parameters were scored with EthoVision© XT 14 software (Noldus Information Technology, Wageningen, NLD). The total time spent in the outer and centre zones of the arena was used as an indicator of anxiety-like behaviour (Saayman, 2019; Steyn *et al.,*2018). Anxious animals tend to spend less time in the centre zone and more time in the outer zones (Saayman, 2019; Steyn *et al.,*2018).

B.2.2 Elevated Plus Maze (EPM)

The EPM is widely used to assess anxiety-like behaviour in rats (Steyn, 2011; Hogman, 2014). Here the test was performed according to previously described methods (Mokoena *et al.,*2015; Regenass *et al.,*2018). The paradigm consists of "+" plus –shaped maze with two open and two closed arms, 50cm x 10cm (length and width), elevated 50cm above the ground. On PND-63, the animals were moved in their home cages to the experimental room and allowed to acclimatize for 30 min (Steyn, 2011). After cleaning the maze with 10% (*v/v*) alcohol, the rats were placed in the centre of the maze facing the open arm opposite to the researcher and allowed to explore the maze for five minutes under red light (80 lx). The total time spent in the open and closed arms were scored by EthoVision© XT 14 software (Noldus Information Technology, Wageningen, NLD). Time spent in a zone was accepted when the centre zone of the animal, as assigned by EthoVision© software, entered the specified zone.

B.3 Results

B. 3.1 Confirmation of face validity of the FSL/FRL Model for anxiety-like behaviour

Regarding anxiety-like behaviour, SAL FSL rats entered the centre zone of the OFT significantly more often than FRL controls ($t_{2.37}$ = 19.82; p = 0.0283) and also spent significantly more time in the zone (not all data normally distributed; U = 28; p = 0.0184).

However, no significant differences were observed between SAL FSL and FRL controls in either time spent in (not all data normally distributed; $U = 56$; p = 0.3777) or entries into ($t_{1.28} = 21.49$; $p = 0.2149$) the open arms of the EPM.

Table B.1: Face validation of the FSL/FRL model

In all instances, animals received SAL treatment. Data is presented as mean ± S.E.M.

Time spent in centre zone (sec)	OFT	5.92	13.38	$p = 0.0184$
Centre zone entries (#)	OFT	3.00 ± 1.15	5.73 ± 2.72	$p = 0.0283$
Time spent in open arms (%)	EPM	12.46	8.12	$p = 0.3777$
Open arm entries (#)	EPM	36.06 ± 8.18	25.61 ± 10.45	$p = 0.2149$

B. 3.2 Monotherapy study: Anxiety-like behaviour (OFT & EPM) results

In *Fig. B.1. (A)* and *(B)*, there were no statistically significant differences between groups for either time spent in centre zone (data not normally distributed; $x^2 = 8.97$; $p = 0.1105$) or frequency into the centre zone of the OFT ($F_{5.00, 54.35} = 0.70$; $p = 0.6271$).

In *Fig. B.1. (C)* and *(D)*, were no statistically significant differences between groups for either time spent (data not normally distributed; $x^2 = 5.83$; $p = 0.3236$) or entries into the open arms of the EPM ($F_{5.00, 58.00} = 1.32$; $p = 0.2698$).

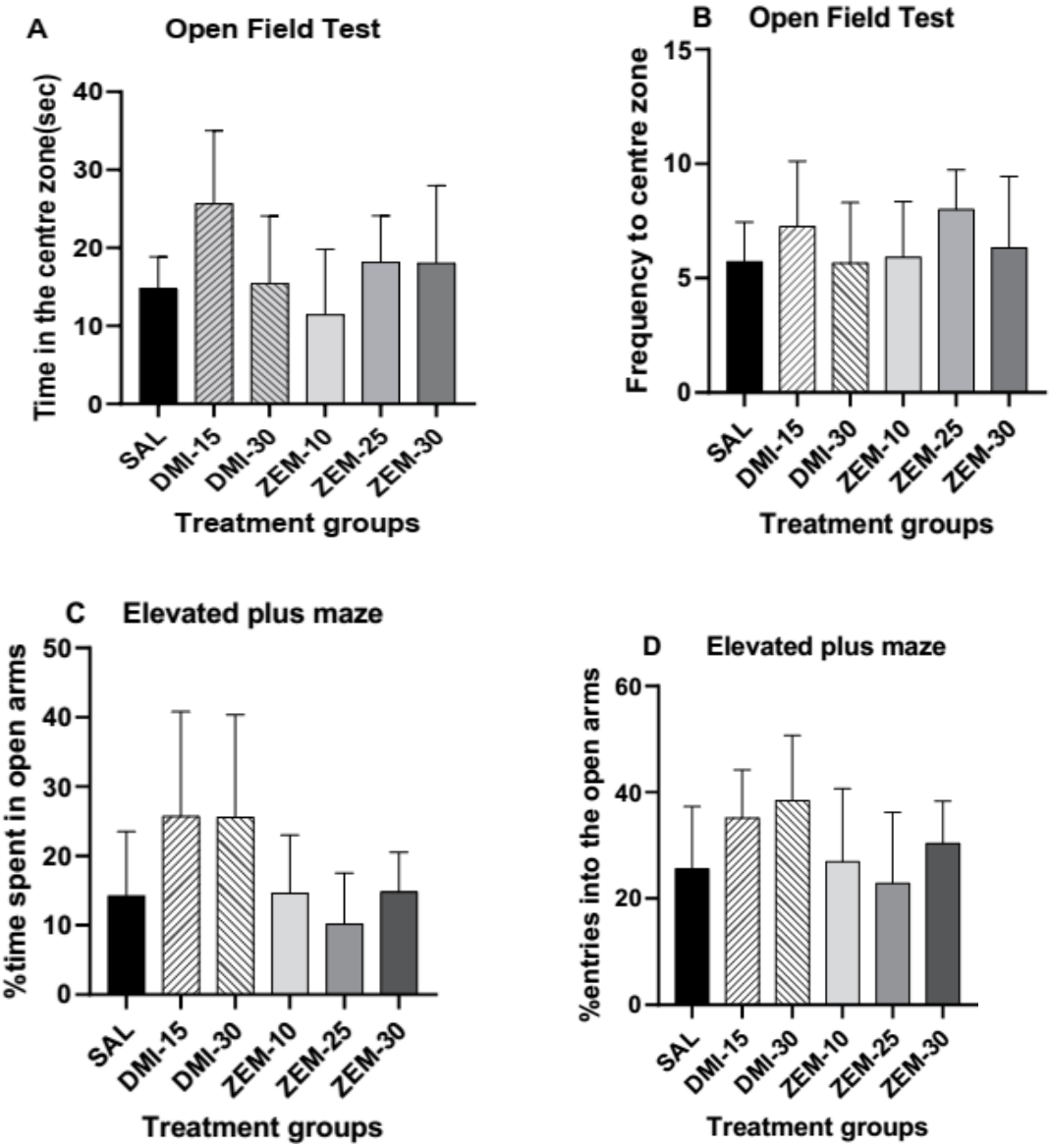


Fig. B.1. *Monotherapy study: Effects of saline, various doses of ZEM (10, 25 & 30 mg/kg/day), low dose DMI (15 mg/kg/day) and DMI (30 mg/kg/day) on anxiety-like behaviour versus FSL saline treated rats following 14 days treatment.* ***(A)*** *Time spent in the centre zone in the OFT (outlier identified and removed from the DMI-15 group; DMI-30 group and ZEM-10 (n = 10) group).* ***(B)*** *Frequency the rats went to the centre zone in the OFT (outlier identified and removed from the FSL-SAL group and DMI-15 group).* ***(C)*** *Percentage time spent in the open arms of the EPM (outlier identified and removed from the ZEM-30 group) and* ***(D)*** *Percentage entries (entries into the open arm/total entries into open and closed arms) in the open arms of the EPM. Data expressed as means ± S.E.M.*

B.3.3 Augmentation study: anxiety-like behaviour (OFT & EPM) results

In *Fig.B.2(A)*, all data sets were normally distributed. Although significant statistical differences in time spent in the centre zone of the OFT were identified between the

treatment groups ($F_{5, 63}$ = 2.64; p = 0.0314), these differences did not reach statistical significance according to the Tukey's multiple comparison test.

In *Fig. B.2(B)*, All data sets were normally distributed. The Ordinary one-way ANOVA showed no statistically significant differences between the treatment groups ($F_{5, 64}$ = 1.50; p = 0.2045).

In *Fig. B.2(C)* and *(D)*, there were no statistically significant differences between groups for either time spent (data not normally distributed; x^2 = 4.86; p = 0.4335) or entries into the open arms of the EPM ($F_{5, 65}$ = 1.10; p = 0.3709).

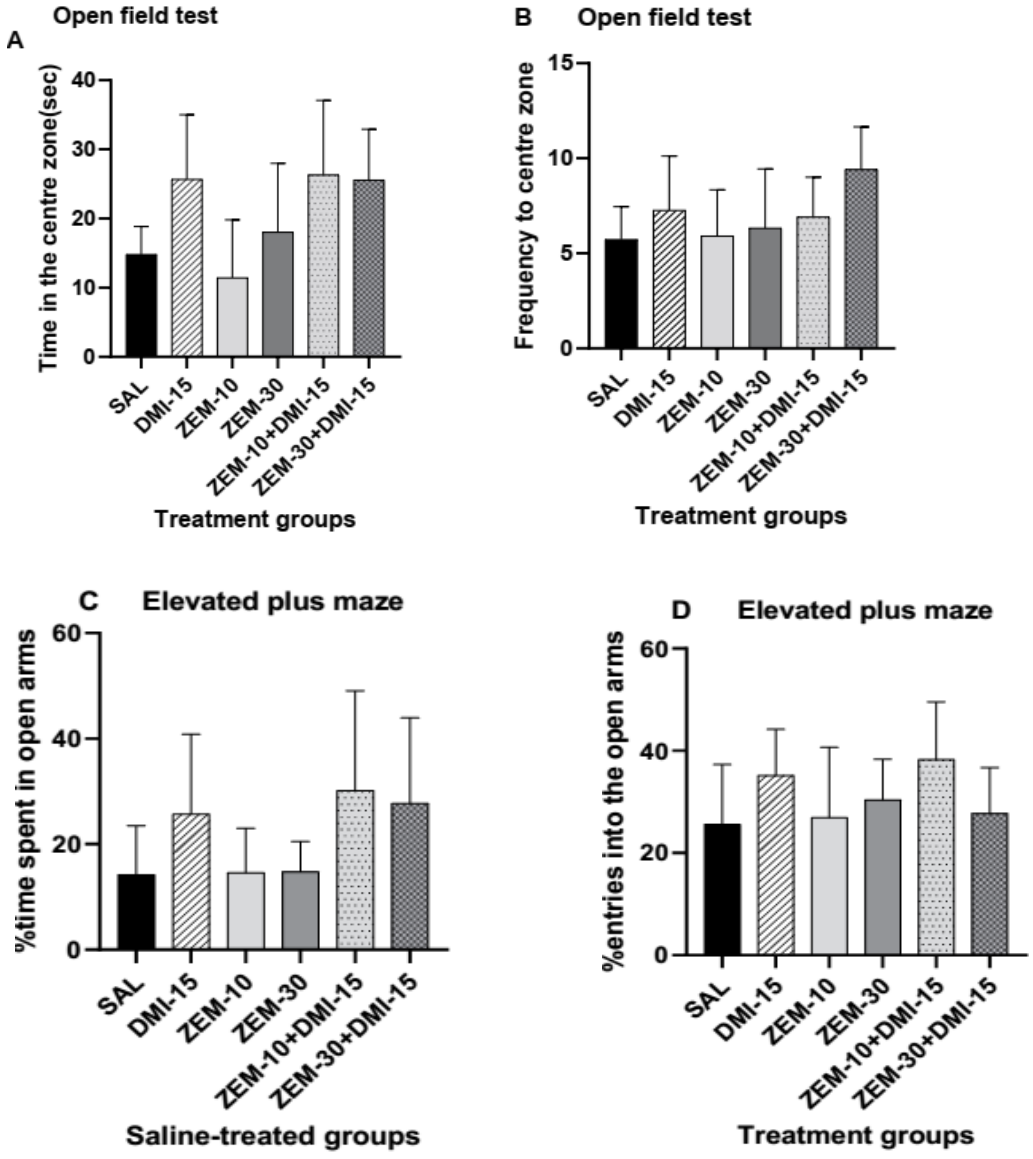


Fig. B.2. *Augmentation study. effects of saline, ZEM (10 & 30 mg/kg/day), low dose DMI (15 mg/kg/day) and combination therapies (ZEM-10+DMI-15 (mg/kg/day) and ZEM-30+DMI-15 (mg/kg/day)) on anxiety-like behaviour versus FSL saline treated rats following 14 days treatment.* ***(A).*** *Time spent (sec) in the*

centre zone in the OFT (Outlier identified and removed from the DMI-15 group and ZEM-10 (n = 10) group). ***(B).*** *Frequency the rats went to the centre zone in the OFT (Outlier identified and removed from the FSL-SAL group and DMI-15 group).* ***(C).*** *Percentage time spent in the open arms of the EPM (Outlier identified and removed from the ZEM-30 group). and* ***(D).*** *Percentage entries in the open arms of the EPM. Data expressed as means ± S.E.M*

B.4 Discussion

Depression and anxiety are most often comorbid conditions (Goddard *et al.,*2010; Altin *et al.,*2014). It was the intension of this study to employ two behavioural assays relevant for MDD to establish the face validity of the model, viz. anxiety and depressive-like behaviours, and so to provide a basis for determining the broad psychotropic actions of the various drug treatments.

Greater anxiety is associated with increased locomotor activity in an open field. Unexpectedly, FSL rats spent significantly more time in the centre zone of the OFT with more centre zone entries, evidence for *less* anxious behaviour, compared to FRL control rats *(Table B.1)*. This is supported by the locomotor results, with FSL rats moving significantly more often than FRL rats (*Table 1*; chapter 3). In general, reduced locomotor activity is more prominent in FSL rats (Wegener *et al.,*2012; Overstreet *et al.,*2005). In order to verify the OFT findings, we undertook additional behavioural analysis in the EPM, widely regarded as a robust test for anxiety in rodents (Walf and Frye, 2007). Here the EPM also showed no significant differences in anxiety-like behaviour between the two strains *(Table B.1)*. These results are in line with previous studies from our laboratory (Steyn, 2011; Kruger, 2014) and other studies (Wegener *et al.,*2012; Overstreet *et al.,*2005; Neumann *et al.,*2011), confirming the FSL rat to be a pure model of MDD. Indeed, the model only displays anxiety-like behaviour following a prior stressor (Overstreet *et al.,*2005) or if combined with another aversive behavioural test (i.e., social interaction) (Overstreet and Griebel, 2005).

Considering the need to assess the possible anxiolytic effects of the various treatments, FSL rats did not present with anxiety-related manifestations versus FRL rats (*Table B.1*). Therefore, any conclusions regarding the possible anti-anxiety-like effects of any of the treatments is not possible or relevant. Further studies are needed, perhaps using other behavioural tests (i.e., social interaction) (Overstreet and Griebel, 2005) or using another animal model of MDD, such as the chronic mild stress model (Gericke,2019).

These data were therefore excluded from Chapter 3 but presented here for completion sake. Behavioural assessment in this study was therefore founded on the FST to assess despair and coping behaviour, and the OFT to assess locomotor activity, as presented in Chapter 3.

References

Altin, M., Harada, E., Schacht, A., Berggren, L., Walker, D., Duenas, H. 2014. Does early improvement in anxiety symptoms in patients with major depressive disorder affect remission rates? A post-hoc analysis of pooled duloxetine clinical trials. *Open Journal of Depression*, 3:112-123

Bay-richter, C., Peterson, E., Liebenberg, N., Elfving, B., Wegener, G. 2019. Latent toxoplasmosis aggravates anxiety- and depressive-like behaviour and suggest a role of gene environment interactions in the behavioural response to the parasite. *Behavioural Brain Research*, 364(9425):133-139

Gericke, J., 2019. Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc thesis)

Goddard, A.W., Ball, S.G., Martinez, J., Robinson, M.J., Yang, C.R., Russel, J.M., Shekhar, A. 2010. Current perspectives of the roles of central norepinephrine system in anxiety and depression. *Depression* and *Anxiety,* 27:339-350

Hershey, J.D., Gifford, J.J., Zizza, L.J., Pavlenko, D.A., Wagner, G., Miller, S. 2018. Effects of various cleaning agent on the performance of mice in behavioural assays of anxiety. *Journal of the American Association for Laboratory Animal Sciences*, 57(4):335-339

Hogman, C. 2014. Explorative stratergies in the open field (OFT), elevated plus maze (EPM) and multivariate concentric square field (MCSF) in adolescent wistar rats. Uppsala University (MSc. dissertation)

Kruger, R. 2014. The effects of early life exposure of rats to venlafaxine on behaviour and neurological markers of antidepressant action in adulthood. North-West University, Potchefstroom. (MSc dissertation)

Mokoena, M.L., Harvey, B.H., Viljoen, F., Ellis, S.M., Brink, C.B. 2015. Ozone exposure of Flinders Sensitive Li ne rats is a rodent translational model of neurobiological oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232(1): 2921-2938.

Neumann, I.D., Wegener, G., Homberg, J.R., Cohen, H., Slattery, D.A., Zohar, J., Olivier, J.D., Mathe, A.A. 2011. Animal models of depression and anxiety; what they tell us about human conditions. *Progress in Neuropsychopharmacology and Biological Psychiatry*, 35:1357-1375

Overstreet, D.H., Friedman, E., Mathe, A.A., Yadid, G. 2005. The Flinders Sensitive Line rat; A selectively bred putative animal model of depression. *Neuroscience and Bio-behavioural Reviews*, 29(4-5): 739-759

Overstreet, D.H., Griebel, G. 2005. Antidepressant-like effects of the vasopressin Vlb receptor antagonist SSR149415 in the Flinders Sensitive Line rat. *Pharmacology, Biochemistry and Behavior*, 82(1): 223-227

Steyn, S.F. 2011. The effects of early-life exposure stress-sensitive rats to the serotonin-norepinephrine reuptake inhibitor venlafaxine on behaviour in adulthood. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Walf A.A., Frye C.A. 2007. The use of the elevated plus maze as an assay of anxiety-related behaviour in rodents. *Nature Protocols,* 2(2): 322‑328.

Wegener, G., Finger, B.C., Elfving, B., Keller, K., Lienbenberg, N., Fischer, C.W., Singewald, N., Slattery, D.A., Neumann, I.D., Mathe, A.A. 2012. Neuropeptide S-alters anxiety, but not depression-like behaviour in Flinders Sensitive Line rats; A genetic animal model of depression*. International Journal of Neuropsychopharmacology*, 15: 375-38

ADDENDUM C

Additional results: monoamines analyses (NE, 5-HT and DA)

This addendum contains additional monoamine data that were not presented in the manuscript (Chapter 3). The monoamine data for the complete study were analysed on two separate occasions, with the augmentation study cohorts analysed at a later date. This approach was necessitated because the results from the FST supports the predictive validity of the FSL rat model using DMI as both doses (15 and 30 mg/kg/day) were effective and DMI-15 had the bigger effect size value, hence was selected for use in the augmentation study, Since none of the doses of ZEM were significantly effective to reverse depressive-like behaviour in the FST, monoamines were partially analysed (ZEM monotherapies vs. FSL SAL groups) to provide direction and to select an appropriate dose for the sub-chronic augmentation study. On critical review, this shortcoming translated into discrepancies between especially basal values when compared across the two dates of analysis. Hence, we were not able to make valid comparisons across all the cohorts. Consequently, the monoamine data for the augmentation studies were removed from chapter 3, with only the validation studies employing monoamine analysis to inform on dose selection of ZEM, together with behaviour, and presented in Chapter 3. The excluded monoamine data are presented here in completion of the aims and objectives of this study.

C.1 Introduction

Major depressive disorder (MDD) has been associated with the depletion of monoamine neurotransmitters in various brain regions, including the hippocampus and frontal cortex (Treadway *et al.,*2015). NE plays an important role in MDD such that a decrease in hippocampal and cortical NE has been associated with symptoms of MDD i.e., drowsiness or fatigue, impaired cognition and executive functions (Maletic *et al.,*2017). Decreased 5-HT levels in the hippocampus and cortex have been associated with impaired working memory and impaired cognitive function (Charnay and Leger, 2010). Dopamine is involved in regulating cognition, motivation and reward (Olguin *et al.,*2016) such that a decrease in the hippocampal and cortical DA concentration in MDD produce symptoms such as anhedonia and psychomotor retardation (Amidfar *et al.,*2018). Chronic treatment with different classes of antidepressants i.e., MAOIs, TCAs, SNRIs, SSRIs, atypical antidepressants (Lieberman and Massey, 2009) and CAMs (Sarris *et al.,*2021)

may elevate monoamines (NE, 5-HT and DA) and reverse symptoms of MDD (Lieberman and Massey, 2009; Maletic *et al.,*2017).

In Chapter 3 we described the monoaminergic effects of ZEM and DMI in FSL rats, noting that ZEM-30 significantly elevates cortical NE and 5-HT compared to the SAL FSL while DMI data was obscure. Subsequently it was necessary to consider low (ZEM-10) and high dose (ZEM-30) in the augmentation studies, where the aim was to assess the augmentation potential of low DMI with ZEM in a dose dependent manner. Here we present the groups in 3 phase , as described below.

C.2. Results

C.2.1 Phase-1 construct validation of the FSL/FRL model

C.2.1.1 Effects of saline on hippocampal and frontal cortical NE levels

All data sets were normally distributed. *In Fig. C.1A,* Unpaired t-test indicated no significant difference on hippocampal NE levels ($t_{14.90}$ = 0.451; p = 0.6582 in FRL rats (260.6 ± 40.18 ng/g) compared to FSL rats (278.7± 18.14 ng/g), whereas, In *Fig C.1B*, Unpaired t-test indicated significantly decreased frontal cortical NE levels ($t_{19.93}$ = 2.51; p = 0.0207) in FSL rats (164.5 ± 43.49 ng/g) compared to the FRL rats (208.0 ± 17.31 ng/g) following intervention over 14 days.

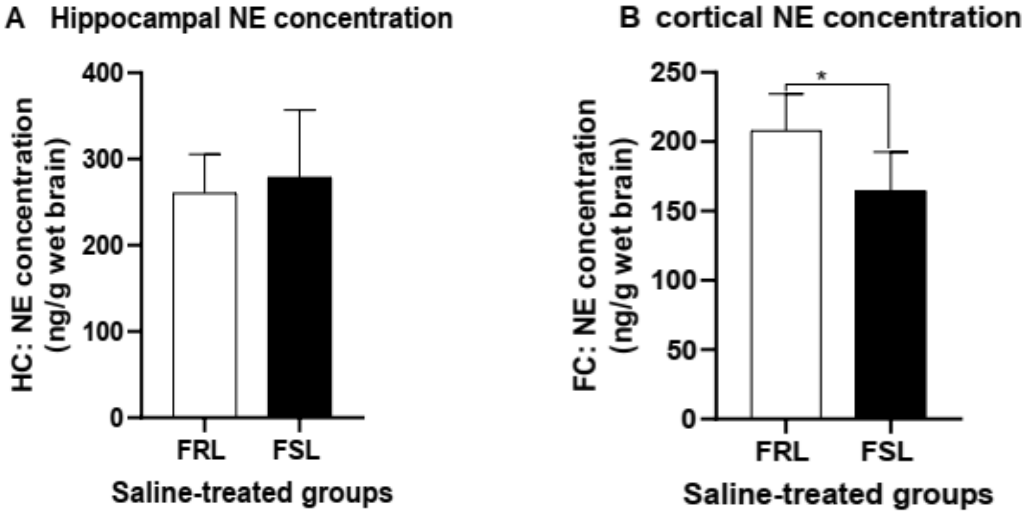


***Fig. C. 1**. Effects of saline on tissue brain NE levels between FRL and FSL rats following 14 days treatment. (A). Hippocampal NE concentration (ng/g) (two outliers identified and removed from the FSL-SAL group). (B). Cortical NE concentration (ng/g) (one outlier identified from each group and removed). Data expressed as means ± S.E.M. with statistical significance taken as *p ≤ 0.05 vs. FSL SAL*

C.2.1.2 Effects of saline on hippocampal and frontal cortical 5-HT levels

Hippocampal 5-HT (Fig. C .2A): Not all data sets were normally distributed. Hippocampal 5-HT levels were detected in 6 (FRL n =6) and 7 (FSL n = 7) samples respectively. Mann-Whitney U-test showed significantly reduced hippocampal 5-HT levels (Mann-Whitney U-value = 2; p = 0.0047) in FRL rats (median = 73.57 ng/g) compared to FSL rats (median = 117.1 ng/g).

Cortical 5-HT (Fig. C. 2B): All data sets were normally distributed. Unpaired t-test indicated no significant difference on frontal cortical 5-HT levels ($t_{18.99}$ = 1.69; p = 0.1078) in FSL rats (116.4± 20.83 ng/g) compared to FRL rats (95.52 ± 12.34 ng/g)

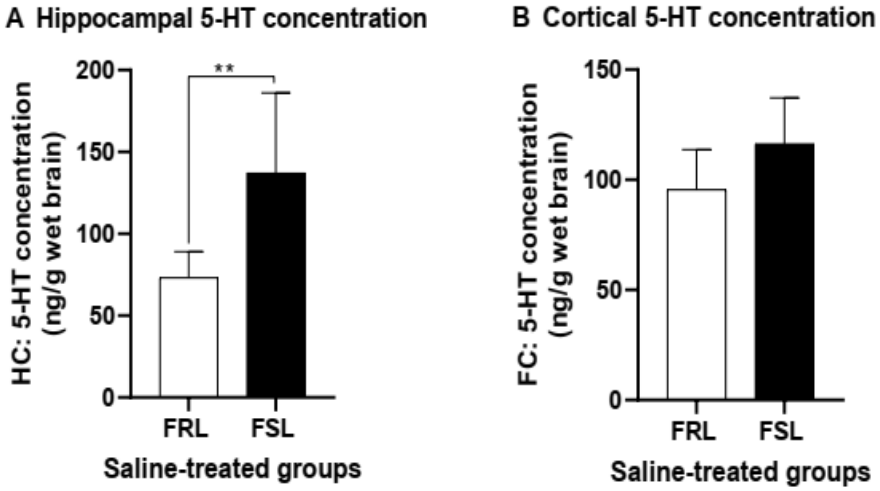


***Fig. C. 2**. Effects of saline on tissue brain 5-HT levels between FRL and FSL rats following 14 days treatment. (A). Hippocampal 5-HT concentration (ng/g) (FRL rats n = 6 and FSL rats n = 7). (B). Cortical 5-HT concentration (ng/g) (one outlier identified and removed from FRL group reducing samples, n = 9 and FSL rats n = 12). Data expressed as means ± S.E.M. with **p ≤ 0.01 vs. FSL SAL*

C.2.1.3 Effects of saline on hippocampal and frontal cortical DA levels

Hippocampal DA (Fig. C. 3A): All data sets were normally distributed. Hippocampal DA levels were detected in 6 (FRL n =6) and 7 (FSL n = 9). Unpaired t-test indicated statistically increased hippocampal DA levels ($t_{6.31}$ = 2.64; p = 0.0368) in FSL rats (118.40 ± 53.73 ng/g) versus FRL rats (64.71 ± 20.35 ng/g).

Cortical DA (Fig. C.3B): All data sets were normally distributed. cortical DA levels were detected in 8 (FRL n = 8) and 10 (FSL n = 10). Unpaired t-test indicated no statistically significant differences in the cortical DA levels ($t_{14.70}$ = 0.48; p = 0.6356) in FSL rats (118.40 ± 8.63 ng/g) versus FRL rats (109.10 ± 17.84 ng/g).

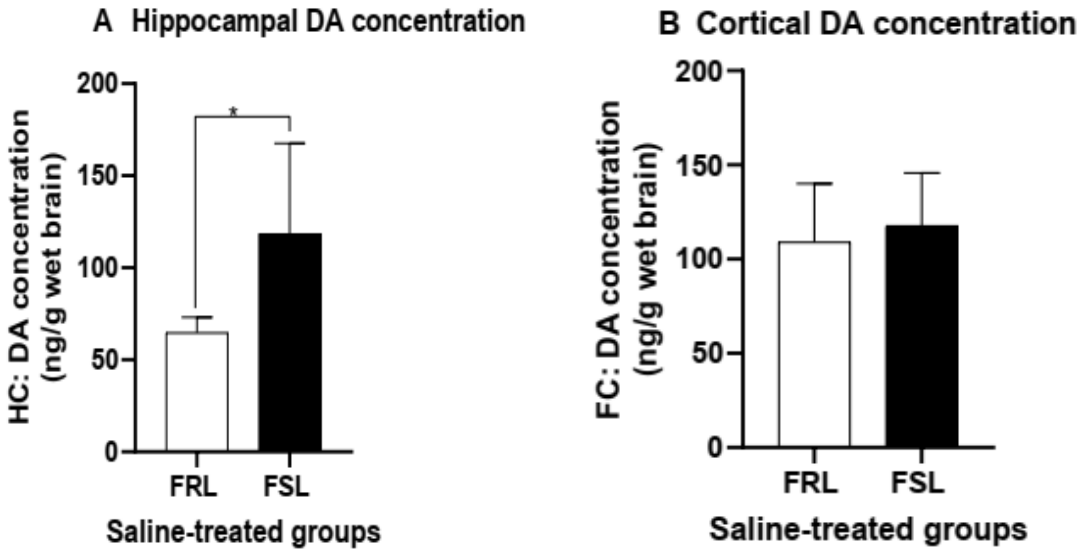


***Fig. C. 3**. Effects of saline on tissue brain DA levels between FRL and FSL rats following 14 days treatment. (A). Hippocampal DA concentration (ng/g) (FRL rats n = 6 and two outliers identified and removed from FSL group reducing samples, n = 7). (B). Cortical DA concentration (ng/g) (FRL rats n = 8. One outlier identified and removed from FSL group reducing samples, n = 9). Data expressed as means ± S.E.M. with *p ≤ 0.05. vs. FSL SAL*

C.2.2 Phase-2 Quantification of monoamines in the monotherapy study

C.2.2.1 Effects of saline, Zembrin® and desipramine on hippocampal and frontal cortical NE levels

Hippocampal NE (Fig. C. 4A): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00,\ 32.53}$ = 14.73; $p \leq$ 0.0001). Compared to SAL FSL rats, the Dunnett multiple comparison post-hoc test indicated significantly reduced hippocampal NE levels in DMI-15 (p = 0.0042) and DMI-30 (p = 0.0028) treated rats.

Cortical NE (Fig. C. 4B): Not all data sets were normally distributed. There were statistically significant differences among the treatment groups (X^2 = 38.60; $p \leq$ 0.0001). The Dunn's multiple comparison post-hoc test showed significantly reduced cortical NE levels in FSL rats treated with DMI-30 (p = 0.0296) compared to the SAL FSL rats . Of note, ZEM-30 significantly increased frontal cortical NE levels in FSL rats compared to the SAL FSL rats (p = 0.0297).

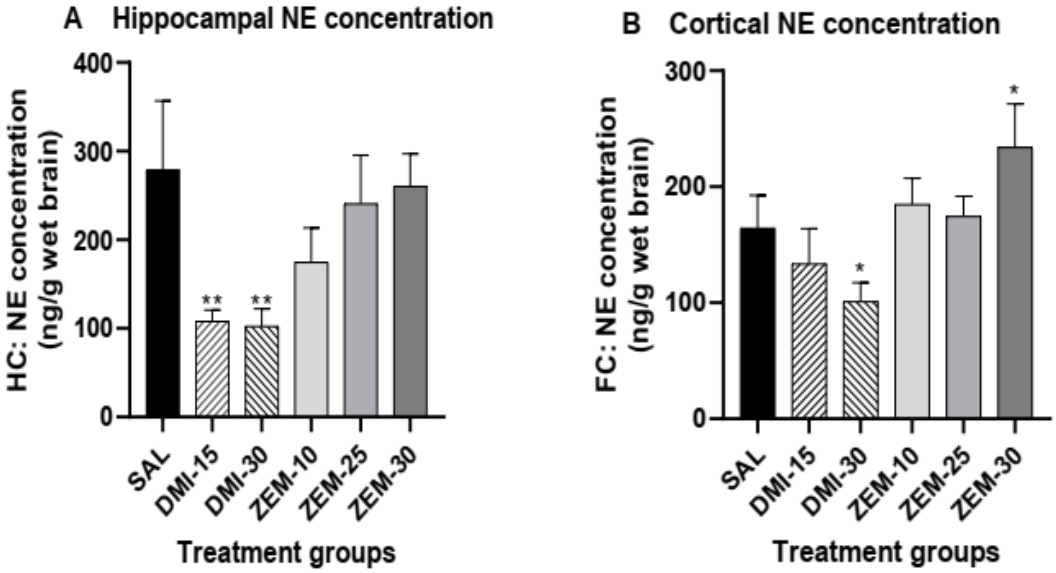


Fig. C. 4*. Effects of saline and escalating doses of ZEM (10, 25 & 30 mg/kg/day) and DMI (15 & 30 mg/kg/day) on tissue brain NE levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal NE concentration (ng/g) (two outliers identified and removed from the FSL-SAL group). (B). Cortical NE concentration (ng/g) (outlier identified and removed from the FSL-SAL group). Data expressed as means ± S.E.M. with *p ≤ 0.05, **p ≤ 0.01vs. SAL*

C.2.2.2 Effects of saline, Zembrin® and desipramine on hippocampal and frontal cortical 5-HT levels

Hippocampal 5-HT (Fig. C. 5A): Not all data sets were normally distributed. In DMI-15 treated rats the hippocampal 5-HT levels were below limit of detection (BLOD). There were statistically significant differences among the treatment groups (x^2 = 18.28; p = 0.0011). Although the Dunn's multiple comparison post-hoc test showed significantly reduced hippocampal 5-HT levels in FSL rats treated with in DMI-30 compared to the SAL FSL rats (p = 0.0082), the hippocampal NE levels were detected in five (5) samples only. Compared to SAL FSL rats, the Dunn's multiple comparison post-hoc test showed significantly reduced hippocampal 5-HT levels in FSL rats treated with ZEM-10 (p = 0.0402).

Cortical 5-HT (Fig. C. 5B): All data sets were normally distributed. In DMI-15 treated rats the hippocampal 5-HT levels were detected in three (3) samples only. There were statistically significant differences among the treatment groups ($F_{5.00,38.20}$= 14.12; p ≤ 0.0001). Of note, ZEM-30 induced a significant increase in frontal cortical 5-HT levels compared SAL FSL rats (p = 0.0027).

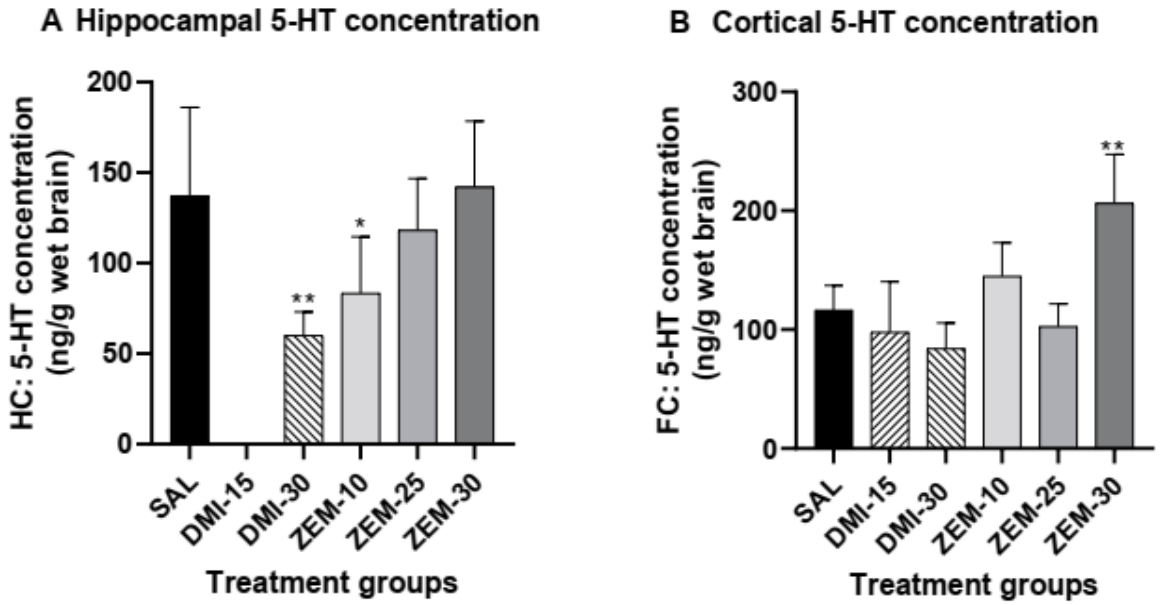


Fig. C. 5. *Effects of saline and escalating doses of ZEM (10, 25 & 30 mg/kg/day) and DMI (15 & 30 mg/kg/day) on tissue brain 5-HT levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal 5-HT concentration (ng/g) (FSL-SAL group (n=7), DMI-15 group (n = 0), DMI-30 group (n = 5), ZEM-10 (one outlier identified and removed reducing sample size n = 9), ZEM-25 (n = 12) and ZEM-30 (n = 12)). (B). Cortical 5-HT concentration (ng/g) (FSL-SAL group (n = 12), DMI-15 group (n = 3), DMI-30 group (n = 9), ZEM-10 (n = 12), ZEM-25 (n = 12) and ZEM-30 (n = 12)). Data expressed as means ± S.E.M. with*p ≤ 0.05, **p ≤ 0.01 vs. SAL.*

C.2.2.3 Effects of saline, Zembrin® and desipramine on hippocampal and frontal cortical DA levels

Hippocampal DA (Fig. C. 6A): Not all data sets were normally distributed. There were no statistically significant differences among the treatment groups (X^2 = 7.14; p = 0.2105).

Cortical DA (Fig. C. 6B): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5.00,13.09}$ = 4.83; $p \leq 0.0102$). The Dunnett multiple comparison post-hoc test indicated significantly reduced cortical DA levels in ZEM-25 (p = 0.0356) treated rats versus SAL control FSL rats.

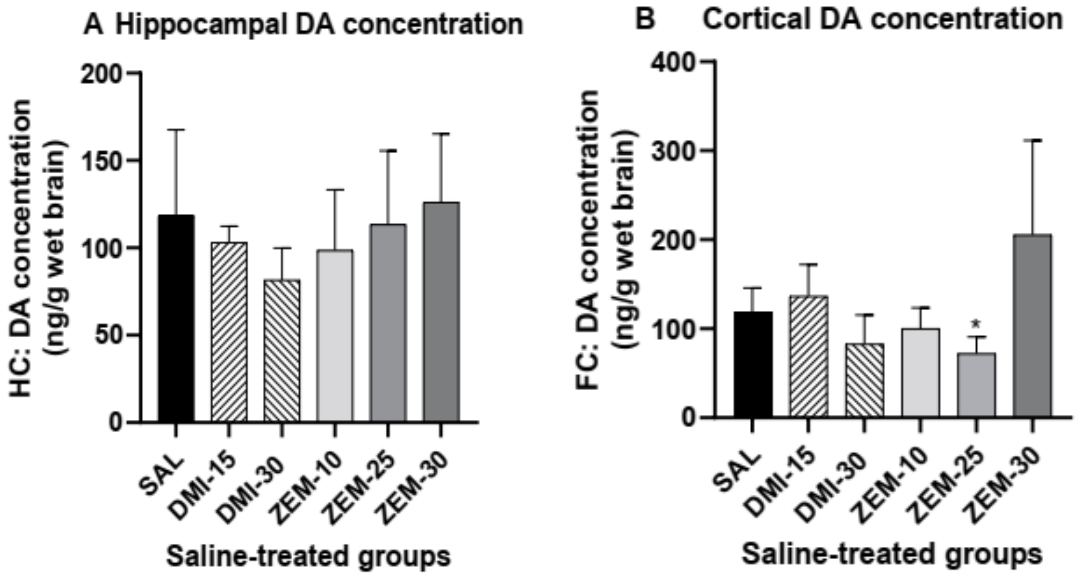


Fig. C. 6. *Effects of saline and escalating doses of ZEM (10, 25 & 30 mg/kg/day) and DMI (15 & 30 mg/kg/day) on tissue brain DA levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal DA concentration (ng/g) (FSL-SAL group, two outliers identified and removed reducing sample size n=7), DMI-15 (n = 11), DMI-30 (n = 7), ZEM-10 (n = 9), ZEM-25 (n = 12) and ZEM-30 (n = 8)). (B). Cortical DA concentration (ng/g) (FSL-SAL group, one outlier identified and removed reducing sample size (n= 9),DMI-15 (n = 8), DMI-30 (n = 5), ZEM-10 (one outlier identified and removed reducing sample size n = 11), ZEM-25 (one outlier identified and removed reducing sample size n = 8) and ZEM-30 (one outlier identified and removed reducing sample size (n = 9)). Data expressed as means ± S.E.M. with*p ≤ 0.05, vs. SAL.*

C.2.3 Phase-3 Quantification of monoamines in the augmentation study

C.2.3.1 Effects of saline, Zembrin® and desipramine alone and as combination therapies on hippocampal and frontal cortical NE levels in FSL rats following 14 days treatment

Hippocampal NE (Fig. C. 7A): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5, 62} = 13.07$; $p \leq 0.0001$). Compared to the SAL FSL rats, the Tukey's multiple comparison post-hoc test indicated a significantly reduced hippocampal NE levels in ZEM-10+DMI-15 ($p = 0.0002$) and ZEM-30+DMI-15 ($p = 0.0001$) treated rats. Compared to the DMI-15 treated rats, the Tukey's multiple comparison post-hoc test showed no significant differences in hippocampal NE levels in ZEM-10+DMI-15 ($p = 0.5195$) and ZEM-30+DMI-15 ($p = 0.0006$) treated rats. Compared to the ZEM-30 treated rats, the Tukey's multiple comparison post-hoc test indicated a significantly reduced hippocampal NE levels in ZEM-10+DMI-15 ($p = 0.0012$)

and ZEM-30+DMI-15 (p = 0.0006) treated rats. The Tukey's multiple comparison post-hoc test showed no significant differences in hippocampal NE levels in ZEM-10+DMI-15 (p = 0.9550) and ZEM-30+DMI-15 (p = 0.8171) treated rats versus ZEM- 10 treated rats.

Cortical NE (Fig. C.7B): Not all data sets were normally distributed. There were statistically significant differences among the treatment groups (x^2 = 34.95; $p \leq 0.0001$). Compared to the FSL rats, the Dunn's multiple comparison post-hoc test showed no significant differences in frontal cortical NE levels in ZEM-10+DMI-15 (p = 0.2214) and ZEM-30+DMI-15 (p = 0.9999) treated rats. Compared to DMI-15 treated rats, the Dunn's multiple comparison post-hoc test showed no significant differences in frontal cortical NE levels in ZEM-10+DMI-15 (p = 0.9999) and ZEM-30+DMI-15 (p = 0.9999) treated rats. ZEM-30 significantly increased frontal cortical NE levels in FSL rats versus ZEM-10+DMI-15 ($p \leq 0.0001$) and ZEM-30+DMI-15 (p = 0.0102) treated rats. ZEM-10 significantly increased frontal cortical NE levels in FSL rats versus ZEM-10+DMI-15 (p = 0.0051) treated rats.

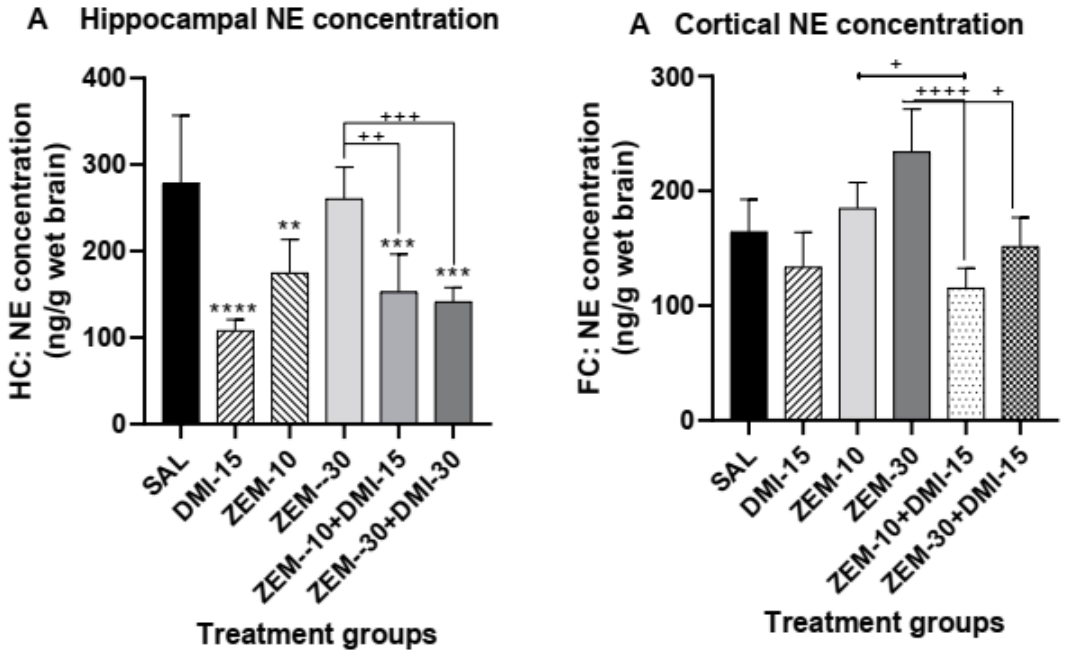


***Fig. C.7**. Effects of saline, low dose DMI (15 mg/kg/day) and escalating doses of ZEM (10, & 30 mg/kg/day) and combination therapies with either ZEM-10 or ZEM-30 on tissue brain NE levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal NE concentration (ng/g) (two outliers identified and removed from the FSL-SAL group (n = 10), one outlier identified and removed from ZEM-30+DMI-15 n = 9) and other groups (n = 12 per group)). (B). Cortical NE concentration (ng/g) (outlier identified and removed from the FSL-SAL group), ZEM-30+DMI-15 group (n = 11) and other groups (n = 12 per group)). Data expressed as means ± S.E.M. with **p ≤ 0.01, ***p ≤ 0.001 ****p ≤ 0.0001, d ≥ 0.8 vs. SAL. $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{+++}p \leq 0.001$, $^{++++}p \leq 0.0001$ vs. ZEM.*

C.2.3.2 Effects of saline, Zembrin® and desipramine alone and as combination therapies on hippocampal and frontal cortical 5-HT levels in FSL rats following 14 days treatment

Hippocampal 5-HT (Fig. C. 8A): Not all data sets were normally distributed. In DMI-15 and ZEM-10+DMI-15 treated rats the hippocampal 5-HT levels were below limit of detection, whereas, in ZEM-30+DMI-15 treated rats, the hippocampal 5-HT levels were detected in two (2) samples only. There were statistically significant differences among the treatment groups (X^2 = 12.68; p = 0.0054).

Cortical 5-HT (Fig. C. 8B): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5,45}$ = 6.99; $p \leq 0.0001$). Compared to the SAL FSL rats, the Tukey's multiple comparison post-hoc test showed no significant differences in cortical 5-HT levels in ZEM-10+DMI-15 (p = 0.1056) rats. Compared to the ZEM-10+DMI-15 treated rats, the Tukey's multiple comparison post-hoc test showed no significant differences in cortical 5-HT levels in FSL rats treated with ZEM-10 (p = 0.8440) and ZEM-30 (p = 0.3558) treated rats.

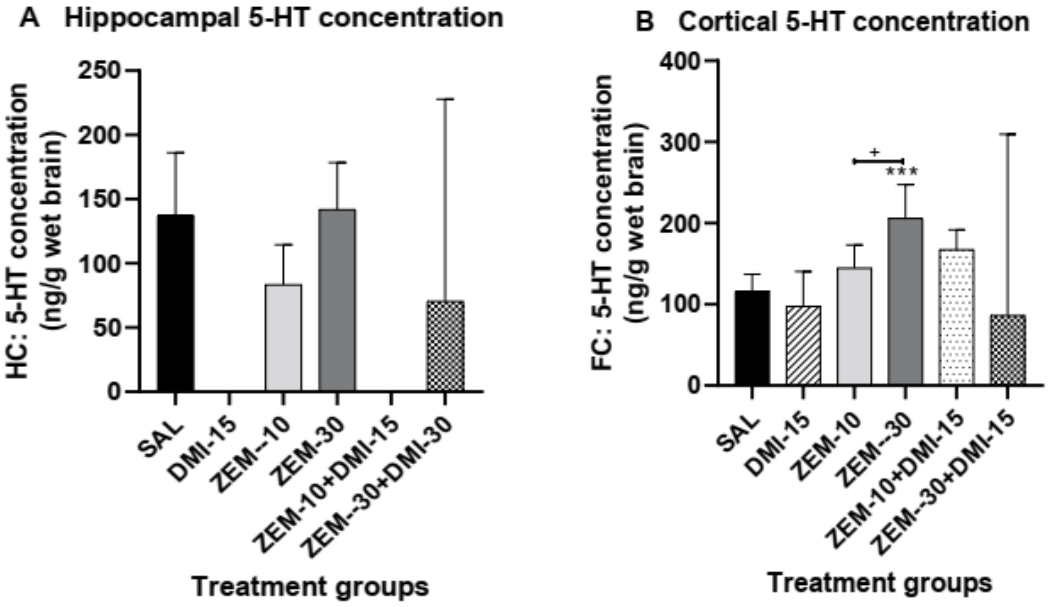


***Fig. C.8**. Effects of saline, low dose DMI (15 mg/kg/day) and escalating doses of ZEM (10, & 30 mg/kg/day) and combination therapies with either ZEM-10 or ZEM-30 on tissue brain 5-HT levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal 5-HT concentration (ng/g) (FSL-SAL group (n = 7), DMI-15 and ZEM-10+DMI-15 samples were below limit of detection (BLOD), ZEM-10 (one outlier identified and removed reducing sample size n = 9), ZEM-30 group (n = 12) and ZEM-30+DMI-15 group (n = 2)). (B). Cortical 5-HT concentration (ng/g) (FSL-SAL group (n = 12), DMI-15 group (n = 3), ZEM-10 group (n = 12), ZEM-30 (n = 12), ZEM-10+DMI-15 group (n = 10) and ZEM-30+DMI-15 (n = 2)). Data expressed as means ± S.E.M. with ***p ≤ 0.001 vs. SAL. $^{+}$p ≤ 0.05 vs. ZEM.*

C.2.3.3 Effects of saline, Zembrin® and desipramine alone and as combination therapies on hippocampal and frontal cortical DA levels in FSL rats following 14 days treatment

Hippocampal DA (Fig. C.9A): All data sets were normally distributed. Although there were statistically significant differences among the treatment groups ($F_{5,32}$ = 2.67; p = 0.0401), these differences did not reach statistical significance according to the the Tukey's multiple comparison post-hoc test.

Cortical DA (Fig. C.9B): All data sets were normally distributed. There were statistically significant differences among the treatment groups ($F_{5,41}$ = 2.62; p = 0.0381). Compared to the SAL FSL rats, the Tukey's multiple comparison post-hoc test showed no significant differences in cortical DA concentration in ZEM-30+DMI-15 (p = 0.9998) treated rats. Compared to DMI-15 treated rats, the Tukey's multiple comparison post-hoc test showed no significant differences in cortical DA concentration in ZEM-30+DMI-15 (p = 0.9997) treated rats. Compared to the ZEM-30+DMI-15 treated rats, the Tukey's multiple comparison post-hoc showed no significant differences in cortical DA concentration in ZEM-10 (p = 0.9619) and ZEM-30 (p = 0.1953) treated rats.

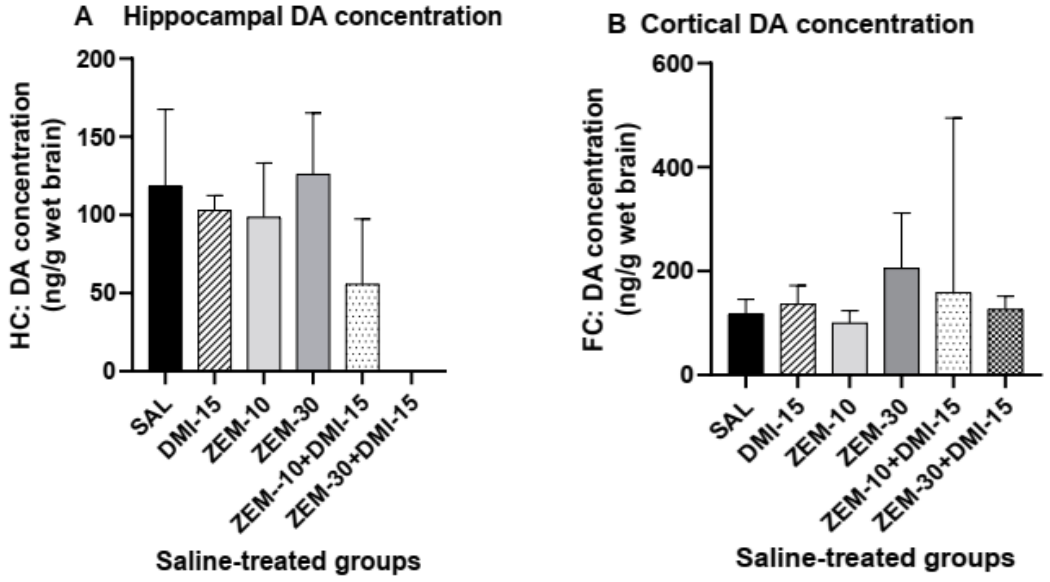


Fig. C. 9*. Effects of saline, low dose DMI (15 mg/kg/day) and escalating doses of ZEM (10, & 30 mg/kg/day) and combination therapies with either ZEM-10 or ZEM-30 on tissue brain DA levels versus FSL saline treated rats following 14 days treatment. (A). Hippocampal DA concentration (ng/g) (FSL-SAL*

group two outliers identified and removed reducing sample size (n=7)), DMI-15 group (n = 11), ZEM-10 (n = 9), ZEM-30 (n = 8), ZEM-10+DMI-15 (n = 2) and ZEM-30+DMI-15 samples were below limit of detection (BLOD). (B). Cortical DA concentration (ng/g) (FSL SAL group, one outlier identified and removed reducing sample size (n= 9), DMI-15 (n = 8), ZEM-10 (one outlier identified and removed reducing sample size (n = 11), ZEM-30 (one outlier identified and removed reducing sample size (n = 9), ZEM-10+DMI-15 (n = 2) and ZEM-30+DMI-15 (n = 8). Data expressed as means ± S.E.M.

C. 3. Discussion

Previous studies reported that variability in some parameters, i.e., temperature changes, sample preparation and the time gap (60 days) between analysis of the samples in an experiment, can significantly affect the sensitivity and reproducibility of the measurements (Klawitter and Hubschmann, 2019). In the current study, the quantification of the monoamines was done in two phases. Monoamine analysed in April 2021 consists of the FSL SAL group and ZEM- (10, 25 and 30 mg/kg/day) monotherapies. This was done because the monoamines were needed for dose selection to be used in the augmentation study, this following failure of ZEM to show antidepressant-like effect in FST as presented in chapter 3 (section 3.2.2). The monoamines in the remaining treatment groups (FRL SAL group, DMI-15, DMI-30, ZEM-10+DMI-15 and ZEM-30+DMI-15 treatment groups) were analysed at the end of the study (August 2021) with more than 60 days in between. This may had affected the monoamines levels in the samples.

Our data showed reduced cortical NE levels in FSL SAL treated rats (Fig. C.1B) and no significant difference in hippocampal NE (Fig. C.1A) levels in FSL SAL treated rats versus FRL SAL treated rats following 14- days interventions. These results are in line with previous studies that reported significantly reduced cortical NE levels in FSL rats (Uys, 2016). Lillethorup *et al.* (2015) revealed upregulated presynaptic α_{-2A} adrenoceptors (auto receptors) in the limbic system in the brain of FSL rats which may support reduced cortical NE levels in FSL rats (Fig. C.1A).

Unexpectedly, we observed increased hippocampal 5-HT levels in FSL rats versus FRL saline rats (Fig. C. 2A) and no significant differences in cortical 5-HT in FSL rats versus FRL SAL rats (Fig. C. 2B. These results are not in line with our earlier study that showed reduced hippocampal 5-HT levels in FSL rats versus FRL rats (Uys, 2016), which reflect the depletion of serotonin theory of MDD (Nutt *et al.,*2008). However, our results correlate with increased locomotor activity in FSL rats versus FRL rats (see chapter 3, Table 1). In fact, Zangen and co-workers (1997) observed significantly increased 5-HT in limbic brain

regions in FSL rats versus FRL rats which may be related to depressive behaviour. Likewise, the DA levels followed the same pattern as with hippocampal and cortical 5-HT levels. We observed significantly reduced hippocampal DA levels in FRL rats versus FSL SAL rats (Fig. C. 3A), and no significant differences in cortical DA levels in FRL rats versus FSL rats (Fig. C. 3B). These findings are not in line with previous study which showed reduced hippocampal DA levels in FSL rats versus the FRL rats (Uys, 2016).

We observed significantly reduced hippocampal and cortical NE in DMI- (15 and 30 mg/kg/day) treated rats versus SAL FSL rats (Fig. C. 4A & B). These results are not in line with previous studies which reported significantly increased hippocampal and cortical NE levels in FSL rats versus FRL rats following sub-chronic treatment with DMI (Overstreet *et al.,*2005). Since our behavioural data in chapter 3 (section 3.3.2, Fig. 3B & C) revealed decreased immobility and increased struggling behaviours in the FST in DMI treated rats versus FSL saline rats, we were expecting DMI to elevate hippocampal and cortical NE levels as the drug blocks reuptake of NE (Ordway *et al.,*2003) with enhanced NA'ergic effects which correlate with increased struggling behaviour in the FST (Mokoena *et al.,*2015; Overstreet *et al.,*2010). However, Mokoena and co-workers (2015) reported no significant change in hippocampal and frontal cortical NE levels in FSL rats treated with DMI in the absence of ozone, despite DMI significantly decreasing immobility and increased struggling behaviours in the FST.

In DMI-15 samples, the hippocampal 5-HT levels were below the limit of detection whereas, in DMI-30 treated rats the hippocampal 5-HT levels were detected in few samples (2) (Fig. C. 5A). We could therefore not draw significant conclusion from such data.

Although hippocampal and cortical DA levels were below the limit of detection in most of the samples, we observed no significant differences in hippocampal DA levels in FSL rats treated with DMI (Fig. C. 6A). We were therefore unable to conclude any further using these results

We observed significantly reduced hippocampal 5-HT levels in ZEM-10 compared to SAL FSL rats (Fig. C. 5A). Acute inhibition of SERT by SSRIs may lead to upregulation and activation of 5-HT_{1A} autoreceptors (Yohn *et al.,*2017). Low dose of ZEM (10 mg/kg/day) may produce partial inhibition of SERT, with low synaptic hippocampal 5-HT level which may stimulate 5-HT_{1A} autoreceptors and further reduce synaptic hippocampal 5-HT

levels. This in fact correlates with reduced locomotor activity in FSL rats treated with low dose of ZEM (10 mg/kg/day), as presented in chapter 3 (Fig. 3A). Most importantly, our data showed increased frontal cortical NE (Fig. C. 4B) but no change in hippocampal NE (Fig. C. 4A) concentration in ZEM-30 compared to SAL FSL rats. Although we have previously shown no such change in either brain region with ZEM (50 mg/kg/day) alone (Gericke, 2019). Coetzee *et al*. (2016) demonstrated that ZEM does present with some noradrenergic effects via NET and MAO-A inhibition, and/or upregulation of VMAT-2. We observed significantly increased cortical 5-HT levels in ZEM-30 treated rats versus FSL saline control (Fig. C. 5B) which correlate with a large effect size increase in the swimming behaviour in the FST in chapter 3 (Fig. 3D). In fact, ZEM- 50 mg/kg/day had a large effect size increase in swimming behaviour in the FST in the earlier acute treatment study (Gericke *et al.,*2022). Thus, high dose ZEM- (30 mg/kg/day) in the current study correlates well with the effective doses for ZEM described in previous acute dose-ranging study in FSL rats (Gericke *et al.,*2022), and further confirm the SERT inhibitory effect of ZEM (Harvey *et al.,*2011).

Although DA levels were detected in few hippocampal samples in all the groups (Fig. C. 6A), we observed no statistically significant differences in the treatment group with regard to hippocampal DA levels (Fig. C. 6A). These findings are in line with earlier studies that showed no significant change in the hippocampal DA levels or failure to detect DA in the hippocampus in most of the samples (Gericke, 2019; Uys, 2016).

Our data showed a significantly decreased cortical DA levels in ZEM-25 treatment group compared to FSL- SAL control (Fig. C.6B). Although our earlier study showed no such change in cortical DA levels following sub-chronic treatment with ZEM (50 mg/kg/day), ZEM-25 (mg/kg/day) may produce partial inhibition of SERT (Harvey *et al.,*2011) and exert some noradrenergic effects via NET and MAO-A inhibition, and/or upregulation of VMAT-2 (Coetzee *et al.,*2016), which may have inhibitory effects on the cortical DA levels in ZEM-25 treated rats. Previous studies demonstrated that an overlap in multiple receptor subtypes may produce excitatory or inhibitory effect in multiple brain regions, including the hippocampus and frontal cortex (Slamlo and Fazlali, 2020; Dean and Keshvan, 2017; Blier, 2001). In fact, Harvey and Slabbert (2014) demonstrated that 5-HT and NA may have inhibitory effects on the DA release via stimulation of the 5-HT_{2c} and α_2- AR heteroreceptors on the ventral tegmental area (VTA) dopaminergic neurons.

In considering the augmentation study, although monoamines were analysed at different times as indicated earlier, we observed reduced hippocampal and cortical NE levels in both combination combinations versus the FSL saline treated rats (Fig. C. 7A). These results are not in line with our earlier studies which showed significantly elevated hippocampal NE with the combination of ZEM (50 mg/kg/day) and ESC (5 mg/kg/day) (Gericke, 2019). We expected to see a dose-dependent increase in hippocampal NE levels in FSL rats treated with combinations of an DMI, an NRI (Mokoena *et al.,*2015; Overstreet *et al.,*2010) and ZEM, a serotonergic agent (Gericke *et al.,*2022). However, this result does correlate with behavioural data in FST (chapter 3, Fig. 7C & D) which showed no significant changes in struggling and swimming behaviour in both combinations versus FSL saline treated rats. Decreased hippocampal NE levels may be related to enhanced 5-HT_{1A} (Yohn *et al.,*2017) and α_2- AR mediated suppression of NE release (Blier, 2001).

We observed no significant difference in hippocampal NE levels in both combinations versus DMI-15 alone treatment (Fig. C. 7A). These results correlate with behavioural data in the FST (chapter 3 Fig. 3B and C) which showed no significant differences in immobility in the FST in DMI-15 rats versus both combinations (chapter 3, Fig. 3B). Likewise, we expected to see a dose-dependent increase in hippocampal NE levels in FSL rats treated with both combinations. Jain (2004) demonstrated that dual action antidepressant produces a better clinical response compared to SSRIs or TCAs alone. In fact, mirtazapine, a noradrenergic and specific serotonergic antidepressant has been suggested to produce a better clinical response compared to a single action antidepressant (Croom *et al.,*2009).

We observed significantly increased hippocampal NE levels in ZEM- 30 (mg/kg/day) treated FSL rats versus both combinations (Fig. C. 7A). These findings are not in line with previous study results which showed no significant differences in hippocampal NE levels in FSL rats treated with ZEM- (50 mg/kg/day) alone versus combination with ESC- (5 mg/kg/day) (Gericke, 2019). In the current study, ZEM-30 alone had no effect on the hippocampal NE (Fig. C. 4A) and 5-HT levels (Fig. C. 5A) which may demonstrate a partial SERT inhibitory action that may indirectly reduce hippocampal NE release (Harvey and Slabbert, 2014). We observed no significant differences in cortical NE levels in ZEM-DMI versus FSL saline treated rats (Fig. C. 6A). These findings are in agreement with earlier study that reported no significant differences in cortical NE levels following

treatment with a combination of ZEM- 50 mg/kg/day and ESC- 5 mg/kg/day (Gericke, 2019). Likewise, DMI-15 treated rats follow the same trend of no change in cortical NE levels versus both combinations. Unexpectedly, we observed significant dose-dependent increases in cortical NE levels in ZEM-10 and ZEM-30 rats versus ZEM-10+DMI-15 treated rats (Fig. C. 7B). These findings are not in line with the previous study which report no significant change in cortical NE levels in ZEM-50+ESC-5 combination versus either ZEM or ESC alone (Gericke, 2019). Although the time factor may had affected the results, ZEM- partial inhibitory actions on SERT may indirectly decreased the cortical NE levels in combinations treated FSL rats compared to the monotherapies alone.

SERT inhibition by SSRIs may elevate 5- HT levels in the hippocampus and cortex and so enhance serotonergic activity (Gericke *et al.,*2022), which correlates with increased swimming behaviour in the FST (Cryan *et al.,*2005). In the current study, the hippocampal and cortical 5-HT levels were below the limit of detection or detected in few samples in most treatment groups which compromises the results and hinders intra-group comparisons. In fact, the hippocampal 5-HT levels in DMI-15 and ZEM-10+DMI-15 were below the limit of detection, whereas in ZEM-30+DMI-15 rats' samples, hippocampal 5-HT levels were only detected in two (2) samples. Likewise, cortical 5-HT levels follow the same trend with only two samples detected in DMI-15 treated rats, whereas in ZEM-10+DMI-15 group, cortical 5-HT levels were detected in ten (10) sample which allows comparison with FSL saline and ZEM- (10 and 30 mg/kg/day) (Fig. C. 8B). We observed no significant changes in cortical 5-HT levels in ZEM-10+DMI-15 treated FSL rats versus both FSL saline and ZEM- (10 and 30 mg/kg/day) treated rats (Fig. C.8B). These findings are in agreement with the previous study which showed no significant difference in cortical 5-HT in FSL rats following treatment with combination of ZEM- 50 mg/kg/day and ESC- 5 mg/kg/day (Gericke, 2019). Likewise, the DA results followed the hippocampal and cortical 5-HT levels, hence we failed to compare the effects of combinations versus monotherapies (Fig. C. 9A& B).

Conclusion

In conclusion the monoamine results were obscure and could not support the behavioural data. The time analysis, the varying basal levels prevented comparison with the earlier analysed groups. Moreover, many samples in the augmentation and DMI groups were below limit of detection. Therefore, the monoamine data presented here could not be

presented in chapter 3. Both ZEM- and DMI can significantly alters hippocampal and frontal cortical monoamines mainly via actions on the SERT or NET or indirectly via 5-HT_{1A} ,5-$HT_{2A/C}$ and α_2- AR receptors which may underlie the unpredictable mechanisms involved and explain the complex results obtained.

Consequently, the monoamine data for the augmentation studies were removed from chapter 3, with only the validation studies employing monoamine analysis to inform on dose selection of ZEM, together with behaviour, and presented in Chapter 3.

References

Blier, P. 2001. Crosstalk between the norepinephrine and serotonin systems and its role in the antidepressant response. *Journal of Psychiatry and Neuroscience*, 26: S3-10

Charnay, Y., Leger, L. 2010. Brain serotonergic circuitries. *Dialogue in Clinical Neuroscience*, 4(12) : 471-487

Coetzee, D.D., Lopez, V.V., Smith, C. 2016. High –Mesembrine Sceletium extract (Trimesemine™) is a monoamine releasing agent, rather than only a selective serotonin reuptake inhibitor. *Journal of Ethnopharmacology*, 177: 111-116

Croom, K.F., Perry, C.M., Plosker, G., L. 2009. Mirtazapine. *CNS Drugs*, 23 (5): 427-452

Cryan, J.F., Valentino, R.J., Lucki, I. 2005. Assessing substrates underlying the behavioural effects of antidepressants using the modified rat forced swimming test. *Neuroscience and Biobehavioural Reviews*, 29 (4-5): 547-569

Gericke, J. 2019. Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment. North-West University (South Africa), Potchefstroom Campus (MSc dissertation)

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, A.M., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:114550

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P. 2011. Pharmacological actions of South African Medicinal and Functional plant *Sceletium tortuosum* and its principle alkaloids. *Journal of Ethnopharmacology*,137(3): 1124-1129.

Harvey, B.H., Slabbert, F.N., 2014. New insight on the antidepressant discontinuation syndrome. *Human Psychopharmacology; Clinical and Experimental*. 299 (6): 503-516

Jain, R. 2004. Single-actions versus dual action antidepressants; Primary care companion. *Journal of Clinical Psychiatry*, 6(1): 7-11

Jung-Klawitter., Hubschmann, O., K. 2019. Analysis of catecholamines and pterins in born errors of monoamines neurotransmitter metabolism-from past to future. *Cells*, 8(8): 867

Lieberman, D.Z., Massey, S.H. 2009. Desvenlafaxine in major depressive disorder: an evidence-based review of its place in therapy. *Core Evidence Dovepress*, 4: 67-82

Lillethorup, T.P., Iversen, P., Wegener, G., Doudet, D.J., Landau, A.M. 2015. Adrenoceptors binding in Flinders Sensitive Line compared with Flinders Resistance Line and Sprague Dawley rats. *ACTA Neuropsychiatrica*, 27(6): 345-352

Maletic, V., Eramo, A., Gwin, K., Offord, S.J and Duffy, R.A. 2017. The role of norepinephrine and its adrenergic receptors in the pathophysiology and treatment of major depressive disorders and schizophrenia. A systematic review. *Frontiers in Psychiatry*, 8 (42): 1-12

Mokoena, M.L., Harvey, B.H., Viljoen, F., Ellis, S.M., Brink, C.B. 2015.Ozone exposure of Flinders Sensitive Li ne rats is a rodent translational model of neurobiological oxidative stress with relevance for depression and antidepressant response. *Psychopharmacology*, 232 (1): 2921-2938

Nutt, D. 2008. Relation of neurotransmitters to the symptoms of major depressive disorder. *The Journal of Clinical Psychiatry*, 69(1): 4-7

Olguin, H.J., Guzman, D.C, Garcia, E.H., Mejia, G.B., 2016. The role of dopamine and its dysfunction as a consequence of oxidative stress. *Oxidative Medicine and Cellular Longevity*, 9730467. https://doi.org/10.1155/2016/9730467 Date of access: 24 Sep. 2020

Ordway, G.A., Schenk, J., Stoclmeier, C.A., May, W., and Klimek, V. 2003. Elevated agonist binding to α_2 adrenoceptors in the locus coeruleus in major depression. *Society of Biological Psychiatry*, 53(4): 315-323

Overstreet, D.H., Friedman, E., Mathe, A.A., Yadid, G. 2005. The Flinders Sensitive Line rat; A selectively bred putative animal model of depression. *Neuroscience and Bio-behavioural Reviews,* 29(4-5): 739-759

Overstreet, D.H., Naimoli, N.M., Griebel, G. 2010.Saredutant, an NK2 receptor antagonist, has both antidepressant-like effects and synergizes with desipramine in an animal model of depression. *Pharmacology, Biochemistry and Behaviour*, 96(2): 206-210

Sarris, J., Marx, W., Ashton, M.M., Ng, C.H., Psych, M., Galvao-coelho, N., Ayati, Z., Zhang, Z., Kasper, S., Ravindra, A., Harvey, B.H. 2021. Plant-based medicines (Phytoceuticals) in the treatment of psychiatric disorders; a meta-review of meta-analyses of randomized controlled trials. *The Canadian Journal of Psychiatry*, 66(10):1-14

Slamloo, Y.R., Fazlali, Z. 2020. Dopamine and noradrenaline in the brain; overlapping or dissociate function?. *Frontiers in Molecular Neuroscience*, 12(334): 1-8

South African Depression and Anxiety Group (SADAG). 2020. Depression, treatment, and referral guide. https://www.sadag.org/. Date of access: 24. Jan.2021

Treadway, M.T., Michael, L.W., Dillon, D.G, *et al.*2015. Illness progression, recent stress and morphometry of hippocampal subfield and medial prefrontal cortex in major depression. *Society of Biological Psychiatry*, 77(3): 285-294

Uys, M.M. 2016. Bio-behavioural characterization of a selective α2c-receptor antagonist in animal model of depression and schizophrenia. North-West University (South Africa),. Potchefstroom campus (PhD Thesis)

Yohn, C.N., Gergues, M.M., Samuels, B.A. 2017. The role of 5-HT receptors in depression. *Molecular Brain*, 10(28): 2-12

Zangen, A., Overstreet, D.H., Yadid, G. 1997. High serotonin and 5-Hydroxyindoleacetic Acid levels in Limbic brains regions in a rat model of depression normalization by chronic antidepressant treatment. *International Society for Neurochemistry*, 69(6): 2477-24

ADDENDUM D

Enzyme -Linked -Immunosorbent-Assay kit for Phosphodiesterase-4BcAMP specific-PDE4B-SEF642Ra

D.1 Brief role of PDE4B in MDD and ADs

Increased PDE4B activity due its overexpression in the hippocampus and prefrontal cortex has been associated with decreased cAMP concentration and/or reduced expression of cAMP response element-binding proteins (CREB) which lead to decreased BDNF expression in the prefrontal cortex and hippocampus significantly leading to atrophy and structural changes in the hippocampus and prefrontal cortex which manifests mostly as impaired learning and memory (Wang *et al.,*2017; Itoh *et al.,*2004;Zhu *et al.,*2001). Furthermore, increased PDE4 activity lead to neuroinflammation via overactivation of the microglia cells which can leads to increase the production of the proinflammatory cytokines such as IL-6 and decreased production of the anti-inflammatory cytokine such as IL-10 (Wang *et al.,*2017). Harvey and colleagues showed in vitro inhibition of PDE4B by standardized extract of *Sceletium tortuosum* plant (Zembrin®) (Harvey *et al.,*2011). Therefore, it was imperative to further assayed the activity of Zembrin® alone and combined with desipramine to correlate with behavioural results to divulge the promising antidepressant of Zembrin® as a potential augmenter in the treatment of MDD.

D.2. The PDE4B ELISA KIT

The hippocampus and frontal cortex samples were analysed using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cloud-Clone Corp.) for phosphodiesterase4B, cAMP specific (PDE4B) SEF642Ra for in vitro quantitative tissue homogenates according to procedures described by the manufacturer.

Specification

- Sensitivity: 0.065ng/ml
- Detection Range: 0.156-10ng/ml
- Specificity: excellent specificity for detection of PDE4B. No significant cross-reactivity or interference between PDE4B and analogues was observed

Test principle

The microtiter plate provided in this kit has been pre-coated with an antibody specific to PDE4B. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to PDE4B. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain PDE4B, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in colour. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of PDE4B in the samples was then determined by comparing the O.D. of the samples to the standard curve.

D3. Reagents and materials

Table D.1 of reagents and material provided with the ELISA KIT

Reagents	**Quantity**	**Reagents**	**Quantity**
	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20ml
Detection Reagent A	1×120µl	Assay Diluent A	1×12ml
Detection Reagent B	1×120µl	Assay Diluent B	1×12ml
TMB Substrate	1×9ml	Stop Solution	1×6ml
Wash Buffer (30 × concentrate)	1×20ml	Instruction manual	1

Other material used but not provided with the KIT

- Microplate reader with 450 ± 10nm filter.
- Precision single or multi-channel pipettes and disposable tips.
- 2 ml Eppendorf Tubes for diluting samples.
- Deionized or distilled water.
- Absorbent paper for blotting the microtiter plate.
- Container for Wash Solution

D4. Sample preparation

1. The day before the assay, hippocampal and frontal tissues were thawed on ice, rinsed with ice-cold phosphate buffer saline (PBS-0.01mol/L, pH 7.0-7.2), weighed and homogenised (in 5-10ml of PBS) with an ultrasonic sonicator for cell disruption.
2. The homogenates were centrifugated for 5 min at 10 000×g and the supernate and stored at -80^{o}C until the day of analysis.

D5. Reagent preparation

1. During the day of the analysis, the reagents and samples were brought to room temperature (18-25^{o}C)
2. 600ml Wash solution (1x) was prepared by diluting 20ml of wash solution concentrate (30x) with 580ml of distilled water.
3. 15 min before the assay, the standard stock solution (10ng/ml) was prepared as follows: Standard was reconstituted with 1.0ml of standard diluent, gently shaken and let for 10 min at room temperature. Seven tubes were initially filled with 0.5ml standard diluent and Serial dilutions were carried from standard diluent solution in a double dilution (10ng/ml,5ng/ml,2.5ng/ml,1.25ng/ml,0.625ng/ml,0.312ng/ml,0.156ng/ml and 0ng/ml) according to the picture shown below

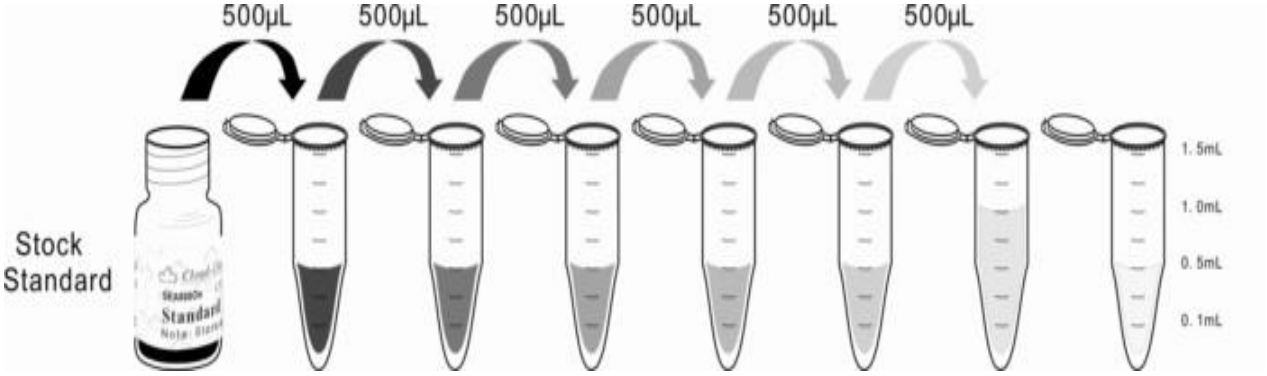


Fig. D.1. Double series dilution according to the ELISA kit manual.

4 The detection working solution AB were prepared. Firstly, the stock detection reagents were centrifuged. The working solutions were diluted with 100x (1,100) concentrated detection AB.

D6. Assay procedure

1. 100 µl of each dilution standard was added in the first two column of the wells
2. A 100 µl of each dilution from each sample was added into the appropriate wells. In this assay, 60 samples from the hippocampus were added in duplicate to 3rd and 4th columns and later followed by 60 samples from frontal cortex which were also added in duplicate in the next columns. The wells were covered with the plate sealer and incubated for 2 h at 37 ^{o}C.
3. The liquid from well was removed
4. A 100 µl of detection reagent A working solution was added to individual well, covered with plate sealer and incubated for 1 h at 37 ^{o}C
5. The well microplates were aspirated and washed three times with 350µL of 1× Wash Solution and allowed to stand for 2 min to drain the solution from the well. The remainder of the solution was removed by snapping the plate on the absorbent paper. The wash buffer was removed by decanting followed by drying on the absorbent paper.
6. 100 µl of detection B working solution was added to each well, covered with the plate sealer and incubated for 30 min at 37 ^{o}C.
7. The microplates were aspirated and washed again for five (5) times as elaborated in step 5 above.

8. 90 µl of TMB substrate solution was added to each well, covered with new plate sealer and incubated for 20 min at 37 ^{o}C. The plates were protected from light to minimise colour changes to blue due to addition of substrate solution.
9. 50 µl of stop solution was added to each microplate well, and colour changes were careful observed to minimise possibility for errors.
10. The microplate reader was preheated 15 min prior measuring the OD (at 450 nm).

D.7 Calculation of Results.

A standard curve was constructed with the mean O.D and concentration of each standard. The computer software was used to ensure a best curve was drawn through the points on the graph. Three samples were required as 141 samples were analysed. Fig D.2 below showed a curve produced from various concentration with PDE4B concentration.

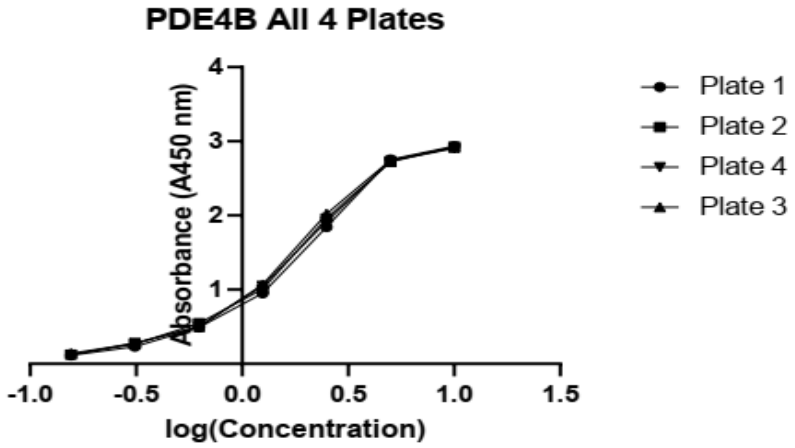


Fig. D.2. Standard curve showing rat hippocampal and cortical PDE4B levels

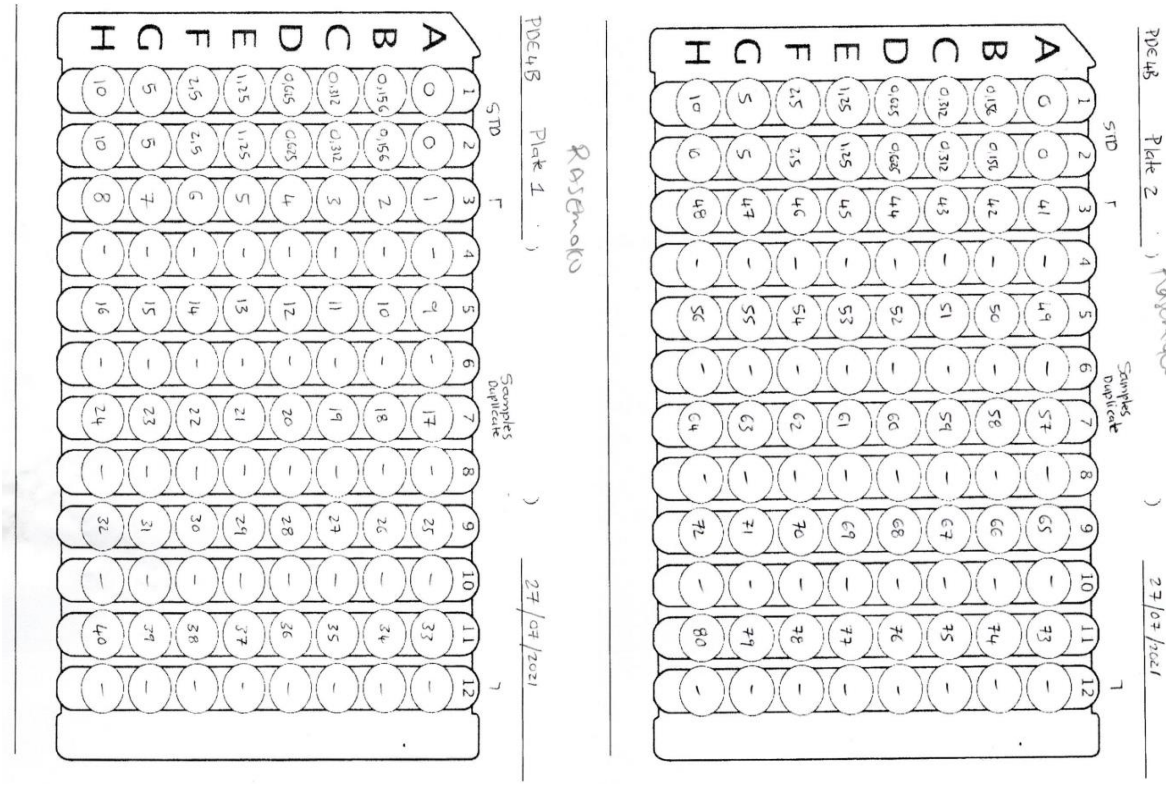
Allocation of the samples on the wells in four plates (plate 1-4) (Fig. D.3)

Hippocampal samples

- 1-9- FSL-SAL
- 10-21-ZEM-10
- 22-33-ZEM-30
- 34-45-DMI-15
- 46-57-ZEM-10+DMI-15
- 58-69-ZEM-30+DMI-15

Cortical (frontal cortex) samples

- 70-81-FSL-SAL
- 82-93-ZEM-10
- 94-105-ZEM-30
- 106-117-DMI-15
- 118-129-ZEM-10+DMI-15
- 130-141-ZEM-30+DMI-15



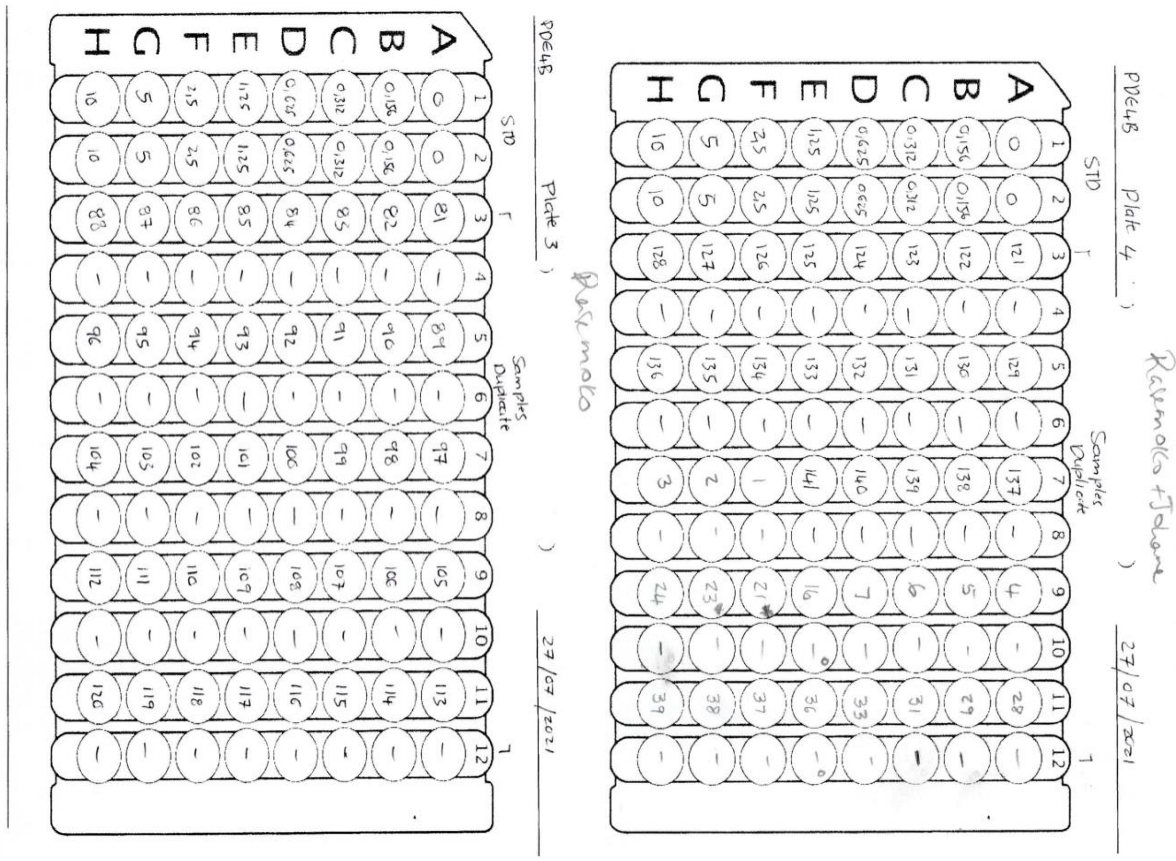


Fig. D.3. Four plates (Plate1-4) sample layout of the rat hippocampal and cortical PDE4B levels with the sample numbers corresponding to the samples illustrated above.

References

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P. 2011. Pharmacological actions of South African Medicinal and Functional plant sceletium tortuosum and its principle alkaloids. *Journal of Ethnopharmacology*,137(3): 1124-1129

Itoh, T., Tokumura, M., Abe, K. 2004. Effects of Rolipram, a phosphodiesterase-4 inhibitor in combination with imipramine on depressive behaviour (CRE-binding activity and BDNF level) in learned helpless rats. *European Journal of Pharmacology*, 498(1-3): 135-142

Wang, C., Wang, Z., Li, M., Li, C., Yu, H., Zhou, D., Chen, Z. 2017, 'Reducing Neuroinflammation in Psychiatric Disorders: Novel Target of Phosphodiesterase 4 (PDE4) and Developing of the PDE4 Inhibitors', in G. E. A. Abreu (ed.), Mechanisms of Neuroinflammation, *IntechOpen*, London. 10.5772/intechopen.69154. http://www.intechopen.com/books/mechanisms-ofneuroinflammation Date of access: 06. Jun.2021

Zhu., J., Mix., E., Winblad., B. 2001. The antidepressant and anti-inflammatory effects of Rolipram in the central nervous drug review.*CNS Drugs Review*, 7(4):387-398

ADDENDUM E

HIGH-PERFOMANCE LIQUID CHROMATOGRAPHY-ELECTROCHEMICAL DETECTION (HPLC-ECD); METHODOLOGY MONOAMINE

E.1. Methodology

The brain samples from the hippocampal and frontal cortex sub-chronic study groups were analysed with High Performance Liquid Chromatography coupled with electrochemical detection (ECD) using a validated method as previously described (Viljoen *et al.,*2018).

E.2. Preparation of internal standard solution

An internal standard as a chemical substance added in a constant amount to sample or blank for calibration during analysis (Oliveira *et al.,*2010). In this study, 5-hydroxy-Nω-methyptryptamine oxalate (5-HMT), a metabolite of serotonin was used as internal standard solution. (5-HMT) was prepared based on calculated dilution factor from the standard (5-HMT) stock solution (100ug/ml) and the solution A (0.1 M perchloric acid;0.5mMsodium metabisulphite; 0.3Mm ethylenediaminetetraacetic acid disodium salt) to produce working internal standard solution containing 1500 ng/ml as a final concentration.

E.3. Sample preparation

- On the day of the analysis, the hippocampal and frontal cortex samples were thawed in ice, weighed and 1 ml of Solution A was added to the sample in the Eppendorf tubes
- The cells were ruptured by sonication (2 x 12s, at an amplitude of 14u) and placed on ice for 20 min in order to allow for completion of perchlorate precipitation of proteins and extraction of monoamines
- The samples were centrifuged at 14 000 rcf (relative centrifugal force) for 25 minutes at 4 ^{o}C.
- Supernatant from each sample was pipetted into 2 ml amber Eppendorf tubes and one drop of 10M potassium acetate was added to each sample to adjust pH to 5.

- The sample solution vortexed to ensure complete mixing of the sample and buffer
- 200 µl of tissue sample extract was added to 1.5 ml Eppendorf tubes and 20 µl of internal standard was added to each sample in the Eppendorf
- The sample solution was vortexed and centrifuged at 14 000 rcf for 5 minutes at 4 ºC
- Samples finally transferred to the UHPLC vial insert
- All excess tissue samples in the 2 ml amber Eppendorf tubes were stored in the -80ºC.

E.4. HPLC conditions

E.4.1 Instrumentation

The instrumentation used, consisted of an Ultimate 3000 UHPLC system, equipped with an ISO-3100SD isocratic pump and WPS-3000TSL analytical autosampler, coupled to an ECD-3000RS rapid separation electrochemical detector with 2-Channel 6011RS ultra Coulometric Analytical Cell and Chromeleon® chromatography management system version 7.2 (purchased from Thermo Fisher Scientific, Waltham, MA USA).

E.4.2. Preparation of mobile phase

The contents of the mobile phase include:

- 0.1 M sodium formate buffer
- 5 mM sodium 1-heptane-sulfonate
- 0.17 Mm ethylenediaminetetraacetic acid (EDTA) disodium salt
- 5% v/v acetonitrile

The mobile phase was filtered through 0.2 µm nylon filter (Agela Technologies) and pH was adjusted to ± 4.0 with ortho-phosphoric acid.

E.4.3. Instrumentation settings

- 20 ul injection
- 1 ml/ml flow rate
- 50 min run time

Electrochemical detection setting:

- ✓ Cell potential settings:
- Test electrode 1 (E1); -150mV (to eliminate background noise)
- Test electrode 2 (E2); + 650mV (to analyse the monoamines)
- Guard Cell (EGC); +350mV
- Detection range; 500nA
- Filter;0.5 sec
- Offset:0%
- Signal output; 0.1v
- ✓ 20 Hz data collection rate

C.5. Standard calibration curve for coefficient calculation

Table E.1. Standard calibration curve

Monoamine/ Metabolite	**Coefficient of Determination(R^2)**	**Standard curve y= mx+ c**
NE	**R^2= 0.9999**	**y= 0.3526x-0.4546**
DA	**R^2=0.9997**	**y= 0.482x-1.2549**
5-HT	**R^2=0.9998**	**y= 0.4905x-0.894**
5-HIAA	R^2=0.9994	y= 0.4307x-1.6948
DOPAC	R^2=0.9993	y= 0.3404x-1.1835
HVA	R^2=0.9991	y= 0.3321x-1.1946
3-MT	R^2=0.9982	y= 0.3331x-0.1372

NB: For this dissertation, NE, 5-HT (presented under manuscript) and DA (addendum B) were used.

E.6. Chromatograms

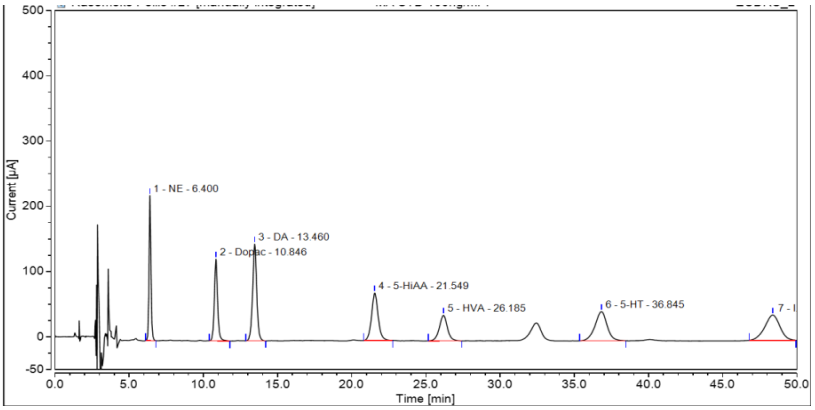


Fig. E.1. Chromatogram Illustrating monoamines internal standard peaks for first time data analysis

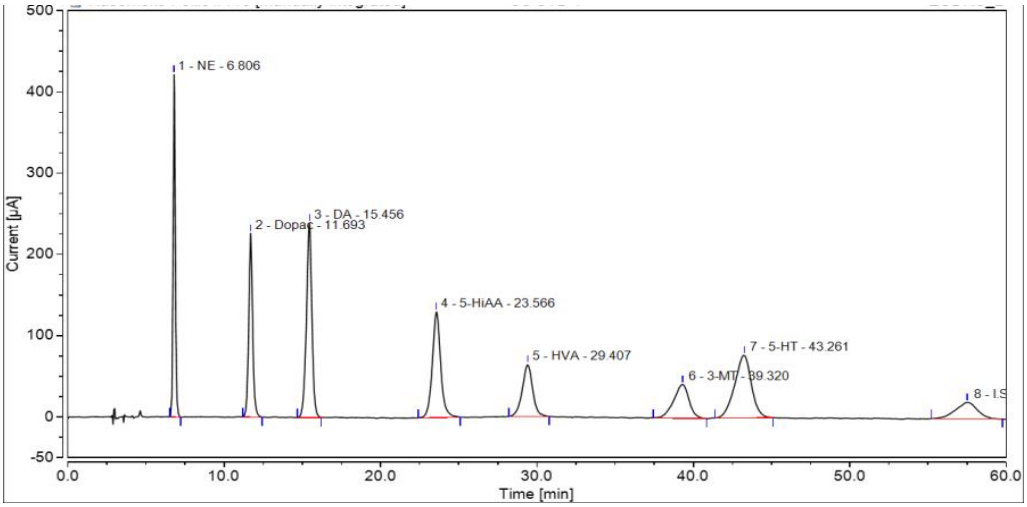


Fig. E.2. Chromatogram Illustrating monoamines internal standard peaks for second time data analysis

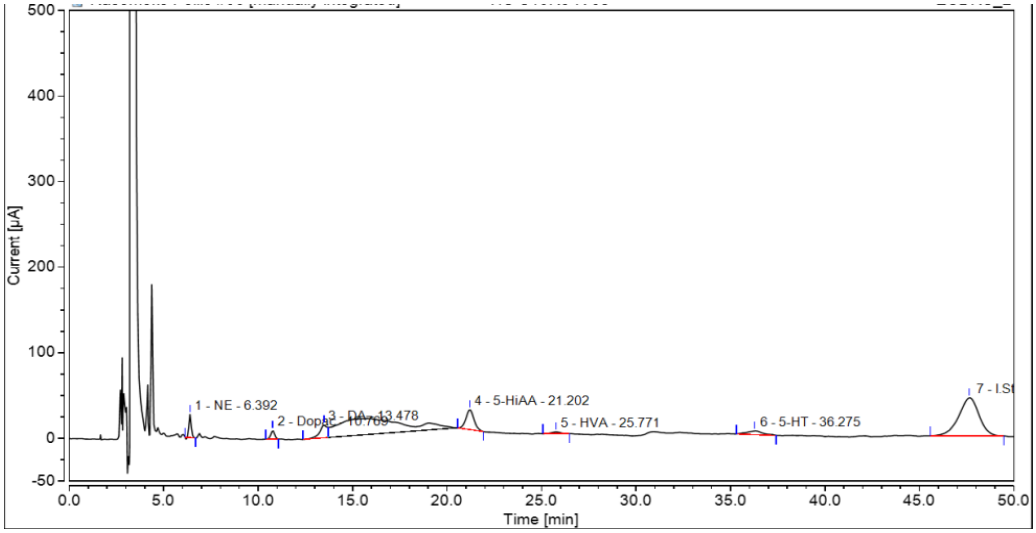


Fig. E.3. Chromatogram Illustrating cortical monoamines peaks in ZEM-30 treated rat sample

References

Oliveira, E., Muller, E., Abad, F., Dallarosa, J. 2004. Internal standard versus external standard calibration: an uncertainty case study of a liquid chromatography analysis. *Quimica Nova*, 33(4): 984–987

Viljoen, F.P., Du Preez, J.L., Wessels, J.C., Aucamp, M.E. 2018. HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples. *Pharmazie,* 73 (10): 563–569

ADDENDUM F

Chromatographic fingerprint of Zembrin®

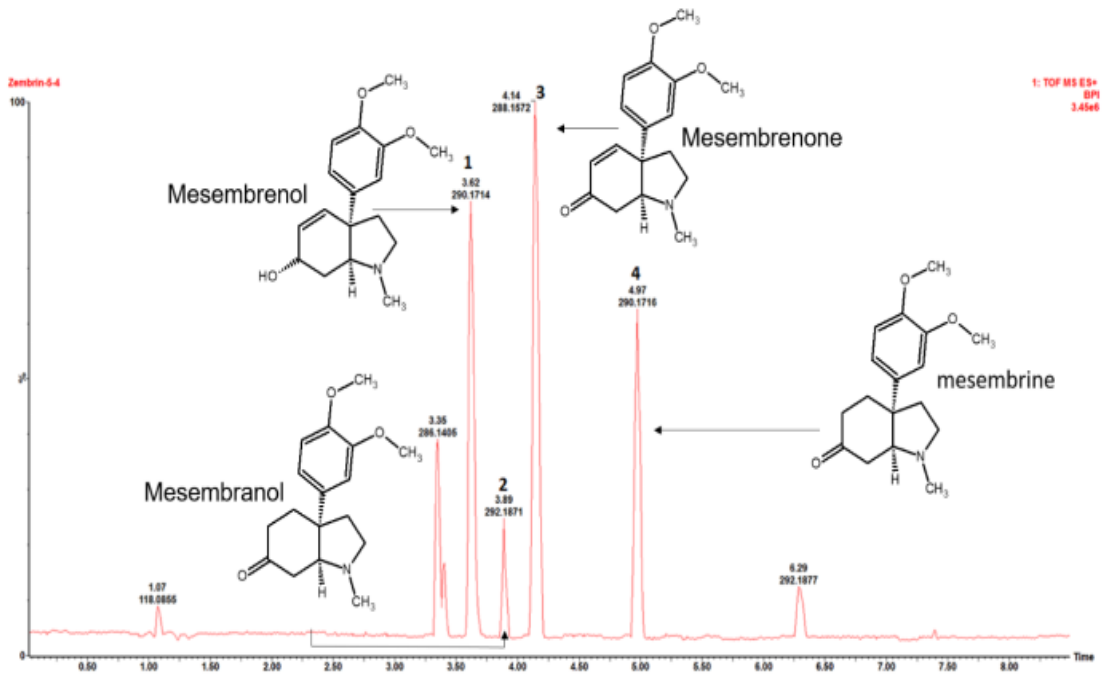


Fig. F.1. chromatographic fingerprint of Zembrin® (Adapted from Gericke *et al.*,2022)

F.1. Chromatogram of Zembrin®

Fig. F.1. below represents the UPLC-MS-PDA chromatographic fingerprint of Zembrin® used in the current study, with four main alkaloids (mesembrenol, mesembranol, mesembrenone and mesembrine) peaks identified. The concentration of the four alkaloids were as follows; mesembrenone (47.9%), mesembrenol (32%), mesembrine (13.2%) and mesembranol (6.8%) of the total alkaloids respectively (Gericke *et al.*,2022). The present Zembrin®seemed to have high mesembrine content (Gericke *et al.*,2022).

References

Gericke, J., Lekhooa, M., Steyn, S.F., Viljoen, A.M., Harvey, B.H. 2022. An acute dose-ranging evaluation of the antidepressant properties of Sceletium tortuosum (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat. *Journal of Ethnopharmacology*, 284:11455

ADDENDUM G

ANIMCARE ANIMAL RESEARCH ETHICS APPROVAL LETTER

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

29 October 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 29/10/2020, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats
Principal Investigator/Study Supervisor/Researcher: Dr M Lekhooa
Student: R Polile - 30980232

Ethics number:

N	W	U	-	0	0	5	2	0	-	2	0	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 22/10/2020
Expiry date: 30/10/2021

Risk: Category 4

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of October annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

ADDENDUM H

Please note! This is a sample monitoring sheet only, which needs to be specifically adjusted as necessary to monitor animal welfare during the project it is designed for. **Instructions:** Keep one sheet per animal in the monitoring file at the relevant Vivarium room's door *(never to leave the Vivarium premises)* and use until the sheet is full, or the study ended. Now scan a copy for your own electronic record and hand in the original at the Vivarium office for record- and safekeeping. **Available at** http://health-sciences.nwu.ac.za/healthethics

Study title:	Title: Antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats			**Year:**	2020/2021
Ethics no.:	**No.NWU-00520-20-A5**	**Project head:**	Name: Dr Makhotso Lekhooa	**Observer / student:**	Name: Rasemoko Polile

Parameter		**Score**	1 Date / Time	2 Date / Time	3 Date / Time	4 Date / Time	5 Date / Time	6 Date / Time	7 Date / Time	8 Date / Time	9 Date / Time	10 Date / Time	11 Date / Time	12 Date / Time	13 Date / Time	14 Date / Time	15 Date / Time	16 Date / Time	17 Date / Time	18 Date / Time	19 Date / Time	20 Date / Time	21 Date / Time
Appearance	Normal	0																					
	Lack of grooming	1																					
	Dull coat, ocular/nasal discharge	2																					
	Piloerection, hunched up	3																					
Food & water intake	Normal	0																					
	<5% weight loss	1																					
	Noted intake, 5-15% weight loss	2																					
	No food or water intake	3																					
Clinical signs	Normal	0																					
	Slight changes	1																					
	Respiratory increase ↑ 30%	2																					
	Respiratory increase ↑ 50%	3																					
Natural behaviour	Normal	0																					
	Minor change	1																					
	Less mobile alert, isolated	2																					
	Vocalisation, restless or still	3																					
Provoked behaviour	Normal	0																					
	Minor depression	1																					
	Moderate change	2																					
	Reacts violently/weakly, precomatose	3																					
	TOTAL SCORE	0-15																					
Project-specific	Criterion 1																						
	Criterion 2																						
	Criterion 3																						
	Criterion 4																						
Other	Observation and/or comment (tick box ☐ if written on reverse side)		1 ☐ revers e	2 ☐ revers e	3 ☐ revers e	4 ☐ revers e	5 ☐ revers e	6 ☐ revers e	7 ☐ revers e	8 ☐ revers e	9 ☐ revers e	10 ☐ revers e	11 ☐ revers e	12 ☐ revers e	13 ☐ revers e	14 ☐ revers e	15 ☐ revers e	16 ☐ revers e	17 ☐ revers e	18 ☐ revers e	19 ☐ revers e	20 ☐ revers e	21 ☐ revers e
Decision	✓ = normal / ? = monitor carefully / ! = seek advice / × = intervene immediately																						
Signature *(please sign/initialise with each observation per column)*																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

ADDENDUM i

CONGRESS PROCEEDINGS

The addendum contains an abstract presented virtual at the 7th All African Congress of Basic and Clinical Pharmacology (ACP 2021) accompanied by the proof of attendance.

The results of the study were presented as an oral presentation under natural product research in a virtual meeting held on the 15th -17th September 2021 by KeSoBAP.

I.1. ABSTRACT

Evaluating the effects of Zembrin® alone and combined with desipramine on PDE4B in Flinders Sensitive Line rats: A bio-behavioural study.

Abstract number: ACP-A-08-21-Mb+1

Presentation type: Oral

Topic :Natural product research

Rasemoko P. Polile[1], 'Makhotso Lekhooa[1,2*], Brian H. Harvey[1,3], Stephan F. Steyn[1], Johane Gericke[1]

[1] Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa

[2] Pre-Clinical Drug Development Platform, North-West University, Potchefstroom, South Africa

[3]SAMRC Unit on Risk and Resilience in Mental Disorders, Department of Psychiatry and Mental Health and Neuroscience Institute, University of Cape Town, Cape Town, South Africa

***Corresponding author:** Dr. Makhotso Lekhooa (Makhotso.Lekhooa@nwu.ac.za)

Tel: (+27) 18 299 2270

BACKGROUND: Comorbid anxiety disorders in patients with major depressive disorder often lead to treatment resistant depression. Augmentation with the standardized extract of *Sceletium tortuosum* (Zembrin®) may be a promising novel augmentation therapy for patients with

depression, who are poor responders to standard antidepressant regimens. Therefore, investigation into its therapeutic potential alone and in combination with a known standard antidepressant, such as desipramine (DMI) is required.

OBJECTIVES: to assess the dose-related antidepressant-like and anxiolytic-like effects of Zembrin® (ZEM) in the Flinders Sensitive Line (FSL) rats alone and as an adjunctive treatment with low dose DMI.

METHODOLOGY: Eight groups (*n*=12) of male FSL were treated with saline (control group), a 3-tier dose of ZEM (10, 25 & 30 mg/kg/day), and a 2-tier dose of DMI (15 & 30 mg/kg/day) for 14 days via oral gavage. To assess the augmentation potential of ZEM (10 and 30 mg/kg/day), ZEM was combined with a low dose of DMI. Following treatment, depressive- and anxiety-like behaviour was assessed in the forced swim test (FST), open field test (OFT) and elevated plus maze (EPM), followed by analyses of cortical and hippocampal phosphodiesterase 4B (PDE4B) levels.

RESULTS: Depressive behaviour, and cortical and hippocampal PDE4 expression were significantly increased in FSL rats, while neither ZEM (10 and 30 mg/kg/day) nor DMI (15 mg/kg/day) monotherapies reduced cortical and hippocampal PDE4B expression, although DMI was antidepressant in the FST. However, the combination of either dose of ZEM plus DMI significantly reduced hippocampal PDE4B expression, whereas only the high dose ZEM-DMI combination significantly increased cortical PDE4B expression.

CONCLUSION: We were unable to confirm the antidepressant and anxiolytic actions of ZEM in this study, either alone or in combination with DMI. These results may be model-related. That said, studies on PDE4 expression do lend some support for the augmentation potential of ZEM as an add-on therapy for patients responding poorly to standard antidepressants, especially where specific actions on PDE4B are required. However, further work is needed.

Keywords: **Zembrin®, PDE4B, Major depressive disorder, Flinders Sensitive line rat**

I.2. Proof of attendance

LETTER OF CONSENT

PCDDP
DST/NWU Preclinical Drug Development Platform

science & technology
Department: Science and Technology
REPUBLIC OF SOUTH AFRICA

9 Dec 2021

The Director: Higher Degrees Administration
North-West University
11 Hoffman Street
Potchefstroom
2520
SOUTH AFRICA

Dear Sir/Madam,

RE: CO-AUTHOR PERMISSION TO SUBMIT CHAPTER 3 OF THIS DISSERTATION FOR EXAMINATION PURPOSES

Hereby I, Dr 'Makhotso Lekhooa, an Supervisor and a co-author of the manuscript titled *" Evaluating the antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats: A bio-behavioural study"*, which is included in Chapter 3 of this dissertation, give permission for this work to be submitted for examination purposes.

Regards,

Dr. M. Lekhooa (Supervisor)

PCDDP, North-West University, Potchefstroom Campus, South Africa

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E Mail brian.harvey@nwu.ac.za

The Director: Higher Degrees Administration
North-West University
11 Hoffman Street
Potchefstroom
2520
SOUTH AFRICA

10 December 2021

Dear Sir/Madam,

RE: CO-AUTHOR PERMISSION TO SUBMIT CHAPTER 3 OF THIS DISSERTATION FOR EXAMINATION PURPOSES

Hereby I, 'Prof. Brian H. Harvey, a co-supervisor and co-author of the manuscript titled *"Evaluating the antidepressant activity of Zembrin® alone and combined with desipramine in Flinders Sensitive Line rats: A bio-behavioural study"*, which is included in Chapter 3 of this dissertation, give permission for this work to be submitted for examination purposes.

Regards,

Prof. B.H. Harvey (Co-supervisor)

BPharm, BSc Hons (Pharmacol) MSc; PhD (Pharmacology)

North-West University, Potchefstroom Campus, South Africa

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Pharmacology Department

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10 December 2021

Dear Sir/Madam,

RE: CO-AUTHOR PERMISSION TO SUBMIT CHAPTER 3 OF THIS DISSERTATION FOR EXAMINATION PURPOSES

Hereby, I Dr Stephanus F Steyn, assistant supervisor and co-author of the manuscript titled "*Evaluating the antidepressant activity of Zembrin® alone and combined with desipramine in Flinders sensitive line rats: A bio-behavioural study*", which is included in Chapter 3 of this dissertation, give permission for this work to be submitted for examination purposes.

Kind regards,

Dr SF Steyn

B.Pharm. (PCDT), M.Sc., Ph.D.
Senior lecturer in Pharmacology
Behavioural Neuroscience and Neuropsychopharmacology