

Extraction-Dependent Immunomodulatory and Angiogenesis-Related Molecular Responses of *Nigella sativa* Oil in Adipose-Derived Mesenchymal Stem Cells

(*Nigella sativa* / black cumin oil / mesenchymal stem cells / anti-inflammatory activity / angiogenesis)

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Abstract. *Nigella sativa* black cumin oil (BCO) exhibits well-documented anti-inflammatory and antioxidant properties; however, the impact of extraction-related compositional variation on its cellular and molecular effects in stem cell systems remains insufficiently characterized. This study investigated the effects of cold-pressed (CP) and supercritical CO₂ (ScCO₂)-extracted BCO on the viability, inflammatory response and angiogenic potential of adipose-derived mesenchymal stem cells (ASCs). The oil composition and antioxidant activity were assessed using GC/MS and DPPH assays, respectively. ASCs were treated with different volumes of BCO, and cell viability was evaluated at 24, 48 and 72 h using the MTT assay. Anti-inflammatory effects

were evaluated by real-time PCR analysis of *IL6* and *IL10* mRNA expression, while angiogenesis-related molecular responses were evaluated based on *VEGF* mRNA expression. Both CP and ScCO₂-extracted BCO significantly increased ASC viability in a volume-dependent manner, with the highest viability consistently observed at 100 µl ($P < 0.05$). Treatment with 50 µl and 100 µl of either oil significantly reduced *IL6* expression and concomitantly increased *IL10* expression at 24 and 48 h ($P < 0.05$). *VEGF* expression was also significantly up-regulated at these time points, with ScCO₂-extracted BCO inducing a more pronounced and sustained angiogenic response ($P < 0.05$). These findings indicate that BCO enhances ASC viability and molecular responses in a volume-dependent manner, while differences associated with extraction-related compositional variation may modulate inflammation- and angiogenesis-related molecular signalling in ASCs.

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Abbreviations: ASCs – adipose-derived mesenchymal stem cells, BCO – black cumin oil, CP – cold-pressed, DPPH – 2,2-diphenyl-1-picrylhydrazyl, GC/MS – gas chromatography-mass spectrometry, HS-GC/MS – headspace GC/MS, *IL6* – interleukin 6 gene, *IL10* – interleukin 10 gene, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, qPCR – quantitative real-time polymerase chain reaction, ScCO₂ – supercritical carbon dioxide, TPC – total phenolic content, *VEGF* – vascular endothelial growth factor gene.

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Introduction

Inflammation plays a pivotal role in regulating tissue homeostasis, repair and regeneration, particularly by shaping the viability and paracrine activity of mesenchymal stem/stromal cells (MSCs). Adipose-derived mesenchymal stem/stromal cells (ASCs) are widely used in regenerative medicine due to their accessibility and immunomodulatory capacity; however, their therapeutic efficacy is strongly influenced by the inflammatory microenvironment. Excessive pro-inflammatory signalling can impair ASC survival and functionality, whereas controlled modulation of inflammatory pathways may enhance regenerative outcomes through improved cell viability and secretion of pro-angiogenic and anti-inflammatory mediators. Natural products with established anti-inflam-

matory and antioxidant properties have therefore gained attention as potential adjuncts in stem cell-based strategies. Among these, *Nigella sativa* L. (black cumin) has been extensively investigated for its broad cellular and molecular profile. Comprehensive reviews and experimental studies consistently report that *N. sativa* and its bioactive constituents exert potent antioxidant and anti-inflammatory effects via modulation of key molecular pathways involved in cytokine production, oxidative stress and immune regulation (Gholamnezhad et al., 2016; Hannan et al., 2021; Wahab and Alsayari, 2023). *In vitro* studies using adipocyte and macrophage models further demonstrate that *N. sativa* extracts suppress pro-inflammatory mediators while promoting a more balanced immunometabolic phenotype (Bordoni et al., 2019; Bashir et al., 2023). Beyond experimental models, translational and clinical evidence supports the anti-inflammatory potential of *N. sativa*. Recent systematic reviews and meta-analyses of randomized controlled trials indicate that *N. sativa* supplementation can significantly reduce circulating inflammatory and oxidative stress biomarkers, reinforcing its relevance as a bioactive natural compound in stem cell biology (Kavyani et al., 2023; Rashwan et al., 2023). In line with these findings, both *in vitro* and human studies demonstrate that *N. sativa* exerts anti-adipogenic and lipid-lowering effects, highlighting its capacity to modulate the inflammatory-metabolic cross-talk in adipose-related cellular systems (Ahmed et al., 2025). Importantly, the biological activity of *N. sativa* oil is strongly influenced by extraction methodology. Cold-pressed (CP) and supercritical carbon dioxide (ScCO₂) extraction techniques yield oils with distinct fatty acid profiles, oxidative stability, and concentrations of bioactive compounds, which may translate into differential cellular responses (Rahim et al., 2022; Choudhury et al., 2023). Comparative studies on other plant oils have demonstrated that extraction-dependent compositional differences can substantially affect antioxidant and biofunctional properties in cell-based systems (Sevim et al., 2013), underscoring the need to evaluate extraction methods as a critical experimental variable. Despite growing interest in the immunomodulatory and regenerative effects of *N. sativa*, data directly examining how extraction-dependent differences in *N. sativa* oil influence ASC viability and inflammatory signalling remain limited. Moreover, standardized dose interpretation in *in vitro* systems remains challenging, as cellular responses may depend not only on nominal concentrations but also on effective cellular dosimetry (Hinderliter et al., 2010; Doskey et al., 2015). Addressing these gaps is essential for improving reproducibility and mechanistic clarity in stem cell research. Therefore, the present study aimed to investigate the effects of CP and ScCO₂-extracted *Nigella sativa* oils on adipose-derived mesenchymal stem cell viability and inflammatory gene expression, with a specific focus on key cytokines involved in inflammation and angiogenesis. By integrating extraction-related compositional variation with a stem cell-relevant inflammatory framework, this work seeks to elucidate inflammation-

and angiogenesis-related gene expression responses relevant to stem cell biology.

Material and Methods

Black cumin oil (BCO) was obtained through cold pressing and ScCO₂ extraction using an ultrahigh-pressure technique. The composition of CP and ScCO₂-extracted BCO was evaluated by gas chromatography-mass spectrometry (GC/MS) and headspace GC/MS (HS-GC/MS), and their antioxidant activities were analysed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The ASCs were cultured with different amounts of CP and ScCO₂-extracted BCO, and their viability was assessed after 24, 48 and 72 h of culture by the MTT assay. In addition, the expression levels of *IL6*, *IL10* and *VEGF* mRNAs were analysed by real-time PCR.

Extraction of BCO

ScCO₂ extraction was performed at pressures ranging from 200–300 bar and temperatures ranging from 40–50 °C, and the yield was approximately 10–20 %. The CP oil was obtained using a pressing machine operated at approximately 40 °C if necessary, to ensure that the oil could flow without solvents.

Chemical analysis of CP and ScCO₂-extracted BCO

The contents of CP and ScCO₂-extracted BCO were analysed by HS-GC/MS and GC/MS at the Natural Products Application and Research Center at Süleyman Demirel University.

Determination of total phenolic content

The total phenolic content (TPC) of BCO was determined according to the methods of Sevim et al. (2013) with minor modifications. An aliquot of every oil sample (1 mg) was dissolved in 1 ml of methanol, and the mixtures were stirred for 1 h on a shaker. The methanol phase was separated by centrifuging at 918 g for 10 min and diluted 20-fold. Subsequently, 500 µl of the diluted sample was mixed with 1000 µl of the Folin-Ciocalteu reagent, and 750 µl of 2 % Na₂CO₃ solution was added to each tube. The samples were incubated at room temperature in the dark for 2 h, and the absorbance at 765 nm was measured against a blank. Gallic acid was used as the standard; the stock solution (100 µg/ml) was serially diluted five times. The above steps were performed for each concentration of the standard. The TPC content was calculated as gallic acid equivalents (mg GAE/g).

Analysis of antioxidant activity

The antioxidant activities of CP and ScCO₂-extracted BCO were evaluated using the DPPH probe in accordance with the methods of Sevim et al. (2013). Briefly, 1 mg of BCO was dissolved in 1 ml of methanol and agitated vigorously for 1 h at room temperature. The mixture was then centrifuged at 918 g for 10 min to separate the polar

and nonpolar components. The methanol phase was aspirated, and 100 µl aliquots of the extracts were combined with 100 mM DPPH solution (1900 µl). Methanol was used for blanks (1900 µl). The mixtures were left undisturbed in the dark at room temperature for 15 min. Serial dilution of the extracts and fixed oils were prepared (100–1000 µg/ml), and their absorbance values (A) were recorded at 517 nm using a spectrophotometer, with methanol as the blank. The Trolox equivalent (TE) of DPPH was calculated against a standard curve prepared with known concentrations of Trolox. The radical scavenging activity (RSA) was calculated as a percentage inhibition using the following formula: (% RSA) = $100 \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}$. The same procedure was repeated for synthetic antioxidant butylated hydroxytoluene (BHT), which was used as a positive control.

Culture of ASCs

Rat ASCs were purchased from Kocaeli University Research and Application Hospital Stem Cell and Gene Therapies Research and Application Center (KOGEM). The same cell line was used by Okcu et al. (2023) in their 2023 study. The cells were seeded in T25 flasks at the density of 1×10^6 cells per flask and cultured in DMEM containing 10 % foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) at 37 °C with 5 % CO₂ and 95 % humidity. After several passages, the cells were harvested through trypsin digestion and seeded in 96-well plates at the density of 5×10^4 cells per well. For preliminary experiments, six different volumes (5 µl, 10 µl, 25 µl, 50 µl, 75 µl and 100 µl) of CP and ScCO₂-extracted BCO were respectively added to the wells, and triplicate wells were prepared for each volume. We selected a volume-based as opposed to a concentration-based dosing approach to ensure homogeneous distribution of the test substances throughout the culture medium (Doskey et al., 2015). This approach also enables clearer evaluation of dose-response relationships by allowing direct comparison between the applied volume and biological outcome (Hinderliter et al., 2010; Kusi-Appiah et al., 2015). Furthermore, several substances exhibit greater stability and functional effectiveness when administered by volume in complex cell-based systems (Kusi-Appiah et al., 2015). The volume-based approach is consistent with previous reports in the literature and is also commonly used in clinical practice (Hinderliter et al., 2010; Doskey et al., 2015). This not only enhances the comparability of our results with that of other studies but also increases their translational relevance. The MTT assay was conducted at 24, 48 and 72 h to assess cell viability, and 10 µl, 50 µl and 100 µl were determined to be the most effective volumes for the subsequent experiments.

MTT assay

ASCs were seeded in 96-well plates at the density of 5×10^3 – 10×10^3 cells per well in 100 µl medium and cultured with 5 µl, 10 µl, 25 µl, 50 µl, 75 µl and 100 µl of CP and ScCO₂-extracted BCO for 24, 48 and 72 h. The cells were harvested at these time points, and their viability

was analysed using the MTT Cell Viability Kit (Cat No: 30006, Biotium, San Francisco, CA) according to the manufacturer's protocol. The absorbance at 570 nm and 630 nm was measured with a spectrophotometer. All assays were performed in triplicate.

Real-time PCR

The ASCs were cultured with 10 µl, 50 µl and 100 µl of CP-fixed and ScCO₂-extracted BCO, respectively. Triplicate wells were set up for each volume. The cells were harvested after 24, 48 and 72 h of culture, and total RNA was isolated from the cells using an RNA isolation kit as per the instructions (Cat No: 301-001, Geneall, Seoul, South Korea). All steps were carried out on ice to preserve RNA integrity. RNA concentration and purity were measured using a Qubit 3 spectrophotometric measuring device, and OD₂₆₀/OD₂₈₀ value ranging from 1.8–2.0 was considered indicative of pure RNA. DNase/RNase-free water was used as the blank. The isolated RNA samples were stored at –80 °C. After equalizing the RNA concentration across the samples by diluting with DNase/RNase-free water, reverse transcription was performed using the WizScript™ cDNA Synthesis Kit according to the manufacturer's protocol (Cat No: W2202, Jungwon-gu, South Korea). The samples were incubated at 65 °C for 5 min and subsequently placed on ice. The cDNA samples were stored at –80 °C until further use. Real-time PCR was performed using the WizPure™ qPCR Master (SYBR) kit (Cat No: W1711R-5, Jungwon-gu) in the Applied Biosystems™ 7500 Fast Real-Time PCR System. The relative expression levels of *IL6*, *IL10* and *VEGF* genes were calculated using the $\Delta\Delta C_t$ method, with β -actin as the endogenous reference gene. Each sample was analysed in triplicate. The primer sequences for the target genes are listed in Table 1.

Statistical analysis

The normality of the data was tested via the Shapiro-Wilk test. The homogeneity of variances in the normally distributed data was tested by the Levene test. All data were expressed as the mean $\pm$ standard deviation. For the MTT assay, homogeneous data of different groups were compared by ANOVA, and non-homogeneous data were analysed with the Welch test. Tukey's post hoc test was used for comparisons between subgroups for homogeneous data, and the Dunnett's T3 test was used for non-homogeneous data. The data of gene expression levels showed normal distribution, and the independent-samples *t*-test was used to compare the control and experimental groups (the different volumes were considered independent variables). All statistical analyses were performed using SPSS software 21, and $P < 0.05$ was considered statistically significant.

Results

GC/MS analysis revealed distinct differences in the fatty acid composition and bioactive profiles of CP and ScCO₂-extracted BCO. The TPC was higher in the ScCO₂-

Table 1. Primer sequences used for real-time PCR analysis

Gene	Primer sequence (5'-3')
<i>IL6</i> (interleukin 6)	Forward: GGTCTTCTGGAGTTCCGTTT Reverse: GAGCATTGGAAGTTGGGGTA
<i>IL10</i> (interleukin 10)	Forward: ATGTTGCCTGCTCTTACTGG Reverse: TCCACTGCCTTGCTTTTATTC
<i>VEGF</i> (vascular endothelial growth factor)	Forward: CAATGATGAAGCCCTGGAGT Reverse: TTTGACCCTTCCCTTTCCT
<i>ACTB</i> (β -actin)	Forward: TTTGGCACCACACTTTCTACAAT Reverse: TTAAATGTCACGCACGATTTC

*The β -actin gene expression level was determined as an endogenous control gene.

extracted oil, whereas the CP-fixed oil exhibited slightly greater radical scavenging activity in the DPPH assay, indicating a divergence between phenolic concentration and antioxidant capacity. To assess the biological relevance of these compositional differences, ASCs were treated with CP-fixed and ScCO₂-extracted BCO, and cell viability together with *IL6*, *IL10* and *VEGF* gene expression was evaluated at 24, 48 and 72 h.

Chemical composition of CP and ScCO₂-extracted BCO

BCO obtained by the cold pressing and ScCO₂ extraction procedures differed in the composition of fatty acids and bioactive compounds, although both methods achieved a total fatty acid content of 100 %. Thymoquinone, a major bioactive compound, was significantly more abundant in the ScCO₂-extracted oil (7.16 mg/g) than in the CP-fixed oil (0.06 mg/g), thus highlighting the efficiency of ScCO₂ extraction in preserving or extracting this key component. Minor fatty acids such as tetradecanoic acid (myristic acid) and pentadecanoic acid were also more abundant in the ScCO₂-extracted BCO (0.34 % and 0.04 %, respectively) compared to those in the CP-fixed BCO (0.01 % and 0.02 %, respectively). For the more common fatty acids, the content of hexadecanoic acid (palmitic acid) was 23.19 % in the ScCO₂-extracted oil and 21.9 % in the CP-fixed oil, while 9,12-octadecadienoic acid (linoleic acid) was slightly more abundant in the CP-fixed oil (44.08 %) than in the ScCO₂-extracted oil (42.51 %). Similarly, 9-octadecenoic acid (oleic acid) was also more abundant in the CP-fixed oil (23.25 %) than in the ScCO₂-extracted BCO (20.64 %). Conversely, some minor fatty acids, such as heptadecanoic acid (margaric acid) and octadecanoic acid (stearic acid), were present at higher concentrations in the ScCO₂-extracted BCO (0.17 % and 9.66 %, respectively) than in the CP-fixed oil (0.10 % and 7.56 %, respectively). Additionally, 11,14-eicosadienoic acid and 11-eicosenoic acid were more concentrated in the ScCO₂-extracted oil (1.43 % and 0.31 %, respectively) than in the CP-fixed BCO (1.16 % and 0.18 %, respectively). The results are summarized in Table 2.

Although cold pressing was more effective in extracting relatively high levels of linoleic acid and oleic acid,

the ScCO₂ method was superior in extracting bioactive compounds such as thymoquinone and several minor fatty acids. These variations suggest that the extraction technique significantly influences the chemical profile of BCO, which may have implications for its biological activity and therapeutic potential.

TPC and antioxidant activity

Notable differences were observed in the TPC and antioxidant capacity of BCO depending on the extraction method. The TPC of ScCO₂-extracted BCO was significantly higher (101 ± 0.03 mg GAE/g) than that of CP-fixed BCO (64.85 ± 0.02 mg GAE/g), indicating a greater enrichment of phenolic compounds following supercritical CO₂ extraction (Table 3). Antioxidant activity, assessed using the DPPH radical scavenging assay, revealed high antioxidant potential for both oil preparations. CP-fixed and ScCO₂-extracted BCO exhibited radical scavenging activities of 92.8 % and 90.3 %, respectively (Table 3). Although ScCO₂ extraction resulted in a higher phenolic content, CP-fixed BCO demonstrated slightly greater radical scavenging activity. Collectively, these findings suggest that while supercritical CO₂ extraction may favour phenolic enrichment, cold pressing more effectively preserves antioxidant capacity, which may be advantageous in applications targeting oxidative stress modulation.

Impact of extraction method of BCO on the viability and bioactivity of ASCs

The ASCs were cultured with different volumes of CP-fixed and ScCO₂-extracted BCO (5 μ l, 10 μ l, 25 μ l, 50 μ l, 75 μ l and 100 μ l) for 24, 48 and 72 h. Cell viability and mRNA expression levels of *IL6*, *IL10* and *VEGF* were assessed at these time points (Figs. 1 and 2).

Effects of CP oil

Cell viability increased significantly in a volume-dependent manner across all time points. At 24 h, the 100- μ l group showed significantly higher viability than the 5- μ l, 10- μ l, 25- μ l and 50- μ l groups ($P < 0.05$; Fig. 1A), while the 75- μ l group also demonstrated significantly greater viability than the lower-volume groups ($P < 0.05$). No significant difference was observed between the 75- μ l

Table 2. Composition of BCO obtained via ScCO₂ extraction and cold pressing methods

Active compounds	Method	Unit	ScCO ₂ extract	CP-fixed oil
Thymoquinone	HS-GC/MS	mg/g	7.16	0.06
Tetradecanoic acid (Myristic acid)	GC/MS	0.01 %	0.34	0.26
Pentadecanoic acid			0.04	0.02
9-Hexadecenoic acid (Palmitoleic acid)			0.17	0.14
Hexadecanoic acid (Palmitic acid)			23.19	21.90
9,10-Methylene hexadecanoic acid			0.07	0.05
Heptadecanoic acid (Margaric acid)			0.17	0.10
9,12-Octadecadienoic acid (Linoleic acid)			42.51	44.08
9-Octadecenoic acid (Oleic acid)			20.64	23.25
Octadecanoic acid (Stearic acid)			9.66	7.56
11,14-Eicosadienoic acid			1.43	1.16
11-Eicosenoic acid			0.31	0.18
Eicosenoic acid (Arachidic acid)			0.23	0.18
13-Docosenoic acid (Erucic acid)			0.03	0.04
Docosanoic acid (Behenic acid)			0.04	0.02
Other compounds			1.16	1.07
TOTAL			100	100

Table 3. TPC and antioxidant activity of BCO obtained via ScCO₂ extraction and cold pressing methods.

	TPC (mg GAE/g)	DPPH (% RSA)
CP	64.85 ± 0.02	92.8
ScCO ₂	101 ± 0.03	90.3

and 100- μ l groups. The 50- μ l group exhibited intermediate viability, significantly higher than that of the 5- μ l, 10- μ l and 25- μ l groups but lower than that of the 75- μ l and 100- μ l groups ($P < 0.05$). At 48 h, a similar volume-dependent pattern was observed, with the 100- μ l group presenting the highest viability compared to all other groups ($P < 0.05$; Fig. 1A). The 75- μ l group also showed significantly greater viability than the 5- μ l, 10- μ l, 25- μ l and 50- μ l groups, while the 50- μ l group maintained moderate but significantly increased viability relative to the lowest-volume treatments ($P < 0.05$). At 72 h, viability remained highest in the 100- μ l group ($P < 0.05$), followed by the 75- μ l and 50- μ l groups, which consistently demonstrated significantly greater viability than the 5- μ l, 10- μ l and 25- μ l groups ($P < 0.05$; Fig. 1A). The 5- μ l and 10- μ l groups consistently exhibited the lowest viability across all time points. Overall, these findings indicate a robust and sustained volume-dependent enhancement of ASC viability, with the most pronounced effects observed at 100 μ l. *IL6* mRNA expression was reduced following BCO treatment in a time- and volume-dependent manner (Fig. 1B). At 24 h, *IL6* expression was significantly decreased in the 50- μ l group compared to the control ($P < 0.05$), while reductions in the 10- μ l and 100- μ l groups did not reach statistical significance. At 48 h, *IL6* expression remained significantly lower in the 50- μ l and 100- μ l groups than in the control group ($P <$

0.05). By 72 h, no significant differences were observed among groups, indicating attenuation of the suppressive effect over time. *IL10* mRNA expression was significantly increased in the 50- μ l and 100- μ l groups at 24 and 48 h compared to the control ($P < 0.05$; Fig. 1C). Although *IL10* expression in the 50- μ l group remained elevated at 72 h, this difference was not statistically significant. No marked changes were observed in the lower-volume groups. *VEGF* mRNA expression was significantly up-regulated in the 50- μ l and 100- μ l groups at 24 h, and this effect persisted at 48 h ($P < 0.05$; Fig. 1D), with the 50- μ l group exhibiting the highest expression level. At 72 h, *VEGF* expression did not differ significantly between treated and control groups, suggesting that the angiogenesis-related gene expression response induced by BCO is most prominent at early time points.

Effects of ScCO₂-extracted oil

Cell viability increased in a volume-dependent manner across all time points following treatment with ScCO₂-extracted BCO (Fig. 2A). At 24 h, viability was significantly higher in the 100- μ l group than in the 5- μ l and 10- μ l groups ($P < 0.05$), while the 50- μ l and 75- μ l groups also demonstrated significantly greater viability compared with the lowest-volume treatments ($P < 0.05$). No significant difference was observed between the 75- μ l and 100- μ l groups. Although the 25- μ l group showed a modest increase in viability relative to the 5- μ l and 10- μ l groups, its viability remained significantly lower than that observed at 50 μ l and higher volumes ($P < 0.05$). At 48 h, this volume-dependent pattern persisted, with the 100- μ l group maintaining significantly higher viability than the 5- μ l, 10- μ l and 75- μ l groups ($P < 0.05$; Fig. 2A). The 50- μ l group again exhibited a marked increase in viability compared with the 5- μ l, 10- μ l and 25- μ l groups ($P < 0.05$), while the 75- μ l group demonstrated an inter-

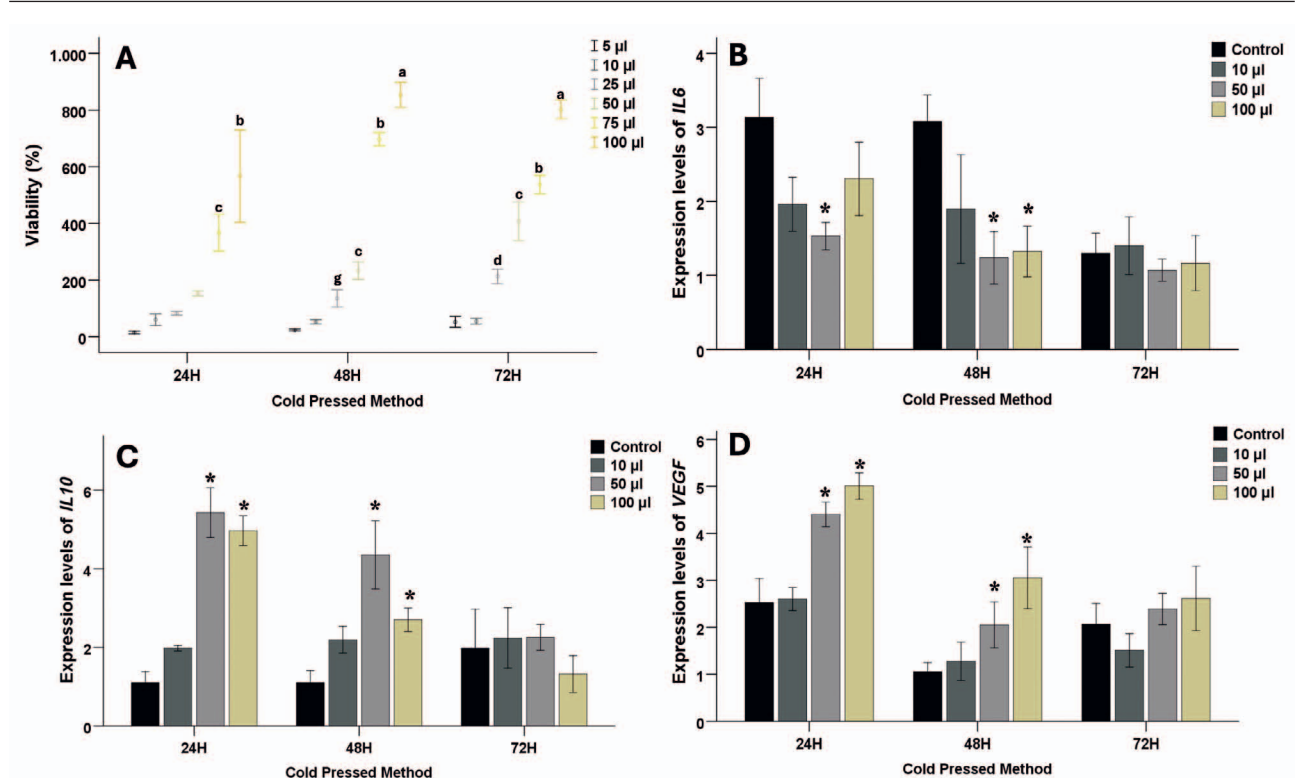


Fig. 1. Effects of CP-fixed BCO on the viability of ASCs and the expression of *IL6*, *IL10* and *VEGF*. (A) Percentage of viable cells in the different treatment groups (5, 10, 25, 50, 75 and 100 µl) at the indicated time points. (B–D) Expression levels of (B) *IL6*, (C) *IL10* and (D) *VEGF* in the indicated groups at 24 h, 48 h and 72 h. The data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined via ANOVA followed by post hoc tests (* $P < 0.05$ vs control group; ^a $P < 0.05$ vs 5, 10, 25, 50 and 75 µl groups; ^b $P < 0.05$ vs 5, 10, 25 and 50 µl groups; ^c $P < 0.05$ vs 5, 10 and 25 µl groups; ^d $P < 0.05$ vs 5 and 10 µl groups; ^e $P < 0.05$ vs 5 µl group).

mediate response. At 72 h, viability remained highest in the 100-µl group ($P < 0.05$), followed by the 75-µl and 50-µl groups, all of which showed significantly greater viability than the 5-µl and 10-µl groups ($P < 0.05$). Overall, these findings indicate that higher volumes of ScCO₂-extracted BCO, particularly 50 µl and 100 µl, consistently enhance ASC viability, with the most robust effect observed at 100 µl. *IL6* mRNA expression was significantly reduced in the 10-µl, 50-µl and 100-µl groups at 24 h compared with the control group ($P < 0.05$; Fig. 2B), and this suppressive effect persisted at 48 h ($P < 0.05$). By 72 h, *IL6* expression levels no longer differed significantly between treated and control groups, suggesting attenuation of the anti-inflammatory effect over time. *IL10* mRNA expression was significantly increased in the 10-µl, 50-µl and 100-µl groups at 24 h and remained elevated at 48 h compared with the control group ($P < 0.05$; Fig. 2C). Although *IL10* expression in the 50-µl and 100-µl groups remained higher at 72 h, these differences did not reach statistical significance. *VEGF* mRNA expression was significantly up-regulated in the 10-µl, 50-µl and 100-µl groups at 24 h ($P < 0.05$; Fig. 2D), with the 50-µl group showing the highest expression. This trend persisted at 48 h ($P < 0.05$). At 72 h, a significant increase in *VEGF* expression was observed only in the 100-µl group compared with the control group ($P < 0.05$), indicating a sustained

angiogenesis-related gene expression response at the highest volume. Collectively, both CP-fixed and ScCO₂-extracted BCO enhanced ASC viability and modulated inflammatory and angiogenic gene expression in a volume-dependent manner. Notably, ScCO₂ extraction resulted in more pronounced and sustained *VEGF* up-regulation at higher volumes, whereas cold-pressed oil promoted more consistent improvements in cell viability across a broader volume range, particularly at 50 µl and 100 µl.

Discussion

The present study demonstrates that *Nigella sativa*-derived black cumin oil, obtained via cold pressing and supercritical CO₂ extraction, exerts extraction- and volume-dependent effects on adipose-derived mesenchymal stem cell viability, inflammatory balance and angiogenic signalling. These findings align with the growing body of evidence indicating that *N. sativa* oils possess strong antioxidant, anti-inflammatory and immunomodulatory properties, while further extending this knowledge into the context of stem cell-based regenerative strategies (Bordoni et al., 2019; Ciesielska-Figlon et al., 2023; Alberts et al., 2024).

A key observation of this study is the dose/volume-dependent enhancement of ASC viability, particularly at

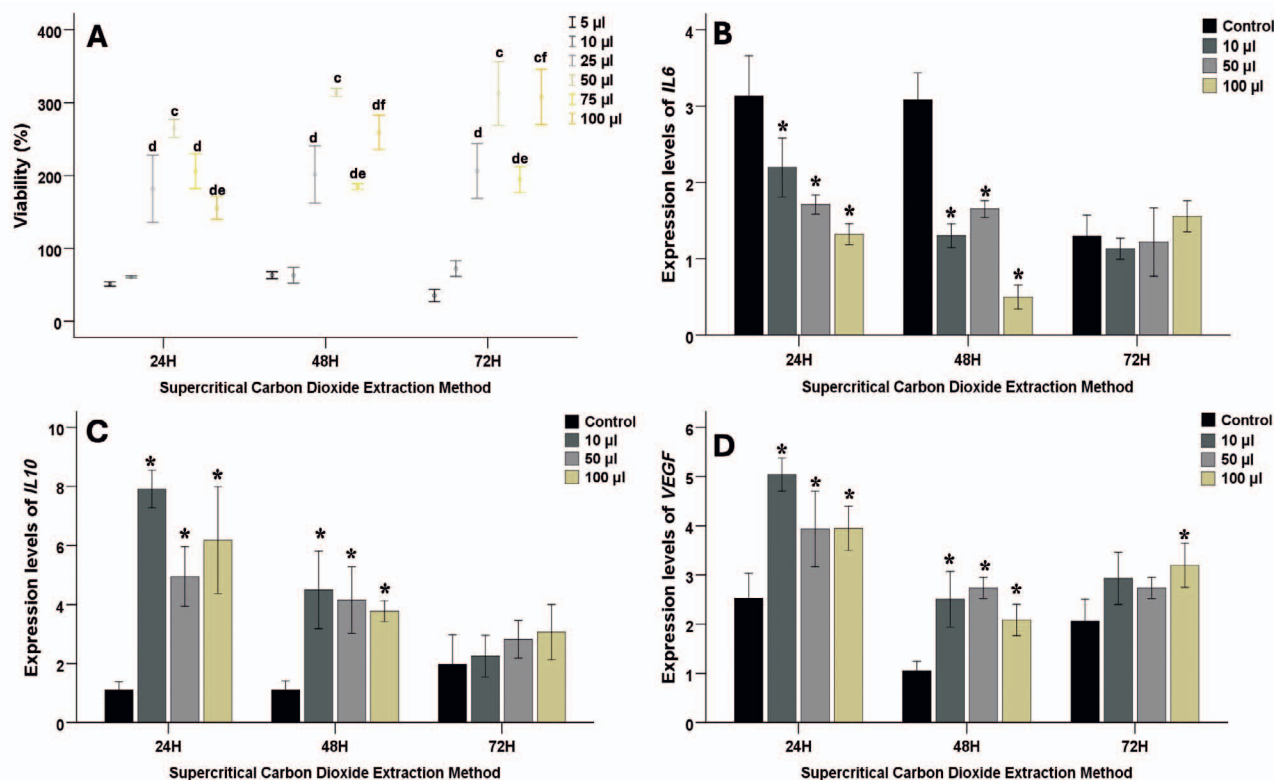


Fig. 2. Effects of ScCO₂-extracted BCO on the viability of ASCs and the expression of *IL6*, *IL10* and *VEGF*. (A) Percentage of viable cells in the different treatment groups (5, 10, 25, 50, 75 and 100 µl) at the indicated time points. (B–D) Expression levels of (B) *IL6*, (C) *IL10* and (D) *VEGF* in the indicated groups at 24 h, 48 h and 72 h. The data are presented as mean ± standard deviation (SD). Statistical significance was determined via ANOVA followed by post hoc tests (*P < 0.05 vs control group; °P < 0.05 vs 5, 10, and 25 µl groups; °P < 0.05 vs 5 and 10 µl groups; °P < 0.05 vs 50 µl group; °P < 0.05 vs 75 µl group).

50 µl and 100 µl for both extraction methods. Similar cytoprotective and metabolic-supportive effects of *N. sativa* extracts have been reported in adipocyte and pre-adipocyte models, where black cumin oil promoted cell survival while attenuating oxidative stress and inflammatory burden (Bordoni et al., 2019; Bashir et al., 2023). Moreover, anti-adipogenic and lipid-modulating effects observed in both cellular systems and human subjects further support the notion that *N. sativa* acts at the intersection of metabolic regulation and inflammation (Ahmed et al., 2025). In the context of ASCs, this dual action is particularly relevant, as lipid homeostasis and inflammatory signalling jointly influence stem cell viability and paracrine function.

The extraction method emerged as a decisive determinant of biological response. Chemical analyses revealed that ScCO₂ extraction yielded higher concentrations of phenolic compounds and thymoquinone, whereas CP-fixed oil retained slightly superior radical scavenging activity. This trade-off has been consistently reported in comparative extraction studies, demonstrating that ScCO₂ extraction enhances recovery of bioactive constituents, while cold pressing better preserves certain antioxidant interactions within the oil matrix (Rahim et al., 2022; Choudhury et al., 2023). Similar extraction-dependent

functional divergence has also been observed in other plant-derived oils used in stem cell biology, reinforcing the importance of tailoring extraction strategies to desired biological outcomes (Ince et al., 2025).

At the inflammatory level, both CP-fixed and ScCO₂-extracted BCO significantly suppressed *IL6* expression while up-regulating *IL10*, particularly at intermediate and high volumes. These findings are consistent with extensive preclinical and clinical evidence indicating that *N. sativa* and its constituents down-regulate pro-inflammatory cytokines while promoting anti-inflammatory mediators (Gholamnezhad et al., 2016; Wahab and Alsayari, 2023; Ridwan et al., 2025). The transient nature of *IL6* suppression observed at later time points likely reflects adaptive cellular feedback mechanisms rather than loss of bioactivity, a phenomenon also described in long-term *in vitro* inflammatory models (Ciesielska-Figlon et al., 2023).

The concomitant increase in *IL10* expression highlights the immunomodulatory capacity of BCO-treated ASCs. IL-10 plays a critical role in maintaining an anti-inflammatory microenvironment conducive to tissue repair and regeneration. Previous studies have demonstrated that *N. sativa* enhances IL-10-mediated immune regulation in both experimental and disease models, supporting its

therapeutic relevance in chronic inflammatory conditions (Kavyani et al., 2023; Rashwan et al., 2023). In the present study, ScCO₂-extracted BCO induced a more sustained IL-10 response, likely reflecting its enriched phenolic and thymoquinone content.

Another notable finding is the up-regulation of *VEGF* expression, particularly in ASCs treated with ScCO₂-extracted BCO. VEGF is a central mediator of angiogenesis and a key component of the regenerative secretome of mesenchymal stem cells. Enhanced *VEGF* expression has previously been linked to *N. sativa*-mediated improvements in endothelial function and angiogenic capacity (Nordin et al., 2019; Ali et al., 2024). The more pronounced and sustained VEGF response observed with ScCO₂-extracted BCO supports the hypothesis that extraction-dependent enrichment of bioactive lipophilic compounds enhances pro-angiogenic signalling.

The use of a volume-based dosing strategy represents an additional strength of this study. In lipid-rich and heterogeneous biological systems, the nominal concentration alone may not accurately reflect effective cellular exposure. Previous work has emphasized the importance of alternative dosing metrics, including volume- and cell-normalized approaches, to improve interpretability and translational relevance of *in vitro* findings (Hindliter et al., 2010; Doskey et al., 2015; Kusi-Appiah et al., 2015). The consistent volume-dependent trends observed here validate this approach for evaluating oil-based bioactives in stem cell culture systems. Beyond its methodological advantages in cell culture systems, the use of a volume-based dosing framework may also have implications for translational research and clinical applications involving lipid-rich bioactives. In lipid-based drug delivery systems and oil-based formulations, dosing strategies are frequently constrained by formulation volume, excipient load and tolerability rather than by the molar amount of a single active compound. Previous studies have shown that in oral lipid-based formulations and injectable lipid depots, clinically acceptable dosing regimens are often defined by capsule fill volume, injection volume and excipient composition, which together determine achievable systemic exposure (Holm et al., 2019, 2023; Williams et al., 2019; Rahnfeld and Luciani, 2020). Similarly, in nutraceutical and natural oil-based systems containing complex mixtures of lipophilic compounds, functional effects are influenced by the total lipid phase and dispersion behaviour rather than by the molar concentration of individual constituents (McClements, 2018; Lazăr et al., 2024). In this context, the volume-dependent trends observed in the present study may better reflect biologically relevant exposure conditions for oil-derived bioactives in cellular systems. Consequently, volume-based dosing approaches may provide a more translationally meaningful framework for evaluating lipid-rich natural products in regenerative and cell-based therapeutic contexts.

Collectively, these findings suggest that cold pressing and ScCO₂ extraction confer distinct but complementary biological advantages. Cold-pressed BCO appears par-

ticularly effective in supporting early ASC viability and antioxidant defence, whereas ScCO₂-extracted BCO favours sustained immunomodulatory and angiogenic responses. This distinction has important implications for stem cell biology, suggesting that compositional differences associated with extraction approach may differentially shape cytoprotective and paracrine signalling responses at the cellular level.

Strengths and limitations

This study is strengthened by its extraction-dependent comparison of cold-pressed and supercritical CO₂-extracted *Nigella sativa* oil, allowing direct evaluation of how processing methodology influences chemical composition and stem cell bioactivity. The integration of GC/MS profiling with antioxidant assays and functional analyses of mesenchymal stem cell viability and inflammatory-angiogenic gene expression provides a comprehensive and mechanistically relevant assessment. In addition, the use of a volume-based dosing strategy enhances the biological interpretability of lipid-rich extracts in cell culture systems. However, several limitations should be acknowledged. The study was conducted under *in vitro* conditions, which may not fully recapitulate the complexity of *in vivo* inflammatory and regenerative environments. Moreover, the analysis was limited to selected molecular markers assessed at the gene expression level (*IL6*, *IL10* and *VEGF*), and angiogenic activity was inferred from *VEGF* mRNA expression. Protein-level validation or functional angiogenesis assays (e.g., tube formation or secretome analysis) were not performed. Future studies should therefore validate these findings at both protein and functional levels to further clarify the angiogenic potential of *Nigella sativa* oil in stem cell systems. Finally, while volume-dependent effects were demonstrated, concentration-normalized dosing and long-term exposure outcomes were not evaluated. Despite these limitations, the findings provide a solid experimental framework and a rationale for future *in vivo* and mechanistic studies aimed at optimizing *Nigella sativa* oil-based strategies in regenerative medicine.

Conclusion

In conclusion, black cumin oil exerts pronounced immunomodulatory and pro-angiogenic molecular effects in adipose-derived mesenchymal stem cells, accompanied by a robust, volume-dependent enhancement of cell viability. Treatment with both cold-pressed and supercritical CO₂-extracted oils consistently promoted ASC survival and shifted inflammatory signalling toward an anti-inflammatory profile, as evidenced by the down-regulation of *IL6* and concomitant up-regulation of *IL10*, particularly at intermediate to high volumes (50–100 µl) and during the early-to-intermediate exposure period (24–48 h). In parallel, both oil preparations induced angiogenesis-related molecular signalling through increased *VEGF* expression, indicating a pro-angiogenic response at the gene expression level. Although these core bio-

logical effects were observed across both oil preparations, differences in the magnitude and temporal persistence of *VEGF* induction suggest that compositional variation associated with extraction approach may modulate specific aspects of ASC bioactivity. In this context, the more pronounced and sustained VEGF response observed with the ScCO_2 -extracted oil is consistent with its higher enrichment in thymoquinone and phenolic constituents, whereas the cold-pressed oil provided stable cytoprotective support across time points, reflected by consistently elevated cell viability. Collectively, these findings support the relevance of *Nigella sativa* oil as a bioactive, inflammation-modulating agent capable of enhancing stem cell viability while simultaneously influencing inflammatory and angiogenesis-related molecular pathways. Rather than defining the overall biological effect, extraction-related compositional differences appear to fine-tune the intensity of specific signalling responses, underscoring the importance of considering oil composition when evaluating the cellular and molecular actions of black cumin oil in stem cell-based regenerative contexts.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Badel Ince, Gokturk Avsar, Cagatay Han Turkseven, Pelin Eroglu, Gamze Ayar and Murat Eser Akyurek. The first draft of the manuscript was written by Cagatay Han Turkseven, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical approval

Ethical approval was not required for this study, as all experiments were conducted using commercially purchased adipose-derived mesenchymal stem cells (ASCs) obtained from the Kocaeli University Research and Application Hospital Stem Cell and Gene Therapies Research and Application Center (KOGEM), a certified provider.

Conflict of interest

The authors declare that they have no conflicts of interest.

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