Evaluating Neem Seed Powder (*Azadirachta indica*) as an Anesthetic for Common Carp (*Cyprinus carpio*) Across Various Weight Groups

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ABSTRACT

A natural alternative anesthetic, such as neem (*Azadirachta indica*) seed powder, may offer safer and more environmentally friendly practices in aquaculture. This study aims to assess its efficacy and safety on common carp (*Cyprinus carpio*) across different weight groups. Seven groups of common carp weighing between 20 and 89 g were exposed to neem seed powder. The time required for each fish to attain stage 3 of anesthesia (total loss of equilibrium) and to recover were recorded. The largest group of fish (80–89 g) was selected for subsequent blood tests due to its sensitivity to the anesthetic action of the neem seed powder. The results showed that the lowest-weight fish group (20–29 g) required more time to attain anesthesia at all dosages (12.98–21.13 min) compared to the maximum weight group (80–89 g) (8.83–16.85 min). Recovery times were not significantly impacted by varying anesthetic doses, but shorter recovery times were associated with large fish size. Although neem seed powder at concentration of 5–10 $g \cdot L^{-1}$ significantly affected blood parameters, such as decreased red blood cell count and increased white blood cell and blood glucose levels, no fish morbidity or mortality was observed. Therefore, neem seed powder (up to 10 $g \cdot L^{-1}$) can be safely and effectively used to sedate common carp as an alternative to synthetic chemical anesthetics.

Keywords: Anesthesia, Fish, Herbal medicine, Medicinal plant, Neem

INTRODUCTION

Anesthesia refers to the use of physical or chemical agents that cause relaxation in animals, leading to the loss of consciousness, movement, and sensation response (Summerfelt and Smith, 1990). As a stress reliever in modern aquaculture, many anesthetics have been utilized recently to assist with fish catching, handling, artificial reproduction, surgical treatments, and transportation (Coyle *et al*., 2004). Fish were previously often anesthetized using chemical anesthetics, but many of these are no longer in use due to health risks, residual issues, accumulation in the fish body, and adverse effects (Yaşar and Yardımcı, 2022) Due to these limitations, the use of plants as natural anesthetics in aquaculture is a recent concept that is gaining popularity (Hoseini *et al*., 2018). Since anesthetics are one of the key inputs that organic farming seeks to replace with non-chemical alternatives, several natural substances were investigated to assess their effectiveness compared to chemical anesthetics (Ramanayaka and Atapatu, 2006).

The neem (*Azadirachta indica*) tree, which belongs to the family Meliaceae, is a bitter, tonic herb that acts as an antipyretic and anti-inflammatory agent (Alzohairy, 2016). Neem is generally considered safe for use in fish farming, but its effects on fish health and behavior may depend on the concentration and duration of exposure. Since ancient times, it has been known that neem leaves,

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which contain nimbin, azadirachtin, and meliantroil, have a range of bioactivities, including insecticidal and antiviral capabilities (Pandey *et al*., 2012). Additionally, it has been observed that neem increases tilapia survival rates, growth, and feed utilization characteristics while not influencing hematological parameters (Gabriel, 2019). According to Ahsan *et al*. (2016), neem leaf extract may function well as an anesthetic at low doses. They also found that 5–10% neem leaf juice provides fish with sufficient sedation time when handling live fish for vaccination or other uses. Studies on the use of plants as anesthetics in aquacultures are becoming more common, but further research is still required to identify the exact process of production, administration, dose, and effects of different medicinal plants on varied fish species. Considering all these issues, the project's goals were to assess the anesthetic effects and safe use of natural agents, like neem seed powder, for various common carp weight groups, as well as to determine the impact on certain blood parameters.

MATERIALS AND METHODS

The Scientific Committee and the Ethics Committee of the University of Sulaimani's College of Veterinary Medicine, Sulaimani, Iraq, approved a study proposal before this experiment was carried out.

Experimental fish

In total, 210 common carp were used in the experiment. They were split into seven groups according to body weight: Group 1 (20–29 g), Group 2 (30–39 g), Group 3 (40–49 g), Group 4 (50–59 g), Group 5 (60–69 g), Group 6 (70–79 g), and Group 7 (80–89 g). The fish were obtained from a nearby farm in Hilla, Iraq. Every fish used for this investigation was kept in rectangular plastic tanks containing approximately 60 L of water at the Fish Diseases Lab, College of Veterinary Medicine, University of Sulaimani. To ensure the fish had properly recovered from any stress associated with capture or transportation, they were acclimated for a minimum of two weeks before the start of the experiments and fed commercial fish feed pellets at 5% body weight, divided into two daily feedings. During the acclimatization period, variables related to the water quality of the aquarium were continuously monitored, and half of the water was changed every two days. The water's temperature was 27±1 °C, pH was 7.5±0.26, and dissolved oxygen content was 6.60±0.44 $mg \cdot L^{-1}$.

Preparation of the experimental agent

Dried neem seeds (5.22% moisture) were gathered from a local herbal shop in Sulaimani, Kurdistan, Iraq. The seeds were thoroughly cleaned to remove any extraneous materials and dirt. The seeds were then ground in the laboratory using a kitchen blender (Nima Electric Grinder, Japan), and the ground neem seeds were sieved into a uniformly fine powder using a 0.5 mm sieve mesh. The powder was placed in a closed container and kept in a dry place before the experiment began.

Experimental procedure

The 35 treatments (with 6 replications) in this experiment are represented by seven different common carp (*Cyprinus carpio*) weights ranging from 20 to 89 g, combined with five different neem seed powder concentrations (0, 2.5, 5, 7.5, and 10 $g \cdot L^{-1}$). Each anesthetic bath was prepared separately by applying 25, 50, 75, and 100 g of neem seed powder directly to 10 L of water at room temperature (~25 °C) for each different fish weight group (Akinrotimi *et al*., 2015). The mixture was stirred with a glass rod for homogenous mixing, and after 15 min, the fish from different weight groups were placed in the anesthetic bath. The tanks were washed before preparing the next concentrations. The fish were not fed for a full day before the studies began (Brown, 2011).

Starting from when each fish was placed in the anesthetic bath until it reached stage 3 of anesthesia, as shown in Table 1, the time needed for each fish was recorded separately. The induction and recovery times were rounded to the nearest minute. Each fish was used individually and only once in the experiment. The test fish that became unbalanced were promptly removed and placed in a recovery tank with fresh, clean, aerated 10 L water as soon as stage 3 anesthesia was achieved. After being recovered, the fish were returned to their plastic tanks immediately, and their survival was monitored at 24 and 72 h after the treatment.

Blood parameters

Based on the results of the induction time to reach stage 3 of anesthesia, the last group of fish weights (80–89 g) was chosen for a blood test because they appeared to be most affected by the neem seed powder. Following the fish's total immobilization with the neem seed powder, 0.5 mL of blood were drawn from the caudal peduncle using separate, disposable, heparinized syringes and placed into heparinized sample vials to measure various blood parameters. The BC-2800 hematology analyzer (Mindray, Shenzhen Mindray Animal Medical Technology Company, China), a fully automated hematology analyzer for the Complete Blood Count test, was used for all hematological testing. A blood glucose test was performed using a clinical chemistry analyzer device, model ACCENT 200 (CORMAY, PZ CORMAY S.A Company, Poland). The following parameters were measured: red blood cell (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and white blood cell (WBC).

Statistical analysis

Normality of distribution and homogeneity of variances among different treatments were confirmed by the Shapiro–Wilk's test and Bartlett's test, respectively. The data obtained from the various parameters (induction and recovery time in minutes) were analyzed with a two-way ANOVA to evaluate the effects of neem seed powder concentrations and different weight groups of fish on the induction and recovery times. The data from hematological tests were subjected to a one-way ANOVA and Duncan's multiple range tests using XLSTAT, Pro 7.5 (Addinsoft company, France). A p-value less than 0.05 was considered statistically significant. All data are presented as mean±standard deviation (SD).

RESULTS

The analysis of variance revealed that the experimental fish reached sedation faster when neem seed concentration increased (Table 2); however, they recovered within similar period regardless of neem seed concentrations (Table 2). Fish size significantly affected both induction time and recovery time, with larger fish reaching sedation and recovering faster (Table 3). Additionally, the analysis showed significant interaction effects of the concentration and fish size ($p<0.05$). The results in Table 4 indicated that fish larger than 59 g treated with 10 $g \cdot L^{-1}$ neem seed powder reached

Table 1. Behavioral observations at various phases of anesthesia (Ross and Ross, 2008).

Stage	Exhibited behavior
0	Normal
1	Fish are relaxed and do not react to physical contact.
2	Fish exhibit unbalanced swimming and lose their equilibrium.
3	Fish show a loss of sensation, a complete loss of swimming motion, and a total loss of equilibrium.
Recovery	The fish restore their balance and swim normally again.

Table 2. Effects of neem seed concentrations on induction time and recovery time, averaged across fish size groups, for the experimental fish exposed to neem seed extract.

Neem seed concentrations	Induction times (min)	Recovery times (min)
2.5 $g \cdot L^{-1}$	18.33^{a}	14.54^{a}
5 $g \cdot L^{-1}$	15.54^{b}	14.87^{a}
7.5 $g \cdot L^{-1}$	12.98^{c}	15.05^{a}
10 $g \cdot L^{-1}$	10.58^{d}	15.48^{a}

Note: Means in each column superscripted with different lowercase letters are significantly ($p<0.05$) different.

Table 3. Effects of fish size groups on induction time and recovery time, averaged across neem seed concentrations, for fish exposed to neem seed extract.

Fish size groups	Induction times (min)	Recovery times (min)
Group 1 (20–29 g)	16.06a	18.08a
Group 2 (30–39 g)	15.15b	16.04b
Group 3 (40–49 g)	14.67b	15.84b
Group 4 (50–59 g)	14.29c	14.96c
Group 5 (60–69 g)	14.03c	14.25c
Group 6 (70–79 g)	13.27d	13.15d
Group 7 (80–89 g)	12.74d	12.88d

Note: Means in each column superscripted with different lowercase letters are significantly ($p<0.05$) different.

Table 4. Effects of treatment combinations (neem seed concentration × fish size group) on induction time and recovery time.

Concentrations	Size groups	Induction times (min)	Recovery times (min)
2.5 g·L^{-1}	Group 1 (20–29 g)	21.13±0.1^{a}	17.34±0.04^{b}
	Group 2 (30–39 g)	19.72±0.05^{b}	15.76±0.07^{cd}
	Group 3 (40–49 g)	18.28±0.05^{c}	15.47±0.06^{d}
	Group 4 (50–59 g)	17.98±0.06^{c}	14.72±0.07^{e}
	Group 5 (60–69 g)	17.66±0.05^{d}	13.83±0.04^{f}
	Group 6 (70–79 g)	17.10±0.04^{de}	12.73±0.05^{g}
	Group 7 (80–89 g)	16.85±0.04^{e}	12.10±0.04^{h}
5 g·L^{-1}	Group 1 (20–29 g)	17.32±0.09^{d}	18.23±0.03^{a}
	Group 2 (30–39 g)	16.00±0.06^{e}	15.95±0.05^{c}
	Group 3 (40–49 g)	15.77±0.05^{f}	15.85±0.05^{c}
	Group 4 (50–59 g)	15.80±0.02^{f}	14.93±0.04^{d}
	Group 5 (60–69 g)	15.83±0.03^{ef}	13.84±0.03^{ef}
	Group 6 (70–79 g)	14.17±0.04^{g}	12.98±0.06^{f}
	Group 7 (80–89 g)	13.81±0.03^{g}	12.23±0.03^{h}
7.5 g·L^{-1}	Group 1 (20–29 g)	13.77±0.02^{g}	18.08±0.02^{a}
	Group 2 (30–39 g)	13.21±0.03^{h}	16.17±0.03^{c}
	Group 3 (40–49 g)	13.30±0.03^{gh}	15.91±0.03^{c}
	Group 4 (50–59 g)	12.95±0.03^{h}	14.98±0.04^{d}
	Group 5 (60–69 g)	12.91±0.04^{h}	14.42±0.06^{e}
	Group 6 (70–79 g)	12.94±0.04^{h}	12.99±0.05^{f}
	Group 7 (80–89 g)	11.55±0.10^{i}	13.21±0.03^{f}
10 g·L^{-1}	Group 1 (20–29 g)	12.98±0.05^{h}	18.67±0.02^{a}
	Group 2 (30–39 g)	11.68±0.05^{i}	16.28±0.02^{c}
	Group 3 (40–49 g)	11.30±0.08^{i}	15.99±0.03^{c}
	Group 4 (50–59 g)	10.36±0.08^{j}	15.23±0.05^{d}
	Group 5 (60–69 g)	9.68±0.04^{jk}	14.73±0.03^{e}
	Group 6 (70–79 g)	8.91±0.06^{k}	13.91±0.06^{ef}
	Group 7 (80–89 g)	8.83±0.06^{k}	13.98±0.04^{e}

Note: Means in each column superscripted with different lowercase letters are significantly ($p<0.05$) different.

sedation in the shortest time (8.83±0.06–9.68± 0.04 min), while recovery time was moderate (13.91±0.06–14.73±0.03 min). Notably, no sick or death fish were observed in any experimental group (survival = 100%).

The blood parameters of common carp (sized 80–89 g) after exposed to the neem seed powder are presented in Table 5. The neem seed powder at doses of 5, 7.5, and 10 g·L^{-1} significantly decreased the RBC, HB, and HCT and increased the MCV, MCH, MCHC, and WBC levels in comparison to the control and the 2.5 g·L^{-1} groups. The fish immobilized with 10 g·L^{-1} of neem seed powder had the lowest values of RBC (0.28×1012 cells·L^{-1}), HB (39 g·L^{-1}), and HCT (5.6%). The greatest levels of MCV, MCH, and MCHC (213.33 fl, 166.66 pg, and 781.25 g·L^{-1}, respectively) were observed in the 7.5 g·L^{-1}group, whereas the highest level of WBC (67.2×109 cells·L^{-1}) and blood glucose (5.20 mmol·L^{-1}) were found when the 10 g·L^{-1}of neem seed powder was used.

Table 5. Effects of the neem seed powder on the blood parameters of common carp (80–89 g).

Blood parameters	Neem seed powder				
	Control	2.5 g·L^{-1}	5 g·L^{-1}	7.5 g·L^{-1}	10 g·L^{-1}
RBC (10^{12} cells·L^{-1})	1.30±0.02^{a}	1.18±0.04^{a}	0.49±0.06^{b}	0.30±0.09^{c}	0.28±0.08^{c}
HB (g·L^{-1})	55.00±0.09^{a}	57.00±0.01^{a}	54.00±0.04^{a}	50.00±0.02^{b}	39.00±0.03^{c}
HCT (%)	18.40±0.03^{a}	19.90±0.06^{a}	9.60±0.05^{b}	6.40±0.04^{c}	5.60±0.05^{c}
MCV (fl)	141.54±0.08^{b}	168.64±0.03^{b}	195.92±0.02^{a}	213.33±0.06^{a}	200.00±0.04^{a}
MCH (pg)	42.31±0.04^{c}	48.31±0.03^{c}	110.20±0.07^{b}	166.66±0.05^{a}	139.29±0.04^{ab}
MCHC (g·L^{-1})	298.91±0.06^{c}	286.43±0.06^{c}	562.50±0.09^{b}	781.25±0.08^{a}	696.43±0.04^{a}
WBC (10^{9} cells·L^{-1})	33.80±0.04^{b}	32.20±0.05^{b}	51.90±0.06^{ab}	65.00±0.08^{a}	67.20±0.06^{a}
Glucose (mmol·L^{-1})	1.51±0.03^{c}	1.82±0.06^{c}	1.30±0.06^{c}	2.80±0.07^{b}	5.20±0.05^{a}

Note: Mean±SD within a row superscripted with different lowercase letters are significantly ($p<0.05$) different.

DISCUSSION

Studies on common carp anesthesia using medicinal plants have been conducted (Taheri *et al*., 2016; Al-Niaeem *et al*., 2017; 2019; Abdulrahman *et al*., 2018 Rahanandeh *et al*., 2022). Yet there is no information available on the anesthetic efficacy of neem in common carp. In this study, the 10 g·L^{-1} of neem seed powder could anesthetize common carp within about 10 min. This finding is consistent with the results of a study done by Ahsan *et al*. (2016), in which they used 5, 10, 15, and 20% neem leaf juice to immobilize climbing perch (*Anabas testudineus*) and spotted snakehead (*Channa punctatus*). They stated that 5 to 10% neem leaf juice provided the fish with adequate time to remain immobile while handling live fish for vaccination or other uses. Comparing the current findings with earlier research on the use of medicinal plants to anesthetize common carp, Al-Hamadany *et al*. (2020) found that the poppy plant, *Papaver nudicaule*, at the concentrations of 200–250 mg·L^{-1} could anesthetize fish within 8 to 10 min, suggesting that its potency was lower than that of the neem seed powder in the current study.

According to Marking and Meyer's (1985) recommendation, fish should be given an anesthetic with an appropriate induction duration of less than 3 min and a recovery period of less than 5 min. This recommendation is often feasible for chemical anesthetics, which produce fast induction and recovery with a general dosage. The concentrations of active compounds in raw materials from natural plant parts (such as seeds, leaves, and flowers) are almost always lower than that of synthetic chemical anesthetics (Ramanayaka and Atapatu, 2006; Fotsing Yannick Stéphane *et al*., 2021) which may be the reason why fish require higher dosages and longer

induction times when natural anesthetics (plant-based) are used (Ramanayaka and Atapatu, 2006; l-Niaeem *et al*., 2017). Additionally, it has been shown that species, size, and water temperature all affect how effective anesthetic medications are (Cardenas *et al*., 2016).

Since quicker tranquilization was attained at greater doses, the neem seed powder's anesthetic activity seemed to be concentration-dependent. This finding is also consistent with recent research, which found that the concentration of anesthetic in fish central nervous system affects the time of anesthesia (Farahi *et al*., 2011; Hassan *et al*., 2016; Kavitha *et al*., 2016). According to Coyle *et al*. (2004), larger fish react more strongly to induced anesthesia. Consequently, smaller fish have longer recovery times following anesthesia induction, as demonstrated by the results of this study. When Jahanbakhshi *et al*. (2012) employed 2-phenoxyethanol as an anesthetic for two sizes of Persian sturgeon, *Acipenser persicus* (100 and 400 g), they saw the same outcome, demonstrating that the bigger fish were anesthetized and recovered faster than their smaller counterparts. This may be attributed to the relatively larger ratio of gill area to body mass in larger common carp (Oikawa and Itazawa, 1985), thus allowing for better penetration of anesthetic agents in larger fish. Additionally, larger fish may have more body fat, increasing the solubility of some anesthetics and resulting in shorter induction times (Coyle *et al*., 2004).

All fish groups showed no significant difference in the recovery times across the four neem seed powder concentrations; this finding is consistent with the other studies (Jahanbakhshi *et al*., 2012; Hoseini *et al*., 2013; Hassan *et al*., 2021). However, the smaller fish group recovered more slowly than the bigger fish group. This might be because of the long exposure to anaesthetic in smaller fish, and the larger fish were able to excrete the anesthetic sooner when put into clean water since they had a shorter induction time (Fries *et al*., 1993; Sladky *et al.*, 2001).

The hematological parameter data is a useful tool for evaluating fish health. Due to their high sensitivity to environmental changes, several hematological indicators can provide information about physiological issues before their external signs appear (Fazio, 2019). RBC, HCT, and HB levels were significantly decreased at higher anesthetic doses than at lower concentrations. This result confirms the findings of Saravanan *et al*. (2011), who reported that when Indian major carp (*Cirrhinus mrigala*) were exposed to neem leaf extract for 24 h, the values of several hematological parameters, including RBC, HB, HCT, MCV, MCH, and WBC, were significantly decreased in comparison to the control group. Higher amounts of neem may be cytotoxic (Saravanan *et al*., 2011) which might explain these changes in blood variables. Additionally, hemolysis, which produces hemodilution, a way to dilute hemoconcentration of the extracts and lessen the impact of the chemicals in their system—could be responsible for this decrease (Akinrotimi *et al*., 2015). It is possible that the erythroblastosis-induced loss of red blood cells, which results in anemia, is another reason for the significant decline in the hemoglobin content, hematocrit values, and RBC count of the treated fish (Saravanan *et al*., 2011). This study found that common carp also had alterations in their MCH, MCV, and MCHC values. These changes might be caused by red blood cell effusion or the discharge of young erythrocytes with lower hemoglobin levels into the bloodstream (Sobecka, 2001).

When fish are exposed to stress and chemical irritants, their white blood cell counts often rise or fall (Pimpao *et al*., 2007). In the current study, it is possible that the fish defensive systems were activated to counteract the anesthetic effect of the high neem seed powder concentrations, which led to an increase in white blood cells at such concentrations. According to Winkaler *et al*. (2007), gill injury caused an increase in WBC levels in the neotropical freshwater fish *Prochilodus lineatus* treated with neem leaf extract compared to the control.

Plasma glucose is a useful indicator in anesthetic research because it plays a significant role in the stress response that fish experience when exposed to various environmental conditions (Teixeira *et al*., 2017). Fish under anesthesia typically suffer

hypoxia from hypoventilation, and this stress increases the plasma glucose (Sneddon, 2012). In the current study, there were dose-dependent and significant alterations in the glucose levels during the induction periods. The findings are consistent with earlier research showing stress reactions brought on by anesthesia with various anesthetics in common carp (Hassan *et al*., 2016), Asian sea bass (*Lates calcarifer*) (Paradewi *et al*., 2021), and Nile tilapia (*Oreochromis niloticus*) (Yousefi *et al*., 2022). Pickering (1981) stated that a rise in corticosteroid hormones and plasma catecholamines might be the cause of the elevated blood glucose level. In research by Mousa *et al*. (2008), Nile tilapia subjected to 0.9 or 0.18 $g \cdot L^{-1}$ of neem leaf extract bath had significantly raised plasma glucose levels in a dose-dependent manner. According to them, neem leaf extract in water might cause a stress reaction, which could explain the rise in blood sugar.

Because some researchers discovered that neem products have some toxic effects on fish (Saravanan *et al*., 2011; Kumar *et al*., 2015; Adamu *et al*., 2018), more research on its safety in different fish species and various environmental conditions is needed before neem seed powder can be generally applied for anesthetic purposes. Furthermore, further studies are needed to evaluate the use of neem seed extracts instead of the whole seed as an ideal anesthetic to reduce the dosage and potential side effects.

CONCLUSIONS

This study showed that different-sized common carp could be successfully anesthetized with neem seed powder at doses of up to 10 $g \cdot L^{-1}$. As fish size increased, we observed reduced induction and recovery times. In all fish size groups, there was no mortality even at the highest tested dose (10 $g \cdot L^{-1}$). From these results, it can be concluded that *Azadirachta indica* seed powder has the potential to be used as a new anesthetic in common carp as an alternative to chemical anesthetics, suggesting a starting point for further investigations of its possible anesthetizing effect on different fish species.

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