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Synergistic Effect of Chlorpyrifos and Mancozeb on The Survival of *Poecilia reticulata*

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Abstract. Pesticides are essential in agricultural pest control, but may also harm non-target organisms. In Temanggung Regency, Indonesia, interviews with tobacco farmers revealed that the insecticide Dursban 200 EC (chlorpyrifos) and the fungicide Manzate 82 WP (mancozeb) are most commonly used, leading to the emission to the aquatic environment of both active ingredients, often resulting in mixtures. The present study aimed at determining the acute toxicity of chlorpyrifos, mancozeb, and their combination to guppy fish (*Poecilia reticulata*). A 96 h survival assay was conducted on male guppies exposed to the individual pesticides and their equitoxic mixtures. Mortality data were analyzed using a logistic dose-response model, and the toxicity of the mixtures was analyzed using the Concentration Addition (CA) and Independent Action (IA) models. Chlorpyrifos was slightly more toxic to *P. reticulata* than mancozeb, with LC_{50}s of 1.81 and 3.45 mg a.i./L, respectively. The combination of chlorpyrifos and mancozeb resulted in enhanced toxicity due to synergistic interaction according to the CA model, which suggests different modes of action of the two active ingredients. When analyzed using the IA model, the interaction, however, was also synergistic. These findings highlight the need to assess both single and combined pesticide exposures in ecological risk evaluations and emphasize cautious use of pesticide mixtures to protect aquatic ecosystems.

Keywords: carbamate; equitoxic mixture; guppy fish; organophosphate; pesticide mixtures

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INTRODUCTION

The application of pesticides in agriculture is considered essential for pest control and optimizing crop production. Ideally, pesticides should act selectively and specifically on target organisms (Lushchak et al., 2018). However, most active ingredients have broad-spectrum activity and lack specificity, making them potentially toxic to non-target organisms (Hill et al., 2017).

Repeated use of pesticides can lead to resistance in organisms (Hawkins et al., 2019), prompting farmers to combine or mix pesticides to delay or overcome the development of pest resistance (Madgwick & Kanitz, 2023). In practice, the use of more than one pesticide active ingredient can pose environmental risks. Pesticide mixtures can produce additive, synergistic (potentiating), or antagonistic effects (Martin et al., 2021). It is estimated that only 0.1% of applied pesticides reach the target pests, while the remainder enter and potentially pollute the environment (de Castro Lima et al., 2020). Once released, pesticides can evaporate, be washed away by rain, and be transported into aquatic ecosystems (de Souza et al., 2020). Due to their stability, many pesticides may persist in water bodies, air, rain, and soil (Lushchak et al., 2018). When pesticides enter aquatic environments, they can cause histopathological changes, developmental disorders, mutagenesis, carcinogenicity, and even mortality of aquatic organisms, including fish (Hamid et al., 2020).

Interviews with 20 tobacco farmers in Windusari Hamlet, Temanggung Regency, Indonesia, revealed that two of the most widely used pesticides contain the active ingredients chlorpyrifos and mancozeb. Chlorpyrifos is an organophosphate insecticide known for its volatility, yet it remains environmentally toxic. Chlorpyrifos is a non-systemic insecticide intended to function by contact with skin, inhalation, or ingestion (Wołejko et al., 2022).

Ambareen & Venkateshwarlu (2023) reported a 96-hour LC_{50} value of chlorpyrifos to *Cyprinus carpio* of 0.318 mg/L, while Jeon et al. (2016) found a 96-hour LC_{50} value to *Danio rerio* of 0.709 mg/L. Chlorpyrifos elicits toxicity through inhibition of the enzyme acetylcholinesterase (AChE), leading to acetylcholine accumulation (Miller et al., 2021) and consequent induction in the central nervous system of excessive parasympathetic effects (Greer et al., 2019). Chlorpyrifos concentrations ranging from 1.8 to 280 µg/L cause the fish to become intoxicated with symptoms including muscular paralysis, hyperactivity, and loss of balance (John & Shaike, 2015). AChE activity was decreased, and lipid peroxidation was raised in adult *Litopenaeus vannamei* exposed to 2.10 µg/L of chlorpyrifos for 96 hours (Duarte-Restrepo et al., 2020). Similarly, 25.0 µg/L of chlorpyrifos reduced AChE and caused oxidative stress in *Oreochromis niloticus* (Majumder & Kaviraj, 2019). *Rasbora lateristriata* fish exposed to chlorpyrifos revealed various behavioral impairments in swimming and feeding responses (Ayu & Retnoaji, 2022).

Mancozeb is the common name of a manganese/zinc-based ethylene-bis-dithiocarbamate fungicide (Costa-Silva et al., 2018; Gürol et al., 2020). Mancozeb completely inhibits the germination of fungal conidia at levels of 2,957.4 µM (Rosero-Hernández et al., 2019). Even though mancozeb is particularly employed for fungal management, it also affects non-target organisms. The predominant effect of carbamate insecticides is that they inhibit AChE in the central nervous system of insects by metabolites such as ethylenethiourea and carbon disulfide (Campanale et al., 2023), so in fact acting in the same way as chlorpyrifos. As a result, mancozeb may also be toxic to aquatic organisms like fish. The 96-hour LC_{50} of mancozeb to *Clarias gariepinus* juveniles was 411 mg/L (Chijioke et al., 2024) and 11.49 mg/L to *O. niloticus*, where it induced behavioral change, neuro-ethology disruption, hepato-renal dysfunction, and immune-oxidative disturbance (Ibrahim et al., 2023). Exposure of goldfish *Carassius auratus* to 3–10 mg/L of mancozeb leads to mild oxidative stress, elevated antioxidant enzyme activity, and high protein carbonyl and lipid peroxide levels (Atamaniuk et al., 2014).

Toxicity testing is employed to determine the possible hazard of pesticide contamination of water bodies (Ceger et al., 2023). Acute toxicity tests establish the harmful effects of a substance within 24–96 hours of exposure (Katsiadaki et al., 2021). Guppy fish (*Poecilia reticulata*) is the most commonly used as a standard test species in toxicity testing (Almeida et al., 2019; Çelebi & and Gök, 2018; Forouhar Vajargah et al., 2020; Rabelo et al., 2021; Salako et al., 2020), because of its good adaptability to contaminated water (Santi et al., 2021) and wide distribution in aquatic ecosystems (Sirimanna and Dissanayaka, 2019).

The objective of this study was to determine the acute toxicity to guppy fish (*P. reticulata*) of single and combined exposures to the insecticide chlorpyrifos and the fungicide mancozeb. The combined effects of chlorpyrifos and mancozeb on adult male guppy mortality have not yet been documented. That gap is filled by this study. Here, we report the acute effects of individual and equitoxic mixtures of chlorpyrifos and mancozeb on the survival of adult male guppies. Our findings indicate that chlorpyrifos is more toxic to guppies than mancozeb. Moreover, we observed a significant synergistic interaction when the two compounds were combined, assessed with the reference models of Concentration Addition (CA) and Independent Action (IA).

METHODS

Test Animal and Pesticides

The test animals used in this study were adult male guppy fish (*P. reticulata*), measuring 1.5–2 cm in length. The fish were collected from an unpolluted river in Salatiga, Indonesia, and acclimated in aquaria filled with well water. All experimental procedures involving live animals were conducted in accordance with ethical standards, including those outlined in the Declaration of Helsinki. During the 14-day acclimation period, guppies were fed *ad libitum* twice daily and provided with continuous aeration. Throughout the toxicity tests, water quality parameters including temperature, pH, and dissolved oxygen were monitored and recorded daily. Two commercial pesticide formulations were used: Dursban 200 EC, containing 200 g/L of chlorpyrifos, and Manzate 82 WP, containing 82 g/L of mancozeb.

Acute Toxicity Tests

Acute toxicity tests were conducted using both the individual pesticides and their binary mixtures. The experiments were performed in 4 L glass aquaria, each containing 2 L of well water and five male guppies. Aeration was provided continuously, and no food was administered during the 96-hour exposure period. To determine LC_{10} and LC_{50} values, five concentrations were tested for each pesticide: chlorpyrifos at 0, 0.375, 0.75, 1.5, and 3 mg a.i./L; and mancozeb at 0, 0.75, 1.5, 3, and 6 mg a.i./L, while the concentrations used for the chlorpyrifos+mancozeb mixture tests were 0+0, 0.375+0.75, 0.75+1.5, 1.5+3, 3+6 mg a.i./L. Each concentration was tested in triplicate.

Data Analysis

The 96-hour mortality data of single pesticide exposures were fitted using a three-parameter logistic dose–response model (Haanstra et al., 1985) in order to estimate the 96-hour LC_{10} and LC_{50} values and the corresponding 95% confidence intervals. All statistical analysis was performed using IBM SPSS Statistics version 22. The Concentration Addition (CA) model, using the Toxic Unit (TU) approach, was applied to analyze the toxicity of pesticide mixtures (Hepditch et al., 2021; Martin et al., 2021). This approach has been widely recognized for its potency in ecotoxicological risk assessment (Adnan et al., 2021; Kim et al., 2025; Li et al., 2020; Omotola et al., 2023). According to this model, the TU is defined as the ratio between the concentration of a chemical in a mixture and a measure of its toxicity, e.g. its LC_{50}. When the sum of the toxic units of all chemicals in a mixture equals 1, the mixture is expected to cause 50% mortality. This is called additivity. If the mixture, however, is less toxic than expected, so producing less than 50% at 1 TU, it is antagonistic, and it is synergistic when it produces a higher toxicity than expected. The CA model is generally applied when chemicals are expected to have the same mode of action. When chemicals in the mixture have a different mode of action, the IA model may be more appropriate. In this study, the mixture toxicity to fish survival was compared with the CA and the IA models using the MixTox framework of Jonker et al. (2005). This model enables the comparison of toxic effects observed with those estimated from these reference models by incorporating deviation functions for detecting and characterizing synergism or antagonism (S/A), and also dose-level dependent (DL) deviations from S/A.

RESULTS AND DISCUSSION

Water Quality

The dissolved oxygen concentrations in the experiments varied from 7.6 to 7.7 mg/L. The water pH varied from 6.24 to 6.37. The water temperature was 26 ± 0.5°C throughout the experimental period.

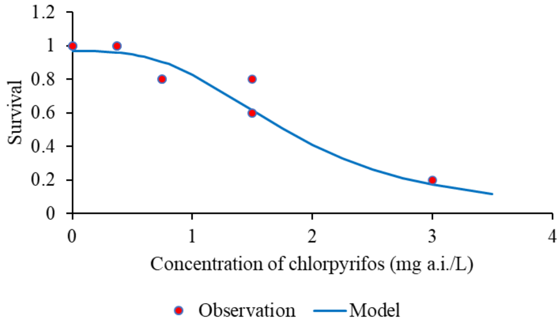
Test conditions in the experiment met the requirements set by OECD Guidelines for Testing of Chemicals for fish acute toxicity tests (OECD, 2019). Water quality parameters remained stable during the exposure period, ensuring the reliability of pesticide toxicity assessments in guppies. Maintaining optimal levels of dissolved oxygen, pH, and temperature is essential, as fluctuations in these parameters can influence the physiological responses of fish and potentially affect pesticide toxicity (Okpala-Ezennia et al., 2025).

Single Toxicity of Pesticides

No mortality was observed in the controls after 96 h, confirming the tests were valid. Figure 1 shows the responses of the guppies to chlorpyrifos and mancozeb. The acute effects of chlorpyrifos and mancozeb could be fitted by a logistic dose-response mode, giving 96 h LC_{10} and LC_{50} and 95% confidence interval values for chlorpyrifos of 0.87 (0.54–1.20) mg a.i./L and 1.81 (1.51–2.10) mg a.i./L, respectively, for mancozeb of 0.66 (0.10–1.21) mg a.i./L and 3.45 (2.35–4.56) mg a.i./L, respectively. This finding is consistent with previous studies that have reported higher toxicity of chlorpyrifos compared to mancozeb in various organisms, including catfish (*Clarias gariepinus*) (Kanu, 2023), Nile tilapia (*O. niloticus*) (Kanu, 2023), the tropical earthworm (*Perionyx excavatus*) (De Silva et al., 2010), and *Allium cepa* (Fatma et al., 2018).

Various studies have provided 96 h LC_{50} values of chlorpyrifos and mancozeb as active ingredients or within formulations for diverse fish species. For example, the 96 h LC_{50} for the toxicity of chlorpyrifos was 3.83 mg/L for zebrafish (*Danio rerio*) (Fan et al., 2021) and 48.6 µg/L for Nile tilapia (*Oreochromis niloticus*) (Hossain et al., 2022). Our findings strongly agree with the 96 h LC_{50} of 1.79 mg/L for chlorpyrifos-methyl in guppies (*P. reticulata*) reported by Selvi et al. (2005). The agreement in this confirms our finding and the sensitivity of guppies to chlorpyrifos.

For mancozeb, while there is limited research on guppies, similar studies with other fish presented 96 h LC_{50} values of 2.29 mg/L for Pacamã (*Lophiosilurus alexandri*) (Silva et al., 2023) and 11.5 mg/L for *O. niloticus* (Ibrahim et al., 2023). The difference in LC_{50} values between guppies and other fish species reflects species-specific sensitivities and perhaps also effects of differences in exposure conditions. These differences can result from variations in metabolic rate, detoxification processes, and habitat conditions.



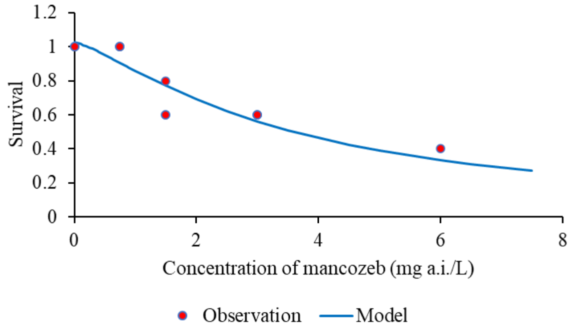


Figure 1. Dose-response curves for the effects of chlorpyrifos and mancozeb on the survival of *Poecilia reticulata* after 96 h exposure. Each dot shows the observed survival

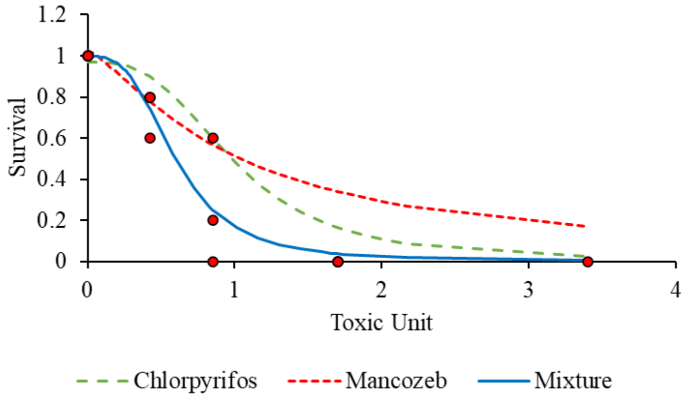


Figure 2. Comparison of the toxicity of chlorpyrifos, mancozeb, and their binary mixtures to the survival of *Poecilia reticulata* after 96 h of exposure, with concentrations expressed as Toxic Unit (TU). Each dot shows the response of the guppies exposed to the equitoxic pesticide mixtures after 96 h of exposure

In addition, physicochemical properties of the test medium and biological factors such as the physiological status and activity of the test species can explain differences in LC_{50} values between species (Ray & Shaju, 2023; Spurgeon et al., 2020). Therefore, even data for a single species cannot capture an integrated perception of the ecological hazards posed by pesticide exposure.

Mixture toxicity of pesticides

The results of the tests with the binary mixtures of chlorpyrifos and mancozeb on the survival of the guppy were first analyzed using the reference model of CA. Results are shown in Table 1. These results suggest a synergistic interaction (a = -2.70, p = 0.000) without significant dose-level dependent deviations. To better enable understanding of this finding, Figure 2 shows the response of the guppies exposed to single pesticides and the equitoxic pesticide mixtures after 96 h exposure, with the exposure concentrations expressed in TU. Chlorpyrifos, mancozeb, and chlorpyrifos + mancozeb LC_{10} and LC_{50} and 95% confidence interval values were 0.48 (0.30–0.67) TU and 1.00 (0.84–1.17) TU, 0.19 (0.03–0.35) TU and 1.00 (0.68–1.32) TU, and 0.30 (0.14–0.45) TU and 0.60 (0.45–0.75) TU, respectively. Therefore, the LC_{10} and LC_{50} values for the toxicity of the mixtures were lower than the values for the individual pesticides. This indicates that the toxicity of the mixture was higher than expected based on the toxicity of chlorpyrifos and mancozeb tested individually, suggesting a synergistic interaction of the compounds.

This synergistic interaction was not expected, as both pesticides share a similar mode of action, namely the inhibition of acetylcholinesterase (AChE) activity in fish (Miller et al., 2021). Therefore, the IA model was run in the MixTox tool, and this also resulted in a synergistic interaction (a = -2,67, p = 0.005), also without further dose-level dependent deviations (Table 1).

Table 1. Results of the analysis of the effects of equitoxic mixtures of chlorpyrifos and mancozeb on the survival of *Poecilia reticulata* exposed for 96 h

			S/A		D/L	
	CA	**IA**	**CA**	**IA**	**CA**	**IA**
Max	1.00	1.00	1.00	1.00	1.00	1.00
$\beta_{Chlorpyrifos}$	3.01	3.01	3.01	3.01	3.01	2.62
$\beta_{Mancozeb}$	1.32	1.32	1.32	1.32	1.32	1.56
LC_{50}Chlor (mg a.i./L)	1.80	1.80	1.80	1.80	1.80	1.63
LC_{50}Manc (mg a.i./L)	3.45	3.45	3.45	3.45	3.45	3.49
a			-2.70	-2.67	-2.70	-2.33
b					0.00	-0.55
Chi = $p(\chi^2)$			0.000	0.005	1.000	0.141
R^2	0.804	0.804	0.835	0.860	0.835	0.875

Note: The data analyzed used equations detailed in Jonker et al. (2005). Chlor = chlorpyrifos; Manc = mancozeb; CA and IA are the reference models of Concentration Addition and Independent Action; S/A is synergism/antagonism, and DL is dose-level deviation from the reference model; *a* and *b* are parameters of the deviation functions; $p(\chi^2)$ is the result of the likelihood ratio test; R^2 is the coefficient of determination.

Synergistic toxicity between organophosphate and carbamate pesticides has previously been documented. For example, studies on juvenile Pacific salmon (*Oncorhynchus* spp.) showed that mixtures of these pesticide classes led to enhanced neurotoxicity (Laetz et al., 2009). Similar synergistic effects between chlorpyrifos and mancozeb have been reported in *C. gariepinus* and *O. niloticus* (Kanu et al., 2022; Kanu, 2023).

Interestingly, Jacobsen et al. (2004) found that while co-administration of mancozeb did not enhance chlorpyrifos-induced AChE inhibition in plasma and brain, effects were evident in other tissues such as the liver, thyroid, thymus, and blood of Wistar rats. Additionally, Banaee et al. (2023) reported that mancozeb significantly reduced lactate dehydrogenase and gamma-glutamyl transferase activities, as well as hepatic glycogen content in zebrafish (*D. rerio*) exposed to a mixture of mancozeb and metalaxyl. Laetz et al. (2009) also documented synergistic interactions of organophosphate and carbamate pesticide mixtures on Pacific salmon, which led to behavioral impairments crucial for survival.

The observed synergistic effects may result from the inhibition of detoxification enzymes (Alejo-González et al., 2018). Chlorpyrifos, as an organophosphate, inhibits AChE, resulting in neurotoxicity. When combined with other pesticides like mancozeb, the toxicity may be exacerbated due to additive or synergistic effects on AChE or interference with other detoxification pathways (Srinivasulu & Rangaswamy, 2013). Notably, some fungicides can inhibit cytochrome P450 enzymes, key mediators of xenobiotic metabolism, potentially enhancing the toxicity of co-applied pesticides through metabolic interference (Nørgaard and Cedergreen, 2010).

It is critical to note that this study did not evaluate the potential influence of adjuvants commonly present in commercial pesticide formulations, such as solvents, dispersants, emulsifiers, and other additives (Ohkouchi & Tsuji, 2022). While these components are often labeled as inert, they may interact with the active ingredients and modify their biological behavior, potentially intensifying their toxic effects. The mechanisms by which these formulation additives contribute to overall toxicity remain poorly understood. Therefore, further studies are required to elucidate the role of these so-called inert ingredients, particularly diluents and emulsifiers, in modifying the toxicological profile of pesticide mixtures (Lackmann et al., 2021).

This research addresses the largely unexplored area of combined pesticide toxicity by studying the effects of chlorpyrifos and mancozeb mixtures on guppy fish. While the individual toxicities of these pesticides are known, their potential synergistic or antagonistic interactions in mixtures have been less examined, particularly in aquatic organisms. Using advanced analytical models, including the CA and IA reference models, this study quantified these interactions and provided the first reported LC_{10} and LC_{50} values for guppies exposed to these pesticide combinations. This provides a useful contribution to addressing challenges in pesticide ecotoxicology and highlights the ecological risk connected with exposure to dangerous pesticides.

The novelty of this current study emanates from the direct connection to pesticide practices among farmers in Indonesia. Chlorpyrifos residue of 0.05 mg/kg (Kurnia et al., 2024) and mancozeb residue of 1.18 mg/kg (Zu'amah et al., 2024) has been detected in agricultural soils in Central Java, Indonesia. Through the linking of laboratory analysis with real field conditions of agriculture, it elucidates the potential ecological effects of pesticide mixtures in water bodies. It also offers essential suggestions for policymakers, urging the adoption of more sustainable and decision-oriented practices for pesticide use.

This research makes significant contributions and yields benefits both to scientific understanding and to the welfare of society. Scientifically, it addresses a significant knowledge gap in ecotoxicology through research on the combined toxicity of chlorpyrifos and mancozeb, two of the most applied pesticides in agriculture. Through the use of sound methodologies like the TU approach in the CA reference model and the IA reference model, this research advances significant details regarding the interaction of these pesticides within aquatic ecosystems. The findings enhance our understanding of pesticide synergism and its implications for aquatic life, paving the way to more accurate ecological risk assessment and more efficient regulatory systems.

For the general public, the study reveals the unseen threats of current farming practices, particularly where widespread use of intensive pesticide application is prevalent. A recent study in Thailand found that exposure to chlorpyrifos, mancozeb and several other pesticides during pregnancy was positively linked to developmental delays in children under the age of 5 (Juntarawijit et al., 2021). By linking laboratory findings with real-world pesticide use, it highlights the potential harm to aquatic life, which is crucial to biodiversity and human health. The study is a wake-up call for policymakers and farmers to urge better pesticide management and regulatory measures. Ultimately, it contributes to the development of safer agricultural practices, promoting environmental conservation and safeguarding the health of both ecosystems and communities dependent on them.

CONCLUSION

Equitoxic mixtures of chlorpyrifos and mancozeb acted synergistically to affect guppy fish survival, leading to a higher than expected ecological risk to aquatic ecosystems. Active ingredient tests should accompany the toxicity testing, with active ingredient tests to be part of together formulations and compare relative toxicities of formulated and pure products in future work. The findings indicated that pesticide mixtures should be employed with care to minimize negative effects on guppy while facilitating effective pest control against crops. Future research should investigate the long-term chronic impacts of chlorpyrifos and mancozeb combinations on aquatic life, such as sublethal impacts on behavior and reproduction. Research should also examine effects at the ecosystem level across trophic levels, conduct field experiments under uncontrolled conditions, and investigate alternative pest management strategies that are safer in order to reduce environmental risks.

AUTHOR CONTRIBUTION STATEMENT

All authors contributed substantially to the work presented in this manuscript. RAN conceived and designed the study, conducted the data analysis, and led the manuscript writing. ARW was responsible for data collection and contributed to data interpretation. DCC assisted with the literature review, supported data visualization, and provided substantial feedback during manuscript drafting. CAMvG reviewed the final manuscript. All authors read and approved the final manuscript and agreed to be responsible for all aspects of the work.

INFORMED CONSENT STATEMENT

All experimental procedures involving live animals were conducted in accordance with ethical standards, including those outlined in the Declaration of Helsinki.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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