The selected concentration range was informed by preliminary range finding observations, which identified the lower threshold for observable behavioural response and the upper threshold associated with toxicity, and this range is consistent with dose selection strategies used in studies of plant derived anesthetics in fish (Ventura et al., 2020; Hoseini et al., 2022).

Fish were fasted for 24 h prior to exposure to reduce metabolic variability and minimise the influence of feeding related physiological processes on anesthetic response (Martos Sitcha et al., 2020; Dawood et al., 2022). During exposure, aeration was suspended to facilitate uptake of anesthetic compounds across the gill surface, a procedure that has been shown to enhance immersion anesthesia efficiency (Brønstad, 2022).

Anesthetic induction was assessed using behavioural criteria including reduced responsiveness, loss of equilibrium, and complete immobility. Following exposure, fish were transferred to clean aerated water, and recovery time was recorded as the time required to regain normal swimming behaviour. Mortality was assessed 24 h after exposure to determine safety margins (Neiffer, 2021; Soldatov, 2021).

The level of replication employed is consistent with established experimental designs in fish anesthesia research, where the tank is treated as the experimental unit because fish within a tank experience identical exposure conditions and are not statistically independent (Neiffer, 2021; Vergneau Grosset and Benedetti, 2022).

2.5 Statistical analysis and welfare assessment

Induction and recovery time data were analysed separately for each fish species. One way analysis of variance was applied within each extract type to evaluate the effect of concentration on induction and recovery time. Only treatments in which recovery occurred were included in the inferential analysis, as the inclusion of treatments with complete mortality can violate the assumptions of normality and homogeneity of variance and may lead to biased statistical outcomes (Neiffer, 2021; Vergneau Grosset and Benedetti, 2022).

Prior to inferential analysis, all datasets were assessed for compliance with parametric assumptions. Normality of data distribution was evaluated using the Shapiro-Wilk test, while homogeneity of variance was examined using Levene's test. These procedures confirmed that the data satisfied the assumptions required for parametric analysis, thereby justifying the application of one way analysis of variance. The use of these diagnostic tests is consistent with established statistical practice in experimental aquaculture research, where verification of distributional properties and variance structure is essential for ensuring the validity of statistical inference (Rodrigues Brandão et al., 2022; Minaz et al., 2025). Where significant differences were detected, mean values were separated using Tukey multiple comparison test, and statistical significance was accepted at p less than 0.05. All statistical analyses were performed using IBM SPSS Statistics software version 26.0.

Although the experimental design incorporated multiple extract types and concentration levels, a factorial analysis of variance was not applied due to the occurrence of complete mortality in some treatment combinations. This resulted in an unbalanced dataset and violated the assumptions required for two way analysis of variance. Consequently, statistical analysis was restricted to biologically recoverable treatments, and one way analysis of variance was applied within these subsets to ensure valid estimation of treatment effects and to avoid distortion of variance structure, in line with recommended analytical approaches in fish anesthesia studies.

Post exposure welfare was assessed through systematic observation of behavioural recovery after transfer to clean water. Observations were conducted at predetermined intervals during the recovery period, with frequent assessments within the first thirty minutes, followed by additional evaluations at one hour and twenty four hours, in order to capture both immediate and delayed behavioural responses. Behavioural criteria included opercular movement, swimming stability, restoration of equilibrium, and time to resumption of feeding. These indicators are widely recognised as reliable measures of post anaesthetic recovery and physiological status in fish (Martos Sitcha et al., 2020; Vergneau Grosset and Benedetti, 2022). Behavioural responses were documented using standardised descriptive criteria to ensure comparability across treatments (Neiffer, 2021).