Modulation of IKK/NF-κB Signaling: A Therapeutic Mechanism of Shilajit in Breast Cancer Cells

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Abstract

Background: Shilajit is a natural pale-brown to blackish-brown phytocomplex that exudes from mountain rocks in various parts of the world. In recent years, numerous pharmacological effects of shilajit have been explored through extensive experiments. Shilajit is known as an antioxidant and anti-inflammatory agent. Activation of the nuclear factor kappa B (NF-κB) signaling pathway could be an important player in inflammation-driven tumor progression. Therefore, the current study evaluated the anti-inflammatory effect of shilajit on a breast cancer cell line (MCF7).

Methods: In this experimental study, the MCF7 cell line was treated with shilajit, and an MTT assay was applied to analyze the half-maximal inhibitory concentration (IC50) after 72 hours of treatment. Following this, the apoptotic rate was assessed using flow cytometry, and p50, RelB, and IKKα/β gene expression were evaluated using real-time PCR assay.

Results: Shilajit had potent cytotoxic activity in a dose- and time-dependent manner with an IC50 of 280 μg/mL. Based on the Annexin-Pi analysis, the IC50 concentration of this compound induced significant apoptosis in the cells, possibly through suppression of NF-κB (p50, RelB, and IKKα/β)-regulated genes. The real-time PCR results indicated that treating MCF7 cells at the IC50 dose of shilajit for 72 hours could reduce the mRNA expression levels of p50, RelB, and IKKα/β in the cells.

Conclusion: The findings of the present experiment showed that shilajit may have promising anti-breast-tumor and anti-inflammatory activity through inhibition of the NF-κB signaling pathway. Therefore, shilajit can help treat breast cancer in combination with other standard treatments.

Keywords: Shilajit, IkB kinase, Nuclear factor kappa B, Inflammation, Breast cancer

Introduction

Breast tumors account for the majority of cancers among females (1). Based on the American Cancer Society’s report, one million women are referred for breast cancer every year. Among them, 1%–6% are diagnosed with the most invasive type. Inflammatory breast cancer (IBC) is a rare and aggressive clinicopathological type of breast cancer. It represents 1% to 6% of all cases of breast cancer (2,3). A causal relationship has been proposed between inflammation and neoplasm, and cancers have been suggested to stem from chronic inflammation (4-9). How accurately the inflammation is linked to carcinogenesis is yet to be completely understood. The activation of the nuclear factor kappa B (NF-κB) signaling pathway could be an important player in inflammation-driven tumor progression (4). Although NF-κB activation is associated with cytotoxic immune reaction against transformed cells (tumor-immunosurveillance), this ubiquitous transcription factor can manage metastasis and vascularization and exert a pro-carcinogenic role (4). The anti-tumorigenic action of the immune system, in which NF-κB plays a critical role, has been called tumor-immunosurveillance. This immune defense against cancer cells, however, is normally not tight enough to
eliminate all the aberrant cells, resulting in a shift to an equilibrium phase, which is often followed by an “escape” phase of the cancer cells, in which they outperform the immune system (4).

Shilajit is a natural pale-brown to blackish-brown phytocomplex with variable consistency, exuding from rocks in many world mountain ranges (10). This substance contains fulvic and humic acids, free and conjugated dibenzo-α-pyrones, proteins, and numerous minerals (10). A growing body of data points to the prophylactic and therapeutic uses of shilajit in treating different diseases and disorders (10,11); however, it is plausible to also count anticancer activity among the functions of this substance (12,13). Ghosal et al. showed the possible effect of shilajit as an anti- ulcerogenic and anti-inflammatory agent. They found that shilajit increased the carbohydrate/protein ratio and decreased the gastric ulcer index (14).

Shilajit, at a dose of 50 mg/kg, was also found to significantly reduce carrageenan-induced hind paw edema in rats. Shilajit has anti-inflammatory qualities recognized in the study by Goel et al. They have shown that Shilajit can reduce inflammation, such as pedal edema and granuloma pouch induced by carrageenan in rats (15). Due to the high prevalence of breast tumors and lack of mechanistic studies on shilajit’s influence on the IκB kinase (IKK)/NF-κB signaling pathway, the present experiment was focused on the anti-MCF-7 effects of shilajit and the corresponding mechanisms.

Materials and Methods
Shilajit was purchased from a local market, and it was verified by the Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran (Herbarium code 1732). After washing (thrice), it was dried, powdered, and dissolved in the culture medium. Finally, it was sterilized using a filter (0.22 μm pore size, Orange Scientific Company).

Cell culture
The human breast cancer cell line, MCF-7 (HTB-22™), was purchased from the Iranian cell bank of Pasteur Institute and was then cultured in Dulbecco’s Modified Eagle’s Medium (Gibco Company) enriched with ten percent fetal bovine serum (Gibco Company) and 1% penicillin/streptomycin (Sigma Aldrich) in a humid atmosphere (5% CO₂ at 37 °C). The medium was replaced every 72 hours, and the cells were given passage using TrypLE™ Select Enzyme (Gibco Company) after reaching 80%-90% confluency.

Metabolic activity evaluation of MCF-7 cells
The cells (5 × 10⁴/well) were seeded in a 96-well plate containing 0.2 mL of medium/well and incubated overnight. Next, the depreciated medium was replaced by 200 μL of new culture medium containing different (100, 200, 400, 600, and 800 μg/mL) concentrations of shilajit (3 wells/each concentration). The mentioned concentrations were selected based on previous studies and confirmed by our pilot dosimetry findings (16,17). Following the stipulated time (24, 48, and 72 hours), the medium was eliminated, and cell survival was analyzed using the MTT assay kit (Sigma Aldrich) according to the manufacturer’s instructions (18). It should be noted that the IC₅₀ concentration was obtained by Prism software and the control groups contained untreated cells.

Apoptosis assay
The effect of shilajit on the death of cells was assayed by the ApoFlowEx™ FITC kit (ExBio Company). The cells were seeded into two cell culture flasks at a density of 5 × 10⁴ cells/flask, and 72 h after shilajit treatment (IC₅₀ concentration), the cells were extracted. Next, the washed cells were suspended in a binding buffer and stained using propidium iodide (PI) and Annexin V, based on the manufacturer’s instructions (11). After incubation time (15 minutes), the apoptotic cell % was quantified using BD FACSCalibur flow cytometer (BD Biosciences Co.). Unstained cells were used to eliminate autofluorescence. Annexin V+ and PI cells were regarded as early apoptotic cells, and cells with positive staining both for Annexin V and PI (Annexin V+/PI+) were considered to be in late apoptosis (18).

RNA isolation and preparation of cDNA
After the 72 hours shilajit treatment of the human breast cancer cell line (MCF-7) according to the instructions, the elicitation of RNA and cDNA synthesis from the treated and untreated cells were done using TRIzol® (Invitrogen, USA) and Revert Aid First Strand cDNA Synthesis Kit (K1621) (Thermo Fisher, Germany – Darmstadt), respectively.

Quantitative real-time PCR
The relative gene expression analysis was conducted by real-time PCR and using RealQ Plus 2× Master Mix Green with high ROX™ (Ampliqon Denmark Company), 1 μL of the cDNA product (50 ng), 0.8 μL of each of the primers (0.2 μM), and 2.9 μL of nuclease-free water (total volume of 12 μL). The PCR amplification steps using a StepOnePlus real-time PCR system (Applied Biosystem) were as follows. Denaturation (95 °C/15 min), 45 cycles (95 °C/30 s), and 63 °C for 1 minute. A melting curve assessment was applied to affirm the specificity of the product. The housekeeping gene, GAPDH, was used as an internal control, and the relative gene expression was calculated by the comparative C(T) method. All trials were repeated three times. The list of the sequences of the primers used is available in Table 1.
Statistical analysis
The mean ± standard error of the mean (mean ± SEM) was calculated, and the statistical differences between multiple groups were assessed by one-way ANOVA using SPSS 20 software. Significant differences between treated and untreated groups in flow cytometry and real-time PCR techniques were analyzed by an unpaired student’s t test. P value ≤ 0.05 was regarded as statistically significant.

Results
Shilajit decreases the metabolic activity of MCF-7 cells
As illustrated in Figure 1, the viability of the cultured cells after exposure to shilajit was reduced in a dose/time-dependent manner. The fifty percent decrease in the viability of treated cells (IC₅₀) is not seen in the concentrations used for 48 hours, indicating the long-term cytotoxic effect of shilajit on MCF-7 cells. Regarding the severe reduction of viability after 72 hours exposure, this exposure time was used in the following experiments. In 72 hours exposure, the cytotoxic effect was concentration-dependent, with approximately 20%, 37%, 57%, 63%, and 69% reduction in metabolic activity after treatment with 100, 200, 400, 600, and 800 μg/mL of shilajit, respectively (IC₅₀ = 280 μg/mL). These findings implied that this ayurvedic substance could exert a long-term and dose-dependent cytotoxic effect on the studied cells.

Shilajit induces apoptosis in MCF-7 cells
As illustrated in Figure 2, 72 h incubation of cells with IC₅₀ concentration significantly (P ≤ 0.01) increased Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ cells in the treated group compared with the control group, implying the apoptotic effect of shilajit on MCF-7 cells (19).

Shilajit downregulates the expression of IKK/NF-κB-related genes
Based on the results, the expression levels of the studied genes were significantly downregulated in 280 μg/mL-treated cells compared with the control cells (Figure 3). The expression level of p50, RelB, IKKα, and IKKβ genes decreased to 0.31 ± 0.03, 0.22 ± 0.05, 0.058 ± 0.006, and 0.04 ± 0.002 respectively. Therefore, the IKKβ and p50 genes had the highest and lowest expression reduction, respectively, in the treatment.

Discussion
Identifying safe and effective compounds is a necessity in cancer drug development. Shilajit, a traditional natural medication, has prevailed for years as a rejuvenating mineral substance for treating several conditions (10). Based on the reports of previous studies, shilajit can induce apoptosis and suppress proliferation in several kinds of cancerous cells (20).

The bioactive potential of this matter is mainly ascribed to easily assimilated minerals, fulvic acids, and humic

Table 1. The used oligonucleotide primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5'-3')</th>
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<tbody>
<tr>
<td>p50.F</td>
<td>AAAGAGCTAATCCGCCCAAGCA</td>
</tr>
<tr>
<td>p50.R</td>
<td>AGCTGTAACATGACCAGC</td>
</tr>
<tr>
<td>RelB.F</td>
<td>TGCGTGGAGGATCTGATCAG</td>
</tr>
<tr>
<td>RelB.R</td>
<td>TCGGCCAATCCCGAGCTCAT</td>
</tr>
<tr>
<td>IKKα.F</td>
<td>ACAGAGTTCTGCGCGTCCCT</td>
</tr>
<tr>
<td>IKKα.R</td>
<td>CTGCCGTAGTCGGGGGCAAGC</td>
</tr>
<tr>
<td>IKKβ.F</td>
<td>CGCCCAATGACCTGCCCTG</td>
</tr>
<tr>
<td>IKKβ.R</td>
<td>GGCACCTTCCGAGACACAC</td>
</tr>
<tr>
<td>GAPDH.F</td>
<td>CCCCTCTGAAAGCTCTTGG</td>
</tr>
<tr>
<td>GAPDH.R</td>
<td>AGTGGATGCAGGGATGATG</td>
</tr>
</tbody>
</table>

F: forward sequence; R: reverse sequence.

Figure 1. Shilajit decreases the viability of the studied cells. The cells were treated for 24, 48, and 72 h with different concentrations of shilajit, and then an MTT assay was applied. After 72 h exposure, the viability was reduced significantly (compared to the control value) in a dose-dependent manner (IC₅₀ = 280 μg/mL). Data are given as the mean ± SEM of the three independent examinations. ***P ≤ 0.001
acids (10). The effectiveness and the non-toxic nature of these organic substances lead to their recommendation for internal and external applications. For instance, humic acids alleviate the functional and structural effects of lead poisoning on the thyroid gland, possibly by impeding its internalization and exerting anti-inflammatory effects (21). In the meantime, different studies have confirmed the anti-inflammatory and neoplastic functions of humic substances (22-24). On the other hand, the anti-inflammatory and anti-allergic effects of fulvic acid have also been proven in sensitized mast cells and basophils (25). Interestingly, fulvic acid can reduce the release of pro-inflammatory mediators and TNF-α expression in U937 cells. Fulvic acid causes a reduction of cyclooxygenase 2 and prostaglandin E<sub>2</sub> secretion in monocytes (26).

Despite the current knowledge of the biological activities of shilajit and its ingredients, studies relating to its anticancer mechanisms are scarce. As presented in Figure 1, MTT results implied that shilajit reduced the viability% of MCF-7 cells in a dose- and time-dependent manner. A significant difference in cell viability was detected after 72 hours incubation of the cells at the tested concentrations. The IC<sub>50</sub> concentration was 280 μg/mL. A previous experiment (13) reported that 24 hours incubation of hepatic cancer cells (Huh-7 cells) with different doses of shilajit causes a concentration-dependent antitumor effect. Considering incubation times (24 vs 72 hours) and the approximate IC<sub>50</sub> dose of shilajit in Pant’s study (~100 μg/mL) and in our experiment (~280 μg/mL), it seems that Huh-7 cells are more shilajit-sensitive than MCF-7 cells.

Exposure to IC<sub>50</sub> concentration caused significant apoptosis (P≤0.01) in shilajit-treated cells (Figure 2). Apoptosis is desirable for an anticancer drug candidate (19,27-29). An increase in both early and late apoptotic cells indicated that apoptosis induction is at least partially responsible for the cytotoxic effect of shilajit. Thus, to ascertain the mechanism of apoptosis, we aimed to analyze the expression of IKK/NF-κB signaling pathway-related genes following treatment. Based on the results (Figure 3), we found that the proposed mechanism suppresses NF-κB activation (down-regulation of p50, RelB, IKKa, and IKKβ genes). Transcription factor NF-κB was identified in 1986 as a nuclear factor that binds to the enhancer element of activated B cells immunoglobulin kappa light chain (NF-κB). The five members of the NF-κB family of proteins are RelA (p65), RelB, c-Rel, NF-κB1 (p105), and NF-κB2 (p100). P105 and p100 are processed to their shorter forms, p50 and p52 (4). When a cell is stimulated by an appropriate extracellular signal, the IkB kinase (IKK) complex, comprised of the regulatory subunits of IKKA and IKKB catalytic subunits, is activated (17). This activated complex phosphorylates IkB, which then undergoes proteasomal-mediated degradation and releases NF-kB dimers that travel to the nucleus and bind to DNA at NF-kB response elements to modulate the expression of a wide variety of genes, including inflammatory cytokines, chemokines, immune...
receptors, and cell adhesion molecules. In contrast to IKKα, which does not play a critical role in NF-kB activation by pro-inflammatory cytokines, the disruption of the IKKβ gene corroborates the essential function of IKKβ in NF-kB activation (30).

In both direct and indirect manner (4), NF-kB, as a master regulator, cooperates with multiple other signaling pathways, kinases (p38 and PI3K), and transcription factors (STAT3 and p53). A large and growing body of data supports the crucial contribution of this activation to the development of cell death resistance and inflammation-associated cancers (4,31,32). Unlike IKKα, the prominent function of IKKβ in NF-kB activation is well-known (33). Interestingly, we hinted at a significant reduction of IKKβ gene expression in the shilajit-treated cells here. Our findings reveal that shilajit is a potential tumor-suppressor compound inhibiting IKK-driven NF-kB activation. However, further molecular and cellular investigations are necessary to recognize other possible antitumor mechanisms of this phytocomplex.

**Limitation**

The present study is only an in vitro experiment. For the widespread use of shilajit in treating breast cancer, further evaluations, including animal studies and clinical trials, are needed.

**Authors’ Contribution**

Conceptualization: Arezoo Saberi, Zeinab Kordestani.

Formal Analysis: Reza Vahidi.

Investigation: Elham Melki.

Methodology: Niloofar Ghorbani.

Project administration: Reza Shideh.

Visualization: Areezo Saberi. Reza Vahidi.

Supervision: Seyede Elmeara Yazdi Rouholamini, Zeinab Kordestani.

Writing—original draft: Areezo Saberi.

Writing—review & editing: Reza Vahidi.

**Competing Interests**

On behalf of all authors, the corresponding author states that there are no conflicts of interest in this study.

**Consent for publication**

Not applicable.

**Data Availability Statement**

The current study’s data are available from the corresponding author upon reasonable request.

**Ethical Approval**

The study was approved by the Ethics Committee of Kerman University of Medical Sciences (ethical approval code: IR. KMU. REC.1395.829). Given that our study is an in vitro experiment, informed consent was not applicable.

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**References**


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