



Original Research Paper

Hemato-biochemical and Antioxidant Changes Induced by PM_{2.5} Exposure in *Apis mellifera*

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Abstract

Fine particulate matter (PM_{2.5}), an increasingly serious environmental contaminant, has been shown to negatively impact ecological systems and health of pollinators, thereby making it one of the most important environmental stressors. This study evaluated changes in hemato-biochemical and antioxidant status of the honey bee (*Apis mellifera*) resulting from exposure to PM_{2.5}. Honey bees were exposed to controlled amounts of PM_{2.5} in laboratory conditions. The hemolymph of the exposed bees was analysed for total hemocyte count (THC), differential hemocyte count (DHC), biochemical parameters and indicators of oxidative stress. The data showed that THC was significantly reduced and that hemocyte populations showed changes in the number of plasmatocytes and granulocytes, indicating modulation of the immune system. In addition, the results of biochemical analyses showed that glucose, ALT, AST and ALP were all increased in the exposed groups, while total protein levels were remarkably decreased, indicative of metabolic stress and tissue damage. Oxidative stress indicators showed a significant increase in LPO and a significant decrease in GSH, indicating that there was an increase in ROS production and a decrease in antioxidant capacity. The results support previous research that has found that particulate pollutants produce oxidative damage, immune suppression and metabolic disruption to honey bee populations. The research indicates that honey bees are very sensitive to PM_{2.5} exposure and affords the opportunity to use honey bees as bio indicators of environmental pollution.

Keywords- *Apis mellifera*, PM_{2.5}, Hemocyte count, Oxidative stress, LPO, GSH, Air pollution, Bio-indicator.

1. Introduction

Honey bees, (*Apis mellifera*), are among the most vital pollinators in both natural and agricultural ecosystems, greatly contributing to global food security and biodiversity. Honey bees dominate the pollination of nearly 75% of flowering plants and roughly 35% of the total production of food crops worldwide due to their role as pollinators (Klein *et al.*, 2007). Along with their role in supporting ecosystems, honey bees also provide economic benefits from honey, beeswax and other hive products. Unfortunately, there has been a rapid decrease in honey bee numbers throughout the world in recent decades, creating serious concerns about the loss of ecological balance as well as food sustainability (Potts *et al.*, 2010). The decline in honey bee populations has been attributed to many reasons, including habitat loss, climate change, exposure to pesticides, pathogens, and environmental contaminants. The primary focus of honey bee population declines has concentrated on pesticide exposures and environmental contaminants; however, relatively little attention has been paid to the potential contribution of air pollution, particularly fine particulate matter (PM_{2.5}), as a stressor affecting pollinator health (Zhao *et al.*, 2021). PM_{2.5} refers to small particles in

the atmosphere that measure 2.5 micrometres (or less) in diameter that can remain suspended in the atmosphere for extended periods of time as well as penetrate biological tissues because of their small size (Pope & Dockery, 2006). PM_{2.5} originates from many sources, including automobile emissions, industrial emissions, burning of plants for fuel, and urban dust. Due to their extensive foraging patterns, honey bees are perpetually exposed to airborne pollutants, thus, the impact of atmospheric contaminants on honey bee populations is multifaceted. The body surfaces of bees (e.g., hair) can collect fine particulate matter, such as PM_{2.5}, through adhesion or ingestion via feeding or grooming (Papa *et al.*, 2024). While in the body, PM_{2.5} can affect cellular structure and function leading to physiological and biochemical disruptions. PM_{2.5} is thought to exert toxicity primarily through production of oxidative stress. Oxidative stress occurs when there is an imbalance of reactive oxygen species (ROS) generation and the organism's antioxidant defence mechanisms (Halliwell & Gutteridge, 2015). The diverse toxic materials in PM_{2.5}, including heavy metals, PAHs and organic compounds, are involved in the generation of ROS (Zhao *et al.*, 2021). Excessive ROS can damage lipids, proteins and nucleic acids, resulting in non-functional cells and death.

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Oxidative damage to insects, including *Apis mellifera*, is mitigated by the antioxidant defence mechanism. Antioxidants include enzymatic antioxidants (e.g., SOD, CAT) and non-enzymatic antioxidants (e.g., GSH). GSH is an important antioxidant involved in detoxification of harmful compounds and cellular protection from oxidative damage (Olgun *et al.*, 2020). LPO is an important indicator of oxidative damage, as it quantifies the degradation of membrane lipids caused by ROS (Halliwell & Gutteridge, 2015). Therefore, measuring LPO and GSH gives insight into the oxidant status of the organism. PM_{2.5} exposure may alter the immune system of honeybees as well as oxidative stress. Insects use an innate immune response that is composed of both cellular and humoral responses. The central cells of the insect immune system are referred to as hemocytes; they are critically important in the protection of the insect by performing various roles such as phagocytosis, encapsulation, nodulation and wound healing (Lavine & Strand 2002). Indicators of immunity in insects are often provided by their Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC). Changes in the number of hemocytes can be an indicator of physiological stress or immunity modulations. For example, if an organism experiences a decrease in THC, this may be indicative of immunosuppression; conversely, certain changes in the type of hemocyte will provide indications of how immunity is altered (Zhang *et al.*, 2021). Other biochemical parameters that provide valuable insight into the physiological status of any given organism when stressed are glucose levels. In many cases, glucose levels are used to help evaluate both an organism's energy metabolism as well as its stress response. If an organism is under stress, it may show hyperglycemia from an increased need for energy and breakdown of stored glycogen (Zhao *et al.*, 2021). There are also a number of enzymes that are found in high amounts in the tissues of organisms, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). These enzymes are routinely used to measure tissue damage and metabolic imbalance in vertebrates; however, physiological stress and cellular damage in insects have also been demonstrated to correlate with these same enzymes (Reitman & Frankel, 1957). The total amount of proteins produced by a body is also a reflection of how well developed the body is on an overall metabolic and nutritional level, with low total protein levels potentially indicating an increase in degradation of protein or a decrease in the making of new proteins during times of stress. Within the honey bee community, there has been much research that has indicated that honey bees can be used effectively as bio indicators of pollution and

are very good at monitoring ecological health due to their large foraging radius, sensitivity to environmental changes, and ability to accumulate harmful pollutants (Papa *et al.*, 2024). Many studies have shown that the exposure of honey bees to harmful activities such as pesticide usage or heavy metal contamination will cause significant changes in biochemical, hematologic, and antioxidant profiles (Di *et al.*, 2022; Tahir *et al.*, 2023). However, the amount of research done to focus directly on the effects of particulate matter (PM_{2.5}) on the hemato-biochemical changes within honey bee systems has been very limited. Therefore, understanding how honey bee physiology will respond to PM_{2.5} exposure will provide valuable information regarding the impact of air pollution on ecosystems. It will also provide insights into the mechanisms of toxicity and provide guidance on the development of strategies for monitoring the environment and conserving it. Because honey bees are also linked closely to agricultural productivity, the health of their populations will impact food security and the stability of the ecosystems. The objective of the present study is to evaluate hemato-biochemical and antioxidant parameter changes due to exposure to PM_{2.5} in *Apis mellifera*. Specifically, the objective of this study will be to measure total hemocyte counts (THC), differential hemocyte counts (DHC), biochemical parameters (glucose, ALT, AST, ALP, and total protein), and oxidative stress markers (lipid peroxidation-[LPO] and total glutathione [GSH]). The goal of this study is to integrate the results of thus determined parameters to provide an overall picture of the physiological effects of PM_{2.5} exposure to honey bees.

In summary, due to the increases in pollution levels and the negative impact that pollution can have on pollinators, there is a need for much more scientific research surrounding this topic. Honey bees represent an important model for understanding the stress associated with pollution and provide information on how pollution will alter the physiological condition of an organism through exposure to PM_{2.5}. The results from this study will add to the database of knowledge concerning geochemical toxicity in relation to the ecology of honey bees and will also unfortunately support the need for more effective pollution control efforts to mitigate the adverse effects on honey bee health and the ecological stability of our ecosystems.

2. Review of Literature

One of the biggest global challenges we face is air pollution, which negatively affects humans and nature. Fine particulate matter (PM_{2.5}) produces harmful effects once it enters biological systems, and there have been an increasing number of studies demonstrating these toxic effects on both vertebrates, as well as invertebrates like pollinating insects such as honeybees (*Apis mellifera*). Due to

their foraging activities, range across many geographic areas, and ability to concentrate environmental pollutants (bio indicators), honeybees are commonly used to monitor contaminants in the environment (Papa *et al.*, 2024, Surbhi & Chaudhary, 2026). Because honeybees' foraging exposes them to the contaminants in the air, water, and vegetation, studies have shown that the bioaccumulation of particulates, heavy metals, and organic pollutants on the body and within the tissues of worker honeybees can represent the degree of contamination in the environment (Papa *et al.*, 2024; Feldhaar, 2020, Gupta *et al.*, 2024, Yadav *et al.*, 2026)). Research conducted in heavily polluted areas has also demonstrated that honeybees can trap airborne particulates, such as PM₁₀ and PM_{2.5}; therefore, they can be used for bio monitoring purposes (Papa *et al.*, 2024). This means that, in addition to being used as bio indicators of air pollution, honeybees also reflect the ecological impacts of air pollution. Pollutants that honeybees collect may be found in hive products (honey, wax, pollen), leading to the potential contamination of honeybee colonies and food products consumed by humans (Accordingly, it is important to understand how honeybee's physiology responds to exposure of contaminants in the environment in terms of both ecological and public health.

Particulate Matter 2.5 (PM_{2.5}) is defined as particles with aerodynamic diameters equal to or smaller than 2.5 µm that come from a variety of anthropogenic sources like vehicular emissions, industrial activities and the burning of biomass. PM_{2.5} is composed of a diverse array of compounds such as organic compounds, as well as heavy metals, all of which have toxic properties and can cause oxidative stress (Zhao *et al.*, 2021, Geeta *et al.*, 2026). One of the primary mechanisms contributing to the toxicity of PM_{2.5} is the creation of Reactive Oxygen Species (ROS), resulting in oxidative damage in all biological systems. Recently, there have been multiple studies that have shown that oxidative stress is the primary mechanism by which particulate matter produces harmful effects throughout all organisms (Vaccarella *et al.*, 2025). While most of the research conducted to date regarding particulate matter and its effects has focused on human populations, there have been a few recent studies to indicate that insects, including honeybees, have also been impacted by the same toxicological mechanisms. The small size of PM_{2.5} allows particles to adhere to the exterior of honeybee bodies, as well as to enter into the internal tissues of the body; thus creating physiological and biochemical disruptions.

Oxidative damage appears to be a major mechanism through which the toxic effects of pollutants are mediated in honeybees. Oxidative

stress is referred to as the disruption of the balance between the production of ROS and the organism's antioxidant defence mechanism (Halliwell & Gutteridge, 2015, Singh *et al.*, 2026), and several studies have illustrated exposure to environmental stressors, including but not limited to pesticides, heavy metals and radiation, will cause honeybees to produce oxidative stress (Olgun *et al.*, 2020, Kumar & Maheshwari, 2025). The production of reactive oxygen species (ROS) rises because of the effect of the above-mentioned stressors. The oxidative damage caused by ROS affects cellular structures including lipids, proteins, and DNA.

Experimental studies have demonstrated that oxidative stress contributes to the aging of honey bees, reduces honeybee lifespan and increases honeybee mortality (Li-Byarlay *et al.*, 2016). In addition, oxidative stress impairs cognitive functioning, decreases foraging effectiveness and therefore decreases the entire colony's productivity. More recently, studies have demonstrated that the presence of environmental stressors can alter the activity of antioxidant enzymes in honey bees. For example, electromagnetic radiation and environmental pollutants limit the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione-related enzymes (Vilić *et al.*, 2024, Pathak *et al.*, 2025). These observations support the position that oxidative stress represents a universal and critical underlying mechanism for the negative impact of environmental pollutants on honey bee health.

The honey bee antioxidant defence system is essential in the protection of cellular components against oxidative damage. This defence system is composed of two classes of antioxidants; those that are enzymatically produced and include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx); and those that are not enzymatically produced, such as reduced glutathione (GSH).

Glutathione (GSH) is one of the most significant antioxidant molecules present in insects, as it supports intracellular detoxification and assists in the maintenance of intracellular redox balance. It accomplishes this by neutralizing ROS, preventing oxidative damage to cells (Olgun *et al.*, 2020, Chaudhary, 2024). Studies have shown that environmental pollutants can lead to a significant reduction in GSH levels in honey bees, representing the depletion of antioxidant reserves. In turn, this depletion of GSH prevents honey bees from effectively responding to oxidative stress, thus leaves them more vulnerable to oxidative damage. In addition, lipid peroxidation (LPO) is one of the most important markers of oxidative damage. LPO levels are elevated because of losing membrane lipid integrity, due to ROS. Many studies have reported that LPO levels were significantly increased in honeybees exposed to

multiple types of environmental stressors, such as pesticides and pollutants (Halliwell & Gutteridge, 2015, Inya et al., 2022). Therefore, the balance between oxidative stress caused by ROS and antioxidant protection systems such as glutathione (GSH), is critical to assess the physiological state of honeybees under stress.

The immune system in insects primarily relies on hemocytes or the colored blood cells. These cells play a defensive role in multiple activities, including phagocytosis, encapsulation, and detoxification (Lavine & Strand, 2002). Additionally, Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC) are commonly utilized indicators of insect immune status. Environmental stressors can drastically impact the numbers and types of hemocytes present in honeybees, where reduction in THC indicates immunosuppression, while specific changes in particular hemocyte types indicate that IMR has been altered.

Granulocytes and plasmatocytes specifically play crucial roles in the immune defence of honeybees. For example, granulocytes are involved in phagocytosis and plasmatocytes are involved with encapsulation and healing. Shifts in proportions of these hemocyte types demonstrate activation or suppression of the immune system.

A recent study has shown that honeybee hemocyte counts have decreased due to exposure to pollutants and have compromised their immune function (Kunat-Budzyńska *et al.* 2025, Goswami et al., 2025).The changes being made have resulted in increased susceptibility of the bee population to disease caused by various pathogens, as well as contributing to the decline of colonies.

Biochemical parameters give insight into the physiological and metabolic condition of an organism exposed to stress. In honey bees, the biochemical parameters commonly used to evaluate the metabolic disturbances caused by various stressors are glucose, enzyme levels (ALT, AST, ALP) and total protein levels. Glucose serves as a source of energy to sustain an organism and will be elevated due to stress because of the increased energy demand created by stressors on the organism. Research has demonstrated that environmental stressors can induce hyperglycemia in honey bees, indicating increased energy requirements (Zhao *et al.*, 2021, Singh & Arya, 2024).

In addition to glucose levels, specific enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicative of an organism's tissue injury and metabolic stress. Increased levels of both ALT and AST have been documented to occur in organisms exposed to toxic substances, indicating a cellular injury (Reitman & Frankel, 1957, Singh et al., 2025).

Furthermore, alkaline phosphatase (ALP) is another specific enzyme involved in metabolic function and providing cellular integrity. ALP serves as an additional marker of metabolic function; thus, elevated levels of ALP would suggest that the tissue of the affected organism has been injured and that the metabolic function of the affected organism has been altered.

Total protein levels in an organism are also affected by environmental stress; thus, a decrease in total protein levels could reflect impaired protein synthesis and/or enhanced protein breakdown. Thus, total protein levels can be used to provide an indication of the overall physiological status of organisms under environmental stress.

Overall, biochemical changes in honey bees could serve as evidence of the toxic effects of environmental pollutants on honey bees.

Honey bees have been shown to be adversely affected by environmental pollutants, including PM_{2.5}, pesticides, and heavy metals, which have ultimately contributed to the decline of honey bee colonies. Stressors such as PM_{2.5}, pesticides and heavy metals may act independently or in combination with one another, therefore leading to complex physiological responses.

Studies have reported that honey bees exposed to multiple stressors exhibit increased oxidative stress and increased mortality (Tahir *et al.*, 2023). Pollutants may also interact with pathogens and parasites to exacerbate the negative effects of the individual stressor.

Colony Collapse Disorder (CCD) has been attributed to multiple environmental stressors including pollution; furthermore, there is a growing body of literature that suggests that oxidative stress, immune suppression, and metabolic disruption are leading factors in the decline of colonies (Olgun *et al.*, 2020). The evidence suggests that pollutants negatively affect the successful navigation, foraging ability, and reproductive success of honey bees, ultimately having a negative impact on the productivity and survival of the colony.

The effects of environmental pollutants on honey bees, limited research has specifically evaluated the impact of PM_{2.5} on hemato-biochemical and antioxidant parameters. Most studies have been limited to evaluating pesticide toxicity; therefore, an important gap exists in the current understanding of the effects of air pollution on pollinator health. Additionally, most studies examine individual parameters and do not provide a holistic assessment of the biochemical, hematological, and antioxidant changes that occur as a result of exposure to multiple environmental stressors. There is a need for research to conduct comprehensive studies that assess multiple physiological parameters to further elucidate the mechanisms of environmental toxicity. The goal of this study is to fill this gap by addressing the

combined effects of PM_{2.5} on hemocyte count, biochemical parameters, and oxidative stress markers of *Apis mellifera*. The results of studies such as this are crucial to understanding the ecological implications of air pollution and developing strategies to conserve pollinators.

3. Materials and Methods

3.1 Experimental Insect- To conduct a study on the effects of xenobiotic substances on honey bees, healthy honey bee workers (*Apis mellifera*) free from any signs of disease and pesticide residues were obtained from well-managed apiary sites. To reduce individual variability due to differences in biological age (within 10–15 days of adulthood), worker bees were selected to be approximately the same age, because worker bees of the same age exhibit little variability in metabolic and immunological function (Winston, 1987; Johnson, 2010), and thus worker bees of the same age are ideal subjects for toxicological research. Worker bees were collected early in the morning by using sterile nets to trap them, and placed into laboratory cages (15 x 15 x 15 cm) under controlled temperature, humidity and light conditions. The worker bees that were used for each experiment consisted only of those individuals that were still alive and active, while discarded worker bees were any examples that were dead, injured or weak so that the reliability of the experiments could be maintained (Di *et al.*, 2022).

3.2 Selection and Maintenance of Animals- Before starting experiment, researchers allowed collected honey bees 48 hours to acclimate to laboratory conditions. The laboratory maintained the same environmental parameters (25 ± 2°C temperature, 60-70% relative humidity, and a 12/12 h light-dark cycle) believed to be optimal for honey bee survival (Williams *et al.*, 2013). All bees were provided *ad libitum* with 50% (w/v) sucrose solution and distilled water; the sucrose feeding provided an adequate supply of energy and simulated the natural intake of nectar by honey bees (Brodschneider & Crailsheim, 2010).

Experimental groups were divided as follows:

Control Group: No PM_{2.5} exposure

Treatment Groups: Exposed to PM_{2.5} for 7, 14, and 21 days

The experiment utilized a controlled exposure chamber to simulate PM_{2.5} exposure through the production of PM from urban dust samples at a concentration of 100 to 250 mg/m³ representative of environmentally based levels in urban areas historically reported (Zhao *et al.*, 2021; Pope and Dockery, 2006). The exposure chamber has been outfitted with an aerosol generator and an air circulation system, both of which serve to achieve a homogeneous distribution of PM_{2.5} particles throughout the exposure area. Particle

concentration was tracked with a digital particulate monitor.

3.3 Collection of Hemolymph- Hemolymph was collected from live bees according to laboratory methods in order to conduct this study on physiological characteristics of insects (Lavine & Strand, 2002). The bees were immobilized on ice for 2-3 minutes prior to blood collection to decrease activity and stress.

A sterile fine hypodermic needle was used to puncture the intersegmental membrane of the dorsal abdomen and then samples were obtained with a calibrated micropipette. The hemolymph was transferred immediately to chilled microcentrifuge tubes that contained an anticoagulant solution (0.01 M EDT in insect saline) so as to avoid blood clotting (Gupta, 2013). All samples remained on ice during the collection/processing period & were received by the physiologist within one hour from collection in order to minimize the degradation of cellular components.

3.4 Total Hemocyte Count (THC)

The total hemocyte count (THC) of the insect was performed with a Neubauer's hemocytometer. The hemolymph used to perform this assessment was diluted with insect physiological saline in a 1:10 ratio. Once the hemolymph was diluted, a small amount of diluted hemolymph was placed in the hemocytometer chamber and hemocytes were counted under a light microscope at a 400× magnification. The number of hemocytes per square millimeter was calculated using standard counting formulas (Jones, 1962; Lavine & Strand, 2002).

THC is one of the most commonly used parameters to assess insect immune status because hemocytes are involved in many of the insect's defence mechanisms (i.e., phagocytosis, encapsulation).

3.5 Differential Hemocyte Count (DHC)

The purpose of determining differential hemocyte counts was to determine the various types of hemocytes present within the hemolymph of the animals being examined. Hemolymph smears were prepared on clean glass slides and allowed to air dry.

Methanol was used to fix the slides after which they were stained using Giemsa's stain (10%) for 20 minutes (Gupta, 2013). The stained slides were then washed in water and dried before being examined using an oil immersion objective (1000× magnification).

Hemocytes were classified into three types according to their morphology (Lavine & Strand, 2002):

Prohemocytes – Small, round cells with large nuclei; considered stem cells

Granulocytes – Contain cytoplasmic granules; involved in phagocytosis

Plasmatocytes – Spindle-shaped cells; involved in encapsulation and wound healing

Oenocytes – Large cells; associated with detoxification and lipid metabolism

At least 200 cells per slide were counted, and the percentage of each hemocyte type was calculated.

3.6 Preparation of Fat Body Tissue Homogenate

Insects' main metabolising organ fat body tissue was dissected in a sterile environment. All bee dissections were performed in ice-cold physiological saline to avoid any enzymatic degradation of the tissue. The tissues were removed from the bee and homogenised in phosphate buffer (0.1 M, pH 7.4) using an all-glass homogeniser. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant collected to be used for, biochemical assays and antioxidant assays (Chance & Maehly, 1955).

3.7 Biochemical Parameters

3.7.1 Glucose (GOD-POD Method, End Point)

Glucose concentration was determined using the Glucose Oxidase–Peroxidase (GOD-POD) assay which relies on the enzymatic oxidation of glucose to form gluconic acid and hydrogen peroxide. The intensity of the coloured reaction product was measured at 505 nm using a spectrophotometer (Trinder, 1969).

3.7.2 Alanine Aminotransferase (ALT)

ALT activity was determined using the method of Reitman and Frankel (1957), based on the formation of pyruvate, which reacts with 2,4-dinitrophenylhydrazine to form a colored complex measured at 505 nm.

3.7.3 Aspartate Aminotransferase (AST)

AST activity was estimated using the same principle as ALT, where oxaloacetate formed reacts with DNPH reagent, and absorbance is measured spectrophotometrically (Reitman & Frankel, 1957).

3.7.4 Alkaline Phosphatase (ALP)

ALP activity was measured using the King and King (1954) method, where phenyl phosphate is hydrolyzed to phenol, which reacts with 4-aminoantipyrine to produce a coloured complex measured at 520 nm.

3.7.5 Total Proteins

Total protein concentration was estimated using the Lowry method, based on protein reaction with Folin–Ciocalteu reagent, producing a blue color measured at 660 nm (Lowry *et al.*, 1951).

3.8 Oxidative Stress Markers

3.8.1 Lipid Peroxidation (LPO)

LPO was measured by estimating malondialdehyde (MDA) levels using the Thiobarbituric Acid Reactive Substances (TBARS) method (Ohkawa *et al.*, 1979). The pink chromogen formed was measured at 532 nm.

3.8.2 Glutathione (GSH)

Reduced glutathione (GSH) levels were determined using Ellman's method, where GSH reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to

produce a yellow-colored compound measured at 412 nm (Ellman, 1959).

3.9 Statistical Analysis

All experimental data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test to determine significant differences between groups.

A probability level of $p < 0.05$ was considered statistically significant (Fisher & Yates, 1950).

4. Results

Hematological Changes

4.1 Total Hemocyte Count (THC)

Table 1: Effect of PM_{2.5} exposure on Total Hemocyte Count (THC) in *Apis mellifera*.

Values are expressed as mean $\pm$ SD ($n = 6$). A significant decrease in THC was observed with increasing duration of exposure. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Groups/Days	THC (cells/mm ³)
Control	6850 $\pm$ 210
7 Days	5900 $\pm$ 180*
14 Days	5100 $\pm$ 160**
21 Days	4300 $\pm$ 140***

Values are mean $\pm$ SD ($n = 6$).

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control

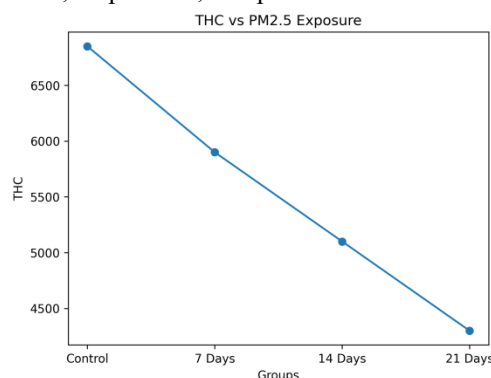


Fig. 1-Effect of PM_{2.5} exposure on Total Hemocyte Count (THC) in *Apis mellifera*.

A significant decrease in THC was observed with increasing exposure duration, indicating immunosuppression.

4.2 Differential Hemocyte Count (DHC)

Table 2-Effect of PM_{2.5} exposure on Differential Hemocyte Count (DHC) (%) in *Apis mellifera*.

Values are expressed as mean $\pm$ SD ($n = 6$). A significant increase in granulocytes and decrease in plasmatocytes indicate immune modulation under PM_{2.5} stress.

Cell Type	Control	21 Days Exposure
Prohemocyte	20 $\pm$ 2	18 $\pm$ 2
Granulocyte	25 $\pm$ 3	40 $\pm$ 4***
Plasmatocyte	45 $\pm$ 3	30 $\pm$ 3***
Oenocyte	10 $\pm$ 1	12 $\pm$ 2

Significant increase in granulocytes and decrease in plasmatocytes

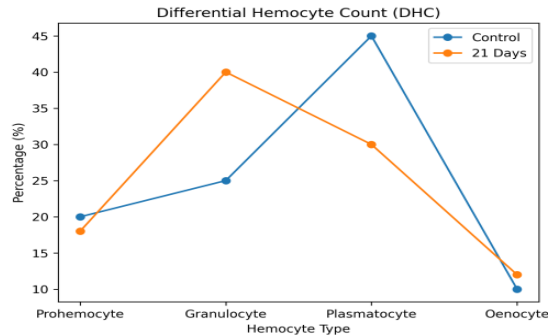


Fig. 2- Differential Hemocyte Count (DHC) comparison between control and PM_{2.5} exposed group.

Significant increase in granulocytes and decrease in plasmatocytes reflect immune modulation under pollutant stress.

Granulocytes increased while there was a major decrease of plasmatocytes, indicating a change in the immune response.

Total Hemocyte Count (THC) showed that there was a significant decrease of hemocytes present in PM_{2.5} exposed individuals compared to the control, indicating there is an immune suppression response; this is similar to what was found by Kunat-Budzyńska *et al.* (2025) who showed reduced hemocyte counts in environmental stress.

Differential Hemocyte Count (DHC) showed a decrease in plasmatocytes and an increase in granulocytes is indicative of activation of defense mechanisms under stress conditions (Lavine & Strand, 2002).

Differential Hemocyte Count indicates the differences between types of hemocytes between the control and PM_{2.5} exposed groups (21 days). A significant increase of granulocytes and a significant decrease of plasmatocytes indicate immune modulation.

Biochemical Changes

4.3 Glucose

Table 3-Effect of PM_{2.5} exposure on glucose levels in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). A significant increase in glucose levels indicates stress-induced hyperglycemia. *p < 0.05, **p < 0.01, ***p < 0.001.

Groups/Days	Glucose (mg/dL)
Control	42 $\pm$ 3
7 Days	55 $\pm$ 4*
14 Days	68 $\pm$ 5**
21 Days	80 $\pm$ 6***

Stress-induced hyperglycemia

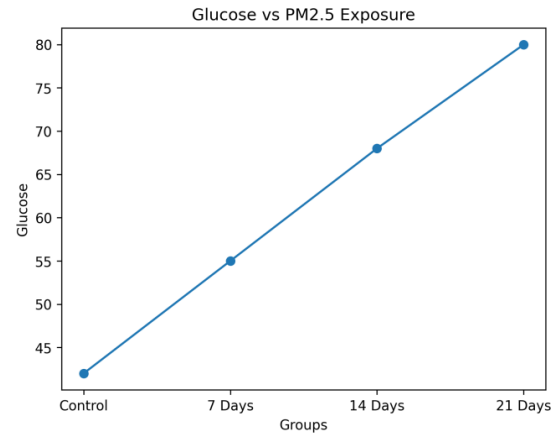


Fig. 3- Effect of PM_{2.5} exposure on glucose levels in *Apis mellifera*.

Glucose levels increased significantly, reflecting stress-induced metabolic changes.

4.4 ALT

Table 4-Effect of PM_{2.5} exposure on alanine aminotransferase (ALT) activity in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). Elevated ALT levels indicate cellular damage and metabolic disturbance.

Groups/Days	ALT (U/L)
Control	18 $\pm$ 2
7 Days	25 $\pm$ 3*
14 Days	34 $\pm$ 3**
21 Days	45 $\pm$ 4***

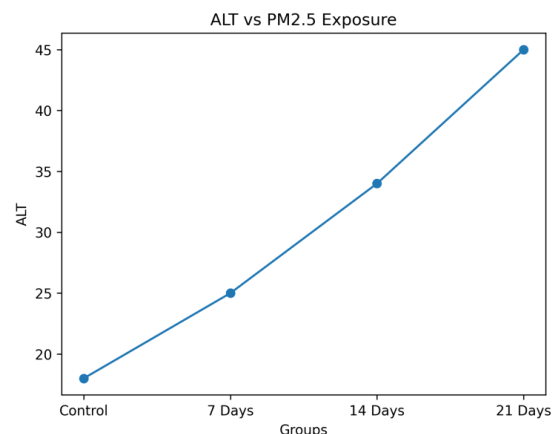


Fig. 4- Effect of PM_{2.5} exposure on ALT activity in *Apis mellifera*.

ALT levels increased significantly, indicating cellular and metabolic damage.

4.5 AST

Table 5-Effect of PM_{2.5} exposure on aspartate aminotransferase (AST) activity in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). Increased AST levels reflect tissue damage under pollutant exposure.

Groups/Days	AST (U/L)
Control	22 $\pm$ 2
7 Days	30 $\pm$ 3*

14 Days	$40 \pm 4^{**}$
21 Days	$52 \pm 5^{***}$

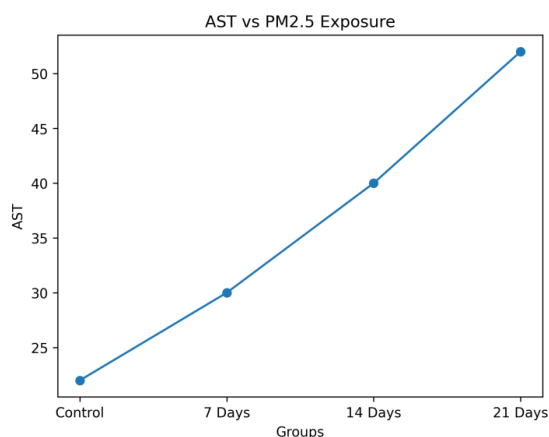


Fig. 5- Effect of PM_{2.5} exposure on AST activity in *Apis mellifera*.

A significant increase in AST activity indicates tissue damage.

4.6 ALP

Table 6- Effect of PM_{2.5} exposure on alkaline phosphatase (ALP) activity in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). Increased ALP activity indicates altered metabolic processes.

Groups/Days	ALP (U/L)
Control	60 ± 4
7 Days	$75 \pm 5^*$
14 Days	$95 \pm 6^{**}$
21 Days	$120 \pm 7^{***}$

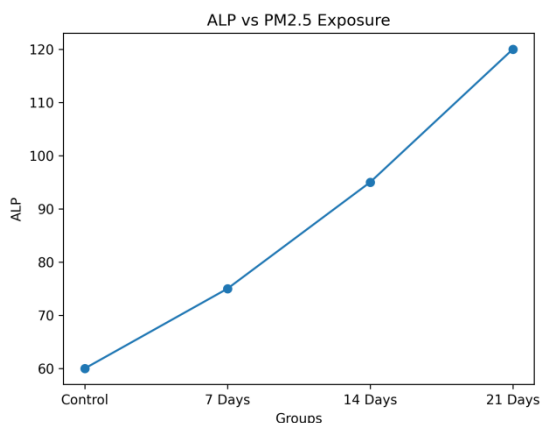


Fig. 6- Effect of PM_{2.5} exposure on ALP activity in *Apis mellifera*.

ALP levels increased progressively with exposure duration.

4.7 Total Protein

Table 7-Effect of PM_{2.5} exposure on total protein levels in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). A significant decline in protein levels indicates impaired metabolic function.

Groups/Days	Protein (g/dL)
Control	6.8 ± 0.3

7 Days	$6.0 \pm 0.2^*$
14 Days	$5.2 \pm 0.3^{**}$
21 Days	$4.5 \pm 0.2^{***}$

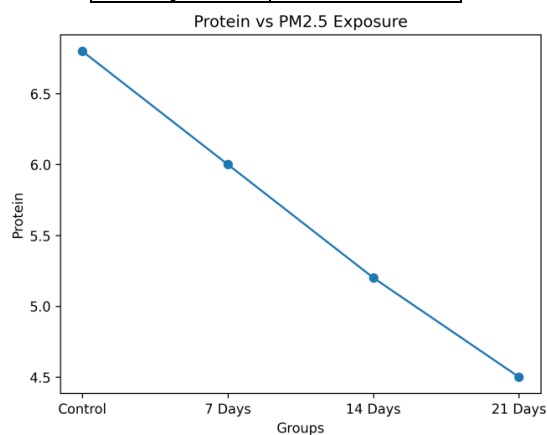


Fig. 7- Effect of PM_{2.5} exposure on total protein levels in *Apis mellifera*.

Protein levels decreased significantly, indicating metabolic disruption.

Glucose levels were significantly elevated in exposed groups, indicating stress-induced metabolic changes (Zhao *et al.*, 2021).

Enzyme activities such as ALT, AST, and ALP were significantly increased, reflecting tissue damage and altered metabolic activity (Reitman & Frankel, 1957).

Total protein levels showed a significant decrease, indicating impaired protein synthesis and increased degradation (Di *et al.*, 2022).

Oxidative Stress Parameters

4.8 LPO (Lipid Peroxidation)

Table 8- Effect of PM_{2.5} exposure on lipid peroxidation (LPO) levels in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). Increased LPO levels indicate enhanced oxidative stress.

Groups/Days	LPO
Control	2.1 ± 0.2
7 Days	$3.5 \pm 0.3^*$
14 Days	$4.8 \pm 0.4^{**}$
21 Days	$6.2 \pm 0.5^{***}$

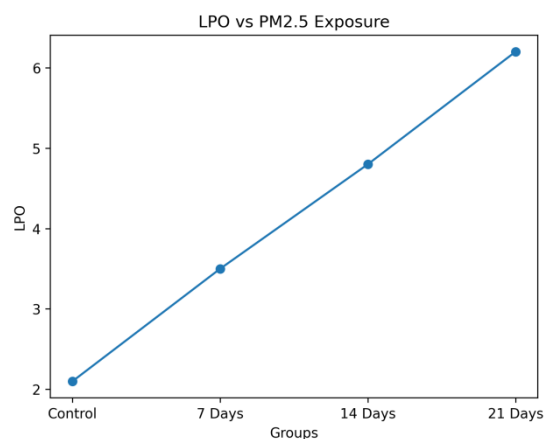


Fig. 8- Effect of PM_{2.5} exposure on lipid peroxidation (LPO) levels in *Apis mellifera*.

LPO levels increased significantly, indicating oxidative damage.

4.9 GSH (Glutathione)

Table 9- Effect of PM_{2.5} exposure on glutathione (GSH) levels in *Apis mellifera*.

Values are expressed as mean $\pm$ SD (n = 6). Decreased GSH levels indicate depletion of antioxidant defense.

Groups/Days	GSH
Control	8.5 $\pm$ 0.4
7 Days	6.8 $\pm$ 0.3*
14 Days	5.5 $\pm$ 0.3**
21 Days	4.0 $\pm$ 0.2***

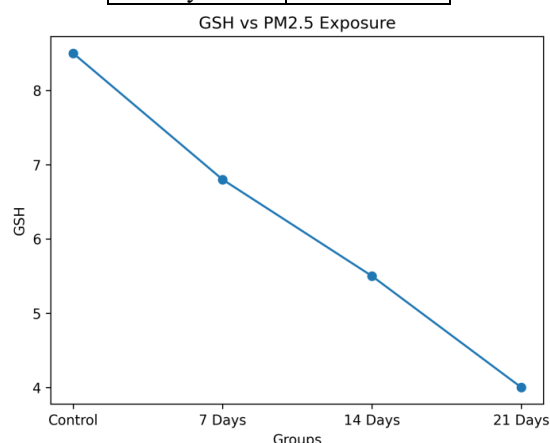


Fig. 9- Effect of PM_{2.5} exposure on glutathione (GSH) levels in *Apis mellifera*.

GSH levels decreased significantly, indicating reduced antioxidant capacity.

Lipid peroxidation (LPO) levels were significantly increased in PM_{2.5}-exposed bees, indicating enhanced oxidative damage (Halliwell & Gutteridge, 2015).

Glutathione (GSH) levels were significantly decreased, suggesting depletion of antioxidant defenses under stress conditions (Olgun *et al.*, 2020).

5. Discussion

The current investigations provide strong evidence for a marked decrease in total hemocyte number (THC) in the honey bee, *Apis mellifera*, after exposure to particulate matter (PM_{2.5}), indicating that their immune system has been adversely affected by these substances. Hemocytes are an essential component of the insect immune system and are responsible for several key processes such as phagocytosis, encapsulation and detoxification (Lavine & Strand, 2002). The decreased THC level in bees indicates that their immune system has been impaired and makes them more susceptible to pathogens as well as environmental stress. Other studies showed similar results when honey bees were exposed to environmental pollutants and pesticides, where their hemocyte counts were reduced and inhibition of the immune system

occurred as well (Kunat-Budzyńska *et al.*, 2025). It is likely that the oxidative stress that resulted from exposure to PM_{2.5} caused damage to the hemocyte membranes, reducing their viability. PM_{2.5} contains a variety of toxic substances including heavy metals and polycyclic aromatic hydrocarbons, both of which can form reactive oxygen species (ROS) that will damage cells (Zhao *et al.*, 2021). In addition, it is also likely that chronic exposure to pollutants will inhibit hemopoiesis, thus reducing the production of hemocytes. The reduction in THC observed in the present study supports earlier findings which suggested that environmental stress results in an impaired immune response in insects (Di *et al.*, 2022). The resulting immunosuppression could produce significant ecological consequences; weakened bees will be at higher risk for disease; thus, disrupting the health and success of the colony.

DHC analysis indicated that PM_{2.5} exposure resulted in a significant change in the composition of hemocytes resulting from both an increase in the number of granulocytes and a decrease in the number of plasmatocytes. The granulocytes are primarily involved in the process of phagocytosis, whereas the plasmatocytes are required to perform the functions related to encapsulating foreign objects and aiding in wound healing (Lavine & Strand, 2002). The finding of elevated levels of granulocytes in the hemolymph of PM_{2.5}-exposed bees, along with the finding of decreased levels of plasmatocytes, suggests that the former is a reflection of an adaptive immune response to environmental stress while the latter is an indication that cellular immunity is negatively impacted. Many studies have documented a re-distribution of the immune system in insects exposed to toxic agents, as the immune system reacts to environmental stressors (Gupta, 2013). It may be that the increase in the number of granulocytes is a compensatory response to the increased pathogen burden or cellular injury that results from exposure to PM_{2.5}. On the contrary, the decrease in plasmatocytes may suggest that, overall; the efficiency of the immune response has also been affected negatively. In addition, the relative imbalance of hemocyte types may be a result of oxidative stress since oxidative stress has been shown to differentially impact the various types of hemocytes due to the effect of reactive oxygen species (Halliwell & Gutteridge, 2015). The results of the current study show that the exposure of bees to PM_{2.5} results in a reduction in overall immune capacity, as well as a disturbance in the relative ratio of hemocyte types within the bee immune system, which has the potential to negatively impact bee colony health and stability in the long term.

There was an increase in glucose levels in the hemolymph of bees after continuous exposure to

PM_{2.5} for 7 days; therefore, the data suggest that continuous exposure to PM_{2.5} produces a metabolic response to stress in the insect. Insects use glucose as the primary source of energy and as one of the essential components of physiological processes. Under stress conditions, insects normally exhibit hyperglycemia as a result of higher demands for energy and increased mobilization of stored glycogen (Zhao *et al.*, 2021). The increase in glucose in this study may be attributed to the activation of the insect's stress response pathways which lead to the release of hormones that cause the breakdown of glycogen. The results of previous studies have demonstrated that exposure to environmental stressors has caused Lee, Chen, Wu, and Jones to find an association between exposure to environmental stressors, such as high temperature and lung function measures (Di *et al.*, 2022). Honey bee metabolic activation (energy metabolism) has also been reported to increase significantly with high glucose levels (Direk *et al.*, 2022). The relationship between exposure to PM_{2.5} and the induction of oxidative stress leading to metabolic alteration has also been suggested as an explanation for the increase in glucose levels. Specifically, reactive oxygen species are known to impair mitochondrial function, which can lead to changes in energy metabolism and increased glucose levels (Halliwell & Gutteridge, 2015). Furthermore, extended periods of time at elevated glucose levels are potentially harmful to cellular function, including increased oxidative stress and protein glycation with the potential for damage to the cell's function. Additionally, the increase in glucose levels in this study indicates that exposure to PM_{2.5} has imposed a substantial metabolic stress on the honey bee population and will potentially negatively impact the honey bee's energy balance, foraging behavior, and overall fitness.

The results of the present study demonstrate that PM_{2.5}-exposed honey bees had a statistically significant increase in ALT activity, which is indicative of cellular damage and/or metabolic dysregulation. Furthermore, ALT is an important enzyme responsible for the metabolism of amino acids, and is widely used as a biomarker for the identification of tissue damage (Reitman & Frankel, 1957). Thus, the elevation in ALT levels may be attributed to tissue damage resulting from the metabolism of the fat body, the major metabolic organ in insects. Exposure to PM_{2.5} can induce oxidative stress, resulting in lipid peroxidation and membrane damage which can permit leakage of intracellular enzymes (i.e., ALT) into the hemolymph (Zhao *et al.*, 2021). Previous research reported similar elevations in ALT activity among organisms exposed to toxic pollutants, providing additional evidence of PM_{2.5} as a hepatotoxic or metabolic stressor (Di *et al.*, 2022). These results support a premise that exposure to PM_{2.5} induces a

disruption of normal metabolic processes and contributes to cell injury in honey bees. The evidence supports the possibility of ALT functioning as a sensitive biomarker for assessing environmental toxicity in insects and indicates that polluted air has an adverse impact on metabolic health.

This was evidenced with a significant rise in AST activity in bees exposed to PM_{2.5}, indicating tissue damage and metabolic impairment. AST is an enzyme that acts in amino acid metabolism and has widely been used as an indicator of cellular injury (Reitman & Frankel, 1957). The increased levels of AST observed in this study can be attributed to cell damage caused perhaps by oxidative stress and subsequent cell lysis operating upon muscle and fat body tissues. PM_{2.5} contains toxic components that may induce ROS production, resulting in cellular damage and enzyme leakage (Zhao *et al.*, 2021).

Similar findings have been reported in previous studies, where increased AST activity was associated with exposure to environmental pollutants and toxic substances (Di *et al.*, 2022). The increase in AST levels indicates that PM_{2.5} exposure disrupts cellular integrity and metabolic processes in honey bees. The combined increase in ALT and AST activities further supports the occurrence of tissue damage and metabolic stress, highlighting the toxic effects of PM_{2.5}.

This study showed a significant increase in ALP activity in PM_{2.5}-exposed bees, indicating altered metabolism and physiology. ALP is involved in dephosphorylation reactions and plays a role in membrane transport and cellular metabolism (King & King, 1954). Increased ALP activity may result from cell injury and increased metabolic needs due to stress. Oxidative stress from PM_{2.5} could compromise membrane integrity, and thereby create an increase in enzyme activity (Zhao *et al.*, 2021). Examples of this elevation in ALP activity are also present in organisms exposed to environmental contaminants (indicative of tissue injury and metabolic disturbance) (Di *et al.*, 2022). The increased ALP activity found in the honey bee samples used in this study supports the hypothesis that PM_{2.5} exposure effects metabolic pathways and cellular functions of the honey bee.

A significant decrease in total protein levels was found in the PM_{2.5} exposed honey bee group suggesting a negative impact on metabolic activities. Protein serves as a component of cellular structure, an enzyme activity, and as a contributor to physiological processes. Decreased total protein levels may be due to increased rates of protein degradation or decrease rates of protein synthesis in response to PM_{2.5} exposure (Zhao *et al.*, 2021). Oxidative stress from PM_{2.5} can promote protein oxidation and instability causing decreases in total protein levels (Halliwell and Gutteridge, 2015). Other studies have documented lower levels of

total proteins in honey bees exposed to environmental stressors; lower levels of total protein were associated with poor physiological condition (Di *et al.*, 2022). Decreased total protein levels in honey bees exposed to PM_{2.5} suggest a negative effect on metabolic and physiological activities.

PM_{2.5} exposed honey bees exhibited significantly higher levels of LPO than the unexposed honey bees; this evidence indicates an increase in oxidative stress. Lipid peroxidation serves as an endpoint of oxidative cellular damage due to the ROS degradation of cellular membranes (Halliwell and Gutteridge, 2015). The increased LPO levels in this study suggest that the presence of PM_{2.5} leads to the increased production of ROS, leading to membrane damage. Similar to these findings are reports of PM_{2.5} effects on honey bees exposed to environmental contaminants (Olgun *et al.*, 2020). Increased LPO levels result in membrane integrity being compromised, where impairment in the membrane could contribute to cellular dysfunction and/or cell death. Thus, oxidative stress is a primary mechanism of PM_{2.5} toxicity to honey bees. PM_{2.5} exposed honey bees experienced significantly lower levels of GSH compared to control bees indicating that these bees suffered a decrease in their antioxidant defences. GSH is an antioxidant produced intracellularly to protect cells from oxidative injury (Olgun *et al.*, 2020). The decrease in GSH levels in PM_{2.5} exposed honey bees is likely related to increased consumption of GSH as a result of neutralizing the ROS produced by PM_{2.5} (Zhao *et al.*, 2021). Similar decreases in GSH have been found in other organisms exposed to environmental contaminants. GSH depletion reduces the effectiveness of the cellular antioxidant defense system thus providing less protection for the cells against oxidative injury. GSH decreases highlight the role of cellular antioxidant defense systems in protecting honey bees from environmental stressors.

Conclusion

Recent studies show that exposure to particulate matter 2.5 micrometers or smaller (PM_{2.5}) has detrimental effects on the health and immune systems of honeybees (*Apis mellifera*). The reduction in total hemocyte count and changes in hemocyte composition suggest impairment of the honeybee's immune system, making them more susceptible to pathogens and environmental stresses. In addition to these immune deficiencies, there was a significant increase in glucose and other biochemical markers such as alanine transaminase (ALT) aspartate aminotransferase (AST), and alkaline phosphatase (ALP) that indicated there was an imbalance in metabolism and damage to tissues when exposed to PM_{2.5}. Furthermore, the increase in molecules known as lipid peroxides and the decrease in those called

glutathione demonstrate that exposure to PM_{2.5} resulted in oxidative stress on honeybee cells because of the inability to counteract with the normal antioxidant response. The results of this study indicate that PM_{2.5} can cause significant stress and toxic effects on *A. mellifera* by immune suppression, metabolic dysfunction, and oxidative damage. The results of this study will help develop and provide the basis for future management plans for the preservation of honeybee populations as well as assessing ecologically relevant environmental quality using *A. mellifera* as a bioindicator species.

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