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Hydroxytyrosol-Mediated Iron Nanoparticles Enhance Erythropoietin And Modulate Immune Response In Phenylhydrazine-Induced Hemolytic Anemia In Rats

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Abstract
Anemia is characterized by a decrease in the quantity or function of red blood cells and hemoglobin together, which impairs the blood's ability to transport oxygen efficiently, it remains one of the most prevalent blood disorders and represents a significant global health challenge affecting a significant proportion of the world's population of all ages. Hemolytic anemia characterized by premature erythrocyte destruction. This study evaluates the therapeutic efficacy of green-synthesized iron nanoparticles (FeNPs) using hydroxytyrosol (HXT) in the treatment of phenylhydrazine (PHZ)-induced hemolytic anemia in male Sprague-Dawley rats. The synthesis of iron nanoparticles was carried out in the laboratories of the College of Science, University of Kirkuk. The synthesis process was successfully completed, and the iron nanoparticles were characterized using various techniques. UV-Vis spectroscopy revealed a strong absorption peak at 294 nm, confirming the synthesis of iron nanoparticles. FESEM images revealed quasi-spherical nanoparticles with an average size of 33 nm, followed by animal housing and experimentation in the animal housing. Forty-eight male Sprague Dawley rats were divided into six groups: control, PHZ, FeNPs-HXT, $FeCl_3$, Feroglobin, and HXT. Biochemical and immunological parameters including erythropoietin (EPO), IL-10, IL-12, TNF-α, and IFN-γ were assessed. FeNPs-HXT significantly restored EPO levels and modulated cytokine profiles more effectively than other treatments. The nanoparticles exhibited higher anti-inflammatory and erythropoietin activity, probably due to the improved bioavailability and antioxidant characteristics of HXT. These findings underscore FeNPs-HXT as a possible therapy candidate for hemolytic anemia.
Keywords: *Cytokines; Erythropoietin; Hemolytic anemia; Hydroxytyrosol; Iron nanoparticles.*

1. INTRODUCTION
Anemia is a prevalent hematological condition that affects oxygen transport and leads to global suffering. Hemolytic anemia, characterized by the early destruction of erythrocytes, results in hypoxia and triggers compensatory synthesis of erythropoietin (EPO) [1], [2]. Phenylhydrazine (PHZ) is used frequently to cause hemolytic anemia in experimental models owing to its oxidative damage to erythrocytes. Latest developments in nanomedicine have presented iron-based nanoparticles (FeNPs) as viable alternatives to traditional iron therapy [4]. The green synthesis utilizing hydroxytyrosol (HXT), a powerful polyphenol derived from olives, presents a biocompatible and environmentally sustainable method [5]. HXT exhibits antioxidant, anti-inflammatory, and metal-chelating characteristics, proving it suitable for the stability of nanoparticles [6-8]. This study aims to assess the relative effectiveness of hydroxytyrosol-mediated iron nanoparticles (FeNPs-HXT) in alleviating phenylhydrazine (PHZ)-induced hemolytic anemia and influencing immunological responses, relative to $FeCl_3$, Feroglobin, and hydroxytyrosol.

2. MATERIALS AND METHODS
Synthesis of FeNPs-HXT
Iron chloride, with a purity of 99.80%, has been purchased from Sigma-Aldrich, a German company, for the green production of iron nanoparticles. A 500 ml solution of 0.01 M iron chloride was prepared. Subsequently, 30 ml of 0.01 M concentration of a hydroxytyrosol solution was added drop by drop to the prepared iron chloride solution, the mixture was refluxed at 50 °C for 1 h. The change in the color of the iron chloride solution confirmed the biosynthesis of iron nanoparticles [6].

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Characterization of FeNPs:

UV-visible spectroscopy is one of the most widely used techniques for characterizing the optical properties of iron nanoparticles. Because of surface Plasmon excitation, iron nanoparticles show a strong absorption peak, making this method very effective for detecting iron colloids. As shown in Figure (1), the absorption spectrum of the iron nanoparticles shows a peak at a wavelength of 294 nm, indicating surface Plasmon resonance, which is consistent with findings from other researchers [9]. FTIR analysis was conducted to identify the functional groups involved in the reduction and stabilization of iron nanoparticles. Identified O–H, C≡C, and C=C functional groups, confirming HXT capping and reduction activity. Reports of "green" synthesis of iron nanoparticles using plant polyphenols have been on the increase in popularity. Recent evaluations indicate that phenolic O–H groups facilitate the reduction of Fe^{2+}/Fe^{3+} to elemental iron, while the aromatic frameworks and remaining unsaturation serve as steric shields around the nanoparticle surface, providing colloidal stability without requiring additional surfactants. The multiple functions of hydroxytyrosol are confirmed by the distinctive retention of its functional-group fingerprints in the FeNPs spectrum [10]. Electron microscopy images are generated based on the interaction between a high-energy electron beam and a solid material. Figure (3) presents a field emission scanning electron microscopy (FESEM) image used to analyze the particle size and shape of the iron nanoparticles created by green method [11]. The nanoparticles exhibit a quasi-spherical shape with an average size of approximately 33 nm. These findings agree with previous reports, such as [12], The mostly uniform and spherical characteristics of the particles are especially beneficial for biological and catalytic applications, where surface area and particle consistency are crucial.

Animals and Experimental Design:

Forty-eight male Sprague Dawley rats (180–220 g), and their ages ranged from (10 – 12) weeks were allocated into six experimental groups, each including eight rats with similar weights: Control, PHZ, FeNPs-HXT, $FeCl_3$, Feroglobin, and HXT. The animals were housed in polypropylene cages measuring 50×35×30 cm with a metal network cover and acclimated under standard circumstances of 25±2 °C, 50±5% humidity, and a 12-hour light-dark cycle within the animal house approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. PHZ was administered intraperitoneally to induce hemolytic anemia. Treatments were given for 21 days.

3.1 Biochemical Analysis:

Blood was collected via cardiac puncture. Serum levels of EPO, IL-10, IL-12, TNF-α, and IFN-γ were measured using ELISA. Data were analyzed using ANOVA ($P \le 0.05$).

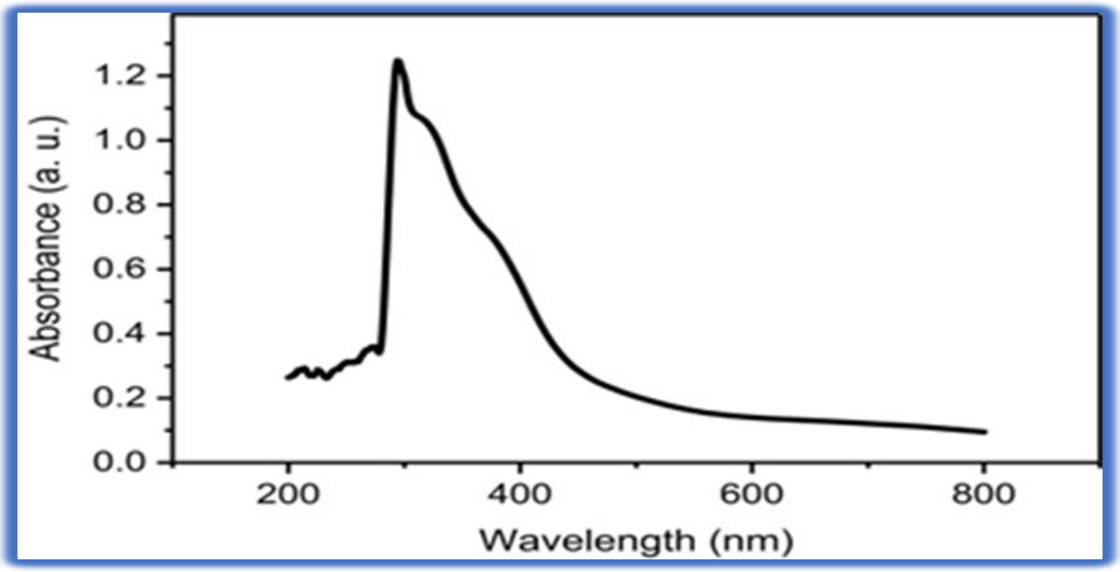

Figure 1: Absorbance spectrum of iron nanoparticles

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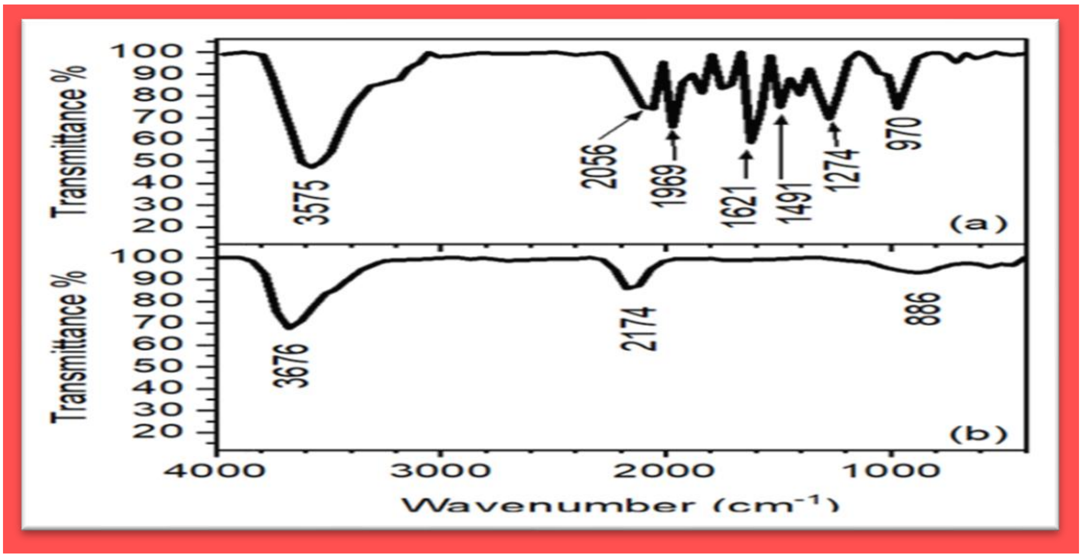

Figure (2) represents the FTIR analysis confirms the key functional groups in both hydroxytyrosol Fe nanoparticles.

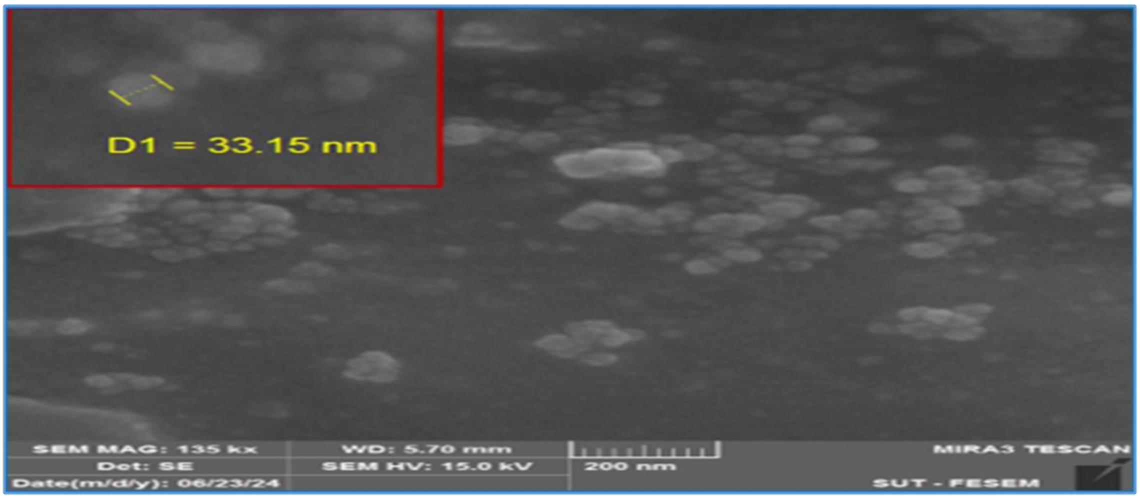

Figure (3) FE-SEM image of iron nanoparticles

Biochemical Analysis:

Blood was collected via cardiac puncture. Serum levels of EPO, IL-10, IL-12, TNF-α, and IFN-γ were measured using ELISA. Data were analyzed using ANOVA ($P \leq 0.05$).

3. RESULTS AND DISCUSSION

PHZ significantly increased EPO as shown in figure (4); (795.8 ± 65.5 pg/mL), TNF-α, IL-12, and IFN-γ, as shown in figures (6-8) while reducing IL-10 as shown in figure (5). This reflects oxidative damage, hypoxia, and systemic inflammation. PHZ induces hemolysis via oxidative stress and JAK/STAT suppression, leading to erythrocyte destruction and immune activation. FeNPs-HXT significantly reduced EPO (717.8 ± 67.1 pg/mL), TNF-α (350 ± 40.4 ng/L), IL-12 (204.6 ± 41 pg/mL), and IFN-γ (155.8 ± 36.7 pg/mL), while increasing IL-10 (38.0 ± 4.37 pg/mL). These effects indicate potent erythropoietic and anti-inflammatory activity. The superior performance is attributed to HXT's antioxidant properties and the enhanced cellular uptake of FeNPs. Studies confirm FeNPs modulate macrophage polarization and suppress NF-κB [13]. According to recent research, FeNPs exceed standard formulations with regard to iron delivery, rapid correction of anemia, and reduced inflammation compared to conventional formulations [14]. Ferric chloride ($FeCl_3$), feroglobin, and hydroxytyrosol significantly reduced EPO levels, these decreases are compatible with improved iron availability and partial maintenance of anemia. Olive polyphenols contain hydroxytyrosol, a potent antioxidant that reduces oxidative stress and inflammation, which may inhibit erythropoiesis in chronic or hemolytic anemia [15]. $FeCl_3$ moderately reduced EPO and IFN-γ but had limited effect on IL-10 and TNF-α. Its inorganic nature may contribute to oxidative stress, limiting its therapeutic benefit [16-117]. Feroglobin showed partial improvement in EPO and TNF-α but lacked significant immunomodulatory effects. Its bioavailability is lower than FeNPs, and it lacks antioxidant support. HXT reduced IL-12 and TNF-α and slightly improved IL-10, confirming its anti-inflammatory role. However, without iron supplementation, its erythropoietic effect was limited.

The delivery of iron nanoparticles significantly elevated serum IL-10 levels, suggesting a possible immunomodulatory effect. This finding agrees with prior studies indicating that iron nanoparticles may contribute to the alleviation of oxidative stress and inflammatory dysregulation [18]. The increase in IL-10 levels may be related to improved bioavailability and cellular absorption of iron, facilitating anti-inflammatory mechanisms and supporting immunological response.

Treatment with iron nanoparticles (FeNPs) markedly diminished IL-12 levels, as studies demonstrate that iron oxide nanoparticles can downregulate pro-inflammatory cytokines like IL-12 by alleviating oxidative stress and inhibiting the activation of nuclear factor kappa B (NF-κB), a principal regulator of cytokine gene expression [19]. Moreover, FeNPs have been documented to promote a transition to an anti-inflammatory macrophage phenotype, correlated with reduced IL-12 production [20]. The hydroxytyrosol-treated group exhibited a notable reduction in IL-12 levels, reinforcing the recognized anti-inflammatory and antioxidant characteristics of this natural phenol. Hydroxytyrosol inhibits essential inflammatory pathways, including MAPKs and NF-κB, thereby reducing the production of IL-12 and other cytokines [21], [22]. The administration of iron nanoparticles (FeNPs) among the treatment groups resulted in a marked decrease in TNF-α levels. This can be ascribed to the immunomodulatory characteristics of FeNPs, which have been shown to downregulate inflammatory cytokines by affecting macrophage polarization and inhibiting pro-inflammatory pathways such as NF-κB [19]. Recent studies indicate that FeNPs enhance the properties of anti-inflammatory M2 macrophages, resulting in reduced levels of TNF-α and other cytokines.

The present study revealed that serum concentrations of interferon-gamma (IFN-γ) were markedly increased in the phenylhydrazine (PHZ)-treated group relative to the healthy control group, as illustrated in Figure (6). This discovery aligns with prior studies demonstrating that PHZ-induced hemolytic anemia correlates with increased oxidative stress and the production of inflammatory cytokines, notably IFN-γ [23]. The elevation of IFN-γ is attributed to immunological activation caused by erythrocyte lysis and oxidative injury, which incites a Th1-dominant immune response marked by heightened IFN-γ secretion [24].

The research conducted by [25] demonstrated that FeNPs therapy decreased IFN-γ levels by controlled iron release and restricted oxidative reactivity, potentially attenuating inflammatory signaling. Similarly, hydroxytyrosol, a powerful antioxidant generated from olive polyphenols, is known, a potent antioxidant derived from olive polyphenols, is known for its immunomodulatory properties and ability to reduce pro-inflammatory cytokine levels, including IFN-γ [26].

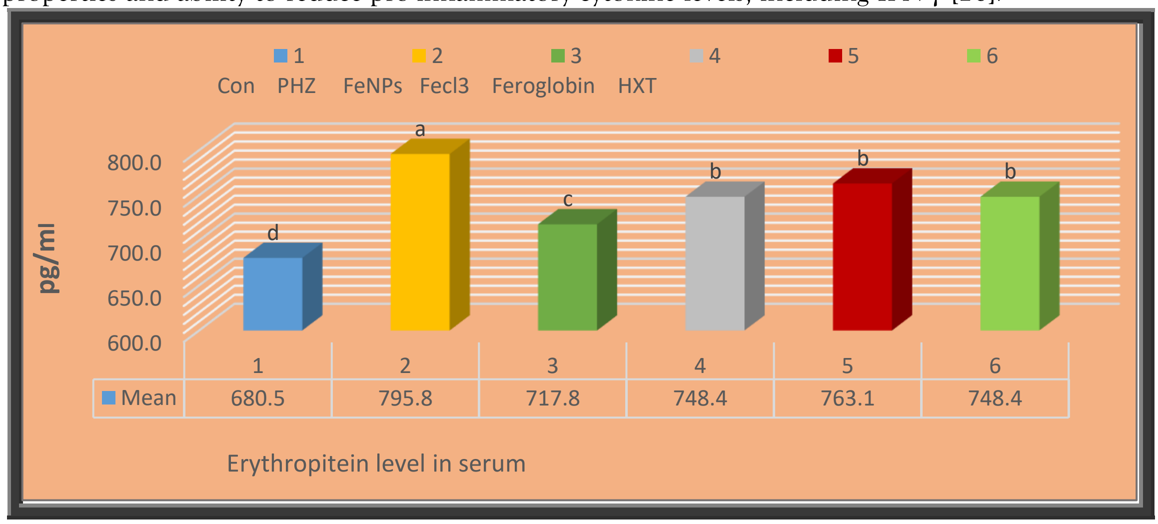

Figure (4): Erythropoietin level in serum rats

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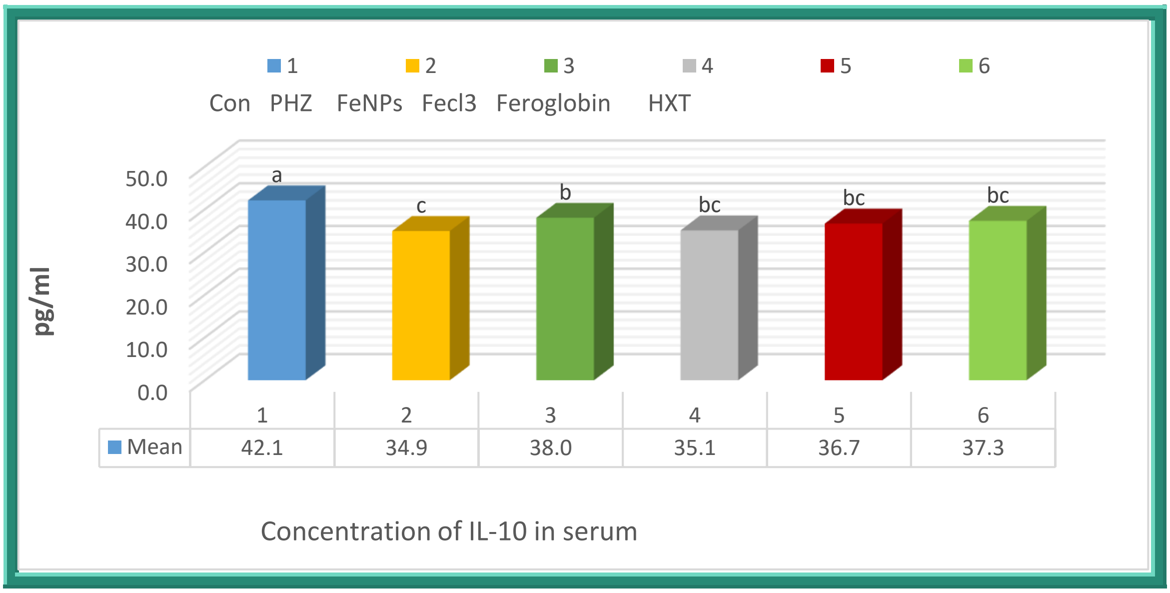

Figure (5): Concentration of IL-10 in serum male rats

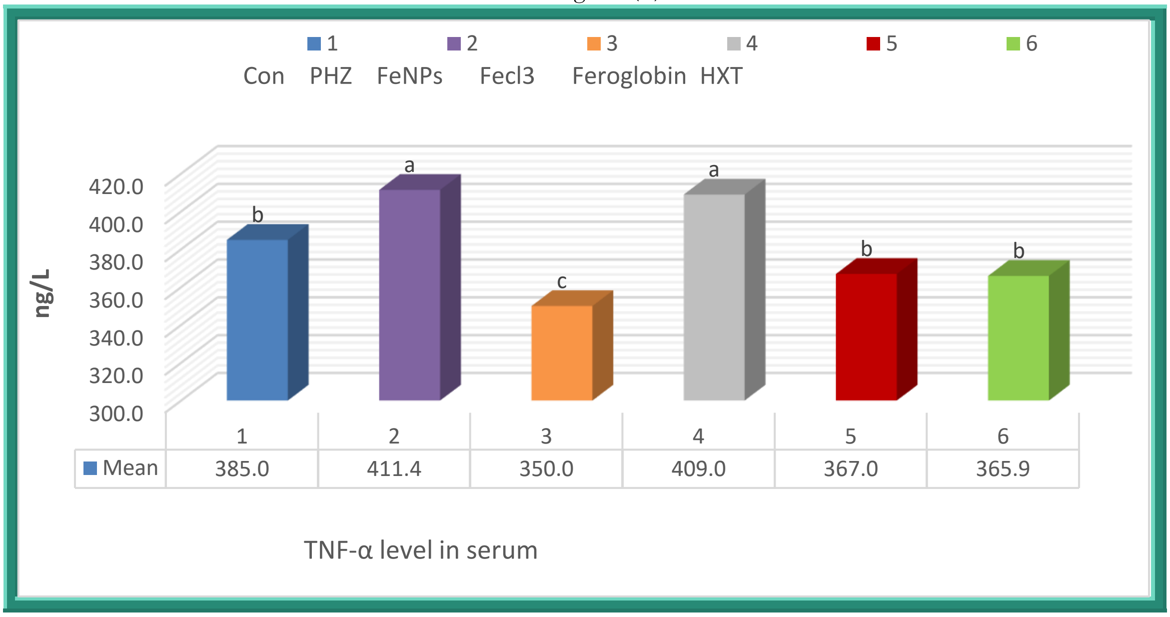

Figure (6): Concentration of IL-12 in serum male rats

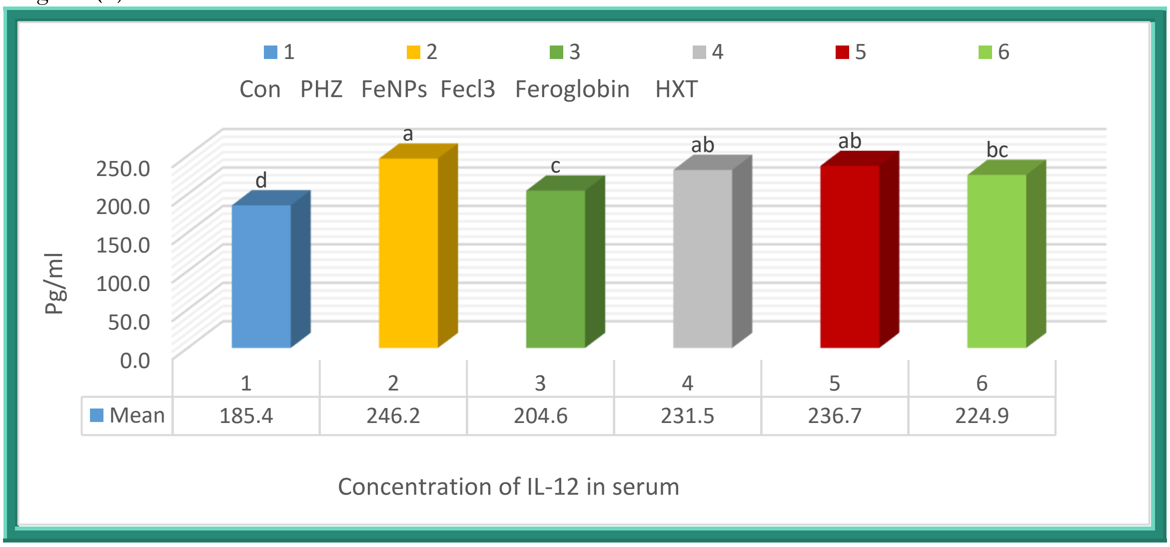

Figure (7): Concentration of TNF-α in serum rats

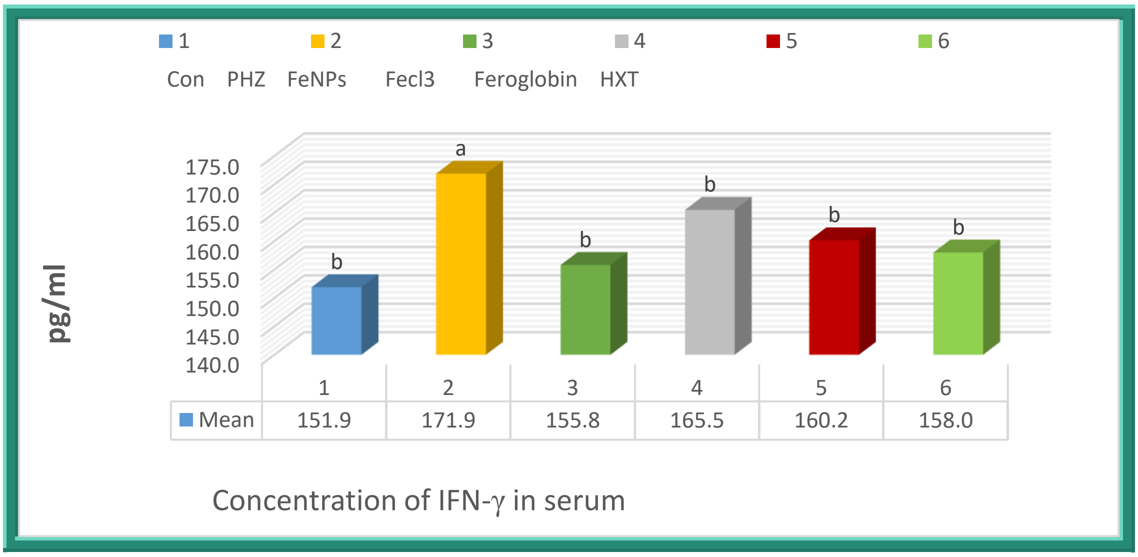

Figure (8): Concentration of IFN-γ in serum male rats.

4. CONCLUSION

Hydroxytyrosol-mediated iron nanoparticles demonstrated superior efficacy in correcting hemolytic anemia and modulating immune responses compared to conventional treatments. Their dual action restoring erythropoiesis and reducing inflammation positions, FeNPs-HXT as a promising therapeutic strategy for anemia-related disorders.

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