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Neuroprotective and neurorestorative properties of *Mesembryanthemum tortuosum* in a Parkinson's disease zebrafish larvae model

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ABSTRACT

Ethnopharmacological relevance: Parkinson's disease (PD) is characterised by the loss of dopaminergic neurons, resulting in reduced dopamine levels in the brain. The exact cause of dopaminergic neuron loss remains unknown but factors such as increased oxidative stress (OS), neuroinflammation and mitochondrial dysfunction have been implicated in the progression of the disease. The behavioural and molecular repertoire of PD can be modelled in zebrafish larvae using a neurotoxin, 6-hydroxydopamine (6-OHDA). Currently, there is no cure for PD, however, medicinal plants such as *Mesembryanthemum tortuosum* may provide neuroprotective or neurorestorative benefits resulting from their psychoactive nature, which has been widely reported.

Aim of the study: This study aimed to evaluate the neuroprotective and neurorestorative effects of *M. tortuosum* extracts on 6-OHDA-induced deficits in zebrafish larvae.

Material and methods: The mesembrine alkaloid profiles of a methanol and acid-base extract of *M. tortuosum* and Zembrin® were obtained using ultra-performance liquid chromatography coupled to mass spectrometry. In the neuroprotection assay, zebrafish larvae at 2 days post-fertilisation (dpf) were treated concurrently with 6-OHDA (250.0 μM) and *M. tortuosum* extracts or Zembrin® (standardised commercial *M. tortuosum*), and incubated for 72 h. At the end of the incubation period, locomotion was monitored using high-throughput EthoVision XT tracking software, and the reactive oxygen species (ROS) and total glutathione content (tGSH) in the larvae were also assessed. In the neurorestoration assay, the 2 dpf larvae were initially pre-treated with 6-OHDA (250.0 μM) for 24 h, and the extracts or Zembrin® were added after 24 h and incubation proceeded for a further 48 h. Locomotion, ROS and tGSH were determined at the end of the 48 h incubation. Both assays utilised L-dopa and selegiline as the positive controls.

Results: The acid-base extract contained higher levels of Δ^7-mesembrenone and mesembrine compared to mesembranol and mesembrenone. Δ^7-Mesembrenone was identified as the major compound in the methanol extract, while Zembrin® contained mesembrenol, mesembranol, mesembrenone and mesembrine as major compounds. Zembrin® displayed the best neuroprotective activity by significantly attenuating locomotor deficits and increasing tGSH content at all the three tested concentrations. The methanol extract displayed the best neurorestorative activity significantly restoring tGSH content, locomotor activity and ROS at varying concentrations.

Conclusions: The *M. tortuosum* extracts and Zembrin® containing various levels of mesembrine alkaloids improved both locomotion and oxidative stress associated with PD in a zebrafish larvae model. Furthermore, the effects of L-dopa and selegiline as core treatments in the management of PD were further confirmed in this PD zebrafish larvae model.

Abbreviations: AREC, Animal Research Ethics Committee; H2DCFDA, 2′,7′–dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's Disease; ROS, reactive oxygen species; MAO-A, monoamine oxidase-A; MAO-B, monoamine oxidase-B; tGSH, total glutathione content; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry.

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1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterised by classical motor symptoms, such as tremors, rigidity, akinesia or bradykinesia and postural instability. Other non-motor symptoms include loss of cognitive function, depression and anxiety. The non-motor symptoms can develop decades before the motor symptoms become apparent (Olanow et al., 2009; Zesiewicz, 2019). The aetiology of PD involves a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) of the midbrain region (Lang and Lozano, 1998). The exact cause of dopaminergic neuron loss remains unknown (Mullin and Schapira, 2015). However, factors such as neuro-inflammation (Amor et al., 2014), increased reactive oxygen species (ROS) (Dias et al., 2013), reduced antioxidant capacity and mitochondrial dysfunction (Nikam et al., 2009; Hassani and Esmaeili, 2024) have also been implicated.

Current treatment of PD is focused on symptomatic dopamine replacement therapy, with L-dopa as the first line drug (Harvey et al., 2012). Apart from L-dopa, other drugs that demonstrate efficacy include dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors such as selegiline. In younger patients with prominent tremors, an anticholinergic agent may be added however, side effects that include impaired cognition have been reported (Olanow et al., 2009; Armstrong and Okun, 2020). Due to the multifaceted nature of PD aetiology, a multi-targeted approach aimed at reducing oxidative stress and inflammation may be beneficial for the management of PD. A dual health care system encompassing traditional medicine practices and western drugs exists in South Africa (Stafford et al., 2008). Plant-derived medicines developed through traditional knowledge, are increasingly sought after due to their natural origin, successful historical use and relative safety. *Mesembryanthemum tortuosum* (L.) N.E.Brown (formerly *Sceletium tortuosum*), is an indigenous South African plant that has a diverse medicinal profile generated through centuries of traditional use (Gericke and Viljoen, 2008). Traditionally, fermented *M. tortuosum*, known as '*kougoed*' is chewed, smoked or inhaled as snuff by the Khoi-san people for its psychoactive properties (Smith et al., 1996). Scientific studies have previously verified the use of *M. tortuosum* as a sedative, anxiolytic and antidepressant herbal remedy (Gericke and Viljoen, 2008). Recently, it has been shown that *M. tortuosum* acts as a neurotransmitter-releasing agent (Luo et al., 2020) and displays both antioxidant and anti-inflammatory properties, which could be beneficial in the treatment of PD (Bennett et al., 2018). Furthermore, the pharmacological properties of *M. tortuosum* have been attributed to the presence of mesembrine-type alkaloids that include mesembrine, mesembrenone, mesembrenol, mesembranol and Δ^7-mesembrenone (Patnala and Kanfer, 2009; Krstenansky, 2017). The dry plant material of *M. tortuosum* contains a total alkaloid content that varies from 0.5% to 2.3%. The wide range of alkaloid content and distribution could be attributed to the variation in preparation methods and the flawed taxonomic authenticity in previous studies of *M. tortuosum* (Gericke and Viljoen, 2008). Nevertheless, it is important for researchers to perform phytochemical profiling of *M. tortuosum* extracts in all research investigating biological activity, to establish a correlation between bioactivity and the chemical constituents.

Zembrin® is a commercially available standardised *M. tortuosum* extract with a defined alkaloid composition. It is an aqueous ethanolic extract prepared in 30% distilled water and 70% ethanol, with a total alkaloid content greater than 0.38% (Nell et al., 2013). Zembrin® consists of 70% mesembrenol and mesembrenone combined, and mesembrine which makes up less than 20% of the product. Mesembranol is present in minute quantities as determined by reliable analytical techniques (Nell et al., 2013). The safety and biological effects (antidepressant, anxiolytic and mood elevating) of Zembrin® have been established *in vitro, in vivo, ex vivo* (Harvey et al., 2011; Murbach et al., 2014; Dimpfel et al., 2018; Gericke et al., 2022; Gericke et al., 2024) and in clinical trials involving healthy participants (Nell et al., 2013; Terburg et al., 2013). This indicates that Zembrin® has the potential to relieve symptoms of various neurodegenerative disorders, including PD.

The vast majority of studies on PD use *in vitro* and *in vivo* animal models to mimic PD hallmarks. A well-known neurotoxin, 6-hydroxydopamine (6-OHDA), can be used to induce dopaminergic neurodegeneration and oxidative stress, as well as impair locomotion, thereby replicating PD manifestations in research models (Kesh et al., 2021). 6-Hydroxydopamine induces oxidative stress through three distinct mechanisms; (1) intra or extracellular auto-oxidation of 6-OHDA which produces hydrogen peroxide, superoxide and hydroxyl radicals; (2) formation of hydrogen peroxide during deamination by monoamine oxidase, and (3) direct inhibition of mitochondrial respiratory chain complex I. These effects can occur independently or in combination to exacerbate oxidative stress which ultimately results in death of dopaminergic neurons. Additionally, dopaminergic neurons remain susceptible to ROS due to inherently limited amount of glutathione (Hernandez-Baltazar et al., 2017; Jin et al., 2023). Locomotor deficits are induced in zebrafish larvae when 6-OHDA selectively destroys dopaminergic neurons, leading to a reduction in dopamine which is essential for motor control (Parng et al., 2007). This results in a reduction in swimming ability and shorter swimming distances. These factors make 6-OHDA a valuable toxin for use in developing a PD-model in zebrafish larvae and evaluating the neuroprotective potential of novel PD drugs (Lal and Chopra, 2024).

Zebrafish (*Danio rerio*) larvae have gained attention as a model for biomedical research as the species share 80% genetic homology with humans (Kalueff et al., 2014). In contrast to rodent models, zebrafish are smaller in size, have high fecundity, faster development and a shorter life cycle. These characteristics make zebrafish an economical and robust high throughput screening animal model for drugs of plant origin. The model is particularly well suited for PD, since zebrafish show early development of the dopaminergic cell networks (Rink and Wullimann, 2002).

Various *M. tortuosum* extracts and products are widely recognized for treating anxiety, depression, and stress, with several studies attributing the psychoactivity to mesembrine-like alkaloids present in the plant. Despite voluminous research on the biological activities of *M. tortuosum* extracts and Zembrin®, none have investigated the possible neuroprotective and neurorestorative effects that could be advantageous in PD. Therefore, the current study primarily focused on investigating the potential of *M. tortuosum* extracts and Zembrin® to alleviate 6-OHDA-induced PD deficits in zebrafish larvae, by assessing locomotion, reactive oxygen species and total glutathione content.

2. Materials and methods

2.1. Chemicals and reagents

The following reagents were obtained from Sigma Aldrich (South Africa): methanol, dichloromethane (AR grade), anhydrous calcium chloride, anhydrous sodium sulphate, dimethyl sulfoxide (DMSO), anhydrous magnesium chloride, anhydrous magnesium sulphate, HEPES sodium salt, bicinchoninic acid (BCA), sulphuric acid, ammonia, sodium sulphate, sodium chloride, sucrose, L-dopa (purity 98%), selegiline (purity 98%), 6-hydroxydopamine hydrobromide, 2′,7′-dichlorofluorescein diacetate (H2DCFDA), potassium chloride and phenylmethylsulphonyl fluoride (PMSF). The alkaloid standards namely mesembrenol (purity 98.4%), mesembranol (purity 95.4%), mesembrenone (purity 98.2%) and mesembrine (purity 95.4%) were provided by HG&H Pharmaceuticals (Pty) (South Africa).

2.2. Plant material sourcing and extraction

2.2.1. Acid-base extraction of Mesembryanthemum tortuosum

Cultivated *M. tortuosum* plant material was obtained as dry fine powders from HG & H Pharmaceuticals and kept at room temperature.

An acid-base extract of the plant material was prepared using the acid-base extraction procedure described by Shikanga et al. (2012a,b). The powdered material (100 g) was mixed with 1.2 L of 0.5 M sulphuric acid and the mixture was placed on a shaker (ESCO Technologies, South Africa) at 150 rpm for 30 min. After shaking, the mixture was filtered through Whatman® filter paper No.1 (Sigma, Germany). The extraction process was repeated twice on the residue and the filtrates were pooled and basified with 400 mL of 20% ammonium hydroxide to pH 8. The mixture was then extracted with 500 mL dichloromethane, which was dried using anhydrous sodium sulphate. The process was repeated four times and the pooled organic phases were evaporated to dryness at 25 °C using a rotavapor (Büchi Rotavapor R-200, Switzerland) and stored at 4 °C for further analysis.

2.2.2. Methanol extraction

The methanol extract was prepared by sonicating 10 g of ground plant material in 100 mL methanol for 30 min, where after it was macerated overnight at 4 °C. The overnight mixture was passed through a Whatman® filter paper No. 1. The extraction process was repeated three times on the residue and the resulting filtrates were pooled and concentrated using vacuum rotary evaporation. The dried extracts were kept at 4 °C until analysis.

2.3. Ultra-performance liquid chromatography-mass spectrometry analysis

Prior to UPLC-MS analysis, the dried plant extracts and commercial Zembrin® powder (Batch no: SCE0419-1402) were dissolved in methanol to a final concentration of 1 mg/mL, filtered through 0.2 µm syringe filters (Millipore®, USA) and injected into a Waters Acquity ultra-performance liquid chromatography system (Waters, Milford, USA). The analysis was performed using the method previously developed in our laboratory (Chen and Viljoen, 2019). The extracts were injected in the mobile phase with an injection volume of 1.0 µL (full-loop injection) and separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters, Milford, USA) at 30 °C. The mobile phase comprised 0.1% ammonium hydroxide (Solvent A) and 90% acetonitrile (Solvent B) at a flow rate of 0.3 mL/min. Gradient elution was carried out as follows: the initial ratio of 80% A, changed to 60% A in 2 min, then changed to 50% A in 4.5 min and finally returning to the starting ratio in 0.2 min. The duration for achieving equilibrium was 1.8 min, and the total run time was 8.5 min. Data were gathered and analysed using Masslynx 4.1® software. For the mass spectrometry, the positive electrospray ionisation mode was used. Nitrogen was used as the desolvation gas at a flow rate of 500 L/h, while the desolvation temperature was maintained at 350 °C. The source temperature was set to 100 °C, and the capillary and cone voltages at 3000 V and 38 V, respectively. Data were collected within the range m/z 100–1000.

2.4. Zebrafish husbandry and embryo production

The research was conducted following the South African National Standards guidelines for the care and use of animals in research (SANS 10386:2021). Ethics approval for the use of zebrafish larvae was obtained from the Animal Research Ethics Committee (AREC) of the Tshwane University of Technology (Ref: AREC2021/10/003). Wild-type laboratory-bred adult zebrafish were housed in polycarbonate tanks at a density of five adult fish per litre of reverse osmosis water in a ZebTec Active Blue standalone self-regulating aquatic system (Tecniplast, Italy). The water parameters were monitored and maintained at pH 6.8–7.5, conductivity 400–800 µS, temperature 26–28.5 °C, ammonia <0.02 mg/L, nitrates <50 mg/L, nitrites <0.1 and a 14/10 h light/dark cycle, in accordance with standards of zebrafish care (Westerfield, 2007). The fish were fed three times a day with dry feed in the morning and at midday, as well as artemia (high protein live feed) in the late afternoon. The fish selected for spawning were placed in breeding tanks at a ratio of 1:2 (female: male), separated with a spacer and kept overnight in the dark. At the end of the dark cycle the following morning, the spacers were removed and the fish allowed to mate for 1 h. Embryos were collected and placed in a petri-dish, suspended in 0.3x Danieau medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 1.5 mM HEPES buffer at pH 7.2) and observed under a microscope (ZEISS, Germany) to remove unfertilised eggs. The healthy embryos were incubated at 28 °C and medium changes performed every 24 h until the larvae reached the required experimental age of 2 days post fertilisation (2dpf).

2.5. Maximum tolerated concentration assay

The maximum tolerated concentrations (MTC) were determined for Zembrin®, acid-base and methanol extracts, to identify the highest concentration that did not produce observable toxic effects in the zebrafish larvae using a modified method of Afrikanova et al. (2013). The plant extracts and Zembrin® powder were dissolved in 100% DMSO to prepare stock solutions, and working solutions were prepared by diluting stocks to the desired concentration in 0.3x Danieau medium (100, 250 and 500 µg/mL) and achieving final DMSO concentration of 0.075% in the solutions. Five larvae/well at 2 dpf were placed in a 48-well plate and treatment groups allocated as follows: **(1)** acid-base extract, **(2)** methanol extract, **(3)** Zembrin®, **(4)** vehicle control (0.075 % DMSO) and **(5)** 0.3x Danieau medium. Fresh solutions were prepared and replaced daily in the treatments and mortality and phenotypic abnormalities were assessed until the end of the experimental period (5 dpf). The MTC was determined as the concentration at which there was ≥ 60% of larvae survival at the end of the treatment period. All treatments were duplicated in two wells resulting in 10 larvae per treatment group. Larvae were monitored under Olympus IX71 microscope mounted with a camera (Olympus, Japan).

2.6. Antiparkinsonian assays

The use of 6-OHDA is a widely used and established method to mimic PD hallmarks in zebrafish larvae. 6-Hydroxydopamine (250 µM) induces dopaminergic neuron death, oxidative stress and locomotor deficits (Feng et al., 2014; Cronin and Grealy (2017)). All treatments (6-OHDA, extracts and drugs) were prepared in 0.3x Danieau medium in a dark room to avoid autoxidation of 6-OHDA by light. The neuroprotective and neurorestorative treatment regimen were adapted from a method of Cronin and Grealy (2017) with slight modifications.

2.6.1. Neuroprotective treatment regimens

To assess the neuroprotective effects of *M. tortuosum*, the treatment regimen followed the method of Cronin and Grealy (2017) with slight

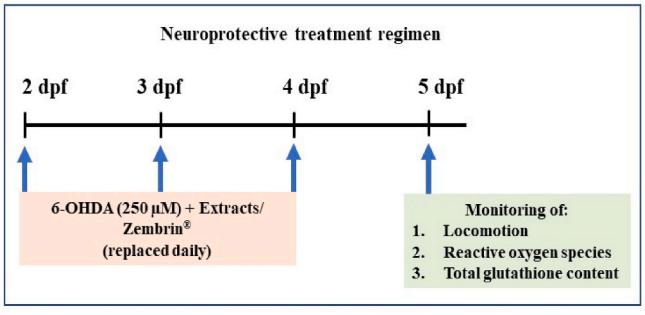

Fig. 1. The neuroprotective treatment regimen applied in the study. At 2 dpf, larvae were treated with extracts and 6-OHDA (250.0 µM), then incubated for an additional 72 h at 28 °C, with fresh treatments administered daily until 5 dpf. At the end of the treatment period (5 dpf), locomotion, total glutathione level, and reactive oxygen species were assessed.

modifications (Fig. 1). Both L-dopa and selegiline were used as positive controls and the untreated control received 0.3x Danieau medium. Zebrafish larvae at 2 dpf were treated in groups as follows: **(1)** acid-base extracts (12.5, 25.0 and 50.0 μg/mL) + 6-OHDA (250.0 μM); **(2)** methanol extracts (12.5, 25.0 and 50.0 μg/mL) + 6-OHDA (250.0 μM); **(3)** Zembrin® (12.5, 25.0 and 50.0 μg/mL) + 6-OHDA (250.0 μM); **(4)** positive control (L-dopa or selegiline) + 6-OHDA (250.0 μM) and **(5)** 0.3x Danieau medium + 6-OHDA (250.0 μM). After treatment began at 2 dpf, larvae were incubated for an additional 3 days at 28 °C, with fresh treatments administered daily until 5 dpf (Fig. 1). At the end of the treatment period (5 dpf), locomotion, total glutathione level, and ROS were measured. Each treatment group included 15 larvae, and the experiment was conducted in triplicate, resulting in a total of 630 larvae.

2.6.2. Neurorestorative treatment regimens

In the neurorestorative regimen, the method of Cronin and Grealy (2017) was used with slight modifications (Fig. 2). Zebrafish larvae at 2 dpf were treated with 6-OHDA (250.0 μM) for 24 h, followed by exposure to various concentrations of the acid-base (12.5, 25.0 and 50.0 μg/mL) and methanol (12.5, 25.0 and 50.0 μg/mL) extracts, Zembrin® (12.5, 25.0 and 50.0 μg/mL) and the controls. Incubation proceeded for a further 2 days, and at the end of the incubation period, locomotion, ROS and total glutathione content were assessed (Fig. 2). Each treatment group included 15 larvae, and the experiment was conducted in triplicate, resulting in a total of 630 larvae.

2.6.3. Monitoring locomotion

To explore locomotion, larvae were individually placed in a 48-well microplate and incurred treatment stipulated in Fig. 1 or Fig. 2. At the end of the incubation period, the larvae in the micro-well plates were assessed for locomotor activity in a temperature controlled DanioVision observation chamber equipped with Noldus EthoVision® XT software. Initially, larvae were allowed 5 min to acclimatise, thereafter locomotion was monitored as total distance travelled and swimming speed, over a period of 10 min (Gomes et al., 2023).

2.6.4. Reactive oxygen species determination

The ROS were quantified following the method described by Lackmann et al. (2018). At the end of the treatment period, 15 larvae per treatment group were pooled, rinsed in 0.3x Danieau medium in petri-dishes and incubated in 5.0 μM of H2DCFDA fluorescent probe for 45 min at 28 °C in the dark. After incubation, larvae were rinsed in 0.3x Danieau medium to remove excess dye. The larvae were euthanised on an ice slurry (20 min) and subsequently transferred to Eppendorf tubes where excess liquid was removed. The larvae were homogenised in ice-cold 60 μL extraction buffer (HEPES buffer, 320 mM sucrose, 0.1 mM $MgCl_2$ and 0.5 mM PMSF at pH 7.4) using a pellet pestle. The homogenate was subsequently centrifuged at 13 000 rpm and 4 °C for 20 min.

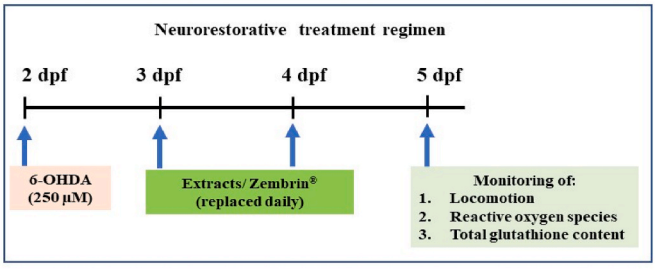

Fig. 2. The neurorestorative treatment regimen applied in the study. At 2 dpf, zebrafish larvae were treated with 6-OHDA (250.0 μM) and incubated for 24 h. After incubation, the larvae were treated with various concentrations of the acid-base and methanol extracts, Zembrin® and the controls. Incubation proceeded for a further 48 h, and at the end of the incubation period (5 dpf), locomotion, ROS and total glutathione contents were assessed.

After centrifugation, 10 μL aliquots of supernatant were added into white opaque 96-well plates in triplicate, and an additional 10 μL for protein quantification using the bicinchoninic acid (BCA) method. A volume of 150 μL of the buffer (30 mM HEPES buffer, 200 mM KCl and 1 mM $MgCl_2$) was added to each well and the plates were covered in aluminium foil and placed on a shaker for 10 min. The fluorescent intensity of each well was measured on a SpectraMax M2 microplate reader (Molecular Device, USA) at the excitation wavelength of 485 nm and the emission wavelength of 515 nm. Reactive oxygen species levels were expressed as arbitrary fluorescent units per mg of protein.

2.6.5. Total glutathione content determination

At the end of the incubation period, 15 larvae per treatment were rinsed with 0.3x Danieau medium in petri-dishes. The larvae were euthanised, homogenised in 100 μL extraction buffer and mixed with 150 μL of 5% sulfosalicylic acid. The homogenate was centrifuged at 13 000 rpm for 10 min at 4 °C and the supernatant collected to measure total glutathione content using a glutathione kit (Sigma Aldrich, USA). Total glutathione levels were measured at the emission wavelength of 412 nm on a SpectraMax M2 microplate reader (Molecular Device, USA). An additional 10 μL of each supernatant was used for protein quantification.

2.7. Data analysis

Data were presented as mean ± standard error of the mean values of triplicate experiments. The data were analysed using the non-parametric Kruskal-Wallis test and group comparisons were determined by Dunns post-hoc test. A p value < 0.05 was considered statistically significant and all statistical analyses were performed using GraphPad Prism version 8.0 (USA).

3. Results

3.1. Phytochemical profiling of Mesembryanthemum tortuosum extracts

The alkaloid profiles of the acid-base and methanol extracts were compared to the standardised Zembrin® profile. Each of the extracts displayed a unique alkaloid profile where the acid-base extract contained the four alkaloids and the peaks for Δ^7-mesembrenone and mesembrine were relatively higher compared to mesembranol and mesembrenone (Fig. 3A). Mesembrenol was not identified in this extract. The methanol extract contained only two alkaloids, Δ^7-mesembrenone which appeared as a major peak and mesembranol among several other minor compounds (Fig. 3B). Notably, mesembrine, mesembrenone and mesembrenol were not detected in the methanol extract. The UPLC-MS profile of Zembrin® indicated that the commercial product contained the four common alkaloids namely mesembrenol, mesembranol, mesembrenone and mesembrine (Fig. 3C), however Δ^7-mesembrenone was not detected.

3.2. Maximum tolerated concentrations of Mesembryanthemum tortuosum extracts

Zebrafish larvae exposed to the acid-base extract at 500.0 μg/mL experienced 60% mortality within 24 h of treatment, while 100% mortality was observed following 48 h exposure to 500 and 250.0 μg/mL concentrations. All the larvae survived in the 100 μg/mL treatment group, with no deformities observed, thus the acid-base extract was subsequently used at concentrations ≤100 μg/mL in the PD assays. The methanol extract at 500.0 μg/mL was moderately toxic with 40% larvae mortality observed after 24 h, which increased to 100% mortality after 48 h. At 72 h, the 250.0 μg/mL treated larvae recorded 40% deaths, whereas the lowest concentration of 100.0 μg/mL did not induce apparent toxicity (100% survival with no deformities) (Fig. 4). The methanol extract was therefore tested at 12.5–50.0 μg/mL in the

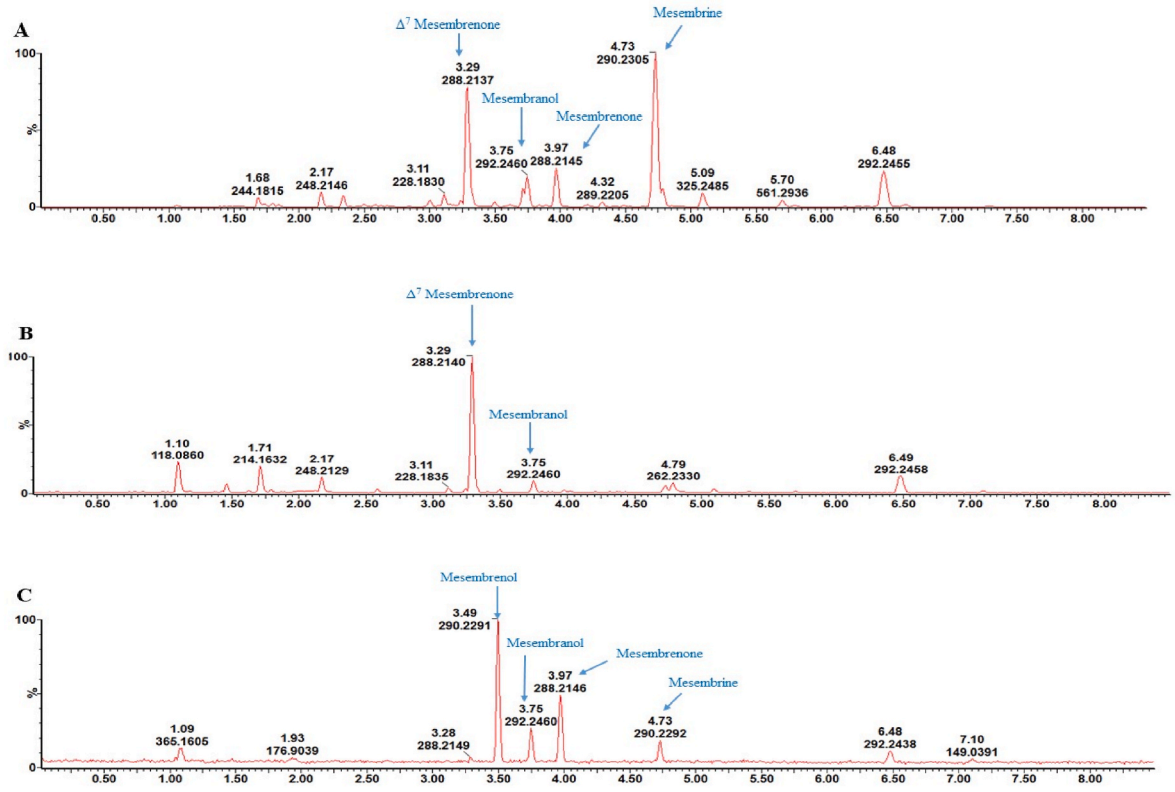

Fig. 3. UPLC-MS profiles of *Mesembryanthemum tortuosum* acid-base extract (A), methanol extract (B) and commercial Zembrin® (C).

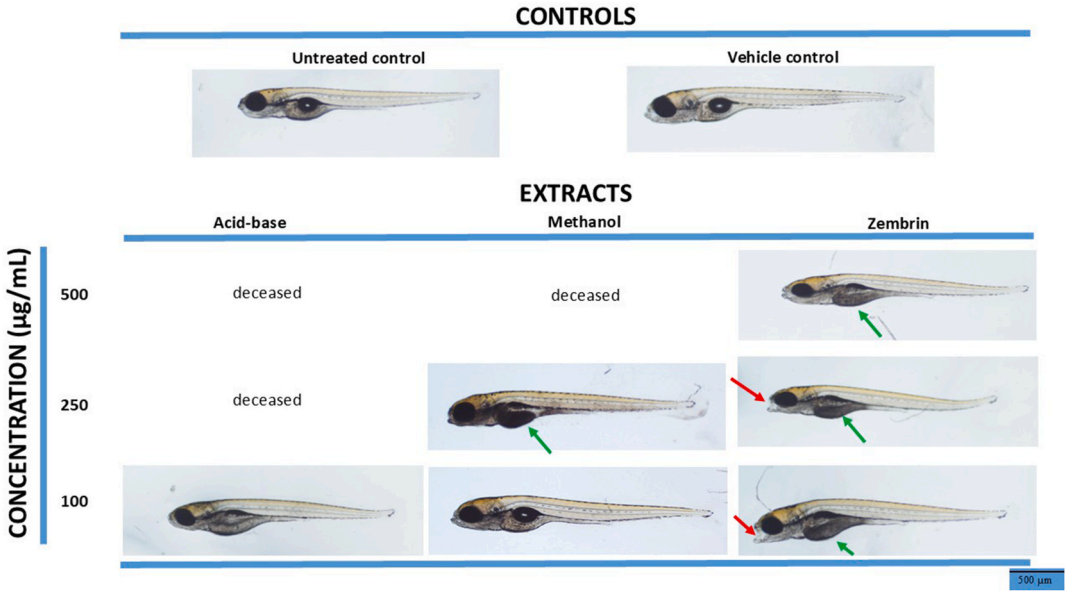

Fig. 4. Maximum tolerated concentration results of *Mesembryanthemum tortuosum* extracts treated zebrafish larvae after 72 h incubation. The green arrows indicate partial liver toxicity and the red arrows indicate jaw deformity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

subsequent PD assays. Zembrin® was relatively safe with no mortality recorded at any of the tested concentrations over the 72 h incubation period (Fig. 4). However, following treatment with Zembrin®, a shortened jaw structure was visible for the 100.0 and 250.0 µg/mL treatments, therefore all subsequent experiments were performed at concentrations lower than 100.0 µg/mL.

3.3. Neuroprotective effects of *Mesembryanthemum tortuosum*

3.3.1. Neuroprotective effect towards locomotor activity

Impaired locomotion is recognized as one of the behavioural repertoires of PD in zebrafish larvae. The effect is exploited to simulate human disease related to movement in PD. In this study, treatment of larvae with the neurotoxin 6-OHDA was able to decrease locomotion to 47%, compared to the untreated control [$D = 109.4$; $p < 0.001$]

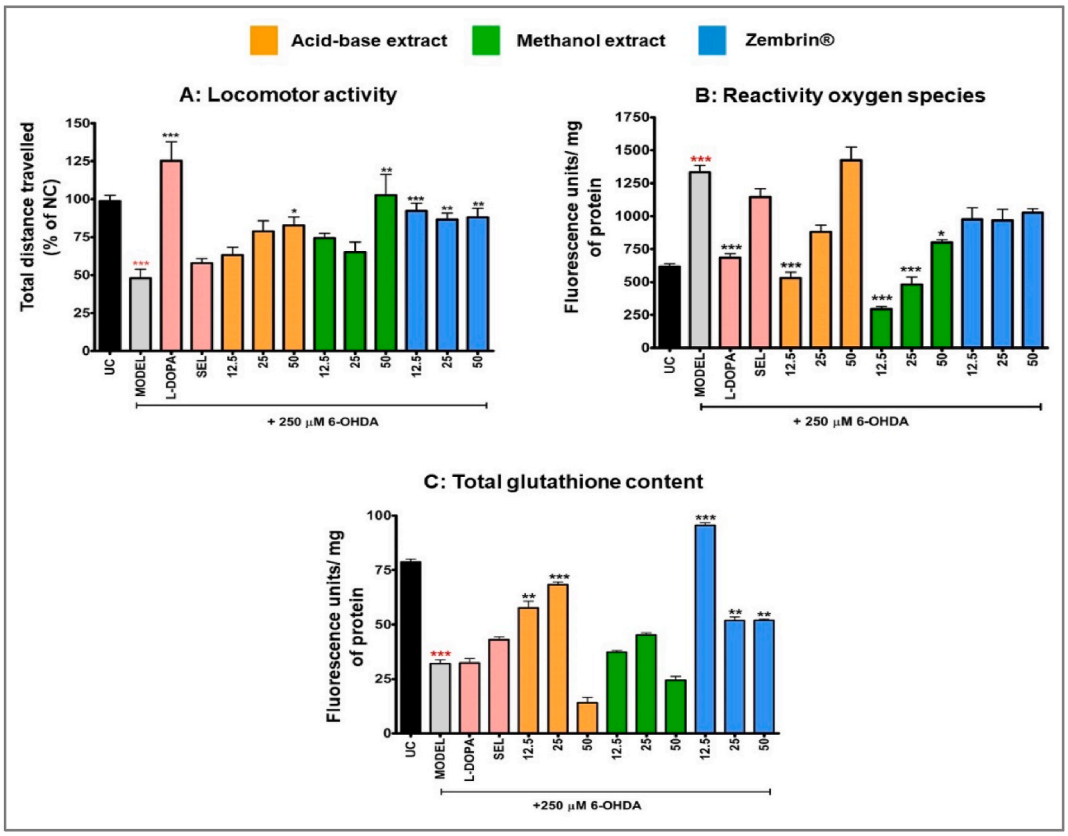

Fig. 5. Bar graphs depicting the total distance travelled (locomotion) (A), reactive oxygen species (B) and total glutathione content (C) of zebrafish larvae after 72 h incubation with *Mesembryanthemum tortuosum* extracts and Zembrin® at three concentrations (12.5, 25.0 and 50.0 µg/mL). UC-Untreated control and SEL-selegiline. Significant difference between the treatments and the model group (6-OHDA), *p < 0.05, **p < 0.01 and ***p < 0.001. Significant difference between the treatments and the untreated control (UC), *p < 0.05, **p < 0.01 and ***p < 0.001.

(Fig. 5A). This result confirms that the 6-OHDA model is suitable to study PD-related locomotor deficits in zebrafish larvae. The positive control, L-dopa significantly improved locomotion to 125% [D = -117.5; p < 0.001]. In contrast, selegiline-treated larvae did not exhibit a significant improvement in locomotion [D = 2.605; p > 0.05]. The acid-base extract improved locomotion at all the three tested concentrations, however, only the 50.0 µg/mL produced a statistically significant improvement compared to the model (6-OHDA) group [D = -68.40; p < 0.05] (Fig. 5A). A similar trend was observed with the methanol extract at 50.0 µg/mL, where a significant improvement in locomotion was observed compared to that of larvae exposed to the extract at lower concentrations [D = -100.0; p < 0.01]. Zembrin® at all the tested concentrations (12.5, 25.0 and 50.0 µg/mL) resulted in a significant improvement in locomotion [D = -91.1; p < 0.001, D = -83.6; p < 0.01, D = -76.7; p < 0.01, respectively]. The results clearly demonstrate that the two *M. tortuosum* extracts at 50.0 µg/mL have neuroprotective potential as they were able to minimise 6-OHDA locomotor deficits. Zembrin® displayed the best activity at all three concentrations showing improved total distance travelled, indicating attenuation of locomotor deficits.

3.3.2. Neuroprotective effect towards reactive oxygen species generation

Exposing zebrafish larvae to 6-OHDA induced a three-fold increase in ROS compared to the untreated control [D = -105.4; p < 0.001] (Fig. 5B). However, pre-treatment of the larvae with L-dopa significantly reduced the effects of 6-OHDA on ROS [D = 86.94; p < 0.001], whereas selegiline achieved only a 10 % decrease in ROS [D = 16.7; p > 0.05] (Fig. 5B). The acid-base extract at 12.5 µg/mL displayed neuroprotective effects by reducing ROS significantly compared to the higher concentrations [D = 114.9; p < 0.001], while the methanol extract showed good activity, with a significant dose-dependent decrease in ROS production (Fig. 5B). Although Zembrin® at 12.5, 25.0 and 50.0 µg/mL caused approximately 27% reduction in ROS production [D = 43.4, p > 0.05; D = 40.9; p > 0.05, D = 26.94; p > 0.05, respectively] the level of ROS remained higher than that of the untreated control group. The results therefore demonstrate that the methanolic extract showed the best neuroprotective effects by reducing ROS production in the presence of 6-OHDA, while the acid-base extract was only active at 12.5 µg/mL and Zembrin® was unable to restore normal ROS levels at all the tested concentrations.

3.3.3. Neuroprotective effect towards total glutathione content

In the presence of 6-OHDA, the total glutathione content was reduced to 32% in zebrafish larvae [D = 116.7; p < 0.001] and pre-treatment with L-dopa did not improve the total glutathione content [D = -0.95; p > 0.05] (Fig. 5C). Although selegiline improved the total glutathione content by 10% [D = -36.27; p > 0.05] it was not able to restore the levels to match the untreated control (Fig. 5C). Larvae treated with the acid-base extract displayed higher levels of total glutathione content up to 57 % [D = -73.17; p < 0.01] and 68% [D = -97.25; p < 0.001] at 12.5 and 25.0 µg/mL, respectively compared to the model group (Fig. 5C). The acid-base extract at 50.0 µg/mL potentiated the decline of tGSH to 14% [D = 36.33; p > 0.05], which was lower than that of larvae treated with 6-OHDA alone (Fig. 5C). Although, the lower concentrations of the methanol extract (12.5 and 25.0 µg/mL) improved the tGSH content [D = -14.2; p > 0.05; D = -43.25; p > 0.05], the extract was not able to restore tGSH to levels comparable to the untreated control group. At 50.0 µg/mL, the methanolic extract potentiated 6-OHDA-induced tGSH depletion, which concurs with the ROS findings, where at this concentration the methanol extract was less efficacious.

Notably, exposure of the larvae to 12.5 μg/mL of Zembrin® significantly increased tGSH by 3-fold [D = -132.7; $p < 0.001$] surpassing all treatment groups and the untreated control. The groups tested with 25.0 and 50.0 μg/mL of Zembrin® significantly reversed the 6-OHDA-induced tGSH depletion by 50% [D = -67.08; $p < 0.01$ and D = -69.63, $p < 0.01$] (Fig. 5C). The results demonstrate that pre-treatment of zebrafish larvae with Zembrin® provides neuroprotective effects, observed as an increase in tGSH, while the acid-base extract exerts potential effects at lower concentrations.

3.4. Neurorestorative effects of *Mesembryanthemum tortuosum*

3.4.1. Neurorestorative effect towards locomotor activity

Treatment of larvae with 6-OHDA in the neurorestorative assay resulted in a decrease in locomotion to approximately 54% of the untreated control [D = 123.7; $p < 0.001$] (Fig. 6A), which was restored to approximately 94% by treating the larvae with L-dopa [D = -102.2; $p < 0.001$]. Treatment with selegiline on the other hand resulted in a slight improvement in locomotion (~10 %) [D = -4.1; $p > 0.05$] (Fig. 6A). Both the acid-base extract and Zembrin® did not significantly improve locomotion at all the three tested concentrations compared to the model larvae [Acid-base extract: D = -11.9; D = -49.7; D = -20.2; $p > 0.05$; Zembrin®: D = -44.0; D = -43.4; D = -59.6; $p > 0.05$] (Fig. 6A)]. The methanol extract at 25.0 μg/mL and 50.0 μg/mL displayed the best activity, restoring locomotion significantly in the larvae compared to the model larvae [D = -74.7; $p < 0.05$, D = -82.9, $p < 0.01$]. These results demonstrate that *M. tortuosum* extracts have neurorestorative potential as they were able to improve 6-OHDA-induced locomotor deficits, thus improving total distance travelled by the larvae.

3.4.2. Neurorestorative effect towards reactive oxygen species generation

In this assay, 6-OHDA treatment did not increase ROS as expected [D = 45.1; $p > 0.05$], but instead resulted in a decrease in ROS compared to the untreated control (Fig. 6B). Treatment with L-dopa resulted in a slight increase in ROS [D = -36.5; $p > 0.05$] and selegiline significantly increased ROS levels by two-fold [D = -101.8; $p > 0.001$] compared to the 6-OHDA model. Both the acid-base extract and Zembrin® maintained ROS at levels comparable to the untreated control (Fig. 6B). The methanol extract however, caused a reduction in ROS at 12.5 μg/mL [D = 30.75; $p > 0.05$], and a significant increase at 25.0 μg/mL [D = -71.8; $p < 0.001$]. Since 6-OHDA did not increase ROS, the *M. tortuosum* extracts maintained ROS levels at baseline comparable to the untreated control, except for 25.0 μg/mL of the methanol extract, which potentiated an increase in ROS.

3.4.3. Neurorestorative effect towards total glutathione content

Treatment of larvae with 6-OHDA (model) showed a reduction in tGSH by approximately 30% [D = 46.1; $p > 0.05$] when compared to the untreated control. When exposed to L-dopa, there was a further reduction in tGSH by 10% [D = 17.5; $p > 0.05$], while selegiline treatment achieved a statistically significant increase in tGSH [D = -55.63; $p < 0.05$] compared to the 6-OHDA treated larvae (Fig. 6C). The acid-base extract displayed the noteworthy activity at 25.0 μg/mL, increasing tGSH [D = -96.2; $p < 0.001$] to a level surpassing the untreated and positive controls. The methanol extract showed an inverse dose-dependent effect, where the lowest concentration (12.5 μg/mL) had

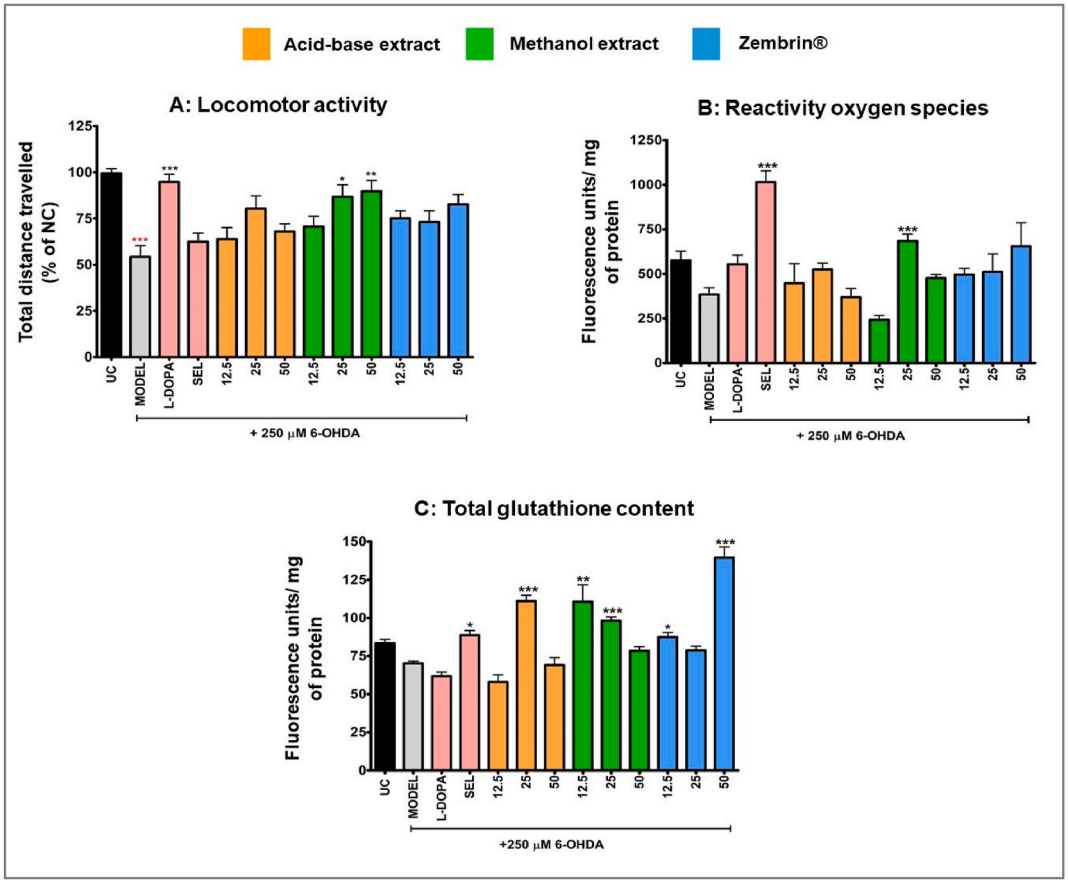

Fig. 6. Bar charts depicting the locomotion, reactive oxygen species and total glutathione content of zebrafish larvae in the neurorestorative treatment regimen following treatment with *Mesembryanthemum tortuosum* extracts and Zembrin® at three concentrations (12.5, 25.0 and 50.0 μg/mL). UC-untreated control and SEL-selegiline. Significant difference between the treatments and the model group (6-OHDA), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Significant difference between the treatments and the untreated control (UC), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

the highest significant effect [D = -67.54; $p < 0.01$], while an increase in the concentration resulted in a reduction in tGSH. Exposure to Zembrin® at 50.0 μg/mL resulted in a significant increase in tGSH [D = -112.1; $p < 0.001$] surpassing that of the untreated and positive controls (Fig. 6C). The results demonstrate that treatment of zebrafish larvae with 50.0 μg/mL Zembrin® provides the best neuroprotective effect, while the methanol extract was effective at lower concentrations.

4. Discussion

Phytochemical profiling of *Mesembryanthemum* extracts is important as the biological/psychoactive properties of the plant have been mostly attributed to the four main mesembrine-type alkaloids which are mesembrine, mesembranol, mesembrenol and mesembrenone (Harvey et al., 2011). The development of standardised commercial *M. tortuosum* extracts, such as Zembrin®, ensures a consistent chemical profile and subsequently, the desired pharmacological effects. In the current study, distinct chemical differences were observed in the methanol and acid-base extracts of *M. tortuosum*. Specifically, the methanol extract had fewer mesembrine-type alkaloids than the acid-base extract. In an earlier study, Shikanga et al. (2012a,b) investigated the impact of three different solvents (water, methanol and acid-base) on the mesembrine-type alkaloids of *M. tortuosum*, and found that the methanol extract had a higher extraction yield with lower concentrations of mesembrine-type alkaloids. In addition, the acid-base extract had the lowest yield with higher amounts of mesembrine alkaloids (Shikanga et al., 2012a,b). It can be inferred that acid-base extraction provides a superior phytochemical profile of mesembrine type-alkaloids in *M. tortuosum* compared to the methanol extract. Although in the current study, Δ^7-mesembrenone was identified in the methanol extract, previous reports by that Chen and Viljoen (2019) and Shikanga et al. (2012a,b), did not identify the compound in the same extract. However, Δ^7-mesembrenone is not new to *M. tortuosum*, as Trimesemine™, a mesembrine-rich standardised extract of *M. tortuosum*, was reported to contain mesembrine, Δ^7-mesembrenone and mesembrenone (Coetzee et al., 2016; Swart and Smith, 2016). The current study confirmed the presence of previously reported mesembrine-type alkaloids which varied depending on the type of extract. In the case of the acid-base extracts, all of the alkaloids are protonated and deprotonated upon addition of the acid and base, respectively. This implies that the alkaloids should dissolve in the acidified aqueous layer from the plant material, and upon deprotonation, be transferred into the organic extractant. This process could be expected to reflect the alkaloid composition of the plant more accurately, unless some alkaloids are unstable under acidic conditions and transform into other alkaloid metabolites. When extracting with methanol, the alkaloids that are more soluble in high polarity solvents will be preferentially extracted and the results seem to suggest that mesembrine is most likely less soluble in methanol.

The current study primarily focused on investigating the neuroprotective and neurorestorative effects of *M. tortuosum* after inducing PD-like effects with 6-OHDA in zebrafish larvae. The locomotor deficits induced by 6-OHDA in the neuroprotective regimen affirm the report of Feng et al. (2014), who observed a decrease in total distance travelled in larvae treated with 250.0 μM 6-OHDA, from 795.84 ± 52.00 mm to 261.66 ± 59.64 mm, corresponding to over 50% reduction in locomotion. Cronin and Grealy (2017) explored the neuroprotective and neurorestorative effects of minocycline and rasagiline in zebrafish larvae (2–5 dpf) and in both assays, 6-OHDA (250.0 μM) reduced locomotion by over 50% (Cronin and Grealy, 2017). Additionally, Zhang et al. (2015) and Wu et al. (2021) reported significantly reduced swimming distance after treatment with 250.0 μM of 6-OHDA from 3 dpf to 7 dpf, while Benvenutti et al. (2018) reported that 6-OHDA induced motor deficits (total distance, mean speed, maximum acceleration, absolute turn angle and immobility time), optomotor response impairment and morphological alterations (total length and head length) in 3 dpf to 7 dpf zebrafish larvae. The current study provides confirmation on the use of 6-OHDA to induce locomotor deficits in zebrafish larvae, which is important for modelling PD in animal models.

L-Dopa alleviated locomotor deficits in zebrafish larvae better than selegiline in the current study and is therefore a more suitable positive control. A previous report by Cronin and Grealy (2017) showed that treatment with 1.0 mM of L-dopa reversed 6-OHDA-induced locomotor deficits in both neuroprotective and neurorestorative treatment regimens. Kesh et al. (2021) demonstrated that treatment with 50.0 μM of L-dopa provided a modest improvement in 6-OHDA-induced locomotor deficits. Vaz et al. (2020) reported that L-dopa (1.25 mM) restored distance travelled, but not the number of immobile events, in zebrafish larvae treated with 750.0 μM of 6-OHDA. In the same study, rasagiline (like selegiline) a monoamine oxidase inhibitor, improved the total distance travelled and reduced the number of immobile events (Vaz et al., 2020). The reports affirm the observation that L-dopa is a suitable positive control in 6-OHDA-induced PD model.

6-Hydroxydopamine-induced neurotoxicity is primarily caused by the generation of ROS through a variety of pathways including the extracellular and intracellular non-enzymatic auto-oxidation of 6-OHDA (Blum et al., 2001). In this study, the methanol extract provided more potent ROS scavenging effects than other extracts, which could be due to the presence of phenolic compounds which can neutralize ROS both intracellularly and extracellularly. There is a direct correlation between a high phenolic content and the ability to scavenge ROS (Shi et al., 2022). Kapewangolo et al. (2016) reported that crude ethanol and ethyl acetate extracts of commercially available *M. tortuosum* had free-radical scavenging activity with IC_{50} values of 49.00 ± 0.2 and 64.7 ± 3.1, respectively using DPPH ROS scavenging assay (Kapewangolo et al., 2016). In a comparative study, Bennett and colleagues (2018) revealed that an extract of *M. tortuosum* high concentration in Δ^7-mesembrenone exhibited a greater capacity to scavenge ROS when compared to an extract that contained high mesembrine (Bennett et al., 2018). This study also highlighted that higher doses of the extract were less efficacious which could be due to antioxidant overload (Bennett et al., 2018). It has been reported that high doses of antioxidants diminish the ROS scavenging activity and may act as pro-oxidants potentially causing cellular damage (Halliwell, 2013). To further highlight the ROS scavenging activity mediated by Δ^7-mesembrenone, in the current study it is evident that Zembrin®, which is devoid of Δ^7-mesembrenone, exhibited a lower ROS scavenging activity in comparison to both the methanol and acid-base extracts which contain Δ^7-mesembrenone. It was further observed in the current study that at lower concentrations (12.5 μg/mL) the extracts exhibited better ROS scavenging activity compared to the 50 μg/mL, which could also highlight antioxidant overload at higher concentrations.

6-Hydroxydopamine also induces an increase in ROS through deamination by monoamine oxidase A (MAO-A), due to structural similarities to dopamine therefore 6-OHDA remains a substrate for MAO-A (Apiraksattayakul et al., 2022; Blum et al., 2001). Monoamine oxidase inhibitors (MAO-I) have long been used in the treatment of PD to prevent metabolism of dopamine. The latter drugs exhibit neuroprotective effects by reducing ROS associated with metabolism of dopamine by MAO (Aluf et al., 2013; Elsherbeny et al., 2021). Aluf et al. (2013) reported that inhibition of MAO-A and MAO-B by clorgyline and rasagiline respectively, reduced oxidative stress and dopamine metabolism in 6-OHDA treated rats. The later study highlights the role of MAO inhibition in 6-OHDA induced PD models. Coetzee et al. (2016) reported that Trimesemine™ (*M. tortuosum*) exhibited moderate inhibition of MOA-A, suggestive of an alternative action mechanism of *M. tortuosum* extracts in reducing oxidative stress associated with PD. A more recent *in vivo* study indicated that *M. tortuosum* fractions containing mesembrine, mesembrenone and Δ^7-mesembrenone showed mild inhibition of MAO-B and more potent ROS scavenging activity (Luo et al., 2022). Aluf and colleagues (2013) reported that inhibition of MAO-A or B enhanced GSH levels in 6-OHDA treated rats. In our study, 6-OHDA induced depletion of tGSH which was reversed by *M. tortuosum* (including

Zembrin®) at varying concentrations in both the neuroprotective and neurorestorative assays. Therefore, the capacity of *M. tortuosum* extracts (including Zembrin®) to elevate GSH levels, thereby counteracting heightened ROS and preventing the death of neuronal cells, presents a potential breakthrough in the treatment of PD. Glutathione (GSH) is a key component of cellular antioxidant defences, since it is involved in the detoxification of ROS, and the conjugation and excretion of toxins. It also plays a role in the control of the inflammatory cytokine cascade (Brown et al., 2004; Martin and Teismann, 2009). Depletion of GSH is one of the first biochemical abnormalities observed in PD patients and is associated with neuronal cell death (Martin and Teismann, 2009). An *in vitro* study by Kesh et al. (2021) highlighted that 6-OHDA caused the depletion of GSH levels, whereas L-dopa was able to restore them. Furthermore, a reduction of GSH was also recorded in brain slices after 6-OHDA treatment of rats (García et al., 2000).

Although the extracts exhibited a greater capacity for neuroprotection than neurorestoration, all the extracts enhanced larval locomotion. We observed that the 6-OHDA-treated larvae displayed a reduction in ROS levels in the neurorestorative experiment, which contradicts the hypothesis regarding PD, that the disease is primarily driven by oxidative stress. This suggests that the neurorestorative treatment regimen failed to induce the entire spectrum of PD after 24 h exposure to 6-OHDA alone. In a similar neurorestorative experiment, Cronin and Grealy (2017) reported that 32 h exposure to 6-OHDA is adequate to induce significant neuronal loss, with no chance of recovery at 5 dpf, or 40 h after 6-OHDA withdrawal. Therefore the 32 h exposure to 6-OHDA could be the minimum required incubation period for neurorestorative studies using 250 μM 6-OHDA. Despite the lack of significant PD-like effects observed in the neurorestorative treatment regimen, the *M. tortuosum* extracts sustained baseline ROS levels and elevated tGSH, suggesting that the extracts may possess neuronal rescuing properties that prevent neuronal cell death.

In the neuroprotective assay, a lower concentration (12.5–50.0 μg/mL) of the methanolic extract reduced 6-OHDA-induced ROS without any subsequent increase in tGSH. This finding suggests that the methanolic extract neutralises ROS via a mode of action that is independent of the tGSH pathway. This phenomenon warrants further investigation. *Mesembryanthemum tortuosum* possesses antioxidant, anti-inflammatory (Bennett et al., 2018), and neurotransmitter-releasing properties (Luo et al., 2020) that may potentially counteract the negative effects of PD progression. Although some alkaloids derived from *M. tortuosum* have been associated with bioactivity, none have been specifically related to activity in PD.

5. Conclusions

The study highlights the neuroprotective effects of *M. tortuosum* extracts to offer a holistic, multi-targeted approach that could be beneficial in treatment of PD. 6-Hydroxydopamine provides a robust PD model that can be established successfully *in vivo* in zebrafish larvae. This study provides evidence that L-dopa improves locomotion, yet it does not increase tGSH levels, thereby potentially compromising the success of treatment in conventional PD management. Moreover, since none of the currently available treatments for PD have demonstrated safety in relation to ROS, the use of *M. tortuosum* extracts (including Zembrin®) presents a unique opportunity to modulate ROS in the brain by their diverse psychoactivity. *Mesembryanthemum tortuosum* extracts have been shown to improve locomotor deficits, with Zembrin® providing superior activity in locomotion and greater safety profile. Although Zembrin® showed superior activity in locomotion, extracts containing Δ^7-mesembrenone have shown better antioxidant potential however, care needs to be taken to avoid antioxidant overload when working with Δ^7-mesembrenone. Even though, *M. tortuosum* extracts showed varying activity profile in combating 6-OHDA-induced PD effects, it is now becoming more prominent to standardise extracts to be more disease specific to receive full benefits of the medicinal plant. Future studies will consider standardization of Zembrin® to improve locomotion and greater safety profile in combination with Δ^7-mesembrenone, to improve oxidative stress associated with PD. Regarding the treatment regimens, the neurorestorative assay showed less induction of ROS and a modest decrease in tGSH, indicating that the effects of 6-OHDA are less prominent when tested in this regimen.

The current findings also support the traditional use of *M. tortuosum* in treating central nervous system disorders and highlight its potential to alleviate PD-like symptoms. However, several other PD indicators should be investigated in the zebrafish model to corroborate the current findings, as well as to investigate possible mechanisms of action. Other biochemical assays to monitor mitochondrial membrane potential, gene expression and malondialdehyde levels should be investigated, as well as neurochemical assays. Mechanistic assays for dopaminergic neuron visualization to monitor changes in dopaminergic neurons should be performed, since neuron loss is a direct indication of PD-like neurodegeneration. Although, the zebrafish larvae model provides valuable insights into biological effects, direct extrapolation to humans is limited and the results must be validated in higher vertebrate models.

CRediT authorship contribution statement

Keagile Lepule: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis. **Maxleene Sandasi:** Writing – review & editing, Writing – original draft, Supervision, Project administration. **Weiyang Chen:** Writing – review & editing, Methodology, Formal analysis. **Alvaro Viljoen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AV declares that he is the EiC of JEP and that the paper will be handled by a senior editor on submission. AV declares that he acts as a scientific advisor to HG&H Pharmaceuticals.

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Data availability

Data will be made available on request.

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