TNF-α and IL-1β Pathway Modulation by a Novel Berberine-Loaded Black Seed Oil Nanoemulsion in C57BL/6J Atherosclerotic Mice

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Abstract

Introduction: Atherosclerosis drives coronary artery disease and related cardiovascular pathologies. This study evaluated the combined therapeutic effects of an innovative co-treatment strategy using berberine and *Nigella sativa L.* (black seed) oil in a C57BL/6J mouse model.

Materials and Methods: A berberine-loaded black seed oil nanoemulsion was prepared via high-speed homogenization and sonication, then characterized using TEM, dynamic light scattering, and zeta potential. Forty-eight male C57BL/6J mice were divided into healthy control, atherosclerosis model (16-week high-fat diet), and four 8-week oral treatment groups (simvastatin, berberine, nanoemulsion, or their combination). Serum lipid profiles, aortic histopathology (H&E), and inflammatory cytokine expression (qRT-PCR) were evaluated.

Results: The nanoemulsion exhibited a 250.4 nm hydrodynamic diameter (PDI: 0.3019), a −18.4 mV zeta potential, and a 117.32 nm TEM core size. Atherosclerosis induction significantly

elevated serum LDL, triglycerides, and total cholesterol while decreasing HDL. All treatments improved lipid profiles, but the combination nanoemulsion demonstrated the highest efficacy, maximizing HDL and reducing atherogenic risks. Histopathologically, the combination therapy attenuated intimal thickening, lipid plaque formation, and endothelial hyperplasia. Furthermore, it most effectively suppressed atherosclerosis-induced upregulation of aortic TNF-α and IL-β expression.

Conclusion: The combined nanoformulation protects against atherosclerosis by normalizing lipid metabolism, mitigating plaque development, and suppressing vascular inflammation, serving as a promising adjunctive cardiovascular therapy.

Keywords: Atherosclerosis, Berberine, Black seed oil, Nanoemulsion, Inflammation, TNF-α, IL-1β, C57BL/6J mice

1. Introduction

Atherosclerosis represents the primary etiological driver behind global cardiovascular morbidity [1]. This disease is a multifactorial process characterized by impaired lipid metabolism, vascular chronic inflammation, oxidative stress, endothelial dysfunction and the formation of atheromatous plaques. Increased total cholesterol, triglycerides, LDL and decreased HDL are key factors in the onset and progression of this disease [2]. Monocyte infiltration, foam cell generation, and the subsequent destabilization of atherosclerotic plaques are heavily driven by the triggering of inflammatory pathways and elevated levels of cytokines like IL-1β and TNF-α [3]. Common treatments such as statins have side effects [4]. Therefore, the development of natural compounds and novel drug delivery systems as complementary or alternative strategies has attracted the attention of researchers.

berberine a naturally occurring alkaloid, as a potent agent possessing hypolipidemic, anti-inflammatory, and antioxidant activities that cuase cardioprotection [5]. However, the therapeutic application of berberine faces challenges due to its low solubility, limited bioavailability, and inadequate oral absorption [6]. black seed oil has anti-inflammatory, antioxidant, and lipid metabolism-modulating effects due to its bioactive compounds, especially thymoquinone [7]. Nigella sativa (black seed) and its bioactive constituents exert beneficial effects by mitigating vascular inflammation, suppressing lipid peroxidation, and normalizing metabolic profiles [8].

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Nanoemulsions have been considered as one of the new drug delivery systems, with increased contact surface area, improved physical stability, improved solubility of hydrophobic compounds, and increased bioavailability. The use of black seed oil nanoemulsions can not only maintain the biological properties of this oil, but also provide its more effective delivery [9]. Also, loading or combining berberine with such a system may enhance its therapeutic effects through pharmacological synergy and improved bioavailability.

Consequently, this study aimed to develop a black seed oil nanoemulsion and evaluate the individual and combined therapeutic effects of berberine and this nanosystem. Anti-atherosclerotic activities by monitoring lipid profiles, aortic histopathology, and inflammatory gene expression pathways, TNF-α and IL-1β in C57BL/6J mice. We hypothesized that the co-administration of berberine and black seed oil nanoemulsion would exert a synergistic therapeutic effect, outperforming either monotherapy in ameliorating dyslipidemia, mitigating aortic histopathological lesions, and suppressing atherosclerosis-linked inflammatory cascades.

2. Materials and Methods

2.1. Nanoemulsion synthesis

For nanoemulsion synthesis, a modified high-speed homogenization method was adopted [9]. Initially, a 2% w/v concentration of berberine hydrochloride was thoroughly incorporated into 10% v/v black seed oil. This oily blend was subsequently combined with a surfactant mixture composed of PEG-400 (10% v/v) and Tween 80 (30% v/v). for optimal homogeneity the combined phases were agitated at 600 rpm for 30 min then subjected to probe-sonication for 10 minutes.

2.2. Characteristics of BBR-loaded black seed oil-based self-nanoemulsion

Zeta potential was determined via a Malvern Zetasizer Nano ZS following a two-step 100-fold serial dilution with deionized water. Viscosity was measured in triplicate at 25 $^{\circ}C$ using an undiluted matrix via a Brookfield LVF viscometer.

2.3. Thermodynamic stability

To evaluate thermal stress endurance, the developed nanoemulsion was subjected to sequential temperature fluctuations, beginning with an incubation period (4 $^{\circ}C$/ 48 hours), immediately followed by storage at 48 $^{\circ}C$ for an identical duration (three consecutive times)

2.4. Stability Index

To assess physical endurance under extreme thermal fluctuations, the formulation was subjected to a rigorous three-cycle freeze-thaw challenge. During each cycle, the samples were kept at -20

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℃ for 24 hours before being allowed to liquefy at ambient temperature, with the final stability index quantified according to **equation 1**.

long-term integrity under accelerated aging conditions was investigated by incubating the essential oil nanoemulsion at an elevated temperature of 40 ℃ for a duration of one month [9].

Equation 1:

Stability index of nanoemulsion = 100 × (Original globule size - Changing globule size) / Original globule size

2.5. Animals

48 adult male C57BL/6J mice (age: 8 weeks) and (weighing: 18-20 g), were purchased from the Pasteur Institute of Iran (Tehran). The ethical guidelines declared by the Institutional Animal Care and Use Committee at Islamic Azad University, Science and Research Branch, Tehran, Iran (ethical approval code: IR.IAU.SRB.REC.1401.229). The mice were randomly distributed into six separate experimental groups (n = 8).

2.6. Induction as a Mouse Model

AS induction in C57BL/6 J mice was modified by the addition of 1.25% (w/w) cholesterol, 0.5% cholate (w/w), 5% corn oil, and 13.75% cocoa oil [10].

2.7. Study Design

The experimental groups received daily treatments via oral gavage. The treatment was defined from the eigth day for 16 weeks in the mice. the mice were administered a diet incorporating 25% dietary fat.

Group 1: The healthy control mouse model (H) (received physiologic saline)

Group 2: The atherosclerosis mouse model (AS) without treatment.

Group 3: AS and received Simvastatin (SIM) (5mg/kg, 0.1 µl).

Group 4: AS and received BBR (B) (1%, 0.1 µl).

Group 5: AS and received a black seed oil-based nanoemulsion (BS) (0.1 µl).

Group 6: AS and received BRR-loaded black seed oil-based self-nanoemulsion (BBS) (0.1 µl).

2.8. Blood sampling

Whole blood was collected by cardiac puncture into plain tubes without anticoagulants and allowed to clot at room temperature. The serum was separated by centrifugation at 3000 rpm for 15 min.

2.9. Tissue Sampling

After 16-week experimental timeline, the animals were subjected to general anesthesia via an intraperitoneal injection ketamine (10 mg/kg) and xylazine (90 mg/kg) (0.1ml). Then the heart and aortic arch were excised from each mouse and immediately transferred into formalin solution for histopathological analysis.

2.10. Measurement of Serum lipid profile

After separating the mouse serum, the concentrations of HDL, LDL, TG, and total cholesterol were measured [11].

2.11. Histopathology

Aortic tissues were fixed in 10% buffered formalin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) [12].

2.12. Measurement of inflammatory factors

Total RNA was isolated from harvested tissues using a standard chloroform-isopropanol phase separation protocol and quantified spectrophotometrically. For cDNA synthesis, 1 μg of purified RNA was reverse transcribed using a commercial kit (45$^{\circ C}$ for 45 min, inactivated at 70$^{\circ C}$ for 10 min). Subsequently, qPCR was performed using SYBR Green master mix to assess the relative expression of target genes (IL-1β and TNF-α) against GAPDH as an internal reference [13]. The primer sequences used are presented in **Table 1**.

Table 1. The primer sequences

Primer Sequence	Direction	Gene
5′-GCAACTGTTCCTGAACTCAACT-3	Forward	*IL-1β*
5′-ATCTTTTGGGGTCCGTCAACT-3	Reverse	
5′-CTTCTGTCTACTGAACTTCGGG-3	Forward	*TNF-α*
5′-CAGGCTTGTCACTCGAATTTTG-3	Reverse	
TGAAGGTCGGTGTGAACGGATTTGGC-3′	Forward	*GAPDH*
CATGTAGGCCATGAGGTCCACCAC-3	Reverse	

2.13. Statistical analysis

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A one-way ANOVA was used for statistical evaluation, followed by Tukey's post hoc analysis by GraphPad Prism (version 10.4.1.). Data are expressed as mean ± SD for nanoemulsion characterization and gene expression, and as mean ± SEM for lipid profile measurements. for histopathological scores were analyzed using the Kruskal–Wallis test. AP value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of BRR-loaded black seed oil-based self-nanoemulsion

3.1.1. Dynamic Light Scattering (DLS) and Zeta Potential Determination

DLS showed the mean size of 250.4 nm with a PDI of 0.3019, indicating a relatively uniform nanoemulsion (**Figure 1A**). The measured zeta potential was −18.4 mV, suggesting satisfactory colloidal stability (**Figure 1B**).

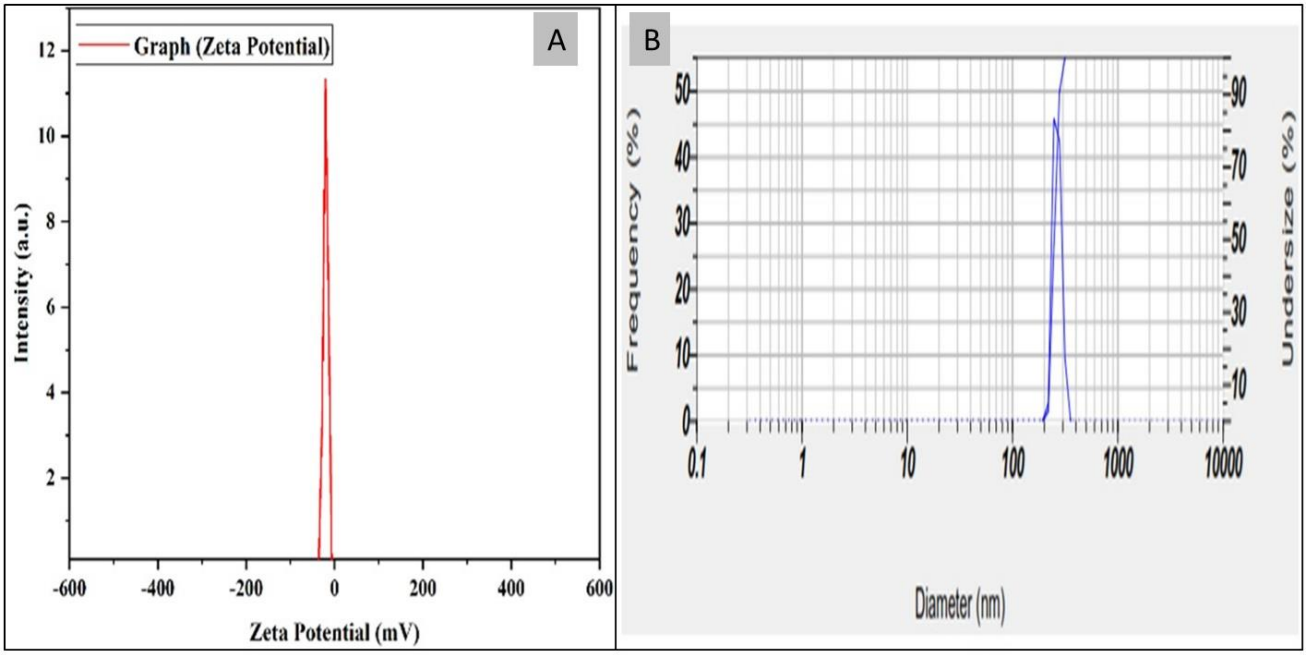

Figure 1. (A) Size characteristics and (B) Zeta potential distribution of nanoemulsion formulations using the DLS technique.

3.1.2. Analysis of Transmission Electron Microscope (TEM) Images

The particle size in TEM images was 117.32 nm (**Figure 2**).

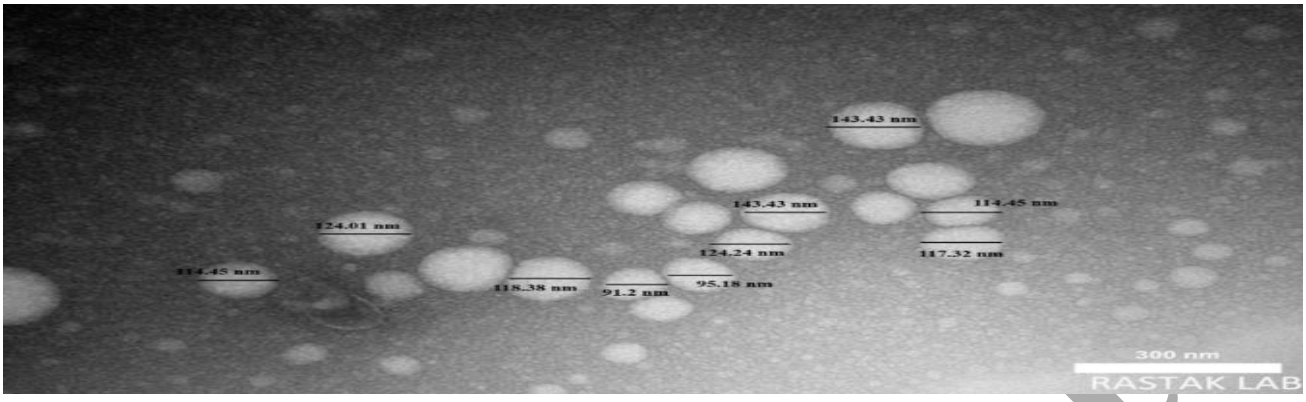

Figure 2. TEM image of the prepared nanoemulsion (at a scale of 300 nm).

3.2. Alterations in serum lipid profile

Analysis of serum lipid profiles showed that SIM, B, BS and BBS significantly increased HDL levels compared to the AS group. the BBS treatment group showed an increase in HDL compared to the SIM, B, and BS groups ($P < 0.0001$) (**Figure 3A**). LDL level alterations in H, SIM, B, BS, and BBS groups exhibited a statistically significant reduction compared to the AS group. BBS showed a significant decrease relative to B ($P<0.0001$) and an increase compared to SIM ($P<0.01$) **(Figure 3B)**. Triglyceride levels in the SIM, B, BS, and BBS groups showed a significant decrease compared to the AS group ($P<0.0001$). BBS and BS exhibited substantially reduced triglyceride level compared to the B group ($P<0.0001$ and $P<0.01$) (**Figure 3C**). cholesterol levels in H, SIM, B, BS and BBS groups exhibited a decrease relative to group AS ($P<0.0001$). the BBS group showed significant reduction in compared to B and BS ($P<0.0001$) (**Figure 3D**).

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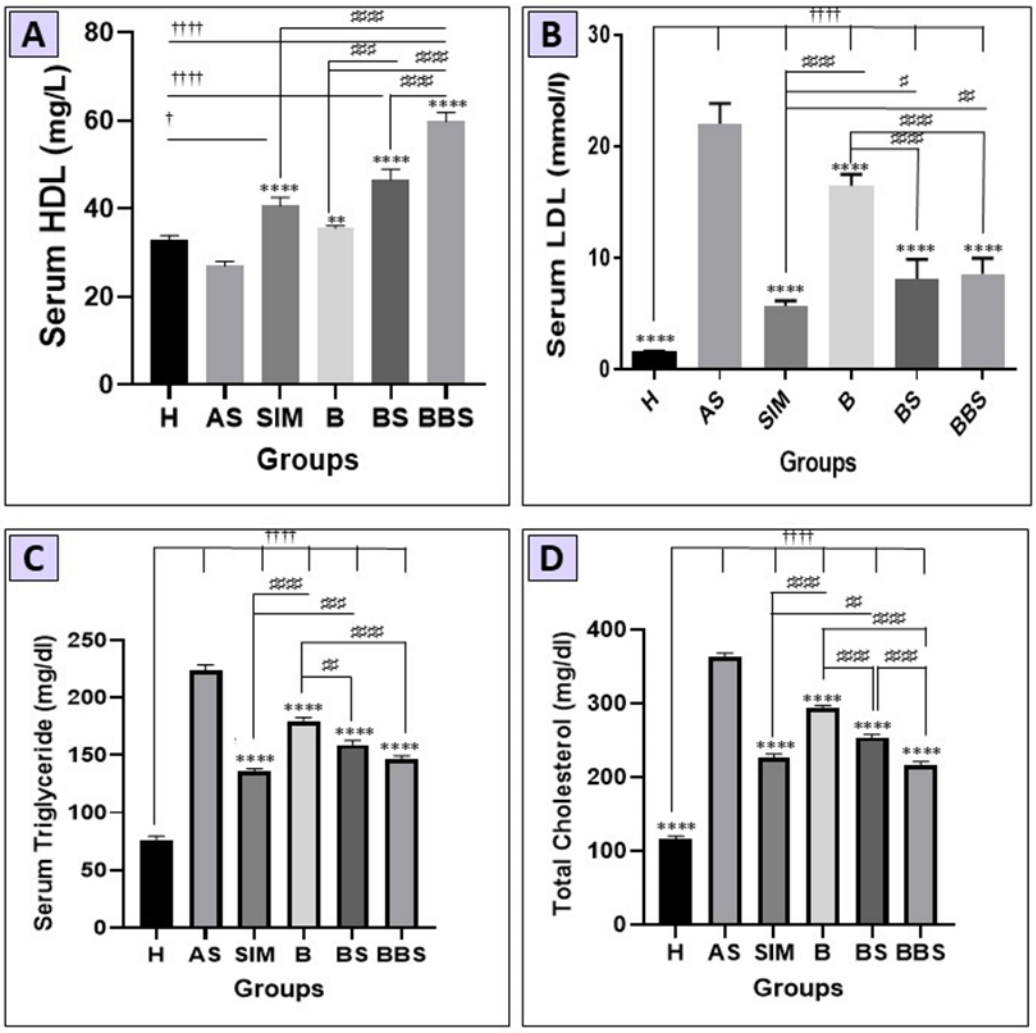

Figure 3. Effects of BBS on C57BL/6 J mice's serum lipid profiles. (A) serum HDL (mg/L), (B) serum LDL (mg/L), (C) serum triglyceride (mg/dl), and (D) total cholesterol (mg/dl). Comparison of H group with AS, SIM, B, BS, and BBS groups † P<0.05 and †††† P<0.0001; Comparison of AS group with H, SIM, B, BS, and BBS groups ** P<0.01 and **** P<0.0001; Comparison of SIM, B, BS, and BBS groups together # P<0.05, ## P<0.01, ### P<0.001, and #### P<0.0001.

3.3. Aorta Histopathological Examination

The results indicated that the aortic cross-section in the healthy control group (H) displayed healthy middle layer of the vessel and normal endothelium (**Figure 4A**). the aortic cross-section in the AS group exhibited numerous cholesterol plaques and significant endothelial hyperplasia in the middle layer (**Figure 4B**). The SIM group showed notable cholesterol plaques and slight endothelial hyperplasia (**Figure 4C**). The B group with a few cholesterol plaques, mild endothelial hyperplasia (**Figure 4D**). the BBS group presented distinct atheromatous plaques and mild

hyperplasia (**Figure 4E**). the BS group exhibited substantial cholesterol plaques and slight hyperplasia (**Figure 4F**).

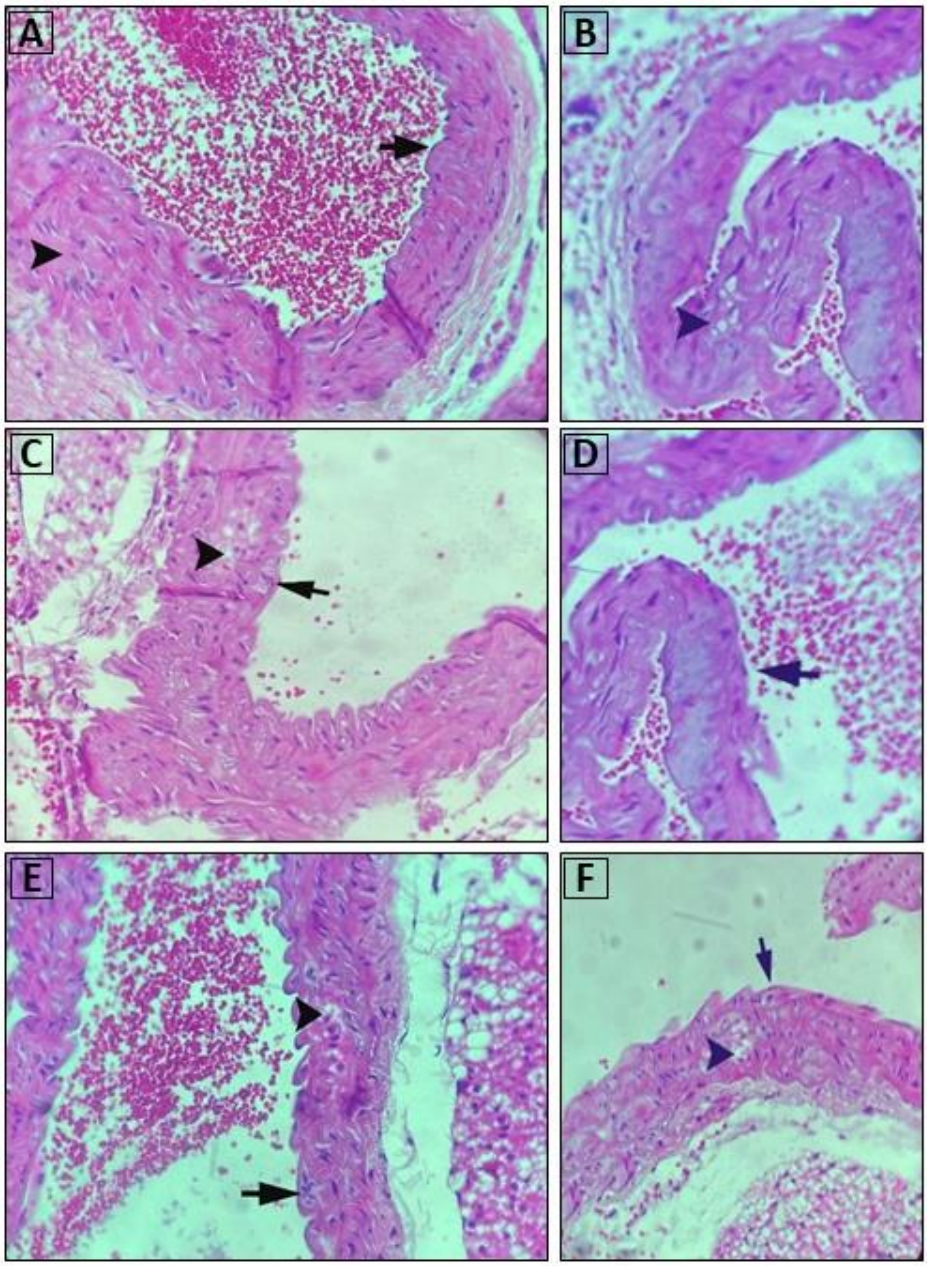

Figure 4. Histopathological evaluation of aortic tissue sections by (H&E) staining (magnification 400×). (A) H group: healthy middle layer of the vessel (arrowhead) and normal endothelial (arrow). (B) AS group: exhibited numerous cholesterol plaques (arrowhead), and severe endothelial hyperplasia (arrow). (C) SIM group: showed notable cholesterol plaques (arrowhead) slight endothelial hyperplasia (arrow). (D) Group B: a few cholesterol plaques (arrowhead) and

mild endothelial hyperplasia (arrow). (E) Group BBS: Optimal protective effects showing minimal lipid/cholesterol deposits (arrowhead) mild endothelial hyperplasia (arrow). (F) Group BS: limited lipid plaques (arrowhead) and slight endothelial hyperplastic (arrow).

Aortic H&E staining revealed severe atherosclerotic lesions in the AS group, like cholesterol accumulation, and endothelial hyperplasia. The B group with a few cholesterol plaques, mild endothelial hyperplasia.The SIM group showed notable cholesterol plaques and slight endothelial hyperplasia. the BS group exhibited substantial cholesterol plaques and slight hyperplasia. the BBS group demonstrated optimal protective effects, displaying only minimal lipid deposition and mild hyperplasia. Overall, these histological findings confirm that the interventions (particularly the BBS) significantly alleviated aortic atherosclerotic changes (**Figure 5**).

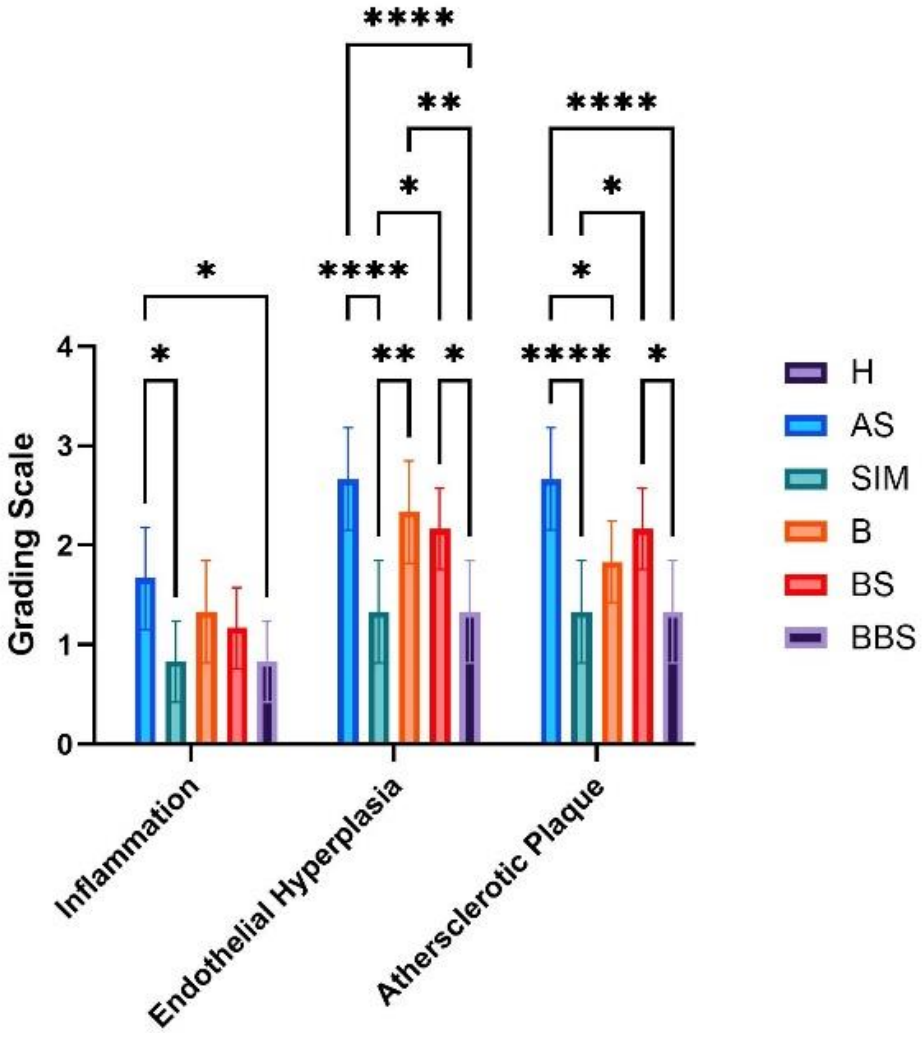

Figure 5. H: Healthy, AS: Atherosclerosis model, SIM: AS and simvastatin (15 mg/kg), B: AS and berberine (30 mg/kg), BS: AS and black seed oil nanoemulsion (10%), BBS: AS and berberine (2%) and black seed oil nanoemulsion (10%). Group comparison * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001;

3.4. Expression of TNF-alpha, IL-1beta genes

Quantitative gene expression analysis revealed a baseline TNF-α transcript level of 1.066 in H group. the AS group showed statistically significant increase in the expression of this inflammatory marker (4.304). in the BS group, the mean expression of the gene was 3.149, which was also significant compared to the AS group (P<0.0001). The results of the Tukey multiple comparison test also showed that the expression of the TNF-alpha gene in the SIM and BBS groups was significantly lower compared to AS (P<0.0001) (**Figure 6A**). The mean expression of the IL-1beta gene in H group was 1.138. but the expression of this gene in the AS and BS groups increased to 5.637 and 3.48, and these increases were significant in both groups compared to H group (P<0.0001). The results of the Tukey test also showed that IL-1beta gene expression was significantly reduced by receiving B, BS and BBS compared to the AS group (P<0.0001) (**Figure 6B**).

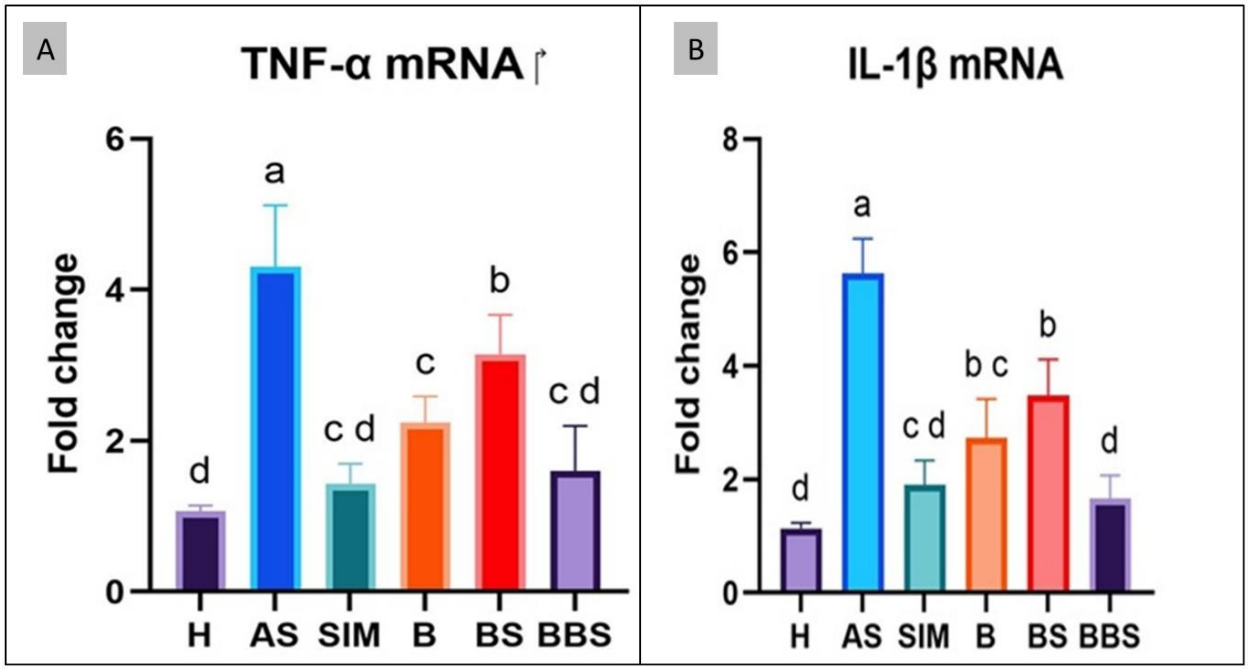

Figure 6. H: Healthy, AS: Atherosclerosis model, SIM: AS and simvastatin (15 mg/kg), B: AS and berberine (30 mg/kg), BS: AS and black seed oil nanoemulsion (10%), BBS: AS and berberine

(2%) and black seed oil nanoemulsion (10%). a, b, c and d indicate significant differences between groups.

4. Discussion

BBS demonstrated favorable physicochemical properties for effective nanodrug delivery. DLS analysis revealed a hydrodynamic diameter of 250.4 nm with a PDI of 0.3019, confirming a homogeneous droplet distribution, while a zeta potential of −18.4 mV indicated adequate colloidal stability. TEM imaging showed a core diameter of approximately 117.32 nm. This discrepancy between DLS and TEM sizes is expected, as DLS measures the hydrodynamic diameter (including the emulsifier and hydration layers) but TEM only core diameter. these data confirm the formulation's suitability for biological applications.

Atherosclerosis induction successfully altered the lipid profile, increasing LDL, TG, total cholesterol (TC) and decreasing HDL. All interventions (simvastatin, berberine, black seed oil nanoemulsion, and particularly the combination therapy) significantly improved most lipid parameters compared to the AS group. Notably, the BBS group demonstrated the greatest HDL level indicating a potent protective effect on lipid metabolism.

The improvement observed in the BBS group is likely due to several simultaneous mechanisms. Berberine may act by regulating LDL receptor expression [14], activating AMPK [15], reducing lipid synthesis [13], and improving cholesterol clearance [14] from the blood. black seed oil and active compounds (thymoquinone) have antioxidant and anti-inflammatory properties and may slow the progression of atherosclerosis by reducing LDL oxidation and inhibiting vascular inflammation [16]. nanoemulsion formulation may have improved the efficacy of the treatment by increasing the solubility and improving the absorption of the active compounds. The relative superiority of the BBS group over the B and BS groups in some indicators may also support the existence of a synergistic effect between berberine and black seed oil [17].

Atherosclerosis induction caused intimal thickening, lipid/cholesterol plaque, and endothelial hyperplasia. Among treatments, berberine alone yielded only partial improvement with remaining plaques, while simvastatin significantly reduced lesion severity. Black seed oil nanoemulsion also demonstrated protective effects by significantly decreasing lipid deposits and limiting hyperplasia.

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Notably, the combination therapy of berberine and black seed oil nanoemulsion most effectively reduced atherosclerotic lesions [13].

We presented the model, which increased the transcriptional expression of proinflammatory cytokines TNF-α and IL-1β in the AS group compared to H group, indicating a strong activation of inflammatory cascades during atherogenesis. Although all treatment regimens successfully reduced the expression of these inflammatory markers compared to the AS, the combined formulation of BBS produced the most suppression and achieved a therapeutic efficacy that was statistically comparable to the standard simvastatin in various parameters. Given that TNF-α and IL-1β act as major upstream regulators of vascular endothelial activation, monocyte recruitment, and subsequent atheromatous plaque progression, their targeted downregulation represents a primary molecular mechanism driving the potent anti-atherogenic properties of the tested nanodrug [18].

The robust alignment of molecular, biochemical, and histological outcomes is a primary strength of this study, demonstrating that suppressed inflammatory gene expression directly correlates with improved lipid profiles and reduced aortic damage. Crucially, the normalized atherogenic coefficients and cardiac risk ratios in the BBS group underscore its clinical potential for cardiovascular risk management. However, several limitations warrant acknowledgment: first, the inflammatory profiling was restricted to TNF-α and IL-1β, meaning that incorporating downstream pathways or complementary biomarkers (such as IL-6, CRP, NF-κB, or oxidative stress markers) would provide a more comprehensive mechanistic overview. Second, the pharmacokinetic parameters and bioavailability of the nanoemulsion-encapsulated berberine were not directly quantified.

5. Conclusions

Overall, this study demonstrates that the black seed oil nanoemulsion, particularly when combined with berberine, exerts significant protective effects in a mouse model of atherosclerosis. These outcomes were driven by improved lipid profiles, decreased atherogenic risk factors, reduced plaque formation and aortic hyperplasia, and the suppression of inflammatory gene expression. Consequently, this formulation represents a promising strategy for the prevention or adjunctive treatment of atherosclerosis.

Ethics Approval

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- **Funding**

The authors declare that no specific funding was received from public, commercial, or non-profit organizations for this study.

- **Data Availability**

All data generated or analyzed during this study are included within this article and its supplementary materials. The experimental procedures were reviewed and approved by the Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran (Approval ID: IR.IAU.SRB.REC.1401.229).

- **Author Contributions**

N.P. and N.D. were responsible for the study conception and design. Data collection, analysis, and interpretation were performed by N.P., M.K.K., and N.D. N.D. prepared the initial manuscript draft, while P.M. provided critical revision of the final version.

- **Conflict of Interest**

The authors declare that they have no competing interests**.**

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