Research Article

Evaluation of the Immunomodulatory Effect of *Moringa oleifera* Leaf Extract in Cold Stress-Induced Rats

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Abstract | Cold stress disrupts immune homeostasis by altering leukocyte dynamics and cytokine signaling, leading to increased inflammatory responses. Natural antioxidants such as *Moringa oleifera* have shown immunomodulatory potential and may mitigate stress-induced immune dysfunction. This study aimed to evaluate the protective effects of *M. oleifera* leaf extract on immunological parameters and cytokine levels (IL-6 and IL-35) in rats subjected to cold stress. Twenty-four male albino rats were randomly divided into four groups: control, extract-only, cold stress, and cold stress with the extract. Cold stress was induced by daily exposure to 4°C for 2 h for 30 days. Rats in the treatment group received 200 mg/kg *M. oleifera* extract. Hematological parameters were analyzed using a hematological analyzer and cytokine levels were measured using ELISA. WBC and granulocyte counts were significantly increased ($P \leq 0.05$), whereas lymphocyte and monocyte numbers were significantly decreased ($P \leq 0.05$) on cold stress. The levels of IL-6 were significantly upregulated, while IL-35 was downregulated in the stress group ($P \leq 0.05$). Administration of *M. oleifera* extract abrogated these effects by restoring the hematological parameters and cytokines to their basal values. Importantly, the IL-6 level was significantly decreased to 3.48 ± 0.6 and IL-35 was enhanced to 232.91 ± 21.8 pg/mL in stress+extract compared with stress-treated rats ($P \leq 0.05$). The findings suggest that *M. oleifera* may provide immunoprotective effects under cold stress, particularly through dual modulation of IL-6 and IL-35. These results support its potential as a natural therapeutic agent for stress-induced immune dysregulation. However, further research is required to confirm its therapeutic efficacy.

Keywords | Cold stress, *Moringa oleifera*, IL-6, IL-35, Hematology

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INTRODUCTION

Stress is a physiologic response that for centuries has been recognized to occur in the face of adversity or hostility in the environment (Castellani and Young, 2016). However, earlier reports did not adequately address stress responses, partly due to limitations related to language barriers among non-native English-speaking contributors. Cold stress, a unique type of stress, is the body's response to low ambient temperatures such as cold air or water (Zhao *et al.*, 2014). Cold temperature is defined as a temperature below 12°C in air or 20°C in water. Such low temperatures may induce a variety of physiological responses in both humans and animals, including cellular and molecular adaptations against cold-induced damage (Li *et al.*, 2024). Furthermore, extreme cold temperatures below 0 °C are able to diminish the body's natural immune defenses, disturb immunological cascades in vivo, and promote susceptibility of an individual to microorganisms (Wu *et al.*, 2022). Physiological stress is known to produce

numerous behavioral changes in animals, such as decreases in overall locomotion and exploration behavior (Campos *et al*., 2013). A principle consequence of oxidative stress, which is a manifestation of physical stress, is generation of ROS that cause damage to cells and tissues (Schieber and Chandel, 2014). Antioxidants represent critical protection factors against reactive oxygen species, and there is a growing wealth of evidence showing an inverse correlation between dietary intake of foods rich in antioxidants and development of several diseases (Jomova *et al*., 2023). From ancient times to the present, herbal drugs have been an important part of society (Gao *et al*., 2024). Though there are various anxiolytics that help in stress management, specialized anti-stress drugs till date have not been developed in the field of modern medicine as a result harmful effects of stress on biological functions (Sestakova *et al*., 2013). New psychotropic drugs, however, have a variety of undesirable side effects and they often fail to treat the psychopathology that underlies stress (Baumeister *et al*., 2016). Thus, it would be beneficial to discover more effective and bringing relief antistress drugs.

The use of medicinal plants in many developing countries have been of great interest to health workers and researchers (Otitoju *et al*., 2014), partly because of an increasing shift toward herbal therapies (Adamu *et al*., 2021). One of the most extensively investigated medicinal plants is *Moringa oleifera*, also known as the "miracle tree" due to its wide range of therapeutic potentials (Gopalakrishnan *et al*., 2016). *M. oleifera*, belonging to the genus *Moringa*, is the most widely cultivated species. This plant has been valued for centuries because of its remarkable nutritional, medicinal, and industrial applications. *M. oleifera* is used as a food source and has long been employed in traditional medicine for the treatment of various ailments, including ear and dental infections, skin disorders, hypertension, respiratory diseases, diabetes, and cancer (Adamu *et al*., 2021; Beker *et al*., 2018). *M. oleifera* leaves have been reported to be a valuable source of both macro- and micronutrients (Islam *et al*., 2021). There are numerous non-chemical, natural approaches that can enhance health and strengthen the immune system, and *M. oleifera* is among the most promising. Its leaves are exceptionally rich in essential nutrients and bioactive compounds, including zinc, selenium, β-carotene, and vitamins C and E, which are often deficient in diets yet play crucial roles in supporting immune function (Drue Jr., 2014).

Interleukin-35 (IL-35) is an anti-inflammatory cytokine predominantly secreted by regulatory T and B cells and is involved in immune tolerance and the suppression of excessive inflammatory responses (Ye *et al*., 2021). Given its immunosuppressive role, IL-35 was assessed alongside pro-inflammatory cytokines such as interleukin-6 (IL-6) to provide insight into the balance between inflammation and immune regulation under stress conditions.

Although the immunomodulatory properties of various natural compounds have been widely investigated, the specific role of *M. oleifera* in regulating the IL-6/IL-35 cytokine axis under cold-stress conditions remains unclear. Therefore, the present study aims to address this gap by evaluating the potential of *M. oleifera* leaf extract to modulate immune responses during cold stress, with particular emphasis on hematological parameters and the balance between the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-35.

MATERIALS AND METHODS

Experimental animals

In this experiment 24 healthy adult male albino rats (200–300 g) were used in this experiment. Male rats were used to minimize variability associated with hormonal fluctuations in females, allowing for more controlled assessment of cold stress–induced immunological changes and the effects of *M. oleifera* extract. Each experimental group consisted of six animals (n= 6), and all analyses represent biological replicates performed under identical experimental conditions. Animals were purchased from the animal house in Najaf Governorate and housed in the animal house of the Biology Department, College of Science, University of Babylon. They were maintained under controlled conditions, of 12 hours of light-dark cycle at 25±3 ˚C. They had been given time to adapt for approximately two weeks. Animals had free access to a standard laboratory pellet diet and water ad libitum throughout the experimental period.

Collection and extraction of plant material

Fresh leaves of *M. oleifera* plant were obtained from a private farm house in Hillah, Babylon, Iraq. The leaves were washed, air-dried in the shade at room temperature, ground into a powder and kept in an airtight sterile container prior to extraction. The process of preparation of plant extract was based on the study of Al-Sultany *et al*. (2019). In brief, 1 gm raw *M. oleifera* leaf powder was mixed with10 ml methanol-water solvent (1:1 v/v). The mixture was rapidly shaken for 1 hour at by using a high-speed shaking device to mix well, and then 40 ˚C water bath incubation of 2 hours. Liquid extract was decanted from solids using a filter paper. The dried filtrated liquid was evaporated in an oven at 45 ˚C to obtain the extract. The dried and concentrated material was powdered, UV sterilized for 20 min and used. The sterilized powder was then stored in a sterile, dark and closed glass container until being used again.

Cold stress model

Animals were housed individually in cages measuring (30

× 15 × 12 cm). Chronic intermittent cold stress was the stress paradigm used in this study, as previously described by Elmarzouki *et al.* (2014) by placing them individually in a plastic container with ice cubes and replacing them at regular intervals to ensure thermal stability. The temperature was routinely monitored using a calibrated digital thermometer to ensure maintenance of the target cold-stress conditions at 4°C, consistent with the established definition of cold stress. The rats in the groups under cold were exposed to a controlled cold environment at 4°C for 2 hours daily over a 30-day period between 8:00 and 10:00 am in order to prevent interference with the circadian cycle of corticosterone. Then animals were returned to their housing cages.

Experimental design

Animals were randomly divided into four groups, with six rats in each group: Control, which served as the negative control and received distilled water only for 30 days without exposure to cold stress; Extract, which received *M. oleifera* extract at 200 mg/kg for 30 days without cold stress (Ruby *et al.*, 2024); stress, which was exposed to cold stress at 4 °C for 2 hours daily for 30 days without treatment; and Stress + Extract, which was exposed to the same cold stress regimen for 30 days followed by administration of *M. oleifera* extract at 200 mg/kg for an additional 30 days. This experimental design was selected to evaluate both the protective and restorative effects of *M. oleifera* against chronic cold stress, as previous studies report cold exposure durations ranging from short-term (1–2 weeks) to long-term (up to 8 weeks). Body weights of all animals were recorded weekly to monitor growth and health, and at the end of the study, animals were sacrificed. Approximately 2 mL of blood was collected into EDTA tubes for the assessment of hematological parameters and cytokine levels.

Immunological study

Complete blood cell count (CBC) was assessed using the Mythic 18 VET, a fully automated benchtop analyzer for hematology that uses impedance technology. The concentrations of IL-6 and IL-35 were measured using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA), following the manufacturer's instructions (Elabscience, China).

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS, Chicago, USA). The sample size for each experimental group was n= 6. Results are presented as mean ± standard deviation (SD). Prior to mean comparisons, the normality of residuals and equality of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. One-way ANOVA was applied to evaluate differences among groups, followed by Duncan's multiple range test as a post-hoc analysis. All data are expressed as mean ± standard error (SE), and differences were considered statistically significant at $P \leq 0.05$.

RESULTS

The effects of cold stress and *M. oleifera* extract on white blood cell (WBC) counts and differential leukocyte percentages (lymphocytes, monocytes, and granulocytes) are summarized in Table 1. There were marked changes in various hematological parameters due to cold stress. The total WBC count was significantly higher ($P \leq 0.05$) in the stress group (51.47 ± 3.4 $\times 10^3/\mu L$) than control (7.33 ± 1.5 $\times 10^3/\mu L$), showing leukocytosis due to stress. Rats treated with *M. oleifera* extract singly, showed a slight but significant ($P \leq 0.05$) marked rise (13.70 ± 4.2 $\times 10^3/\mu L$) in WBC when compared to control while the stress +extract group revealed no such rising trend from control (8.33 ±1.1 $\times 10^3/\mu L$) which implies prospective normalization of WBC count after administration of extract protocol alone or in combination. The lymphocyte percentage was significantly reduced ($P \leq 0.05$) in the stress group (1.75 ± 0.4%) in contrast to the control (73.13 ± 3.9%), extract (70.75 ± 6.2%), and stress+extract (72.63 ± 2.5%) groups. Similarly, monocyte percentage was significantly reduced ($P \leq 0.05$) in the stress group (4.52 ± 1.0%) compared to the control group, whereas treatment with *M. oleifera* alone (10.95 ± 2.8%) or in combination with stress (13.08 ± 2.7%) resulted in a significant increase ($P \leq 0.05$) compared to the control group, with the stress + extract group showing the highest value. In contrast, the granulocyte percentage was significantly increased ($P \leq 0.05$) in the stress group (92.78 ± 4.9%) compared to the control (16.93 ± 3.1%), extract (17.47 ± 1.5%), and stress + extract (15.20 ± 3.7%) groups, all of which maintained similar levels.

Table 1: Effect of *M. oleifera* extract on some immunological parameters (mean ± SD) in rats exposed to cold stress for 30 days.

Groups/ Parameters	Control	Treatment groups		
		Stress	Extract	Stress + Extract
Mean±S.D				
WBC $10^3/\mu l$	7.33 ± 1.5^{A}	51.47 ± 3.4^{C}	13.70 ± 4.2^{B}	8.33 ± 1.1^{A}
Lym, %	73.13 ± 3.9^{B}	1.75 ± 0.4^{A}	70.75 ± 6.2^{B}	72.63 ± 2.5^{B}
Mon, %	9.93 ± 1.2^{B}	4.52 ± 1.0^{A}	10.95 ± 2.8^{BC}	13.08 ± 2.7^{C}
Gran, %	16.93 ± 3.1^{A}	92.78 ± 4.9^{B}	17.47 ± 1.5^{A}	15.20 ± 3.7^{A}

Different letters in a row indicate significant difference at ($P \leq 0.05$). WBC, white blood cell; Lym, lymphocyte; Mon, monocyte; Gran, granulocyte.

In the present study, the serum levels of interleukin-6 in

Figure 1 showed significant ($P \leq 0.05$) variation among the experimental groups. Rats exposed to cold stress exhibited a marked increase in IL-6 levels (81.24 pg/mL), which were significantly higher ($P \leq 0.05$) than those in the control group (47.59 pg/mL). In contrast, rats treated with *M. oleifera* extract alone showed a significant decrease ($P \leq 0.05$) in IL-6 levels (32.34 pg/mL) compared to the control and stress groups. Moreover, the Stress + Extract group showed a marked attenuation of IL-6 concentration (40.7 pg/mL) compared with the stress group.

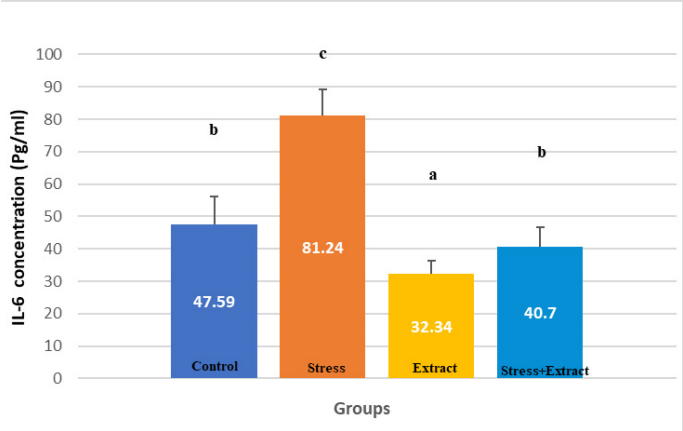

Figure 1: Effect of *M. oleifera extract* on serum IL-6 levels in rats exposed to cold stress. Data are presented as mean ± SD (n = 6). Bars with different letters indicate statistically significant differences ($P \leq 0.05$).

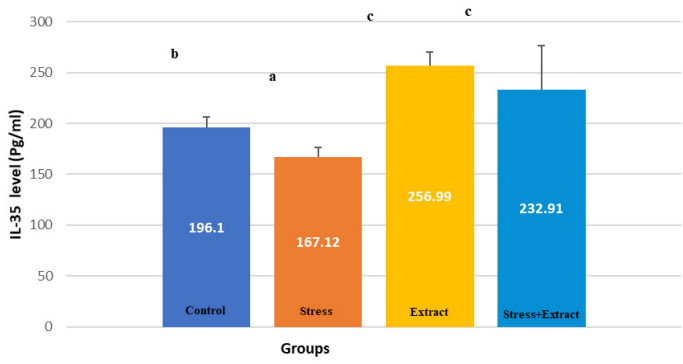

Figure 2: Effect of *M. oleifera extract* on IL-35 levels in rats exposed to cold stress. Data are presented as mean ± SD (n= 6). Bars with different letters indicate statistically significant differences ($P \leq 0.05$).

In Figure 2 serum IL-35 was significantly ($P \leq 0.05$) altered between experimental groups. A significant reduction ($P \leq 0.05$) on IL-35 level was observed when compared with the control (196.1 pg/mL 167.12 pg/mL) upon cold treatment, indicating a down regulation of this anti-inflammatory cytokine in stress status. On the other hand, *M. oleifera* extract administration significantly raise ($P \leq 0.05$) the IL-35 (256.99 pg/mL) levels, group co-treated stressed rats with extract also increased in IL-35 (232.91 pg/mL) levels when compared to control and stress groups. Statistical analysis demonstrated that extract-treated and stress+extract groups were significantly higher IL-35 level than the stress and control groups at the level of ($p \leq 0.05$), however they did not differ significantly among themselves.

DISCUSSION

The present study demonstrated that chronic intermittent cold stress induced significant immunological and hematological disturbances in rats, including leukocytosis, granulocytosis, lymphopenia, and shifts in pro-inflammatory cytokines, with increased IL-6 and decreased IL-35 levels. These alterations indicate systemic immune dysregulation under stress conditions. Notably, the reduction in IL-35 represents a novel finding, suggesting a previously underexplored mechanism by which cold stress disrupts immune homeostasis. Administration of *M. oleifera* leaf extract effectively restored these parameters, highlighting its potent immunomodulatory and protective effects. These results support and extend previous findings on stress-induced immunopathology and have potential implications for phytomedicine-based interventions (Alotiby, 2024; Mubeen *et al.*, 2025).

Hematological changes in the presented study, including the marked elevation of total WBC and granulocyte levels and remarkable decline of lymphocyte counts, consistent with an adaptive stress response. These results are consistent with other studies reporting that cold stress produces a fight-or-flight response physiology, stimulating the hypothalamic pituitary adrenal (HPA) axis and, finally, release of stress hormones corticosterone (Chu *et al.*, 2024). Elevated levels of glucocorticoids and catecholamines promote the mobilization of neutrophils from the marginal pool and bone marrow, while simultaneously causing the sequestration of lymphocytes into lymph nodes. This leads to leukocytosis dominated by neutrophilia and stress-induced lymphopenia as accessory features, which is indicative of acute adaptive redistribution before injury or infection (Dhabhar, 2014). Other research has shown that cold temperatures (4°C and -12°C) modulate immune parameters in rats, including shifts in T-lymphocyte populations (Hu *et al.*, 2016). Eimonte *et al.* (2021) also observed a prolonged hysteresis, with a sustained increase in neutrophil percentage and a concomitant decrease in lymphocyte percentage following cold-water immersion.

Treatment with *M. oleifera* extract, both alone and after cold stress, appeared to alleviate the stress-induced immune alterations. Total WBC counts were normalized, and leukocyte balance was restituted by the extract demonstrating its potent immunomodulatory and antioxidant activities. Changes in lymphocytes and monocytes indicate an improved adaptive immunity

with less overactivation of the innate immune system in Stress + Extract. This effect is likely attributed to the high phytochemical content of non-chemical extracts (NCEs), including flavonoids, phenolic acids, ascorbic acid, and carotenoids, which can scavenge reactive oxygen species (ROS) and protect hematopoietic tissues (Xiao *et al*., 2020). Further decrease in granulocytes percentage and restoration of lymphocyte/granulocyte ratio confirmed that *M. oleifera* mediate the attenuation in stress induced neutrophilia and systemic inflammatory response (Drue and Minor, 2018). These hematological alterations were accompanied by pronounced changes in inflammatory cytokine profiles, further confirming systemic immune dysregulation under cold stress conditions.

Immunologically, under cold stress levels of IL-6 were dramatically increased in the immunological parameters, indicating a switch to pro-inflammatory mode. The pleiotropic cytokine IL-6 plays a critical role in inflammatory response, hematopoiesis and immune reaction. It is a widely reported biomarker of systemic inflammation and is activated in the presence several physical and environmental stress factors including exposure to cold (Vialard and Olivier, 2020). Cold exposure is a physiological challenge that induces oxidative stress, modulates the activity of immune cells and promotes systemic secretion of proinflammatory cytokines such as IL-6 (Wu *et al*., 2022). The increase in IL-6 levels in cold-stressed animals could also be a product of stress-induced activation of nuclear factor kappa B (NF-κB), a transcriptional activator for several inflammatory mediators (Su *et al*., 2018). In addition, induction of IL-6 by cold stress is enhanced with an increase in the release of catecholamines through β-adrenergic receptor-pathways (Balakin *et al*., 2025).

The administration of *M. oleifera* leaf extract was associated with a significant reduction in serum IL-6 levels under both normal and cold stress conditions. This observation supports its potential anti-inflammatory properties, which have been attributed to polyphenols, flavonoids, and isothiocyanates that may suppress NF-κB activation and cytokine expression (Padayachee and Baijnath, 2020; Hamdy, 2024). The decrease in IL-6 indicates inhibiting the inflammation induced by the cold stress for *M. oleifera*, a higher level of vitamin C and β-carotene could contribute to immune regulation and cellular redox homeostasis (Srivastava *et al*., 2023; Sulastri *et al*., 2023). The herein reported decrease of IL-6 (from 81.24 pg/mL in the stress group to 40.7 pg/mL in the stress + extract group) constitutes direct local *in vivo* evidence of these anti-inflammatory effects.

Conversely, few studies reported that IL-35 is involved in cold stress, which is a new mechanism of this study. IL-35 is a powerful anti-inflammatory and immunosuppressive cytokine of the IL-12 family, produced mainly by regulatory T cells (Tregs) as well as regulatory B cells (Bregs), which plays an essential part in immune tolerance, and inhibition of over reactive inflammatory responses (Ye *et al*., 2021). Under cold stress, the reduction of IL-35 indicates that stress is not only an inducer of pro-inflammatory cytokines (like IL-6), but also a suppressor of immunoregulatory components, resulting in immune imbalance. This may be due to the fact that hyperactivation of HPA axis and over release of glucocorticoids may cause damage to regulatory T-cells function, so as to reduce IL-35 production (Li *et al*., 2025). Hence, the decreased levels of IL-35 in stress group can be an indicator of an imbalance between pro-and anti-inflammatory signals which will create favoritism towards inflammatory reactions and possibility to tissue damage or disease (Picant *et al*., 2025).

Interestingly, the observed increase in the anti-inflammatory cytokine IL-35 in the groups receiving the extract (256.99 pg/mL in the extract-only group and 232.91 pg/mL in the stress + extract group) is a novel finding. This suggests that *M. oleifera* may not only suppress inflammation but could also stimulate the body's natural defense and restorative systems. The stimulation of IL-35 by *M. oleifera* is very important because cytokines can suppress proliferation and cytokine secretion from Th1 and Th17 cells (Su *et al*., 2018). The above mentioned phenomenon indicates *M. oleifera* may help alleviate inflammation and restore immune homeostasis by up-regulating anti-inflammatory IL-35 expression, another marker we have not noticed on cold stress. It also guards against stress-induced inflammation-related disease, autoimmune diseases and tissue damage. Collectively, these results indicate that *M. oleifera* has a dual regulation effect on immunoregulation through decreasing pro-inflammatory cytokines such as IL-6 and increasing anti-inflammatory cytokines such as IL-35 in order to maintain immune homeostasis in stress conditions (Saleem *et al*., 2020).

Though the findings of this study offer some clues on *M. oleifera* modulating the immune system in cold stress, some limitations should be noted. The present study used only male rats, so our findings might be limited to generalization in females because of differences between sexes in immune and stress reactions. The short 30-day period over which the present study spanned gives a glimpse of the momentary effects but not much insight into long-term beneficial/side effects of *M. oleifera* supplementation. Future studies should also attempt to clarify these points, including female rats in the research, researching the appropriate dose (not just one sub-dose as this study), and extending observation times to look at chronic effects. An important next step will be to determine the bioactive component within the extract and their specific active compounds which are responsible for immunomodulatory activity. While these

preclinical results are encouraging, caution must be used in extrapolating to humans. Placebo-controlled clinical trials on *M. oleifera* are urgently needed to definitely establish its safety and efficacy for managing stress-induced immune dysfunction within human populations.

CONCLUSION

In conclusion, this study provides compelling evidence that chronic intermittent cold stress induces a significant pro-inflammatory state in rats, characterized by leukocytosis, granulocytosis, and a critical cytokine imbalance marked by the upregulation of IL-6 and downregulation of IL-35. Administration of *M. oleifera* leaf extract effectively counteracted these immunopathological changes, restoring hematological parameters and, most notably, exerting a dual immunomodulatory effect on the IL-6/IL-35 axis. The observed suppression with a concomitant induction of the pro-inflammatory (IL-6) and anti-inflammatory (IL-35) cytokines, respectively indicates that *M. oleifera* is not only suppressing inflammation but also contributing to promotion of an immunoregulatory phenotype. These results highlight the IL-6/IL-35 signaling equilibrium as a pharmacological target for diminishing stress-induced immunomodulatory imbalance and support the potential of *M. oleifera* as phytomedical agent. Our results should be confirmed in further studies able to dissect the specific bioactive compounds probably involved and translated to a clinical setting.

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NOVELTY STATEMENT

This study provides novel experimental evidence on the immunomodulatory role of *Moringa oleifera* methanolic leaf extract in mitigating cold stress-induced immune and hematological disturbances. Specifically, it demonstrates, for the first time, the modulatory effects of *Moringa oleifera* on both pro-inflammatory (IL-6) and anti-inflammatory regulatory cytokine (IL-35) responses under chronic cold stress conditions. The study also integrates hematological profiling with cytokine analysis to provide a comprehensive understanding of the protective immunophysiological mechanisms of *Moringa oleifera* in stress-induced immunopathology

AUTHOR'S CONTRIBUTION

NMJ: Conceptualization, methodology, validation, investigation, resources, writing/original draft, writing review and editing, supervision, project administration, funding acquisition. AJH: Methodology, validation, investigation, writing/original draft, writing-review, and editing. JOA: Validation, formal analysis, investigation, data curation, writing original draft, visualization. All the authors have read and agreed to the published version of the manuscript.

Ethics approval

All procedures on animals were performed in connection with the ethical principles for animal experimentation and were approved by the Animal Ethics Committee of College of Science, University of Babylon, Department of biology (Approval No. Z240104; January 30, 2024).

Generative AI and AI-assisted technology statement

During the preparation of this manuscript, the authors used AI-assisted language tools solely for the purpose of improving English language clarity, grammar, and readability. The authors take full responsibility for the originality, integrity, accuracy, and scientific content of the work. No AI tools were used for data generation, data analysis, or interpretation of results.

Conflict of interest

The authors have declared no conflict of interest.

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