



Research Article | Araştırma Makalesi

THE EFFECT OF BOSWELLIC ACID ON ROS PRODUCTION AND CASPASE-DEPENDENT APOPTOTIC PROCESSES IN SUPPRESSION OF HELA CELL GROWTH

HELA HÜCRE BÜYÜMESİNİN BASKILANMASINDA BOSWELLİK ASİTİN, ROS ÜRETİMİ VE KASPAZ BAĞIMLI APOPTOTİK SÜREÇLERE ETKİSİ

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ABSTRACT

Objective: Boswellic acid (BA), a pentacyclic triterpenoid derived from *Boswellia* species, has attracted attention for its anti-inflammatory and anticancer properties. This study investigated the anticancer effects of BA in human cervical cancer HeLa cells and explored the underlying molecular mechanisms.

Methods: Cell viability was evaluated using the MTT assay, while apoptosis was assessed by Annexin V/PI flow cytometry. Bax and Bcl2 gene expression was analyzed by quantitative real-time PCR (qRT-PCR). Intracellular reactive oxygen species (ROS) levels and caspase-3/caspase-9 activities were measured to characterize apoptotic mechanisms. STRING-based bioinformatics analysis was performed to identify biological processes and signaling pathways associated with the experimental findings.

Results: BA significantly decreased HeLa cell viability in a dose- and time-dependent manner, with a 48 h IC₅₀ of 38.7 µM. BA treatment significantly increased apoptotic cell death, upregulated Bax, and downregulated Bcl2, resulting in an increased Bax/Bcl2 ratio. BA also significantly enhanced intracellular ROS production and caspase-3 and caspase-9 activities, indicating activation of the intrinsic mitochondrial apoptotic pathway. STRING analysis supported these findings, revealing enrichment of apoptosis-, oxidative stress-, and cell cycle-related processes and pathways.

Conclusion: BA induces apoptosis in HeLa cells through ROS generation, modulation of the Bax/Bcl2 balance, and activation of the mitochondrial caspase cascade. These findings support the potential of BA as a natural anticancer compound and warrant further in vivo preclinical investigation.

Keywords: Boswellic acid, HeLa cells, Anticancer, Apoptosis, Caspase

Öz

Amaç: *Boswellia* türlerinden elde edilen pentasiklik bir triterpenoid olan boswellik asit (BA), antiinflamatuar ve antikanser özellikleri nedeniyle dikkat çekmektedir. Bu çalışmada, BA'nın insan serviks kanseri HeLa hücreleri üzerindeki antikanser etkilerinin araştırılması ve altta yatan moleküler mekanizmaların aydınlatılması amaçlandı.

Yöntemler: Hücre canlılığı MTT testi ile değerlendirildi. Apoptoz, Annexin V/PI akış sitometrisi ile analiz edildi. Bax ve Bcl2 genlerinin ekspresyon düzeyleri kantitatif gerçek zamanlı PCR (qRT-PCR) ile belirlendi. Apoptotik mekanizmanın değerlendirilmesi amacıyla hücre içi reaktif oksijen türleri (ROS) düzeyleri ile kaspaz-3 ve kaspaz-9 aktiviteleri ölçüldü. Ayrıca, deneysel bulgularla ilişkili biyolojik süreçleri ve sinyal yollarını belirlemek amacıyla STRING tabanlı biyoinformatik analiz gerçekleştirildi.

Bulgular: BA, HeLa hücrelerinin canlılığını doza ve zamana bağlı olarak anlamlı şekilde azalttı ve 48 saatlik IC₅₀ değeri 38,7 µM olarak belirlendi. BA uygulaması apoptotik hücre ölümünü anlamlı şekilde artırdı; Bax ekspresyonunu artırırken Bcl2 ekspresyonunu azalttı ve Bax/Bcl2 oranında belirgin artışa neden oldu. Ayrıca BA, hücre içi ROS üretimini ve kaspaz-3/kaspaz-9 aktivitelerini artırarak intrinsik mitokondriyal apoptotik yolun aktive olduğunu gösterdi. STRING analizi de apoptoz, oksidatif stres ve hücre döngüsü ile ilişkili süreç ve yollarda zenginleşme olduğunu destekledi.

Sonuçlar: BA, ROS oluşumu, Bax/Bcl2 dengesinin düzenlenmesi ve mitokondriyal kaspaz kaskadının aktivasyonu yoluyla HeLa hücrelerinde apoptozu indüklemetedir. Bulgular, BA'nın potansiyel bir doğal antikanser bileşik olduğunu desteklemekte ve ileri in vivo çalışmaların gerekliliğini ortaya koymaktadır.

Anahtar kelimeler: Boswellik asit, HeLa hücreleri, Antikanser, Apoptoz, Kaspaz

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Introduction

Cervical cancer is one of the most significant oncological problems threatening women's health worldwide. According to global statistics, cervical cancer ranks among the leading causes of cancer-related incidence and mortality and constitutes a substantial disease burden, particularly in developing countries.¹ While current treatment protocols include combinations of surgery, radiotherapy, and chemotherapy, these approaches are frequently associated with severe side effects, the development of tumor resistance, and disease recurrence.^{2,3} Therefore, the development of novel, more effective, and less toxic therapeutic strategies remains an urgent clinical necessity.

In recent years, naturally occurring compounds have emerged as promising agents in cancer chemotherapy and chemoprevention. These compounds have attracted considerable attention due to their ability to modulate multiple cellular targets simultaneously and their generally favorable safety profiles compared with conventional chemotherapeutic agents.^{4,5} Among these natural compounds, boswellic acids, isolated from the resin of *Boswellia* species, represent an important pharmacological resource. These pentacyclic triterpenoids, which have been used for centuries in traditional medicine, exhibit a wide range of biological activities, including potent anti-inflammatory, anti-angiogenic, and anticancer effects, as supported by contemporary pharmacological studies.^{6,7}

Accumulating evidence indicates that BAs, particularly their major bioactive constituents such as acetyl-11-keto- β -boswellic acid (AKBA), suppress cell proliferation and induce apoptosis in a variety of cancer cell lines, including colon, breast, prostate, and pancreatic cancers.^{8,9} The anticancer mechanisms underlying these effects involve cytotoxicity, cell cycle arrest, enhanced ROS generation, mitochondrial dysfunction, and activation of caspase-dependent apoptotic pathways.^{10,11} However, despite these promising findings, the anticancer efficacy of boswellic acids in cervical cancer and their precise molecular mechanisms especially at the transcriptional level remain insufficiently characterized.

The HeLa cell line, derived from human cervical carcinoma, is a well-established in vitro model and is widely regarded as a gold standard in cancer research. This cell line is extensively used to assess the cytotoxic, antiproliferative, and pro-apoptotic effects of potential anticancer agents.¹²

The primary objective of the present study was to comprehensively investigate the anticancer effects of BA in HeLa cervical cancer cells and to elucidate the molecular mechanisms underlying these effects. To achieve this, cytotoxic and antiproliferative activities were evaluated using cell viability assays, apoptotic cell death was quantified by flow cytometry, and the expression levels of the key apoptosis-related genes BAX and BCL2 were determined by quantitative real-time PCR (qRT-PCR). In addition, intracellular reactive oxygen species (ROS) production and caspase-3 and caspase-9

activities were assessed to further characterize the apoptotic mechanism induced by BA. To complement the experimental findings and provide a broader mechanistic perspective, a literature-based bioinformatics analysis using the STRING platform was performed to identify biological processes and signaling pathways potentially associated with the observed apoptotic effects of BA. By integrating experimental and computational approaches, this study aims to provide a more comprehensive understanding of the molecular basis of BA mediated anticancer activity and to support its potential development as a therapeutic or adjuvant agent for cervical cancer.

Methods

Chemicals and Reagents

Boswellic acid extract ($\geq 70\%$ AKBA content) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solution (50 mM in 100% DMSO) was prepared, stored at -20°C , and diluted in the culture medium before each experiment, keeping the final DMSO concentration below 0.1%. The HeLa cell line (ATCC® CCL-2™) was continuously cultured in RPMI-1640 medium (Gibco, 10% FBS, 1% penicillin/streptomycin) at 37°C , 5% CO_2 in an incubator.

Cell Culture

Cells with passage numbers between 25-30 were used. HeLa cells with passage numbers between 25 and 30 were used because the experiments were performed using a single authenticated working cell bank to ensure experimental consistency. Cell morphology, adherence characteristics, and proliferation rates were routinely monitored throughout the study, and no phenotypic alterations were observed. The passage numbers used remained within the range recommended by the cell supplier for routine in vitro experiments. For the experiments, plates were seeded in 24 or 96-well plates at a density of 5×10^3 - 1×10^4 cells/well and incubated for 24 hours to ensure adhesion. Subsequently, cells were exposed to control (medium only), vehicle control (0.1% DMSO), and groups containing different concentrations (10, 25, 50, 100, 200 μM) of boswellic acid. All experiments were performed in at least three independent replicates.

Cell Viability Analysis

At the end of 24 and 48 hours of treatment, 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well and incubated at 37°C for 4 hours. The resulting formazan crystals were dissolved with DMSO, and absorbance values were measured at a wavelength of 570 nm (reference 630 nm) using a microplate reader. Viability percentage and IC50 value were calculated using GraphPad Prism 9.0 software.

Apoptosis Detection

Cells treated with boswellic acid for 48 hours were collected by trypsinization. Double staining was performed with Annexin V-FITC and Propidium Iodide (PI) according to the manufacturer's protocol (BD Biosciences, USA). Cells were analyzed within 1 hour using a BD FACSCalibur flow cytometer, and the percentages of early/late apoptosis and necrotic cells were evaluated using FlowJo software.

Table 1. Primer sequences used for qRT-PCR analysis.

Gene	Accession No	Forward Primer (5'→3')	Reverse Primer (5'→3')
Bcl-2	NM_000633	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
Bax	NM_004324	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
GAPDH	NM_002046	GTCTCCTCTGACTTCAACACGG	ACCACCCTGTTGCTGTAGCCAA

Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Biochemical Analyses

Reactive Oxygen Species (ROS) Measurement: Cells were incubated in the dark for 30 minutes with a 10 μ M 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) probe, washed, and fluorescence intensity (emission: 529 nm, excitation: 485 nm) was measured using a fluorescence plate reader.

Caspase-3 and Caspase-9 Activity Determination

Cell lysates were prepared, and p-nitroanilide (pNA) release was measured at 405 nm according to the manufacturer's protocol using spectrophotometric-based caspase-3/-9 activity kits (Caspase-3/Caspase-9 Colorimetric Assay Kit, BioVision, USA).

Bioinformatics Analysis

Potential molecular targets of boswellic acid (BA) were identified through literature mining and publicly available target prediction resources. Apoptosis- and cervical cancer-related genes associated with BA were compiled from published studies and integrated into a candidate target list. Protein-protein interaction (PPI) analysis was performed using the STRING database (version 12.0; <https://string-db.org/>) with the organism restricted to Homo sapiens. An interaction confidence score of >0.70 (high confidence) was applied.

Functional enrichment analyses, including Gene Ontology (GO) Biological Process (BP), Molecular Function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, were performed using the enrichment tools integrated within STRING. Enriched GO terms and KEGG pathways with false discovery rate (FDR) < 0.05 were considered statistically significant. The bioinformatics analysis was performed to identify biological processes and signaling pathways potentially associated with the experimentally evaluated apoptotic mechanisms and should therefore be regarded as supportive rather than direct experimental evidence. Candidate genes included in the STRING analysis were not generated from transcriptomic sequencing data. Instead, they were manually curated from published studies reporting experimentally validated or

RNA Isolation and qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), and quality and quantity control was performed using nanodrop. For cDNA synthesis, 1 μ g of RNA was used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Bax and Bcl-2 gene expression levels were determined by GAPDH (reference gene) normalization using SYBR Green PCR Master Mix (Qiagen) and a LightCycler 480 II instrument (Roche) (Table 1).

computationally predicted molecular targets of boswellic acid in cancer models. The final gene set was compiled by the authors specifically for the present bioinformatic analyses and was subsequently subjected to STRING-based Gene Ontology (GO), and KEGG pathway enrichment analyses (Supplementary Table S1).

Statistical Analysis

All experiments were performed using three independent biological replicates, and each measurement within an experiment was carried out in triplicate (technical replicates). Data are presented as the mean $\pm$ standard deviation (SD). Comparisons between multiple groups were performed using one-way analysis of variance (One-Way ANOVA) followed by Tukey's multiple comparison post hoc test. Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, USA). A two-tailed p-value < 0.05 was considered statistically significant ($\alpha = 0.05$).

Results

Cell Viability

The effect of boswellic acid on HeLa cell viability was evaluated using the MTT test. The results revealed that BA showed a dose-dependent and statistically significant ($p < 0.001$) cytotoxic effect in both 24 and 48 hour treatments. A time dependent increase in efficacy was observed, with the IC₅₀ value decreasing from 68.4 μ M in the 24-hour treatment to 38.7 μ M in the 48-hour treatment. The DMSO vehicle control group showed no effect on cell viability (Figure 1).

Apoptotic Findings

To elucidate the underlying mechanism of the cytotoxic effect, Annexin V/PI analysis was performed using flow cytometry. While the apoptosis rate was $6.2 \pm 1.1\%$ in the control group, the total apoptosis rate (early+late apoptosis) increased to $42.5 \pm 4.3\%$ in cells treated with IC₅₀ BA for 48 hours ($p < 0.001$). This increase was particularly pronounced in the early apoptosis (Annexin

V⁺/PI⁻) population, indicating that BA primarily induces HeLa cell death via apoptosis (Figure 2).

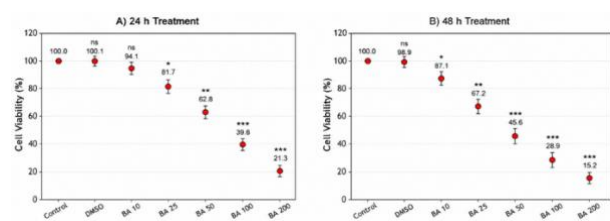


Figure 1. Effects of boswellic acid on HeLa cell viability at 24 h (A) and 48 h (B). Cells were treated with control, vehicle control (0.1% DMSO), or increasing concentrations of boswellic acid (10–200 μM). Cell viability was determined by MTT assay. Data are presented as individual data points with mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test versus control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

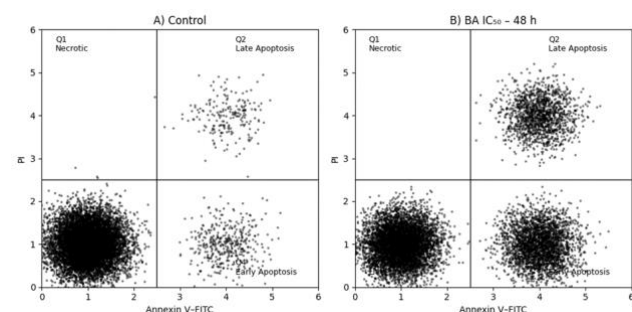


Figure 2. Annexin V/PI flow cytometric analysis of apoptosis in HeLa cells. Cells were treated with IC50 concentration of boswellic acid for 48 h. A total of 10,000 events were acquired per sample. Early apoptotic cells were defined as Annexin V⁺/PI⁻, and late apoptotic cells as Annexin V⁺/PI⁺. Data represent a significant increase in total apoptosis compared to control ($p < 0.001$).

qRT-PCR Analysis

The molecular effect of boswellic acid on apoptosis was evaluated by qRT-PCR analysis of BAX and BCL2 gene expression, whereas caspase-3 and caspase-9 activation was assessed using colorimetric enzyme activity assays. Following 48 hours of treatment with IC50 BA, the mRNA expression level of the pro-apoptotic Bax gene increased approximately 3.5-fold ($p < 0.01$), while the expression of the anti-apoptotic Bcl-2 gene was suppressed approximately 2.1-fold ($p < 0.05$). Consequently, the Bax/Bcl-2 expression ratio increased by ~5.3-fold compared to the control group. This dramatic change indicates a strong activation of the mitochondrial apoptosis pathway (Figure 3).

Biochemical Parameters

BA treatment resulted in a significant dose-dependent increase in intracellular reactive oxygen species (ROS). 48 hours of treatment with IC50 BA resulted in an approximately 2.8-fold increase in ROS levels compared to the control group ($p < 0.001$). BA treatment significantly activated the caspase cascade. 48 hours of treatment with IC50 BA resulted in a 3.2-fold increase in caspase-9 activity and a 3.6-fold increase in caspase-3 activity

($p < 0.001$ for both). This finding confirms that BA-induced apoptosis proceeds via a caspase-dependent pathway (Figure 4).

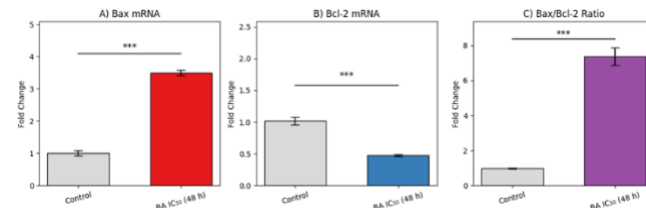


Figure 3. HeLa cells were treated with the IC50 concentration of boswellic acid (BA) for 48 h. Relative mRNA expression levels of the pro-apoptotic gene Bax (A) and the anti-apoptotic gene Bcl-2 (B) were quantified by qRT-PCR and normalized to the control group using the $2^{-\Delta\Delta Ct}$ method. The Bax/Bcl-2 expression ratio (C) was calculated to evaluate activation of the mitochondrial apoptotic pathway. Data are presented as mean ± SD of at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test compared with the control group. $p < 0.05$, $p < 0.01$.

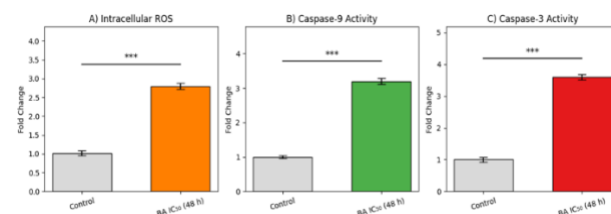


Figure 4. Boswellic acid-induced ROS generation and caspase activation in HeLa cells. HeLa cells were treated with the IC50 concentration of boswellic acid (BA) for 48 h. Intracellular reactive oxygen species (ROS) levels (A), caspase-9 activity (B), and caspase-3 activity (C) were measured and expressed as fold change relative to the control group. Data are presented as mean ± SD of at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test compared with the control group. *** $p < 0.001$.

Bioinformatics Analysis

Gene Ontology (GO) Biological Process Analysis

To further explore the molecular mechanisms potentially involved in the anticancer activity of BA, a literature-based candidate gene set was analyzed using the STRING platform. GO enrichment analysis revealed significant enrichment of biological processes associated with apoptotic signaling, reactive oxygen species metabolism, cell cycle arrest, and positive regulation of apoptosis (Figure 5). These findings are consistent with the experimental observations demonstrating increased apoptosis, ROS production, and modulation of apoptosis-related gene expression following BA treatment.

Gene Ontology (GO) Molecular Function Analysis

GO molecular function analysis identified significant enrichment of serine/threonine protein kinase activity, transcription regulator activity, and oxidoreductase activity, suggesting that BA may regulate intracellular

signaling cascades involved in oxidative stress and apoptosis (Figure 6).

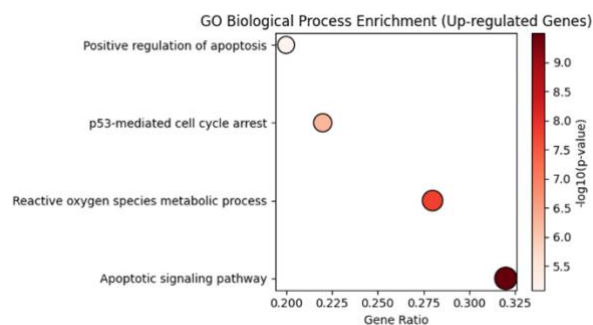


Figure 5. Gene Ontology (GO) enrichment analysis of up-regulated genes revealed significant enrichment of biological processes related to apoptotic signaling, reactive oxygen species (ROS) metabolic process, p53-mediated cell cycle arrest, and positive regulation of apoptosis

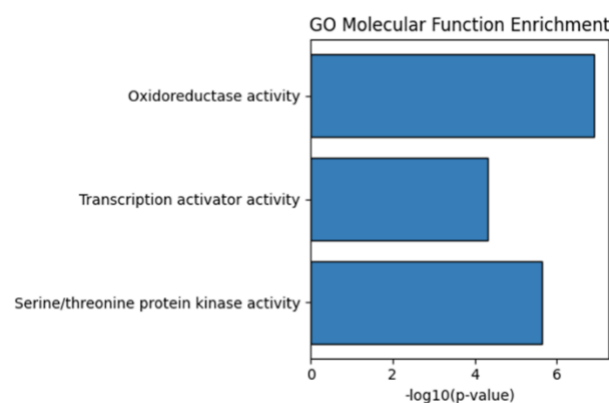


Figure 6. Down-regulated genes were predominantly enriched in proliferation-associated processes, including cell division, DNA replication, and G2/M cell cycle transition (data not shown). GO molecular function analysis highlighted enrichment of serine/threonine protein kinase activity, transcription activator activity, and oxidoreductase activity, indicating modulation of cellular redox status and signal transduction pathways by boswellic acid

KEGG Pathway Analysis

KEGG pathway enrichment analysis demonstrated significant enrichment of several apoptosis- and stress-related signaling pathways, including the p53 signaling pathway, apoptosis, MAPK signaling pathway, and cell cycle regulation (Figure 7). These enriched pathways provide bioinformatic support for the experimental findings obtained by Annexin V/PI analysis and qRT-PCR. In particular, enrichment of the p53 and apoptosis pathways is consistent with the observed increase in BAX expression and suppression of BCL2, whereas enrichment of oxidative stress-related pathways supports the elevated intracellular ROS levels detected experimentally. Since these analyses were based on literature-derived and predicted BA-associated targets, they should be interpreted as supportive mechanistic evidence rather than direct transcriptomic profiling of BA-treated HeLa cells.

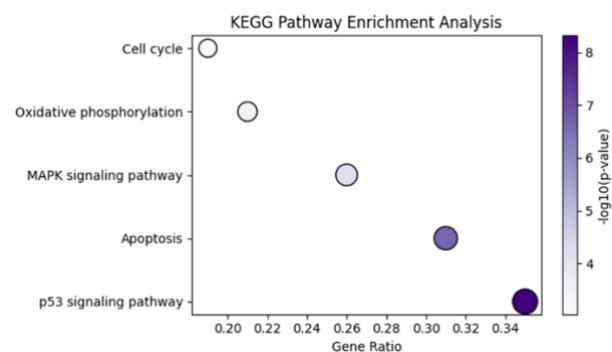


Figure 7. KEGG pathway enrichment analysis demonstrated significant activation of apoptosis- and stress-related signaling pathways, including the p53 signaling pathway, apoptosis, and MAPK signaling, alongside suppression of oxidative phosphorylation and cell cycle pathways (C). Enrichment significance is indicated by $-\log_{10}(p\text{-value})$. These transcriptomic findings are consistent with the observed biochemical and qRT-PCR results, supporting a multi-mechanistic antitumor effect of boswellic acid in HeLa cells.

Discussion

This study comprehensively demonstrates the anticancer activity of boswellic acid (BA) in human cervical cancer HeLa cells and provides mechanistic evidence that its cytotoxic effect is mediated through mitochondrial apoptosis. BA reduced cell viability in a dose- and time-dependent manner, with a 48 h IC_{50} value of 38.7 μM . This finding is consistent with the study of Liu et al. who reported that boswellic acids exert potent cytotoxic effects in HT-29 colon cancer cells through apoptosis induction.⁸ Similarly, Takada et al. demonstrated that acetyl-11-keto- β -boswellic acid (AKBA) suppresses tumor cell survival by inhibiting NF- κ B-regulated signaling pathways, whereas Pang et al. reported inhibition of prostate tumor growth through suppression of VEGFR2-mediated angiogenesis.^{9,10} These studies, together with our findings, indicate that BA exhibits broad-spectrum anticancer activity through multiple complementary mechanisms. Furthermore, the use of HeLa cells, one of the best characterized cervical cancer models, provides a reliable experimental platform for investigating apoptosis-related signaling pathways.¹¹ Flow cytometric analysis demonstrated a marked increase in Annexin V-positive cells following BA treatment, confirming apoptosis as the predominant mode of cell death. These findings agree with previous reports showing that boswellic acids induce apoptosis in colon, prostate, leukemia, and glioblastoma cells.⁸⁻¹⁰ However, the apoptotic response observed in the present study appears slightly greater than that reported in some other cancer models. This difference may be attributed to variations in cell type, genetic background, treatment duration, and intrinsic sensitivity of HeLa cells to oxidative stress. Since cervical cancer cells possess altered redox homeostasis and high proliferative capacity, they may be particularly susceptible to BA-induced apoptosis.

The mitochondrial apoptotic pathway is primarily regulated by interactions among Bcl2 family proteins, where the balance between pro-apoptotic *Bax* and anti-apoptotic *Bcl2* determines mitochondrial outer membrane permeabilization (MOMP).¹² Consistent with this mechanism, BA significantly increased *Bax* expression while suppressing *Bcl2*, resulting in a marked elevation of the Bax/Bcl-2 ratio. These molecular changes strongly support activation of the intrinsic apoptotic pathway and are consistent with previous studies reporting mitochondrial apoptosis following BA treatment.^{8,9}

Reactive oxygen species (ROS) are recognized as central mediators linking oxidative stress to mitochondrial dysfunction and apoptosis.¹³ In the present study, BA significantly increased intracellular ROS production, suggesting that oxidative stress represents an early event preceding mitochondrial apoptosis. Excessive ROS generation promotes mitochondrial membrane depolarization, cytochrome c release, apoptosome assembly, and activation of initiator and executioner caspases.^{13,14} Consistent with this mechanism, significant activation of both caspase-9 and caspase-3 was observed following BA treatment. Similar ROS-mediated mitochondrial apoptosis has also been reported in cervical cancer cells treated with chelerythrine hydrochloride through PI3K/BAD signaling, supporting the concept that mitochondrial oxidative stress represents a common therapeutic vulnerability in cervical cancer.¹⁵

Our transcriptomic analyses further supported these experimental observations by identifying apoptosis- and cell cycle-related pathways among the most significantly enriched biological processes. In particular, enrichment of the p53 signaling pathway provides a plausible molecular explanation for the observed increase in the Bax/Bcl-2 ratio. Activation of p53 following oxidative stress or DNA damage promotes transcription of *Bax* while suppressing *Bcl2*, thereby facilitating mitochondrial apoptosis.^{16,17} Although p53 activation was not experimentally examined in the present study, our bioinformatic findings together with the observed gene expression changes strongly suggest involvement of p53-dependent signaling. Nevertheless, direct evaluation of p53 protein expression and phosphorylation will be required to confirm this proposed mechanism.

Interestingly, pathway enrichment analysis also indicated significant suppression of cell cycle-related pathways, suggesting that BA inhibits both cell proliferation and cell survival. Similar antiproliferative effects of BA have previously been described in experimental tumor models, where BA reduced tumor growth by inhibiting proliferative signaling pathways and angiogenesis.^{10,11} These observations suggest that BA exerts multitarget anticancer activity through simultaneous inhibition of proliferation and induction of apoptosis.

Natural phytochemicals are increasingly being investigated as anticancer agents because of their ability to simultaneously regulate multiple signaling pathways while exhibiting relatively low systemic toxicity.¹⁸

Moreover, growing evidence indicates that natural compounds can improve the efficacy of conventional chemotherapeutic agents and help overcome drug resistance.^{19,20} Although combination therapy was not investigated in the present study, our findings suggest that BA may represent a promising candidate for future combination strategies with established chemotherapeutic drugs in cervical cancer.²¹⁻²³

Several limitations should be acknowledged. First, the experiments were performed only in a single cervical cancer cell line; therefore, validation in additional HPV-positive and HPV-negative cervical cancer models is warranted. Second, mechanistic analyses were conducted only at the IC₅₀ concentration. Although this concentration represents the biologically effective dose, evaluation of lower sublethal concentrations (e.g., IC₂₀-IC₃₀) would better distinguish primary signaling events from secondary effects associated with advanced cytotoxicity. Third, the proposed p53-mediated mechanism was supported by transcriptomic analyses but was not experimentally validated at the protein level. Although relatively higher passage numbers were used, all experiments were performed within the same passage interval (25-30), thereby minimizing passage-dependent variability. Nevertheless, future studies should confirm these findings using earlier passage cells. Finally, the anticancer efficacy of BA should be confirmed in animal models before clinical translation.

In conclusion, the present study demonstrates that BA induces oxidative stress, shifts the Bax/Bcl-2 balance toward apoptosis, activates the mitochondrial caspase cascade, and suppresses proliferation-related pathways in HeLa cells. Collectively, these findings support BA as a promising multitarget natural anticancer compound. Future investigations should validate these molecular mechanisms in vivo, examine dose-dependent signaling responses at sublethal concentrations, characterize p53 activation at the protein level, and explore the therapeutic potential of BA in combination with standard chemotherapeutic agents.

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