Linalool and *trans*-nerolidol prevent pentylenetetrazole-induced seizures in adult zebrafish

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ABSTRACT

Ethnopharmacological relevance: linalool (LIN) and *trans*-nerolidol (NER) are terpene alcohols found in high concentrations in plant-derived essential oils used as a traditional treatment in many cultures. Several studies evaluating the effects of these essential oils and their isolated compounds have shown antiseizure, sedative, neuroprotective, and antioxidant properties in acute seizure models both *in vitro* and *in vivo*. Therefore, this study aimed to evaluate the effects of acute exposure to LIN or NER on a pentylenetetrazole (PTZ)-induced seizure model in adult zebrafish (*Danio rerio*).

Methods: 240 adult wild-type zebrafish were randomly allocated to the experimental groups: control (dechlorinated water), 1% DMSO (vehicle), diazepam (50 µM DZP), and LIN or NER (4, 40 and 400 µM). Animals were exposed to the treatment solution (400 mL) for 10 minutes, taken to a washout beaker (dechlorinated water, 400 mL) for 5 minutes, and recorded in the test apparatus containing 10 mM PTZ for 20 minutes. Experienced researchers conducted a blinded analysis of behavioral seizure phenotypes using BORIS® software. Additionally, locomotor activity was evaluated through ANY-Maze™ tracking software, ensuring precise and unbiased assessment of movement behaviors.

Results: the highest concentration of LIN (400 µM) and NER (40 and 400 µM) increased the latency to reach clonic and tonic-like seizure stages while decreasing the seizure intensity. Interestingly, NER has shown a larger effect size than the positive control group DZP, and at a concentration of 40 µM, increased locomotor behavior.

Conclusion: our findings show the antiseizure properties of LIN and NER in zebrafish acutely exposed to PTZ, delaying the time to reach the highest seizure stages and decreasing seizure intensity. These results are consistent with previous experiments conducted in mice and amplify the external validity of these compounds, highlighting their potential as antiseizure remedy candidates.

Keywords: linalool, *trans*-nerolidol, essential oil, terpene, seizure, acute seizure model, pentylenetetrazole, zebrafish, *Danio rerio*.

Graphical abstract. Created using BioRender.

1. INTRODUCTION

Essential oils (EOs) are mixtures of plant-derived volatile compounds, such as alcohol terpenes and phenylpropanoids (Elaine Elisabetsky and Domingos S. Nunes, 2020; Wang and Heinbockel, 2018). Historically, EOs have been used as a complementary treatment in traditional therapies for several neuropsychiatric conditions, such as anxiety, depression, and epilepsy (Lee et al., 2010; Nóbrega de Almeida et al., 2011; Wang and Heinbockel, 2018). An example of this application is found in the traditional treatment of caboclo folk in the Brazilian Amazon, which uses *Aeollanthus suaveolens* (Mart. ex Spreng) leaves' EO as an antiseizure medication (Elisabetsky and Brum, 2003). Similarly, the EOs of sour orange (*Citrus aurantium* L.) have been reported as an alternative therapy for anxiety, insomnia, and seizure-like symptoms in Brazilian, Chinese, and Haitian folk medicines (Paul, 1995; Carvalho-Freitas, 2002).

Recent studies propose that EOs' mechanism of action is related to the GABAergic system and voltage-gated channel's modulation (Wang and Heinbockel, 2018). Since the therapeutic effects of EOs stem from the active compounds, neurological diseases related to these biological mechanisms, such as epilepsy and Alzheimer's disease, may be modulated by EOs (Elaine Elisabetsky and Domingos S. Nunes, 2020). Supporting this concept, previous ethnopharmacological studies identified the antiseizure and sedative properties of several EOs and their active compounds in both pre-clinical models and clinical trials (Carvalho et al., 2018; Lee et al., 2011; Wang and Heinbockel, 2018). Most antiseizure medications approved by the U.S. Food and Drug Administration (US-FDA) modulate GABAergic receptors (e.g. Diazepam, Clonazepam, Lorazepam) or voltage-gated Na^+ channels (e.g. Carbamazepine, Lamotrigine and Topiramate). However, about 30% of patients with epilepsy do not respond to any clinically available antiseizure medication (Boleti et al., 2022), highlighting the need for developing new drugs.

Linalool (LIN) and nerolidol (NER) are acyclic terpene alcohols synthetized as secondary metabolites in protein metabolisms from several plants, including rosemary (*Baccharis dracunculifolia* DC.), lavender (*Lavandula angustifolia* Mill.), and sour orange (*Citrus aurantium* L.) (Azanchi et al., 2014; Cláudia Maria Oliveira Simões et al., 2016; Klopell et al., 2007; Pokajewicz et al., 2021; Queiroga et al., 1990). Traditional use of EOs containing high

concentrations of these compounds has been reported in the context of antiseizure, anxiolytic, analgesic, anti-inflammatory, and antimicrobial activities (Elaine Elisabetsky and Domingos S. Nunes, 2020; Fabiane et al., 2008; Gazim et al., 2022; Moslemi et al., 2019; Wang and Heinbockel, 2018). However, the current literature on these compounds and their antiseizure properties is limited, consisting primarily of studies conducted in rodents, with one report utilizing zebrafish as the model organism (Coelho de Souza et al., 1997; Elisabetsky et al., 1999; Elisabetsky and Shanley, 1994; Kaur et al., 2016; Thornton et al., 2020). Such scarce information regarding LIN and NER's antiseizure effects culminates in literature gaps and a possible species-specific bias, emphasizing the need for further studies to expand the understanding of the therapeutic properties of both compounds.

Zebrafish (*Danio rerio* Hamilton) have been increasingly used in antiseizure drug screenings (Chitolina et al., 2023). The beginnings of the seizure-like model establishment in zebrafish go back to the 2000s, when the first study using PTZ in zebrafish larvae showed chemically-induced seizure-like abnormal electric discharges and behavioral phenotypes relevant to epilepsy research (Baraban et al., 2005). Almost a decade later, behavioral phenotype characterization of the PTZ-induced seizure model was adapted in adult zebrafish to improve our understanding of the behavioral alterations induced by $GABA_A$ receptor antagonism in this species (Mussulini et al., 2013). Additionally, a recently published systematic review identified PTZ as the main chemical agent used to induce seizures in zebrafish, followed by kainic acid and pilocarpine models (Chitolina et al., 2023). Therefore, our study aimed to evaluate the behavioral alterations induced by LIN and NER exposure in adult zebrafish submitted to the acute PTZ-induced seizure model, providing further information and direct comparison regarding their potential as antiseizure drugs and increasing the external validity of previous findings.

2. MATERIALS AND METHODS

2.1. Animals

240 adult wild-type zebrafish (6 months old, 300-500 mg) with a similar distribution of males and females were used. Animals were obtained from a local commercial supplier (Distribuidora Flower Pet, Porto Alegre, Brazil) and habituated for at least 2 weeks in 14-L

housing tanks (maximum density of 5 animals/L) until the test day. Facility conditions were controlled and settled as ideal for the species: temperature 28 ± 1 °C, pH 7.0 ± 0.5, conductivity 500 uS/cm, luminosity 260 ± 5 lux, and light/dark cycle of 14:10 hours. Fish were fed twice a day with commercial feed (Poytara®, Brazil) and *Artemia salina*. The protocol was approved by the Ethics Committee for Animal Use (CEUA) at the Universidade Federal do Rio Grande do Sul (#36307) and conducted according to the guidelines set by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Chemicals and drugs

Linalool (LIN, CAS 78-70-6), *trans*-nerolidol (NER, CAS 40716-66-3), dimethyl sulfoxide (DMSO, CAS 67-68-5), and pentylenetetrazole (PTZ, CAS 54-95-5) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diazepam (DZP), used as a positive control, was obtained from União Química Nacional S/A (SP, Brazil). Pre-treatment stock solutions, prepared in 10% DMSO (vehicle), were adjusted to a final concentration of a maximum of 1% DMSO, which was shown not to modify toxicological and behavioral outcomes in zebrafish (Sackerman, 2010; Kolesnikova, 2017; Hoyberghs, 2021). Exposure to DMSO at this concentration did not induce locomotor changes when compared with the control group (Fig. 1-3, supplementary material).

The concentration range of tested compounds (4, 40, and 400 µM) was defined by a logarithmic curve in which the intermediate concentration was established based on the LIN concentration reported as anxiolytic in a previous study conducted in zebrafish (Szaszkiewicz, 2021).

2.3. Experimental design

The experimental design was adapted from previous studies (Szaszkiewicz, 2021). Experiments were conducted in the morning, between 8 a.m. and 12 p.m., in two batches for each compound. On the test day, fish were grouped in sets of 4 animals and allocated to the pre-treatment solutions (400 mL): control (dechlorinated water), vehicle (1% DMSO), positive control (50 µM DZP), 4, 40, and 400 µM LIN or NER for 10 minutes. Twenty animals were used per experimental group. Immediately after, animals were taken to a washout beaker (400 mL of dechlorinated water) for 5 minutes; since the PTZ solution was not changed during each

experiment, this step was necessary to minimize the test solution contamination by different treatments. After the washout period, animals were individually submitted to the PTZ-induced acute seizure test, which consists of a tank (13 cm high × 15 cm long × 10 cm wide) containing 1 liter of 10 mM PTZ where fish were recorded (Logitech® C920 HD webcam) for 20 minutes (Figure 1). At the end of the test, the fish were immersed in cold water (0-4 °C) until opercular cessation, and death was confirmed by decapitation, following the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA). Seizure-like phenotype behavioral and locomotor behavior were assessed as described below. The different housing tanks were carefully placed side by side under similar conditions of luminosity, temperature, and vertical position. The allocation sequence to the treatment beakers and the test apparatus was randomized according to block randomization performed using random.org, and the primary source was counterbalanced to avoid treatment allocation from only one housing tank.

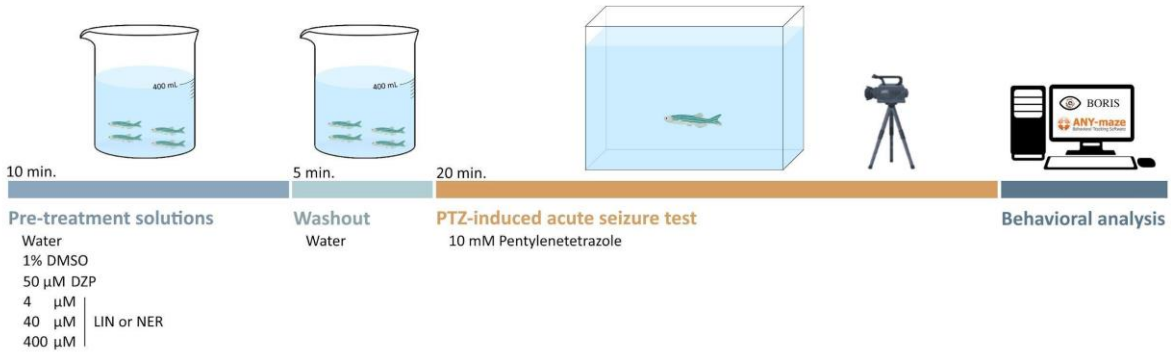

Figure 1: Experimental design showing LIN and NER pre-treatment followed by the acute PTZ-induced seizure test. DMSO = dimethyl sulfoxide; DZP = diazepam; LIN = linalool; NER= *trans*-nerolidol; PTZ = pentylenetetrazole.

2.4. Seizure-like behavioral phenotype assessment

Seizure-like behavioral stages were analyzed by researchers blinded to the experimental groups according to PTZ-induced seizure-like phenotypes previously established

in the literature (Mussulini et al., 2013): stage 0, basal locomotor activity; stage 1, increase in swimming activity and opercular movements frequency; stage 2, whip-like swimming activity and erratic movements; stage 3, whirlpool-like behavior, circular movements; stage 4, clonic-like seizures, abnormal muscle contractions; stage 5, tonic-like seizure stage, loss of posture and sinking to the bottom of the aquarium; stage 6, death. Researchers analyzed and manually scored latencies for animals reaching the highest seizure-like stages: clonic and tonic-like seizure stages (4 and 5, respectively), and the maximum seizure-like stage reached every 30 seconds for 20 minutes for each animal. Experimental groups were manually encoded by a third researcher till the end of the analysis to ensure blinded outcome assessment. Seizure intensity was calculated by the area under the curve of the maximum seizure stage reached over time. Analyzes were performed using BORIS® v. 7.7.3 (Behavioral Observation Research Interactive Software) (Friard and Gamba, 2016).

2.5. Locomotor behavioral assessment

The locomotor behavior analysis from the acute seizure tests was assessed using ANY-Maze™ v. 7.4 tracking software (Stoelting Co., Wood Dale, IL, USA) by a researcher blinded to the experimental groups. Experimental groups were encoded by the software till the end of the analysis to ensure a blinded outcome assessment. Total distance traveled, mean swimming speed during the test and distance traveled across time (m/30-second bin) were assessed.

2.6. Statistical analysis

The sample size estimation was calculated using G*Power 3.1.9.6 software based on the seizure intensity as the primary outcome. It was applied an ANOVA fixed effect model with the values of α=0.05, statistical power=0.95, and effect size=0.4, considering 6 experimental groups. Therefore, the total sample size was 114, n=19 fish per group, and we added one extra animal per group to account for eventual tracking losses (n=20). Raw data are available in the Open Science Framework database at DOI 10.17605/OSF.IO/8QG4R (Silva et al., 2023). All fish were included in the analysis.

The normality of variances was assessed using the D'Agostino & Pearson test. Latencies for clonic and tonic-like seizure stages were analyzed with Kruskal-Wallis tests followed by Dunn's post hoc analysis. Seizure-like stages over time and distance traveled over time were analyzed using repeated measures two-way ANOVA (Factor 1: time; Factor 2: pre-treatment drug), followed by Tukey's post hoc test. Seizure intensity, total distance traveled, and mean swimming speed were analyzed by one-way ANOVA followed by Tukey's post hoc. Effect sizes of seizure intensity analyses were done by Cohen's d test using an online calculator (https://www.cem.org/effect-size-calculator). Parametric data are expressed as mean ± standard deviation, non-parametric data as median ± interquartile range, and effect size as standardized mean difference ± 95% confidence interval. Statistical analysis and graphics were performed using GraphPad Prism 8.0.1.

3. RESULTS

Initially, we checked whether exposure to 1% DMSO would have any effect on PTZ-induced phenotype behaviors as compared to dechlorinated water. No significant effects were observed between the negative control groups in the latency to the highest seizure-like stages and seizure intensity (Supplementary Figures 1-3). Therefore, data are expressed relative to the DMSO group, given that all compounds were dissolved in 1% DMSO.

3.1 Effects of LIN on PTZ-induced seizure-like behavior phenotype

As expected, DZP increased the latency to reach clonic and tonic-like seizure stages (Figures 2a and 2b). Additionally, DZP pre-treatment attenuated the seizure intensity (Figure 2c), calculated as the area under the curve of the maximum seizure-like stage reached every 30-second interval, (Figure 2d), resulting in the higher effect size in seizure attenuation (4.20) (Figure 2e). LIN pre-treatment at the highest concentration (400 µM) increased the latency to reach both clonic and tonic-like seizure stages (Figure 2a and 2b). At this concentration, LIN also decreased the seizure intensity (Figure 1d) but showed a smaller effect size than DZP (1.34 vs 4.20, respectively, Figure 2e).

3.2 Effects of NER on PTZ-induced seizure-like behavior phenotype

DZP showed a similar effect compared to the LIN experiment, increasing the latency to reach the clonic and tonic-like seizure stages (Figure 3a and 3b, respectively), and decreasing the seizure intensity (Figure 3d). Interestingly, both 40 and 400 µM NER increased the latency to reach the clonic and tonic-like seizure stages (Figure 3a and 3b, respectively), and decreased the PTZ-induced seizure intensity (Figure 3d). Cohen's d of seizure intensity data showed a larger effect size for 400 µM NER as compared to DZP (3.58 vs 2.57, respectively, Figure 3e).

3.3 Effects of LIN and NER on PTZ-induced locomotor alterations

At the tested concentrations, neither LIN nor DZP pre-treatment showed alterations in total distance traveled or mean swimming speed (Figure 4a and 4b). The distance traveled across time was modulated in a time-dependent manner (Time Factor: $F(39, 3705) = 10.69$, $p=0<0.0001$), as an interaction between pre-treatment drug and time was observed (Interaction between factors: $F(156, 3705) = 1.284$, $p=0.0112$). In the NER experiment, 40 µM NER increased the total distance traveled and mean swimming test (Figures 5a and 5b). DZP did not modulate any of the evaluated outcomes. The distance traveled across time was modulated in a drug and time-dependent manner (Figure 5c; Drug Factor: $F(4, 95) = 2.598$, $p=0.0410$; and Time Factor: $F(39, 3705) = 1.488$, $p=0.0263$) and an interaction between these factors was observed (Interaction between factors: $F(156, 3705) = 1.371$, $p=0.0019$).

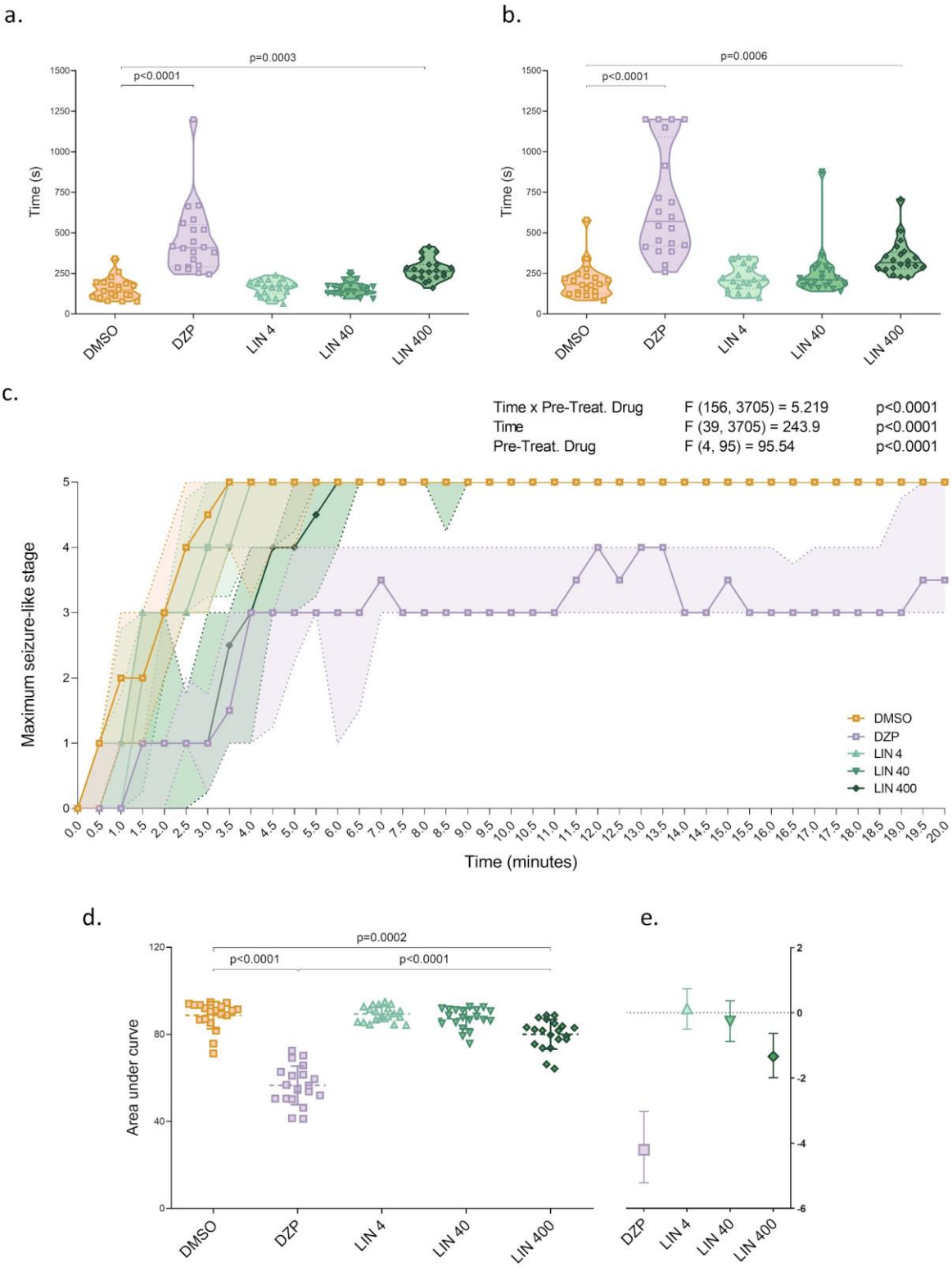

Figure 2: Effects of LIN (4, 40, and 400 µM) and DZP on (a) latency to reach clonic-like seizure stage, (b) latency to reach tonic-like seizure stage, (c) maximum seizure-like stage reached across time, (d) seizure intensity, as calculated by the area under the curve of maximum seizure-like stage across time, and (e) effect size of seizure

intensity. Data are expressed as median ± interquartile range (a-c), mean ± S.D. (d), and standardized mean difference ± 95% confidence interval (e). Data were analyzed by Kruskal-Wallis followed by Dunn's test (a, b), repeated measures Two-way ANOVA followed by Tukey's post hoc (c), One-way ANOVA followed by Tukey's post hoc test (d), and Cohens's d effect size test (e). n= 20. DMSO = 1% dimethyl sulfoxide; DZP = 50 µM diazepam; LIN = linalool.

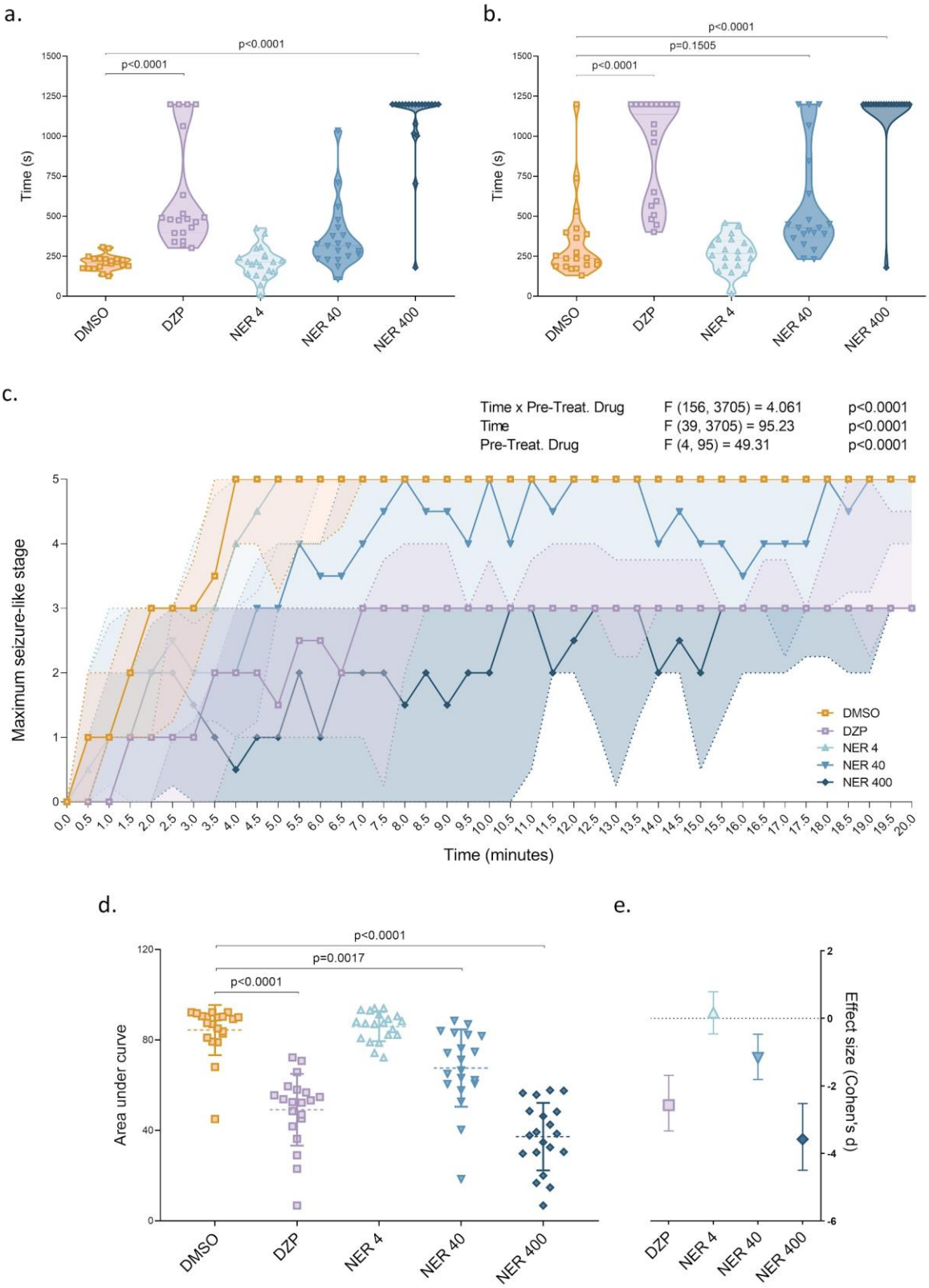

Figure 3: Effects of NER (4, 40, and 400 µM) and DZP on (a) latency to reach clonic-like seizure stage, (b) latency to reach tonic-like seizure stage, (c) maximum seizure-like stage reached across time, (d) seizure intensity, as

calculated by the area under the curve of maximum seizure-like stage across time, and (e) effect size of seizure intensity. Data are expressed as median ± interquartile range (a-c), mean ± S.D. (d), and standardized mean difference ± 95% confidence interval (e). Data were analyzed by Kruskal-Wallis followed by Dunn's test (a, b), repeated measures Two-way ANOVA followed by Tukey's post hoc (c), One-way ANOVA followed by Tukey's post hoc test (d), and Cohens's d effect size test (e). n= 20. DMSO = 1% dimethyl sulfoxide; DZP = 50 µM diazepam; NER = *trans*-nerolidol.

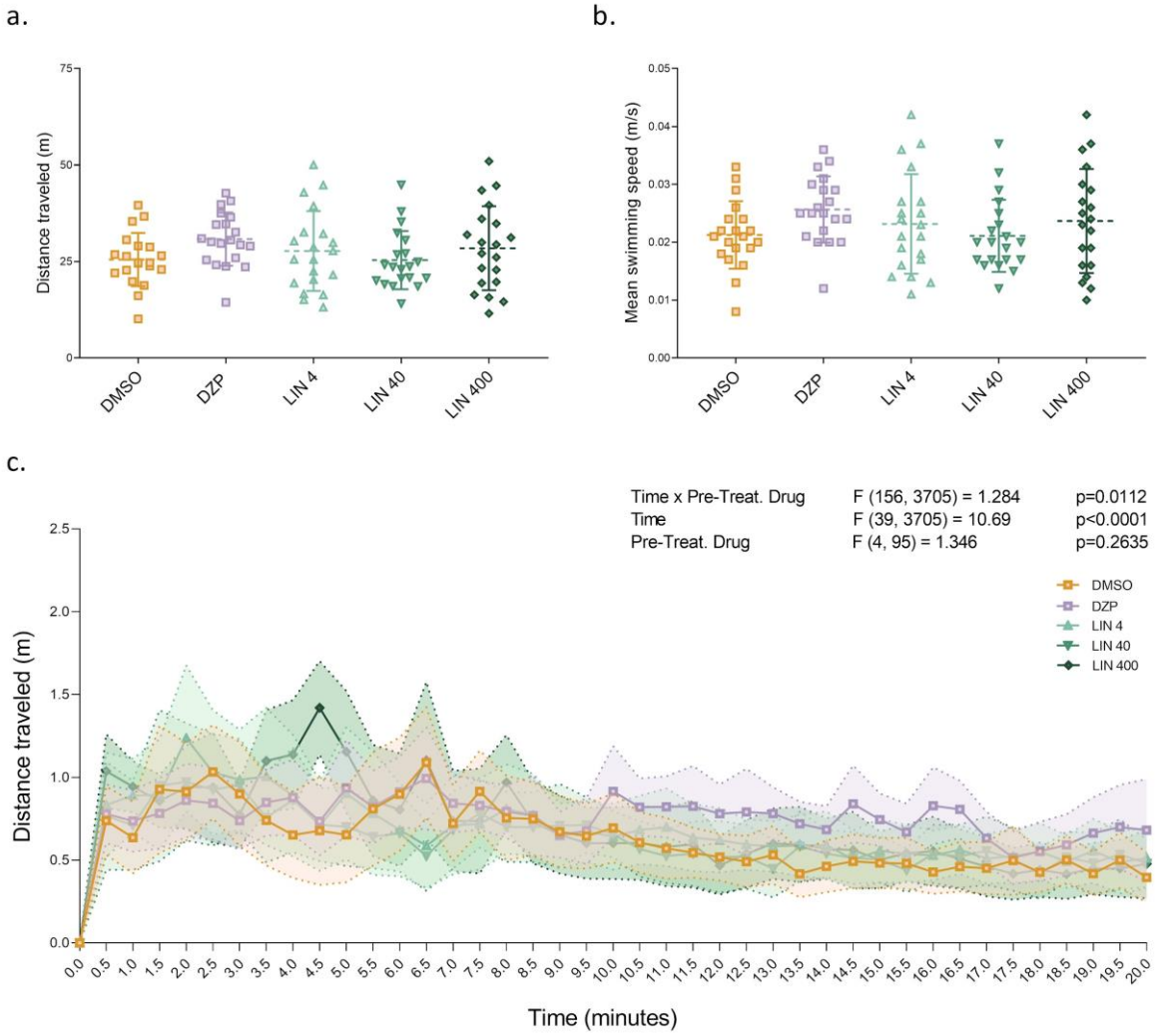

Figure 4: Effects of LIN (4, 40, and 400 µM) and DZP on (a) total distance traveled (m), (b) mean swimming speed, and (c) distance traveled across time (m). Data are expressed as mean ± S.D. and were analyzed by One-way ANOVA followed by Tukey's post hoc (a,b) and repeated measures two-way ANOVA (c). n= 20. DMSO = 1% dimethyl sulfoxide; DZP = 50 µM diazepam; LIN = linalool.

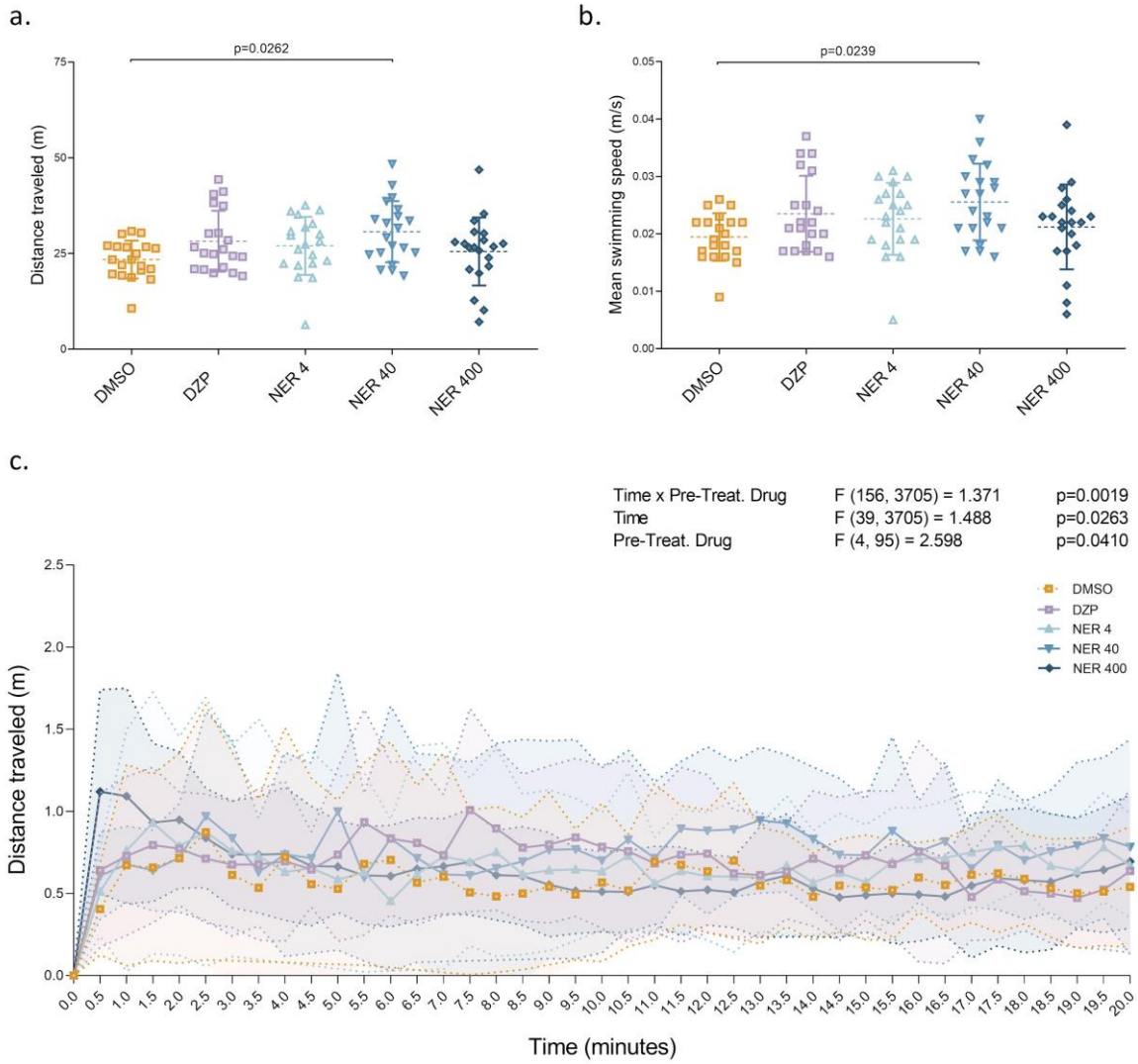

Figure 5: Effects of NER (4, 40, and 400 µM) and DZP on (a) total distance traveled (m), (b) mean swimming speed, and (c) distance traveled across time (m). Data are expressed as mean ± S.D. and were analyzed by One-way ANOVA followed by Tukey's post hoc (a,b) and repeated measures two-way ANOVA (c). n= 20. DMSO = 1% dimethyl sulfoxide; DZP = 50 µM diazepam; NER = *trans*-nerolidol.

4. DISCUSSION

The use of zebrafish as a model organism to mimic neurological disorders is an important tool to provide insights into potential new drugs. Our study demonstrated the antiseizure properties of LIN and NER in an acute seizure-like model using zebrafish. Acute exposure to LIN and NER increased the latency to reach the clonic and tonic-like seizure stages and attenuated the seizure intensity, showing large effect sizes in this outcome.

Linalool is a monoterpene synthesized in the mevalonate pathway, which plays an essential role in plant metabolism converting acetyl-CoA into isoprenoids, such as cholesterol and steroid hormones (Buhaescu and Izzedine, 2007; Cláudia Maria Oliveira Simões et al., 2016; Liao et al., 2016). It has been shown to have anti-seizure properties in both rats and mice (Almeida et al., 2009; Bahr et al., 2019). In rodents, LIN has been shown to increase the latency for seizure-like behavioral alterations and decrease its duration in seizure models related to chemically-induced alterations on glutamatergic and GABAergic synapses (de Sousa et al., 2010; Elisabetsky et al., 1999). In a study involving zebrafish larvae, LIN did not exhibit antiseizure properties in either chemically induced seizure models or scn1a-mutant zebrafish models at the tested concentrations (0.3–4 µM) (Thornton et al., 2020). Our results may be aligned with the previous findings, once the effective concentration of LIN (400 µM) of this study was 100x higher than the concentration previously evaluated by Thornthon and colleagues, and the 4 µM LIN concentration did not induce behavioral alteration in both experiments.

Trans-nerolidol is the *trans* isomer of a naturally occurring sesquiterpene biosynthesized in the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. This mevalonate-independent pathway also plays an essential role in isoprenoid biosynthesis by pyruvate conversion, as an alternative to the pathway cited above (Eisenreich et al., 2004; Hunter, 2007; Rohmer, 1999). Despite the therapeutic potential of nerolidol, the literature on the isolated compound effects in an antiseizure context is limited to only one study in mice. Kaur and colleagues (2016) evaluated the effects of chronic administration of nerolidol in PTZ-induced kindling in mice and showed that NER pre-treatment decreased seizure severity. Likewise, studies conducted evaluating the behavioral effects of Neroli (an EO containing high concentration of NER) administration prevented tonic and clonic-like seizures in PTZ and

maximal electroshock-induced seizure models (Azanchi et al., 2014). Our study is the first article to show the antiseizure properties of NER in zebrafish, by increasing the latency to the most severe seizure-like stages and attenuating seizure intensity. Interestingly, Cohen's d effect size test showed a larger effect size of 400 µM NER in the seizure intensity analysis compared to 50 µM DZP. This result may be explained by NER's higher lipophilicity and lower molecular weight (Elaine Elisabetsky and Domingos S. Nunes, 2020), meaning that NER may have greater bioavailability and may cross the blood-brain barrier more easily than diazepam. However, these conclusions are merely speculative, and more studies are necessary to better comprehend the results here described.

The underlying mechanisms of antiseizure effects observed in this study have not been fully described. LIN was shown to potentiate GABAergic currents through the $GABA_A$ receptors α1β2γ2 subunits *in vivo* (Hossain et al., 2002; Kessler et al., 2014; Milanos et al., 2017) and to bind to NMDA receptors and serotonin transporters in a dose-dependent manner (López et al., 2017). In antinociceptive studies, the effects of NER are reversed by a $GABA_A$ antagonist, bicuculline (Fonsêca et al., 2016). This combined overview highlights a potential mechanism of action of LIN and NER, probably by modulating $GABA_A$ receptors. However, the specific binding site of these compounds and their specificity remains unclear. Flumazenil prevented the antiseizure effects of a EO containing high concentration of NER (Neroli) in mice (Azanchi et al., 2014). Therefore, these compounds may act on the benzodiazepine binding site of $GABA_A$ receptors. The lack of further information in this context highlights the need for further investigations.

In a wide scope, the beneficial effects of both LIN and NER may be related to different biological mechanisms, not restricted to the attenuation of PTZ-induced seizure models (Hosseini et al., 2021; Linck et al., 2010). The pathophysiology of epilepsy encompasses a variety of biological alterations and comorbidities beyond seizures, such as neuroinflammation (Devinsky et al., 2018), increased oxidative stress (Ramos-Riera et al., 2023), as well as cognitive, physiological, and social impairments (Fisher et al., 2014). The potential therapeutics of LIN and NER may be interesting in preventing comorbidities and related psychiatric and neurocognitive impairments. As redox imbalance and neuroinflamation are hallmarks of several neurological disorders, such as several epilepsy

syndromes, and Alzheimer and Parkinson's disease (Borowicz-Reutt and Czuczwar, 2020; Chinta and Andersen, 2008; Di Domenico et al., 2024; Rey et al., 2023), studies showing both LIN and NER's EOs (*Peperomia serpens* and *Piper aleyreanum*) and isolated compounds' antioxidant and anti-inflammatory properties highlights its therapeutic potential not restricted to symptomatic impairments (Balakrishnan et al., 2022; de Moura et al., 2021; Lima et al., 2012; Pinheiro et al., 2011). Additionally, LIN was shown to ameliorate cognitive deficits in mouse models of Alzheimer's disease (Sabogal-Guáqueta et al., 2016; Xu et al., 2017) and to counteract social impairment in mice submitted to a social defeat protocol (Caputo et al., 2018). *Trans*-nerolidol has also been shown to have beneficial effects on outcomes related to ameliorating cognitive dysfunction and neuroprotective effects in rat models of Alzheimer's disease (Taheri et al., 2022); and anxiolytic effects in mice (Goel et al., 2016).

As reported in previous studies, DZP is known to attenuate seizure-like behavioral phenotypes and epileptic discharges in rodents (Mandhane et al., 2007; Watanabe et al., 2010) and zebrafish (Afrikanova et al., 2013; Mussulini et al., 2013). Here, 50 µM DZP was chosen as the positive control drug due to its effects on modulating seizure-like stages previously described in zebrafish (Afrikanova et al., 2013). In both experiments, DZP induced similar behavior alterations attenuating the acute PTZ-induced seizures, proving its reliability as the appropriate positive control drug in preclinical studies.

Studies evaluating PTZ-induced alterations in locomotor behavior showed increasing distance traveled and swimming speed in both larvae and adult zebrafish (Afrikanova et al., 2013; Lee et al., 2010). Interestingly, 40 µM *trans*-nerolidol had the opposite effect than expected, increasing distance traveled and swimming speed. Different from most of the antiseizure drugs, which are sedatives, decreasing zebrafish locomotor activity (Afrikanova et al., 2013). However, its biological meaning remains unclear and needs further investigations to be elucidated.

Despite the interesting findings obtained here that contribute with new data to the literature, we used a chemical-induced model of acute seizures and not a model of epilepsy, which is distant from the patients with spontaneous and recurrent seizures (Devinsky et al., 2018). Acute exposure to PTZ was chosen once it is the most commonly used chemoconvulsant for drug screenings in zebrafish (Chitolina et al., 2023). However, this

protocol has important limitations, since PTZ does not induce comorbidities and secondary neurocognitive impairments observed in clinical trials. Models of epilepsy, such as kindling and genetic models, better mimic neurological alterations observed in patients with epilepsy, therefore being alternative models to improve potential drug screening (Löscher, 2017). We opted to perform the present protocol once the current zebrafish literature does not have standardized kindling protocols.

To enhance the construct validity of our findings, incorporating cross-species studies presents a promising approach. Utilizing diverse animal models can help reduce species-specific biases and improve external validity (Maximino et al., 2015), offering a broader assessment of the antiseizure effects of LIN and NER. Moreover, given that epilepsy is characterized by abnormal neuronal discharges, the evaluation of electrophysiological outcomes—such as the quantification and characterization of epileptiform spike patterns (ictal and interictal discharges)—could provide valuable insights into the pharmacodynamics of LIN and NER. Future studies aim to further explore the effects of LIN and NER on neurological activity, employing adult zebrafish (Pineda et al., 2011) and other model organisms, such as rodents, to deepen our understanding.

5. CONCLUSION

Our study showed linalool and *trans*-nerolidol antiseizure effects in zebrafish submitted to an acute seizure model. It is the first study to show the behavioral effects of these compounds in adult zebrafish under an antiseizure context. Besides the limited literature, our findings are in line with studies conducted in rodents, increasing the external validity of these compounds as antiseizure remedy candidates. However, our data are preliminary and exclusively based on behavioral alterations. Therefore, more studies are warranted to improve LIN and NER characterization, mainly regarding electrophysiological and pharmacodynamics assays.

AUTHORS CONTRIBUTION

Conceptualization: D.S.N., E.E., A.P.H., and A.P. Data curation: A.L.S. and L.M.B. Formal analysis: A.L.S., L.M.B., M.G.-L. and M.E.C. Funding acquisition: A.P. Investigation: A.L.S., L.M.B., R.C., C.G.R., and M.G.-L. Methodology: A.L.S., L.M.B., R.C., C.G.R., and M.G.-L. Project administration: A.P. Supervision: A.P. Writing - original draft: A.L.S., L.M.B., and A.P. Writing - review & editing: L.M.B., A.L.S., M.G.-L., R.C., C.G.R., D.S.N., E.E., A.P.H., M.E.C., and A.P.

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CONFLICT OF INTEREST

The authors claim no conflicts of interest.

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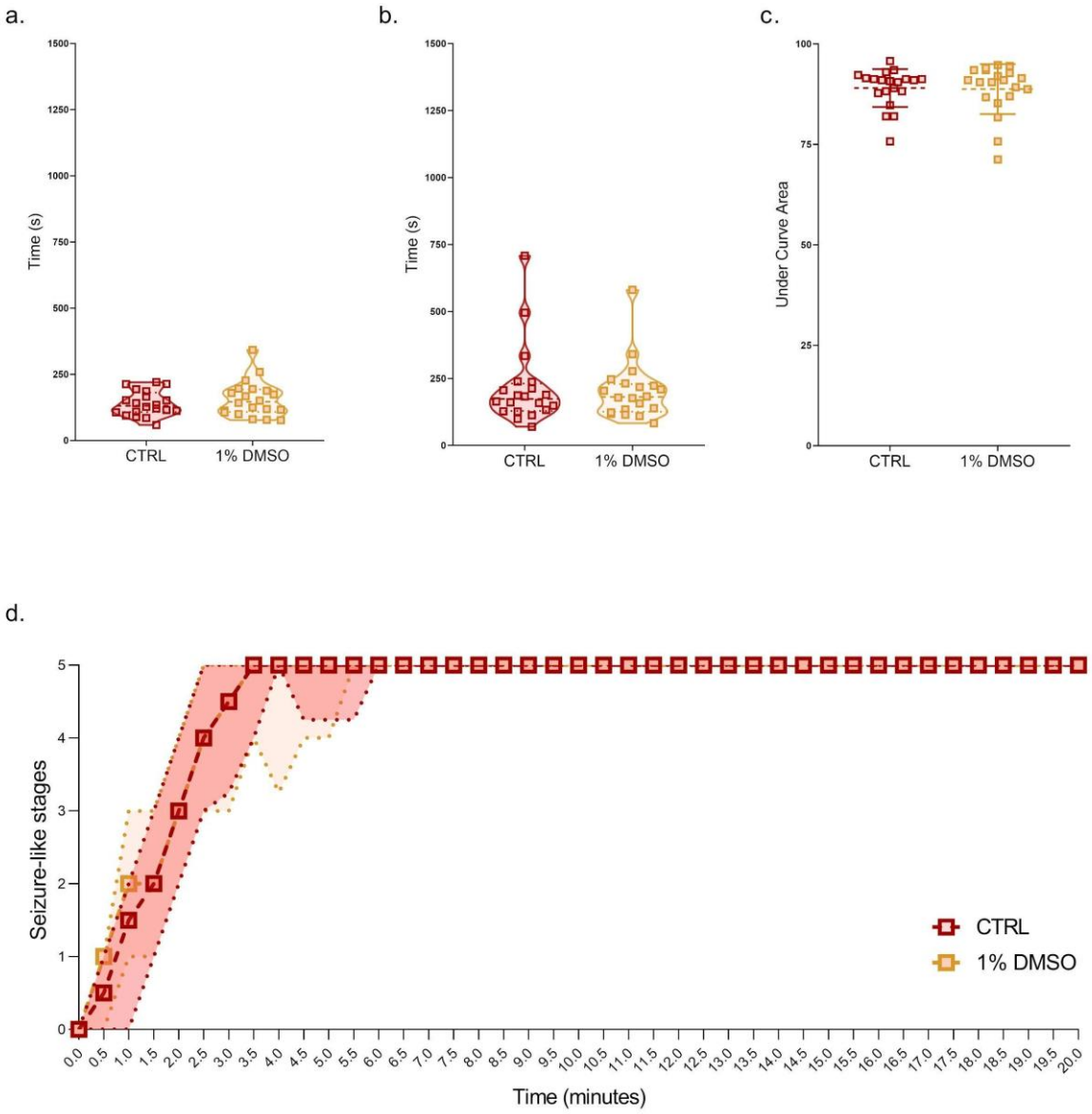
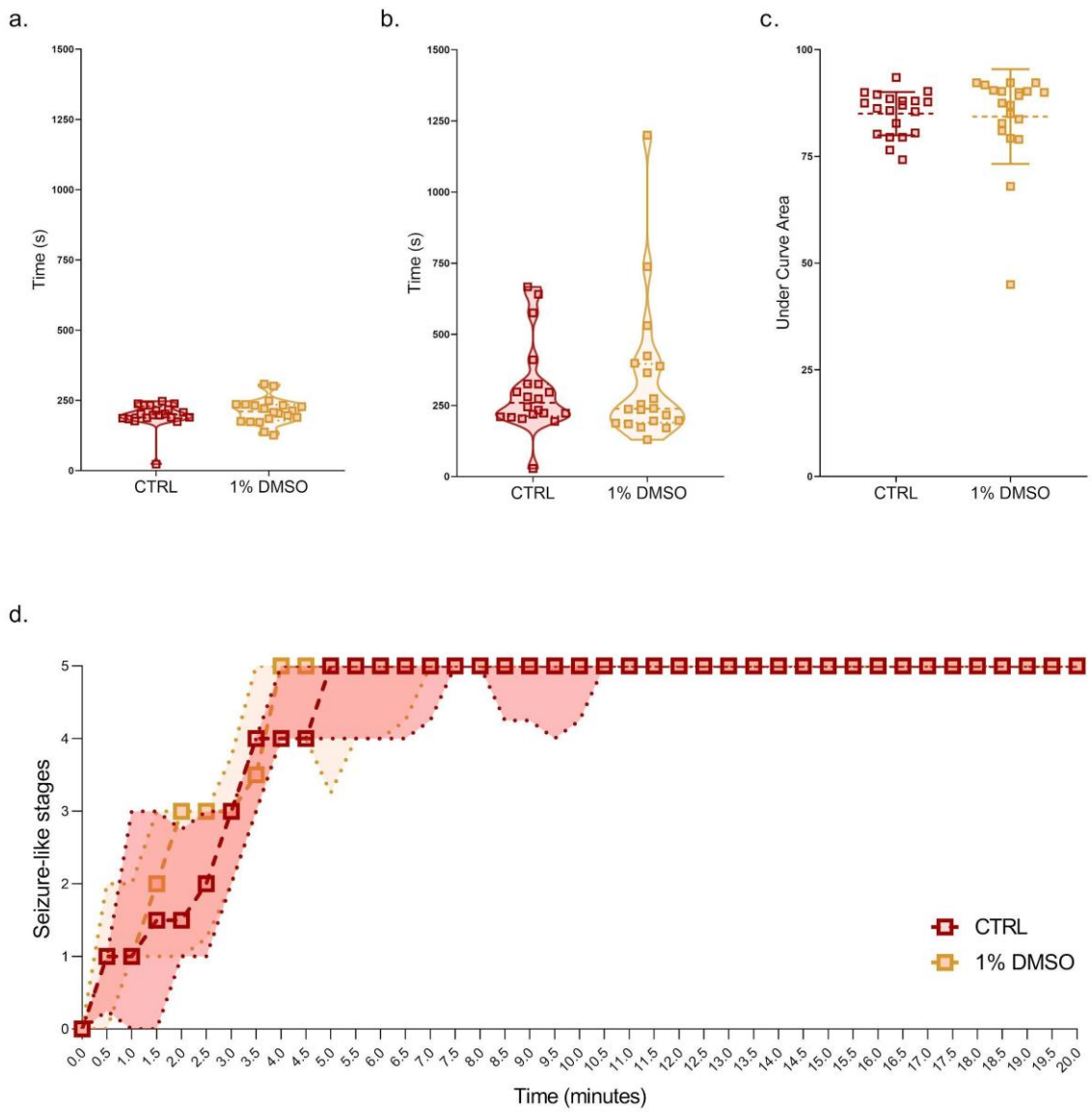
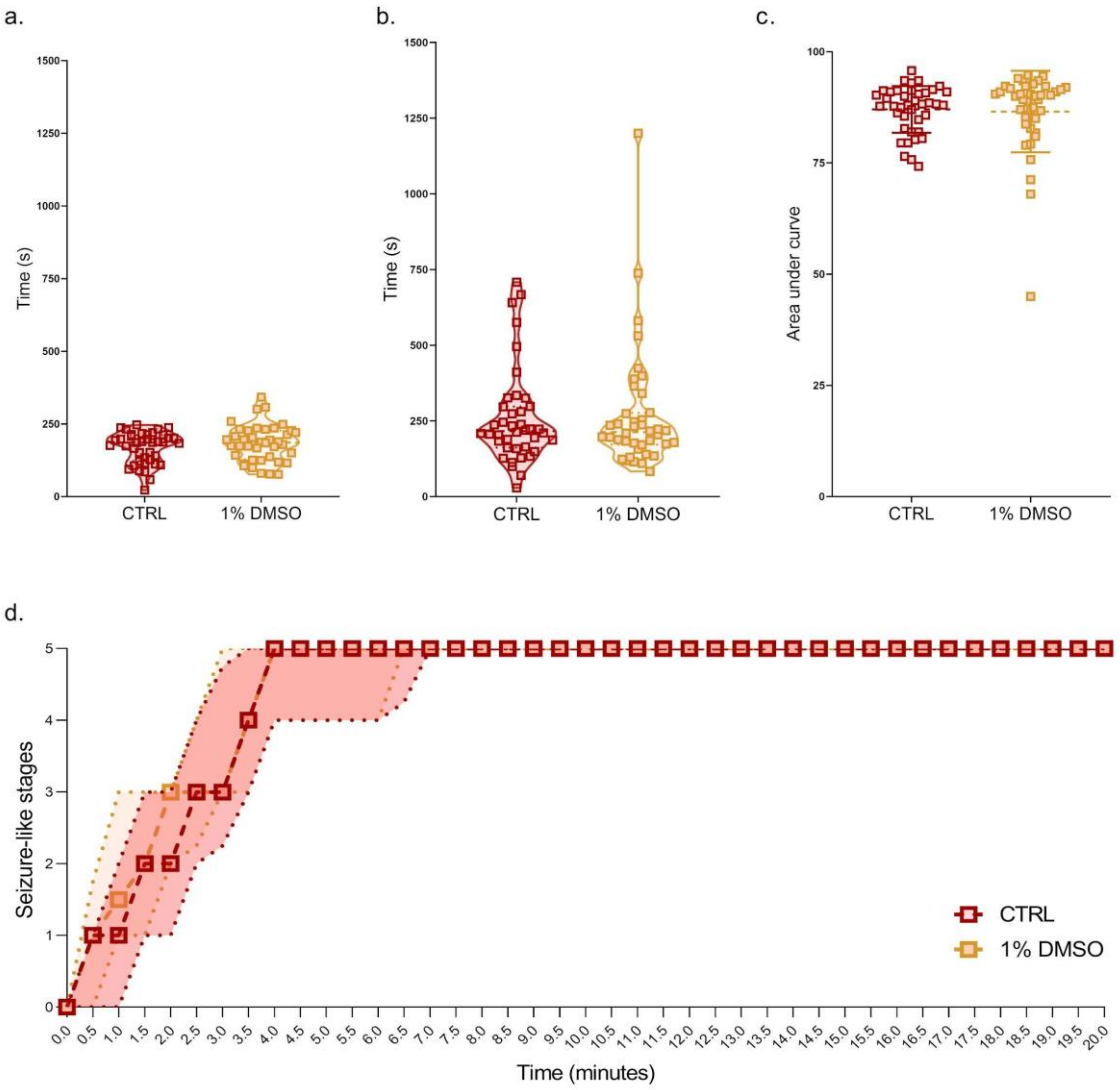
Supplementary Material



Supplementary Figure 1: Linalool experiment: effects of 1% DMSO (vehicle) on (a) latency to clonic-like seizure stage (score onset 4), (b) latency to tonic-like seizure stage (score onset 5), (c) seizure intensity and (d) seizure-like stages scored across time. Data were analyzed for normality by D'agostino & Pearson test, and for variance differences between groups by T-test (Supp. Fig. 1a and 1b) and Mann-Whitney test (Supp. Fig. 1c) according to data distribution. n=20.



Supplementary Figure 2: *Trans*-nerolidol experiment: effects of 1% DMSO (vehicle) on (a) latency to clonic-like seizure stage (score onset 4), (b) latency to tonic-like seizure stage (score onset 5), (c) seizure intensity and (d) seizure-like stages scored across time. Data were analyzed for normality by D'agostino & Pearson test, and for variance differences between groups by T-test (Supp. Fig. 2a and 2b) and Mann-Whitney test (Supp. Fig. 2c) according to data distribution. n=20.



Supplementary Figure 3: Linalool and *trans*-nerolidol experiments cumulative data - Effects of 1% DMSO (vehicle) on (a) latency to clonic-like seizure stage (score onset 4), (b) latency to tonic-like seizure stage (score onset 5), (c) seizure intensity and (d) seizure-like stages scored across time. Data were analyzed for normality by D'agostino & Pearson test, and for variance differences between groups by T-test (Supp. Fig. 3a and 3b) and Mann-Whitney test (Supp. Fig. 3c) according to data distribution. n=40.