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Preliminary Data Regarding the Neurobehavioural Modulatory Effect of *Acmella Oleracea* L. Extract in an Zebrafish Model of Autism Spectrum Disorder

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Abstract: Plant-derived extracts, such as those obtained from *Acmella oleracea* (L.) R.K. Jansen (AO), have drawn increasing interest due to possible neuroprotective properties. Several reports of neuroprotective potential were attributed to the main bioactive component of AO extract, spilanthol. Due to its capacity of spilanthol to cross the blood-brain barrier and to modulate serotonergic and GABAergic systems, AO extract may represent a promising experimental candidate for investigating effects on autism spectrum disorder (ASD) relevant behavioural symptoms, such as anxiety and aggression. The most common drug to model core symptoms of autism spectrum disorder (ASD) in zebrafish is the antiepileptic valproic acid (VPA). The current study aimed to evaluate the behavioural effects of AO extract in zebrafish, as compared to or in combination with VPA, to assess their effects on anxiety-like and aggressive behaviour, as assessed by the novel tank test (NTT) and the mirror-biting test (MBT). Our findings suggest that VPA-treated zebrafish show an increased frequency of aggressive and anxiety-like behaviours, as compared to controls. Similar anxiety-like, but not aggressive, behavioural changes were seen in AO-treated zebrafish. These effects may be connected with the modulation of GABAergic neurotransmission. Moreover, we observed that acute AO extract treatment was not able to modulate aggressive behaviour in VPA-treated or in healthy zebrafish.

Keywords: zebrafish; valproic acid; *Acmella oleracea*; aggressive and anxiety-like behaviours.

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1. Introduction

Acmella oleracea (L.) R.K. Jansen (known as "jambu") (AO) is widely used in folk medicine due to its pharmacological properties, such as anti-inflammatory, antioxidant, antibacterial, and local analgesic effects (Rodrigues et al., 2023; Chakraborty et al., 2010; Wu et al., 2008). Recent reports have shown that AO extracts are rich in various secondary metabolites, such as alkaloids, flavonoids, saponins, steroid glycosides, and tannins (Spinozzi et al., 2022), the main one being spilanthol, a N-alkylamide, also known as "acmellonate", or "affinin" (Rodrigues et al., 2023; Déciga-Campos et al., 2012; Custódio de Souza et al., 2019). In addition to impressive regenerative effects on skin, bones, and muscle, studies have reported that spilanthol also has a significant neuroprotective potential and the capability to cross the blood-brain barrier (Dubey et al., 2013; Barbosa et al., 2016; Boonen et al., 2010; Maxim et al., 2024). In this way, it was shown that spilanthol improves anxiety and cognitive processes by modulating serotonergic and GABAergic systems (Déciga-Campos et al., 2012; Barbosa et al., 2016; Maxim et al., 2024).

Anxiety is an important symptom of neuropsychiatric diseases, including neurodevelopmental impairments such as those being characteristic to autism spectrum disorder (ASD) (Tarver et al., 2021). Thus, ASD is frequently associated with socio-affective impairments, such as aggressive behaviour and altered response to external stimuli, that limit normal behaviour and prevent patients' social inclusion (Ambler et al., 2015). Usually, anxiety and aggressive behaviours are pharmacologically managed by GABA and serotonergic regulation in ASD patients (Tarver et al., 2021; Wang & Sun, 2025).

Multiple animal models for ASD research are currently based on valproic acid (VPA) treatments in all life stages of rodents and zebrafish (*Danio rerio*) (Camussi et al., 2025). The latter are mainly used for understanding the neurobiology of ASD and underlying pathological mechanisms due to their similarity to the complex human cognitive pathways, highly conserved neurobiological mechanisms, and the capacity to perform complex behavioural tasks (Li et al., 2024; Oliveira et al., 2011; de Abreu et al., 2021). VPA is an analogue of valeric acid (a natural product extracted from *Valeriana officinalis*) widely used as an antiepileptic drug and mood stabiliser. In epilepsy, migraine, and bipolar disorder, VPA modulates the GABAergic system (Camussi et al., 2025; Singh et al., 2021). On the other hand, preclinical and clinical studies have emphasised teratogenic and neurotoxic effects of VPA that cause neurodevelopmental impairments and altered socio-affective behaviour when administered maternally or in excessive doses, disregarding the developmental stage (Taleb et al., 2021; Zarate-Lopez et al., 2024; Lotufo-Denucci et al., 2025; Dorsey et al., 2024; Washbourne et al., 2023).

In this context, the current study aimed to evaluate the behavioural effects of AO extract in zebrafish, as compared to or in combination with VPA, to evaluate their modulation of anxiety-like and aggressive behaviour.

2. Materials and Methods

2.1. Zebrafish Accommodation

Twenty wild-type zebrafish (*Danio rerio*, 3–4 months, sex ratio 1:1) were obtained from a local breeder and accommodated for 2 weeks in a 50 L glass tank. Subsequently, four groups designated by random assignment were habituated for 5 days in 6 L tanks under controlled environmental conditions (water temperature: 27 ± 1 °C; pH 7.5; dissolved oxygen: 7.2 mg/L; with daily water renewal; 14:10 hours light–dark cycles). Standard fish food formulation (TetraMin flakes, Germany) was provided twice daily, according to the recommended food/weight ratio. All the procedures were performed according to the Recommendation of the European Union Commission of 18 June 2007 and the Directive of the European Parliament and of the Council of the European Union of 22 September 2010, and were approved by the Ethics Committee of the Faculty of Biology of "Alexandru Ioan Cuza" University of Iasi (122/12.05.2025).

2.2. Experimental Design

Following the accommodation period, 4 experimental groups were formed ($n = 5$ fish/group): CTR – control, VPA (200 μ M valproic acid), AO (250 μ g/L *Acmella oleracea* extract), VPA + AO (200 μ M VPA and 250 μ g/L *Acmella oleracea* extract) (Figure 1). Dried *Acmella oleracea* aerial part samples (at a total mass of 2 g) were extracted by shaking with 50 mL of solvent (ethanol:water 70:30, v/v) at 150 rpm for 2 h at room temperature. The extracts were subsequently filtered using 0.22 μ m PTFE syringe filters before use. Valproic acid (Sigma-Aldrich, Germany) and AO extract were administered continuously in the housing tank water for 48 hours (at a final volume of 5 L) (Custódio de Souza et al., 2019; Li et al., 2024). *Acmella oleracea* extract was obtained according to previous procedures described by Csabai et al. (2024). Also, the extract was analysed for content by UHPLC, revealing the presence of 77 compounds, including spilanthol, phaselic acid (2-O-caffeoylmalic acid), quercetin-O-dihexoside-O-rhamnoside; quercetin-O-(rhamnosyl) dihexoside; quercetin-O-dihexoside; N-p-coumaroyl3-hydroxytyrosine; quercetin-O-pentoside; astragalin (kaempferol-3-O-glucoside); isorhamnetin-3-O-glucoside; and dihydropilanthol isomer (Csabai et al., 2024).

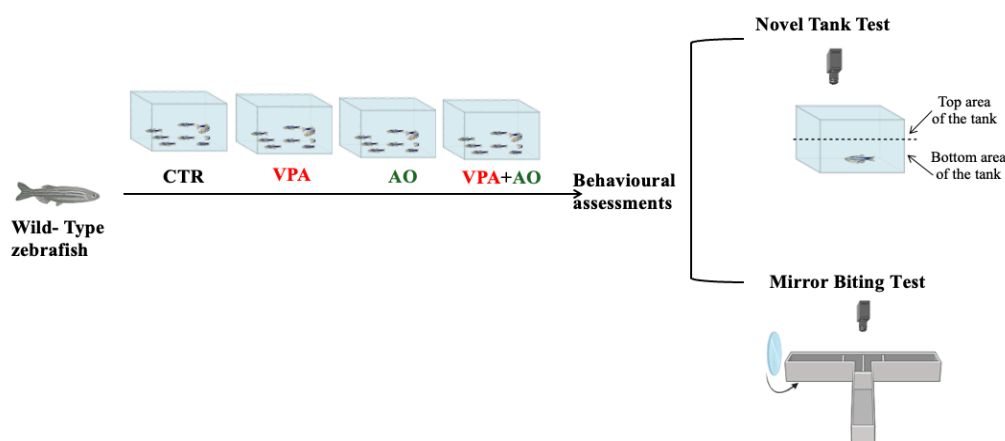


Figure 1. Schematic representation of the experimental design (VPA – valproic acid; AO – *Acmella oleracea*)

2.3. Behavioural Assessment

At 24 hours after the last VPA and AO extract administration, behavioural assessments were performed to evaluate the exploratory, aggressive, and anxiety-like behaviours using the novel tank test and the mirror biting test.

2.3.1. Novel Tank Test

The Novel Tank Test (NTT) allows the evaluation of vertical exploration patterns that are relevant to anxiety-like behaviours (Rosemberg et al., 2011; Golushko et al., 2025). A 6 L rectangular glass tank was divided into two observational zones from a lateral view–top and bottom areas. After a short accommodation period (30 seconds), the fish were left to freely explore the tank for 5 minutes, while their activity was recorded and processed using EthoVision XT 16 (Wageningen, Netherlands). The following general behavioural parameters were recorded: total swimming distance (cm), average swimming velocity (cm/s), total activity/inactivity time (s), and counter-clockwise rotation frequency (#) (Robea et al., 2024). The anxiety-like behaviour parameters recorded were the time spent in the top area (s); latency to the first entry in the top area, and crossing between top and bottom areas (Rosemberg et al., 2011; Golushko et al., 2025).

2.3.2. Mirror Biting Test

The mirror biting test (MBT) was used to assess the aggressive behaviour, as previously described (Li et al., 2024; Robea et al., 2024). A T-shaped maze consisting of three arms: central, left and right, with a mirror placed on the left side, was used to simulate the presence of a conspecific. After a short accommodation period (30 seconds), the fish were left to freely explore the T-shaped maze for 5 minutes, while their activity was recorded and processed using EthoVision XT 16 (Wageningen, Netherlands). Specific behavioural parameters for aggressive behaviour were: frequency of mirror biting, the time spent in the mirror proximity (s) and the time spent in other maze arms (right and central arms, decision point). In addition, the standard locomotion parameters (total swimming distance (cm), average swimming velocity (cm/s), total activity/inactivity time (s), maximum acceleration (cm/s²), and counter-clockwise rotation frequency (#) were measured to evaluate the general locomotor activity (Li et al., 2024; Robea et al., 2024).

2.4. Statistical Analysis

The numerical data obtained by analysing the behavioural parameters were statistically processed using the GraphPad PRISM software (version 10.0.0 for Windows, Boston, MA, USA). The results were expressed as mean $\pm$ SEM. A one-way ANOVA test, followed by post-hoc analyses (Tukey's HSD test), was performed to test the differences between the groups. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Novel Tank Test

The statistical analysis of our NTT results suggests increased locomotor activity and anxiety-like behavioural trends. The total distance swam was increased in the VPA-treated group, while it was significantly increased after AO extract treatments in both VPA-treated and healthy zebrafish, as compared to the CTR group (AO versus CTR, $p < 0.05$; AO + VPA versus CTR, $p < 0.01$ in one-way ANOVA test) (Figure 2a). Similarly, the average swimming velocity significantly increased in all groups, as compared to the CTR group (VPA versus CTR, $p < 0.05$; AO versus CTR, $p < 0.05$; AO + VPA versus CTR, $p < 0.05$, in one-way ANOVA test) (Figure 2b). On the other hand, we observed that AO and VPA treatments significantly increased freezing behaviour frequency (VPA versus CTR, $p < 0.05$; AO versus CTR, $p < 0.05$, in one-way ANOVA test) (Figure 2d) while significantly decreasing the activity total time, as compared to CTR group (VPA versus CTR, $p < 0.05$; AO versus CTR, $p < 0.01$; AO + VPA versus CTR, $p < 0.05$ in one-way ANOVA test) suggesting a significant anxiogenic effect of both VPA and AO extract, as compared to healthy controls (Figure 2c). On the other hand, a potential synergistic effect of VPA and AO was observed in modulating anxiety-like behaviour (activity total time, AO + VPA versus CTR, $p < 0.05$) and impairing decision-making behaviour (counter-clockwise rotation frequency, AO + VPA versus CTR, $p < 0.05$ in one-way ANOVA test) (Figure 2 d).

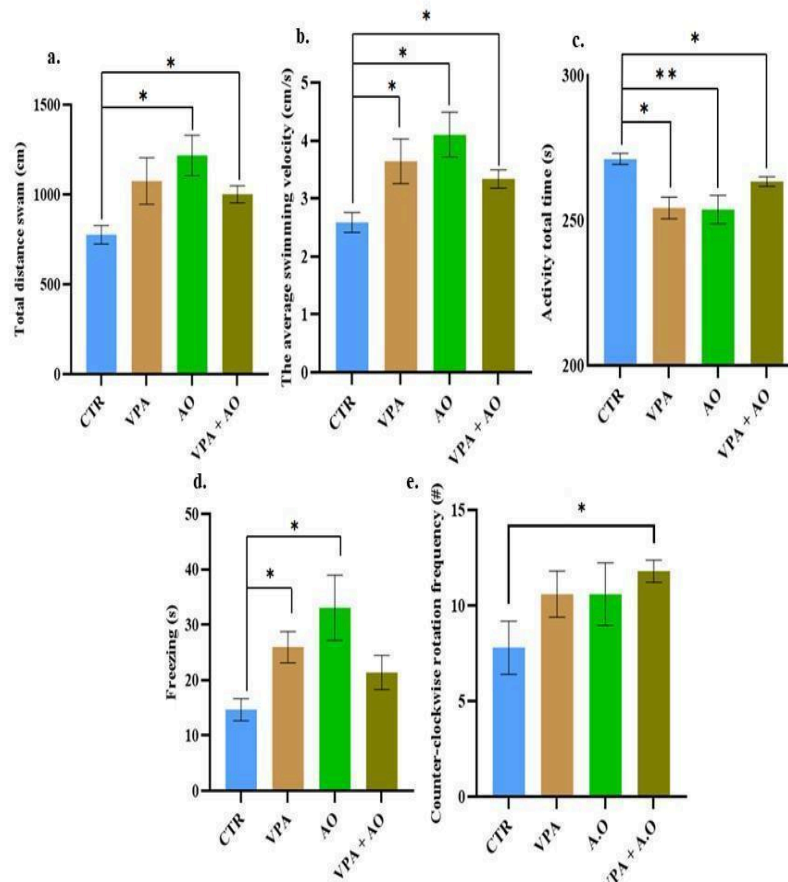


Figure 2. Locomotor performance, as evaluated using NTT: a. Total distance swam (cm); b. Average swimming velocity (cm/s); c. Activity total time (s); d. Inactivity total time (s); e. Freezing (s); f. Counter-clockwise rotation frequency (#). The results are expressed as mean $\pm$ SEM (CTR – control; VPA – 200 μ M valproic acid treatment for 48 hours; AO – *Acmella oleracea* extract for 48 hours; VPA + AO – 200 μ M valproic acid and 250 μ g/L *Acmella oleracea* extract for 48 hours; * p < 0.05; ** p < 0.01 in one-way ANOVA)

Additionally, our results showed significant anxiety-like behaviours, as the time spent in the top area significantly decreased after VPA and AO treatments, as compared to the CTR group (VPA versus CTR, p < 0.05; AO versus CTR, p < 0.05, in one-way ANOVA) (Figure a). Interestingly, the combined treatment (VPA + AO) did not follow the same variation pattern for this behavioural parameter (time spent in the top area, VPA versus VPA + AO, p < 0.05; AO versus VPA + AO, p < 0.05, in one-way ANOVA) (Figure 3 a). Similarly, significant decreases in crossing frequency in treated groups, as compared to the CTR group, was observed (VPA versus CTR, p < 0.05; VPA versus VPA + AO, p < 0.05; AO versus CTR, p < 0.05; AO versus VPA + AO, p < 0.05, in one-way ANOVA test) (Figure 3c). Despite the latency to the first entry in the top area of the NTT not being significantly impaired, the behavioural trends observed in NTT suggest increased anxiety and poor exploratory behaviours (Figure 3b).

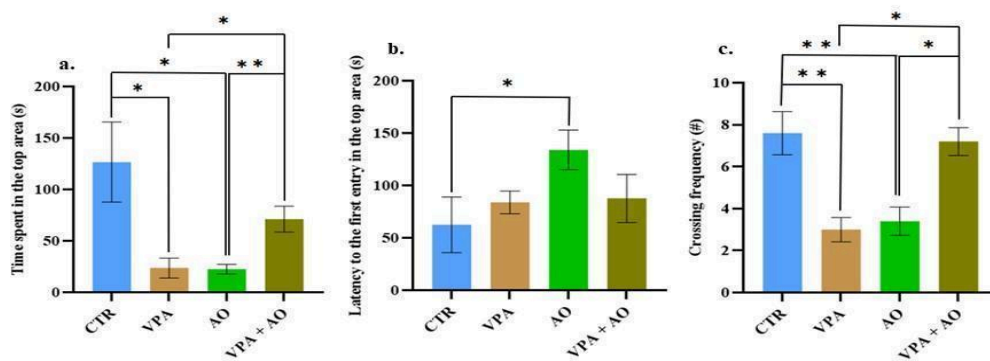


Figure 3. Specific behavioural parameters evaluated using NTT: a. Time spent in the top area (s); b. Latency to the first entry in the top area (s); c. Crossing frequency (#). The results are expressed as mean $\pm$ SEM (CTR – control; VPA – 200 μ M valproic acid treatment for 48 hours; AO – *Acmella oleracea* extract for 48 hours; VPA + AO – 200 μ M valproic acid and 250 μ g/L *Acmella oleracea* extract for 48 hours; * $p < 0.05$; ** $p < 0.01$ in one-way ANOVA)

3.2. Mirror Biting Test

In the MBT test, our results suggest the presence of aggressive behaviour and hyperlocomotion following VPA and AO extract treatments. Similar to NTT, the total swimming distance significantly increased after VPA and VPA + AO treatments, as compared to the healthy control group (VPA versus CTR, $p < 0.05$; VPA + AO versus CTR, $p < 0.05$, in one-way ANOVA) (Figure 4 a). In addition, increases in average swimming velocity, activity total time, and maximum acceleration were seen in all treated groups, as compared to the CTR group, suggesting hyperlocomotion (Figure 4 b, c, d).

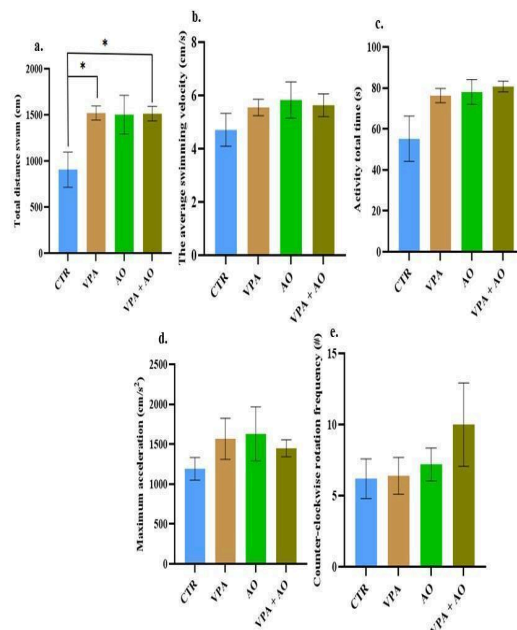


Figure 4. Locomotor performance parameters evaluated in MBT: a. Total distance swam (cm); b. The average velocity (cm/s); c. Activity total time (s); d. Inactivity total time (s); e. Maximum acceleration (cm/s²); f. Counter-clockwise rotation frequency (#). The results are expressed as mean $\pm$ SEM (CTR – control; VPA – 200 μ M valproic acid treatment for 48 hours; AO – *Acmella oleracea* extract for 48 hours; VPA + AO – 200 μ M valproic acid and 250 μ g/L *Acmella oleracea* extract for 48 hours; * $p < 0.05$ in one-way ANOVA test)

Regarding aggressive behaviour assessment, we observed a significant increase in the mirror biting frequency following solely acute VPA treatment (VPA versus CTR, $p < 0.05$ in one-way ANOVA) and in combination with AO (VPA + AO versus CTR, $p < 0.05$, in one-way ANOVA) (Figure 5a). AO extract had no significant effect on mirror biting frequency when administered to healthy zebrafish (Figure 5). However, a non-significant decrease in the time spent in the proximity to the mirror was observed in all treated groups, as compared to the CTR group, suggesting potential anxiety states characterised by avoidance of the stimulus proximity area (Figure 5b). The significant increases in the time spent in the right and central arms (right arm: AO versus CTR; central arm: VPA versus CTR, $p < 0.05$ in one-way ANOVA), further support the anxiety-like behavioural pattern characterised by avoidance of the stimulus-containing arm. Moreover, the increased time spent in the decision point of the AO treated group, as compared to the CTR group, indicates impairments in decision-making behaviour ($p < 0.05$ in one-way ANOVA).

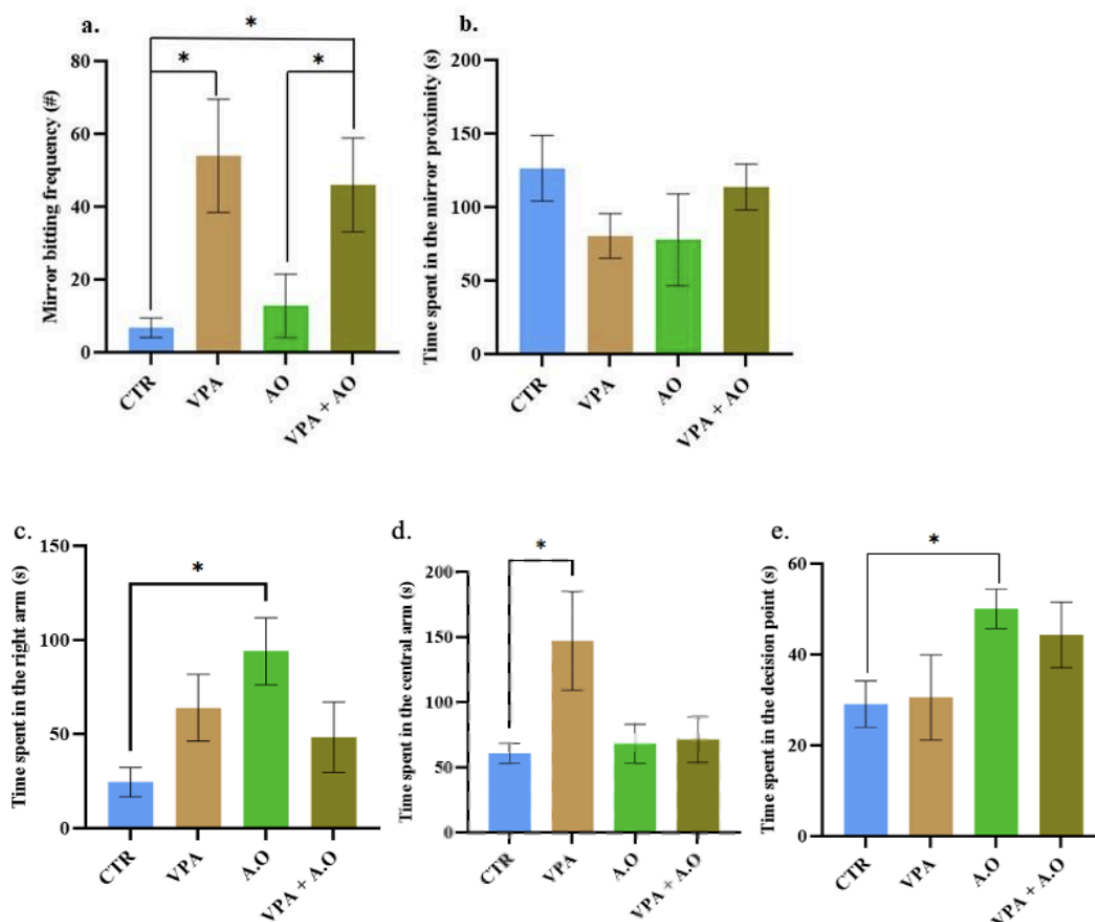


Figure 5. Aggressive behaviour parameters evaluated using MBT: a. Mirror biting frequency (#); b. Time spent in the mirror proximity (s); c. Time spent in the right arm (s); d. Time spent in the central arm (s). e. Time spent in the decision point (s). The results are expressed as mean $\pm$ SEM (CTR – control; VPA – 200 μ M valproic acid treatment for 48 hours; AO – Acmella oleracea extract for 48 hours; VPA + AO – 200 μ M valproic acid and 250 μ g/L Acmella oleracea extract for 48 hours; * $p < 0.05$ in one-way ANOVA).

4. Discussion

The present study aimed to evaluate and describe the behavioural modulatory effects of AO extract and acute short-term VPA treatment in zebrafish, with special regard to aggressive and anxiety-like behaviours. We aimed to provide additional information regarding the potential of AO extract to modulate neurocognitive processes and behavioural patterns. In this context, our results showed that AO extract induced similar anxiety-like behavioural patterns, as compared to a dedicated VPA-based ASD zebrafish model. Consequently, AO extract may exert anxiogenic

effects, impaired locomotory performance and decision-making processes, with a possible potentiating effect when administered in combination with VPA. Moreover, we observed that acute AO extract treatment was not able to modulate aggressive behaviour in VPA-treated nor in healthy zebrafish.

In the current experimental design, we showed that acute treatment with 200 μ M VPA resulted in hyperlocomotion (significantly increased total swimming distance, average velocity, and significantly decreased total active time), anxiety-like (significantly increased freezing episodes, and decreased time spent in the top area and crossing between areas), and aggressive behaviours (significantly increased mirror biting frequency). Previous studies described impaired swimming behaviour in zebrafish after a short-term VPA treatment (50-265 μ M VPA exposure for up to 120 hours) (Blazina et al., 2013; Bailey et al., 2016). Furthermore, an increase in anxiety-like behaviours was reported in zebrafish treated with 500 μ M VPA for 4 days, suggesting that VPA could worsen the negative effects of environmental stress, leading to hyperlocomotion and anxiety-like behaviours (Li et al., 2024). Furthermore, we observed that acute treatment with 200 μ M VPA continuously for 48 hours could trigger aggressive behaviour, as indicated by the significantly increased mirror biting frequency. Furthermore, a "fight or flight" reaction is suggested by the decreased time spent in the mirror proximity area (near the stimulus). Our findings are consistent with those of Li et al. (2024), who reported that anxiety-like behavioural responses are strongly inversely correlated with the time spent away from the mirror. In this context, several studies have shown that VPA could alter the hypothalamic-pituitary-adrenal axis (HPA), which controls stress response stimuli and triggers the "fight or flight" response (Oliveira et al., 2011; de Abreu et al., 2021; Tringali et al., 2004; Colwill & Creton, 2011; Jones & Norton, 2015).

Consistent with the behavioural profile observed in VPA-treated zebrafish, AO-treated zebrafish exhibited similar behavioural alterations, except for aggressive behaviour. The anxiogenic effect could be suggested by the significantly decreased time spent in the top area and crossing frequency between areas, as seen in NTT. Furthermore, significantly increased exploratory behavioural parameters (e.g., total distance swam, velocity, activity total time and freezing frequency) suggest hyperlocomotory patterns, partly as a result of increased anxiety-like behaviours. In line with our results, Custódio de Souza et al. (2019) reported similar behaviour patterns (e.g., longer time spent at the bottom of the tank, spasms) after exposure to a similar dose of 250 μ M AO extract for 48 hours. Additionally, Ribeiro (2013) reported hyperlocomotion in zebrafish exposed to "jambú" extract. On the other hand, other studies have reported the potential of treatment with AO extract in reducing aggressive behaviour when administered to healthy individuals (Déciga-Campos et al., 2012; Barbosa et al., 2016; Déciga-Campos et al., 2010); nonetheless, the "flight" response to MBT stimuli showed a substantial anxiogenic effect. In this context, previous studies have explained these behavioural patterns by the neuroactive effects of spilanthol in modulating the nociception and HPA axis. In a rodent model of nociception, Déciga-Campos et al. (2012; 2010) have consistently demonstrated that spilanthol (136 μ M, i.p.) shows dose-dependent anxiolytic and antinociceptive effects based on GABAergic and serotonergic systems modulation.

Moreover, we observed a possible synergistic effect between AO extract and VPA, as suggested by the potentiation of the anxiogenic effect when administered in combination. In both NTT and MBT, we observed that combined treatment (VPA+AO) led to significantly increased anxiety-like behaviours and impaired decision-making processes. The possible mechanism of interaction could be based on both targeting GABA-mediated neurotransmission (Déciga-Campos et al., 2012; Déciga-Campos et al., 2010; Singh et al., 2021; Hepsomali et al., 2020). While VPA enhances the inhibitory effect of GABA by preventing GABA degradation, suppressing GABA transamination, and promoting GABA synthesis (Singh et al., 2021) the precise mechanisms of spilanthol on GABA are still unclear, research on rodents indicating GABAergic regulation by increasing GABA release contributing to altered response to stress stimuli (Déciga-Campos et al., 2012; Déciga-Campos et al., 2010).

Several limitations of the present study should be addressed. The small sample size ($n = 5$ /group) and the acute (48h) exposure could limit statistical power and detection sensitivity. Secondly, our preliminary results only focused on limited observation of some aggressive and anxiety-like responses, as they could be assessed by NTT and MBT. Although locomotor activity was recorded during these tests, the relationship between locomotor changes and other behavioural endpoints was not specifically investigated. As zebrafish behavioural patterns are rather complex, further behavioural evaluation (e.g. social behaviour, conspecific interaction or learning), as well as analyses exploring the interaction between different behavioural domains, could shed more light upon the effects of AO extract treatment on animal models. Also, the limited and cautious translation between animal models and humans could prevent other conclusions except for those regarding the understanding of the neurobiological context of VPA and AO extract effects in zebrafish behaviour. In this way, future research should focus on larger sample sizes and longer treatment periods, together with molecular and biochemical analyses, including neurochemical, transcriptomic, protein expression assessments and whole-body cortisol quantification. These approaches would strengthen the mechanistic interpretation of the findings and allow for a better understanding of the neurobiological pathways underlying the effects of AO extract on core ASD-related behavioural impairments.

5. Conclusions

Acmella oleracea (L.) R.K. Jansen (known as "jambu") is widely used in folk medicine due to its pharmacological properties, such as anti-inflammatory, antioxidant, antibacterial, and local analgesic effects. Our results showed that AO extract produced similar anxiety-like behavioural patterns, as compared to a dedicated VPA-based ASD zebrafish model. Consequently, AO extract appeared to induce anxiogenic-like behavioural changes and induced hyperlocomotion, with a possible potentiating effect when administered in combination with VPA. The similarities seen in the behavioural patterns following both VPA and AO treatments suggest a possible shared neurobiological process that could involve GABAergic regulation. Moreover, we observed that acute AO extract treatment was not able to modulate aggressive behaviour in either VPA-treated or healthy zebrafish.

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