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An acute dose-ranging evaluation of the antidepressant properties of *Sceletium tortuosum* (Zembrin®) versus escitalopram in the Flinders Sensitive Line rat.

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ABSTRACT

Ethnopharmacological relevance: *Sceletium tortuosum* (L.) N.E.Br. (ST) has been used by the Khoisan people of South Africa as a mood elevator. Its various pharmacological mechanisms of action suggest distinct potential as an antidepressant. Clinical studies in healthy individuals suggest beneficial effects on mood, cognition, and anxiety.

Aim of the study: To obtain a chromatographic fingerprint of a standardized extract of *S. tortuosum* (Zembrin®), and to evaluate the acute antidepressant-like properties of Zembrin® versus the reference antidepressant, escitalopram, in the Flinders Sensitive Line (FSL) rat, a genetic rodent model of depression.

Materials and methods: The chemical profile of Zembrin® was determined by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) chromatogram method using alkaloid standards. Twelve saline treated FSL and six Flinders Resistant Line (FRL) control rats were used to confirm face validity of the FSL model using the forced swim test (FST). Thereafter, FSL rats ($n = 10$) received either 5, 10, 25, 50 or 100 mg/kg of Zembrin®, or 5, 10 or 20 mg/kg escitalopram oxalate (ESC), both via oral gavage, and subjected to the open field test (OFT) and FST.

Results: Four main ST alkaloids were identified and quantified in Zembrin® viz. mesembrenone, mesembrenol, mesembrine, and mesembranol (47.9%, 32%, 13.2%, and 6.8% of the total alkaloids, respectively). FSL rats showed significantly decreased swimming and climbing (coping) behaviours, and significantly increased immobility (despair), versus FRL controls. ESC 5 mg/kg and Zembrin® 25 mg/kg and 50 mg/kg showed significant dose-dependent reversal of immobility in FSL rats and variable effects on coping behaviours. Zembrin® 50 mg/kg was the most effective antidepressant dose, showing equivalence to ESC 5.

Conclusions: Zembrin® (25 and 50 mg/kg) and ESC (5 mg/kg) are effective antidepressants after acute treatment in the FST, as assessed in FSL rats. Moreover, Zembrin® 50 mg/kg proved equivalent to ESC 5. Further long-term bio-behavioural studies on the antidepressant properties of Zembrin® are warranted.

1. Introduction

Approximately 300 million people around the globe suffer from major depressive disorder (MDD) (World Health Organization, 2018; Willner et al., 2013). Due to the complex neurobiology of MDD, only 30–40% of patients respond to current antidepressants, emphasizing a pressing need for improved novel and alternative treatments (Trivedi et al., 2006; Willner et al., 2013). The pathophysiological mechanisms implicated in MDD variably include diminished central brain monoamines, neuroinflammation, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, genetic predisposition, and impaired neuroplasticity

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List of abbreviations

5-HT	Serotonin
5-HT_{1A}	Serotonin 1A receptor
5-$HT_{2A/C}$	Serotonin 2A and serotonin 2C receptors
ANOVA	Analysis of variance
DA	Dopamine
DAT	Dopamine transporter
ESC	Escitalopram
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
FST	Forced Swim Test
HPA	Hypothalamic-pituitary-adrenal
MAO-A	Monoamine oxidase inhibitor A
MDD	Major depressive disorder
NA	Noradrenaline
NAT	Noradrenaline transporter
NWU	North-West University
OFT	Open Field Test
PDE4	Phosphodiesterase 4
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
ST	*Sceletium tortuosum*
TUT	Tshwane University of Technology
UPLC-MS	Ultra-performance liquid chromatography-mass spectrometry
UPLC-MS-PDA	Ultra-performance liquid chromatography-mass spectrometry coupled to a photodiode array detector
VMAT-2	Vesicular monoamine transporter 2
ZEM	Zembrin®

among others (Dean and Keshavan, 2017; Jesulola et al., 2018). Due to their selective actions to bolster monoamine levels, currently prescribed antidepressants appear insufficient to engender full and sustained remission of the illness (Akil et al., 2018; World Health Organization, 2018). Furthermore, side effects and a delayed onset of action (Bet et al., 2013; Carhart-Harris and Nutt, 2017) compromises patient compliance. Together, this highlights a need for safer and more effective antidepressant treatment strategies.

Many herbal substances have demonstrated antidepressant effects in preclinical and clinical studies, often with similar mechanisms to that of standard antidepressants (Fajemiroye et al., 2016; Sarris, 2018). Additionally, these preparations may exhibit multi-target mechanisms of action on many different neurobiological processes implicated in MDD (Fajemiroye et al., 2016; Sarris, 2018). While most of these natural substances show potential as monotherapy, others indicate more favourable results when combined with traditional antidepressants (Akhondzadeh et al., 2003). Consequently, the World Health Organization initiated a traditional medicine strategy for 2014–2023 stating that evidence-based research be utilized to evaluate the safety and efficacy of complementary medicines (World Health Organization, 2013). Furthermore, in resource constricted countries, up to 80% of the population depends on herbal medicines for primary healthcare, albeit with a lack of supporting scientific data (Oyebode et al., 2016). A significant deterrent in studying herbal preparations is the lack of standardization, which leads to inconsistency in results (Sarris et al., 2021). It is with this motivation that a standardized extract of the indigenous South African plant, *Sceletium tortuosum* (ST), has attracted a great deal of interest (Olatunji et al., 2021; Brendler et al., 2021).

For generations, the indigenous Khoisan people have used ST as a mood-elevator, analgesic, hypnotic, anxiolytic, thirst and hunger suppressant, and for its intoxicating/euphoric effects (Gericke and Viljoen, 2008; Brendler et al., 2021). The characteristic alkaloid profile of *Sceletium tortuosum*, including mesembrine, mesembrenone, mesembrenol, and mesembranol (Krstenansky, 2017), presents with subtly different neuro-psycho-pharmacological actions (Olatunji et al., 2021) that may underlie the above-mentioned therapeutic properties. These actions include up-regulating vesicular monoamine transporter 2 (VMAT-2), serotonin transporter (SERT) inhibition, inhibition of phosphodiesterase 4 (PDE4) activity, anti-inflammatory properties, inhibition of monoamine oxidase A (MAO-A) and inhibition of the noradrenaline (NA) and dopamine (DA) transporters (NAT, DAT) (Olatunji et al., 2021). Clinically, ST also shows a relatively low side effect profile (Krstenansky, 2017; Murbach et al., 2014) although elevated blood pressure, headache, nausea, irritability, insomnia and anxiety are associated with its use (Schifano et al., 2015), while some studies suggest further investigation into possible dose-dependent adverse effects (Smith, 2011; Loria et al., 2014). Clearly, further exploration of the potential antidepressant actions of ST could prove invaluable due to its multi-modal mechanisms of action that align well with the pathophysiological mechanisms of MDD (Brand et al., 2015).

Zembrin® is a hydroethanolic extract standardized to 0.40% total alkaloids. This propriety extract allows for consistency regarding the above constituents, and hence allows parity across studies. Using a validated rodent model of depression, viz. BALB-c mice, Schell (2014) found that an isolated alkaloid extract of ST induced dose-dependent antidepressant effects in the FST, with the lower dose of the extract *viz.* 10 mg/kg, having greater antidepressant effects. However, to our knowledge, no studies have specifically analysed Zembrin® in such a translational model. Furthermore, broader dose-response studies in other translational models need to be performed, as well as studies comparing the efficacy of Zembrin® to a reference antidepressant. Such studies will enable proof of concept and establish effective dose ranges for further pre-clinical and clinical evaluation.

Considering genetic predisposition in the development of MDD (Dean and Keshavan, 2017; Jesulola et al., 2018), the Flinders Sensitive Line (FSL) rat is a genetic animal model that presents with robust construct, face, and predictive validity for MDD (Overstreet et al., 2005; Overstreet, 2012; Overstreet and Wegener, 2013). While the model is recognised to show preferential response to chronic antidepressants (Overstreet and Wegener, 2013), acute treatments have also been applied successfully (Du Jardin et al., 2018; Oberholzer et al., 2018). This model is also useful in studying the antidepressant actions of novel plant extracts (Oberholzer et al. (2018)), and hence is a valuable platform to explore the antidepressant activity of Zembrin®.

This study firstly aimed to obtain a fingerprint profile of Zembrin®, and to confirm the depressive phenotype of the FSL rats under our conditions of study. An acute dose response study was then conducted with five escalating doses of Zembrin®, as well as three doses of the reference antidepressant escitalopram (ESC), a selective serotonin reuptake inhibitor (SSRI). To elaborate on antidepressant-like activity, behavioural parameters akin to MDD were analysed using the forced swim (FST) and open field tests (OFT).

2. Materials and methods

2.1. Plant materials and chemical profiling

Zembrin® (HG&H Pharmaceuticals, Johannesburg, South Africa; Lot: SCE0416-1407), a standardized dry hydroalcoholic extract of the aerial parts of *Sceletium* (local names: kanna, channa, kougoed), *Sceletium tortuosum* (L.) N.E. Br. (Syn. *Mesembryanthemum tortuosum* L., (http://www.worldfloraonline.org.; accessed July 2021); drug-to-extract

ratio: 2:1 (w/w); extraction solvents: water and alcohol; standardized to 0.40% total alkaloids. The botanical material used for manufacture of the extract was authenticated by a botanist (A. Viljoen) and a voucher specimen is retained at the Tshwane University of Technology.

2.2. Chromatographic fingerprinting

Alkaloid profiling and quantification was performed on an ethanolic extract of Zembrin® using a validated ultra-performance liquid chromatography-mass spectrometry coupled to a photodiode array detector (UPLC-MS-PDA), as described previously (Zhao et al., 2018).

2.3. Animals

Male adult Flinders Sensitive Line (FSL) rats ($n = 92$) served as the experimental model. FRL control rats ($n = 6$), used to confirm the depressive phenotype of the FSL rat (Overstreet and Wegener, 2013), received vehicle treatment but no drug treatment. All animals were bred, housed and supplied at the DSI/NWU Vivarium (SAVC reg: FR15/13458) of the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University. Original colonies were acquired from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. A constant ambient temperature of 22 ± 2 °C and relative humidity of 40–60% was maintained, with a full spectrum of light in a 12:12h light/dark cycle (switched on at 06:00 and off at 18:00). At the start of experiments, all animals weighed between 200 and 230g. The animals were group housed (3–4 rats per cage) in polypropylene cages (380 x 380 × 230 mm) containing corncob bedding, and polyvinyl chloride pipes and standard Vivarium nesting material as environmental enrichment. Positive air pressure was maintained throughout at an air exchange rate of 18/h. Standard rat pellets and water was supplied *ad libitum*, and the animals were weighed and monitored daily. All experiments were approved by the NWU-AnimCareREC animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University, and all steps of animal handling conformed to the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). Animals were maintained and procedures performed in accordance with the code of ethics in research, training, and testing of drugs in South Africa and complied with national legislation (ethics approval number NWU-00168-18-A5). Importantly, these studies were performed in accordance with ARRIVE guidelines (McGrath et al., 2010).

2.4. Drug treatment

Drugs used in this study were escitalopram oxalate (ESC), kindly gifted by Lundbeck AG (Copenhagen, Denmark), and Zembrin® sponsored by HG&H. Both drugs were dissolved in 3 ml physiological saline (also used in the control treatment) according to manufacturer guidelines (escitalopram: Wolmarans et al. (2013); Zembrin® : HG&H). After the rats were weighed, drug doses were calculated and administered via oral gavage, to assess the acute dose response of both drugs. Based on previous ST studies (Carpenter et al., 2016; Loria et al., 2014; Schell, 2014), five doses of Zembrin® were tested, *viz.* 5, 10, 25, 50, 100 mg/kg (ZEM 5, ZEM 10, ZEM 25, ZEM 50, ZEM 100), while three doses of ESC were evaluated to establish the appropriate therapeutic dose of ESC in this model, *viz.* 5, 10, 20 mg/kg (ESC 5, ESC 10 and ESC 20). ESC 10 was used as a reference dose (Eren et al., 2007; Hui et al., 2010; Wang et al., 2014).

2.5. Study layout

Chromatographic fingerprint of ST: A sample of Zembrin® used in this study was analysed via UPLC-MS-PDA.

Confirmation of depressive phenotype of FSL rats: FRL ($n = 6$) and FSL ($n = 12$) rats were treated (intervals of 24 h, 6 h, and 1 h) with saline, with behaviour compared and analysed according to the OFT and FST methods described below.

Acute dose response study: As mentioned above, a three-tier dose response for ESC and a five-tier dose response for Zembrin® was performed. When the animals reached the required weight (200–230 g), treatment of drug groups ($n = 10$) occurred at intervals of 24 h, 6 h and 1 h before being subjected to the OFT and the FST, as described below. To conserve animals, saline-treated FSL rats used during the confirmation of the model, also served as the vehicle control group for the acute dose response study. The following day, the animals were euthanized via CO_2 overdose, according to standard vivarium protocols.

2.6. Behavioural tests

2.6.1. Open field test (OFT)

The OFT is widely used to determine locomotor activity in rodents (Kraeuter et al., 2019). In this instance, reduced locomotor performance is a commonly reported symptom of MDD (American Psychiatric Association, 2013). Moreover, the test is also used to exclude any confounding drug treatment effects on locomotor activity that could otherwise complicate interpretation of the FST data (Lavi-Avnon et al., 2005).

All animals were handled from weaning during weighing and later during dosing to familiarize the animals to handling stress. Experiments commenced from 19:00 when rodents are most active and were performed 30 min prior to the FST. The OFT apparatus presents with a black open field box measuring100 x 100 × 50 cm (Oberholzer et al., 2018). Each rat was allowed to explore the OFT for 5 min, with behaviour recorded under a 40-lux red light using a ceiling-mounted digital camera. Video footage was subsequently analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, Netherlands). General locomotor activity of the rat was scored as total distance moved (cm).

2.6.2. Forced swim test (FST)

The FST is a widely used behavioural protocol to measure depressive-like behaviour (despair) in rodents (Slattery and Cryan, 2012). A previously published method by Brand and Harvey (2017) was implemented. Following a 5 min habituation period in the test room, rats were placed in a Perspex® cylinder (*diameter* 18 cm*, height* 60 cm) containing 30 cm of 25 °C water and allowed to swim for a total of 7 min. A water depth of 30 cm was necessary to prevent rats from touching the bottom of the cylinder. Side-view video footage was recorded, with the first and last minutes excluded from scoring, as described previously (Oberholzer et al., 2018). Randomly assigned video codes were used to ensure unbiased scoring. Immobility (floating) was defined as the necessary movements to keep the rat's head above the water. Swimming (horizontal movement across quadrants) and climbing/struggling (upward movement against the side of the cylinder) behaviours inform on possible serotonergic and noradrenergic-mediated activity, respectively (Cryan et al., 2005). Thereafter, the rats were removed from the cylinders, gently dried with a clean towel and returned to their home cage (Brand and Harvey, 2017; Oberholzer et al., 2018). Since FSL rats display pronounced hang-time upon first being introduced to the FST (Du Jardin et al., 2018; Overstreet et al., 2005), the animals were not subjected to a pre-swim, as is customary in the FST (Cryan et al., 2002; Slattery and Cryan, 2012).

2.7. Statistical analyses

All statistical analyses and graphical presentations were undertaken using GraphPad Prism 7 software for Windows (GraphPad software, San Diego, USA) with effect magnitudes calculated in Exploratory Software for Confidence Intervals (Cumming, 2014). Statistical analyses were performed in consultation with the Statistical Consultation Service of the NWU.

For verification of the FSL model, comparison of behaviour between the FSL and FRL groups required unpaired student's t-tests for normal or Mann-Whitney U-tests for non-normal distributed data (Shapiro-Wilk test for normality, $p < 0.05$). An ordinary one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis (for normally distributed data), or Kruskal-Wallis ANOVA followed by Dunn' multiple comparisons (for non-normal distributed data), was used to analyse behaviour between treated and untreated FSL animals, as previously described (Harvey et al., 2021; Liebenberg et al., 2015; Uys et al., 2017). In all instances, $p < 0.05$ was deemed significant. Effect sizes were calculated using Cohen's *d* values, with only large effect size differences ($d \geq 0.8$) accepted as significant in the acute dose studies. Importantly, no outliers were removed.

3. Results

3.1. The chemical profile of Zembrin®

The Zembrin® UPLC-MS-PDA chromatogram (Fig. 1) displays four main peaks at retention times (Rt) 3.35, 3.62, 4.14, and 4.97 min, their identify confirmed by comparison with alkaloid standards as, mesembranol, mesembrenol, mesembrenone and mesembrine respectively. The sample comprised, in descending order, mesembrenone (47.9%) mesembrenol (32%), mesembrine (13.3%) and mesembranol (6.8%), which contributed to 3.84 μg/mg of total plant material.

3.2. Confirmation of depressive phenotype of the FSL rat

Table 1: According to the Shapiro-Wilk test, all except swimming data were normally distributed. The Mann-Whitney test indicated significant differences in swimming behaviour between the FSL and FRL groups, with FSL rats displaying significantly decreased swimming behaviour compared to FRL rats ($U = 5$, $p = 0.002$). An unpaired *t*-test indicated that FSL rats spent significantly less time in struggling behaviour than FRL controls ($t(16) = 4.89$, $p = 0.0002$). FSL rats also spent significantly more time immobile than FRL rats (unpaired *t*-test, $t(16) = 7.05$, $p < 0.0001$). Finally, an unpaired *t*-test indicated that FSL rats showed significantly more movement in the OFT than FRL rats ($t(16) = 3.416$, $p = 0.003$).

Table 1
Verification of the FSL rat as a model of MDD. Time spent immobile (immobility), swimming, and struggling as measured in the FST, and distance moved in the OFT as a measure of locomotor activity. Data represent saline treated FSL ($n = 12$) versus FRL ($n = 6$) rats, expressed as mean [95% confidence interval].

	FRL-SAL	FSL-SAL	*p*-value
Swimming	43.82 s [30.88, 56.76]	26.6 s [22.15, 31.4]	0.002
Struggling	94.68 s [68.66, 120.7]	48.51 s [38.69, 58.33]	0.0002
Immobility	160 s [134.8, 186.2]	220.1 s [212.5, 227.7]	<0.0001
Distance moved (OFT)	2284 cm [1611, 2958]	3247 cm [2921, 3573]	0.003

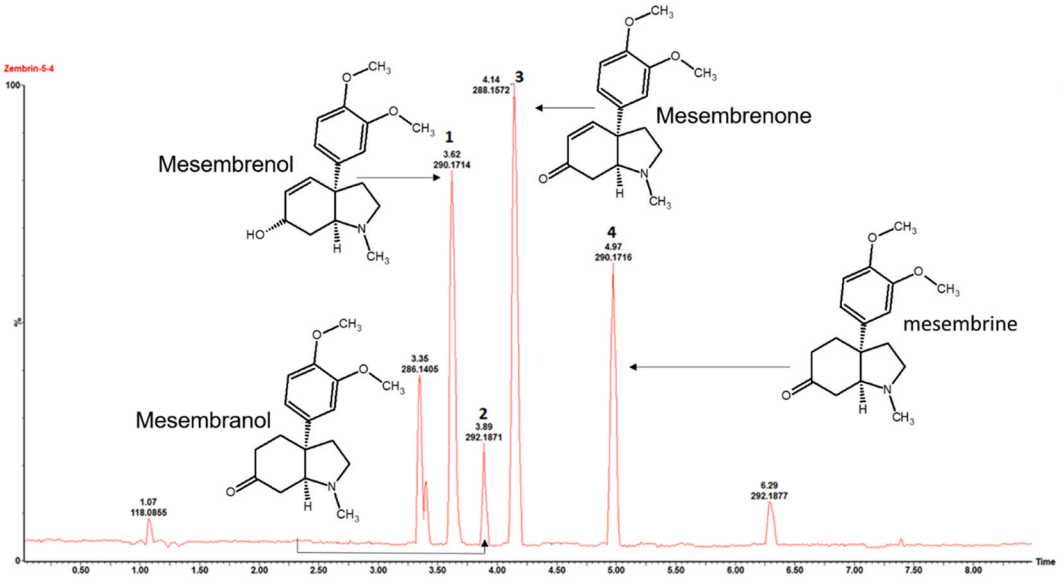

Fig. 1. UPLC-MS-PDA chromatographic fingerprint of Zembrin®.

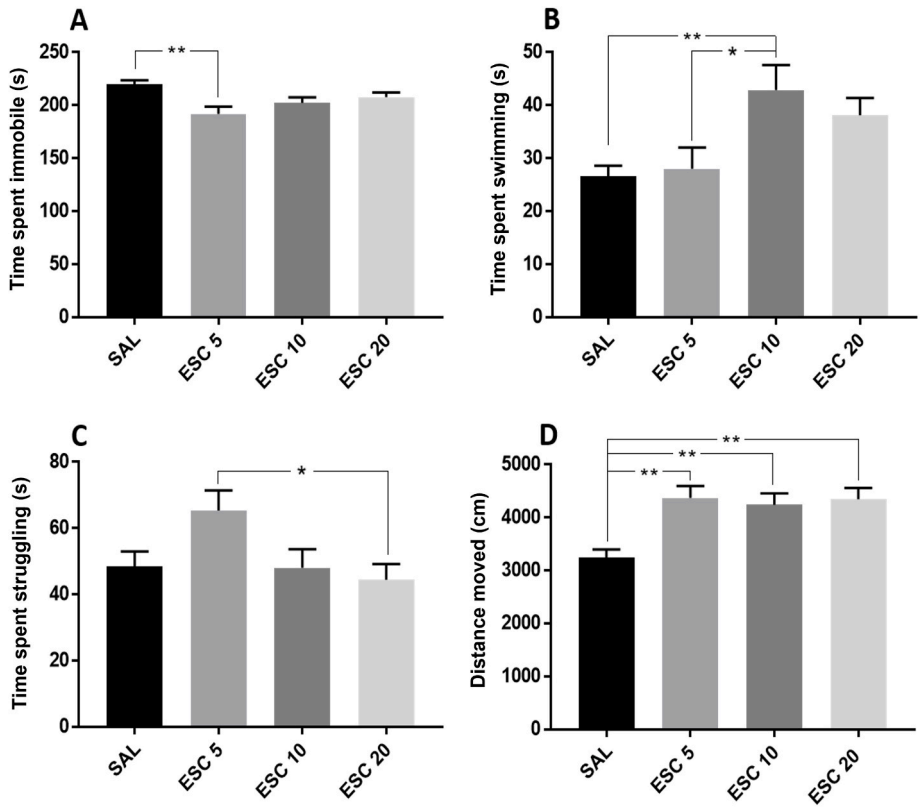

Fig. 2. *Acute ESC dose response in the FSL rat in the FST and OFT:* Acute antidepressant-like effects of various doses of ESC (n = 10), as shown, compared to saline-treated FSL rats. Parameters measured include time (s) spent A) immobile B) swimming and C) struggling in the FST, and D) locomotor activity (distance travelled (cm)) in the OFT. Data are expressed as means [95% CI]. *$p \leq 0.05$, **$p \leq 0.01$. ESC: Escitalopram. SAL: Saline.

3.3. Acute dose response studies

3.3.1. Acute ESC dose response in the FST

Immobility (Fig. 2A)*:* A one-way ANOVA displayed significant differences between the groups (F (3.38) = 5.62, $p = 0.003$), with only the ESC 5 group spending significantly less time immobile than FSL-SAL control rats ($p = 0.002$; $d = 1.6$ [0.6, 2.6]).

Swimming (Fig. 2B)*:* A Kruskal-Wallace test highlighted significant group differences (χ^2 (3) = 13.1, $p = 0.004$), with only ESC 10 significantly increasing swimming behaviour, relative to FSL-SAL control rats ($p = 0.026$, $d = 1.4$ [0.4, 2.4]) and ESC 5 treated groups ($p = 0.036$, $d =$ 1.1 [0.1, 2.0]).

Struggling (Fig. 2C): A one-way ANOVA showed significant differences between groups (F (3,38) = 3.1, $p = 0.038$). ESC 5 significantly increased struggling behaviour when compared to ESC 20 treated rats (p = 0.041, d = 1.2 [0.2, 2.2]), with a similar trend observed when compared to FSL-SAL control rats ($p = 0.11$, $d = 1.0$ [0.1, 1.9]).

Locomotor activity (Fig. 2D)*:* A one-way ANOVA showed significant differences between groups (F (3.39) = 8.18, $p = 0.0002$), with all three ESC 5 ($p = 0.0012$, $d = 1.806$ [0.804,2.778]), ESC 10 ($p = 0.0045$, $d =$ 1.635 [0.661, 2.581]) and ESC 20 ($p = 0.016$, d =1.8 [0.779, 2.772]) displaying significantly greater movement in the OFT, compared to FSL-SAL animals (statistical values reported in figure legend).

3.3.2. Acute Zembrin® dose response in the FST

Immobility (Fig. 3A)*:* A one–way ANOVA showed significant group differences (F (5.56) = 4.73, $p = 0.001$). Both ZEM 25 (p =0.022, $d = 1.7$ [0.7, 2.6]) and ZEM 50 (p = 0.006, d = 1.8 [0.8, 2.8]) displayed significantly reduced immobility time in the FST compared to SAL-treated controls. Additionally, ZEM 25 (p =0.041, d = 1.1 [0.7, 2.1]) and ZEM 50 (p =0.013, d = 1.3 [0.3, 2.2]) significantly reduced immobility time relative to ZEM 5 treated animals.

Swimming (Fig. 3B)*:* Although the Kruskal-Wallace analysis revealed significant differences between groups (χ^2 (5) = 11.8, $p = 0.038$), no significant intergroup differences were identified following the post-hoc test. ZEM 50 treated group showed a strong trend to increase swimming behaviour, compared to SAL treated controls (p -0.31, d - [0.0, 1.8]), although failed to reach significance.

Struggling (Fig. 3C)*:* A one–way ANOVA revealed significant differences between groups (F (5.56) = 3, $p = 0.018$). Struggling was significantly increased in rats treated with either ZEM 25 ($p = 0.027$, $d = 1.4$ [0.4, 2.4]) or ZEM 50 ($p = 0.036$, $d = 1.3$ [0.3, 2.2]) compared to ZEM 5 treated rats.

Locomotor activity (Fig. 3D)*:* A one way ANOVA revealed no significant differences between the groups (F (5.57) = 2.28, p = 0.059). However, ZEM 25 treatment significantly increased locomotor activity versus the ZEM 5 group ($p = 0.045$, d = 1.07 [0.062, 2.051]).

3.3.3. Most effective dose comparison of ESC versus Zembrin®

Immobility (Fig. 4A)*:* A one-way ANOVA revealed significant differences between the groups (F (2.29) = 9.34, $p = 0.0007$). ESC 5 (p = 0.004, $d = 1.6$ [0.6, 2.6]) and ZEM 50 ($p = 0.002$, $d = 1.8$ [0.8, 2.8]) significantly decreased time spent immobile compared to FSL-SAL but were each other equivalent.

Swimming (Fig. 4B)*:* The Kruskal-Wallis test indicated no significant differences between groups (χ^2 (2) = 4.47, p = 0,11).

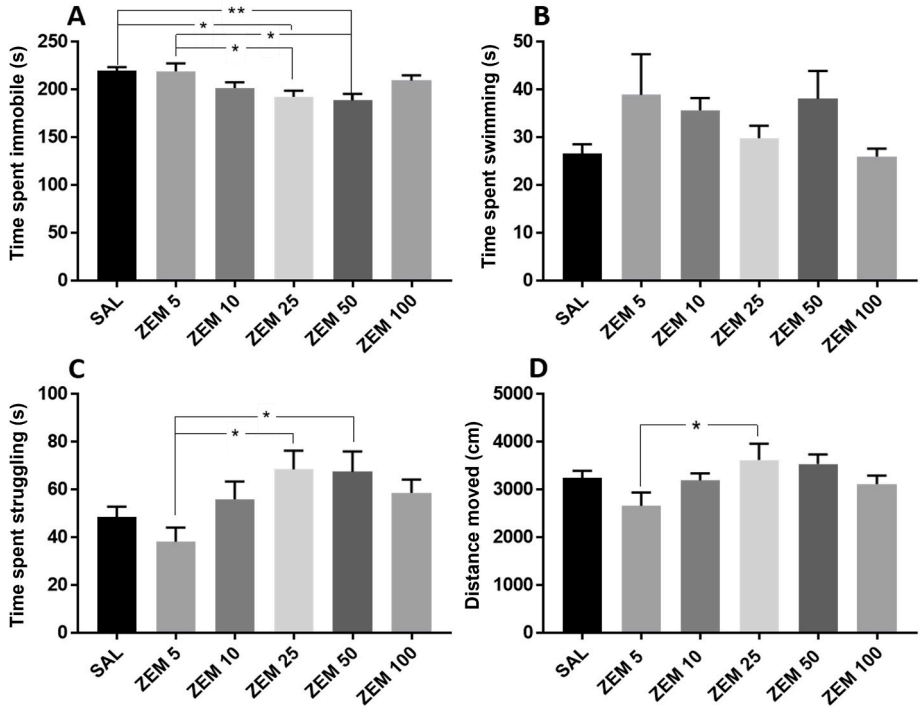

Fig. 3. *Acute Zembrin® dose response in the FSL rat in the FST and OFT:* Acute antidepressant-like effects of various doses of Zembrin® (ZEM) ($n = 10$ per group) in the FST, as shown, compared to saline-treated FSL animals ($n = 12$). Parameters measured include time (s) spent A) immobile B) swimming and C) struggling in the FST, and D) locomotor activity (distance travelled (cm)) in the OFT. Data are expressed as means [95% CI]. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. SAL: Saline. ZEM: Zembrin®.

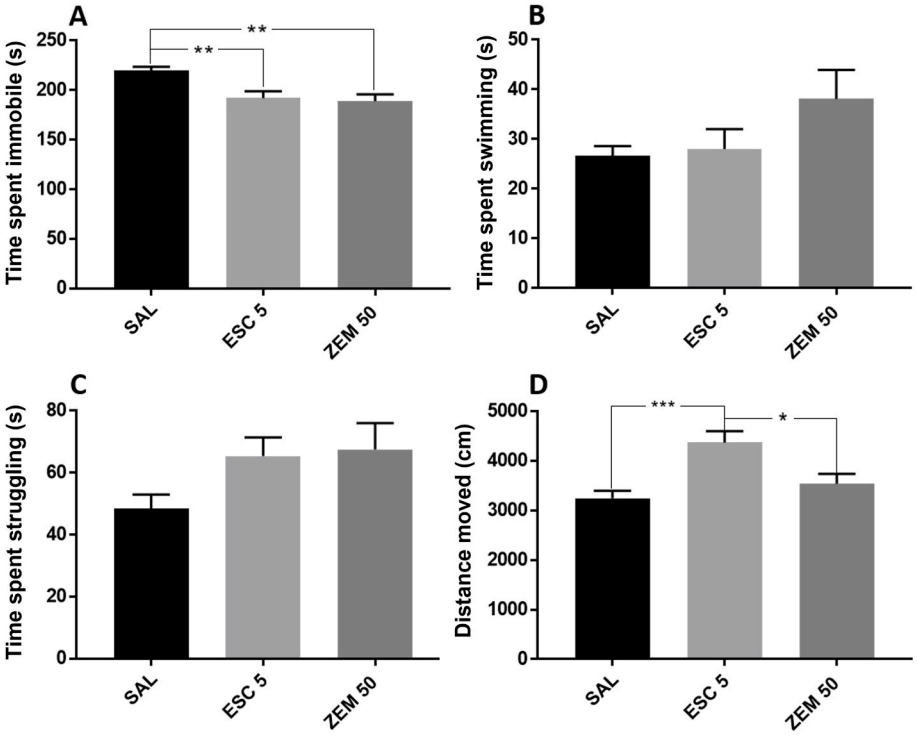

Fig. 4. *Comparison of the most effective doses of ESC and Zembrin® in the FSL rat in the OFT and FST:* Comparison between the acute effects of ESC 5 and Zembrin® 50 ($n = 10$ per group) as compared to saline-treated FSL rats ($n = 12$). Parameters measured include time (s) spent A) immobile B) swimming and C) struggling in the FST, and D) locomotor activity (distance travelled (cm)) in the OFT. Data are expressed as means [95% CI]. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ESC: Escitalopram. SAL: Saline. ZEM: Zembrin®.

Struggling (Fig. 4C)*:* A one-way ANOVA indicated no significant differences between groups (F (2.29) = 2.85, p = 0.074).

Locomotor activity (Fig. 4D)*:* A one-way ANOVA showed significant differences between the groups ($F(2.30) = 9.44, p = 0.0007$), with ESC 5 significantly increasing total distance moved in the OFT compared to both SAL ($p = 0.001$, $d = 1.8$ [0.8, 2.8]) and ZEM 50 ($p = 0.016$, $d = 1.2$ [0.2, 2.2]) treated groups.

4. Discussion

The most important findings of this study are that oral administration of both ESC and Zembrin® show dose-dependent antidepressant-like effects in the FST, with doses of ESC 5 mg/kg and ZEM 25 and 50 mg/kg effective in an acute treatment paradigm in FSL rats. Moreover, oral ZEM 50 proved equal in efficacy with respect to decreased immobility versus oral ESC 5 in a head-to-head comparison.

The fingerprint analysis for Zembrin® (Fig. 1) confirms its authenticity and composition (Liu et al., 2020). The typical Zembrin® chemotype is displayed, *viz*. mesembrenone (47.9%) mesembrenol (32%), mesembrine (13.3%) and mesembranol (6.8%), which concurs with previous Zembrin® studies (Krstenansky, 2017; Patnala and Kanfer, 2015; Shikanga et al., 2012). Since each alkaloid has different pharmacological mechanisms (Olatunji et al., 2021), batch variances and chromatographic fingerprint need to be factored in to control for species, climatic conditions, analytic methods *etc*. However, the standardized Zembrin® extract avoids the impact of these extraneous confounders, allowing for parity across different Zembrin® studies.

The FST and OFT data confirm the depressive phenotype of the FSL rats compared to FRL control rats, with FSL rats presenting with significantly decreased swimming and struggling behaviour, and significantly increased immobility in the FST (Table 1). Although MDD can present with either hyper- or hypoactivity (American Psychiatric Association, 2013), the significant increase in locomotor activity in FSL rats (Table 1) is unexpected. Indeed, decreased locomotor activity is said to underlie decreased reactive coping in a novel environment (immobility and passivity) (Magara et al., 2015a,b; Overstreet, 1993; Overstreet et al., 2005). This may be a novel observation, or an anomaly of inbreeding, requiring further study.

Since Zembrin® is active as an SSRI (Olatunji et al., 2021), it was necessary to utilize a known SSRI as reference agent in the FST. ESC shows dose-dependent antidepressant-like effects in the FST after acute oral treatment (Fig. 2) and confirms the predictive validity of the FSL model which also corresponds with previous studies (Bech et al., 2006; Loria et al., 2014; Murdoch and Keam, 2005; Schell, 2014). These data also confirm the FSL model to be a valid preparation with which to test the antidepressant properties of Zembrin®. ESC 5 proved the more effective antidepressant dose by significantly decreasing immobility in the FST (Fig. 2A). Interestingly, ESC 5 failed to significantly increase swimming behaviour, a noted serotonergic response (Fig. 2B), although this was significantly increased following a higher dose, viz. ESC 10 (Fig. 2B), thus reaffirming its serotonergic enhancing actions. In fact, ESC failed to significantly increase climbing behaviour, a noradrenergic response (Fig. 2C), versus control animals, in keeping with an SSRI (Cryan et al., 2005). The ability of all doses of ESC to increase locomotor activity (Fig. 2D) may be related to an increase in swimming behaviour and hence a possible serotonergic action (Kitamura et al., 2016). That said, increased locomotor activity may imply a false positive response in the FST (De Kloet et al., 1999; Slattery and Cryan, 2012), although this would have entailed a general increase in swimming and struggling and reduced immobility across all doses, which did not happen. Moreover, despite a sustained increase in locomotor acitivty at all doses of ESC, no accompanying reversal of immobility was observed with ESC 10 or ESC 20 (Fig. 2B). While many studies suggest 10 mg/kg to be a nominal therapeutic dose for ESC via oral gavage (Hui et al., 2010, 2016), ESC 20 and ESC 10 presented with similar, albeit less robust responses across all FST parameters (Fig. 2). These observations probably reflect over-activation of pre-synaptic 5-HT_{1A} auto-receptors thus lowering 5-HT levels with reduced antidepressant-like response as a consequence (Blier and Ward, 2003; Carhart-Harris and Nutt, 2017). Based on these data, ESC 5 was selected as the reference antidepressant for the Zembrin® comparative study.

Zembrin® exerted dose-dependent antidepressant-like effects in the FST (Fig. 3A), with ZEM 25 and ZEM 50 significantly reducing immobility compared to the saline-treated group. Our data suggests that ZEM 50 ($d = 1.7$ [0.7, 2.6]) displays a slightly greater effect size decrease in immobility than ZEM 25 ($d = 1.8$ [0.8, 2.8]), in relation to saline-treated controls, thereby supporting an earlier paper that Zembrin® has dose-dependent antidepressant-like effects (Schell, 2014). Zembrin® 50 was therefore selected as the most effective dose and used in a head-to-head ESC-Zembrin® comparison.

In vitro studies by Harvey et al. (2011) and Zhong et al. (2012) suggest that ST shares some mechanistic properties of SSRIs as both target 5-HT through potent inhibition of SERT (Harvey et al., 2011; Brendler et al., 2021). In fact, alkaloid components of ST, specifically mesembrine, mesembrenol and mesembrenone, present with dose-dependent inhibitory actions on SERT, similar to that of citalopram and fluoxetine (Coetzee et al., 2016; Gericke and Viljoen, 2008; Krstenansky, 2017). ST also possesses monoamine releasing properties by increasing VMAT-2 and is also a mild MAO-A inhibitor (Coetzee et al., 2016; Gericke and Viljoen, 2008; Krstenansky, 2017). Although not immediately evident with respect to the actions of Zembrin® on serotonergic-based swimming behaviour (Fig. 3B), ESC and Zembrin® can broadly be regarded as serotonergic drugs, indirectly activating pre-and post-synaptic 5-HT_{1A} and 5-$HT_{2A/C}$ receptors via SERT inhibition. Moreover, the most effective dose of ESC, *viz*. 5 mg/kg, *also* did not increase swimming yet did so at a 10 mg/kg dose, while ESC 5 still significantly reversed immobility in FSL rats (Fig. 2B). Taken together these findings suggest a dose-dependent action on swimming behaviour that may not correlate with reversing immobility in the FST.

Zembrin® can also induce NA release by increasing the expression of VMAT-2 (Coetzee et al., 2016; Krstenansky, 2017). Increased locomotor activity in the OFT is purported to reflect more noradrenergic mechanisms of action for an antidepressant (Chen and Reith, 1995), and coincides with increased noradrenergic-driven coping behaviour (i.e. climbing/struggling) observed in the FST (Fig. 3C) (Cryan et al., 2005). On the other hand, Zembrin® did not alter locomotor activity at any dose in relation to saline controls (Fig. 3D), thus confirming more specific antidepressant-like actions. Interestingly, ESC (Fig. 2C) and Zembrin® (Fig. 3C) significantly enhanced time spent struggling, with direct comparative analysis showing similarity in this regard (Fig. 4C), and hence a common noradrenergic mode of action despite their SSRI-like properties. Further work, however, is needed to confirm this assumption.

As alluded to earlier, ZEM 50 and ESC 5 proved the most effective antidepressant doses for either compound. This is especially true with regard to attenuating immobility in the FST (Figs. 2 and 3), with both compounds showing therapeutic equivalence with regard to reversing immobility (Fig. 4A) and coping deficits (struggling) (Fig. 4C). That said, ZEM 50 ($d = 1.8$) induced a slightly greater effect size reduction on immobility than ESC 5 ($d = 1.6$). Although speculative, this improved therapeutic benefit may reflect additional target engagement presented by Zembrin®, viz. actions on phosphodiesterase 4, SERT, NAT and immune–inflammatory processes that simultaneously address multiple deficits in central brain monoamines, neuroinflammation, impaired neuroplasticity and others (Olatunji et al., 2021).

5. Conclusion

Escitalopram and Zembrin® display dose-dependent antidepressant-like effects in an acute treatment paradigm in FSL rats, with 25 and 50 mg/kg Zembrin® significantly better than placebo, and the latter dose comparable to 5 mg/kg ESC. These preliminary findings prompt interest

in the therapeutic potential of Zembrin® in the treatment of MDD. However, further studies need to consider chronic treatment paradigms (Carhart-Harris and Nutt, 2017), evaluate concomitant effects on appropriate biomarkers of MDD, e.g. monoamines, neurotrophins, immune-inflammatory components etc (Brand et al., 2015), study additional behavioural parameters akin to MDD, e.g. anxiety, anhedonia, cognition, and investigate its possible synergistic actions when combined with traditional treatments (Dodd et al., 2005; Sarris, 2018). Albeit preliminary, this is the first investigation of Zembrin® versus an SSRI in a genetic rodent model of MDD that provides an evidence-based ethnopharmacological basis for the traditional and modern day use of *Sceletium tortuosum* in the treatment of mood disorders.

CRediT authorship contribution statement

Johané Gericke: contributed to the planning of the study, obtaining ethical approval, performed all experiments, performed the data collection, analysis and interpretation, and prepared the first draft of the manuscript.

Makhotso Lekhooa: co-supervised Johané Gericke, planned the study, reviewed and edited the manuscript, and assisted in data interpretation.

Stephan F. Steyn: assisted with behavioural scoring and processing of the results, assisted with the statistical analyses and interpretation of results, and reviewed and edited the article.

Alvaro M. Viljoen: contributed to the planning of the study, provided Zembrin® fingerprint analysis, as well as provided funding.

Brian H. Harvey: was principle investigator, supervised Johané Gericke, designed the study, assisted in data analysis and interpretation, reviewed and edited the manuscript, and acquired funding for the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

HG&H (manufacturers of Zembrin®) provided an unrestricted educational grant towards this study and provided the Zembrin®. HG&H had no further role in this study. BH Harvey has acted as a scientific advisor to HG&H. AM Viljoen (Tshwane University of Technology) provided funding for this study. Although AM Viljoen acts as a scientific advisor to HG&H, this study was undertaken in a facility not affiliated to AM Viljoen. AM Viljoen also declares his position as editor-in-chief for Journal of Ethnopharmacology.

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Glossary

Behavioural despair: Interpreted as the susceptibility of a rodent to negative mood in response to threat of drowning in the forced swim test

Construct validity: Neurochemical abnormalities bearing resemblance to those seen in humans

Face validity: Behavioural abnormalities bearing resemblance to symptoms of disorders as seen in humans

Locomotor activity: Movement of a human or animal from one location to another. It can be used to examine exploration and spontaneous activity

Neuroplasticity: The ability of the brain and nervous system to adapt to internal and external changes by changing its structure, connectivity, and function

Predictive validity: Ability of a model to respond to treatments widely used for the disorder