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PYRETHRUM EXPOSURE COMPROMISES CELLULAR IMMUNE RESPONSES IN *GALLERIA MELLONELLA* L. (LEPIDOPTERA: PYRALIDAE)

| Serhat Kaya* | Tuğba Söküt Açar |**

* Department of Biology, Faculty of Science, Çanakkale Onsekiz Mart University, Çanakkale, TÜRKİYE. E-mail: serhatkaya@comu.edu.tr; ORCID ID: 0000-0002-0984-2824
** Department of Statistics, Faculty of Science, Çanakkale Onsekiz Mart University, Çanakkale, TÜRKİYE. ORCID ID: 0000-0002-4444-1671)

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ABSTRACT: Pyrethrum extract, a plant-derived insecticide obtained from *Chrysanthemum cinerariaefolium*, is widely recognized for its neurotoxic activity against insects. However, its potential effects on insect immune function remain poorly understood. The present study investigated the impact of pyrethrum on hemocyte-mediated immune responses using *Galleria mellonella* L. as a model organism. Pyrethrum dissolved in acetone was topically applied to last instar larvae, and LC_{10}, LC_{25}, and LC_{50} concentrations were determined. Following exposure, total hemocyte count, encapsulation, and melanization responses were assessed using phase-contrast microscopy. Total hemocyte count increased significantly at LC_{25} and LC_{50} doses compared to controls, whereas LC_{10} did not induce a significant change. At LC_{25}, a shift from strong to weak encapsulation responses was observed after 24 h. Melanization responses were enhanced at LC_{10} and LC_{25}, as indicated by reduced numbers of non-melanized beads. These results demonstrate that pyrethrum induces dose-dependent alterations in cellular immune responses, suggesting that beyond neurotoxicity, pyrethrum may modulate immune balance in exposed insects.

KEY WORDS: Pyrethrum extract, *Galleria mellonella,* total hemocyte count, encapsulation, melanization

Global food security is increasingly threatened by pest-associated losses, which account for an estimated 20–40% of agricultural production. This reality underscores the critical need for effective and sustainable pest control strategies within Integrated Pest Management (IPM) frameworks. A central goal of modern IPM is to reduce reliance on broad-spectrum synthetic pesticides by incorporating effective natural alternatives (Angon et al., 2023).

Pyrethrum, a natural insecticide extracted from *Chrysanthemum cinerariaefolium* flowers, represents one such alternative. Its insecticidal activity is attributed to six pyrethrin esters that primarily target insect nervous systems (Valentine, 1990; Jayaprakas et al., 2023). Although its environmental lability led to the development of more stable synthetic pyrethroids (Power et al., 2007), growing ecological and health concerns have revived interest in the original botanical extract.

However, the biological profile of natural pyrethrum, particularly concerning non-target physiological systems, remains incompletely understood. While its neurotoxicity is well-characterized, emerging evidence suggests that pyrethroid

compounds can have broader systemic effects, including modulation of the immune system in both vertebrates and invertebrates (Tang et al., 2018; Bao et al., 2020). Specifically, the effects of natural pyrethrum extract on cellular immune parameters in insects are poorly defined, creating a significant knowledge gap for its risk-benefit assessment within IPM.

The greater wax moth, *Galleria mellonella* L. serves as an established and ethically favorable invertebrate model for innate immunity research. Its defense system relies on hemocytes, which orchestrate key cellular responses such as encapsulation and melanization—processes directly quantifiable through hemocyte counts and functional assays (Dubovskiy et al., 2008; Grizanova et al., 2014). Changes in these parameters are sensitive indicators of immunomodulation (Altuntaş et al., 2012), making *G. mellonella* an ideal organism to probe the immunological impact of xenobiotics.

Encapsulation represents a rapid and effective cellular immune response against large invaders in insects, making it a prominent parameter for assessing cell-mediated immunity. In *G. mellonella*, this process, along with the closely associated humoral melanization response, has been extensively used to understand innate immunity (Dubovskiy et al., 2008; Grizanova et al., 2014; Ono et al., 2020). The response is primarily executed by granulocytes and plasmatocytes, which sequentially adhere to and layer around foreign targets, forming a capsule whose strength is graded based on cell layer thickness (Pech & Strand, 1996; Richards & Dani, 2008). Given their functional synergy, encapsulation and melanization are often evaluated concurrently on the same implanted objects, as performed in the present study, providing a comprehensive view of cellular defense efficacy.

Changes in total hemocyte count (THC) are intrinsically linked to the capacity for encapsulation and melanization. Fluctuations in THC—whether an increase, decrease, or stability—directly influence these immune outcomes, as demonstrated in *G. mellonella* studies involving various stressors, including chemical agents, nanoparticles, and plant extracts (Altuntaş et al., 2012; Gwokyalya & Altuntaş, 2019; Eskin et al., 2022; Demirtürk et al., 2023). Consequently, THC serves as a fundamental and sensitive biomarker for immunomodulatory effects within this model system (Kaya, 2023). Therefore, simultaneous assessment of THC alongside functional encapsulation and melanization responses—as undertaken in this investigation—provides a robust framework for elucidating the impact of toxicants on cellular immunity.

Beyond their role in encapsulation and melanization, hemocytes are also the primary site for the synthesis of key immune signaling molecules, such as the pattern recognition receptor hemolin, which is critically involved in organizing cellular immune responses (Kaya et al., 2025). Given this central role of hemocytes, any compound that alters their count or function could potentially disrupt this critical coordination node of the immune system.

To investigate this potential disruption, this study aimed to test the hypothesis that sublethal exposure to natural pyrethrum extract disrupts hemocyte homeostasis, leading to dysfunctional encapsulation and melanization responses in *G. mellonella* larvae. By simultaneously evaluating total hemocyte count, encapsulation capacity, and melanization activity—three interconnected pillars of cellular defense—we sought to provide a comprehensive analysis of pyrethrum's immunomodulatory potential beyond its canonical neurotoxic role.

MATERIALS AND METHODS

Insect Rearing

G. mellonella larvae were obtained from an ongoing laboratory colony maintained at the Insect Physiology Laboratory, Department of Biology, Faculty of Science, Çanakkale Onsekiz Mart University. Colony maintenance was achieved by placing four female and two male moths into 1 L glass jars containing 2 g of naturally darkened honeycomb. Larvae were reared at 29±1 °C, 65±5% relative humidity, and under complete darkness.

Following egg hatching, larvae were fed daily with 10 g of an artificial diet developed by Bronskill (1961), consisting of naturally darkened honeycomb, wheat bran, honey, water, and glycerin. Final instar larvae with a mean body weight of 0.18±0.02 g were selected for experimental use.

Preparation of Experimental Doses

Due to the discontinuation of the previously planned in-house pyrethrum extract production, a commercially available pyrethrum extract containing ≥50% Pyrethrin I and II (Product No: 82670, Sigma-Aldrich, Germany) was used in this study. Detailed product specifications and the Certificate of Analysis are provided as Supplementary Material (Supplementary File 1). Owing to its oily nature, the extract was soluble only in acetone, as recommended by the manufacturer. Preliminary range-finding tests were conducted to estimate lethal concentrations. Groups of 20 larvae per concentration were topically treated with 20 µL of pyrethrum solutions at various concentrations, and mortality was recorded after 48 h. Based on the mortality data, three sublethal doses were selected for immunotoxicity assessment: 0.0015, 0.0018, and 0.002 µL/mL, approximating LC_{10}, LC_{25}, and LC_{50} values, respectively.

Application of Experimental Doses

Prior to treatment, larvae were surface-sterilized using 70% ethanol. Sterilized larvae were placed in Petri dishes, and prepared pyrethrum extract solutions or acetone (control group) were applied externally using an automatic pipette at a volume of 20 µL per larva. Following application, larvae were incubated for 24 h to allow the extract to exert its effects. All experimental procedures were conducted after this incubation period.

Total Hemocyte Count

Hemolymph samples were collected by puncturing the anterior segment of the abdominal proleg using a sterile needle. A volume of 4 µL hemolymph was immediately transferred into Eppendorf tubes containing 36 µl anticoagulant solution and gently mixed by pipetting. Subsequently, 10 µL of the hemolymph–anticoagulant mixture was loaded into a Neubauer hemocytometer. Total hemocyte counts were performed under a phase-contrast microscope (SOPTOP CX-40, China).

Encapsulation and Melanization Assays

Encapsulation and melanization responses were assessed using Sephadex A-25 chromatography beads. Beads were stained with 1% Coomassie Brilliant Blue G and suspended in 10 µL phosphate-buffered saline (PBS) at a concentration of

approximately 15–20 beads per injection. The bead suspension was injected into larvae using a microsyringe (50 µL capacity, 22s gauge needle; Hamilton, USA).

Larvae were dissected at 4 h (short-term response) and 24 h (long-term response) post-injection, and the beads were recovered. Encapsulation responses were evaluated under a phase-contrast microscope and classified according to Er et al. (2010). Beads surrounded by 0–2 hemocyte layers were classified as non-encapsulated, those with 2–8 layers as weakly encapsulated, and those with nine or more layers as strongly encapsulated.

Melanization was assessed simultaneously on the same beads used for encapsulation analysis. Based on the method described by Kaya et al. (2021b), melanization responses were categorized as non-melanized (no darkening), weak (1–20% darkening), moderate (20–70% darkening), or strong (≥70% darkening).

Statistical Analysis

Encapsulation and melanization data were collected at 4 h and 24 h time points. Statistical analyses were performed using SPSS software and the R programming language. Descriptive statistics were expressed as mean ± standard error, and 95% confidence intervals were generated for data visualization. Violin plots were used to illustrate central tendency and variability.

Encapsulation responses were analyzed across three ordered levels (non-encapsulated, weak, strong), while melanization responses were evaluated across four ordered levels (non, weak, moderate, strong). Differences among dose groups at each time point, as well as THC, were assessed using one-way analysis of variance (ANOVA). In addition, the combined effects of dose and exposure time on total encapsulation and melanization responses were evaluated using two-way ANOVA, including the dose × time interaction term. When significant effects were detected and the assumptions of normality and homogeneity of variances were satisfied, Tukey's post hoc test was applied to perform multiple pairwise comparisons. Because encapsulation and melanization responses are inherently ordinal in nature, ordinal logistic regression models were additionally employed to evaluate how dose and exposure time influenced the probability of responses shifting toward higher or lower categorical levels. The significance level was set at $\alpha = 0.05$. The statistical power of the tests, conducted with 16 samples per group across five dose groups at both time points, was calculated as 80.3%.

RESULTS

Total Hemocyte Count

Total hemocyte count (THC) in *G. mellonella* larvae responded to pyrethrum exposure in a dose-dependent but non-linear manner (Fig. 1). The most pronounced effect was observed at the LC_{25} dose, which elicited a significant increase in circulating hemocytes compared to all other groups, including both control groups (untreated and acetone) and the other pyrethrum-treated doses (LC_{10} and LC_{50}) ($p < 0.001$). The LC_{10} dose did not result in a statistically significant alteration in THC relative to the control groups. In contrast, the LC_{50} dose induced a moderate but significant increase in THC compared to the control groups, although this increase remained significantly lower than that observed in the LC_{25} group ($p < 0.001$).

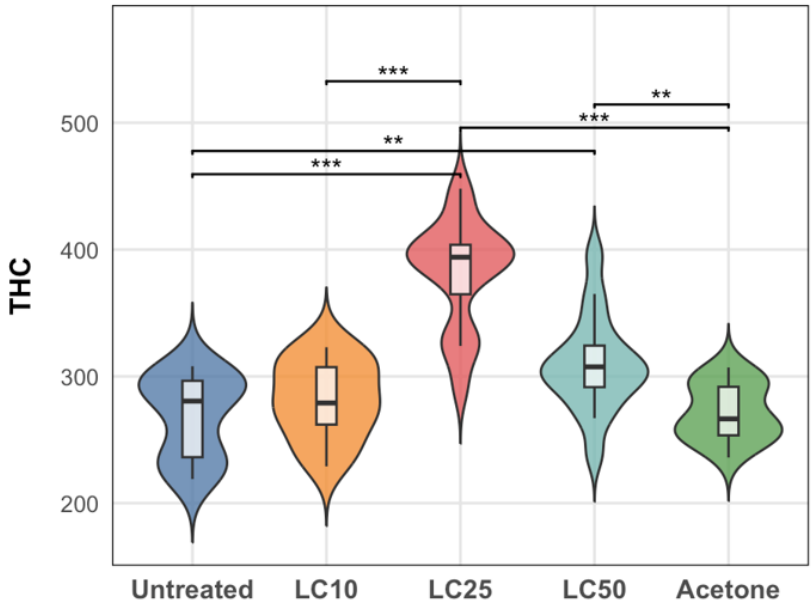


Figure 1. Violin plots showing the distribution of THC measurements across different treatment groups. Each violin shape represents the probability density of the data at different values, with wider sections indicating higher probability density. Inside each violin, a boxplot is embedded showing the median (center line), interquartile range (box boundaries; middle 50% of data), and data distribution. The treatment groups are: Untreated (control group without any treatment), LC10 (treatment with 10% lethal concentration dose), LC25 (treatment with 25% lethal concentration dose), LC50 (treatment with 50% lethal concentration dose), and Acetone (solvent control group, acetone-treated). The y-axis (THC) represents the measured THC values in appropriate units. Each group contains 16 biological replicates (n=16). Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post-hoc test. Brackets with asterisks above the violins indicate statistically significant differences between groups (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

One-way analysis of variance revealed a statistically significant effect of treatment on total hemocyte count (THC) among the experimental groups ($F(4,75) = 31.238$, $p < 0.001$; Table 1). As the assumption of homogeneity of variances was met, Tukey's post hoc test was applied for pairwise comparisons. The highest mean THC value was recorded in the LC_{25} group, which differed significantly from both control groups. The LC_{50} group also exhibited a significant increase in THC relative to the controls, whereas the LC_{10} group showed only a limited and comparatively weaker response. Together, these results indicate that pyrethrum exposure induces a dose-dependent yet non-linear hemocytic response.

The visual differences in THC distributions among treatment groups observed in Figure 1 were subsequently tested for statistical significance using one-way ANOVA, as summarized in Table 1.

The one-way analysis of variance presented in Table 1 demonstrated that pyrethrum dose resulted in statistically significant differences in total hemocyte count (THC) among the experimental groups ($F(4,75) = 31.238$, $p < 0.001$). As the assumptions of homogeneity of variances and normality were satisfied, group comparisons were conducted using Tukey's post hoc test. Accordingly, the highest mean THC value was observed in the LC25 group, which exhibited significantly higher THC levels compared to both the Untreated and Acetone control groups. In addition, the LC50 group also showed a significant increase in THC relative to the control groups. In contrast, the change in THC observed in the LC10 group was limited and represented a weaker response compared to the [MOU1.1] other pyrethrum doses.

Table 1. One-way ANOVA results showing the effects of pyrethrum dose on THC in *G. mellonella* haemolymph at 24 h post-exposure.

THC	N	Mean	Std. Error	ANOVA			
				F	Df1	Df2	p
Untreated	16	268.688	8.058	31.238	4	75	<.001
LC10	16	281.438	7.579				
LC25	16	381.938	10.629				
LC50	16	309.188	9.477				
Acetone	16	271.688	5.533				

Homogeneity of variances was assessed using Levene's test ($F = 1.074$, $p = 0.376$), and the assumption of normality was evaluated with the Shapiro–Wilk test ($W = 0.989$, $p = 0.742$). No statistically significant violations of either assumption were detected.

Encapsulation Response

The cellular encapsulation response against implanted beads was significantly altered by pyrethrum, with effects becoming prominent only after 24 hours of exposure (Fig. 2).

At the 4-hour time point, no statistically significant differences were found among any dose groups for non-encapsulated, weakly encapsulated, or strongly encapsulated beads (Fig. 2a).

To formally evaluate the effects of dose, exposure time, and their interaction on total encapsulation response, a two-way ANOVA was performed (Table 2). The analysis revealed a significant main effect of group ($F(4,150) = 4.587$, $p = 0.002$), whereas the main effect of time was not significant ($F(1,150) = 0.806$, $p = 0.371$). In contrast, the group × time interaction was significant ($F(4,150) = 6.202$, $p < 0.001$), indicating that temporal changes in the encapsulation response were not uniform across all groups and were more pronounced at specific doses. Post hoc Tukey comparisons demonstrated that the significant group-level differences in total encapsulation response were primarily driven by differences between the LC25 dose and the LC50 and acetone control groups. Furthermore, post hoc analyses of the interaction term revealed that these differences became

particularly evident at the 24-hour time point. While a significant change between 4 and 24 hours was observed in the LC25 group, no statistically significant time-dependent differences were detected in the untreated, LC10, or LC50 groups. Two-way ANOVA was used to test changes in overall encapsulation magnitude, whereas ordinal logistic regression analysis was applied to examine dose-dependent shifts in the ordered encapsulation response categories (none, weak, strong). The results of the ordinal logistic regression analysis are summarized in Table 3.

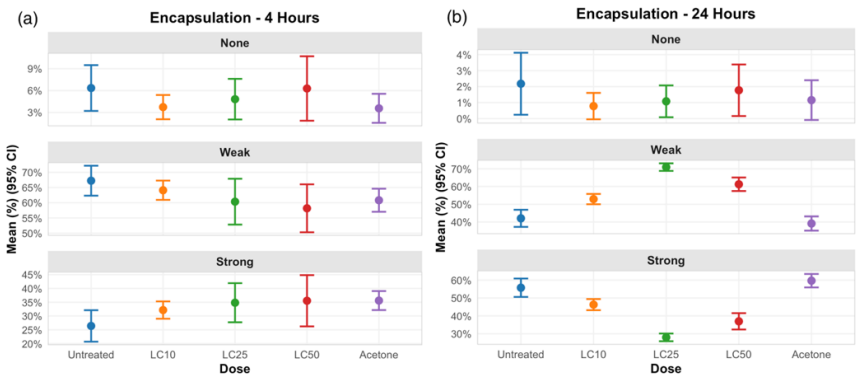


Figure 2. Encapsulation responses at 4 (a) and 24 (b) hours post-treatment. Data are presented as mean percentage with 95% confidence intervals for five treatment groups: Untreated (negative control), LC10 (10% lethal concentration), LC25 (25% lethal concentration), LC50 (50% lethal concentration), and Acetone (solvent control). Encapsulation is categorized as None (minimal or no encapsulation), Weak (partial encapsulation), or Strong (complete encapsulation). Points represent mean encapsulation percentage, error bars indicate 95% confidence intervals. Statistical analysis was performed using one-way ANOVA with Tukey's HSD post-hoc test.

Table 2. Effects of dose and exposure time on total encapsulation response: Two Way Anova.

	Sum of Squares	df	Mean Square	F	p	η^2
Group	384.788	4	96.197	4.587	0.002	0.095
Time	16.900	1	16.900	0.806	0.371	0.04
Group*Time	520.288	4	130.072	6.202	< .001	0.128
Residuals	3146.000	150	20.973			

Homogeneity of variances was assessed using Levene's test ($F = 1.247$, $p = 0.165$), and the assumption of normality was evaluated using the Shapiro–Wilk test ($W = 0.991$, $p = 0.451$). No statistically significant violations were detected for either assumption.

Table 3. Ordinal logistic regression results for encapsulation response.

	Estimate	SE	z	p-value	Odds Ratio
Group (Reference=Untreated)					
Acetone	0.426	0.174	2.45	0.014	1.53
LC10	0.310	0.172	1.80	0.072	1.36
LC25	0.324	0.177	1.83	0.068	1.38
LC50	0.467	0.174	2.69	0.007	1.60
Time (Reference=4 h)					
24 h	1.254	0.175	7.16	<0.001	3.51
Group*Time					
Acetone × 24 h	−0.258	0.247	−1.05	0.295	0.77
LC10 × 24 h	−0.660	0.236	−2.80	0.005	0.52
LC25 × 24 h	−1.351	0.239	−5.65	<0.001	0.26
LC50 × 24 h	−1.197	0.246	−4.86	<0.001	0.30

Reference categories: Group = Untreated, Time = 4 h. Odds ratios represent the likelihood of shifting toward higher encapsulation categories (non → weak → strong)

The encapsulation response (non, weak, strong) was modeled as an ordered dependent variable using ordinal logistic regression, with Group, Time, and their interaction (Group × Time) included as predictors (Table 3). The untreated group and the 4-hour time point were defined as reference categories. Accordingly, the main effect coefficients represent differences relative to the untreated group at 4 hours, while the Time24 coefficient reflects the change between 24 and 4 hours within the untreated group. Odds ratios (ORs) greater than 1 indicate an increased likelihood of shifting toward higher encapsulation categories (non → weak/strong or weak → strong), whereas ORs less than 1 indicate a reduced likelihood of such shifts.

Considering the reference condition (Untreated–4 h), the 24-hour measurement in the untreated group was associated with a significantly increased likelihood of shifting toward higher encapsulation categories compared to 4 hours (Time24: OR = 3.51, $p < 0.001$), corresponding to an approximately 251% increase in the odds of higher encapsulation responses. However, this temporal increase was specific to the untreated group. In the pyrethrum-treated groups, significant negative group × time interaction terms indicated a reversal of this trend. Specifically, the 24-hour interaction terms for LC10 (OR = 0.52, $p = 0.005$), LC25 (OR = 0.26, $p < 0.001$), and LC50 (OR = 0.30, $p < 0.001$) demonstrate a reduced likelihood of shifting toward higher encapsulation

categories at 24 hours compared to the untreated group, corresponding to decreases of approximately 48%, 74%, and 70%, respectively.

In line with these results, all pyrethrum-treated groups (LC_{10}, LC_{25}, LC_{50}) differed significantly from the control groups in both weak and strong encapsulation responses at 24 hours (Fig. 2b). Notably, the LC_{25} group, despite exhibiting the highest total hemocyte count, displayed the lowest proportion of strongly encapsulated beads and a concurrent increase in weakly encapsulated beads compared to the other doses.

Melanization Response

Melanization of the implanted beads was significantly influenced by pyrethrum in a time- and dose-dependent fashion.

Early Response (4 h): At 4 hours, all pyrethrum-treated groups showed a significant increase in the proportion of non-melanized beads compared to controls ($p < 0.05$). This was accompanied by a general shift in response profiles toward weaker melanization categories across all doses (Fig. 3a).

Late Response (24 h): The melanization pattern shifted considerably by 24 hours. The initial suppression was overcome, and the LC_{10} and LC_{25} dose groups now exhibited a significantly higher proportion of strongly melanized beads than control groups ($p < 0.05$). The LC_{10} group showed the most distinct response, differing significantly not only from controls but also from the other pyrethrum-treated groups (LC_{25} and LC_{50}) in the intensity of melanization. In summary, sublethal pyrethrum exposure induced an initial delay in melanization, followed by a potentiated response at 24 hours (Fig. 3b).

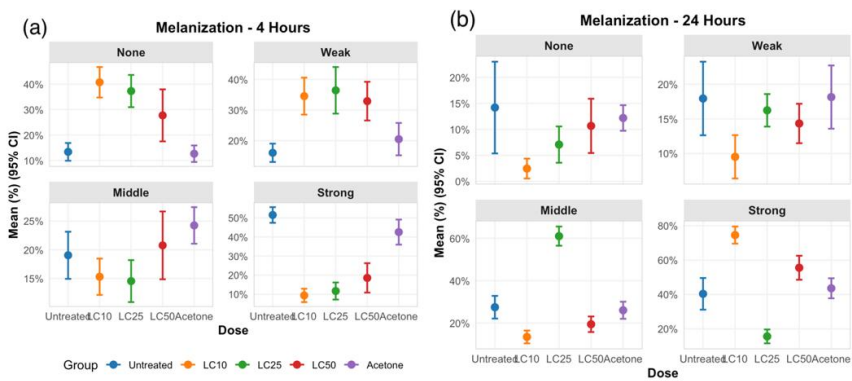


Figure 3. Melanization responses at 4 and 24 hours post-treatment. Data are presented as mean percentage with 95% confidence intervals for five treatment groups: Untreated (negative control), LC_{10} (10% lethal concentration), LC_{25} (25% lethal concentration), LC_{50} (50% lethal concentration), and Acetone (solvent control). Melanization is categorized as None (no melanin deposition), Weak (light melanization), Middle (intermediate melanization), or Strong (heavy melanization). Points represent mean melanization percentage, error bars indicate 95% confidence intervals. Statistical analysis was performed using one-way ANOVA with Tukey's HSD post-hoc test.

To statistically evaluate the combined effects of pyrethrum dose, exposure time, and their interaction on total melanization response, a two-way ANOVA was performed (Table 4). The analysis revealed a statistically significant main effect of group ($F = 4.605$, $p = 0.002$, $\eta^2 = 0.094$), whereas the main effect of time alone was not significant ($F = 0.813$, $p = 0.369$, $\eta^2 = 0.004$). These results indicate that the group × time interaction represents the dominant source of variation in melanization response, with the group main effect contributing at a secondary level.

Table 4. Effects of dose and exposure time on total melanizasyon response: Two Way Anova.

	Sum of Squares	df	Mean Square	F	p	η^2
Group	382.838	4	95.709	4.605	0.002	0.094
Time	16.900	1	16.900	0.813	0.369	0.004
Group*Time	535.287	4	133.822	6.439	<0.001	0.132
Residuals	3117.375	150	20.783			

Homogeneity of variances was assessed using Levene's test ($F = 1.457$, $p = 0.169$), and normality was evaluated using the Shapiro–Wilk test ($W = 0.992$, $p = 0.561$). No statistically significant violations of either assumption were detected.

Tukey post hoc comparisons for the group factor showed that the LC_{25} group differed significantly from the LC_{50} and acetone control groups, whereas comparisons between the untreated group and the other dose groups were not statistically significant. However, because main-effect comparisons do not capture temporal changes in response, biological interpretation was primarily based on the results of the group × time interaction. Post hoc analyses of this interaction demonstrated that the observed differences in melanization were largely driven by the 24-hour measurements of the LC_{25} group. Specifically, melanization responses in the LC_{25} group decreased significantly between 4 and 24 hours, and at 24 hours this group exhibited significantly lower melanization compared to the untreated, LC_{50}, and acetone control groups (all comparisons $p < 0.001$).

To further characterize dose- and time-dependent shifts in melanization intensity, ordinal logistic regression analysis was applied to the ordered melanization response categories (none, weak, moderate, strong) (Table 5). Using the untreated group and the 4-hour time point as reference categories, the analysis revealed that melanization responses in the LC_{10}, LC_{25}, and LC50 groups were significantly shifted toward lower melanization categories compared to the untreated group. For example, the odds ratio for the LC_{25} dose was 0.135, indicating an approximately 87% reduction in the likelihood of exhibiting strong melanization relative to the untreated group. Similar reductions were observed in the LC_{10} (OR = 0.118) and LC_{50} (OR = 0.221) groups.

The main effect of time further indicated that melanization responses at 24 hours were less likely to occupy higher categories compared to 4 hours (OR = 0.674, $p = 0.014$). In addition, significant group × time interaction terms

demonstrated that the temporal dynamics of melanization differed markedly among dose groups. Collectively, these findings confirm that melanization is not only dose dependent but is also strongly shaped by the interaction between dose and exposure time.

Table 5. Ordinal logistic regression results for melanization response.

	Estimate	SE	z	p-value	Odds Ratio
Group (Reference=Untreated)					
Acetone	-0.344	0.158	-2.179	0.029	0.709
LC10	−2.138	0.158	−13.561	<0.001	0.118
LC25	−2.000	0.162	−12.371	<0.001	0.135
LC50	−1.509	0.157	−9.588	<0.001	0.221
Time (Reference=4 h)					
24 h	−0.394	0.160	−2.463	0.014	0.674
Group*Time					
Acetone × 24 h	0.451	0.226	1.994	0.046	1.569
LC10 × 24 h	3.661	0.232	15.789	<0.001	38.891
LC25 × 24 h	1.764	0.213	8.279	<0.001	5.837
LC50 × 24 h	2.079	0.229	9.061	<0.001	8.000

Reference categories: Group = Untreated, Time = 4 h. Odds ratios represent the likelihood of transitioning toward higher ordered melanization response categories (none → weak → moderate → strong). Odds ratios greater than 1 indicate an increased probability of occupying higher melanization categories, whereas odds ratios less than 1 indicate a reduced probability.

DISCUSSION AND CONCLUSIONS

Together, the encapsulation and melanization data demonstrate that pyrethrum exposure reshapes cellular immune responses in a dose- and time-dependent manner rather than exerting a uniform suppressive or stimulatory effect. The pronounced group × time interactions observed for both immune parameters indicate that temporal dynamics are a critical determinant of how sublethal pyrethrum doses modulate host defense responses. These findings suggest that immune outcomes following pyrethrum exposure cannot be inferred from dose alone but emerge from the combined influence of dose intensity and exposure duration.

This study provides clear evidence that natural pyrethrum extract, beyond its primary neurotoxic action, exerts significant dose- and time-dependent immunomodulatory effects on *G. mellonella* larvae. The most notable finding is the dissociation between cellular abundance and function. Specifically, while the LC_{25} dose elicited the highest total hemocyte count (THC), it concurrently resulted in the lowest proportion of strong encapsulation responses after 24 hours, alongside a significantly higher proportion of weakly encapsulated beads. This suggests that an elevated hemocyte number does not necessarily translate to enhanced cellular immune capacity and may even indicate a loss of functional competence in these key effector cells presenting a form of 'immuno-paradox' where abundance contradicts efficacy (Dubovskiy et al., 2008; Ono et al., 2020). Furthermore, melanization responses indicated clear alterations between control and pyrethrum-treated groups at both 4- and 24-hour exposure periods, underscoring the broad impact on cellular immune pathways.

Due to the limited number of studies investigating natural pyrethrum extracts, the results of the present study were primarily evaluated in comparison with findings reported for synthetic pyrethroids, while available data on natural pyrethrum were also considered. Studies conducted in Wistar rats have demonstrated that the pyrethroid cypermethrin induces oxidative stress by increasing malondialdehyde levels while reducing antioxidant enzyme activities, including superoxide dismutase, catalase, and glutathione peroxidase (Adeniyi et al., 2024). In insects, cypermethrin has been shown to reduce mitotic activity and increase apoptotic hemocytes in the parasitoid Pimpla turionellae (Uçkan & Sak, 2010). Similarly, deltamethrin exposure in *G. mellonella* larvae resulted in a decrease in total hemocyte count at 72 h followed by an increase at 96 h, accompanied by elevated micronucleus formation, indicating genotoxic activity (Kurt & Kayış, 2015). Kalita et al. (2017) reported that sublethal cypermethrin exposure in Philosamia ricini induced apoptosis, activated caspase responses, increased DNA damage, and reduced total hemocyte counts. Collectively, these studies indicate that synthetic pyrethroids induce oxidative stress and genotoxic effects, ultimately impairing hemocyte function.

The observed pattern—increased THC coupled with suppressed encapsulation—aligns with the concept of 'stress-induced hematopoiesis' followed by functional exhaustion, a phenomenon reported for other stressors (Ghoneim et al., 2021). For instance, while cypermethrin induces apoptosis in parasitoid hemocytes (Uçkan & Sak, 2010), and deltamethrin causes fluctuations in THC and genotoxicity in *G. mellonella* (Kurt & Kayış, 2015), our data with natural pyrethrum highlight a specific disruption in the effector phase of the cellular response, namely encapsulation. This distinction may arise from the complex phytochemical profile of natural pyrethrum, which includes synergistic compounds not present in synthetic analogues, potentially influencing immune signaling pathways differently (Hodoşan et al., 2023).

The discrepancy between elevated hemocyte counts and reduced encapsulation efficiency at the LC_{25} dose points towards a compromise in hemocyte functionality rather than availability. Several non-exclusive mechanisms could underlie this effect. First, pyrethrum-induced oxidative stress, well-documented for synthetic pyrethroids (Adeniyi et al., 2024; Kalita et al., 2017), could impair hemocyte metabolism and cytoskeletal dynamics essential for adhesion, spreading, and migration—processes critical for effective encapsulation

(Pech & Strand, 1996). Second, sublethal activation of apoptotic pathways may render hemocytes non-viable or less responsive despite being present in circulation. Third, the modulation of key immune signaling pathways, such as the phenoloxidase (PO) cascade, is likely involved. Our observed time-dependent changes in melanization, corroborated by increased PO activity reported at 24 hours post-pyrethrum exposure (Kaya, 2020), provide clear evidence of this dysregulated state and suggest that immune activation may not be optimally coordinated with other cellular defense mechanisms like encapsulation (Jiravanichpaisal et al., 2006).

This mechanistic complexity supports the broader concept that plant-derived pesticides may either stimulate hemocyte proliferation as a defensive response or disrupt immune homeostasis depending on dose and exposure conditions (Ghoneim et al., 2021). Our findings with pyrethrum clearly reflect this duality: the LC_{25} dose triggered proliferation (high THC) yet ultimately led to functional disruption (low encapsulation).

This observed functional disruption is known to lead to reduced cellular immune responses in *G. mellonella* (Demirtürk et al., 2023), given that encapsulation and melanization responses are largely dependent on the functional capacity of hemocytes (Ono et al., 2020). Therefore, the concurrent reduction in strong encapsulation despite elevated THC at the LC_{25} dose indicates a loss of hemocyte functionality rather than an enhancement of immune competence. This interpretation is consistent with previous reports highlighting that immune suppression may occur even in the presence of elevated hemocyte numbers. Consequently, the LC_{25} dose appears to represent a critical immunomodulatory threshold where hemocyte proliferation is stimulated while effective cellular immune functions are compromised.

From both biological and applied perspectives, the immunosuppressive effect of pyrethrum at sublethal doses presents a dual implication for Integrated Pest Management (IPM). Biologically, the compromised hemocyte-mediated defenses can significantly alter host–pathogen dynamics in insects, highlighting the importance of including immune-related endpoints in the risk assessment of plant-derived insecticides. Practically, this immunosuppression can be strategically leveraged within IPM. Pyrethrum could be used to weaken the cellular immunity of target pests, thereby increasing their susceptibility to subsequent infection by entomopathogens such as Bacillus thuringiensis or fungi (Grizanova et al., 2014; Kryukov et al., 2020). This approach could enhance biocontrol efficacy and reduce the required doses of both agents, aligning with the principle of exploiting sublethal stress rather than relying solely on direct lethality. However, this very characteristic constitutes a significant ecological risk. The same mechanism that suppresses immunity in pests may also impair vital defense responses in non-target organisms, including essential pollinators and natural enemies like parasitoid wasps, whose fitness and survival depend on a fully functional cellular immune system (Diogo et al., 2023). Consequently, the integration of natural pyrethrum into IPM protocols necessitates comprehensive environmental risk assessments that specifically evaluate its sublethal immunomodulatory impacts across a broader ecological scale.

While this study establishes a clear link between pyrethrum exposure and altered cellular immunity in *G. mellonella*, certain limitations should be acknowledged and addressed in future work. First, our analysis measured total

hemocyte count without differentiating sub-populations (e.g., granulocytes, plasmatocytes), which have distinct roles in encapsulation. Future studies should employ flow cytometry or specific staining to identify which populations are proliferating or declining (Gwokyalya & Altuntaş, 2019). Second, direct functional assays such as phagocytosis efficiency, reactive oxygen species (ROS) production, and cell viability assays (apoptosis/necrosis) would provide a more direct measure of hemocyte health beyond morphological encapsulation. Third, molecular investigations into the expression of genes related to oxidative stress (e.g., SOD, catalase), apoptosis (e.g., caspases), and immune pathways (e.g., PO cascade genes, antimicrobial peptides) are needed to elucidate the underlying mechanisms (Kaya et al., 2021a). Finally, examining the effects of chronic, low-dose exposure scenarios would better reflect potential real-world environmental impacts.

In conclusion, this study demonstrates that natural pyrethrum extract significantly modulates hemocyte-mediated immunity in *G. mellonella*, with sublethal doses inducing a state characterized by elevated hemocyte numbers but reduced functional output—an immuno-paradox that underscores how pyrethrum's biological impact extends beyond neurotoxicity to include complex immunomodulatory effects. By disrupting hemocyte functionality, particularly encapsulation efficiency and the coordination of melanization responses, pyrethrum may alter host–pathogen interactions and compromise effective cellular immune defenses. These findings highlight the importance of incorporating immune-related endpoints into the evaluation of plant-derived insecticides and suggest that, under carefully controlled conditions, pyrethrum-induced immunosuppression could be strategically exploited to enhance biological control efficacy within integrated pest management (IPM) frameworks. However, this same property also raises ecological concerns, as sublethal immunomodulation may adversely affect non-target organisms whose fitness relies on intact cellular immunity. Overall, this work provides novel insight into the dose- and time-dependent immunological effects of natural pyrethrum and emphasizes the need for future studies integrating hemocyte subtyping, functional assays, and molecular analyses to fully elucidate the mechanisms underlying pyrethrum-induced immune dysregulation and to support its sustainable and ecologically informed use in pest management.

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