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RESEARCH ARTICLE

Oprea et al: Plant extracts and zebrafish behaviour

Effects of plant extract formulations on anxiety-like behavior, sociability, and gut microbiota diversity in paracetamol-treated adult zebrafish

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ABSTRACT

Plant extract-based formulations may influence behavior and gut microbiota, but their effects following acute paracetamol (PAR) exposure remain unclear. This preliminary study evaluated the behavioral effects of two formulations, coded MOD and TEL, and explored TEL-associated microbiota changes in adult zebrafish. Sixty fish were randomized to six groups (10/group). PAR exposure (5 mM for 24 h) was followed by formulation treatment (500 mg/L for five days), alongside untreated, PAR-only, and formulation-only controls. Behavior was assessed using the novel tank test (NTT) and social behavior test. Full-length 16S ribosomal ribonucleic acid (rRNA) gene sequencing was performed in untreated, PAR-only, and PAR-plus-TEL groups (three fish/group). Compared with PAR alone, MOD increased swimming distance and velocity during social testing (p = 0.028 and p = 0.003, respectively), whereas TEL reduced vertical crossings in the NTT (p = 0.035) and time spent near social stimuli (p = 0.0033). Reductions in freezing duration with both formulations were not statistically significant compared with PAR alone. No significant differences in alpha or beta diversity were detected, and no genus-level differences remained significant after multiple-testing correction. These exploratory findings suggest formulation- and test-dependent behavioral responses without statistically supported microbiota changes. Larger studies with replicated tanks and deeper sequencing are needed to clarify these associations.

Keywords: Zebrafish, paracetamol, plant extracts, anxiety, sociability, gut microbiota.

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INTRODUCTION

Paracetamol (PAR, known as acetaminophen) is one of the most common over-the-counter analgesic and antipyretic drugs [1,2,3]. However, excessive use of PAR could lead to acute and chronic liver failure, cirrhosis. In some cases, the accumulation of neurotoxic metabolites together with severe liver damage and intestinal dysbiosis [4,5,6,7]. PAR overdose was previously associated with neuropsychiatric impairment, including confusion, irritability, and agitation [4,5,6]. Recent studies reported a consistent correlation between neurobehavioral changes and PAR hepatotoxicity via dopaminergic impairment [8]. According to recent studies [7,9-12], PAR could disrupt intestinal microbiota and damage gut barrier integrity, promoting bacterial translocation to the liver and worsening liver damage. Several bacterial groups (*Bacteroides, Firmicutes, Fusobacteriota, Deinococcota*) have been linked to PAR-induced dysbiosis and hepatotoxicity, though some of these associations remain poorly understood [7,13,14]. Due to the disruptive effects associated with PAR consumption [15,16] the specialised literature is oriented to different compounds able to restore the balance of the microbiota and reduce the neurological impact of dysbiosis such as plant-based formulations and extracts were considered potential alternative therapeutics due to their antioxidant, anti-inflammatory, immunomodulatory, and hepatoprotective properties due to their richness in polyphenols [17-22]. Several studies reported that plant extract could alleviate the PAR-induced effects and regulate the intestinal microflora [9,23–25]. Moreover, some plant extracts (*e.g.*, *Rubus idaeus, Vaccinium spp., Cichorium intybus, Astragalus membranaceus, Silybum marianum, Centella asiatica*) have been reported to exhibit hepatoprotective effects due to the rich content of antioxidant, anti-inflammatory biomolecules [26-41]. Additionally, several studies [27,33,34] have reported improved socio-affective behavior, cognition, and anxiety/depression-like symptoms in various animal models receiving chronic treatments with plant extracts. Furthermore, promising results regarding the beneficial effect of plant products, such as *Galla chinensis* extract, were reported to improve gut dysbiosis associated with PAR-induced acute liver injury.[9] Our most recent study already evaluated the potential of one of the natural products of interest in regarding behavioural changes and gut microbiota diversity in another zebrafish model (MOD) [42]. However, major shifts in gut microbiota diversity were not observed, thus we focused on the novel product of interest [TEL]. on the other hand, we showed that MOD could induce

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relevant behavioural changes in a neuro-psychiatric model (of autism spectrum disorder) by modulating social-affected behaviour thus we evaluated its potential in another type of neuropsychiatric impairment (*i.e*
neurotoxic acute PAR treatment). Several previous studies have reported validated zebrafish (*Danio rerio*) models for PAR-induced hepatotoxicity [43,44]. Furthermore, neurotoxic effects of PAR were also studied in zebrafish models and impairments of locomotor behaviour, socio-affective processes, memory, and cognitive functions were repeatedly reported [45-48]. On the other hand, the PAR effects on intestinal dysbiosis throughout the alteration of the balance and shape of the microbiota composition, barrier impairment, microbiota diversity and abundance were reported [7, 49]. In this context, our study aimed to evaluate the possible potential of two novel formulations of plant-based extracts to modulate behavioural changes and gut dysbiosis induced by acute exposure to PAR in adult zebrafish.

MATERIALS AND METHODS

Zebrafish accommodation

Sixty wild-type zebrafish (*Danio rerio*, 2 – 3 months, sex ratio 1:1) were acquired from a certified local breeder and accommodated for 2 weeks in a 50 L tank in standard conditions (water temperature: 27 ± 1˚C, pH: 7.5, dissolved oxygen: 7.20 mg/L, daily replacement, with a 14:10 h light/dark cycle). Fish were fed daily with standard fish food formulation (TetraMin flakes, Germany) according to the recommended food ratio [50].

Experimental design

Following the 2 weeks accommodation period, fish were randomly assigned to 6 experimental groups (n = 10 fish/group): CTR – control, PAR – 5 mM, CTR+MOD – 500 mg/L, CTR+TEL - 500 mg/L; PAR+MOD – 5 mM PAR and 500 mg/L, PAR+TEL – 5 mM + 500 mg/L (Figure 1). Each group was housed in a separate 5 L tank under the same environmental conditions as described above throughout the treatment period. After the accommodation period, the PAR, PAR+MOD, and PAR+TEL groups were treated with PAR for 24 h according to previous studies [51-53], while the CTR, CTR+MOD, and CTR+TEL tanks were not exposed to any substance during this initial interval. Following this 24 h PAR exposure, PAR treatment was stopped, and treatment with both plant-based formulation products,

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received directly from the producer, began in the CTR+MOD, CTR+TEL, PAR+MOD, and PAR+TEL groups for 5 days (at a final volume of 5 L), following the method previously described by Păduraru et al. [54]. The CTR and PAR groups were kept and untreated until the end of the study.

The selected concentration (5 mM, 24 h) is markedly lower than the maximum concentration reported as tolerable in adult zebrafish over a short treatment period (20 mM for 3 h [49], and remains within a comparable range to concentrations tested over similar or longer exposure durations (up to 10 mM for up to 48 h [53]. Additionally, our selected concentration corresponds to a mid-range dose within the concentration interval tested in zebrafish larvae (1.25–10 mM, 2–5 dpf [52]).

The first plant-based formulation (MOD) consists in a mixture of plant extracts: *Betula alba* (11,15% stem sap-originating xylitol), aqueous extracts of *Rubus idaeus* (29,25% 20:1 leaf extract), *Vaccinium vitis-idaea* (28,25%, 20:1 leaf extract), *Vaccinium myrtillus* (15,45% 20:1 leaf extract), *Cichorium intybus* (10,5% root extract containing 97% oligofructose), *Saccharomyces cerevisiae* (0,75% extract containing 60% nucleosides), *Hericium erinaceus* (0,75% fruit body extract containing 30% polysaccharides), *Tremella fuciformis* (0,75% 8-10:1 fruit body extract containing 30% polysaccharides), *Pleurotus ostreatus* (0,75% 11-14:1 fruit body extract containing 30% polysaccharides), *Citrus paradisi* (0,6% dried hydroalcoholic seed extract) and essential oils: *Citrus paradisi* (0,6%), *Mentha viridis* (0,6%), and *Citrus sinensis* (0,6%). The second plant-based formulation (TEL) consists in a mixture of plant extracts:*Astragalus membranaceus* (23,26%,18-20:1 hydroalcoholic root extract on maltodextrin, containing 70% polysaccharides), *Silybum marianum* (23.26%, dry fruit extract containing 80% silymarin), *Centella asiatica* (18.60%, dry herb extract containing minimum 20% asiaticosides), *Taraxacum officinale* (9.30%, leaf powder), *Cynara scolymu*s (9.30%, dry aqueous leaf extract on maltodextrin, containing 2.5% caffeic acid derivatives), *Olea europaea* (9.30%, dry hydroalcoholic leaf extract containing minimum 18% oleuropein), *Astragalus membranaceus* (4.58%, root powder), *Chlorella vulgaris* (2.33%, powder containing 50% protein) and the essential oils of *Boswellia serrata* (0.023%), *Copaifera officinali*s (0.023%), *Elettaria cardamomum* (0.012%) and *Laurus nobilis* (0.012%). To avoid conflicts of interest, the producer's brand names were kept anonymous and will therefore be encoded as MOD (Lot No. 2865, manufactured on

23.06.2025, expires in June 2028) and TEL (Lot No. 6514, manufactured on 21.05.202, expires in May 2028).

Additional quality-control information was available for the raw materials used in the formulations. Microbiological analyses showed low microbial loads, with Escherichia coli and Salmonella spp. not detected, and the tested samples complied with the relevant warning values of the German Society for Hygiene and Microbiology (DGHM). Additional analyses included screening for ethylene oxide and 2-chloroethanol, pesticide residues, and polycyclic aromatic hydrocarbons (PAHs). The investigated parameters were either below the respective limits of detection or quantification or, where applicable, complied with the relevant regulatory criteria. Fresh treatment solutions were prepared daily, and tank water was regularly renewed to maintain consistent exposure conditions.

Behavioural assessments

At 24 hours after the exposure to PAR and plant-based formulations, behavioural patterns were assessed and the exploratory, social, and anxiety-like behaviours were described. All 10 fish per group were included in the behavioural assessments. An investigator, other than the one performing the treatments, blindly conducted the behavioral assessments.

Novel Tank test

The Novel Tank test was performed to evaluate the exploratory and anxiety-like behaviours [55-57].The test was performed in a 6 L rectangular tank, equally divided in two observational areas, from lateral view (bottom and upper areas) and from top view (in peripheral and central areas). After a short 30 seconds accommodation, zebrafish were left to freely explore the new environment for 4 minutes. To evaluate the exploratory behaviour, several general locomotory parameters were recorded: the total swimming distances (cm), the average swimming velocity (cm/s), inactivity total time (s), clockwise and counter-clockwise rotation frequency (#) and freezing behaviour (s). The specific anxiety-like behaviour parameters recorded were: the time spent in the top area (s), the time spent in central area (s), thigmotaxis (s), crossing between top and bottom areas (#), and crossing

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between walls and central areas (#). The fish activity was recorded and processed with the EthoVision XT 16 (Wageningen, Netherlands).

Social behaviour test

The Social Behaviour Test was used to assess the social behaviour characteristics. The social behaviour test was performed using a T-maze, divided into 3 arms (left, right and central), according to literature guidelines [58,59]. The social stimulus, consisting of 3 conspecifics, was placed at the end of the left arm and separated by a plexiglass wall. After a short 30 seconds accommodation at the start point, the social behaviour test was performed for 4 minutes. The specific parameters for social behaviour were recorded: time spent near social stimuli (s) and the presence in the other maze arms (s)(right arm, central arm, and decision point). In addition, the general locomotory parameters: the total swimming distance (cm),the average swimming velocity (cm/s), activity total time (s), clockwise and counter-clockwise rotation frequency (#) were recorded to evaluate the general locomotor activity. The zebrafish activity was video recorded and processed with the EthoVision XT 16 (Wageningen, Netherlands) software.

Microbiota analysis

At the predefined experimental endpoints, fish were euthanized in accordance with approved ethical protocols. For the microbiota analysis, 3 fish per group were randomly selected across three experimental groups of interest (CTR, PAR and PAR+ TEL), yielding a total of 9 distinct biological samples. To preserve biological individuality and capture true intra-group variance, all samples were processed as individual, non-pooled specimens. The entire intestine was aseptically dissected using sterile forceps and scissors, avoiding contamination from surrounding tissues. When necessary, intestinal samples were gently rinsed with sterile phosphate-buffered saline to remove external debris. Each intestine was immediately transferred into a sterile, labeled microcentrifuge tube and snap-frozen without cryoprotectant. Samples were stored at −80 °C until DNA extraction. All samples were processed individually to minimize cross-contamination.

Microbial genomic DNA extraction

Genomic DNA was extracted from intestinal samples using the Maxwell® RSC Fecal Microbiome DNA Kit (Promega, Madison, WI, USA) on a Maxwell RSC

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Instrument, following the manufacturer's instructions. The concentration and purity of microbial DNA were assessed using a SpectraMax QuickDrop UV–Vis spectrophotometer (Molecular Devices, San Jose, CA, USA).

16S rRNA gene amplification and library preparation

Bacterial community profiling was performed by sequencing the 16S rRNA gene using the Rapid Sequencing DNA—16S Barcoding Kit 24 V14 (SQK-16S114.24, Oxford Nanopore Technologies, Oxford, UK). For each sample, 10 ng of genomic DNA was subjected to barcoded PCR amplification using kit-provided primers. Amplified products were quantified using the Equalbit 1× dsDNA HS Assay Kit (EQ121-01, Vazyme International LLC, Nanjing, China) on a QFX Fluorometer (DeNovix Inc., Wilmington, DE, USA), pooled in equimolar concentrations, and purified using AMPure XP beads. Rapid sequencing adapters were ligated according to the manufacturer's protocol. Prepared libraries were kept on ice and loaded immediately onto the flow cell.

Sequencing and bioinformatic analysis

Sequencing was performed on a MinION Mk1B device using an R10.4.1 flow cell (FLO-MIN114, Oxford Nanopore Technologies, Oxford, UK). Raw sequencing data generated in POD5 format were basecalled using Dorado basecaller (v5.2.0; Oxford Nanopore Technologies) with the dna_r10.4.1_e8.2_400bps_hac model. Basecalling was performed using GPU acceleration with CUDA support, with recursive processing enabled and the SQK-16S114-24 kit specified. Reads were output in FASTQ format.

Demultiplexed FASTQ files were analyzed using the wf-16s Nextflow pipeline (epi2me-labs, Oxford Nanopore Technologies) executed with the standard profile. Taxonomic classification was performed using the minimap2 aligner against the NCBI 16S/18S reference database. Default workflow parameters were applied unless otherwise specified. Relative abundance tables were generated at multiple taxonomic ranks (phylum to genus), and alpha diversity indices, including Shannon diversity, Simpson index, and richness, were calculated for each sample. Output files were used for downstream statistical analysis and visualization.

Ethical statement

All 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" Iasi (1671/30.04.2026).

Statistical analysis

The GraphPad PRISM software (version 10.0.0 for Windows, Boston, MA, USA) was used to statistically analyze all numerical data. The results were expressed as mean ± SEM. One-way ANOVA test, followed by post-hoc analyses (Tukey's HSD test), was performed to test the differences between the groups. A p-value of less than 0.05 was regarded as statistically significant for a F value, while p-values ≥ 0.05 were considered non-significant and were interpreted as trends or tendencies where appropriate.

For microbiota analysis, samples with a sequencing depth below a minimum threshold of ≥ 10 genus-level classified reads were excluded from diversity analyses. To account for variations in sequencing depth, alpha-diversity indices were calculated after rarefying samples to 100 reads (the lowest sequencing depth among samples) using 1,000 rarefaction iterations. Group differences in alpha-diversity indices were evaluated using the Kruskal–Wallis test, with Benjamini–Hochberg false discovery rate (FDR) correction applied across indices. Beta diversity was assessed at the genus level using Bray–Curtis dissimilarity derived from total-sum-scaled relative abundance data, and group separation was evaluated via PERMANOVA with 9,999 permutations. Differential abundance testing at the genus level was performed using the Kruskal–Wallis test on relative abundance data. Taxa were retained for analysis if they were present in at least two samples and exhibited a maximum relative abundance of ≥ 1%. Resulting p-values were adjusted using the Benjamini–Hochberg FDR procedure.

RESULTS

We observed hypolocomotion and reduced exploratory behavior during NTT. Total swimming distance and average swimming velocity were decreased in the PAR-treated group, as compared to the CTR group (Figure 2 a, b). Similarly, the total swimming distance and average swimming velocity tended to decrease after TEL

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treatment in both healthy and PAR-treated zebrafish, as compared to CTR and PAR-treated groups, respectively (CTR+TEL *versus* CTR, p = 0.07; PAR+TEL *versus* CTR, p = 0.0520 in one-way ANOVA test) (Figure 2 a, b). No significant changes were observed after MOD treatment neither in healthy nor in PAR-treated zebrafish (Figure 2 a, b). Yet, we observed significant differences in locomotory parameters while comparing the treatments (MOD *versus* TEL) in healthy zebrafish (total swimming distance, average swimming velocity, inactivity total time: CTR+TEL versus CTR+MOD, p = 0.0086, p =0.00855, p = 0.0097, respectively in one-way ANOVA test) (Figure 2 a- c).

Significant decrease of immobility total time was observed after MOD treatment in PAR-treated fish, as compared to CTR+MOD group (PAR+MOD *versus* CTR+MOD, p = 0.039 in one-way ANOVA test) (Figure 2 c). A decrease in immobility total time was observed following TEL treatment in both healthy and PAR-treated zebrafish, although this differences did not reach statistical significance (CTR+TEL *versus* CTR, p = 0.07; PAR+TEL *versus* CTR, p = 0.05 in one-way ANOVA test) (Figure 2 c). Furthermore, a significant difference in the immobility total time was observed between MOD and TEL treatments in healthy zebrafish (CTR+TEL *versus* CTR+MOD, p = 0.009 in one-way ANOVA test)(Figure 2 c).

On the other hand, changes of clockwise rotation frequency was observed in PAR-treated groups receiving MOD, as compared to healthy and PAR-treated groups (PAR+MOD *versus* CTR, p = 0.065; PAR+MOD *versus* CTR+MOD, p = 0.005 in one-way ANOVA test)(Figure 2d). Significant changes in counterclockwise rotations frequency was observed following TEL treatment in PAR-treated zebrafish (PAR+TEL *versus* PAR, p = 0.027; PAR+TEL *versus* CTR+TEL, p = 0.021; PAR+TEL *versus* PAR+MOD, p = 0.033 in one-way ANOVA test)(Figure 2e).

Regarding the manifestation of an associated anxiety-related behaviour, freezing duration significantly decreased in the PAR-treated groups receiving MOD and TEL, as compared with healthy and PAR-treated groups (PAR+MOD *versus* CTR, p = 0.043; PAR+MOD *versus* CTR+MOD, p = 0.041; PAR+TEL *versus* CTR, p = 0.035 in one-way ANOVA test) (Figure 2 f). Moreover, a tendency toward decreased freezing duration was observed following MOD and TEL treatment in PAR-treated zebrafish (PAR+MOD *versus* PAR, p = 0.07; PAR+TEL *versus* PAR, p = 0.07 in one-way ANOVA test)(Figure 2 f).

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We observed that acute PAR treatment may indicated a trend towards a reduction in anxiety-like behavior, as suggested by the tendency of increased time spent swimming in the central area and significantly decreased thigmotaxis, as compared to CTR group (PAR *versus* CTR, $p = 0.07$; PAR *versus* CTR, $p = 0.05$ in one-way ANOVA test) (Figure 3 a- c). Similarly, increased time spent swimming in the top area and significantly decreased thigmotaxis were observed in the PAR + MOD group, as compared to CTR group (PAR+MOD *versus* CTR+MOD, $p = 0.007$; PAR+MOD versus CTR, $p = 0.041$ in one-way ANOVA test) (Figure 3 a, c).

Regarding the vertical crossings, we observed that MOD treatment in healthy zebrafish could reveal an anxiogenic-like tendency, as compared to CTR group (CTR+MOD *versus* CTR, $p = 0.07$ in one-way ANOVA test), while MOD treatment in PAR-treated fish could suggest a possible anxiolytic-like behaviour, as compared to CTR+MOD group (PAR+MOD *versus* CTR+MOD, $p = 0.029$ in one-way ANOVA test) (Figure 3 d). Opposite effects were observed after TEL treatment, as treated healthy individuals showed a significantly increased crossing frequency, as compared to CTR+MOD (CTR+TEL *versus* CTR+MOD, $p = 0.048$ in one-way ANOVA test)(Figure 3 d). While an anxiogenic-like effect was suggested by significantly decreased crossing frequency in the PAR+TEL group, as compared to model group and MOD-treated models (PAR+TEL *versus* PAR, $p = 0.035$; PAR+TEL *versus* PAR+MOD, $p = 0.04$ in one-way ANOVA test) (Figure 3 d). However, no significant changes were observed in the horizontal exploration behaviours (Figure 3 e).

The behavioural trends observed in social behaviour results could suggest locomotor and social-affective impairments. Increases of total swimming distance and average velocity were observed after MOD treatment in both healthy and PAR-treated fish, as compared to CTR groups (PAR+MOD *versus* CTR, $p = 0.041$; PAR+MOD *versus* PAR, $p = 0.028$ in one-way ANOVA test; CTR+MOD *versus* CTR, $p = 0.052$; PAR+MOD *versus* CTR, $p = 0.041$; PAR+MOD *versus* PAR, $p = 0.003$ in one-way ANOVA test for both behavioural parameters) (Figure 4a, b). Similarly, increases of total swimming distance and average velocity were observed in the PAR-treated fish (PAR+TEL *versus* CTR, $p = 0.037$; PAR+TEL *versus* PAR, $p = 0.086$ in one-way ANOVA test; PAR+TEL *versus* CTR, $p = 0.037$; PAR+TEL *versus* PAR, $p = 0.01$ in one-way ANOVA test for both behavioural parameter) (Figure 4a, b). In contrast, significant decreases in total swimming distance and average velocity were observed

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after TEL treatment healthy fish, as compared to the CTR+MOD group (CTR+TEL *versus* CTR+MOD, $p = 0.032$; CTR+TEL *versus* CTR+MOD, $p = 0.031$ in one-way ANOVA test for both behavioural parameter) (Figure 4a, b).

Moreover, decreased activity total time was observed in all treated groups, as compared to CTR group (CTR+MOD *versus* CTR, $p = 0.07$; PAR+MOD *versus* CTR, $p = 0.046$; PAR+TEL *versus* CTR, $p = 0.046$ in one-way ANOVA test), suggesting affected locomotion and exploratory behaviour (Figure 4 c). On the other hand, increased clockwise rotation was noted after MOD and TEL treatments in both healthy and PAR-treated zebrafish, as compared with CTR group (CTR+MOD *versus* CTR, $p = 0.016$; CTR+TEL *versus* CTR, $p = 0.045$; PAR+MOD *versus* CTR+MOD, $p = 0.0008$; PAR+TEL *versus* CTR+TEL, $p = 0.066$ in one-way ANOVA test), along with visible increased counter-clockwise rotation frequency in all treated group, suggesting alterations of locomotor performance (Figure 4 d, e).

Regarding the social behaviour assessments, we observed that the time spent near social stimuli significantly increased in the PAR group, as compared to the CTR group (PAR *versus* CTR, $p = 0.005$ in one-way ANOVA test), while the time spent in other maze arms decreased (central arm, PAR *versus* CTR, $p = 0.007$ in one-way ANOVA test) (Figure 5 a - d). Similarly, visible increased time spent near stimuli were observed after MOD administration in healthy individuals, along with a tendency towards increased time spent in decision point, as compared to CTR group (CTR+MOD *versus* CTR, $p = 0.06$ in one-way ANOVA test), possibly suggesting alteration in social-affective and decision-making behaviour (Figure 5 a-d). Moreover, while significantly decreased time spent near social stimuli and in the central arm were observed in the PAR+MOD group, as compared to CTR and PAR groups, significantly increased time spent in the right arm were recorded (time spent near stimulus, PAR+MOD *versus* CTR+MOD, $p = 0.02$ in one-way ANOVA test; right arm, PAR+MOD *versus* CTR, $p = 0.004$; PAR+MOD *versus* PAR, $p = 0.003$; PAR+MOD *versus* CTR+MOD, $p = 0.002$ in one-way ANOVA test; central arm, PAR+MOD *versus* CTR, $p = 0.003$ in one-way ANOVA test) (Figure 5 a- d). Similar changes were observed after TEL treatment in both healthy and PAR-treated zebrafish, as compared to CTR and PAR groups (time spent near social stimulus, CTR+TEL *versus* PAR, $p = 0.08$; CTR+TEL *versus* CTR+MOD, $p = 0.038$; PAR+TEL *versus* PAR, $p = 0.0033$; PAR+TEL *versus* PAR+MOD, $p = 0.043$ in one-way ANOVA test;

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right arm, PAR+TEL *versus* PAR+MOD, $p = 0.0079$ in one-way ANOVA test; central arm, PAR+TEL *versus* CTR, $p = 0.012$ in one-way ANOVA test) (Figure 5 a-d).

To investigate shifts in the intestinal microbiota following PAR and PAR+TEL treatments, 16S rRNA profiling was performed via Nanopore sequencing. The sequencing run generated a total of 24,790 reads, corresponding to an estimated yield of 52.38 Mb, with a median N50 read length of 1.73 kb that is consistent with near full-length 16S rRNA gene amplicons. Following basecalling and demultiplexing using the wf-16s pipeline, raw genus-level counts were mapped to their respective barcoded samples. Although a 24-barcode library was deployed, only barcodes 01–03 (CTR) and 19–24 (PAR and PAR+TEL) corresponded to the biological samples within the experimental design, representing three randomly selected individuals from each of the three groups. Genus-level read depths exhibited substantial variation across these barcodes, ranging from 100 to 6,600 reads. To mitigate potential bias introduced by low-yield sequencing, a strict inclusion threshold of ≥10 genus-level classified reads was instituted. Across all analysed samples, the proportion of unclassified or unmapped reads remained low (generally <2%), indicating high basecalling accuracy and efficient reference-based classification. Most reads were retained and successfully classified at least to the phylum level, confirming that the sequencing depth and overall data quality were adequate for the intestinal microbial community's characterization.

Taxonomic profiling at the phylum level revealed that the intestinal microbiota was overwhelmingly dominated by *Pseudomonadota*, which accounted for 83.00% of all classified reads across samples (Figure 6a). Minor components of the bacterial community included the phyla *Fusobacteriota* (1.87%) and *Bacteroidota* (0.94%), whereas *Bacillota* and *Deinococcota* were detected only at trace relative abundances (<0.05% each). Consistent with these phylum-level trends, the genus-level community structure was largely composed of taxa belonging to *Pseudomonadota*. *Aeromonas* was identified as the most abundant genus, comprising 52.15% of total reads, followed by *Acinetobacter* (13.87%) and *Plesiomonas* (8.41%). Additional genera present at lower relative abundances included *Roseateles* (2.93%), *Aquipseudomonas* (2.88%), *Cetobacterium* (1.86%), *Pseudomonas* (1.29%), *Flavobacterium* (0.93%), and *Shewanella* (0.85%). Reads that could not be

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confidently assigned to a specific genus accounted for 13.93% of the dataset. Collectively, these results indicate that the intestinal microbiota was characterized by a small number of highly dominant genera, with the remaining taxa contributing marginally to the overall community composition. Because of the exploratory design and variable sequencing depth, taxonomic patterns are presented as descriptive trends rather than definitive treatment-induced differences.

Alpha-diversity indices calculated at the genus level after rarefaction to 100 reads (corresponding to the lowest read depth among retained samples using 1,000 rarefaction iterations) revealed distinct descriptive trends in microbial richness, dominance, and evenness across the treatment groups (Table 1). Descriptively, a progressive increase in community complexity was observed from the control to the combination treatment. The PAR group showed higher mean richness (5.84) and Shannon diversity (1.05) than the CTR group (richness: 5.05, Shannon: 0.83), alongside a reduction in the Berger–Parker dominance index (0.63 vs. 0.68). This trend was most pronounced in the PAR+TEL group, which exhibited the highest community richness (6.60), Shannon diversity (1.34), and Pielou's evenness (0.71), coupled with the lowest Berger–Parker index (0.48) and the highest effective number of species (4.10). Taken together, these descriptive metrics suggest a more diverse and evenly distributed microbial community structure following the combined PAR+TEL treatment.

However, Kruskal–Wallis testing demonstrated that these observed differences were not statistically significant among the experimental groups for any alpha-diversity index after false discovery rate (FDR) correction: Berger–Parker index ($H = 1.6889$, $p = 0.4298$, FDR-adjusted $p = 0.4835$), effective number of species ($H = 3.2000$, $p = 0.2019$, FDR-adjusted $p = 0.4835$), inverse Simpson 1/D ($H = 1.8667$, $p = 0.3932$, FDR-adjusted $p = 0.4835$), Pielou's evenness ($H = 0.8000$, $p = 0.6703$, FDR-adjusted $p = 0.6703$), richness ($H = 1.6889$, $p = 0.4298$, FDR-adjusted $p = 0.4835$), Shannon diversity ($H = 2.2222$, $p = 0.3292$, FDR-adjusted $p = 0.4835$), Simpson ($H = 1.8667$, $p = 0.3932$, FDR-adjusted $p = 0.4835$).

Community clustering assessed by Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity revealed that the first two axes explained 65.6% of the total variance in microbial community composition (Figure 6b). The ordination space showed only partial separation according to experimental conditions, characterized by a substantial spatial overlap among the cohorts. Descriptively, the CTR and PAR

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groups exhibited a wider, more dispersed distribution, suggesting high intra-group beta-diversity and individual variability. In contrast, the combined PAR+TEL treatment group displayed a more localized and cohesive clustering pattern, hinting at a potentially more uniform microbial community configuration under combined treatment.

However, PERMANOVA confirmed that the observed group separation was not statistically significant (pseudo-F = 1.707, R2 = 0.3627, p = 0.1845 under 9,999 permutations). Mirroring the beta-diversity outcomes, differential abundance testing at the genus level using the Kruskal–Wallis test did not identify any taxa that remained statistically significant after Benjamini–Hochberg FDR correction. The lowest unadjusted p-value was observed for the genus *Aquipseudomonas* (p = 0.0459), which lost statistical significance upon multiplicity adjustment (FDR-adjusted p = 0.1679).

Consequently, the statistical evidence from this dataset does not support confirmed treatment-induced shifts in alpha diversity, beta diversity, or specific genus-level relative abundances. Overall, these patterns should be treated as exploratory descriptive trends. Given the substantial intra-group variation, validation in larger cohorts with higher and more balanced sequencing depths remains necessary to definitively evaluate the impact of PAR and TEL administration on the intestinal microbiota structure.

DISCUSSION

The present study aimed to evaluate and describe the potential of two novel products based on plant extracts to modulate behavioural changes and gut dysbiosis induced by acute exposure to PAR in adult zebrafish. In the current experimental design, adult zebrafish were acutely exposed to 5 mM PAR solution for 24 hours, according to previous studies that acknowledged the hepatotoxic effects of acute PAR treatment [4-7]. We observed hypolocomotor tendencies and impaired exploratory behaviour (*e.g.* visible decreased total swimming distance and the average swimming velocity, counter and counterclockwise rotation frequencies). Yet we observed that PAR treatment could indicate a trend towards reduction in anxiety-like behavior (*e.g.* decreased freezing behaviour duration, significantly increased time in the central area, significantly decreased thigmotaxis, and no changes in time spent in the top area) and increased vertical crossing and decreased horizontal crossing. PAR treatment showed

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significantly increased time spent near the social stimuli, while the time spent in other maze arms decreased. Moreover, the visible increased time spent in the decision point alongside with increased counterclockwise frequency and no changes in clockwise rotation frequency could suggest possible alterations of locomotor and decision-making associated behaviours.

Previous zebrafish study reported reduced locomotor behaviour (*e.g.* reduced total distance moved, swimming speed and maximum acceleration) after 96 h exposure to 2mM and 8mM PAR of zebrafish [60]. Moreover, anxiolytic-like behavior and alleviation of stress-evoked anxiety (*e.g.* increased time on top), without effect on locomotor activity (*e.g.* distance traveled, freezing episodes, time in freezing, and entries in top of tank) were also showed after acute exposure to 150 mg/L PAR for 1 hour [61]. Previous studies have highlighted that PAR, either acutely and sub-chronically administered, could significantly change time spent near the social stimuli through its analgesic effect and reduce physical pain, empathy for pain and psychological reactivity as rodent studies reported [62-64].

Regarding the potential of plant-based formulations and traditional medicinal practices, several studies have described the antioxidant, anti-inflammatory, and hepatoprotective properties of plant extract-based products [65-68]. These diverse and often complementary bioactivities suggest that plant-derived molecules may act as multi-target agents within interconnected biological networks, simultaneously influencing pathways related to cell protection, metabolism, inflammation, and immune escape, rather than through a single isolated mechanism [69, 70]. Beyond these effects, bioactive metabolites, such as phenolic compounds and flavonoids derived from plant extracts have been shown to modulate the central nervous system and behavioural responses [71]. In this way, several studies involving plant extracts constituents of the current products, have reported effects on modeling behavioural outcomes. Improvements in socio-affective behaviours, cognitive and motor performance, learning and memory performance were reported following administration and supplementation with *Rubus idaeus, Vaccinium myrtillus, Vaccinium vitis-idaea, Cichorium intybus, Astragalus* membranaceus administration, *Silybum marianum* and *Centella asiatica* (Table 1) [26-41]. Reductions in anxiety- and depression-like behaviour were also observed, including in models of post-traumatic stress disorder and chronic unpredictable mild stress [27,33,34]. Moreover, improvements in behaviours associated with sleep disturbances, acute and chronic

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stress, and also fear were reported after administration of *Cichorium intybus* (Table 2)[33,34].

Furthermore, Paduraru et al. [54] reported that a compositionally similar product did not have significant changes in social behaviour or locomotor activity (*e.g.* total distance travelled, mean speed, immobility time) in zebrafish, with the exception of increased inactivity time. In the present study, it was observed that MOD treatment did not show major changes of locomotor and exploratory behaviours in both healthy and PAR-exposed zebrafish. However, different outcomes regarding the socio-affected behaviours were noticed. The behavioural observations following MOD treatment in PAR-exposed zebrafish could be associated with decreased anxiety-like behaviour (e.g. significantly decreased freezing behaviour and thigmotaxis, and crossing frequency significantly increased). In contrast, the MOD treatment in healthy zebrafish could reveal a trend towards increased anxiety-like behavior (*e.g.* crossing frequency significantly decreased and decreased time spent in the top area). Regarding the social behaviour assessments, we observed that MOD administration in healthy individuals tends to change time spent near the social stimuli (*e.g.* increased time spent near stimuli). However, the presence of social stimuli could also appear to influence locomotor behaviour and possible anxiety-like states by increasing the general locomotory behavioural parameters (*e.g.* total swimming distance and average velocity, activity total time). In addition, the increased time spent in the decision point and clockwise rotation frequency could also suggest impairments of locomotor behaviour and decision-making processes. Similar locomotor behaviour outcomes were also observed after MOD administration to PAR- exposed zebrafish, along with reduced social behaviour and a possible increased anxiety-like states (*e.g.* significantly increased time spent in right time).

On the other hand, the TEL treatment in healthy zebrafish could lead to hypolocomotor behavior tendencies and a possible affected exploratory behavior (*e.g.* decreased the total swimming distance and the average swimming velocity, decreased immobility total time, decreased clockwise and counterclockwise rotation frequencies). Moreover, the social behaviour assignment results showed that TEL administration in healthy individuals could be associated to impaired locomotor behaviour (*e.g.* decreased total swimming distance, average velocity and activity time, significantly increased clockwise rotation and increased counter-clockwise rotation) and, no significant changes in the time spent near social stimuli. Moreover, TEL

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treatment in PAR-treated zebrafish may be related to impaired locomotor behaviour (*e.g.* significantly increased total swimming distance and average swimming velocity, significantly decreased activity time), possible impairments in decision-making processes (*e.g*. increased counter-clockwise rotation and time spent in the decision point), and anxiety-like states (*e.g.* significantly decreased freezing behaviour, significantly decreased bottom-top crossing frequency). No significant changes in social behaviour were observed. Nevertheless, these modifications of behavioural parameters (evaluated through locomotor and social-affected behaviour assessments) could also be attributed to altered locomotor capacity, reduced fear response, general behavioural disinhibition, changes in motivation, or stress-related activity, rather than specifically reflecting social or anxiety-like behaviours. Notably, correlations between whole-body cortisol levels and behavioural changes following exposure to various toxins have also been reported, suggesting a stress-related activity response [72].

Regarding the overall intestinal microbiota status, previous studies described the correlation between PAR treatments and intestinal dysbiosis [10,11]. Moreover, due to intercommunication between liver and gut, intestinal dysbiosis could increase the permeability of intestinal barriers and facilitate the bacterial translocation to liver, which is strongly connected to multiple hepatic disorders [9]. In a rodent model, PAR-treatment was associated with altered intestinal barrier integrity, gut microbiota composition and decrease of the hepatic microbiota diversity [7,12]. In a previous study conducted by Li et al., [9] *Galla chinensis* alleviated reported that PAR-induced acute liver injury could disturb intestinal flora and increase the population of pathogens, such as *Bacteroides* [9]. While, a similar study reported that decreased abundance of *Firmicutes psyllium* after PAR treatment could be a significant indicator of hepatotoxicity development [7]. Similarly, *Fusobacteriota* species presence indicated intestinal mucosal barrier function, liver inflammation, and acute liver failure [13]. Moreover, a correlation between PAR hepatotoxicity, oxidative stress excessively, and *Deinococcota* phylum presence was previously established [14], yet their association with gut microbiota dysbiosis were not described, except for their resistance to oxidative stress [73].

Overall, the present study finding regarding the intestinal microbiota of *Danio rerio* characterized under multiple experimental conditions using full-length 16S rRNA gene sequencing and Nanopore-based analysis indicate that the

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applied experimental treatments could be associated with subtle variations in the internal structure and diversity of the intestinal microbiota, without inducing shifts in overall community composition. Nanopore sequencing enabled reliable taxonomic profiling, revealing a microbiota dominated by a limited number of prevalent taxa, primarily within the phylum *Pseudomonadota*. While taxonomic composition at higher ranks remained largely conserved across groups, alpha diversity metrics indicated differences in richness, dominance, and evenness. In contrast, community clustering analysis suggests substantial overlap among experimental groups with no clear evidence of substantial shifts in overall community composition. Notably, the PAR+TEL group displayed a more cohesive clustering pattern compared to the CTR and PAR groups, which exhibited wider, more dispersed distributions, suggesting higher intra-group variability. Collectively, these findings suggest subtle variations in microbial diversity and dominance patterns, while the overall intestinal microbiota structure remains relatively stable in *Danio rerio.* Consequently, the statistical evidence does not support confirmed treatment-induced shifts in alpha diversity, beta diversity, or specific genus-level relative abundances. Overall, these patterns should be treated as exploratory descriptive trends. Given the substantial intra-group variation, validation in larger cohorts with higher and more balanced sequencing depths remains necessary to definitively evaluate the impact of PAR and TEL administration on the intestinal microbiota structure.

Several limitations of the present study need to be acknowledged. First, the small sample size (n = 10/ group for behavioural testing and n = 3/group initially selected for exploratory microbiota profiling) could limit statistical power and detection sensitivity. Additionally, the relatively short experimental duration, 24 h of PAR exposure followed by 5 days of the plant-based formulations (MOD, TEL) could have constrained the modulation of the targeted behaviors and gut microbiota, as indicated by literature. Therefore, the results should be interpreted with caution within the time frame of the present study, and long-term investigations are needed to confirm the persistence of the observed effects.

Furthermore, the biological interpretation of the observed effects should remain cautious, as no mechanistic markers, such as those related to oxidative stress, inflammation, liver injury, gut barrier integrity, or neurotransmitter changes, were assessed. Therefore, the biological mechanisms underlying the observed effects should be explored in future studies.

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Additionally, only one dose of each formulation was evaluated according to the selected literature; therefore, dose-response relationships and optimal therapeutic dose could not be determined. Future studies investigating multiple dose levels are needed to further characterize the efficacy and safety of the tested formulations. Moreover, MOD and TEL are multi-component botanical formulations. Therefore, the observed effects cannot be attributed to any individual plant species or bioactive compound, and potential synergistic interactions (e.g. possible a network basis) among the ingredients cannot be excluded. The complex composition of the tested formulations hinders the identification of specific compound-target interactions. Therefore, future studies need to investigate individual components that are warranted to identify the compounds responsible for the observed effects and integrating network-based approaches could help elucidate the coordinated molecular pathways underlying the observed effects.

Furthermore, genetic analyses could only be performed for three of the six experimental groups due to previous analyses that reveal non-significant differences in gut microbiota diversity following treatment with one of the interesting natural products (MOD) thus redundant analyses were avoided. Moreover, due to a similar trend with other natural products evaluated, analyses on gut microbiota diversity in healthy individuals following this treatment were also avoided. These results should therefore be considered preliminary, and future studies should aim to validate findings across the full set of experimental groups.

In addition, in the social preference assay, the social stimulus was placed in the same arm (left) for all fish, without counterbalancing its position across trials, and no control group without a stimulus was included to assess potential baseline side bias. Therefore, an innate lateral preference cannot be fully excluded as a contributing factor to the observed differences in time spent near the stimulus, and this should be considered when interpreting the social preference results. Future studies should incorporate counterbalanced stimulus placement and appropriate no-stimulus controls to control for this potential confound.

Finally, each treatment group was maintained in a single experimental tank, without replicate tanks, therefore, potential tank-related effects cannot be fully disentangled from treatment effects, and the independence of individual fish as experimental units may have been reduced due to their shared housing environment.

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CONCLUSION

Previous preclinical studies have reported behavioral effects of plant extract products, including improvements in affective, cognitive, and motor outcomes in various experimental models through bioactive phenolic compounds and flavonoids derived from these extracts. Thus, this evidence could contribute to their possible role as adjuvant supplements. In the present study, the preliminary behavioural observations suggest that both plant-based formulations could be associated with modulatory socio-affective behaviors and may exert subtle, descriptive trends on the internal structure and diversity of the intestinal microbiota without inducing consistent shifts in overall community composition in the PAR-treated zebrafish group. Therefore, these preliminary findings should be considered exploratory, interpreted with caution, and require validation in larger, longer-term, and mechanistic studies.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Summary of alpha diversity indices across experimental groups

Diversity Index	Berger Parker index	Effective number of species	Inverse Simpson's index	Pielou's evenness	Richness	Shannon diversity index	Simpson's index
CTR	0.68	2.34	1.91	0.56	5.05	0.83	0.44
PAR	0.63	2.93	2.31	0.64	5.84	1.05	0.52
PAR+TEL	0.48	4.10	3.42	0.71	6.60	1.34	0.64

Values represent group means calculated from rarefied genus-level data after applying a minimum read-depth threshold (>=10 reads). **Abbreviations**: CTR, untreated control group; PAR, paracetamol; PAR+TEL, paracetamol exposure followed by TEL treatment; TEL, code for the second plant extract-based formulation.

Table 2. Behavioral effects of plant extracts in current formulations

Plant sp.	Experimental model	Behavioural outcomes	References
Rubus idaeus	aged rat model	cognitive and motor performance, specifically balance and psychomotor coordination deficits	[26]
Vaccinium myrtillus	-	reduced anxiety levels associated to post-traumatic stress disorder; improved depression and anxiety symptoms	[27]
Vaccinium myrtillus	kainate-treated rat model; rat model of post-traumatic stress disorder	attenuated impaired learning performance; decreased anxiety.	[28,29]
Vaccinium vitis-idaea	adult zebrafish model	not affected locomotor behaviour (e.g. total swimming distance and total activity time behavioural parameters).	[30]

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chicoric acid extracted from *Cichorium intybus*	Parkinson's disease zebrafish model	improved motor function	[31,32]
oligopolysaccharide from *Cichorium intybus*	chronic sleep deprivation-stressed mice model; chronic sleep deprivation-stressed mice model; acute despair and chronic unpredictable mild stress mouse model	reduced anxiety-like behaviours associated with sleep disturbances and fear, enhanced exploratory behaviour in chronic sleep deprivation-stressed mice; depression-like behaviours	[33,34]
Astragalus membranaceus	restraint stress rats model	improved spatial learning, memory and reduced stress-induced anxiety	[35]
Astragalus membranaceus	Alzheimer mice model	improved working, fear avoidance and spatial memory	[36]
Silybum marianum	adult zebrafish model	mitigate acute stress-induced anxiety (e.g. spending more time in the upper level of the tank)	[37]
silibinin from *Silybum marianum*	zebrafish model treated with Bisphenol A	reversed scototaxis behaviour	[38]
Centella asiatica	larvae zebrafish model adult zebrafish mode	alleviates neurotoxicity and improves locomotor activity;	

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		memory deficits; antidepressant activity; freezing duration.	[39-41]

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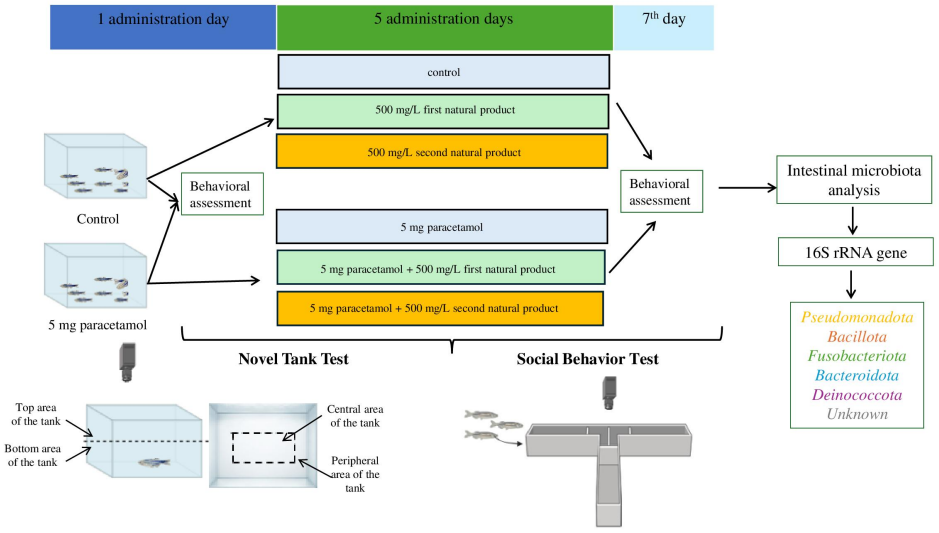


Figure 1. Schematic representation of experimental design

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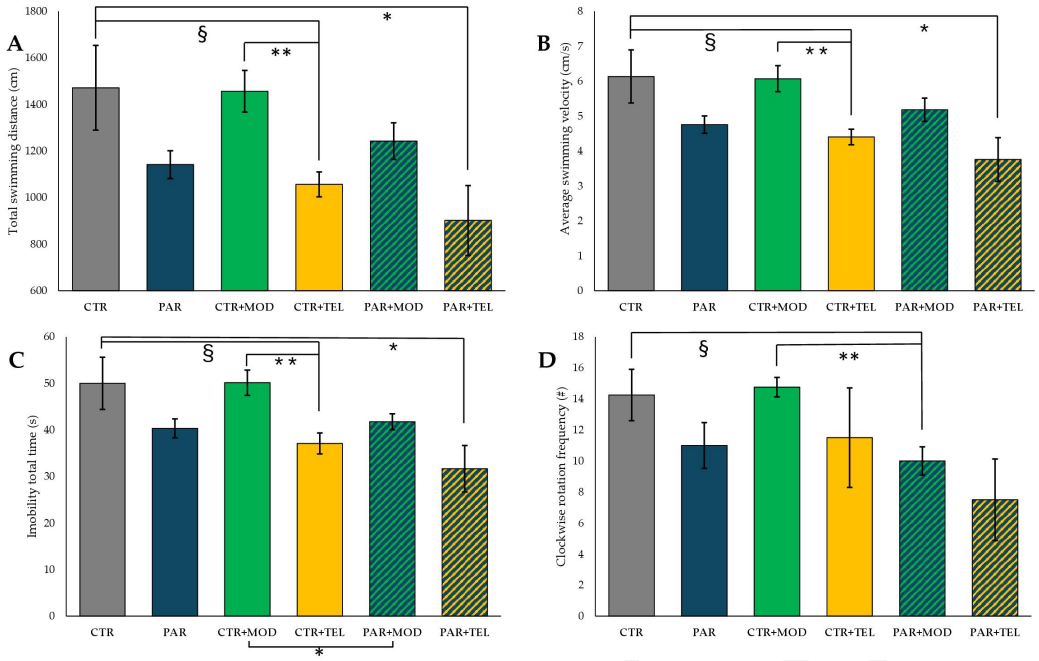


Figure 2. Locomotor activity and freezing behaviour in adult zebrafish assessed using the NTT. (A) Total swimming distance (cm); **(B)** average swimming velocity (cm/s); **(C)** total immobility time (s); **(D)** clockwise rotation frequency (number of rotations); **(E)** counterclockwise rotation frequency (number of rotations); **(F)** total freezing duration (s). Behaviour was recorded for 4 min following a 30-s acclimation period. PAR exposure was performed at 5 mM for 24 h, and MOD or TEL was administered at 500 mg/L for 5 days. In the PAR+MOD and PAR+TEL groups, formulation treatment began after PAR exposure had ended. The CTR+MOD and CTR+TEL groups received the respective formulation without prior PAR exposure. CTR fish remained untreated, whereas PAR-only fish received no further treatment after PAR exposure. Data are presented as mean ± SEM (n = 10 fish per group). Between-group differences were analysed using one-way ANOVA followed by Tukey's HSD test. Brackets indicate the groups compared: * $p < 0.05$; ** $p < 0.01$; §, non-significant comparisons highlighted in the figure. **Abbreviations**: ANOVA, analysis of variance; CTR, untreated control group; CTR+MOD, MOD-treated group without prior PAR exposure; CTR+TEL, TEL-treated group without prior PAR exposure; HSD, honestly significant difference; MOD, code for the first plant extract-based formulation; NTT, novel tank test; PAR, paracetamol; PAR+MOD, PAR exposure followed by MOD treatment; PAR+TEL, PAR exposure followed by TEL treatment; SEM, standard error of the mean; TEL, code for the second plant extract-based formulation.

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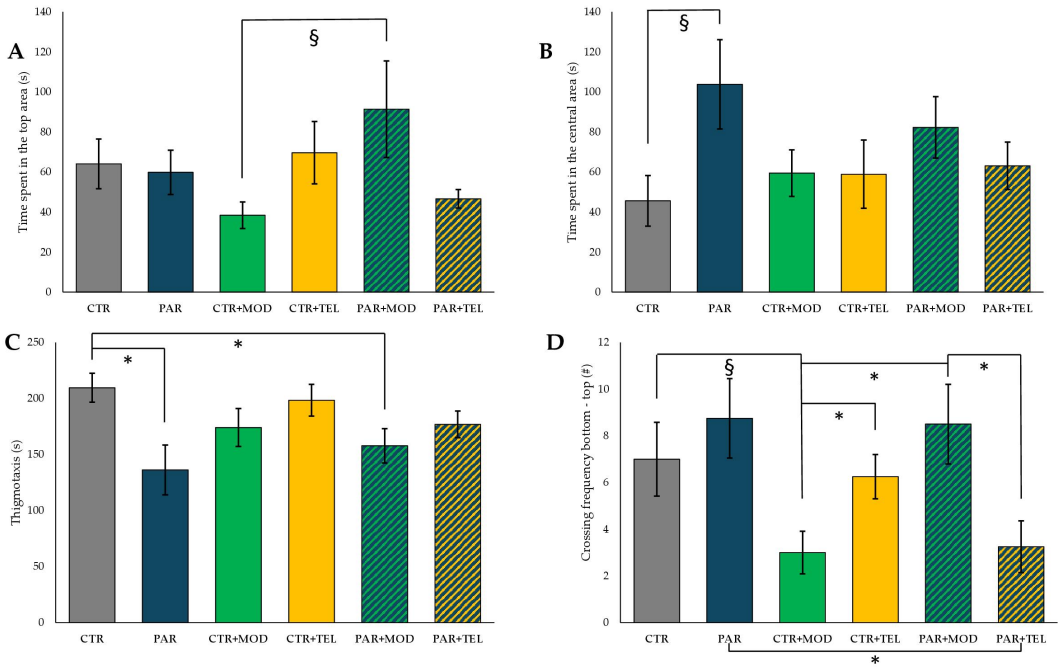


Figure 3. Exploratory and anxiety-related behavioural parameters in adult zebrafish assessed using the NTT. (A) Time spent in the top area (s); **(B)** time spent in the central area (s); **(C)** thigmotaxis duration (s); **(D)** vertical crossing frequency between the bottom and top areas (number of crossings); **(E)** horizontal crossing frequency between the central and peripheral areas (number of crossings). Behaviour was recorded for 4 min following a 30-s acclimation period. PAR exposure (5 mM for 24 h) was followed by MOD or TEL treatment (500 mg/L for 5 days) in the PAR+MOD and PAR+TEL groups, respectively. The CTR+MOD and CTR+TEL groups received the respective formulation without prior PAR exposure. CTR fish remained untreated, whereas PAR-only fish received no further treatment after PAR exposure. Data are presented as mean ± SEM (n = 10 fish per group). Between-group differences were analysed using one-way ANOVA followed by Tukey's HSD test. Brackets indicate the groups compared; * $p < 0.05$; §, non-significant comparisons highlighted in the figure. **Abbreviations**: ANOVA, analysis of variance; CTR, untreated control group; CTR+MOD, MOD-treated group without prior PAR exposure; CTR+TEL, TEL-treated group without prior PAR exposure; HSD, honestly significant difference; MOD, code for the first plant extract-based formulation; NTT, novel tank test; PAR, paracetamol; PAR+MOD, PAR exposure followed by MOD

treatment; PAR+TEL, PAR exposure followed by TEL treatment; SEM, standard error of the mean; TEL, code for the second plant extract-based formulation.

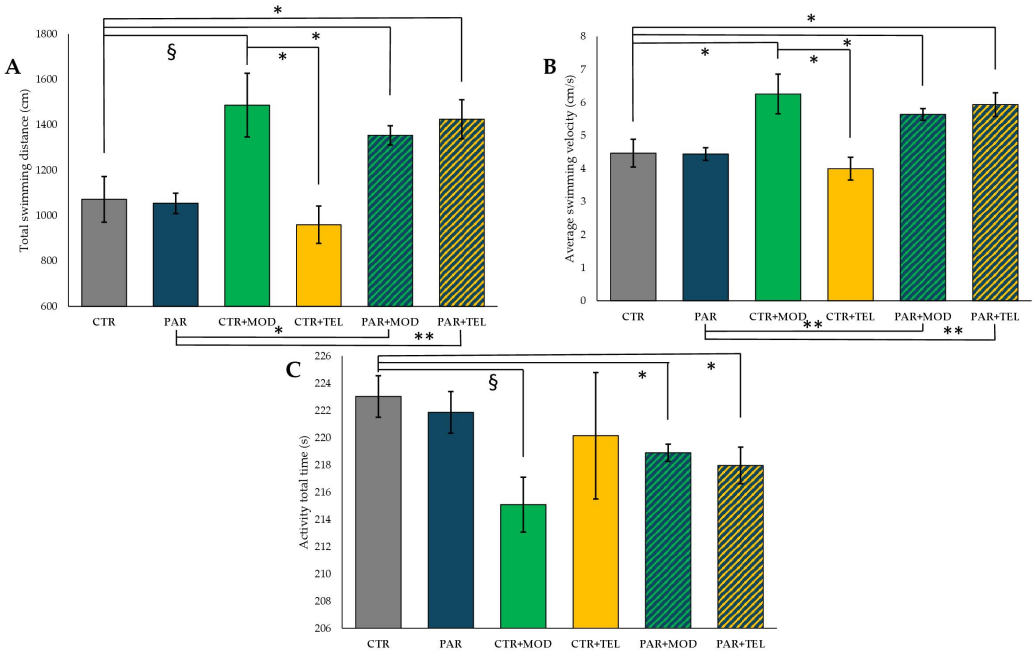


Figure 4. Locomotor activity in adult zebrafish assessed during the social behaviour test. (A) Total swimming distance (cm); **(B)** average swimming velocity (cm/s); **(C)** total activity time (s); **(D)** clockwise rotation frequency (number of rotations); **(E)** counterclockwise rotation frequency (number of rotations). Behaviour was recorded for 4 min following a 30-s acclimation period in a T-maze, with three conspecifics positioned behind a plexiglass partition at the end of the left arm. PAR exposure (5 mM for 24 h) was followed by MOD or TEL treatment (500 mg/L for 5 days) in the PAR+MOD and PAR+TEL groups, respectively. The CTR+MOD and CTR+TEL groups received the respective formulation without prior PAR exposure. CTR fish remained untreated, whereas PAR-only fish received no further treatment after PAR exposure. Data are presented as mean ± SEM (n = 10 fish per group). Between-group differences were analysed using one-way ANOVA followed by Tukey's HSD test. Brackets indicate the groups compared: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; §, selected non-significant comparisons highlighted in the figure. **Abbreviations**: ANOVA, analysis of variance; CTR, untreated control group; CTR+MOD, MOD-treated group without prior PAR exposure; CTR+TEL, TEL-treated group without prior PAR exposure; HSD, honestly significant difference; MOD, code for the first plant extract-based formulation; PAR, paracetamol;

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PAR+MOD, PAR exposure followed by MOD treatment; PAR+TEL, PAR exposure followed by TEL treatment; SEM, standard error of the mean; TEL, code for the second plant extract-based formulation.

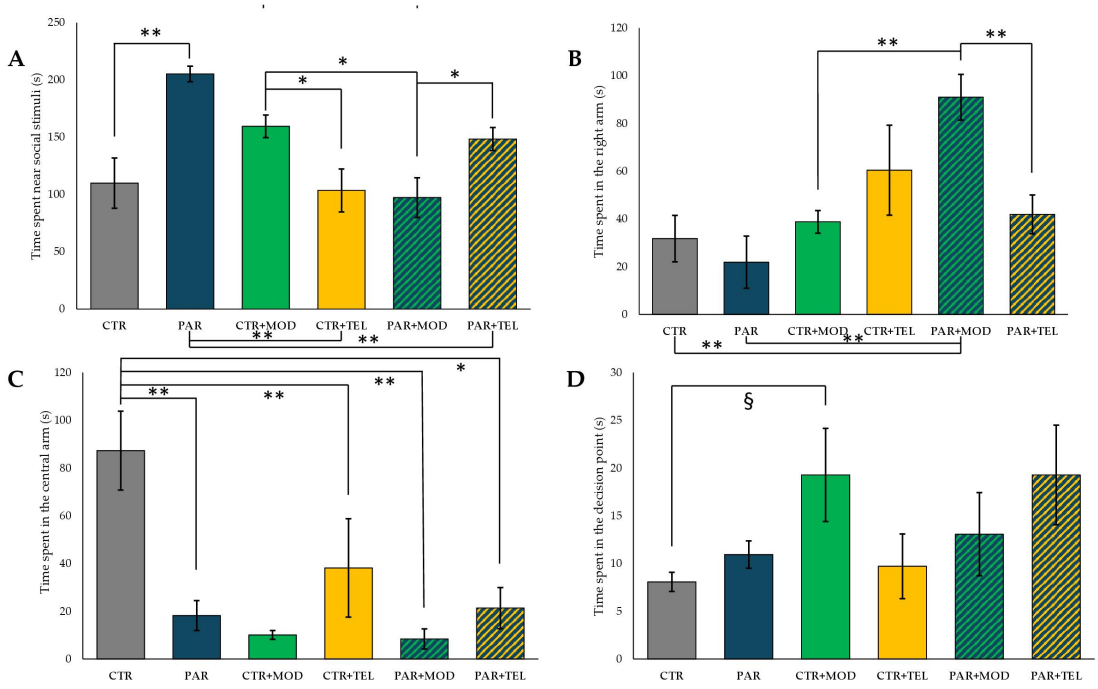


Figure 5. Social preference-related behavioural parameters in adult zebrafish assessed using the social behaviour test. (A) Time spent near the social stimuli (s); **(B)** time spent in the right arm (s); **(C)** time spent in the central arm (s); **(D)** time spent in the decision point (s). Behaviour was recorded for 4 min following a 30-s acclimation period in a T-maze, with three conspecifics positioned behind a plexiglass barrier at the end of the left arm. PAR exposure (5 mM for 24 h) was followed by MOD or TEL treatment (500 mg/L for 5 days) in the PAR+MOD and PAR+TEL groups, respectively. The CTR+MOD and CTR+TEL groups received the respective formulation without prior PAR exposure. CTR fish remained untreated, whereas the PAR group received no additional treatment after PAR exposure. Data are presented as mean ± SEM (n = 10 fish per group). Between-group differences were analysed using one-way analysis of variance followed by Tukey's honestly significant difference test. Brackets indicate the groups compared: * $p < 0.05$; ** $p < 0.01$; §, non-significant comparison highlighted in the figure. **Abbreviations**: CTR, untreated control group; CTR+MOD, MOD-treated group without prior PAR exposure;

CTR+TEL, TEL-treated group without prior PAR exposure; MOD, code for the first plant extract-based formulation; PAR, paracetamol; PAR+MOD, PAR exposure followed by MOD treatment; PAR+TEL, PAR exposure followed by TEL treatment; SEM, standard error of the mean; TEL, code for the second plant extract-based formulation.

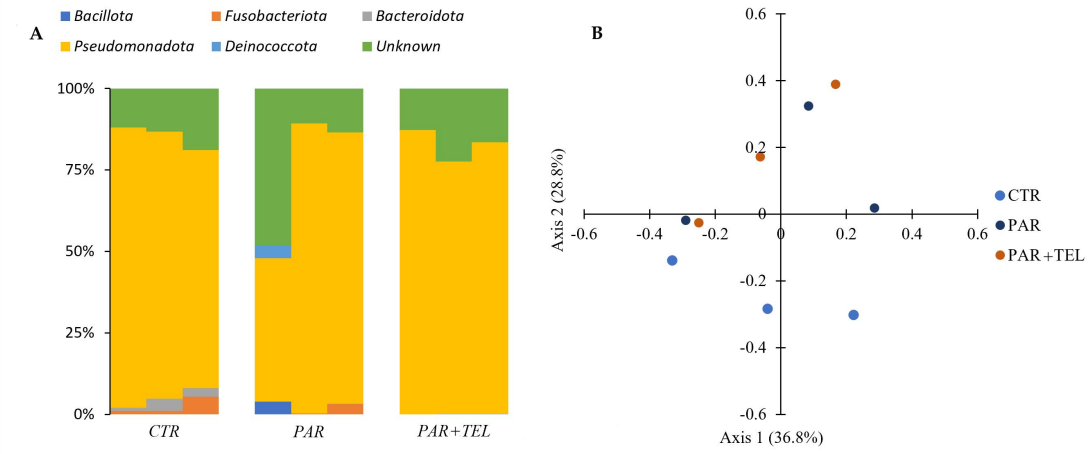


Figure 6. Intestinal microbiota composition and beta-diversity patterns in adult zebrafish. (A) Phylum-level relative abundance of the intestinal microbiota in the CTR, PAR, and PAR+TEL groups. Stacked bars show the relative proportions (%) of the detected bacterial phyla and unclassified taxa in each experimental group. **(B)** PCoA plot of genus-level microbiota composition based on Bray–Curtis dissimilarity. Each point represents one individual intestinal sample, coloured according to the corresponding experimental group (n = 3 per group). Axis 1 and Axis 2 explain 36.8% and 28.8% of the total variance, respectively. In line with the exploratory analysis, the plot indicates substantial overlap among groups, without clear separation of community structure. **Abbreviations**: CTR, untreated control group; PAR, paracetamol; PAR+TEL, PAR exposure followed by TEL treatment; PCoA, Principal Coordinates Analysis; TEL, code for the second plant extract-based formulation.