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Acute and sub-acute toxicity of botanical oils on mortality, respiratory physiology and histopathology in *Poecilia sphenops*

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Abstract
The rising use of botanical biopesticides in integrated pest management demands rigorous evaluation of their ecotoxicological impacts on non-target aquatic organisms. This present study investigates the toxic effects of three plant-derived oils—neem oil (*Azadirachta indica*), pungam oil (*Pongamia pinnata*), and coconut oil (*Cocos nucifera*) on the *Poecilia sphenops* (Molly fish). Thus, commercial oils and fish are purchased from the Palayamkottai market and local aquariums from Tirunelveli, Tamil Nadu, India (latitude 8°43059 N and longitude77°4425 E). All the fish are maintained under the laboratory conditions. The acute toxicity (96 h) and subacute toxicity (21 days) assays were conducted at concentrations of different ppm (1, 5, and 10). And operculum movement monitoring and histopathological examination of gill and muscle tissues were carried out. The neem oil exhibited the highest toxicity value, with LC_{50} = 6.52 and LC_{90} = 12.84, followed by the Pungam Oil value of LC_{50} = 4.18 and LC_{90} = 8.92, while coconut oil proved negligible toxicity, with LC_{50} > 100. The operculum movement decreased significantly in a concentration- and time-dependent manner, with maximum respiratory depression observed at 10 ppm in Pungam oil 50 beats/min compared to 77 from the control group on the 4th day. During the subacute exposure, there was 100% survival for neem and coconut oils, whereas Pungam oil at 10 ppm caused 20% mortality. This study providing the botanical oil biopesticide like, the neem, Pungam, and coconut oil. And their differential toxicity profiles, particularly pungam oil's potent effects on *Poecilia sphenops*, demand careful dosage regulation in Agro-aquatic ecosystems to safeguard non-target fish species.

Keywords: *Poecilia sphenops*, biopesticide Azadirachtin acute toxicity histopathology operculum movement and ecotoxicology

Introduction

Ornamental fish production represents one of the fastest-growing sectors within global aquaculture, with trade volumes exceeding 15 billion annually and involving over 2,500 species worldwide (King, 2019; Uribe *et al*., 2018) [10,29]. The *Poecilia sphenops*, a euryhaline live-bearing species native to Central America, commands significant market demand due to its striking coloration, hardiness, and adaptability to diverse environmental conditions (Patel *et al*., 2017; Oliveira *et al*., 2014) [21, 19]. The species' physiological resilience, including tolerance to salinities ranging from freshwater to 80% and sulfidic habitats toxic to most metazoans, makes it an excellent model organism for ecotoxicology (Koutsikos, 2018; Riesch *et al*., 2015) [11, 24]. The increase of agricultural practices and domestic wastes mixing in the water bodies has caused widespread contamination of aquatic ecosystems through pesticide runoff, posing severe threats to non-target organisms. (Chaudhary *et al*., 2017; Roy Choudhury, 2016; Islam *et al*., 2026, Azhagu Raj *et al*., 2022, Azhagu Raj *et al*., 2023) [5,7,25,32,33,34]. Synthetic pesticides are effective, induce persistent environmental damage, bioaccumulation, and resistance development, necessitating alternative pest management strategies and need Eco-compatible alternative biopesticides (Pant *et al*., 2016; Mukherjee *et al*., 2026) [20, 14]. Botanical biopesticides have emerged as promising alternatives, with neem oil (*Azadirachta indica*) representing one of the most extensively studied natural products. Neem oil contains diverse bioactive limonoids, principally azadirachtin, which exhibits insecticidal, antifungal, and antibacterial properties through ecdysone receptor disruption and feeding deterrence (Nagini *et al*., 2024) [16].

The operculum's role in creating pressure differentials for water flow across gill lamellae makes it particularly sensitive to toxicants that impair gas exchange (Nilachandana, 2024) [18]. Operculum movement frequency constitutes a widely recognized indicator of respiratory health in aquatic toxicology, providing real-time insights into metabolic rate and stress responses (Sopinka *et al*., 2016) [27]. The Pungam oil (*Pongamia pinnata*), derived from a nitrogen-fixing leguminous tree native to the Indian subcontinent, contains furanoflavonoids such as karanjin and pongamol that demonstrate potent insecticidal and acaricidal activity (Ahmed *et al*., 2022; Raghav *et al*., 2019) [1,22].

The oil's non-edible nature and high calorific value have historically restricted its use to biodiesel production, although emerging applications in pest control raise environmental safety concerns (Mukta and Sreevalli, 2010) [15]. The coconut oil (*Cocos nucifera*), rich in medium-chain fatty acids, particularly lauric acid (45–52%), has nutritional benefits in fish diets without apparent toxicity (Wang *et al*., 2024) [30]. Therefore, this study aims to analyze the acute

and subacute effects like, mortality, respiratory physiology, and histopathology on *Poecilia sphenops* using botanical biopesticides.

Materials and Methods

Collection and Maintenance of Test Organisms

The *Poecilia sphenops* of uniform size and weight were purchase from a private local aquarium in Tirunelveli, Tamil Nadu, India (latitude 8°43059 N and longitude77°4425 E) (Figure. 1). Fish were acclimatized for four weeks in 70-L glass aquaria under controlled laboratory conditions: temperature 25 ± 1°C, 12:12 h light: dark photoperiod, and continuous aeration. During acclimatization, fish were fed commercial flake food twice during the study period. Dechlorinated tap water (pH 7.2 ± 0.2, dissolved oxygen 6.5–7.0 mg/L, hardness 120 ± 10 mg/L as $CaCO_3$) was used throughout the study.

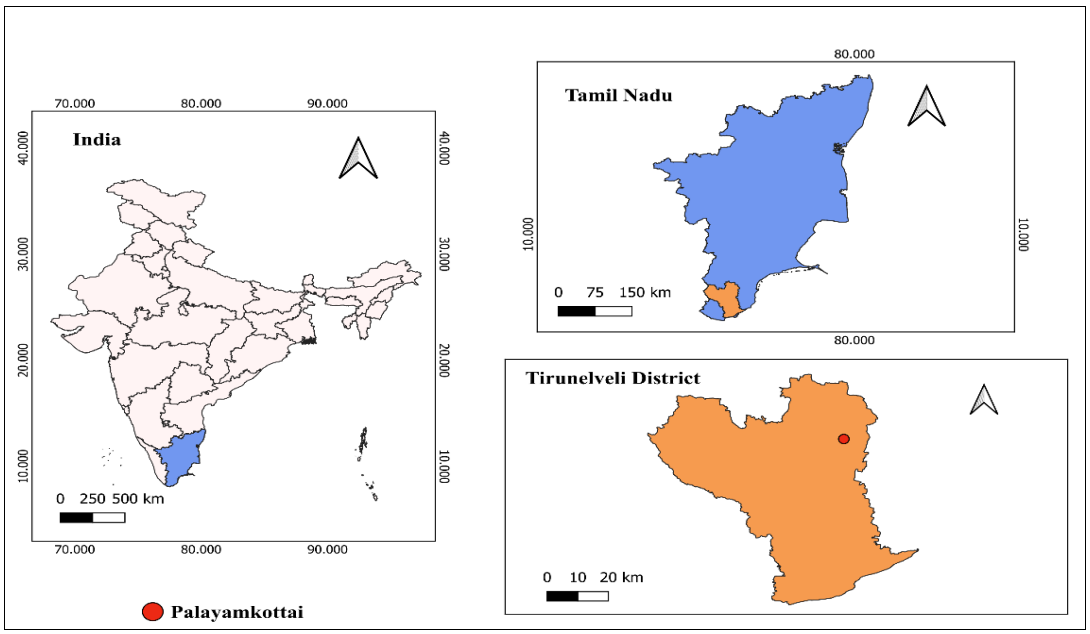


Fig 1: Study area Palayamkottai.

Morphometric Analysis

The total length (TL) was measured from the snout tip to the caudal fin end (±0.1 cm precision). Wet weight (WW) was determined using an electronic balance (±0.01 g precision) after blotting excess water.

Operculum Movement Assessment

The Operculum beats per minute (BPM) were counted visually using a stopwatch for 60-second intervals. Baseline measurements were recorded prior to exposure and subsequently at 24-hour intervals (Day 1–4) for acute toxicity and on Days 1, 7, 14, and 21 for subacute toxicity. Each fish was assessed individually in triplicate, with mean values calculated per treatment group.

Test Substances and Preparation

The commercial-grade neem oil (*Azadirachta indica*), pungam oil (Pongamia pinnata), and coconut oil (Cocos nucifera) were purchased from Palayamkottai market, Tirunelveli. Stock solutions (0.1 mL) were prepared by dissolving 10 mL emulsified oil with 2% Tween-20 emulsifier in 1 L distilled water. Working concentrations were prepared by serial dilution.

Experimental Design

Acute Toxicity Assay

The static renewal bioassays were conducted in 1.5-L trays containing 1 L test solution and 5 fish per replicate (n = 3 replicates per treatment). The concentrations tested were 1 ppm (low), 5 ppm (medium), and 10 ppm (high) for each oil, plus an emulsifier-only control (Fig. 1). Mortality was recorded, and dead fish were removed immediately during 24, 48, 72, and 96 hrs. Water was renewed every 24 hours with freshly prepared solutions. LC_{50} and LC_{90} values were calculated using probit analysis (Finney, 1971) [6] with 95% confidence limits.

Sub-Acute Toxicity Assay

The subacute concentrations (1:10 dilution of acute concentrations: 0.01, 0.05, and 0.1 mL/L) were administered for 21 days in static renewal systems. Test medium was renewed every 48 h; fish were fed daily. Survival was monitored throughout.

Histopathological Examination

The three fishes per treatment and control were sampled at 96 h (acute) and 21 days (subacute). The Gill and muscle tissues were dissected, washed in neutral saline and fixed in 10% neutral buffered formalin, and tissues were dehydrated through a graded ethanol series (70–100%), cleared in methyl benzoate, embedded in paraffin wax, sectioned at 5 µm thickness, and stained with hematoxylin-eosin (H&E) (Bancroft and Stevens, 1982; Bancroft *et al*., 1996) [3,4]. The sections were examined under the Olympus CH30 microscope at 10× magnification, and photographs were taken.

Statistical Analysis

The mortality data were analyzed by probit regression using SPSS v.16.0. Operculum movement and morphometric data were subjected to one-way ANOVA at the $p < 0.05$ significance level. All values are expressed as mean ± standard deviation (SD).

Results

Morphometric Characteristics

The experimental fish exhibited a mean total length of 4.12 ± 0.26 cm and a wet weight of 1.25 ± 0.12 g (n = 5), with a length-weight of healthy specimens (Table 1 and Figure 2 A and B).

Table 1: Length and weight parameters of *Poecilia sphenops*

Fish Sample	Total Length (cm)	Wet Weight (g)
Molly 01	4.2	1.25
Molly 02	3.8	1.10
Molly 03	4.5	1.42
Molly 04	4.0	1.18
Molly 05	4.1	1.30
Mean (±SD)	4.12 ±0.26	1.25 ±0.12

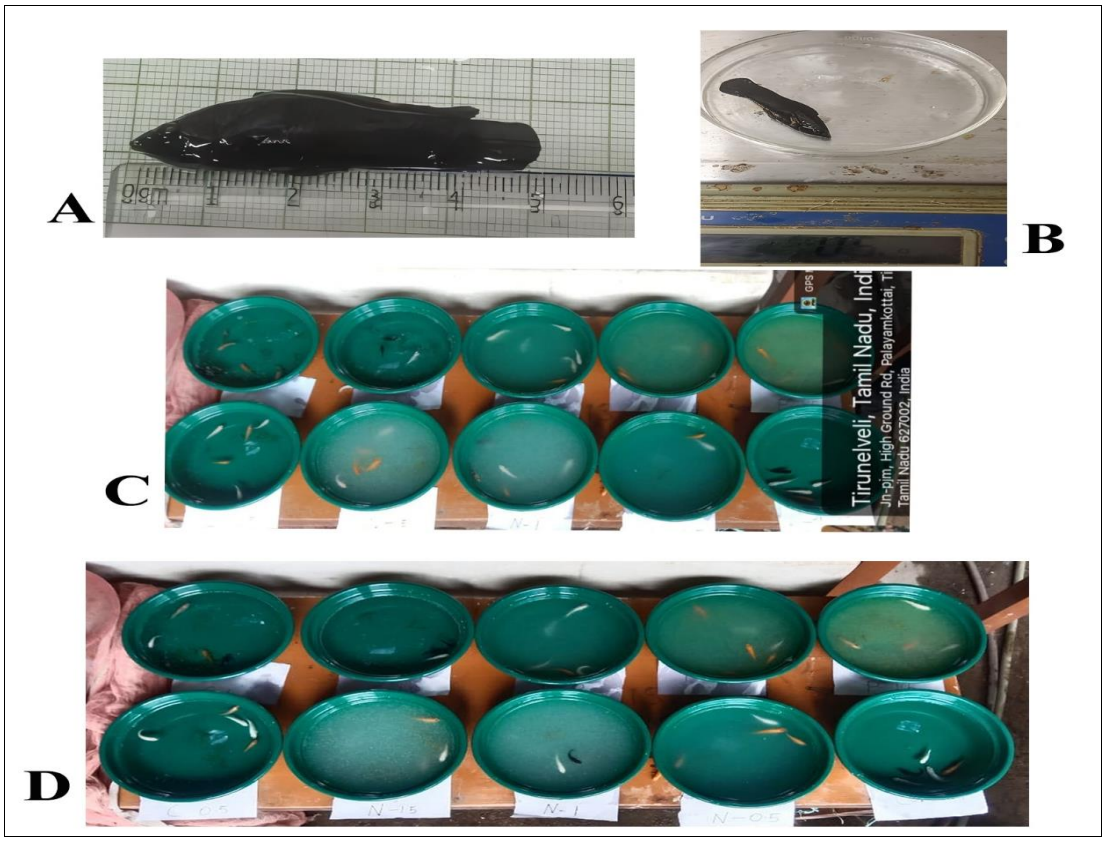


Fig 2: A – B *Poecilia sphenops* (Measuring the length and wight); C - Acute and D - Subacute Toxicity of Molly fish against Neem, Pungam and Coconut Oil.

Operculum Movement Response

The baseline operculum movement ranged from 78 to 80 BPM across all groups. The control fish maintained stable respiratory rates (77–80 BPM) throughout the 96-hour observation period, and treated groups exhibited concentration- and time-dependent depression of operculum movement (Table 2; Fig. 3). The Pungam oil induced the most severe respiratory depression, with high-concentration groups reaching 50 BPM on Day 4 (37.5% reduction from baseline). Neem oil showed intermediate effects (35% reduction), while coconut oil produced minimal changes (18.75% reduction), consistent with its lack of bioactive pesticidal compounds.

Table 2: Operculum movement (beats/min) of *Poecilia sphenops* exposed to botanical oils.

S. No	Treatment	Concentration	Before Exposure	Day-1	Day-2	Day-3	Day-4
1	Control	Nil	78	80	79	78	77
		Low	78	75	74	72	70
		Medium	79	72	70	66	62
2	Neem oil	High	80	68	64	58	52
		Low	78	74	73	71	69
		Medium	79	71	69	64	60
3	Pongam oil	High	80	66	62	56	50
		Low	78	77	76	75	74
		Medium	79	75	74	72	70
4	Coconut oil	High	80	72	70	68	65

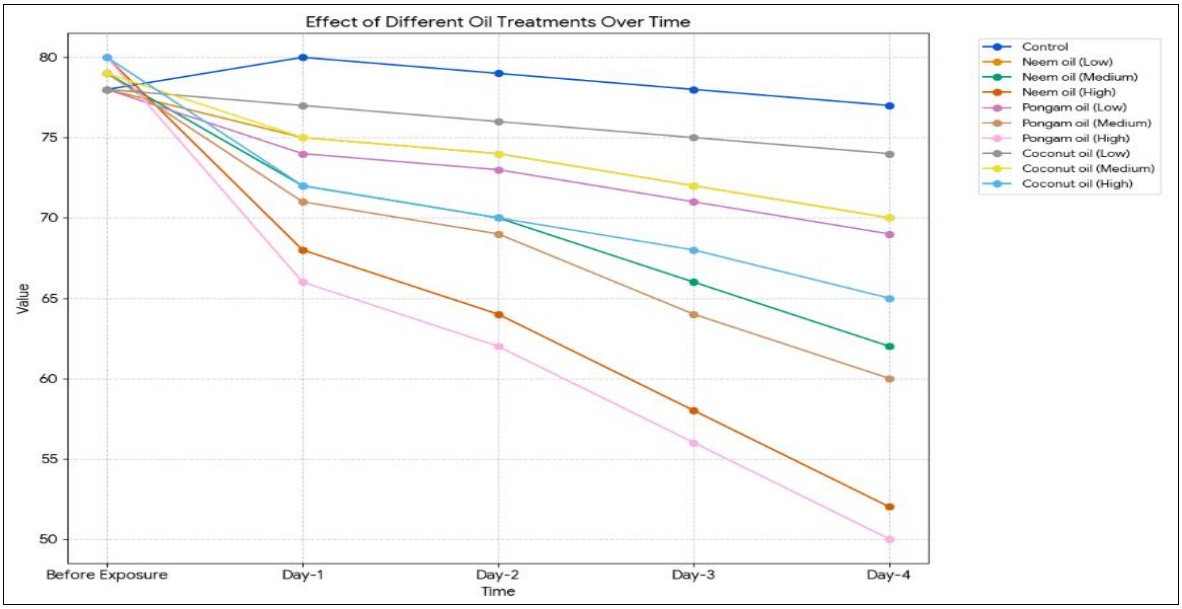


Fig 3: Operculum movement of *Poecilia sphenops*

Acute Toxicity and LC_{50} Analysis

The mortality patterns revealed marked differential toxicity among the three oils (Table. 3). The toxicity hierarchy was Pungam oil > Neem oil >> Coconut oil (non-toxic). The LC_{50} of Pungam oil (4.18 ppm) is 1.56-fold lower than that of neem oil's, indicating substantially higher acute lethality. The steep slope values (1.42–1.67) reflect rapid dose-response transitions, with small concentration increments producing large mortality increases.

Table 3: Cumulative mortality of *Poecilia sphenops* during 96-h acute exposure

S. No	Treatment Oil	Concentration	24 Hours	48 Hours	72 Hours	96 Hours
1	Neem Oil	Low (1 ml)	0	0	0	0
		Medium (5 ml)	1	0	0	0
		High (10 ml)	3	1	1	0
2	Pungam Oil	Low	0	0	0	1
		Medium	1	1	2	1
		High	5	0	0	0
3	Coconut Oil	Low	0	0	0	0
		Medium	0	0	0	0
		High	0	0	0	0

Table 4: The Probit-derived LC_{50} and LC_{90} values of botanical biopesticides.

Treatment Oil	LC50	95% Confidence Limit		LC90	95% Confidence Limit		Slope ± SE	Intercept ± SE	χ^2
		LL	UL		LL	UL			
Neem Oil	6.52	4.21	9.88	12.84	9.45	21.62	1.42 ± 0.32	2.11 ± 0.48	0.84*
Pungam Oil	4.18	2.76	6.35	8.92	6.11	15.74	1.67 ± 0.41	1.83 ± 0.52	0.71*
Coconut Oil	>100	–	–	–	–	–	–	–	–

Sub-Acute Toxicity

The subacute exposure at 1/10th acute concentrations revealed high survival rates across most treatments (Table 5). Pungam oil at 0.1 mL/L was the only treatment causing mortality (20%), confirming its threshold-dependent toxicity. Neem and coconut oils demonstrated complete safety at tested subacute levels (Figure 2A).

Table 5: Survival rates during 21-day sub-acute exposure

S. No	Treatment Oil	Concentration	No. of Fish Exposed	No. of Fish Dead	Survival (%)
1	Neem Oil	Low	5	0	100
		Medium	5	0	100
		High	5	0	100
2	Pongam Oil	Low	5	0	100
		Medium	5	0	100
		High	5	1	80
3	Coconut Oil	Low	5	0	100
		Medium	5	0	100
		High	5	0	100

Histopathological Analysis

Acute Toxicity (Gill Tissue)

The control gills displayed normal architecture with well-defined gill filaments (GF), regular lamellar arrangement, and intact epidermal epithelium (EP) (Fig. 4A). In Pungam oil exposure induced progressive damage: 1 ppm (low): mild congestion, dilatation of filaments, lamellar aneurysms, cartilage inflammation, epithelial lifting (EL), initial lamellar fusion (LF), and mild hyperplasia (Fig. 4B). The medium concentration of 5 ppm was significant hyperplasia in filament epithelia, degeneration of lamellae (DL), slight edema (SE), and pronounced congestion and dilation of blood vessels (BV) (Fig. 4C). The widespread necrosis (N), complete rupture of lamellae (RL), extensive tissue damage, vascular congestion (V), and total degeneration of gill filaments (DF) from high concentrations of 10 ppm (Fig. 4D).

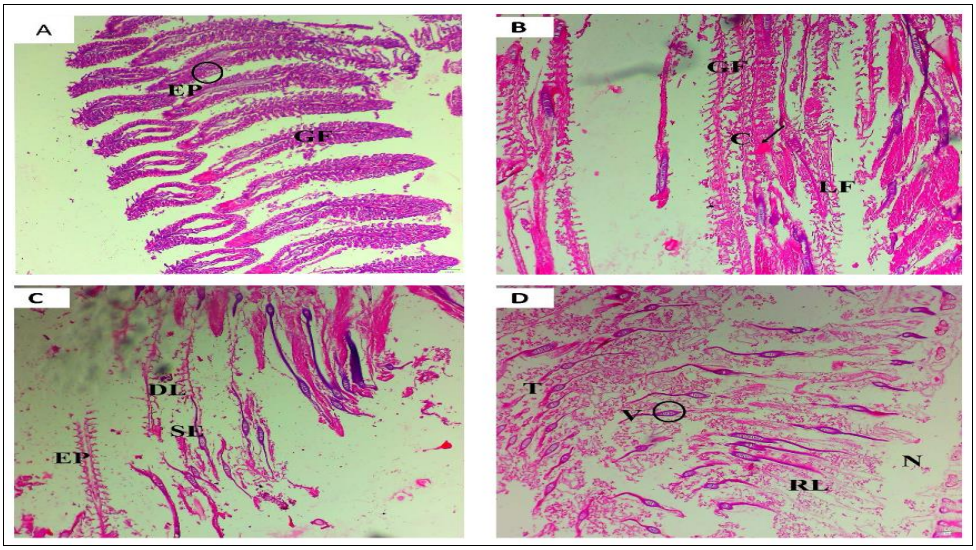


Fig 4: A – D Microscopic view of L.S section of gills from *Poecilia sphenops* at 10X magnification. (A) – Control group gill, B – D; Acute toxicity of Pungam oil experimental group from *P. sphenops* gill (B) - 1ppm, (C) – 5ppm, and (D) - 10 ppm. (GF - Gill filament, EP - Epithelial proliferatio, L - Lamellar fusion, T - Telangiectasia, V - Vacuolation, LF - Lamellar fusion, RL -Ruptured lamellae, SE – Slight edema, DL - Degenerated lamellae, N – Necrosis).

Acute Toxicity (Muscle Tissue)

The control muscle exhibited organized striated muscle (SM) fibers, distinct muscle fibers (MF), and regular nuclei (Nu) distribution (Fig. 5A). In Pungam, oil-induced damage is in all the ppm concentrations. From 1 ppm was loss of connective tissue (CT), myofibril clumping (MC), and broken muscle fibers (Fig. 5B). Almost in 5 ppm wide vacuoles (V), thickened muscle fibers (MP), hemocytic infiltration (HI), and continued fiber breakage (Fig. 5C). Furthermore, in 10 ppm, extensive necrosis (N), hemorrhage (H), hyalinization and fragmentation (HF), and persistent hemocytic infiltration are noted (Fig. 5D).

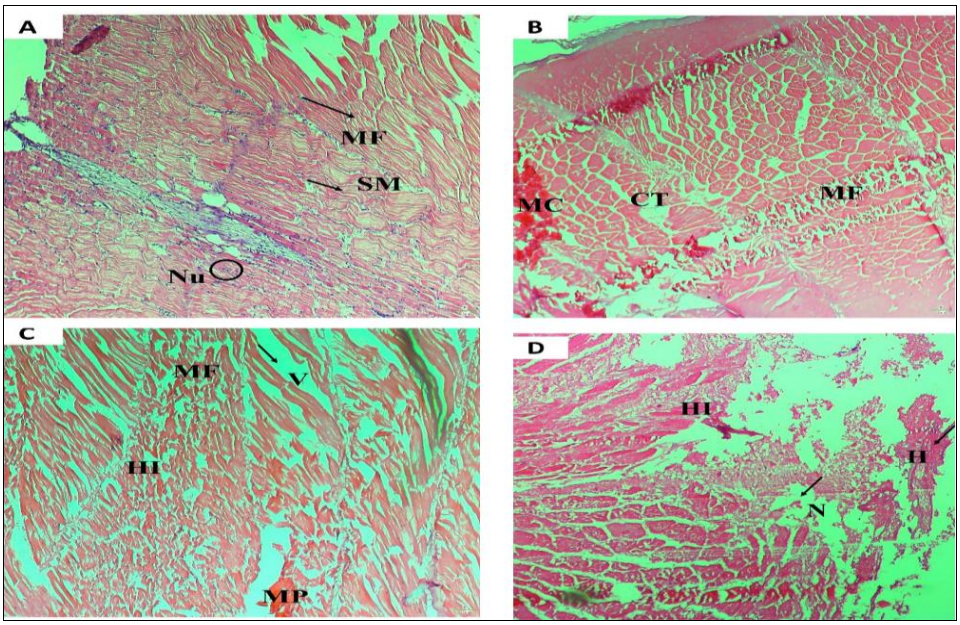


Fig 5: A – D Microscopic view of L.S section of muscles tissue *Poecilia sphenops* at 10X magnification. (A) – Control group, B – D; Acute toxicity of Pungam oil experimental group from *P. sphenops* muscle (B) - 1ppm, (C) – 5ppm, and (D) - 10 ppm. (MF – Muscle fibre, N - Necrosis, SM - Skeletal muscle, CT - Connective tissue, MP - Muscle separation, V - Vacuolation, HI – Haemocyte infiltration, H – hyalinization, HF - Hypertrophied fibre, and Nu - Nucleus).

Sub-Acute Toxicity

The subacute exposure to 10 mL/L Pungam oil caused severe lamellar fusion (LMF) with secondary lamellae merging into continuous tissue masses and degeneration of lamellae (DL) (Fig. 6 A and B). The muscle tissue showed architectural disruption (AD) with wavy, disconnected bundles and fragmented muscle fibers (FMF) (Fig. 6 AA and BB), indicating permanent myopathic damage.

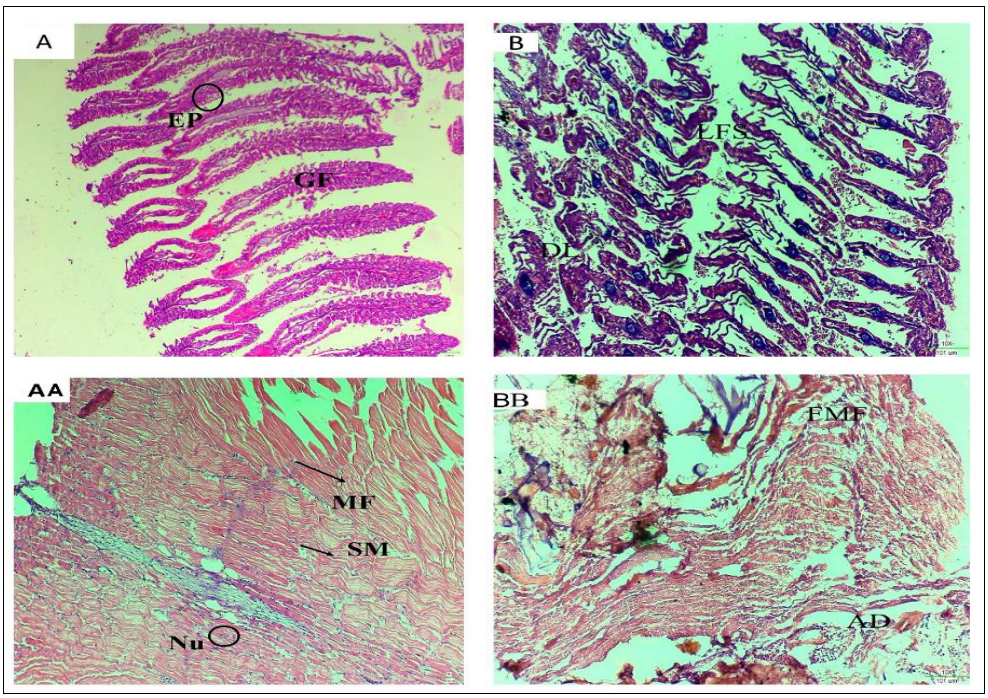


Fig: 6. A – B: The microscopic view of the *Poecilia sphenops* gills, **(A)** – Control group and **(B)** – Pungam oil experimental group at 1/10 ml. **AA – BB**: The microscopic view of the Muscle tissue of *P. sphenops*, (**AA** – Control group and **BB** - Pungam oil experimental group at 1/10 ml). (GF – Gill filament, EP – Epithelial proliferation, DL - Degenerated lamellae, LF – Lamellar fusion, MF – Muscle fibre, Nu – Nucleus, SM – Skeletal muscle, and FMF – Fragmented muscle fibres, and AD - Adipose deposits).

Discussion

The present study shows the clear toxicity hierarchy among botanical oils tested on *P. sphenops*, with pungam oil exhibiting the highest acute lethality, followed by neem oil, while coconut oil proved essentially non-toxic. The normal operculum movement range of 60–120 BPM in teleosts reflects adequate gas exchange efficiency (Nilachandana, 2024) [18]. During the operculum movement study, it has served as a critical physiological indicator of respiratory distress in toxicant-exposed fish, and particularly the 37.5% reduction in pungam oil-treated fish suggests impaired branchial oxygen uptake likely stemming from gill epithelial damage and increased diffusion distances. This pattern aligns with established toxicological responses where fish initially increase ventilation to compensate for hypoxic stress, followed by exhaustion-mediated depression as cellular energy reserves are depleted (Svobodova *et al*., 2003) [28]. Sarkar and Saha (2018) [26] Observed that *Cyprinus carpio* fry exposed to spinosad, where operculum movement increased at 24–48 h but declined significantly at 72–96 h, reflecting the compensatory-exhaustion sequence. The pungam oil potency approaches that of spinosad, which recorded a 96-h LC_{50} of 1.77 mg/L in common carp fry (Sarkar and Saha, 2018) [26] and induced neural excitation through nicotinic acetylcholine receptor disruption (Amaral *et al*., 2024) [2]. Yadav *et al*. (2019) [31] reported that the 1.717 mL/L (96 h) in *Danio rerio*, while Moghadam *et al*. (2023) [12] found 1.19 mg/L for Neem Azal in *Cyprinus carpio*, the similar probit slopes (1.42–1.67) indicate narrow safety margins where small concentration elevates the produce's uneven mortality, which increases a characteristic shared with other biopesticides. Raghav *et al*. (2019) and Ahmed *et al*. (2022) [1,22] reported that Pungam oil's superior toxicity likely derives from its furanoflavonoid constituents, particularly karanjin, which exhibits potent insecticidal activity through mitochondrial disruption and oxidative stress generation. The "green" reputation of biopesticides, as their rapid biodegradation and non-persistence prevent long-term bioaccumulation (Kilani-Morakchi *et al*., 2021) [9]. Wang *et al*. (2024) [30] reported that the dietary coconut oil replacement of fish oil did not affect growth or survival in orange-spotted groupers, corroborating our finding of coconut oil's aquatic safety. Mohamed *et al*. (2023) and Ramachandramohan and Mamatha (2015) [13,23] analyze that the lamellar aneurysms and vascular congestion reflect pillar cell rupture and circulatory disruption was noted in *Glossogobius giuris* exposed form neem oil. Using the biopesticides are not toxic in rat and it's compared with control groups (Ismail and Mohamed, 2012) [8] in some case of biopesticides it causing damages a tissue at higher concentration level (Nasiruddin *et al*., 2013) [17].

Conclusion

This study provides the first comprehensive toxicological profile of neem, pungam and coconut oils on the ornamental fish *Poecilia sphenops*, integrating acute lethality, sub-acute survival, respiratory physiology and histopathological. These results demonstrate that the "natural" designation does not confer universal safety for botanical biopesticides in aquatic ecosystems. Pungam oil, in particular, requires rigorous management to prevent non-target fish mortality in integrated agro-aquatic systems. Future research should

explain the specific mechanisms of karanjin toxicity in fish, further strengthen environmental protection for non-target aquatic species.

Declaration of AI Use

The author(s) acknowledge the use of Quillbot to assist with grammar checking.

Competing Interests

Authors have declared that no competing interests exist.

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Authors' Contributions

Conceptualization: RA. Data curation: RA. Formal analysis: GSM, RA. Investigation: RA, Methodology: GSM. Resources: RA. Supervision: RA. Visualization: RA, GSM. Project administration: RA. Software: GSM, IK. Validation: GSM. Writing – original draft: GSM. Writing – review and editing: GSM, RA.

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