




# Modulation of IKK/NF- $\kappa$ B 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- $\kappa$ B) 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 (IC<sub>50</sub>) after 72 hours of treatment. Following this, the apoptotic rate was assessed using flow cytometry, and p50, RelB, and IKK $\alpha/\beta$  gene expression were evaluated using real-time PCR assay.

**Results:** Shilajit had potent cytotoxic activity in a dose- and time-dependent manner with an IC<sub>50</sub> of 280  $\mu$ g/mL. Based on the Annexin-PI analysis, the IC<sub>50</sub> concentration of this compound induced significant apoptosis in the cells, possibly through suppression of NF- $\kappa$ B (p50, RelB, and IKK $\alpha/\beta$ )-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 IKK $\alpha/\beta$  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- $\kappa$ B signaling pathway. Therefore, shilajit can help treat breast cancer in combination with other standard treatments.

**Keywords:** Shilajit, I $\kappa$ B kinase, Nuclear factor kappa B, Inflammation, Breast cancer

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## 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- $\kappa$ B) signaling pathway could be an important player in inflammation-driven tumor progression (4). Although NF- $\kappa$ B 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- $\kappa$ B 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- $\alpha$ -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 I $\kappa$ B kinase (IKK)/NF- $\kappa$ B 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  $\mu$ m pore size, Orange Scientific Company).

### Cell culture

The human breast cancer cell line, MCF-7 (HTB-22<sup>TM</sup>), 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% CO<sub>2</sub> at 37 °C). The medium was replaced every 72 hours, and the cells were given passage using TrypLE<sup>TM</sup> Select Enzyme (Gibco Company) after reaching 80%–90% confluency.

### Metabolic activity evaluation of MCF-7 cells

The cells (5  $\times$  10<sup>3</sup>/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  $\mu$ L of new culture medium containing different (100, 200, 400, 600, and 800  $\mu$ g/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 IC<sub>50</sub> 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<sup>®</sup> FITC kit (ExBio Company). The cells were seeded into two cell culture flasks at a density of 5  $\times$  10<sup>4</sup> cells/flask, and 72 h after shilajit treatment (IC<sub>50</sub> 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 cytometry (BD Biosciences Co.). Unstained cells were used to eliminate autofluorescence. Annexin V<sup>+</sup> and PI<sup>-</sup> cells were regarded as early apoptotic cells, and cells with positive staining both for Annexin V and PI (Annexin V<sup>+</sup>/PI<sup>+</sup>) 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  $\times$  Master Mix Green with high ROX<sup>TM</sup> (Ampliqon Denmark Company), 1  $\mu$ L of the cDNA product (50 ng), 0.8  $\mu$ L of each of the primers (0.2  $\mu$ M), and 2.9  $\mu$ L of nuclease-free water (total volume of 12  $\mu$ L). 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  $\pm$  standard error of the mean (mean  $\pm$  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  $\leq 0.05$  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 ( $IC_{50}$ ) 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  $\mu\text{g/mL}$  of shilajit, respectively ( $IC_{50} = 280 \mu\text{g/mL}$ ). 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  $IC_{50}$  concentration significantly ( $P \leq 0.01$ ) increased Annexin V<sup>+</sup>/PI<sup>-</sup> and Annexin V<sup>+</sup>/PI<sup>+</sup> 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- $\kappa$ B-related genes

Based on the results, the expression levels of the studied genes were significantly downregulated in 280  $\mu\text{g/mL}$ -treated cells compared with the control cells (Figure 3). The expression level of p50, RelB, IKK $\alpha$ , and IKK $\beta$  genes decreased to  $0.31 \pm 0.03$ ,  $0.22 \pm 0.05$ ,  $0.058 \pm 0.006$ , and  $0.04 \pm 0.002$  respectively. Therefore, the IKK $\beta$  and p50 genes had the highest and lowest expression reduction, respectively, in the treatment.

### Discussion

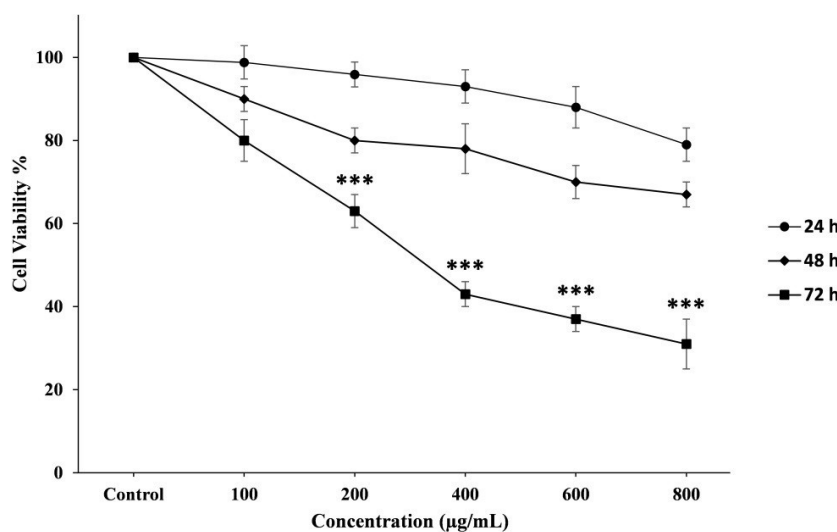
Identifying safe and effective compounds is a necessity in cancer drug development. Shilajit, a traditional natural medication, has prevailed for years as a rejuvenating mineral substance for treating several conditions (10). Based on the reports of previous studies, shilajit can induce apoptosis and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

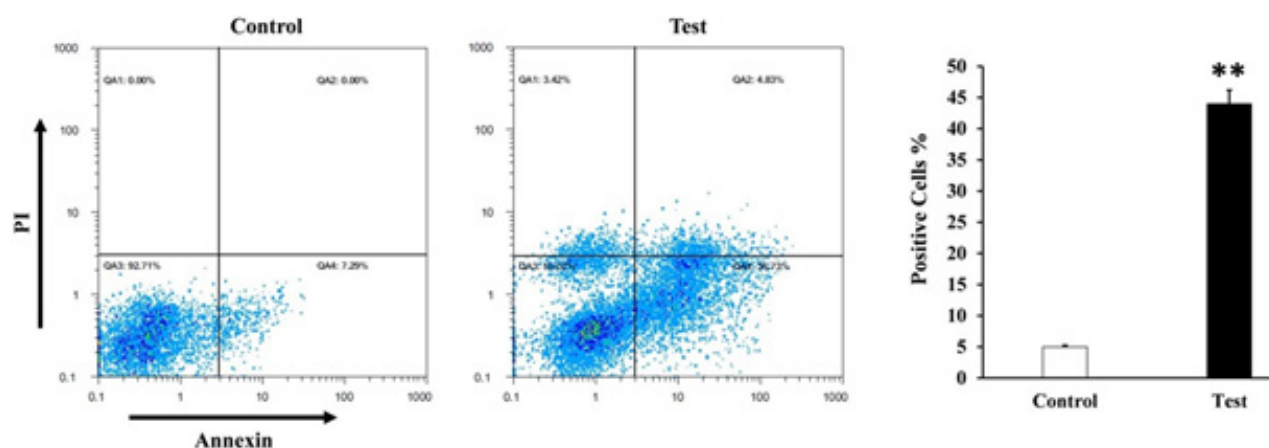
**Table 1.** The used oligonucleotide primers

Primers	Sequences (5'-3')
p50.F	AAAGAGCTAATCCGCAAGCA
p50.R	AGCTGTAAAACATGAGCCGCAC
RelB.F	TGTGGTGAGGATCTGCTCCAG
RelB.R	TCGGCAAATCCGCAGCTCTGAT
IKK $\alpha$ .F	ACAGAGTTCGCCCGGTCCCT
IKK $\alpha$ .R	CTGCTGAAGTCGGGGGCGAC
IKK $\beta$ .F	CGCCCAATGACCTGCCCTG
IKK $\beta$ .R	GGCACCTCCCGCAGACCAC
GAPDH.F	CCCTCTGGAAGCTGTGG
GAPDH.R	AGTGGATGCAGGGATGATG

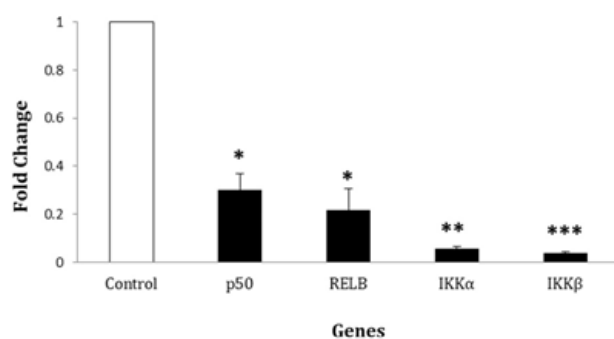
F: forward sequence; R: reverse sequence.



**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 ( $IC_{50} = 280 \mu\text{g/mL}$ ). Data are given as the mean  $\pm$  SEM of the three independent examinations. \*\*\* $P \leq 0.001$



**Figure 2.** Apoptotic effect of shilajit on MCF-7 cells. The MCF-7 cells were exposed to  $IC_{50}$  concentration of shilajit (280  $\mu\text{g}/\text{mL}$ ) for 72 h, and then the level of Annexin V binding and PI uptake were investigated using a flow cytometer. Approximately 43% increase ( $P \leq 0.01$ ) in Annexin V<sup>+</sup>/PI<sup>+</sup> and Annexin V<sup>+</sup>/PI<sup>-</sup> cells was observed after treatment. One representative analysis of the three independent analyses ( $n=3$ ) is shown. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$



**Figure 3.** Analyzing gene expression using the qRT-PCR technique. The mRNA expression levels of Nfκ (p50 and RelB, \* $P < 0.05$  in both) and IKK (IKK $\alpha$ , \*\* $P < 0.01$ ; IKK $\beta$ , \*\*\* $P < 0.001$ ) were lower in the treatment group compared with the control group

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- $\alpha$  expression in U937 cells. Fulvic acid causes a reduction of cyclooxygenase 2 and prostaglandin  $E_2$  secretion in monocytes (26). Besides the activities above, the immune-boosting and -modulating and antioxidant properties of fulvic and humic acids have been evidenced in various studies (22).

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  $IC_{50}$  concentration was 280  $\mu\text{g}/\text{mL}$ . 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  $IC_{50}$  dose of shilajit in Pant's study ( $\sim 100$   $\mu\text{g}/\text{mL}$ ) and in our experiment ( $\sim 280$   $\mu\text{g}/\text{mL}$ ), it seems that Huh-7 cells are more shilajit-sensitive than MCF-7 cells.

Exposure to  $IC_{50}$  concentration caused significant apoptosis ( $P \leq 0.01$ ) 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- $\kappa\text{B}$  signaling pathway-related genes following treatment. Based on the results (Figure 3), we found that the proposed mechanism suppresses NF- $\kappa\text{B}$  activation (down-regulation of p50, RelB, IKK $\alpha$ , and IKK $\beta$  genes). Transcription factor NF- $\kappa\text{B}$  was identified in 1986 as a nuclear factor that binds to the enhancer element of activated B cells immunoglobulin kappa light chain (NF- $\kappa\text{B}$ ). The five members of the NF- $\kappa\text{B}$  family of proteins are RelA (p65), RelB, c-Rel, NF- $\kappa\text{B}1$  (p105), and NF- $\kappa\text{B}2$  (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 I $\kappa\text{B}$  kinase (IKK) complex, comprised of the regulatory subunits of IKK $\alpha$  and IKK $\beta$  catalytic subunits, is activated (17). This activated complex phosphorylates I $\kappa\text{B}$ , which then undergoes proteasomal-mediated degradation and releases NF- $\kappa\text{B}$  dimers that travel to the nucleus and bind to DNA at NF- $\kappa\text{B}$  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 IKKA, which does not play a critical role in NF- $\kappa$ B activation by pro-inflammatory cytokines, the disruption of the IKKB gene corroborates the essential function of IKKB in NF- $\kappa$ B activation (30).

In both direct and indirect manner (4), NF- $\kappa$ B, 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 IKK $\alpha$ , the prominent function of IKK $\beta$  in NF- $\kappa$ B activation is well-known (33). Interestingly, we hinted at a significant reduction of IKK $\beta$  gene expression in the shilajit-treated cells here. Our findings reveal that shilajit is a potential tumor-suppressor compound inhibiting IKK-driven NF- $\kappa$ B 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:** Arezoo Saberi, Reza Vahidi.

**Supervision:** Seyede Elmeira Yazdi Rouholamini, Zeinab Kordestani.

**Writing—original draft:** Arezoo Saberi.

**Writing—review & editing:** Reza Vahid.

### 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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