ORIGINAL INVESTIGATION

Screening selected medicinal plants for potential anxiolytic activity using an in vivo zebrafish model

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Abstract

Rationale Medicinal plants are used extensively in many countries to treat conditions related to the central nervous system (CNS), and there is renewed interest to explore natural products, which may exhibit CNS activity.

Objective In this study, seven plants were selected based on literature reports of their ethnopharmacological use in treating anxiety-related conditions and assayed in a zebrafish model.

Methods Crude extracts were prepared with solvents of different polarities, and the maximum tolerated concentration (MTC) of these crude extracts was established. The anxiolytic activity of the crude extracts was determined using 5-day post-fertilization (dpf) zebrafish larvae. General locomotor activity and reverse-thigmotaxis behavior (indicative of anxiolytic activity) were analyzed under continuous illumination and alternating light-dark challenges, which induced anxiety in the zebrafish larvae.

Results Of the 28 extracts tested, 13 were toxic according to the MTC values obtained. Larvae were subsequently treated with the 15 non-toxic extracts, at a dose determined in the MTC assay or with 1% DMSO as control. The anxiolytic activity (reverse-thigmotaxis) was demonstrated by an increase in the percentage time spent by the larvae in the central arena of the well. Of the 15 non-toxic extracts tested, the *Sceletium tortuosum* water extract exhibited the most promising anxiolytic activity.

Conclusions The zebrafish model was effective in studying anxiety-related behavior. Thus, the study confirmed that *S. tortuosum* mitigates anxiety in zebrafish larvae, a step towards the full in vivo validation of the traditional use of the plant.

Keywords Anxiety · Zebrafish model · Anxiolytic activity · Light-dark transitions

Introduction

Anxiety is an intense or overwhelming feeling which threatens psychological and physical well-being (Sousa et al. 2015). When the feeling of anxiety continues far beyond the presence of the stimulus and disrupts normal functioning it is defined as an anxiety disorder. The intensity and duration may differ from one person to the other and reach a point where it affects normal daily functioning (Koda-Kimble et al. 2012). According to Romana and co-workers (Romana et al. 2017), the neurobiology of anxiety disorder is unknown. However, anxiety, like other psychiatric disorders including depression and mood disorders, is linked to neurotransmitter imbalances within the central nervous system (CNS). Four neurotransmitters play a major role in mood regulation; serotonin, dopamine, gamma-aminobutyric acid (GABA), and norepinephrine. The most widespread neurotransmitter is glutamate, which is assisted by three catecholamines, dopamine, epinephrine, and norepinephrine. Inhibitory neurotransmitters include GABA, glycine, and serotonin. GABA is a major inhibitory neurotransmitter, which assists neurons to recover after impulse transmission and in so doing reducing stress and anxiety, particularly after excessive stimulation.. Epinephrine and norepinephrine are also regulated by GABA in decreasing the excitability in the neural transmission chain (Romana et al. 2017). Anxiolytic drugs encompass a number of drug classes; the barbiturates are no longer used due to their narrow therapeutic index and potential for toxicity; benzodiazepines and

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their derivatives are commonly used to treat anxiety but also have undesirable effects such as sedation and their propensity to produce dependence; a third major class is the selective serotonin reuptake inhibitor (SSRI) drugs, but often initially worsen anxiety and have a delayed onset of action (Benneh et al. 2017).

Benzodiazepines, including alprazolam, lorazepam, and diazepam, remain a first line pharmacological anxiolytic treatment (Koda-Kimble et al. 2012). The target receptors for benzodiazepines are the γ-aminobutyric acid (GABA$_A$) receptors present in the neuronal membranes in the CNS. At low doses benzodiazepines selectively enhance GABAergic transmission in the GABA$_A$ receptor, thereby inhibiting neuronal circuits in the limbic system of the brain (Finkel et al. 2009).

The SSRI drugs inhibit the serotonin transporter protein (5-HTT), the regulator of serotonergic neurotransmission, which is, as such, the main molecular target for these commonly used antidepressant drugs (Lesch et al. 1993). Inhibition of 5-HTT increases the serotonergic tone in the brain to regulate emotions (Thakker et al. 2005).

Available anxiety treatment is expensive, is very often not ideal due to side effects, and has to be individualized for every patient (Bandelow et al. 2008). There are an increasing number of individuals who prefer using complementary and alternative medicines in the treatment of physical and psychiatric symptoms (Van der Watt et al. 2008). Most patients believe that complementary medicines cause fewer side effects than allopathic medicines and complementary alternatives are often less expensive (Bandelow et al. 2008). Furthermore, traditional medicines are still an integral part of culture and customs in many countries, especially in the majority of African communities (Fennell et al. 2004). According to the World Health Organization (WHO) 2000, 70–95% of the population in developing countries still use traditional medicines. The knowledge of using traditional medicine has been passed on from one generation to the next based on cognitive features, ecological factors, and cultural history (Leonti 2011). These indigenous knowledge systems emphasize the advantages of ethnopharmacologically guided bioprospecting to conserve both the medicinal plant resources as well as the healer-expert cultural wisdom and to facilitate culturally relevant health promotion (Pesek et al. 2009).

A number of medicinal plants have been studied based on their putative anxiolytic activity and use in traditional medicine systems. *Sceletium tortuosum* (L.) N.E. Br. (Mesembryanthemaceae) enhances well-being and relieves anxiety, stress, or depression (Gericke and Viljoen 2008). Naringenin, a flavonoid isolated from *Mondia whitei* (Hook.f.) Skeels (Apocynaceae), displayed affinity for the GABA$_A$- receptor, the same site at which benzodiazepines act (Stafford et al. 2008). The roots of *Xysmalobium undulatum* (L.) W.T. Aiton (Asclepiadaceae) are used by the Xhosa people to treat exaggerated or uncontrollable emotions (Nielsen et al. 2004; Stafford et al. 2008) and has been reported to have anti-depressant activity (Hutchings and Van Staden 1994).

Stress and anxiety can now be studied in the zebrafish model, which has been validated as a neurobehavioral science model (Kalueff et al. 2014). The zebrafish has been used for decades as an animal model for biomedical research, especially in developmental and genetic studies (Zon and Peterson 2005). The zebrafish, which has shares a highly homologous genome with vertebrate species (rodents and humans), as well as brain modeling, structure, and function, provides the added advantages of high fecundity, small size and allows rapid high-volume cost-effective screening. Furthermore, zebrafish have numerous neural and physiological systems (stress regulating system) in common with vertebrates (Burne et al. 2011; Guo 2004, 2009; Lieschke and Currie 2007) and share with them some key receptors that play an important role in the etiology of neurological disorders (Guo 2004). The model is ideally suited for large-scale analysis as hundreds of fertilized eggs can be harvested daily, 4 to 5-dpf larvae demonstrate a broad range of behaviors, and their small size allows for easy handling (Schnörr et al. 2012).

The light-dark emergence task is one of the tests commonly used to determine anxiety-like behavior in rodents (Arrant et al. 2013; Crawley 1985; Peng et al. 2016). The animal's choice between the dark or bright side of a chamber demonstrates changes in anxiety level. This test, together with the open field exploration test, was adapted to the zebrafish model (Belzung and Grieble 2001; Ramcharitar and Ibrahim 2013). Locomotion and thigmotaxis are two important parameters in the zebrafish model that are indicative of anxiety levels. Additionally, light-dark transitions also elevate anxiety levels in the fish (Blaser et al. 2010; Maximino et al. 2010). Thigmotaxis (a preference for peripheral areas of the tank or well) is a behavioral endpoint commonly used to measure anxiety. Rodents were commonly used before this behavior was successfully adapted to a number of models, including 5-dpf zebrafish larvae. The zebrafish larvae demonstrate thigmotaxis by avoiding the central arena and moving to the peripheral areas of a novel environment (Schnörr et al. 2012; Sharma et al. 2009; Treit and Fundytus 1988). The assessment of thigmotaxis behavior is dependent on adequate physical exploration of all spatial zones (centre and periphery) of a novel environment (Bouwknecht and Paylor 2008; Schnörr et al. 2012).

There is a need for the validation of the ethnopharmacological use and development of safe, pharmacologically effective, and cost-efficient medicines to treat mental health disorders, such as anxiety. In the current study crude extracts of medicinal plants reported in literature for their use in traditional medicine to alleviate anxiety and other CNS conditions, namely, *Melia azedarach* L. (Meliaceae), *Mondia whitei* (Hook.f.) Skeels (Apocynaceae), *Piper*

methysticum G.Forst. (Piperaceae), *Sceletium tortuosum* (L.) N.E. Br. (Mesembryanthemaceae), *Sutherlandia frutescens* (L.) R.Br. (Fabaceae), *Withania somnifera* (L.) Dunal (Solanaceae), and *Xysmalobium undulatum* (L.) W.T. Aiton (Asclepiadaceae), were screened for potential anxiolytic activity using an effective in vivo zebrafish assay.

It was hypothesized that medicinal plants used traditionally in the treatment of anxiety-related conditions would exhibit potential anxiolytic activity in the in vivo zebrafish model. This would cement the value of a simple, efficient, and reliable assay in evidence-based ethnopharmacological research, while simultaneously identifying those plants with potential toxicity.

Methods

Plant preparation

Plants that have been reported to have CNS effect were identified based on their ethnopharmacological use (Tables 1 and 2). No endangered (Red List) species were included in the study. Voucher specimens and retention samples are stored in the Department of Pharmaceutical Sciences, Tshwane University of Technology, Arcadia, Pretoria, South Africa.

Extraction of plant material

Plant materials were dried at 30 °C and ground into a powder. Four different extracts were prepared (distilled water, dichloromethane, ethyl acetate and methanol). A sonicator was used to accelerate the extraction process, and the extract was filtered using Whatmann filter paper (No: 1). The extraction process was repeated twice on the residue using 40 mL and 20 mL of fresh solvent for second and third extractions, respectively, to maximize the yield. A rotary evaporator was used to concentrate the extract. The crude extracts were further dried in a fume hood, transferred to Eppendorf micro tubes, tightly sealed, and stored in a refrigerator at 4–8 °C until required for analysis when they were dissolved in the required solvent immediately prior to use.

Zebrafish husbandry and egg collection

Husbandry and maintenance of the wild type zebrafish (AB) strain were carried out according to standard protocol (Westerfield 2000). The zebrafish were maintained in a controlled environment at a temperature of 26–28.5 °C, conductivity of 550–700, and pH ranging between 6.9 and 7.5 on a 14-h light and 10-h dark cycle. Zebrafish male and female adult fish (3 months and older) were used for mating; each pair was used once every 2 weeks to maximize egg production. Mating tanks were prepared a day before reproduction by

natural spawning. Females were kept separated from males in mating tanks overnight (dark cycle). As soon as the lights were turned on, the separator in the tank was removed and fish were left for an hour to reproduce. Embryos were collected and placed in a petri dish. System water was used to remove debris from the embryos, and thereafter, the medium (containing nutrients important for normal development of embryo) was added to the petri dish. The embryos were observed under a microscope (ZEISS, Germany) to distinguish between fertilized and none fertilized embryos. Cleaning and sorting were done daily until 5-dpf; afterwards larvae were used in experiments. All behavioral tests were performed in a room with an ambient temperature of 28.5 °C.

Determination of the maximum tolerated concentration

Stock solutions of 500 mg/L of each extract were prepared in dimethyl sulfoxide (DMSO), sonicated for complete dissolution and stored at − 20 °C. Concentrations required for MTC determinations were prepared each day from stock solutions ranging from 12.5 to 500 mg/L. 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 initial plate for MTC determinations was set up with four concentrations of each test sample (in duplicate) ranging from maximum to minimum dissolvable concentration. The second plate was set up to verify the MTC results obtained in the first plate. The third plate was prepared in lower concentrations depending on the MTC results obtained in the first and second plates in accordance with the method of Berghmans et al. (2007). 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, slow heartbeat, edema, precipitation, and death.

Anxiolytic activity assay

The anxiolytic activity assay was performed at 5-dpf with one larva in each well of a 24-well plate. Stock solutions of each crude extract were prepared at 50 mg/L, and working solutions were prepared each day immediately prior to the assay, in accordance with the results obtained from MTC determinations. Larvae were transferred from a petri dish into a 24-well plate gently using a plastic Pasteur pipette (one larva per well). The medium was removed, and 1 mL of each test extract was

Table 1 Ethnopharmacological use of plants investigated for anxiolytic activity

Family Species Common name	Origin	Plant part used	Traditional use	Pharmacological activity reported from several studies	References
Meliaceae Melia azedarach L. Chinaberry	Asia, Africa, America and Australia	Flowers, leaves and seeds	Ayurveda medicine in India	Calmative properties, anxiolytic activity, anti-diuretic, anti-rheumatic, anti-fungal and anti-oxidant activity	Hurst 1942; Jain et al. 2006; Grundmann et al. 2009
Apocynaceae Mondia whitei (Hoof.F) Skeels White ginger	Most African countries expect the northern parts	Roots	Stimulates appetite, aphrodisiac, stress and tension relief	Anti-depressant activity	Pedersen et al. 2008
Piperaceae Piper methysticum G.Forst Kava	Western Pacific (Fiji, Samoa, Tonga, Vanuata)	Rhizomes and roots	Traditionally kava drink is prepared from the roots which have anesthetic, sedative, anti-convulsive, muscle relaxant properties	Aqueous kava preparation produced significant anxiolytic and antidepressant activity	Van Wyk and Wink 2004; Sarris et al. 2009; Gomes et al. 2009
Mesembryanthemaceae Sceletium tortuosum (L.) N.E. Br. Kougoed (chewing substance)	South Africa	Whole plant	Relieves hunger and thirst, used as therapy for alcoholics, treating mood swings	Mesembrine acts as a serotonin re-uptake inhibitor. Has anti-depressant and anxiolytic effects	Gericke and Van Wyk 2001; Van Wyk and Wink 2004
Fabaceae Sutherlandia frutescens (L.) R.Br. Cancer bush	Southern Africa	Twigs and leaves	Extracts are used in treatment of burns, wounds and inflammatory skin conditions	Stress reducing properties, anti-cancer, anti-viral, anti-diabetic activity	Van Wyk and Wink 2004; Smith and Myburgh 2004
Solanaeceae Withania somnifera (L.) Dunal Winter cherry	Africa, southern Europe and Asia	Roots and leaves	Infusions, decoctions and tinctures are taken as adaptogenic tonics, sedatives and hyptonics	Plant enhances memory and learning acquisition, anxiolytic and antidepressant activity	Van Wyk and Gericke 2000; Howes et al. 2003; Van Wyk and Wink 2004
Asclepiadaceae Xysmalobium undulatum (L.) W.T. Aiton Ishongwe/Uzara	Southern Africa	Roots and leaves	Administered by Xhosa people to treat hysteria	Leaf extract demonstrates antidepressant activity	Nielsen et al. 2004; Van Wyk and Wink 2004

immediately added to each well. The negative control group was treated with 1 mL of 1% DMSO. A 100-mM stock solution of diazepam was prepared to make a working solution of 10 μM diazepam, the positive control, using the zebrafish system water prior the experiment.

The plate was incubated for 30 min prior 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 (Schnörr et al. 2012).

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 activity, immediately followed by three light-dark transitions, to induce anxiety-like behavior in the zebrafish larvae (15 min each, i.e., 10-min illumination and 5 min of darkness) as shown in Fig. 1 (Peng et al. 2016). Due to the length of time it took to conduct experiments and to reduce the risk of unwanted variation, all doses of a single extract were subjected to the assay on day one and the same experiment repeated on the following day, day two.

This protocol was adopted after Schnörr et al. (2012). The response of the zebrafish larvae to various doses of the test extracts and dark-light transitions (i.e., the independent variables) in terms of thigmotaxis (wall-hugging behavior) and increased locomotor activity (i.e., the dependent variables) was measured in a series of experiments on each extract. The inner zone of the well, an inner circular area 4 mm from the wall of the well, the central arena, was marked to study anxiolytic activity (reverse-thigmotaxis), and a threshold was established to detect larval movement.

Table 2 Plant extracts tested for maximum tolerated concentration (MTC) and anxiolytic activity

Plant	Source	Voucher specimen	Code	Solvent used	Yield (mg)
Melia azedarach L.	Parceval		Ma(DW)	Distilled water	646.4
			Ma(DCM)	Dichloromethane	210.5
			Ma(EA)	Ethyl acetate	75.3
			Ma(MEOH)	Methanol	351.8
Mondia whitei (Hoof.F) Skeels	Parceval		Mw(DW)	Distilled water	245.6
			Mw(DCM)	Dichloromethane	322.5
			Mw(EA)	Ethyl acetate	225.3
			Mw(MEOH)	Methanol	351.8
Piper methysticum G.Forst	Hawaii	KVLHa-20	Pm(DW)	Distilled water	75.4
			Pm(DCM)	Dichloromethane	153.7
			Pm(EA)	Ethyl acetate	218.4
			Pm(MeOH)	Methanol	221.1
Sceletium tortuosum (L.) N.E. Br.	Afrigetics Botanicals	NANST1016	Sc(DW)	Distilled water	192.9
			Sc(DCM)	Dichloromethane	36.2
			Sc(EA)	Ethyl acetate	28.3
			Sc(MeOH)	Methanol	156.5
Sutherlandia frutescens (L.) R.Br.	Kruishof	TUT0001070	Sf(DW)	Distilled water	66.1
			Sf(DCM)	Dichloromethane	58.5
			Sf(EA)	Ethyl acetate	51.6
			Sf(MeOH)	Methanol	244.2
Withania somnifera (L.) Dunal	Muthi Market		Ws(DW)	Distilled water	48.7
			Ws(DCM)	Dichloromethane	13.1
			Ws(EA)	Ethyl acetate	16.8
			Ws(MeOH)	methanol	80.3
Xysmalobium undulatum (L.) W.T. Aiton	Thaba Chweu	L8 R577-10D	U(DW)	Distilled water	98.1
			U(DCM)	Dichloromethane	58.3
			U(EA)	Ethyl acetate	46.4
			U(MeOH)	Methanol	461.9

The threshold for inactivity and small movements was set at 0.2 cm/s, while threshold for large movements was set at 0.8 cm/s. The anxiolytic activity was defined as reverse-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 were acquired for the analysis. The percentage distance moved by zebrafish larvae and percentage time spent in the central arena was calculated as shown below:

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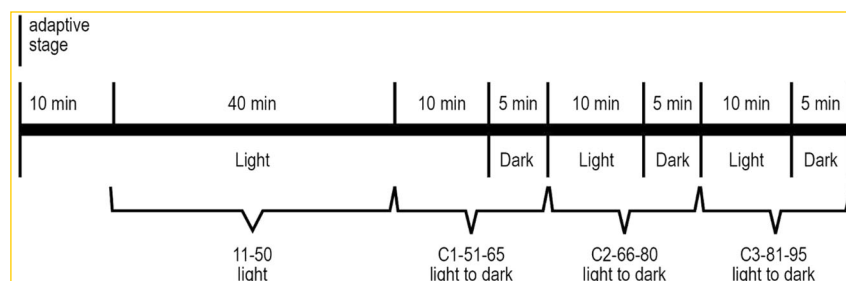
$$\text{Anxiolytic activity (\%distance moved in central arena)} = [\text{distance moved in central arena/distance moved in outer region} + \text{central arena}] \times 100$$

The locomotor activity was calculated using the tracking mode of ZebraLab software with recorded videos. The videos of zebrafish larvae were taken at the rate of 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 accurately detect the movement of the larvae.

Statistical analysis

To determine the statistical differences between various drugs and dilutions, the Prism software (GraphPad Software, San Diego, CA) was used. Data were presented as mean ± SEM. Data were analyzed using one-way or two-way analysis of variance (ANOVA) to compare the pretreatment and treatment effects. One-way ANOVA was followed by the Tukey's post hoc test. Two-way ANOVA was followed by the Bonferroni's post hoc test. Dose-dependent influence on

Fig. 1 The lighting conditions and light-dark transitions of the locomotor activity and thigmotaxis experiment (Peng et al. 2016)

adaptive stage								
10 min	40 min	10 min	5 min	10 min	5 min	10 min	5 min
	Light		Dark	Light	Dark	Light	Dark

11-50 light   C1-51-65 light to dark   C2-66-80 light to dark   C3-81-95 light to dark

spontaneous locomotor activity or thigmotaxis behavior was evaluated when one-way ANOVA statistical analysis was performed. Light-dark response, dose-dependent response (treatment), or interaction between light-dark condition and dose was calculated when two-way ANOVA was performed. According to GraphPad and Statistica software Tuckey post hoc is dedicated to one-way, whereas Bonferoni test is dedicated to two-way ANOVA. The confidence limit of $p < 0.05$ was considered statistically significant.

Results

According to the MTC values obtained, 13 out of 28 extracts were toxic. Thus, 15 extracts were tested in an initial screening study. In the first step of the experiment, spontaneous locomotor activity was measured, as total distance moved by the larvae in the whole well area, within each 1-min time bin during the 40 min of continuous illumination (Table 3). Locomotor activity data were summarized for each individual larvae replicate and analyzed. The next step was to evaluate the level of thigmotaxis, as a validated index of anxiety (Table 4). Only the *Sceletium tortuosum* water extract exhibited significant ($p < 0.001$) anxiolytic activity (reverse-thigmotaxis) compared with control group during light-dark transitions. This extract was therefore subjected to further analysis for more detailed behavioral tests.

The effect of SC-DW on the spontaneous locomotor activity and thigmotaxis behavior of the zebrafish larvae

One-way ANOVA revealed that the diazepam and tested extract at all concentrations (12.5, 25, 50 mg/L) did not influence the locomotor activity of the zebrafish larvae at 5-dpf ($F(4,160) = 0.8933$, $p = 0.4726$) (Fig. 2a).

One-way ANOVA showed statistically significant differences between groups when the distance moved during 40 min under continuous light was considered ($F(4,160) = 6.801$, $p = 0.002$). Indeed, post hoc Tukey's test confirmed that diazepam ($p < 0.01$) and SC-DW at the concentration of 12.5 and 25 mg/L increased the distance moved in the central arena ($p < 0.05$, $p < 0.01$, respectively) in comparison with

DMSO-treated groups (Fig. 2b). When the time spent in the central arena was considered, one-way ANOVA showed statistically significant effect ($F(3,106) = 6.062$, $p = 0.0007$). Post hoc Tukey's test confirmed that diazepam ($p < 0.05$) and SC-DW at the concentration of 12.5 and 25 mg/L increased the percentage of time spent in the central arena ($p < 0.01$, $p < 0.05$, respectively) in comparison with DMSO-treated groups (Fig. 2c). This result indicated a decrease of thigmotaxis behaviors in response to diazepam and SC-DW incubation.

The effects of SC-DW on thigmotaxis behaviors of the zebrafish larvae during the light-dark challenge

In order to characterize the effects of SC-DW treatment on the locomotor activity of the zebrafish larvae in response to light-dark challenge, the average distance traveled per minute in all three light-dark cycle was determined as shown in Fig. 3a. Two-way ANOVA showed statistically significant changes in light-dark condition response ($F(1,320) = 2.95$, $p = 0.0129$), as well as interaction ($F(4,320) = 5.99$, $p < 0.0001$) without treatment effect ($F(4,320) = 1.78$, $p = 0.1835$) (Fig. 3a). The post hoc Bonferroni's analysis showed increase of locomotor activities during the dark challenge phase after DMSO treatment ($p < 0.01$) in comparison with DMSO-treated group in light phase (Fig. 3). Also, during the dark challenge phase significant decreases in locomotor activities were observed when the 5-dpf larvae were treated with diazepam 10 μM ($p < 0.01$) and SC-DW in all concentrations ($p < 0.01$) compared with DMSO-treated group in dark phase. During the light challenge phase significant increases in locomotor activities were observed when the 5-dpf larvae were treated with SC-DW (12.5 mg/L) ($p < 0.01$) and SC-DW (25 mg/L) ($p < 0.05$) compared with DMSO-treated group in light phase (Fig. 3a).

The light-dark transitions were also studied for thigmotaxis behavior on the zebrafish larvae. Figure 3b represents the percentage of distance traveled by zebrafish larvae in the centre arena in each minute. For the zebrafish larvae at 5-dpf, diazepam and SC-DW treatment in the dark challenge influenced on thigmotaxis behaviors of the larvae, when the traveling distances were considered (two-way ANOVA light-dark condition ($F(1,320) = 102.85$, $p < 0.0001$), treatment
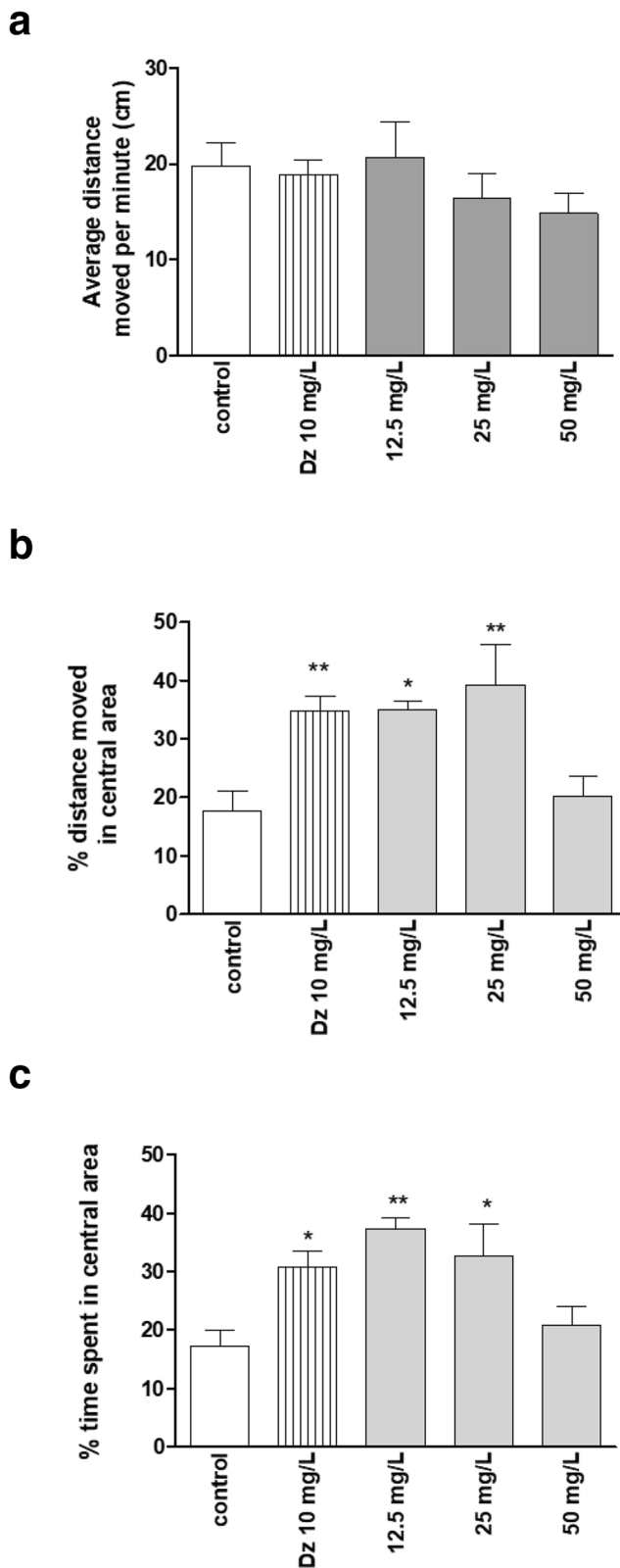
Table 3 The effects of the selected plant extracts on distance moved (in cm) by zebrafish larvae under continuous illumination. Average distance moved by zebrafish larvae during the 40 min is reported. Data are presented as mean ± SEM, $n = 18$; $*p < 0.05$ vs control group (post-hoc Tukey's test)

DMSO (control)	Extract	DOSE (mg/L)										F	P
		500	250	200	150	100	75	50	25	12.5	6		
10.12±1129	Ma-DW	19.44±1.736	18.45±2.158	19.99±2.521	8.37±0.743	10.24±1.299	13.78±1.874	15.86±2.760	15.74±2.063	9.85±0.625	-	(9180)=1.1116	0.1645
14.67±0.869	Mon-DW	-	18.27±1.208	16.33±1.307	17.82±1.640	14.87±1.582	-	10.37±1.023	14.04±1.419	11.14±1.169	-	(8.162)=3.563	0.0011
14.92±1.724	Pm-EA	12.21±1.380	11.27±2.275	15.36±0.656	12.68±2.058	18.57±2.395	16.53±2.102	17.63±1.726	11.88±1.600	15.66±1.435	-	(8.162)=1.971	0.0578
10.65±1.485	Sc-DW	12.48±2.086	14.61±2.323	17.30±2.736	12.04±1.731	12.16±1.393	-	5.84±0.649	9.77±1.217	11.58±1.557	15.16±1.547	(10.198)=2.096	0.0296
15.21±0.754	Sc-DCM	-	14.89±1.465	15.84±1.302	18.60±1.425	18.66±1.846	19.25±1.608	19.78±1.999	18.65±1.654	18.42±2.892	-	(8.162)=1.1696	0.1078
16.04±0.749	Sc-EA	-	14.89±1.465	15.84±1.302	18.60±1.425	20.33±2.006	20.91±2.038	19.78±1.999	18.65±1.654	21.75±3.446	18.42±1.949	(8.162)=0.1874	0.0710
14.44±1.241	Sc-MEOH	-	4.50±1.635 *	5.41±0.9807 *	5.69±2.162 *	7.507±0.851	13.27±4.623	20.89±1.805	13.76±1.649	15.08±1.890	-	(7.144)=6.817	<0.0001
15.69±0.792	Sf-DW	17.69±0.791	15.60±2.296	-	16.03±1.897	13.73±1.616	15.22±1.009	15.30±1.080	18.44±1.282	16.95±1.152	-	(8.162)=1.021	0.4252
17.39±0.907	Sf-DCM	-	-	-	-	-	-	-	19.54±0.938	21.73±1.133 *	20.69±1.903	(3.72)=2.889	0.0418
19.97±4.464	Sf-EA	-	-	-	-	-	-	-	17.92±1.019	21.26±1.814	-	(2.162)=0.7561	0.4825
21.05±1.138	Sf-MEOH	-	-	-	-	-	-	20.36±1.450	-	-	-	(5.162)=1.146	0.3421
17.5±1.592	Ws-DW	23.29±1.686	22.5±1.719	23.26±1.328	19.37±1.715	20.37±1.837	20.95±2.000	18.43±1.555	21.07±2.112	16.61±2.305	16.91±1.485	(9.180)=1.834	0.0642
15.16±0.527	Ws-DCM	-	-	-	-	11.78±1.006	13.82±1.225	18.63±1.379	12.43±1.134	12.59±1.269	-	(5.162)=5.269	0.004
16.76±1.485	Ws-EA	-	-	-	-	17.29±2.021	15.00±0.9627	17.76±1.455	23.67±1.969	22.33±1.790	-	(5.162)=4.349	0.018
24.62±1.483	Ws-MEOH	-	25.30±1.275	27.14±1.33	29.23±1.240	26.09±1.522	25.53±1.822	23.78±1.375	23.16±1.634	25.30±1.275	-	(8.162)=0.1899	0.0614

Table 4 The evaluation of reverse-thigmotaxis behavior represented by the percentage time (minutes) spent in the central arena during light-dark cycle transitions under the influence of different concentrations of extracted plants

DMSO (control)	Extract	DOSE (mg/L)										F	P
		500	250	200	150	100	75	50	25	12.5	6		
17.79±1.875	Ma-DW	16.40±1.889	18.74±1.848	19.22±2.356	21.36±2.480	20.10±1.964	-	16.36±1.445	12.51±1.595	12.41±0.818	-	(9180)=1.871	0.0723
17.34±1.289	Mon-DW	-	14.77±2.028	18.41±0.968	15.5±2.131	17.45±2.323	22.05±3.252	20.61±2.442	14.48±2.254	21.78±3.685	-	(8162)=1.543	0.1514
19.12±1.086	Pm-EA	10.31±1.450	10.91±4.489	13.85±1.091	17.89±2.378	14.60±1.453	14.36±1.663	16.98±1.786	18.92±1.813	15.95±3.040	-	(8162)=1.923	0.063
20.03±1.829	Sc-DW	14.26±1.501	18.73±3.587	14.85±2.037	16.49±1.091	17.05±1.322	21.46±2.692	23.13±1.813	24.56±2.813	25.52±2.164 *	16.43±2.021	(10.198)=4.575	<0.0001
18.90±1.955	Sc-DCM	-	11.59±1.741	11.45±1.382	18.24±3.561	22.06±8.622	24.30±6.029	15.12±2.807	13.95±2.106	19.41±5.045	-	(8162)=1.195	0.3092
17.11±1.173	Sc-EA	-	12.51±3.125	19.82±3.589	11.11±1.918	17.91±3.483	14.21±1.917	19.95±4.995	17.83±4.184	17.74±4.516	19.15±3.863	(8162)=0.4309	0.9014
16.17±1.332	Sc-MEOH	-	13.40±6.322	5.21±1.107	15.42±8.635	11.37±2.293	10.15±2.989	12.18±1.884	13.45±1.366	13.92±2.782	-	(7144)=0.8444	0.5538
20.22±1.888	Sf-DW	20.77±3.085	22.26±1.467	-	21.77±1.970	20.61±1.802	23.09±2.062	19.67±2.082	14.47±1.556	15.57±0.788	-	(8162)=1.979	0.0559
19.48±1.984	Sf-DCM	-	-	-	-	-	-	-	12.15±2.183	15.71±3.666	-	(3.72)=1.573	0.2039
18.94±2.193	Sf-EA	-	-	-	-	-	-	-	23.43±1.819	19.04±2.085	-	(2.54)=1.747	0.1987
21.18±1.479	Sf-MEOH	-	-	-	-	-	-	-	15.07±1.347	18.70±2.019	23.07±5.872	(5108)=2.175	0.0607
19.02±1.250	Ws-DW	16.18±1.495	17.08±1.515	15.55±1.700	18.23±1.854	17.16±1.653	16.00±1.658	17.86±1.300	20.74±2.046	19.10±3.138	-	(9180)=0.7999	0.6168
19.53±2.159	Ws-DCM	-	-	-	-	15.47±2.811	16.36±2.069	16.01±1.434	16.17±1.653	17.90±0.838	-	(5108)=0.6188	0.6859
19.10±1.763	Ws-EA	-	-	-	-	13.42±2.050	14.56±1.698	17.81±1.965	14.28±1.829	13.70±1.744	-	(5108)=1.734	0.1397
16.23±1.263	Ws-MEOH	-	16.70±0.939	17.68±1.837	16.35±1.648	17.07±2.138	15.51±1.826	16.6±1.920	15.83±1.840	15.49±1.675	-	(8.62)=0.1787	0.9936

The percentage time spent by a zebrafish larva in the central arena under light-dark is shown. Data are presented as mean ± SEM. $n = 18$. $*p < 0.05$. vs the DMSO-control group (post-hoc Tukey's test)

a

b

c

Fig. 2 a The effects of diazepam (DZ 10 mg/L) and SC-DW (12.5, 25, 50 mg/L) on distance moved by zebrafish larvae (in cm) under continuous illumination. Average distance moved by zebrafish larvae within each 1-min time bin was plotted. b The thigmotaxis behavior was observed as the percentage of the distance moved in the central arena in different concentrations of SC-DW (12.5, 25, 50 mg/L) under continuous lighting. Data are presented as mean ± SEM, $n = 32$. c The thigmotaxis behavior under continuous lighting was evaluated by the percentage of the time spent in the centre arena under the influence of SC-DW (12.5, 25, 50 mg/L) in different concentrations Data are presented as mean ± SEM, $n = 32$. $*p < 0.05$, $**p < 0.01$, vs control group (post-hoc Tukey's test)
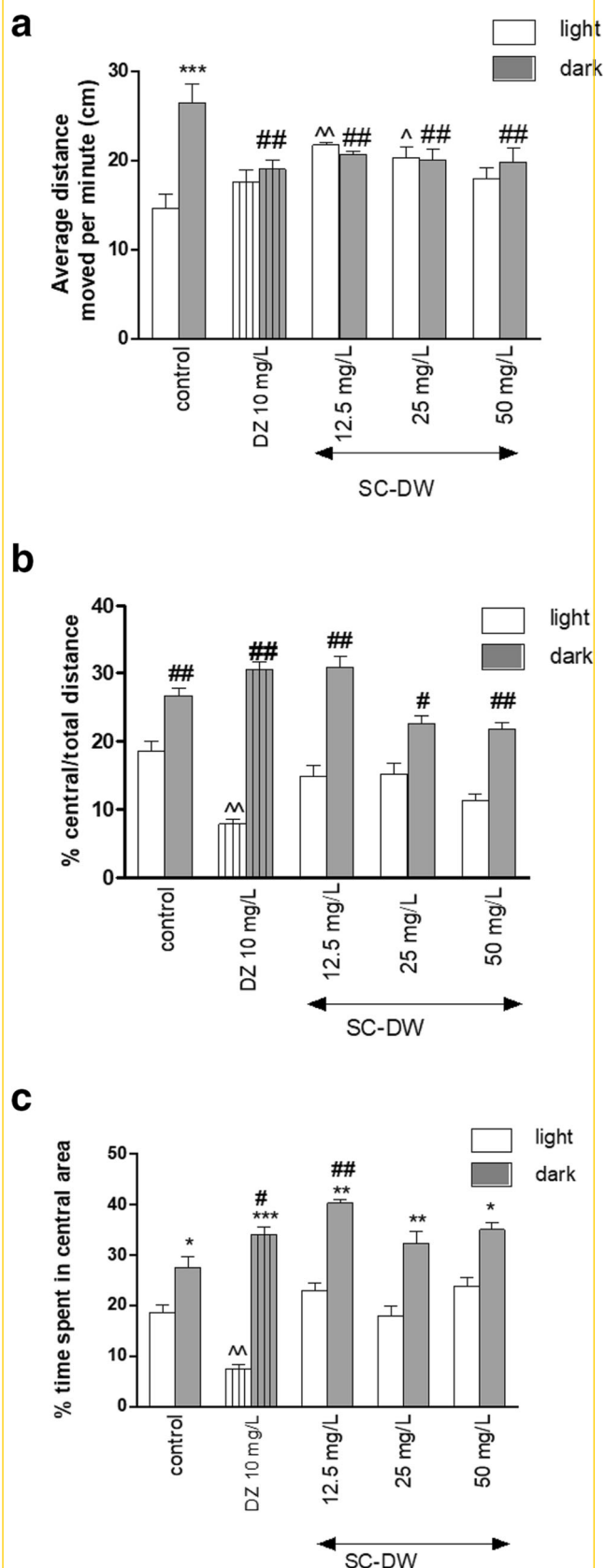
and SC-DW at the concentrations 12.5 mg/L ($p < 0.01$), 25 mg/L($p < 0.05$), and 50 mg/L ($p < 0.01$) in the dark phase as compared with the light phase. As compared with the DMSO-treated group, the SC-DW-treated larvae demonstrated no changes in locomotor activity during the dark phase. As compared with the DMSO-treated group, the diazepam-treated larvae demonstrated decrease in locomotor activity during the light phase ($p < 0.01$) (Fig. 3b).

Figure 3 c shows the effect of different concentration of diazepam and SC-DW on thigmotaxis behaviors of the larvae, when the durations of the activities in the centre arena were measured (two-way ANOVA light-dark condition ($F(1,320) = 63.75$, $p < 0.0001$), treatment effect ($F(4,320) = 11.96$, $p < 0.0001$), and interaction ($F(4,320) = 14.76$, $p = <0.0001$). Post hoc Bonferroni's analysis showed that diazepam ($p < 0.05$) and SC-DW at the concentration 12.5 mg/L increased percentage of time spent in the centre arena during dark phase in comparison with control group ($p < 0.01$).

Also, in the dark phase, significant increases in the durations of centre activities were observed when larvae were incubated in DMSO ($p < 0.05$) and diazepam ($p < 0.001$) as well as when the SC-DW concentrations reached 12.5 ($p < 0.01$), 25 ($p < 0.01$), and 50 mg/L ($p < 0.05$) as compared with the light phase within each concentration group. As compared with the DMSO-treated group, the diazepam-treated larvae demonstrated decrease in time spent in centre arena the light phase ($p < 0.01$) (Fig. 3c).

Discussion

Zebrafish larvae are able to respond to light-dark conditions as early as 5-dpf (Ji et al. 2017) and can detect the difference in light intensities as their visual system is well developed at this stage (Richendrfer et al. 2012; Shaw et al. 2009). Anxiety in humans is characterized by restlessness and increased motor activity (Sousa et al. 2015). Neurobehavioral animal models such as zebrafish larvae are well suited to study anxiety as they have well-developed systems for locomotor activity (Ji et al. 2017). It has been proven that light-dark transitions (stressful situations) affect the locomotor activity of the zebrafish larvae and the transition from light to darkness

($F(4,320) = 40.99$, $p = <0.0001$), and interaction ($F(4,320) = 9.32$, $p < 0.0001$)). Post hoc Bonferroni's test showed an increase in the percentage of the distance traveled by larvae in the centre arena after DMOS ($p < 0.001$), diazepam ($p < 0.01$),

Fig. 3 **a** The effects of diazepam (10 mg/L) and SC-DW treatment (12.5, 25, 50 mg/L) on locomotor activity during all three light-dark challenge phase. Average distance moved by zebrafish larvae within each 1-min time bin under either light (open bars) or dark (filled bars) was plotted. Data are presented as mean ± SEM, $n = 32$. **b** The thigmotaxis behavior was assessed by determining the percentage of the distance moved in the centre arena under the influence of different concentrations of SC-DW during the light-dark transitions. The total distance moved by zebrafish larvae in the centre arena under either light (open bars) or dark (filled bars) was plotted. Data are presented as mean ± SEM, $n = 32$. **c** The evaluation of thigmotaxis behavior represented by the percentage time spent in the centre arena during the first light-dark cycle under the influence of different concentrations of SC-DW. The percentage of time spent by a zebrafish larva in the centre arena under either light (open bars) or dark (filled bars) was plotted. Data are presented as mean ± SEM, $n = 32$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs the light conditions within the same SC-DW concentration group; $\#p < 0.05$, $\#\#p < 0.01$ vs control group under dark condition; $^\wedge p < 0.05$, $^{\wedge\wedge}p < 0.01$ vs control group under light condition (post-hoc Bonferroni's test)

Plant extracts are well known to be complex mixtures of unknown composition. Torres-Hernandez and colleagues (Torres-Hernández et al. 2016) demonstrated the robustness and sensitivity of zebrafish larvae to plant extracts in order to verify their psychoactive efficacy. In the current study, extracts which were shown to exhibit no toxic effects on zebrafish larvae were screened for anxiolytic activity. As outlined from literature, all plants listed in Tables 1 and 2 were reported as having anxiolytic activity. However, in this study *Sceletium tortuosum* demonstrated the highest anxiolytic activity as compared with other extracts. Different concentrations of *Sceletium tortuosum* demonstrated anxiolytic activity (reverse-thigmotaxis) on the zebrafish larvae when spontaneous locomotor activity and light-dark transitions were considered. The water extract of *Sceletium tortuosum* had a significant anxiolytic effect on the zebrafish larvae at the 12.5 and 25 mg/L concentrations during the 40 min of continuous illumination as compared with the control group. Over the years *Sceletium tortuosum* has been recognized as a plant of interest due to its anti-stress effects in healthy individuals as well as alleviating a wide-ranging of psychological and psychiatric disorders (Gericke and Van Wyk 2001; Gericke and Viljoen 2008; Shikanga et al. 2013). The results obtained were compared with other studies where either rodents or other mammals were used to study the anti-anxiety/stress effects of *Sceletium tortuosum*. An example of one such study is that conducted by Smith (2011). One of the most interesting observations in this study was that *Sceletium tortuosum* reduced the production of interleukin-2 (IL-2) in rats. Normally IL-2 increases in response to stress, thereby activating the hypothalamic-pituitary adrenal axis (HPA-axis), which in turn produces hormones, which are responsible for reducing stress. Furthermore, IL-2 levels were higher in low-dose *Sceletium tortuosum* treated rats but lower in higher-dose–treated rats. This suggested that this extract might have had a similar effect

induces hyper-locomotion as a result of increased stress/ anxiety level (Peng et al. 2016).

on the zebrafish larvae; hence, higher anxiolytic activity was observed in low-dose extract–treated groups. The zebrafish has both the brain sympathetic-chromaffin cell axis and hypothalamic–pituitary–interrenal (HPI) axis, which functions in a similar manner to the sympathetic–adrenal and hypothalamic–pituitary adrenal (HPA) axis found in higher vertebrates like humans. The anatomy, connectivity, and molecular elements of the HPA (higher vertebrates) and HPI (fish) share a homologous functional organization and physiology in terms of stress response (Bonga 1997; Alsop and Vijayan 2008, 2009). As compared with human beings, zebrafish react towards stressful situations via increased levels of cortisol (Bonga 1997; Ramsay et al. 2009; Verbeek et al. 2008). Furthermore, neurotransmitters such as serotonin play a major role in anxiety. Serotonin is one of the most widespread neurotransmitters and has numerous behavioral functions (Lowry and Hale 2010; Maximino et al. 2012, 2013; Robbins and Crockett 2010). Moreover, the synthesizing enzyme tryptophan hydroxylase, serotonin 1A receptor (5-HT$_{1A}$), and serotonin transporter were found to be similar in zebrafish to higher vertebrates (Bellipanni et al. 2002; Maximino et al. 2013; Norton et al. 2008; Wang et al. 2006). Certain regions homologous to the amygdala, in zebrafish such as the dorsomedial, pre- and post-commissural ventral telencephali, and the periventricular gray zone of the tectum (Maximino et al. 2013; Norton et al. 2008) are triggered following exposure of zebrafish to the light/dark transitions and are linked with fear and anxiety behavior (Lau et al. 2011). However, there are differences between the serotonergic system of zebrafish and mammals. Nonetheless, the therapeutic effects of drugs on targeted proteins are closely related between these vertebrates (Maximino et al. 2013). *Sceletium tortuosum* produces mesembrine-type alkaloids (mesembranol, mesembrenol, mesembrine, and mesembrenone) as secondary metabolites; these alkaloids are responsible for the anxiolytic activity (Shikanga et al. 2013; Smith et al. 1996). Additionally, these alkaloids inhibit serotonin-reuptake and phosphodiester-4 (PDE-4) enzyme, thus treating a variety of central nervous system–related disorders (Gericke and Viljoen 2008; Harvey et al. 2011). The results of this study justify further research into the anxiolytic activity of these alkaloids using an in vivo zebrafish model.

Conclusion

As previously mentioned in several studies, the zebrafish model is effective in studying anxiety related behaviors. In this study, *Sceletium tortuosum* greatly attenuated thigmotaxis behavior on 5-dpf zebrafish larvae. Moreover, to gain a better understanding of this important ethnomedicinal plant and its pharmacological activity compounds responsible for anxiolytic activity on 5-dpf zebrafish larvae should be isolated to provide future leads in order to develop standardized products and consistently provide reliable medication for patients.

Compliance with ethical standards

Conflict of interest A Viljoen has acted as a consultant to HG&H Pharmaceuticals on the phytochemistry of *Sceletium tortuosum* from which a commercial product Zembrin® has been developed. HG&H Pharmaceuticals however is not involved as a collaborator and has not provided any funding for the presented research.

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