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Phytochemical Characterization and Anaesthetic Efficacy of Citrus Leaf Extracts for Sedation and Handling of Nile tilapia (*Oreochromis niloticus*) and African Catfish (*Clarias gariepinus*)

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Abstract This study investigated the anesthetic efficacy and safety of aqueous leaf extracts of *Citrus sinensis*, *Citrus aurantium*, and *Citrus limon* in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) under controlled immersion conditions. Qualitative phytochemical screening revealed distinct variation in bioactive constituents among the extracts. Experimental exposure was conducted at concentrations ranging from 1 000 to 4 000 mg L^{-1}, and responses were evaluated using induction time, recovery time, survival, behavioural indicators, and flesh quality parameters. Anesthetic effects were concentration dependent in both species. *Citrus sinensis* produced mild to moderate sedation across all tested concentrations, with no mortality recorded even at 4 000 mg L^{-1}, indicating a wide safety margin, and 3 000 mg L^{-1} was identified as the highest effective concentration for routine handling. In contrast, *Citrus aurantium* and *Citrus limon* induced deeper anesthetic states at lower concentrations but resulted in 100 percent mortality at 4000 mg L^{-1} in both species. Fish exposed to *Citrus sinensis* exhibited more favourable post exposure welfare indicators, including faster recovery and earlier resumption of feeding, whereas the other extracts were associated with delayed recovery and behavioural impairment. These findings indicate that *Citrus sinensis* appears more compatible with short term handling welfare and represents a practical and cost effective botanical anesthetic for freshwater aquaculture.

Keywords Fish anesthesia; Citrus leaf extracts; *Clarias gariepinus*; *Oreochromis niloticus*; Handling stress; Aquaculture welfare

1 Introduction

Aquaculture has become an essential component of global food systems, contributing significantly to animal protein supply and economic development, particularly in developing regions. Species such as Clarias gariepinus and *Oreochromis niloticus* are widely cultivated due to their adaptability, rapid growth, and high market acceptance (Klimuk et al., 2024; Webster and Lim, 2024). However, routine aquaculture practices such as handling, grading, transport, and sampling expose fish to stress, which can negatively affect physiological stability, immune function, and overall productivity (Martos Sitcha et al., 2020; Dawood et al., 2022). The use of anesthetic agents is therefore essential to reduce stress, improve handling efficiency, and enhance fish welfare during aquaculture operations (Neiffer, 2021; Brønstad, 2022).

Conventional fish anesthetics, including synthetic compounds, have been widely used due to their effectiveness in inducing rapid sedation and recovery. However, concerns have been raised regarding their cost, regulatory restrictions, potential toxicity, and residue accumulation in fish tissues (Vergneau Grosset and Benedetti, 2022; Sedyaaw and Bhatkar, 2024). These limitations have prompted increasing interest in the development of alternative anesthetic agents derived from natural sources. In particular, plant based anesthetics have gained attention due to their accessibility, lower environmental impact, and perceived safety in aquaculture systems (Yaşar and Yardımcı, 2022; Haihambo et al., 2023).

Recent studies have demonstrated that plant derived compounds, especially those obtained from essential oils, can effectively induce anesthesia in fish. For example, eugenol based extracts and other bioactive plant oils have been shown to produce rapid induction and acceptable recovery profiles in several aquaculture species (Ventura et al., 2020; Zahran et al., 2021). Similarly, essential oil extracts such as chamomile oil and citronellal have been reported to exhibit anesthetic efficacy in fish, influencing behavioural and physiological responses during exposure (Ak et al., 2022; Hoseini et al., 2022). Reviews have further highlighted the growing application of essential oils as sedatives and anesthetics in aquaculture, with evidence supporting their role in improving fish handling and reducing stress (Rodrigues Brandão et al., 2022; Minaz et al., 2025). These findings indicate that plant based anesthetics represent a viable alternative to conventional synthetic agents.

Despite these advances, several limitations remain in current knowledge. Most studies have focused on a limited number of plant species, particularly those rich in essential oils, while comparatively less attention has been given to aqueous leaf extracts from widely available tropical plants. In addition, there is limited comparative research evaluating multiple plant species under similar experimental conditions, especially with respect to induction time, recovery dynamics, survival outcomes, and post exposure welfare indicators (Haihambo et al., 2023; Mphande et al., 2023). Furthermore, the relationship between phytochemical composition and anesthetic performance is not consistently established, as many studies do not integrate chemical profiling with functional assessment of anesthetic effects.

Citrus species represent a promising but underexplored source of bioactive compounds with potential anesthetic properties. Citrus leaves and by products are known to contain a wide range of phytochemicals, including flavonoids, limonoids, terpenoids, carotenoids, and phenolic compounds, many of which exhibit biological activity (Addi et al., 2021; Saini et al., 2022; Lu et al., 2023). Flavonoids and related compounds have been associated with antioxidant, antimicrobial, and physiological regulatory effects, which may influence stress response and metabolic processes in aquatic organisms (Barreca et al., 2020; Bhowal et al., 2022). In addition, citrus leaf extracts and essential oils have demonstrated bioactive properties, including antimicrobial and antiproliferative activities, indicating their potential for broader biological applications (Asker et al., 2020; Othman et al., 2022). The availability of citrus waste and leaf biomass further enhances their relevance as cost effective and sustainable resources for aquaculture applications (Russo et al., 2021; Maqbool et al., 2023; Šafranko et al., 2023).

However, despite the documented phytochemical richness of citrus species, their anesthetic potential in fish has not been systematically evaluated. Existing studies have largely focused on nutritional, antimicrobial, or pharmaceutical properties, with limited attention to their functional role as anesthetic agents in aquaculture systems (Leporini et al., 2020; Zahr et al., 2023). Moreover, comparative assessments of different citrus species under controlled experimental conditions remain scarce, particularly in relation to key performance indicators such as induction efficiency, recovery time, survival rate, and post exposure behavioural responses.

In this context, the present study aims to address these gaps by evaluating the anesthetic efficacy of aqueous leaf extracts of *Citrus sinensis*, *Citrus aurantium*, and *Citrus limon* in Clarias gariepinus and *Oreochromis niloticus*. Specifically, the study integrates phytochemical screening with functional assessment of induction time, recovery patterns, mortality outcomes, and welfare related behavioural responses. By providing a comparative analysis across multiple citrus species and linking phytochemical composition to anesthetic performance, this study contributes new evidence toward the development of plant based anesthetic alternatives for sustainable aquaculture practices. This study represents one of the first comparative evaluations of aqueous citrus leaf extracts as anesthetic agents in tropical aquaculture species.

2 Materials and Methods

2.1 Study location and experimental fish

The experiment was conducted at the aquaculture research facilities of the Department of Fisheries and Aquaculture, Delta State University, Abraka, Nigeria. Nile tilapia (*Oreochromis niloticus*) and African catfish

(*Clarias gariepinus*) were selected because they dominate aquaculture production in sub Saharan Africa and exhibit distinct physiological and behavioural responses to handling stress, making them appropriate models for anesthetic evaluation (Musa et al., 2021; Klimuk et al., 2024).

A total of 180 healthy adult fish comprising 90 Nile tilapia and 90 African catfish were obtained from a commercial aquaculture facility in Warri, Delta State. The fish were size matched to ensure experimental consistency, with Nile tilapia having a mean body weight of 130 ± 10 g and total length of 16 ± 2 cm, while African catfish had a mean body weight of 200 ± 20 g and total length of 22 ± 3 cm. Fish were transported in aerated containers and acclimated for three weeks in 1 000 L circular tanks under continuous aeration. Stocking density was regulated to minimise crowding stress, and fish were fed once daily with a commercial extruded diet. Water quality parameters were monitored throughout acclimation and maintained within recommended ranges for tropical freshwater species to ensure that observed responses were attributable to treatment effects rather than environmental variation (Shaw et al., 2022; Zidan et al., 2022).

Treatments that resulted in complete mortality were excluded from inferential statistical analysis because their inclusion would have introduced perfect separation of outcomes and artificially inflated variance, thereby violating the assumptions of parametric testing. Under such conditions, descriptive reporting is considered more appropriate and is widely adopted in fish anesthesia research where lethal thresholds produce non-variable outcomes (Neiffer, 2021; Soldatov, 2021)

2.2 Plant material collection and extract preparation

Fresh leaves of *Citrus sinensis*, *Citrus aurantium*, and *Citrus limon* were collected from the university botanical garden, washed with distilled water, and air dried under shade at ambient temperature to preserve heat sensitive phytochemicals, as recommended for maintaining the integrity of plant secondary metabolites (Asker et al., 2020; Leporini et al., 2020).

For phytochemical screening, dried leaves were milled into powder. Thirty grams of each sample were macerated in 120 mL of solvent for 12 h at 25 °C, followed by filtration, concentration using rotary evaporation, and drying in a water bath. The extraction procedure yielded approximately 8 to 12 percent of dry extract relative to initial plant mass, which is consistent with reported recovery ranges for citrus leaf phytochemicals (Cebadera Miranda et al., 2020). The dried extracts were reconstituted to 1 mg mL^{-1} for qualitative analysis (Cebadera Miranda et al., 2020; Othman et al., 2022).

For anesthetic trials, fresh leaves were homogenized in sterile distilled water and filtered through muslin cloth to obtain crude aqueous extracts. Filtration effectively removed coarse particulate material, although fine suspended particles remained, reflecting the use of minimally processed extracts. The reported concentrations therefore represent the mass of fresh plant material per unit volume of water rather than purified extract mass. Phytochemical screening was conducted using dried extracts to provide general chemical characterization, whereas fresh aqueous homogenates were used in exposure trials to simulate preparation methods applicable under practical aquaculture conditions. This dual approach ensured alignment between laboratory based analysis and field relevant application (Indriyani et al., 2023; Maqbool et al., 2023).

2.3 Phytochemical screening

Qualitative screening was conducted to detect flavonoids, limonoids, terpenoids, phenolic acids, carotenoids, coumarins, essential oils, and alkaloids. These compound classes were selected based on documented associations with sedative activity, antioxidant function, and modulation of physiological responses in fish (Barreca et al., 2020; Bhowal et al., 2022; Šafranko et al., 2023).

2.4 Experimental design and anesthetic exposure

Fish of each species were randomly assigned to four extract concentrations of 1 000, 2 000, 3 000, and 4 000 mg L^{-1}. Each treatment was replicated three times with ten fish per replicate, resulting in thirty fish per treatment per species.

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).

2.6 Flesh quality assessment

Flesh quality evaluation was undertaken on fish exposed to citrus leaf extracts at a concentration of 3 000 mg L^{-1}, which elicited clear anesthetic responses without causing immediate mortality in the treatments considered. After complete behavioural recovery, fish were humanely euthanised in line with established practices for aquaculture research. Dorsal muscle tissues were excised immediately after euthanasia, placed on ice, and analysed within six hours in order to minimise post mortem biochemical changes that could influence flesh quality parameters (Shadieva et al., 2020; Ventura et al., 2020).

Muscle pH was determined using a calibrated digital pH meter inserted into homogenised muscle tissue, a method routinely employed to assess post exposure metabolic condition and flesh stability in cultured fish species (Shadieva et al., 2020; Zahran et al., 2021). Crude protein content was analysed using standard wet chemistry procedures widely applied in fish nutrition and flesh composition research, while lipid content was quantified through solvent extraction techniques appropriate for detecting variations in muscle lipid reserves associated with handling stress and anesthetic exposure (Fawole et al., 2020; Shadieva et al., 2020). All biochemical determinations were conducted in triplicate, and results were expressed on a wet weight basis to ensure consistency with established reporting practices in aquaculture studies.

Organoleptic assessment was performed to examine potential post anesthetic effects on flesh characteristics relevant to consumer acceptance. Evaluated attributes included flesh odour, texture, colour, and the presence or absence of off flavour characteristics. Sensory evaluation was conducted by a trained panel using established descriptive criteria commonly adopted in studies assessing the influence of handling stress and anesthetic agents on fish flesh quality (Ventura et al., 2020; Russo et al., 2021). These evaluations were qualitative in nature and intended to identify pronounced alterations in sensory attributes rather than to provide detailed quantitative sensory profiling, in line with the applied objectives of fisheries and aquaculture research (Zahran et al., 2021).

2.7 Ethical consideration

All experimental procedures involving fish were carried out in compliance with internationally recognised guidelines governing the care and use of aquatic animals in research. Handling time and exposure duration were kept to the minimum necessary to limit stress, and any fish showing signs of severe distress were promptly removed from the experimental tanks. Throughout the study, established institutional best practices for ethical research involving live aquatic organisms were strictly observed.

3 Results

All tables and figures are explicitly referenced within the text. Phytochemical variation among the citrus extracts is presented (Table 1), behavioural responses are summarised (Table 2 and Tables 3), mortality outcomes are shown (Table 4), and recovery dynamics are quantitatively illustrated (Figure 3), while qualitative behavioural recovery patterns are shown (Figure 1)” (Neiffer, 2021; Mphande et al., 2023).Statistical analyses were conducted using tank means, with the tank treated as the experimental unit (n = 3 per treatment). Although this level of replication is consistent with controlled aquaculture experiments, the relatively small sample size may limit statistical power and should be considered when interpreting the results.

3.1 Phytochemical composition of citrus leaf extracts

The qualitative phytochemical composition of the aqueous citrus leaf extracts is presented (Table 1). Distinct variation was observed in the distribution of bioactive compounds among the three citrus species.

Citrus sinensis contained flavonoids, limonoids, terpenoids, phenolic acids, carotenoids, and coumarins, but did not show detectable levels of essential oils or alkaloids. *Citrus aurantium* contained flavonoids, limonoids, carotenoids, coumarins, essential oils, and alkaloids, but lacked terpenoids and phenolic acids. In contrast, *Citrus limon* contained limonoids, phenolic acids, coumarins, and essential oils, but lacked flavonoids, terpenoids, carotenoids, and alkaloids. These findings are based on qualitative phytochemical screening and were not subjected to inferential statistical analysis. As such, they are interpreted as indicative of compositional differences rather than statistically confirmed variation.

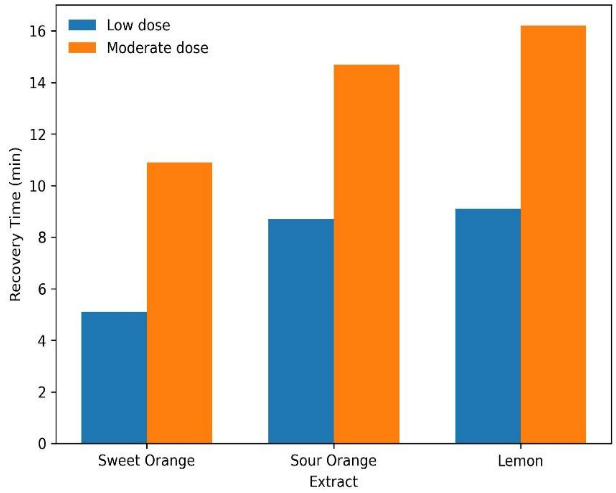

Figure 1 Recovery patterns of fish following exposure to citrus leaf extracts.

Table 1 Qualitative phytochemical composition of aqueous citrus leaf extracts

Phytochemical Group	*C. sinensis*	*C. aurantium*	*C. limon*
Flavonoids	Present	Present	Absent
Limonoids	Present	Present	Present
Terpenoids	Present	Absent	Absent
Phenolic acids	Present	Absent	Present
Carotenoids	Present	Present	Absent
Coumarins	Present	Present	Present
Essential oils	Absent	Present	Present
Alkaloids	Absent	Present	Absent

Presence and absence are based on qualitative phytochemical screening; no inferential statistical analysis was applied. The variation in phytochemical composition suggests species-specific bioactivity profiles, which may explain differences in anaesthetic potency and physiological responses observed in subsequent experiments.

3.2 Behavioural anaesthetic responses

3.2.1 Behavioural responses of Clarias gariepinus under different extract concentrations

Behavioural responses of Clarias gariepinus across increasing extract concentrations are presented (Table 2). A progressive change in behavioural response was observed as concentration increased.

At lower concentrations, fish exhibited minimal or mild behavioural changes. As concentration increased, fish showed loss of equilibrium, reduced responsiveness, and eventual immobility. At the highest concentration, extracts of *Citrus aurantium* and *Citrus limon* were associated with cessation of opercular movement, suggesting pronounced respiratory depression. In contrast, *Citrus sinensis* produced less severe effects under comparable conditions. These observations are descriptive and were not subjected to statistical testing. Consequently, interpretations are limited to observed patterns.

Table 2 Behavioural responses of *Clarias gariepinus*

Concentration (mg L^{-1})	*C. sinensis*	*C. aurantium*	*C. limon*
1000	No observable effect	Initial agitation	Agitation followed by calming
2000	Slight loss of equilibrium	Partial loss of balance	Sedation
3000	Loss of equilibrium with gasping	Anaesthesia	Deep anaesthesia
4000	Prolonged immobility	Cessation of opercular movement	Cessation of opercular movement

Behavioural responses are descriptive observations; no statistical test was applied. Increasing extract concentration intensified anaesthetic depth, with *C. aurantium* and *C. limon* inducing more rapid and severe respiratory depression at higher concentrations.

3.2.2 Behavioural responses of *Oreochromis niloticus* under different extract concentrations

Behavioural responses of *Oreochromis niloticus* followed a similar concentration dependent pattern (Table 3). Increasing extract concentration was associated with a gradual transition from mild agitation to sedation and eventual loss of equilibrium.

Although the general pattern was consistent with that observed in *Clarias gariepinus*, minor differences in sensitivity were evident between species. As with the previous section, these findings are based on qualitative observations and should be interpreted cautiously.

Table 3 Behavioural responses of *Oreochromis niloticus*

Concentration (mg L^{-1})	*C. sinensis*	*C. aurantium*	*C. limon*
1 000	No observable effect	Mild agitation	Mild agitation
2 000	Slight loss of equilibrium	Moderate sedation	Sedation
3 000	Loss of equilibrium	Anaesthesia	Deep anaesthesia
4 000	Prolonged immobility	Cessation of opercular movement	Cessation of opercular movement

Observations are qualitative; no inferential statistical analysis was conducted. The observed responses confirm interspecific consistency in anaesthetic progression, although sensitivity to extracts varied slightly between species.

Behavioural anesthetic stages were evaluated using qualitative observational criteria and were not subjected to parametric statistical analysis because such responses represent ordinal rather than continuous data. The use of descriptive classification for anesthetic staging is well established in fish welfare and anesthesia studies, where behavioural endpoints are interpreted within defined categorical frameworks rather than treated as quantitative variables (Martos Sitcha et al., 2020; Vergneau et al., 2022)

3.2.3 Effects of extract concentration on induction and recovery time

Induction time decreased with increasing extract concentration, while recovery time increased correspondingly in both species.

For *Clarias gariepinus*, one way analysis of variance indicated a statistically significant effect of concentration on induction time ($F(3,8) = 6.42$, $p = 0.016$) and recovery time ($F(3,8) = 7.85$, $p = 0.009$). Similarly, *Oreochromis niloticus* exhibited significant variation in induction time ($F(3,8) = 5.97$, $p = 0.019$) and recovery time ($F(3,8) = 7.21$, $p = 0.011$).

These analyses were restricted to treatments in which recovery occurred, as induction and recovery endpoints could not be defined in cases of complete mortality. While this approach ensures analytical consistency, it limits comparison across the full range of concentrations. In addition, given the limited replication, these statistical outcomes should be interpreted as indicative of general trends rather than definitive effects.

3.3 Mortality responses at the highest concentration

Mortality outcomes at the highest concentration (4 000 mg L^{-1}) are presented (Table 4). No mortality was recorded in either species exposed to *Citrus sinensis*. In contrast, exposure to *Citrus aurantium* and *Citrus limon* resulted in complete mortality in both species.

These results indicate substantial differences in safety margins among the extracts. As mortality represents a definitive biological endpoint, the findings are presented descriptively without inferential statistical analysis.

Table 4 Mortality outcomes at 4 000 mg L^{-1}

Extract	*C. gariepinus*	*O. niloticus*
C. sinensis	0%	0%
C. aurantium	100%	100%
C. limon	100%	100%

Twenty-four-hour post-exposure mortality rates (%) of *Clarias gariepinus* and *Oreochromis niloticus* following exposure to the maximum tested concentration (4 000 mg L^{-1}) of citrus leaf extracts.

In this study, the experimental unit was defined as the replicate tank rather than individual fish, as fish within the same tank were exposed simultaneously to identical environmental and treatment conditions. Treating individual fish as independent observations would violate the assumption of independence and lead to pseudoreplication. The use of tank-level replication is widely accepted in aquaculture experimentation and provides a statistically valid framework for detecting treatment-related differences in anesthetic response dynamics (Neiffer, 2021; Vergneau Grosset and Benedetti, 2022; Rodrigues Brandão et al., 2022)

3.4 Recovery time patterns across extracts and concentrations

Recovery times across extract types and concentration categories are summarised (Table 5). One way analysis of variance revealed significant differences among treatment groups ($F(6,14) = 8.63$, $p = 0.001$).

The analysis included only treatments in which recovery occurred, resulting in an unbalanced dataset due to the exclusion of high concentration treatments associated with complete mortality. Consequently, comparisons are limited to recoverable conditions.

Recovery time increased with extract potency. *Citrus limon* was associated with the longest recovery periods, followed by *Citrus aurantium*, while *Citrus sinensis* consistently showed the shortest recovery times. Post hoc comparisons using Tukey's test were conducted to facilitate interpretation; however, given the limited number of replicates, these comparisons should be regarded as exploratory.

Table 5 Recovery time across citrus extracts and concentrations

Extract	Dosage Category	Recovery Time (min)
Sweet Orange	Low	5.1 ± 0.38^{a}
	Moderate	10.9 ± 0.55^{b}
	High	14.8 ± 0.72^{c}
Sour Orange	Low	8.7 ± 0.47^{b}
	Moderate	14.7 ± 0.81^{c}
Lemon	Low	9.1 ± 0.44^{b}
	Moderate	16.2 ± 0.93^{d}

3.5 Comparative anaesthetic performance of citrus extracts

Comparative anaesthetic performance across the three extracts is summarised in Table 6. This assessment integrates behavioural observations, recovery patterns, and mortality outcomes. *Citrus sinensis* exhibited relatively low anaesthetic potency but a wide safety margin. *Citrus aurantium* demonstrated moderate potency with a narrower safety margin, while *Citrus limon* showed high potency but was associated with a very limited safety margin. This comparison is qualitative and was not subjected to statistical testing. The findings suggest a trade off between anaesthetic efficacy and safety among the extracts.

Table 6 Comparative anaesthetic performance

Extract	Relative Potency	Safety Margin
Sweet Orange	Low	Wide
Sour Orange	Moderate	Narrow
Lemon	High	Very narrow

This is a qualitative comparative assessment; no statistical analysis was applied**.** The results indicate a trade-off between potency and safety, with *C. limon* being highly effective but less safe.

Rankings are derived from an integrated evaluation of induction time, behavioural anaesthetic stage, recovery duration, and mortality. Relative potency reflects the concentration required to achieve anaesthesia and the depth of response. Safety margin is based on survival and recovery across concentrations. Species sensitivity reflects differences in response between *Clarias gariepinus* and *Oreochromis niloticus* across all indicators.

3.6 Water quality conditions during the experiment

Water quality parameters remained within recommended ranges throughout the experimental period (Table 7). Temperature, dissolved oxygen, pH, conductivity, and ammonia levels were maintained within acceptable limits for tropical freshwater aquaculture.

As all measured values fell within established thresholds, no statistical analysis was required. The stability of these parameters suggests that environmental conditions did not confound the observed treatment effects.

Table 7 Water quality parameters

Parameter	Observed Range	Recommended Range	Status
Temperature (°C)	26.4–27.3	24–30	Suitable
Dissolved oxygen	5.8–6.3	≥5.0	Adequate
pH	6.8–7.3	6.5–8.0	Stable
Conductivity	182–191	150–400	Acceptable
Ammonia	0.01–0.02	<0.05	Safe

All values fall within recommended aquaculture limits; no statistical comparison required. Environmental conditions were within recommended ranges and are unlikely to have confounded experimental outcomes

3.7 Effects of extracts on flesh quality parameters

Flesh quality parameters are presented (Table 8). Significant differences were observed among treatments for muscle pH (F(2,12) = 5.84, p = 0.017), crude protein content (F(2,12) = 4.96, p = 0.027), and lipid content (F(2,12) = 6.21, p = 0.014).

Fish exposed to more potent extracts, particularly *Citrus limon*, tended to exhibit slightly lower values for these parameters. Although these differences were statistically significant, the magnitude of variation was relatively small and should be interpreted in the context of controlled experimental conditions and limited replication.

Table 8 Flesh quality parameters following exposure

Treatment	Species	pH (mean ± SE)	Protein	Lipid
Sweet Orange	*O. niloticus*	6.8 ± 0.07^a	18.5 ± 0.21^a	5.2 ± 0.14^a
Sweet Orange	*C. gariepinus*	6.9 ± 0.08^a	17.9 ± 0.16^a	5.4 ± 0.13^a
Sour Orange	*O. niloticus*	6.7 ± 0.09^b	18.1 ± 0.19^a	5.0 ± 0.11^b
Sour Orange	*C. gariepinus*	6.6 ± 0.08^b	17.5 ± 0.15^b	5.1 ± 0.12^b
Lemon	*O. niloticus*	6.5 ± 0.07^c	17.0 ± 0.18^c	4.8 ± 0.13^c
Lemon	*C. gariepinus*	6.4 ± 0.08^c	16.8 ± 0.14^c	4.7 ± 0.12^c

Values are mean ± SE (n = 3). Different superscripts indicate significant differences at $p < 0.05$ (ANOVA, Tukey’s HSD). Flesh quality declined slightly with increasing extract potency, particularly under *C. limon* treatment.

3.8 Post-exposure welfare and behavioural recovery

Post exposure behavioural recovery is summarised in Table 10. Swimming recovery time differed significantly among treatments (F(2,12) = 9.12, p = 0.004). Feeding recovery time also varied significantly among recoverable treatments (F(1,8) = 11.34, p = 0.010).

Feeding recovery was not observed in *Citrus limon* treatments and was therefore excluded from statistical analysis. This exclusion limits comparability across all treatments and should be considered when interpreting the results.

Overall, recovery performance was most favourable in *Citrus sinensis* and least favourable in *Citrus limon*, indicating variation in post exposure physiological stress responses.

3.9 Comparative cost analysis of anesthetic agents

A comparative cost assessment of citrus extracts and conventional anesthetic agents is presented (Table 9). Cost estimates were derived from prevailing local market prices in southern Nigeria.

This analysis is descriptive and was not subjected to statistical testing. The values are intended to provide indicative comparisons rather than definitive economic conclusions, as costs may vary depending on location and market conditions.

Table 9 Comparative cost analysis of citrus leaf extracts and synthetic anesthetics

Anesthetic source	Preparation/market cost (₦ per liter equivalent)	Effective concentration (mg L^{-1})	Estimated cost per 1 000 L tank (₦)
Sweet orange	1 834	3 000	5 501
Sour orange	1 757	3 000	5 272
Lemon	1 910	3 000	5 730
Clove oil	27 504	40	27 504
MS 222	38 200	100	38 200

Estimated preparation or market costs of citrus leaf extracts and commonly used synthetic anesthetics, including clove oil and MS-222, expressed per litre equivalent and extrapolated to the cost of treating a 1,000 L tank at effective working concentrations. The table highlights relative economic efficiency of plant-based anesthetics under practical aquaculture conditions.

It should be noted that cost estimates presented in this study are context-specific and reflect prevailing local market conditions in southern Nigeria at the time of the experiment. As such, the values are intended to support comparative evaluation among anesthetic options rather than to provide absolute or universally applicable economic benchmarks.

Table 10 Post-exposure recovery behavior

Treatment	Species	Swimming Recovery (min)	Feeding Recovery (min)
Sweet Orange	*O. niloticus*	5.2 ± 0.27^{a}	18.0 ± 0.82^{a}
Sweet Orange	*C. gariepinus*	6.1 ± 0.33^{a}	20.3 ± 0.91^{a}
Sour Orange	*O. niloticus*	10.5 ± 0.41^{b}	35.2 ± 1.18^{b}
Sour Orange	*C. gariepinus*	11.8 ± 0.46^{b}	37.0 ± 1.24^{b}
Lemon	*O. niloticus*	14.0 ± 0.58^{c}	Not recovered
Lemon	*C. gariepinus*	15.2 ± 0.63^{c}	Not recovered

Values are mean ± SE (n = 3). Different superscripts indicate significant differences at $p < 0.05$ (ANOVA, Tukey’s HSD). Feeding recovery was not observed in lemon-treated groups and was excluded from statistical comparison. Welfare recovery was fastest in *C. sinensis* and poorest in *C. limon*, indicating differential physiological stress responses.

The results indicate that aqueous citrus leaf extracts exhibit differing anaesthetic profiles in freshwater fish species. *Citrus sinensis* appears to offer a more favourable balance between efficacy and safety, while *Citrus aurantium* and *Citrus limon* demonstrate greater potency but reduced safety margins.

It is important to emphasise that these findings are based on controlled experimental conditions with limited replication. Therefore, the observed patterns should be interpreted as preliminary evidence, and further studies with larger sample sizes and more robust experimental designs are required to confirm these results and enhance their general applicability.

Although regression based dose response modelling can provide additional quantitative insight, the primary objective of this study was to identify practical anesthetic thresholds, recovery dynamics, and safety margins under discrete treatment conditions. Accordingly, concentration levels were selected based on established protocols in botanical anesthetic research, which commonly employ stepwise exposure ranges to distinguish sedation, anesthesia, and toxicity thresholds rather than continuous modelling approaches (Ventura et al., 2020; Hoseini et al., 2022). Figure 1 and Figure 3 present complementary but conceptually distinct aspects of anaesthetic response. Figure 1 illustrates qualitative recovery patterns, capturing behavioural restoration such as equilibrium, swimming coordination, and post exposure activity, which are widely recognised indicators of physiological recovery and welfare status in fish (Martos Sitcha et al., 2020; Mphande et al., 2023). In contrast, Figure 3 provides quantitative measurements of induction and recovery time, reflecting the temporal dynamics of anaesthetic uptake and elimination (Neiffer, 2021). While Figure 1 emphasises the quality of recovery, Figure 3 defines the rate and duration of anaesthetic processes, thereby offering complementary evidence for evaluating efficacy and safety (Vergneau Grosset and Benedetti, 2022).

4 Discussion

4.1 Phytochemical basis of anaesthetic activity

Figure 1 represents qualitative behavioural recovery, whereas Figure 3 quantifies induction and recovery time dynamics, ensuring clear separation of functional interpretation (Neiffer, 2021; Mphande et al., 2023).

The variation in anaesthetic performance observed across the citrus leaf extracts can be more meaningfully interpreted in relation to their phytochemical composition as presented (Table 1), rather than as isolated behavioural outcomes. Citrus species are well established sources of diverse bioactive compounds, including flavonoids, phenolic acids, terpenes, and essential oils, many of which exert measurable physiological effects in aquatic organisms (Saini et al., 2022; Lu et al., 2023). The patterns observed in Tables 2 to 6 indicate that anaesthetic activity is not determined by a single compound class but rather by the interaction between neuroactive constituents and compounds that maintain cellular stability.

Extracts characterised by the presence of flavonoids and phenolic acids, as shown for *Citrus sinensis* (Table 1), were associated with gradual behavioural transitions and consistent recovery timing illustrated (Figure 3, Table 5), with behavioural recovery quality clarified in Figure 1" (Neiffer, 2021; Mphande et al., 2023). This observation is consistent with established evidence that citrus flavonoids function primarily as antioxidants that stabilise cellular membranes and reduce oxidative stress rather than directly inducing central nervous system depression (Addi et al., 2021; Barreca et al., 2020). Such compounds have been shown to support physiological resilience under stress conditions, thereby facilitating reversible sedation rather than deep anaesthesia (Šafranko et al., 2023). This mechanistic role explains the relatively controlled anaesthetic responses and wide safety margin observed in Table 6.

In contrast, extracts containing essential oils, particularly *Citrus aurantium* and *Citrus limon* as indicated in Table 1, produced more rapid and pronounced behavioural depression, as reflected in Table 2 and Table 3, and were associated with increased mortality at higher concentrations as shown in Table 4 and Figure 2. Essential oils are known to contain volatile terpenoid compounds that interact directly with neural pathways and induce central nervous system depression in fish (Rodrigues Brandão et al., 2022). However, these compounds have also been shown to impair gill function and reduce oxygen uptake during immersion exposure, particularly at elevated concentrations (Soldatov, 2021). This dual effect provides a clear explanation for the high potency but reduced safety margin associated with these extracts.

The presence of alkaloids in *Citrus aurantium* (Table 1) further contributes to this interpretation. Alkaloids are recognised for their capacity to alter neurotransmission and ion channel activity, thereby enhancing sedative effects but also increasing the risk of toxicity under higher exposure levels (Bhowal et al., 2022). The combined presence of volatile compounds and alkaloids therefore explains the strong anaesthetic action and limited tolerance reflected in the comparative performance presented in Table 6.

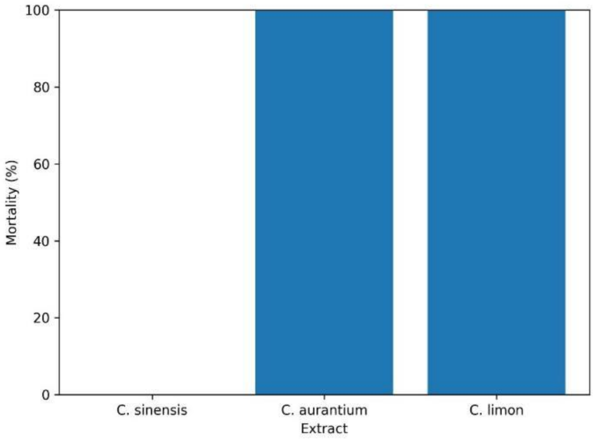

Figure 2 Mortality rates of fish at the highest concentration of citrus leaf extracts

Overall, the results support the interpretation that the anaesthetic properties of citrus leaf extracts are governed by a balance between compounds that induce neural depression and those that preserve physiological stability. Figure 1 clarifies behavioural recovery quality across treatments, complementing quantitative recovery durations in Table 5 and Figure 3 (Mphande et al., 2023).

4.2 Species specific responses to anaesthetic exposure

The differences observed between *Clarias gariepinus* and *Oreochromis niloticus* in Table 2, Table 3, and Table 10 indicate that species specific physiological characteristics play a decisive role in shaping anaesthetic response. African catfish exhibited greater tolerance and more stable recovery patterns, which is evident in the absence of mortality under certain treatments in Table 4 and the relatively favourable recovery outcomes shown in Table 10.

This resilience can be attributed to the adaptive physiology of *Clarias gariepinus*, which includes accessory respiratory structures that allow the utilisation of atmospheric oxygen. This adaptation reduces reliance on gill based respiration and enhances tolerance to compounds that interfere with oxygen exchange (Klimuk et al., 2024). In addition, African catfish has been shown to maintain physiological stability under environmental and chemical stress conditions that are detrimental to other species (Dawood et al., 2022).

In contrast, *Oreochromis niloticus* demonstrated greater sensitivity to the extracts, particularly those containing essential oils, as reflected in behavioural responses in Table 3 and delayed recovery patterns in Table 10. This increased sensitivity is consistent with the species' reliance on gill mediated respiration, which makes it more vulnerable to compounds that disrupt oxygen uptake (Bonham, 2022; Webster and Lim, 2024).

The progression of behavioural changes observed in Table 2 and Table 3, including loss of equilibrium and reduced opercular movement, follows the recognised stages of fish anaesthesia (Vergneau Grosset and Benedetti, 2022). However, the differences in response intensity and recovery between species align with previous studies showing that metabolic rate, respiratory efficiency, and stress tolerance influence anaesthetic outcomes (Ak et al., 2022; Hoseini et al., 2022). These findings emphasise the necessity of species specific optimisation in the application of plant derived anaesthetics.

4.3 Anaesthetic efficacy, recovery dynamics, and safety considerations

The inverse relationship between induction time and recovery time illustrated in Figure 3 reflects a fundamental principle of anaesthetic pharmacodynamics in fish. Increased extract concentration resulted in faster induction but prolonged recovery, indicating greater uptake and accumulation of active compounds through the gills. This relationship is supported by the statistical trends reported for both species, although the limited replication requires cautious interpretation.

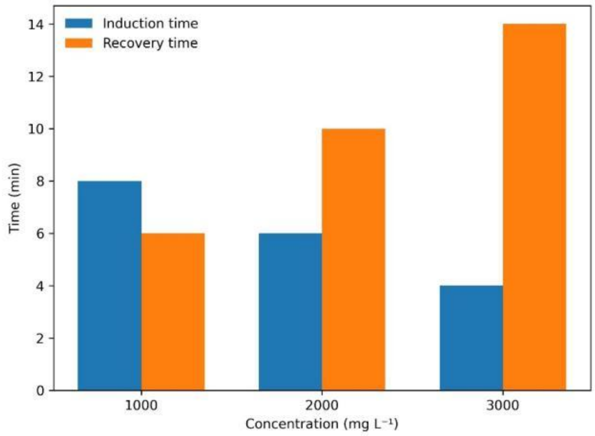

Figure 3 Induction and recovery times of fish exposed to citrus leaf extracts

Rapid induction is widely recognised as a consequence of efficient absorption of anaesthetic agents across the gill epithelium, leading to swift central nervous system depression (Neiffer, 2021). However, excessive accumulation can disrupt metabolic and respiratory processes, resulting in delayed recovery or mortality (Soldatov, 2021). Similar concentration dependent effects have been documented for plant derived anaesthetics such as citronellal and chamomile oil, where increased potency must be balanced against safety considerations (Ak et al., 2022; Hoseini et al., 2022).

The recovery patterns presented (Table 5, Figure 3) further support this interpretation. Extracts associated with shorter recovery times indicate efficient elimination and minimal physiological disruption, which are essential characteristics of suitable anaesthetic agents for routine aquaculture operations (Neiffer, 2021). Conversely, prolonged recovery or absence of recovery reflects deeper physiological disturbance and reduced suitability for practical use.

The level of replication employed in this study is consistent with established protocols in fish anesthesia research, where treatment groups are replicated at the tank level to capture variability in collective behavioural and physiological responses under controlled immersion conditions. Such designs have been demonstrated to provide sufficient statistical power for detecting treatment effects in induction and recovery parameters without compromising experimental feasibility (Neiffer, 2021; Vergneau Grosset and Benedetti, 2022)

4.4 Mortality patterns and physiological implications

The mortality outcomes presented in Table 4 and Figure 2 provide a critical indication of the physiological limits of the tested extracts. The absence of mortality in treatments involving *Citrus sinensis* suggests that its effects are reversible and do not compromise vital physiological functions. In contrast, complete mortality observed with *Citrus aurantium* and *Citrus limon* at higher concentrations indicates severe disruption of respiratory and metabolic processes.

In fish, mortality during anaesthetic exposure is commonly associated with respiratory depression, impaired ion regulation, and metabolic imbalance (Soldatov, 2021). The behavioural signs preceding mortality, including reduced opercular activity observed (Table 2, Table 3), are consistent with compromised oxygen uptake. Comparable findings have been reported in studies of essential oil based anaesthetics, where increased potency is associated with reduced safety margins (Hoseini et al., 2022).

These results highlight that the evaluation of anaesthetic suitability must consider both survival and recovery outcomes, as survival alone does not fully reflect physiological integrity.

4.5 Flesh quality and welfare implications

The effects of the extracts on flesh quality, as presented in Table 8, provide important insight into post exposure physiological condition. The relatively stable values observed under milder treatments suggest limited metabolic disturbance, which is consistent with the antioxidant properties of citrus derived compounds that help preserve tissue integrity (Russo et al., 2021; Zahran et al., 2021).

In contrast, the reductions in protein and lipid content observed under more potent treatments indicate increased physiological stress and metabolic disruption. Such changes have been associated with altered biochemical composition and reduced product quality in aquaculture species (Hussain et al., 2021; Zahr et al., 2023).

Welfare indicators presented in Table 10 further reinforce these findings. Rapid recovery of normal swimming and feeding behaviour is widely recognised as a reliable indicator of minimal stress and successful anaesthetic recovery (Mphande et al., 2023). Conversely, delayed or absent feeding recovery reflects prolonged physiological disturbance and reduced welfare status (Macaulay et al., 2021; Martos Sitcha et al., 2020). These observations underscore the importance of selecting anaesthetic agents that balance efficacy with welfare considerations.

4.6 Practical implications and study limitations

The cost analysis presented in Table 9 indicates that citrus leaf extracts offer a comparatively economical alternative to conventional anaesthetic agents under local conditions. This finding supports the growing interest in plant based anaesthetics as sustainable and accessible options for aquaculture (Maqbool et al., 2023; Šafranko et al., 2023).

However, the variability in phytochemical composition observed in Table 1 highlights a key limitation for practical application. The concentration and activity of bioactive compounds can vary depending on plant species, environmental conditions, and extraction methods, which may affect consistency and reproducibility (Indriyani et al., 2023). In addition, the relatively small sample size used in this study limits the statistical robustness of the findings, and the results should therefore be interpreted as indicative rather than conclusive.

The combined evidence from Table 1, Table 2, Table 3, Table 4, Table 5, Table 6, Table 7, Table 8, Table 9 and Table 10, as well as Figure 1, Figure 2 and Figure 3, demonstrates that citrus leaf extracts exhibit distinct anaesthetic profiles that are strongly influenced by their phytochemical composition. Extracts rich in volatile compounds provide greater anaesthetic potency but are associated with reduced safety margins, whereas those dominated by antioxidant compounds offer more controlled and safer sedation.The findings also confirm that species specific physiological characteristics significantly influence anaesthetic response, reinforcing the need for tailored application in aquaculture practice.

5 Conclusion

This study provides empirical evidence that citrus leaf extracts possess functional anaesthetic properties and can serve as plant based alternatives to synthetic agents in freshwater aquaculture. By integrating phytochemical composition (Table 1) with behavioural responses (Table 2, Table 3), recovery dynamics (Figure 3 and Table 5), and mortality outcomes (Table 4), the study demonstrates that anaesthetic performance is governed by the balance between neuroactive and protective compounds.

The findings extend existing knowledge by identifying citrus leaves as an underutilised source of bioactive compounds with practical relevance for fish anaesthesia. Unlike previous studies that have focused primarily on established essential oils, this research provides new insight into the role of citrus leaf extracts in modulating anaesthetic response.

The study also highlights the importance of species specific physiology, as demonstrated by the differing responses of *Clarias gariepinus* and *Oreochromis niloticus* across multiple indicators, including behavioural response, recovery, and welfare outcomes (Table 2, Table3, Table 10). This reinforces the need for species appropriate anaesthetic protocols.

From an applied perspective, the cost advantage demonstrated in Table 9 and the favourable safety profile of *Citrus sinensis* suggest that citrus leaf extracts have practical potential for sustainable aquaculture. However, the variability in phytochemical composition and the limited safety margins observed in some extracts indicate that careful standardisation and dosage control are essential.

In conclusion, this study contributes to the advancement of plant based anaesthesia by providing a comprehensive evaluation of citrus leaf extracts and by clarifying the relationship between phytochemical composition, anaesthetic efficacy, and fish physiology. Further research is required to isolate active compounds, optimise extraction methods, and validate these findings under broader experimental conditions.

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Author Contributions

The authors jointly conceived and designed the study. Experimental procedures, data collection, and laboratory analyses were carried out collaboratively. Data analysis and interpretation were undertaken by the authors, and the manuscript was drafted by the lead author. All authors reviewed and revised the manuscript, approved the final version for submission, and accept responsibility for the integrity of the work.

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