



## *Mesembryanthemum tortuosum* L. alkaloids modify anxiety-like behaviour in a zebrafish model



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### ABSTRACT

**Ethnopharmacological relevance:** *Mesembryanthemum tortuosum* L. (previously known as *Scelletium tortuosum* (L.) N. E. Br.) is indigenous to South Africa and traditionally used to alleviate anxiety, stress and depression. Mesembrine and its alkaloid analogues such as mesembrenone, mesembrenol and mesembranol have been identified as the key compounds responsible for the reported effects on the central nervous system.

**Aim of the study:** To investigate *M. tortuosum* alkaloids for possible anxiolytic-like effects in the 5-dpf *in vivo* zebrafish model by assessing thigmotaxis and locomotor activity.

**Materials and methods:** Locomotor activity and reverse-thigmotaxis, recognised anxiety-related behaviours in 5-days post fertilization zebrafish larvae, were analysed under simulated stressful conditions of alternating light-dark challenges. Cheminformatics screening and molecular docking were also performed to rationalize the inhibitory activity of the alkaloids on the serotonin reuptake transporter, the accepted primary mechanism of action of selective serotonin reuptake inhibitors. Mesembrine has been reported to have inhibitory effects on serotonin reuptake, with consequential anti-depressant and anxiolytic effects.

**Results:** All four alkaloids assessed decreased the anxiety-related behaviour of zebrafish larvae exposed to the light-dark challenge. Significant increases in the percentage of time spent in the central arena during the dark phase were also observed when larvae were exposed to the pure alkaloids (mesembrenone, mesembrenol, mesembrine and mesembrenol) compared to the control. However, mesembrenone and mesembranol demonstrated a greater anxiolytic-like effect than the other alkaloids. In addition to favourable pharmacokinetic and physicochemical properties revealed via *in silico* predictions, high-affinity interactions characterized the binding of the alkaloids with the serotonin transporter.

**Conclusions:** *M. tortuosum* alkaloids demonstrated an anxiolytic-like effect in zebrafish larvae providing evidence for its traditional and modern day use as an anxiolytic.

### 1. Introduction

*Mesembryanthemum tortuosum* L. (Aizoaceae) is a succulent plant indigenous to the southwestern parts of South Africa (Loria et al., 2014) and is of scientific interest due to its possible therapeutic effects, such as the enhancement of physical well-being and the treatment of anxiety, stress, and depression (Gericke and Viljoen, 2008). Traditionally, pastoralists and hunter-gatherers have used this plant for managing mood-swings and improving general well-being (Van Wyk and Gericke,

2000). Several alkaloids have been isolated from *M. tortuosum*, namely; mesembrine, mesembranol, mesembrenone, and mesembrenol. The modulation of the stress response is mediated via inhibition of serotonin reuptake into raphe nuclei neurons; after repeated administration this results in increased serotonin levels throughout the brain (Smith et al., 1996; Shikanga et al., 2013, Yohn et al., 2017) in the management of anxiety, depression, bulimia nervosa, and obsessive-compulsive disorder (Patnala and Kanfer, 2017). A recent report from our group also predicted the moderation of anxiety-like behaviour in zebrafish larvae by *M. tortuosum*; in which, amongst the various medicinal plants studied

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Abbreviations	
5-dpf	5 days post fertilization
5-HT	5-hydroxytryptamine;
5-HTT	5-hydroxytryptamine transporter
ADMET	absorption, distribution, metabolism, excretion and toxicity
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood brain barrier
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
DZ	diazepam;
DMSO	dimethyl sulfoxide;
GABA	gamma aminobutyric acid
HPCCC	high performance counter current chromatography
MTC	maximum tolerated concentration
PDE4	phosphodiester-4
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor
SAR	structure-activity relationships
RCSB PDB	Research Collaboratory for Structural Bioinformatics Protein Data Bank
TPSA	topological polar surface area
UCSF	University of California San Francisco
UPLC-MS	ultra-performance liquid chromatography coupled with mass spectrometry

for anxiolytic activity, the water extract of *M. tortuosum* exhibited the most favourable anxiolytic-like activity compared to other extracts (Maphangwa et al., 2020).

*M. tortuosum* introduced to the market under the commercial name Zembrin®, it is a standardised 70% alcoholic extract, containing 0.35–0.45% total *Sceletium* alkaloids. The reported pharmacological effects of Zembrin® include inhibition of the phosphodiester-4 (PDE4) enzyme as well as the inhibition of the serotonin (5-hydroxytryptamine, 5-HT) transporter (5-HTT, also known as SERT) (Harvey et al., 2011). Inhibition of PDE4 results in the elevation of the intracellular cyclic adenosine monophosphate (cAMP) levels with resultant modulation of second messenger effects and has been implicated in the regulation of anxiety and depression in animals models (O'Donnell and Zhang, 2004). For several decades serotonin and serotonin receptors have been linked to the aetiology of depression and the mechanisms fundamental to the response to antidepressant treatment (Yohn et al., 2017; Bowman and Daws, 2019; Butler and Meegan, 2008; Dell'Osso et al., 2005; Reimold et al., 2008).

Selective 5-HT reuptake inhibitors (SSRIs) are widely used for the treatment of anxiety disorders and depression (Pringle et al., 2011; Garakani et al., 2021). A combination treatment using an SSRI and a PDE4 inhibitor has been suggested, due to the potential synergistic effects, in treating central nervous system (CNS) disorders. Chronic treatment with SSRIs has been reported to upregulate PDE4 (Ye et al., 2000), resulting in tolerance and reduced sensitivity to SSRIs, suggesting that combination treatment with SSRIs and PDE4 inhibitors is a rational treatment option (Cashman et al., 2009). In a double-blind, cross-over, placebo-controlled study (Terburg et al., 2013) the potential synergistic effects of a PDE4 inhibitor and SSRI combination were investigated. A standardised extract of *M. tortuosum*, Zembrin® (25 mg) a dual 5-HT reuptake and PDE4 inhibitor, administered to 16 healthy study participants subjected to an anxiety-related activity demonstrated the anxiety-attenuating effects of *M. tortuosum*, providing supporting evidence for the effectiveness of the combination of a PDE4 inhibitor and an SSRI. (Terburg et al., 2013).

By 5 days post fertilisation (5-dpf) zebrafish larvae exhibit a number of quantifiable behaviours such as hunting, avoidance, startle response, scototaxis, thigmotaxis, and increased locomotor activity in response to light-dark transitions (Fetcho and Liu, 1998; Colwill and Creton, 2011; Schnörr et al., 2012; Basnet et al., 2019). Scototaxis refers to the preference for darker over brightly lit environments by teleost fish (Maximino et al., 2010), whilst thigmotaxis is the preference for peripheral areas of a novel environment as opposed to the central area (Schnörr et al., 2012). Both behaviors, in addition to the high homology of zebrafish larva with mammals, present them as valuable models to perform many behavioral analysis, including anxiolytic-likeness. Other species such as rodents (Treit and Fundytus, 1988; Prut and Belzung, 2003; Sousa et al., 2006; Belzung and Philippot, 2007), other fish species (López-Patiño et al., 2008; Peitsaro et al., 2003; Sharma et al., 2009;

Champagne et al., 2010; Colwill and Creton, 2011) and humans (Kallai et al., 2005, 2007) also demonstrate thigmotaxis. These species all tend to prefer peripheral areas of a novel environment rather than the central area in response to stressful situations (Treit and Fundytus, 1988; Sharma et al., 2009). Thigmotaxis is the normal behavioural response of zebrafish larvae when exposed to anxiety-inducing light-dark transitions (de Esch et al., 2012; Vignet et al., 2014). In this study, a positive control drug (diazepam), which acts as an agonist of gamma aminobutyric acid (GABA) receptors in the CNS, was used to attenuate thigmotaxis behaviour.

Therefore, in furtherance of our previous investigation on *M. tortuosum*, this study aims to investigate the *M. tortuosum* alkaloids for possible anxiolytic-like behavior by assessing thigmotaxis and locomotor activity using the 5-dpf *in vivo* zebrafish model. Molecular modelling techniques were also employed to confirm and provide structural perspectives into any possible anxiolytic-like behavior of the alkaloids via the inhibition of SERT.

## 2. Materials and methods

### 2.1. *Mesembryanthemum tortuosum* alkaloids

*Mesembryanthemum tortuosum* L. alkaloids were made available from previous studies in our group as described by Shikanga et al. (2011). The four alkaloids were analysed for purity (>85%) prior to use in the *in vivo* assay. The analytical methods and typical spectra of *M. tortuosum* and the isolated alkaloids are shown in supplementary files S1 and S2.

### 2.2. Zebrafish husbandry and embryo collection

The zebrafish larvae experiments were carried out at the Medical University of Lublin in Poland. The facility is under the European and Polish law regulations, especially Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes and Act of 15th January of 2015 on the protection of animals used for scientific or educational purposes. According to the practice, all experiments on zebrafish larvae not capable of self-feeding (five days or younger) are exempted from applying for local ethical commission for experiments on animals. *Danio rerio* of the AB strain (Experimental Medicine Centre, Medical University of Lublin, Poland) were maintained at 28.5 °C, on a 14/10 h light/dark cycle, under standard aquaculture conditions. Fertilized eggs were collected via natural spawning. Embryos were reared in E3 embryo medium (pH 7.1–7.3; 17.4 µM NaCl, 0.21 µM KCl, 0.12 µM MgSO<sub>4</sub> and 0.18 µM Ca(NO<sub>3</sub>)<sub>2</sub>) in an incubator (IN 110 Memmert GmbH, Germany) at 28.5 °C until 5-dpf.

### 2.3. Determination of the maximum tolerated concentration (MTC)

Stock solutions of 50 mM for each alkaloid were prepared in 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), sonicated for complete dissolution, and stored at -20 °C. Concentrations required for MTC determinations were prepared each day from stock solutions ranging from 10 to 150 µM. Larvae at 5-dpf were gently transferred into a 48 well plate using a plastic Pasteur pipette. Five larvae were transferred into each well. The medium was removed from each well, and immediately 600 µL of each test sample was added to duplicate wells. The control group of larvae was exposed to 600 µL of 1% DMSO. The MTC plates were placed in an incubator for 18 h at 28.5 °C overnight and observed under a microscope (ZEISS, Germany) the following morning to detect the concentration at which sedation occurred, and signs of acute locomotor impairment were observed, including hypo-activity, absence of touch response, decreased touch response, loss of posture, body deformations (kyphosis, lordosis, scoliosis, and deformities), slow heartbeat, oedema, precipitation, and death.

### 2.4. Anxiolytic-like activity assay

The anxiolytic-like activity assay measuring thigmotaxis and locomotor activity of the isolated compounds of *M. tortuosum* was performed according to the method previously described by Maphangwa et al. (2020) and briefly summarised below. The anxiolytic-like activity assay was performed at 5-dpf on the zebrafish larvae. Stock solutions of each isolated compound were prepared at 100 mM in 1% DMSO, and working solutions (10, 15, 30, and 50 µM) were prepared in E3 medium each day immediately prior to the assay, in accordance with the results obtained from MTC determinations. A 100 mM stock solution of diazepam (DZ) (Sigma-Aldrich, Germany), dissolved in DMSO was prepared to yield a working solution of 10 µM using the zebrafish system water before the experiment. Three dilutions (2.5, 5, and 10 µM) were prepared to determine the most effective anxiolytic-like concentration of diazepam in 5-dpf zebrafish larvae as a positive control. The negative control group was treated with 1% DMSO. The plate was incubated for 30 min prior to the experiment and initiation of video tracking. Zebrabox (Viewpoint, Lyon, France) with ZebraLab software was used for video tracking. The plate was held in a multi-well plate holder located in the automated video recording bench station (Viewpoint) connected to a temperature control unit maintaining the temperature between 27 and 29 °C (Schnör et al., 2012).

### 2.5. Thigmotaxis and locomotor activity

This experiment was conducted over a period of 95 min with 10 min of acclimatization, 40 min of continuous lighting to study spontaneous locomotor activity, immediately followed by three light-dark transitions, to induce anxiety-like behaviour in the zebrafish larvae (15 min each, i.e., 10 min illumination and 5 min of darkness) (Peng et al., 2016). The protocol of Schnör et al. (2012) was adopted; the inner zone of the well was marked to study reverse-thigmotaxis, and a threshold was established to detect larval movement. The threshold for inactivity and shorter movements was set at 0.2 cm/s, while the total duration spent in longer movements was set at 0.8 cm/s. The model for the assessment of the thigmotaxis and locomotor activity was in accordance with the thorough descriptions outlined in our previous report (Maphangwa et al., 2020). Anxiolytic-like activity was defined as reversed thigmotaxis and was indicated by an increase in the time spent in the central arena. Measurement of the distance moved and the time spent in the central arena was acquired for the analysis. The percentage distance moved by zebrafish larvae and percentage time spent in the central arena were calculated as shown below:

$$\text{Anxiolytic-like activity (\% time in central arena)} = [\text{time in central arena} / \text{time in outer region + central arena}] \times 100 \quad (1)$$

$$\text{Anxiolytic-like activity (\% distance moved in central arena)} = [\text{distance moved in central arena} / \text{distance moved in outer region + central arena}] \times 100 \quad (2)$$

The locomotor activity was calculated using the tracking mode of ZebraLab software with recorded videos. The videos of zebrafish larvae were acquired at 25 frames per second (fps) and were pooled into 1-min time bins. The detection threshold was set at 25, an arbitrary level that allowed the software to detect the larvae movement accurately.

### 2.6. Computational methodology

#### 2.6.1. System preparation

The X-ray crystal structures of SERT complexed with paroxetine were retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (Berman et al., 2002) with identification code 6W2B (Coleman et al., 2020). The structure also contained a heavy chain antibody fragment, chain B, while the human SERT was labelled chain A. The antibody fragment was subsequently deleted during the preparation of the system to reduce computational cost. The presence of paroxetine in the SERT structure allowed for the identification of the inhibitor binding site of the studied inhibitors, the *M. tortuosum* alkaloids. The SERT complex was prepared for the molecular docking of the four alkaloids. The structures of the alkaloids, as shown in Fig. 1 were generated using Marvin Sketch 6.3.0 (ChemAxon, 2013). The 3D structure generation, energy minimization, and optimisation of the alkaloids were performed using the Avogadro software 2.0.8 (Hanwell et al., 2012) before preparation on University California San Francisco (UCSF) Chimera 1.11.2 (Pettersen et al., 2004) for molecular docking. Preparation of the complex for molecular docking involved removing hydrogen atoms and adding corresponding Gasteiger charges to each alkaloid using UCSF Chimera 1.11.2. Subsequently, the ligands were saved in mol2 formats for molecular docking.

#### 2.6.2. Molecular docking

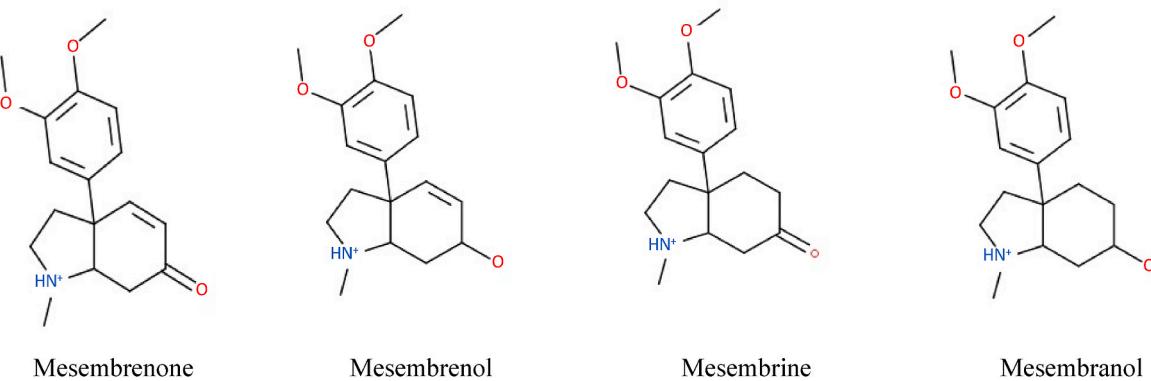
AutoDock Vina 1.1.2 (Trott and Olson, 2010; Nguyen et al., 2019) embedded in the software PyRx (Dallakyan and Olson, 2015) was employed for the molecular docking of the four alkaloids into the paroxetine binding pocket on SERT. AutoDock Vina was used because of its known scoring power and reliability in predicting accurate binding poses (Nguyen et al., 2019) (A grid box with coordinates X = 30.62, Y = 180.21, Z = 143.45 for centre and X, Y, and Z dimensions of 9.66, 9.50, and 7.57 respectively, for the size, were used. Generated docked results were displayed in Protein Data Bank, Partial Charge (Q), & Atom Type (T)) (pdbqt) format, and the optimal geometric position with the best pose and energy score was selected for each compound and saved in a complex form with reference to SERT. Intermolecular interactions were visualized using Discovery studio visualizer 3.0 (BIOVIA, 2017).

#### 2.6.3. Cheminformatics screening of the physicochemical and pharmacological properties of alkaloids

The physicochemical and pharmacokinetic properties of the alkaloids predicted on the SwissADME platform were used to ascertain their possible adherence to Lipinski's rules of five (Lipinski et al., 2001; C. A. Lipinski et al., 2012), a set of rules widely employed in assessing the drug-likeness of chemical compounds (Lipinski, 2000; Lipinski et al., 2012). An assessment of the oral toxicities of the alkaloids through the prediction of their LD<sub>50</sub>'s was performed using the ProTox webserver, which evaluated the toxicities based on structural similarities to identify over-represented fragments in toxic compounds (Drwal et al., 2014; Hurmath Unnissa and Rajan, 2016). AdmetSAR 2.0 (Hongbin et al., 2019) was subsequently employed to access the alkaloids' metabolic properties and predict their absorption and distribution properties.

### 2.7. Statistical analysis

The Prism software (GraphPad 7.04 Software, San Diego, CA) was



**Fig. 1.** Structures of *Mesembryanthemum tortuosum* alkaloids.

used to determine the statistical differences between various drugs and dilutions. Data were presented as mean  $\pm$  SEM. Light-dark response, dose-dependent response (treatment), or interaction between light-dark condition and dose was calculated using two-way ANOVA. According to GraphPad and Statistica software, Tukey Post-hoc is dedicated to one-way, whereas the Bonferroni test is dedicated to two-way ANOVA. The confidence limit of  $p < 0.05$  was considered statistically significant.

### 3. Results

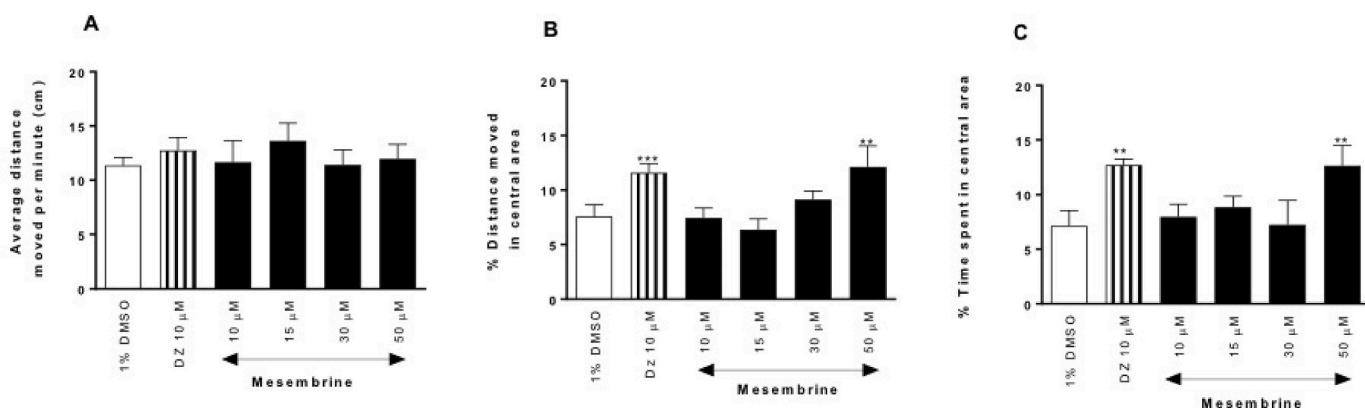
#### 3.1. Anxiolytic activity

##### 3.1.1. The effect of mesembrine on spontaneous locomotor activity of the zebrafish larvae during continuous illumination

Three different concentrations of diazepam (2.5, 5, and 10  $\mu$ M), a well-known anxiolytic drug, were tested to identify the most effective anxiolytic-like response in 5-dpf zebrafish larvae. The 10  $\mu$ M DZ concentration demonstrated the best anxiolytic-like activity (reverse-thigmotaxis) and was accordingly used in all further studies at this concentration for the positive control. Mesembrine is a well-known major compound found in the aerial parts of *M. tortuosum*; thus, the results of mesembrine are used as an example to demonstrate its effect on spontaneous locomotor activity of the zebrafish larvae during the 40 min of continuous illumination. Similar results were obtained for the other three alkaloids, mesembrenone, mesembrenol, and mesembranol. To avoid repetition only results for mesembrine are shown. These results are provided to demonstrate the response of larvae to alkaloid

treatment, the effect on locomotor activity, and the stimulatory effects of the alkaloids on the zebrafish larvae prior to exposure to the anxiety-mimicking light-dark transitions. The concentrations of alkaloids used in the test groups were informed by the results of the MTC studies, and encompassed the MTC and dilutions thereof. Concentrations between 75 and 150  $\mu$ M significantly impaired locomotor activity of the zebrafish larvae, so these concentrations were excluded in further studies; 50  $\mu$ M was determined as the MTC and accordingly data for 50, 30, 15 and 10  $\mu$ M test groups is presented. Neither diazepam (10  $\mu$ M) nor mesembrine at concentrations of 10, 15, 30, and 50  $\mu$ M impaired the locomotor activity of 5-dpf zebrafish larvae compared to the negative control (1% DMSO)(one way ANOVA:  $F(5,192) = 0.3016, p = 0.9995$ ) (Fig. 2A); no significant differences in total locomotor activity (reported as average distance moved per min in cm) were observed between test groups and the control. Reverse thigmotaxis behaviour was, however, noted to be significant between test and control groups. One-way ANOVA revealed statistically significant differences between the percentage of the total distance that was moved in the central arena during the 40 min of continuous lighting conditions ( $F(5,192) = 4.165, p = 0.0006$ ). Post-hoc Tukey's test confirmed that both 10  $\mu$ M diazepam ( $p < 0.001$ ) and mesembrine at a concentration of 50  $\mu$ M ( $p < 0.01$ ) resulted in statistically significant reverse-thigmotaxis behaviour when compared to the negative control, where diazepam and mesembrine showed a 78% and 77% increase in the total distance moved respectively (Fig. 2B).

One-way ANOVA also revealed statistically significant differences in the percentage of time spent in the central arena ( $F(5,192) = 4.117, p = 0.0028$ ). A post-hoc Tukey's test confirmed that diazepam (positive control) ( $p < 0.01$ ) and mesembrine (50  $\mu$ M) ( $p < 0.01$ ) significantly



**Fig. 2.** (A) The effects of mesembrine (10, 15, 30 and 50  $\mu$ M) and diazepam (10  $\mu$ M) on average distance moved by zebrafish larvae (cm) under continuous illumination. (B) The reverse-thigmotaxis behaviour observed as the percentage distance moved in the central arena under the influence of mesembrine (10, 15, 30 and 50  $\mu$ M) and diazepam (DZ, 10  $\mu$ M) under continuous illumination. (C) The thigmotaxis behaviour under continuous lighting was assessed by the percentage of time spent in the central arena under the influence of mesembrine (10, 15, 30 and 50  $\mu$ M) and diazepam (10  $\mu$ M). Data are presented as mean  $\pm$  SEM,  $n = 32$ . \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs positive control group (Post-hoc Tukey's test).

increased observed parameter compared to the negative control group (Fig. 2C). These results indicated statistically significant decreased thigmotaxis during continuous illumination after treatment with both diazepam and mesembrine ( $50 \mu\text{M}$ ), indicative of potential anxiolytic-like activity of mesembrine, where diazepam and mesembrine showed a 52% and 60% increase in the time spent in the central arena respectively.

### 3.1.2. The effect of the four alkaloids on distance travelled by the zebrafish larvae during the light-dark challenge assay

Changes in locomotor activity were observed in zebrafish larvae in all four alkaloid treatment groups in response to light-dark challenges. No obvious habituation was observed across all three light-dark cycles for all alkaloid test groups (data not shown). In order to better characterise the treatment effect for each alkaloid on the locomotor activities of the zebrafish larvae in response to light-dark challenge, the average distances travelled per min were determined. When the average distances were observed, two-way ANOVA showed statistically significant changes in light-dark condition response ( $F(1, 204) = 36.61, p < 0.0001$ ), treatment effect ( $F(5, 204) = 2.88, p = 0.0154$ ) as well as interaction ( $F(5, 204) = 3.69, p = 0.0032$ ). The Post-hoc Bonferroni's analysis demonstrated a statistically significant decrease in locomotor activity of zebrafish larvae during the dark challenge phase at 30 and  $50 \mu\text{M}$  concentrations of mesembrenone ( $p < 0.01$ ), respectively, in comparison with the DMSO-treated group during the dark phase (Fig. 3). The observed decrease in locomotor activity was 60% and 80%, respectively. Also, during the dark challenge phase significant increase in locomotor activity was observed when the 5-dpf larvae were treated with DMSO ( $p < 0.001$ ), diazepam  $10 \mu\text{M}$  ( $p < 0.01$ ) and mesembrenone -  $10 \mu\text{M}$  ( $p < 0.01$ ) and  $15 \mu\text{M}$  ( $p < 0.05$ ) compared with the light phase.

On exposure to mesembrenol, the zebrafish larvae demonstrated changes in locomotor activities in response to dark challenges. For the average distance moved by larvae, two-way ANOVA showed statistically significant changes in the light-dark condition response ( $F(1, 384) = 104.98, p < 0.0001$ ), treatment effect ( $F(5, 384) = 3.89, p = 0.0010$ ) as well as interaction ( $F(5, 384) = 2.89, p = 0.0057$ ). The Post-hoc Bonferroni's analysis showed a significant increase in locomotor activities during the dark challenge phase when the 5-dpf larvae were treated with

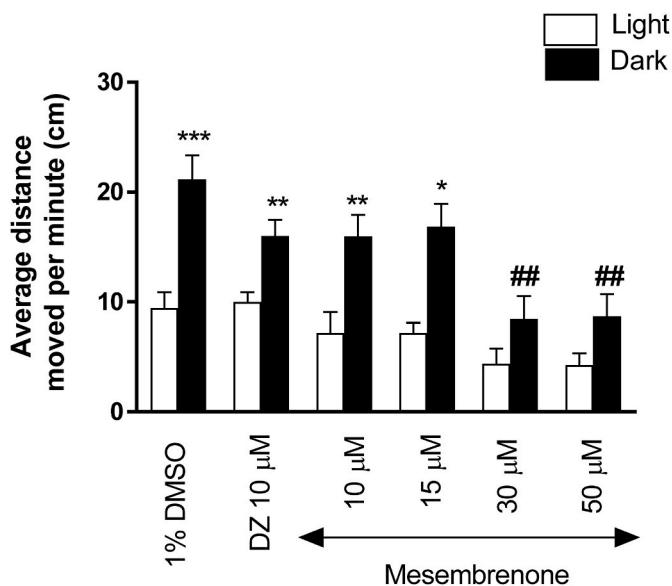
DMSO ( $p < 0.001$ ), and mesembrenol -  $10 \mu\text{M}$  ( $p < 0.05$ ),  $15 \mu\text{M}$  ( $p < 0.01$ ),  $30 \mu\text{M}$  ( $p < 0.01$ ) and  $50 \mu\text{M}$  ( $p < 0.05$ ) compared with the light phase. However, there was a significant statistical decrease in locomotor activities during the dark challenge phase when larvae were treated with diazepam and mesembrenol  $50 \mu\text{M}$  ( $p < 0.01$ ), where a 32% and 26. % decrease was observed respectively (Fig. 4).

When average distances travelled were observed, two-way ANOVA for mesembrine showed statistically significant changes in light-dark condition response [ $F(1, 204) = 36.61, p < 0.0001$ ], treatment effect [ $F(5, 204) = 2.88, p = 0.0154$ ] as well as interaction [ $F(5, 204) = 3.69, p = 0.0032$ ]. The post-hoc Bonferroni's analysis during the dark challenge phase demonstrated significant increases in locomotor activities when the 5-dpf larvae were treated with 1% DMSO ( $p < 0.001$ ), and mesembrine at concentrations of  $10 \mu\text{M}$  ( $p < 0.001$ ),  $15 \mu\text{M}$  ( $p < 0.001$ ),  $30 \mu\text{M}$  ( $p < 0.001$ ),  $50 \mu\text{M}$  ( $p < 0.01$ ) compared with the light phase. A significant 27.5% decrease in locomotor activities during the dark challenge phase was observed only on diazepam, the positive control ( $p < 0.01$ ) compared to the DMSO treated group (Fig. 5).

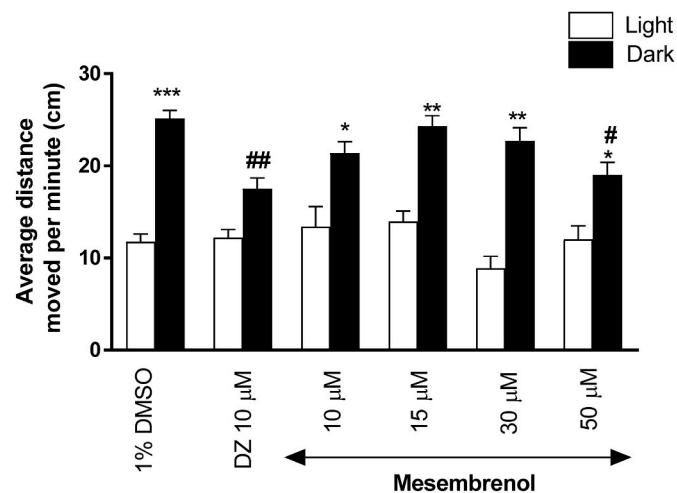
When average distance were considered, two-way ANOVA for mesembranol showed statistically significant changes in light-dark condition response ( $F(1, 384) = 104.98, p < 0.0001$ ), treatment effect ( $F(5, 384) = 3.89, p = 0.0010$ ) as well as interaction ( $F(5, 384) = 2.89, p = 0.0057$ ). The Post-hoc Bonferroni's analysis showed a decrease of locomotor activities during the dark challenge phase after diazepam and mesembranol  $10 \mu\text{M}$  and  $15 \mu\text{M}$  in comparison with DMSO-treated group in dark phase ( $p < 0.01$ ). The percentage decrease in the average distance moved by larva was 30.57% for diazepam while the percentage decrease for mesembranol  $10$  and  $15 \mu\text{M}$  was 40.5% and 41% respectively. During the dark challenge phase significant increases in locomotor activities were observed when the 5-dpf larvae were treated with DMSO ( $p < 0.001$ ), and mesembranol -  $10 \mu\text{M}$  ( $p < 0.05$ ),  $15 \mu\text{M}$  ( $p < 0.01$ ),  $30 \mu\text{M}$  ( $p < 0.001$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with the light phase as shown in Fig. 6.

### 3.1.3. The effects of the four alkaloids on percentage distance travelled in the central arena

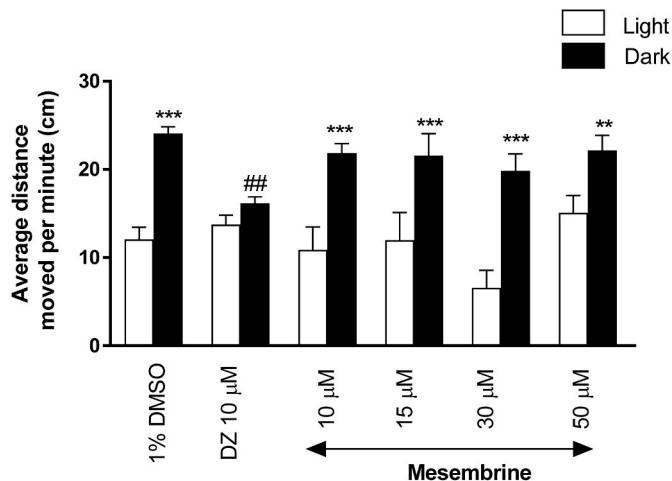
The effects of the four alkaloid treatments were studied during the light-dark challenges on reverse-thigmotaxis behaviour of the 5-dpf zebrafish larvae as they demonstrated an increased percentage on the distance travelled in the central arena. Two-way ANOVA for mesembrenone demonstrated the changes in light-dark response ( $F(1, 384) = 9.93, p = 0.0001$ ), treatment ( $F(5, 384) = 159.46, p = 0.0001$ ) and



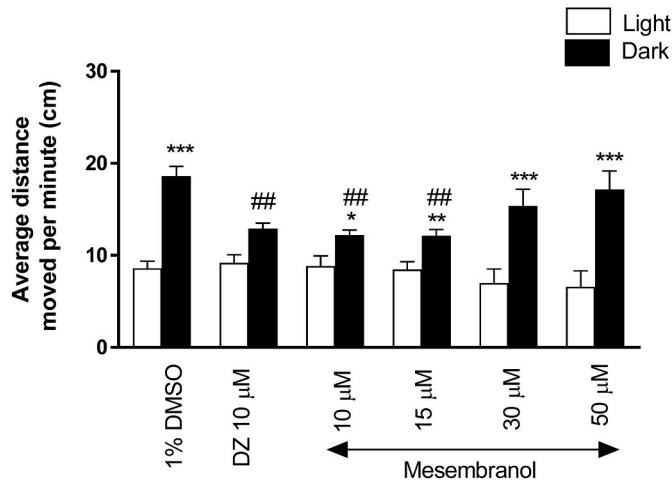
**Fig. 3.** Mesembrenone: Average distances moved by zebrafish larvae within each 1-min time bin under either light (open bars) or dark (filled bars) were plotted. Data are presented as mean  $\pm$  SEM,  $n = 32$  animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs the same group under light condition; # $p < 0.01$  vs control group under dark condition (Post-hoc Bonferroni).



**Fig. 4.** Mesembrenol: Average distances moved by zebrafish larvae within each 1-min time bin under either light (open bars) or dark (filled bars) were plotted. Data are presented as mean  $\pm$  SEM,  $n = 32$  animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs the same group under light condition; # $p < 0.01$  vs control group under dark condition (Post-hoc Bonferroni).



**Fig. 5.** Mesembrine: Average distances moved by zebrafish larvae within each 1-min time bin under either light (open bars) or dark (filled bars) were plotted. Data are presented as mean  $\pm$  SEM,  $n = 32$  animals per group. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , 1 vs the same group under light condition; # $p < 0.01$  vs control group under dark condition (Post-hoc Bonferroni).



**Fig. 6.** Mesembranol: Average distances moved by zebrafish larvae within each 1-min time bin under either light (open bars) or dark (filled bars) were plotted. Data are presented as mean  $\pm$  SEM,  $n = 32$  animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$  vs the same group under light condition; # $p < 0.01$  vs control group under dark condition (Post-hoc Bonferroni).

interaction ( $F(5, 384) = 4.18, p = 0.0011$ ). Post-hoc Bonferroni showed an increased percentage on the distance moved in the central arena after diazepam ( $p < 0.001$ ) and mesembrenone at  $10 \mu\text{M}$  ( $p < 0.001$ ),  $15 \mu\text{M}$  ( $p < 0.01$ ),  $30 \mu\text{M}$  ( $p < 0.001$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) in comparison with the light phase. During the dark challenge phase significant increases in locomotor activities were observed when the 5-dpf larvae were treated with diazepam ( $p < 0.001$ ), mesembrenone -  $10 \mu\text{M}$  ( $p < 0.05$ ),  $15 \mu\text{M}$  ( $p < 0.05$ ),  $30 \mu\text{M}$  ( $p < 0.01$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with DMSO-treated group in dark phase (Fig. 7A). Diazepam showed 120.27% increase in the distance moved in the central arena whiles mesembrenone  $10 \mu\text{M}$ ,  $15 \mu\text{M}$ ,  $30 \mu\text{M}$ , and  $50 \mu\text{M}$  showed a percentage increase of 28.3%, 32.5%, 67.36% and 106.4%, respectively.

Two-way ANOVA in light-dark condition for mesembranol showed the following statistics for response ( $F(1, 384) = 9.36, p < 0.0001$ ), treatment ( $F(5, 384) = 168.25, p = 0.0001$ ) and interaction ( $F(5, 384) = 9.98, p = 0.0001$ ). Post-hoc Bonferroni showed an increase in the percentage distance moved in the central arena after DMSO ( $p < 0.01$ ), diazepam ( $p < 0.001$ ) and mesembranol at  $10 \mu\text{M}$  ( $p < 0.001$ ))  $30 \mu\text{M}$  ( $p <$

$< 0.01$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with the light phase. During the dark challenge phase a significant increase in locomotor activities were observed when the 5-dpf larvae were treated with diazepam ( $p < 0.01$ ) and mesembranol at the dose of  $10 \mu\text{M}$  ( $p < 0.05$ ) when compared with 1% DMSO-treated group in dark phase, where a percentage increase of 56.4% and 49% was observed, respectively, as shown in Fig. 7B.

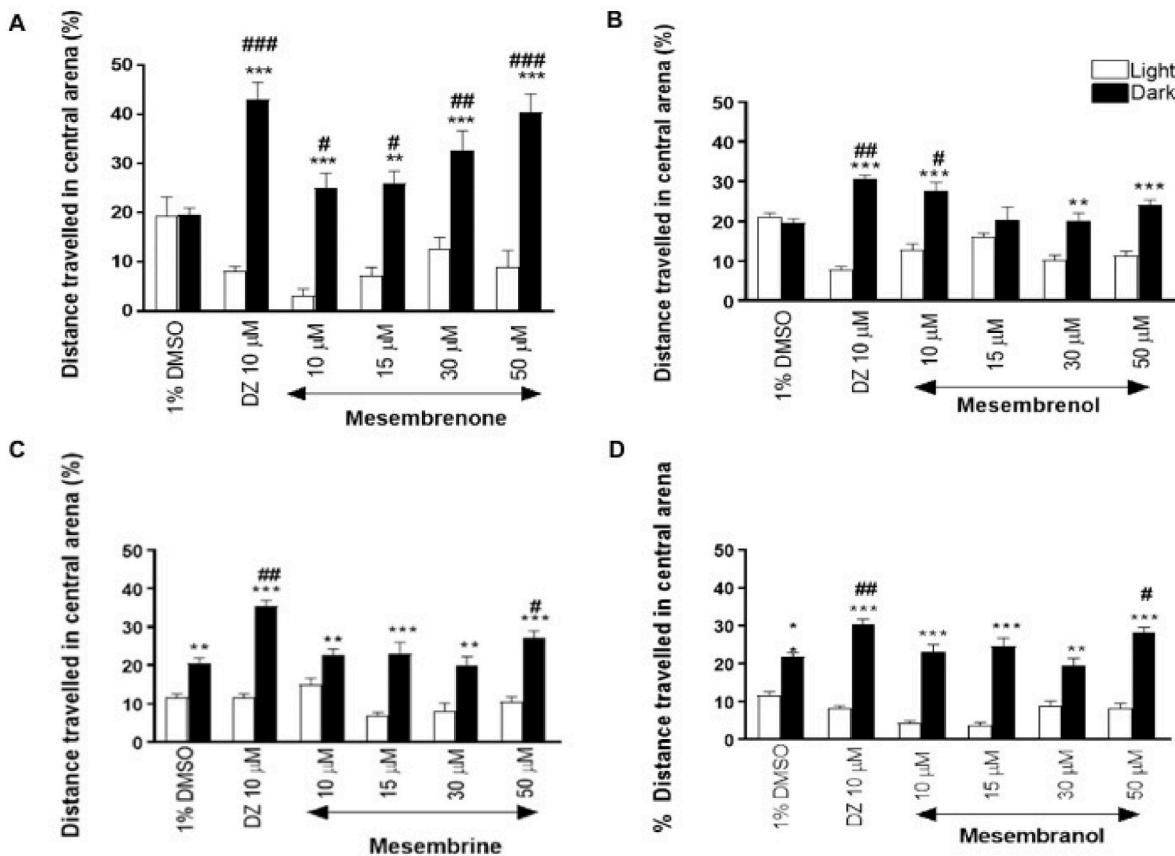
Two way ANOVA in light-dark condition for mesembrine showed statistically significant changes in light-dark condition response ( $F(1, 204) = 36.61, p < 0.0001$ ), treatment effect ( $F(5, 204) = 2.88, p = 0.0154$ ) as well as interaction ( $F(5, 204) = 3.69, p = 0.0032$ ). The Post-hoc Bonferroni's analysis showed a decrease in locomotor activities during the dark challenge phase after diazepam ( $p < 0.01$ ) in comparison with DMSO-treated group in dark phase and mesembrine at a concentration of  $50 \mu\text{M}$  ( $p < 0.05$ ). Diazepam showed a decrease of 73.5% whiles mesembrine  $50 \mu\text{M}$  showed a decrease of 31.16% in the distance moved in the central arena. Also, during the dark challenge phase significant increases in locomotor activities were observed when the 5-dpf larvae were treated with DMSO ( $p < 0.001$ ), diazepam (0.001) and mesembrine -  $10 \mu\text{M}$  ( $p < 0.001$ ),  $15 \mu\text{M}$  ( $p < 0.001$ ),  $30 \mu\text{M}$  ( $p < 0.001$ ),  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with the light phase as demonstrated in Fig. 7C.

For the zebrafish larvae at 5-dpf, mesembranol treatment and dark challenge altered thigmotaxis behaviours of the larvae, when the travelling distances in the central arena were considered. Two-way ANOVA light-dark condition ( $F(1, 384) = 19.36, p < 0.0001$ ), treatment [ $F(5, 384) = 168.25, p < 0.0001$ ] and interaction ( $F(5, 384) = 9.98, p < 0.0001$ ). Post-hoc Bonferroni showed an increase in percentage distance moved in the central arena after DMSO ( $p < 0.01$ ), diazepam ( $p < 0.001$ ) and mesembranol  $10 \mu\text{M}$  ( $p < 0.001$ ),  $15 \mu\text{M}$  ( $p < 0.001$ ),  $30 \mu\text{M}$  ( $p < 0.01$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with the light phase. During the dark challenge phase significant increases in locomotor activities were observed when the 5-dpf larvae were treated with diazepam ( $p < 0.01$ ) and mesembranol at the dose of  $50 \mu\text{M}$  ( $p < 0.05$ ) when compared with DMSO-treated group in dark phase, where the observed percentage increase was 38% and 12.3%, respectively (Fig. 7D).

#### 3.1.4. The effect of the four alkaloids on reverse-thigmotaxis behaviour with respect to time spent in the central arena, by the zebrafish larvae during the light-dark challenge

In addition to the distance travelled by the zebrafish larvae in the central arena of the well, the time spent in the center also plays a role in assessing reverse-thigmotaxis behaviour of the zebrafish larvae as an indication of potential anxiolytic-like activity. Two-way ANOVA and post-hoc Bonferroni's test revealed statistically significant results for the effects of mesembrenone, mesembranol, mesembrine, and mesembranol on the zebrafish larvae with respect to the time spent in the central arena. When reverse-thigmotaxis behaviour of the larvae for mesembrenone treated groups were observed, two-way ANOVA under the light-dark condition showed an increase of response [two way ANOVA light-dark condition as ( $F(1, 384) = 5.64, p < 0.0001$ ), treatment [ $F(5, 384) = 235.93, p < 0.0001$ ] and interaction ( $F(5, 384) = 4.18, p < 0.0001$ ]. Post-hoc Bonferroni showed an increase in the percentage of time spent on the central arena the during dark phase after DMSO-treatment ( $p < 0.01$ ), diazepam and mesembrenone at all doses ( $p < 0.001$ ) compared with the light phase. During the dark challenge phase a significant increase in the percentage time spent on the central arena was observed when the 5-dpf larvae were treated with diazepam ( $p < 0.01$ ) and mesembrenone at  $10 \mu\text{M}$  ( $p < 0.05$ ),  $15 \mu\text{M}$  ( $p < 0.05$ ),  $30 \mu\text{M}$  ( $p < 0.01$ ) and  $50 \mu\text{M}$  ( $p < 0.001$ ) compared with DMSO-treated group in dark phase as shown in Fig. 8A. Thus diazepam showed an increase of 120.3% and mesembrenone  $10 \mu\text{M}$ -28.34%,  $15 \mu\text{M}$ -32.5%;  $30 \mu\text{M}$ -67.4%,  $50 \mu\text{M}$ -106.4% in time spent in the central arena (Fig. 8A).

Two-way ANOVA demonstrated the effect of mesembranol under the light-dark condition when the time spent in the central arena was observed on an increased response [ $F(1, 384) = 8043, p < 0.0001$ ],



**Fig. 7.** Reverse-thigmotaxis behaviour demonstrated by the distance moved in the central arena throughout the three light-dark cycles as influenced by compounds of different concentrations. Open bars signify light cycle while shaded bars are for dark cycle were plotted to demonstrate the percentage distance moved by zebrafish larvae in the central arena. (A) Mesembrenone. (B) Mesembrenol. (C) Mesembreine (D) Mesembranol. Data are presented as mean  $\pm$  SEM,  $n = 32$ . \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs the similar group under light condition; # $p < 0.05$ , ### $p < 0.01$ , #### $p < 0.001$  vs positive control group under dark condition (Post-hoc Bonferroni's test).

treatment ( $F(5, 384) = 110.56 p < 0.0001$ ) and interaction ( $F(5, 384) = 10.42, p < 0.0001$ ). Post-hoc Bonferroni showed an increase in the percentage of time spent on the central arena during dark phase after DMSO-treatment ( $p < 0.05$ ), diazepam ( $p < 0.001$ ), and mesembrenol at the dose of 10  $\mu$ M ( $p < 0.01$ ), 30  $\mu$ M ( $p < 0.01$ ) and 50  $\mu$ M ( $p < 0.001$ ) compared with the light phase. During the dark challenge phase, significant increase in the percentage of time spent on the central arena was observed when the 5-dpf larvae were treated with diazepam ( $p < 0.01$ ) and mesembrenol - 10  $\mu$ M ( $p < 0.05$ ) when compared with the DMSO-treated group in the dark phase as shown in Fig. 8B. The observed percentage increase was 55.4% and 38.4%, respectively.

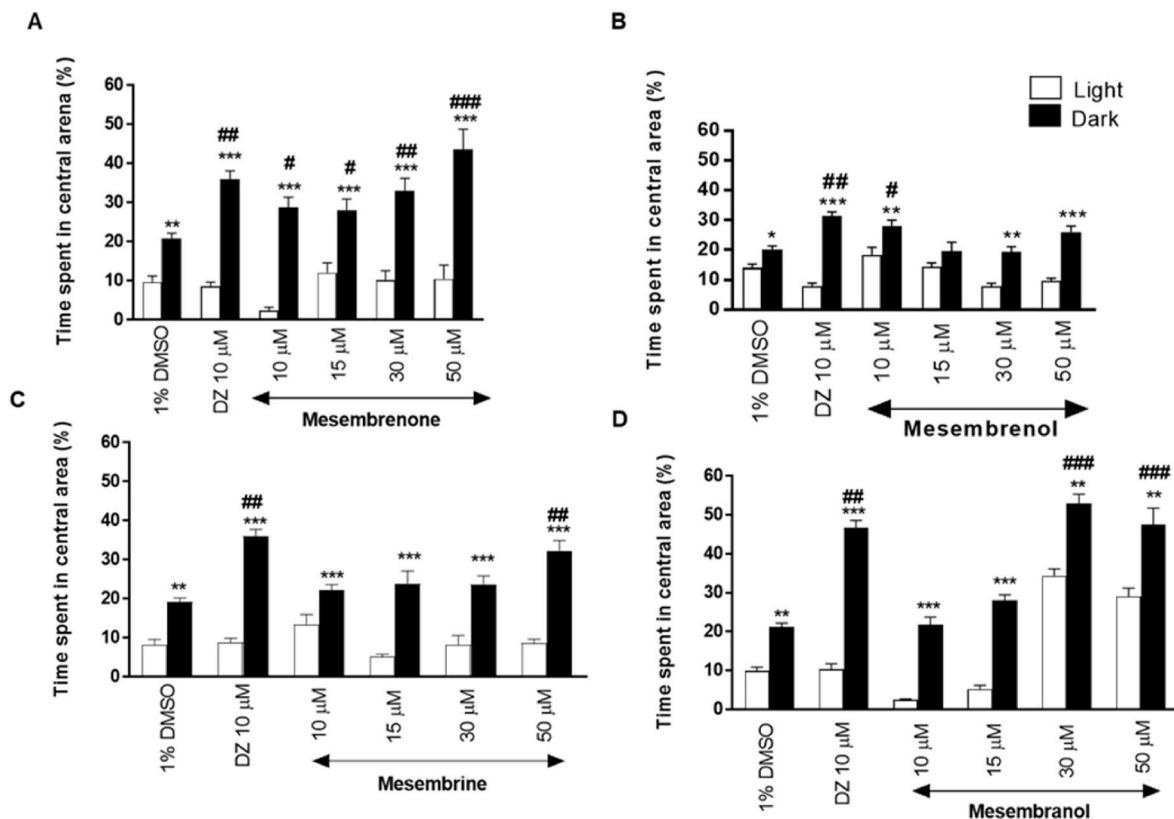
Mesembreine at different concentrations also demonstrated an effect under the light-dark condition when the time spent in the central arena was observed [two-way ANOVA light-dark condition response ( $F(1, 384) = 5.64, p < 0.0001$ ), treatment ( $F(5, 384) = 235.93 p < 0.0001$ ) and interaction ( $F(5, 384) = 4.18, p < 0.0001$ ). Post-hoc Bonferroni showed an increase in the percentage time spent on the central arena during the dark phase after DMSO-treatment ( $p < 0.01$ ), diazepam and mesembrenone at all doses ( $p < 0.001$ ) compared with the light phase. During the dark challenge phase a significant increase in the percentage time spent on the central arena was observed when the 5-dpf larvae were treated with diazepam and mesembreine - 50  $\mu$ M ( $p < 0.01$ ) compared with DMSO-treated group in dark phase shown in Fig. 8C. The observed percentage increase was 87.4% and 68.3%, respectively.

Mesembranol also demonstrated an effect on reverse-thigmotaxis behaviours on the larvae, when the durations of the activities in the central arena were observed [two-way ANOVA light-dark condition response ( $F(1, 384) = 8043, p < 0.0001$ ), treatment ( $F(5, 384) = 110.56 p < 0.0001$ ) and interaction ( $F(5, 384) = 10.42, p < 0.0001$ ).

Post-hoc Bonferroni showed an increase in the percentage time spent in the central arena during dark phase after DMSO-treatment ( $p < 0.01$ ), diazepam ( $p < 0.001$ ) and mesembranol at the dose of 10  $\mu$ M ( $p < 0.001$ ), 15  $\mu$ M ( $p < 0.001$ ), 30  $\mu$ M ( $p < 0.01$ ) and 50  $\mu$ M ( $p < 0.01$ ) compared with the light phase. During the dark challenge phase a significant increase in the percentage time spent in the central arena was observed when the 5-dpf larvae were treated with diazepam ( $p < 0.01$ ) and mesembranol at 30 and 50  $\mu$ M ( $p < 0.001$ ) when compared with DMSO-treated group in dark phase (Fig. 8D). Diazepam showed a 120.27% increase in the time spent in the central arena while mesembranol 30  $\mu$ M and 50  $\mu$ M showed a percentage increase of 150.5% and 124.7% respectively.

### 3.2. Differential physicochemical, pharmacokinetic and toxicity characteristics of the four alkaloids using *in silico* techniques

Several *in silico* software applications were employed to predict crucial properties of the alkaloids that would affect their absorption, distribution, metabolism, and potential toxic effects. The physicochemical properties and drug-likeness assessment of the alkaloids were predicted using the SwissADME platform and presented in Table 1. These were subsequently validated using AdmetSAR 2.0. All the alkaloids have a molecular weight below 500 g/mol, and based on the *in silico* predictions, all the alkaloids were shown to possess the following properties: an octanol-water partition coefficient below 5, hydrogen bond donors below 5, and hydrogen bond acceptors below 10. This suggested an adherence of all the alkaloids to Lipinski's rules of five (Lipinski et al., 2012), thus predicting their drug-likeness and potential suitability for bioactivity. Adherence of the alkaloids to Lipinski's rule of



**Fig. 8.** Reverse-thigmotaxis behaviour represented by the percentage time spent in the central arena during the three light-dark cycles under the influence of different concentrations of compounds. The percentage of time spent by a zebrafish larva in the central arena under either light (open bars) or dark (filled bars) was plotted. Data are presented as mean  $\pm$  SEM, n = 32 animals per group. (A) Mesembrenone. (B) Mesembrenol. (C) Mesembrine. (D) Mesembranol \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs the same group under light condition; #p < 0.05, ##p < 0.01, ###p < 0.001 vs positive control group under dark condition (Post-hoc Bonferroni's test).

**Table 1**  
Differential estimations of physicochemical properties of the *M. tortuosum* alkaloids.

Compound	Mesembrenone	Mesembrenol	Mesembrine	Mesembranol
Chemical formula	C <sub>17</sub> H <sub>21</sub> NO <sub>3</sub>	C <sub>17</sub> H <sub>23</sub> NO <sub>3</sub>	C <sub>17</sub> H <sub>23</sub> NO <sub>3</sub>	C <sub>17</sub> H <sub>25</sub> NO <sub>3</sub>
Molecular weight (g/mol)	287.35	289.37	289.37	291.39
Number of heavy atoms	21	21	21	21
Number of aromatic heavy atoms	6	6	6	6
Number of rotatable bonds	3	3	3	3
Number of H-bond acceptors	4	4	4	4
Number of H-bond donors	0	1	0	1
TPSA (Å <sup>2</sup> )	38.77	41.93	38.77	41.93
Molar Refractivity	85.04	86.00	85.51	86.48
LogP <sub>O/W</sub>	2.84	2.13	2.32	2.26
LogS (moles/L)	-1.96	-1.91	-2.09	-2.21
LD <sub>50</sub> (mg/kg)	580	420	369	340
Bioavailability Radar Summary	Lipo Flex Insatu Size Polar Insolu	Lipo Flex Insatu Insolu Polar Size	Lipo Flex Insatu Insolu Polar Size	Lipo Flex Insatu Insolu Polar Size

five further suggested favourable oral bioavailability and a tendency to cross various aqueous and lipophilic barriers such as the blood-brain barrier (BBB) and the gastrointestinal tract (Lipinski et al., 2012). The prediction of the lipophilicity of the alkaloids on SwissADME was based on the Brain Or Intestinal EstimateD permeation method (BOILED-Egg) concept (Daina and Zoete, 2016). As shown in Table 1, each of the

alkaloids was shown to fall within the suitable physicochemical space (colored zone) correlating with oral bioavailability. The ability of the alkaloids to cross lipophilic barriers was also evidenced by a LogPow of less than 5, representing favourable lipophilicity (Lipinski et al., 2001) and a topological polar surface area (TPSA) below 140 Å. A predicted TPSA below 140 Å for all the alkaloids suggested the ability of the

alkaloids to be transported across the lipid bilayer and the BBB since TPSA takes into account polar atoms on the surfaces of the compounds (Ertl et al., 2000; Prasanna and Doersken, 2009; Shityakov et al., 2013). To augment their predicted drug-likeness and lipophilicity, the LD<sub>50</sub> of the alkaloids, a parameter that assesses the differential toxicities when orally administered, was also predicted using the ProTox platform. Compounds with an oral LD<sub>50</sub> of 0–50 mg/kg are considered highly toxic, whereas compounds with LD<sub>50</sub> greater than 2000 mg/kg are less toxic (Morris-Schaffer and McCoy, 2021). Accordingly, it could be deduced that the alkaloids would present no oral toxicity since they each showed LD<sub>50</sub> values above 0–50 mg/kg, as shown in Table 1.

All the alkaloids were predicted to have the ability to permeate the BBB, to be absorbed through the human intestinal wall, possessed Caco-2 permeability, were potential substrates of P-glycoprotein, and were non-inhibitors of P-glycoprotein. The favourable predictions towards these key markers of drug absorption (Hubatsch et al., 2007; Lin, 2004; Lin and Yamazaki, 2003; Van Breemen and Li, 2005) further suggest their therapeutic potential and thereby warrant further investigation. These findings corroborated reports by Shikanga et al. (2012) in which the purified or crude extract form of the *M. tortuosum* alkaloids was shown to permeate across intestinal, buccal, and sublingual mucosal tissue.

The prediction of the metabolic properties of the alkaloids using key biological markers, notably CYP450 2D6 and CYP450 2C9 on the AdmetSAR platform, also showed that all the alkaloids were non-substrates and non-inhibitors of CYP450 2C9, a crucial drug-metabolising enzyme that accounts for about 18% of cytochrome P450 protein content in the human microsomes (Van Booven et al., 2010). Also, all the alkaloids were predicted to be potential substrates and inhibitors of CYP450 2D6, except mesembrine, which was the only non-inhibitor of CYP450 2D6. As substrates of CYP450 2D6, an enzyme implicated in the metabolism of about 25% of current drugs in clinical use, including known antidepressants like paroxetine, suggests a guarantee of the metabolism of the alkaloids upon absorption (Ingelman-Sundberg et al., 2007; Vuppulanchi, 2011). The predicted potential inhibition of CYP450 2D6 is characteristic of cotreatment with SERT inhibitors, leading to a decrease in the metabolism of substrate drugs (Tirona and Kim, 2017). Overall the alkaloids were shown to possess favourable pharmacokinetic and physicochemical properties, which warrants further investigation of the *M. tortuosum* alkaloids as potential inhibitors of SERT.

### 3.2.1. Exploring the binding mechanisms of *M. tortuosum* alkaloids to the serotonin reuptake transporter (SERT)

Having established the anxiolytic-like effects of these alkaloids in the previous sections of the study, we further investigated the potential SERT inhibitory mechanism of the alkaloids by comparing their binding mechanism with the known SERT inhibitor, paroxetine (Nevels et al., 2016; Pollack et al., 2001). Molecular docking of the four *M. tortuosum* alkaloids; mesembranol, memsembrane, mesembrenone, and mesembranol, revealed docking scores of -7.9 kcal/mol, -8.1 kcal/mol, -8.1 kcal/mol, and -7.9 kcal/mol, respectively, within the paroxetine binding pocket as shown in Table 2. Redocking of paroxetine also revealed a docking score of -11.1 kcal/mol. The similarity in the docking scores of the alkaloids suggests a similarity in binding pocket stability and affinity towards SERT. The alkaloids were also shown to engage in an extensive network of interactions with SERT pocket residues similar to paroxetine, suggesting a similarity in a binding mechanism. These extensive interactions anchor the alkaloids within their respective binding pockets, which intend to favour the formation of high-affinity binding interactions.

According to a report by Coleman et al., in 2019 and 2020 and a separate report by Slack et al. (2019), residues Asn177 and Ala169 are crucial to therapeutic inhibition of SERT by paroxetine. These particular residues were shown to engage in van der Waals and π-alkyl interactions with paroxetine, as shown in Table 2. Other residues crucial to drug

binding of SERT include; Asp98, Try95, and Ile172 (Barker et al., 1998; Henry et al., 2006; Sørensen et al., 2012). Interaction with these residues is known to establish high-affinity recognition of antidepressants. Interestingly, the alkaloids elicited strong interactions with these crucial residues, as shown in Table 2, thereby suggesting their inhibitory potential and possible high-affinity recognition similar to known antidepressants. Specifically, mesembranol engaged in salt bridge interaction with Asp98, whereas van der Waals interactions were formed with Try95, Ile172, Asn177, and Ala169. Mesembrenol engaged in salt bridge interaction with Asp98, a conventional hydrogen bond with Tyr95, and a π-alkyl with Ile172. Mesembrenone, on the other hand, elicited a salt bridge interaction with Asp98, a conventional hydrogen bond with Tyr95, and a π-alkyl with Ile172. Mesembrine was also shown to exhibit a conventional hydrogen bond with Tyr95, π-alkyl with Ile172, and a van der Waals interaction with Asp98. The similarity in the dynamics of the alkaloids' interactions suggests a similarity in their binding mechanisms, whereas their collective similarity with paroxetine also suggests a similarity in binding mechanisms and a possible SERT inhibitory activity.

As shown in Table 2, the unique orientations of the alkaloids could have also contributed to the favourable interactions observed. Mesembranol and mesembrenol assumed unique orientations that allowed for the formation of salt bridges between the indole ring and Asp98 and possibly accounted for the similar docking score of -7.9 kcal/mol. The observed peculiar orientations of both mesembranol and mesembrenol could be attributed to the conventional hydrogen bond-mediated hydroxyl group on C6, a moiety previously reported by Dimpfel and colleagues in 2018 as a crucial determinant of their mechanism of action. Mesemberine and mesembrenone assumed unique orientations characterized by their anchorage at opposing ends of the binding pocket by conventional hydrogen bond interactions between Tyr95, Val97, and the indole rings and π-alkyl between the dimethoxy-phenyl moiety and Ile172, Val501 and Phe335. These peculiar orientations and associated interaction on both mesembrenone and mesembrine collected accounted for the similarity in docking score of -8.1 kcal/mol, particularly, the interactions mediated by the unique carbonyl group on the C6 of both alkaloids. Collectively these varying binding modes could favour binding pocket stability and binding affinity of the alkaloids.

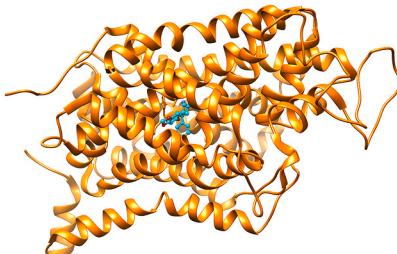
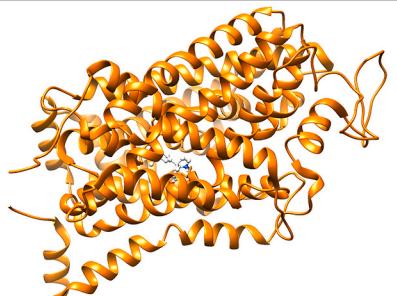
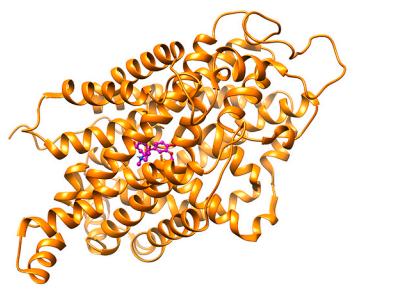
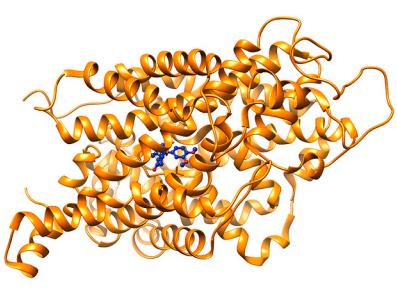
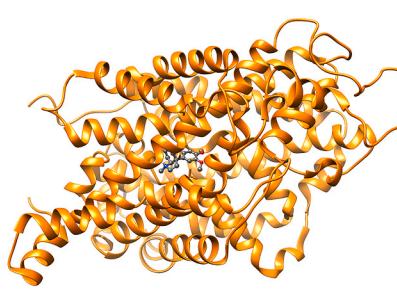
## 4. Discussion

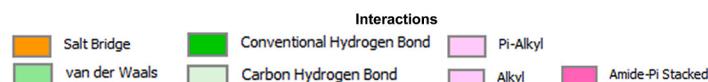
*Mesembryanthemum tortuosum*, known as 'kougoed' or 'channa' in South Africa, traditionally used for its tranquilizing and anxiolytic properties (Gericke and Viljoen, 2008; Smith, 2011), is marketed as Zembrin®, a standardized ethanolic extract (Shikanga et al., 2012). Since 2010 *M. tortuosum* has been the subject of much *in vitro* and *in vivo* research, as well as clinical studies, with respect to its CNS activity. All these studies, with the exception of Fountain (2016), corroborated its mood elevation, antidepressant or antiepileptic activity (Gericke and Viljoen, 2008; Harvey et al., 2011; Loria et al., 2014; Schell, 2014; Carpenter et al., 2014; Dimpfel et al., 2018). The anxiolytic-like effects of *M. tortuosum* have also been studied and substantiated by some research groups (Dimpfel et al., 2018; Fountain, 2016). In a recent study by Maphanga et al. (2020) on various extracts of *M. tortuosum*, the aqueous extract exhibited the highest anxiolytic-like activity in the larval zebrafish light-dark challenge model of anxiety; reverse-thigmotaxis behaviour was evaluated, and the increased time spent in the central arena demonstrated the superior anxiolytic-like activity of the water extract to that of other less polar extracts. These promising results prompted further analysis of compounds potentially responsible for the bioactivity of the water extract.

Accordingly, in this study, the psychoactive properties of alkaloids (mesembrenone, mesembranol, mesembrine, and mesembranol) obtained from *M. tortuosum* were investigated for anxiolytic-like activity using zebrafish larvae subjected to light-dark challenges as an assay for anxiolytic-like effects; the findings were further confirmed using *in silico*

**Table 2**

Docking score, 3D docked conformations and residue interaction profiles of the *M. tortuosum* alkaloids bound to SERT.

Compound	Docking Score (Kcal/mol)	Docked Complex	Residue interaction profile
Paroxetine	-11.1		
Mesembranol	-7.9		
Mesembrenol	-7.9		
Mesembrenone	-8.1		
Mesembrene	-8.1		



modelling techniques. During the dark challenge, the *M. tortuosum* alkaloids applied at different concentrations exhibited anxiolytic-like effect as evidenced by the reverse-thigmotaxis behaviour of the zebrafish larvae compared to the control group. When the activity of each of the alkaloids was compared with the other alkaloids and with the positive control, diazepam, a well-known anxiolytic benzodiazepine, all the alkaloids demonstrated an anxiolytic-like effect; however mesembrenone and mesembranol demonstrated greater anxiolytic-like activity than mesembrace and mesembrenol.

The pharmacokinetics and physicochemical properties of an administered drug are crucial to its absorption, distribution, metabolism, excretion, and toxicity (ADMET) (Klopman et al., 2002; Lin and Lu, 1997; Lombardo et al., 2017; Tahir ul Qamar et al., 2019; Van de Waterbeemd and Gifford, 2003). These properties affect the bioavailability of the drug and, consequently, its safety and efficacy as a therapeutic agent. The ADMET properties can be investigated via experimental methods; however, these are usually time-consuming and expensive. Therefore, multiple computational tools were employed, namely; Molinspiration Cheminformatics (Ertl, 2002), SwissADME (Daina et al., 2017), AdmetSAR 2.0 (Cheng et al., 2012; Hongbin et al., 2019), and ProTox (Drwal et al., 2014), to evaluate the physicochemical and pharmacokinetic properties of the compounds under investigation, as these relate to the possible inhibitory activity of the alkaloids. The application of multiple predictive tools was to allow for the reproducibility of the results while validating the methods. Harvey et al. (2011), in a study of the effects of a standardised ethanolic extract of *M. tortuosum*, commercially available as Zembrin® and the purified isolated alkaloids, found that the extract exhibited potent inhibitory effects on the SERT ( $IC_{50}$  4.3  $\mu$ g/mL) as well as on PDE4 ( $IC_{50}$  8.5  $\mu$ g/mL), but no effect was observed on other PDEs. Mesembrenol, mesembrenone, and mesembrace inhibited binding to the SERT but showed minimal effect at GABA receptors. Mesembrace was the most potent inhibitor of the SERT ( $K_i$  1.4 nM). Of the isolated alkaloids, mesembrenone exhibited the most potent inhibitory effect on PDE4 ( $IC_{50} < 1 \mu$ m), as well as an inhibitory effect on the SERT ( $IC_{50} < 1 \mu$ m). None of the alkaloids displayed cytotoxicity. The authors concluded that while all three alkaloids are responsible for the observed anxiolytic-like and anti-depressant effects of Zembrin®, mesembrace, and mesembrenone contribute the greatest portion to the physiological effect, as per binding assay predictions. Fountain (2016) studied the effects of *M. tortuosum* in a chick anxiety-depression model and showed that an alkaloid enriched *M. tortuosum* extract reduced anxiety but did not show any effect on depression in this model.

Dimpfel et al. (2018) are of the opinion that all four alkaloids contribute to the activity of Zembrin®. In an *ex vivo* (Zembrin®) and direct *in vitro* (individual alkaloids) study in rat hippocampal slices, the excitability of the tissue was attenuated. In addition, the action of AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) agonist fluoro- willardine was inhibited by the full extract and by mesembranol and mesembrace, leading the authors to speculate that these two alkaloids present promising therapeutic leads for the development of antiepileptic medicines. Glutamate gated ion channel receptors of the AMPA receptors subtype mediate the fast excitatory synapse transmission in the CNS (Geiger et al., 1995) and so represent a potential therapeutic target in the treatment of CNS disorders.

The pharmacological activity of the alkaloids at particular target receptors reported by Harvey et al. (2011) with resultant anxiolytic effects was further established in the current study in which the alkaloids were shown to demonstrate a similar binding mechanism to that of paroxetine (Nevels et al., 2016; Pollack et al., 2001), a known SERT inhibitor. The binding mechanisms of alkaloids were particularly characterized by high-affinity interaction with crucial residues highlighted in previous reports by Coleman et al. (2020, 2019) and Slack et al. (2019) as pertinent residues in the inhibition of SERT. Another interesting factor to consider is the structural properties of the alkaloid molecules. Mesembrenol and mesembranol have a hydroxyl group on

carbon six, while mesembrace and mesembrenone have a carbonyl group on the same carbon. Thus, there is a connection between the structural activity of these alkaloids (Dimpfel et al., 2018). The importance of these groups to the structural activity of the respective alkaloids was corroborated in this report by the implication of the groups in the formation of peculiar intermolecular interactions with SERT binding pocket residues observed in the molecular docking performed, and consequently accounted for the favourable SERT-binding affinity of the respective alkaloids.

Nonetheless, the current study provides some indication that mesembrenone and mesembranol may potentially exhibit greater anxiolytic-like effects than diazepam, even though they differ structurally. A further study, comprising full dose-response effects, would be required to provide comprehensive substantiating evidence for this. Since most of the compounds showed activity at the higher concentrations applied, this suggests that mesembrenone and its derivatives may exert their anxiolytic-like activity in a concentration-dependent manner. Further studies using a wider concentration range will be necessary to confirm this. Based on these facts, *M. tortuosum* alkaloids have demonstrated a possible anxiolytic-like effect as evidenced by their effects on hyper-locomotor activity and thigmotaxis behavior of zebrafish larvae in the light-dark challenge.

## 5. Conclusions

*M. tortuosum* alkaloids demonstrated a potential anxiolytic-like effect on the zebrafish larvae under light-dark transitions, an assay that induces an anxiety-like response in zebrafish larvae. This study provides evidence that the four alkaloids (mesembrenone, mesembranol, mesembrace, and mesembranol) produce anxiolytic-like effects in *M. tortuosum*; the extract may however contain yet other compounds that also exhibit anxiolytic-like effects in zebrafish. Screening these alkaloids in higher vertebrates might be considered in future studies to further validate the potential anxiolytic-like activity. Further computational studies may be valuable in determining qualitative structure-activity relationships (SAR) for the alkaloids, followed by compound optimisation and experimental validation of the constructed SAR model.

## CRediT authorship contribution statement

**Veronica B. Maphanga:** Masters student performed the *in vivo* work, Formal analysis, Writing – original draft, Writing – review & editing. **Krystyna Skalicka-Wozniak:** Project administration, Supervision, Writing – review & editing. **Barbara Budzynska:** Assisted with statistical, Formal analysis. **Andriana Skiba:** Assisted with *in vivo* zebrafish assays. **Weiyang Chen:** Assisted with the phytochemistry analytical work. **Clement Agoni:** Assisted with *in silico* predictions, Writing – original draft. **Gill M. Enslin:** Project administration, Supervision, Writing – review & editing. **Alvaro M. Viljoen:** Conceptualization, Supervision.

## Declaration of competing interest

A. Viljoen declares his role as Editor-in-Chief of the Journal of Ethnopharmacology. A. Viljoen also acts as a scientific advisor to HGH Pharmaceuticals, the producers of Zembrin®. However, all assays were conducted at an independently facility in Poland with whom A. Viljoen has no affiliation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2022.115068>.

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