

Antineoplastic Effects of Ankaferd Hemostat on Hep-2 Laryngeal Cancer Cells: Involvement of Apoptosis and PI3K/Akt/mTOR Signaling

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ABSTRACT

To investigate the cellular and molecular effects of Ankaferd Hemostat on human laryngeal squamous carcinoma Hep-2 cells, with particular emphasis on cell viability, apoptosis, oxidative stress, cell cycle progression, migration, invasion, and PI3K/Akt/mTOR signaling.

Hep-2 cells were treated with increasing concentrations of Ankaferd Hemostat (50-1600 nL/mL) for 24, 48, and 72 h. Cell viability, clonogenic survival, apoptosis, cell cycle distribution, migration, invasion, nuclear morphology, DNA fragmentation, intracellular reactive oxygen species (ROS) production, and expression of apoptosis- and signaling-related proteins were evaluated using established in vitro assays.

Ankaferd Hemostat significantly reduced Hep-2 cell viability and clonogenic survival in a dose- and time-dependent manner. It induced apoptosis, as demonstrated by Annexin V/PI staining, nuclear fragmentation, DNA laddering, and activation of caspase-3 and PARP. Treatment also resulted in G2/M phase cell cycle arrest and increased intracellular reactive oxygen species (ROS) levels. In addition, Ankaferd Hemostat inhibited cell migration and invasion and modulated metastasis-associated proteins by downregulating MMP-2, MMP-9, and VEGF, while upregulating E-cadherin. At the signaling level, Ankaferd Hemostat suppressed the activation of the PI3K/Akt/mTOR pathway.

These findings provide preclinical in vitro evidence that Ankaferd Hemostat exerts multifaceted antineoplastic effects on Hep-2 laryngeal cancer cells by modulating apoptosis, oxidative stress, cell cycle progression, migratory and invasive behavior, and intracellular survival signaling. Validation in additional laryngeal cancer models and in vivo systems is required before the translational relevance can be established.

Keywords: Ankaferd Hemostat, Hep-2 cells, Laryngeal squamous cell carcinoma, Apoptosis, PI3K/Akt/mTOR signaling

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INTRODUCTION

Laryngeal cancer is a malignant neoplasm of the head and neck region and is one of the most common malignancies in this anatomical area [1]. It accounts for approximately 2-5% of all malignancies and is the second most frequent head and neck cancer after skin cancer [1,2]. Despite advances in surgery, radiotherapy, chemotherapy, and combination treatment strategies, laryngeal cancer remains associated with substantial morbidity and mortality [3]. Disease recurrence and distant metastasis continue to pose major clinical challenges [3].

Ankaferd Blood Stopper (ABS) is a standardized herbal hemostatic agent that has been widely used for the management of minor and major bleeding. ABS is composed of extracts from *Urtica dioica* (nettle), *Vitis vinifera* (grape), *Glycyrrhiza glabra* (licorice), *Alpinia officinarum* (galangal), and *Thymus vulgaris* (thyme) [4]. Previous studies have demonstrated that these components exert diverse biological effects on endothelial cells, erythrocytes, angiogenesis, inflammation, and cellular proliferation [5,6]. In addition to its hemostatic properties, accumulating evidence indicates that ABS exhibits antineoplastic activity against several solid tumors, including bladder, breast, colon, and bone cancers [4-7]. At the molecular level, ABS regulates multiple transcription factors and signaling pathways involved in cell cycle control, apoptosis, angiogenesis, and immune responses, including AP-2, CREB, p53, SMAD2/3, and PI3K/Akt-related pathways [5-7].

Despite the growing number of studies investigating the antineoplastic properties of Ankaferd Blood Stopper in various malignancies, its effects on laryngeal carcinoma remain unclear [4-7]. To our knowledge, no previous in vitro study has comprehensively characterized the anti-cancer activity of ABS in a laryngeal squamous cell carcinoma model. Previous studies on ABS have primarily focused on growth inhibition, general cytotoxicity, or other tumor types, whereas the present study integrates viability and clonogenic assays with apoptosis, cell cycle analysis, oxidative stress, migration and invasion, metastasis-associated proteins, and PI3K/Akt/mTOR signaling in Hep-2 cells. Therefore, this study aimed to investigate the antineoplastic effects of ABS on Hep-2 laryngeal cancer cells in vitro and provide a pathway-level mechanistic characterization of its cellular effects.

METHODS

Ethical Statement and Cell Line

This study was conducted in accordance with the institutional research policies. Because the study was

performed exclusively using an established in vitro cell line and did not involve human participants or animals, formal ethical committee approval was not required. The human laryngeal squamous carcinoma Hep-2 cell line was obtained from the Cell Culture Collection of the Oral and Dental Diseases Institute Cell Bank (registration no. 92041501).

Cell Line Authentication

The Hep-2 human laryngeal squamous carcinoma cell line was obtained from a certified cell bank and used within six months of receipt. The cell lines were expanded and maintained according to the supplier's recommendations. As the cells were cultured for less than six months after resuscitation, additional re-authentication was not performed. No cross-contamination or morphological abnormalities were observed during the routine cell culture monitoring.

Cell Culture and ABS Treatment

Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin and maintained at 37 °C in a humidified incubator with 5% CO₂. Cells were seeded at a density of 3×10^4 cells/mL for all experiments.

Ankaferd Blood Stopper (ABS) stock solution (10 µL/mL) was prepared in DMEM and further diluted to obtain final concentrations ranging from 50 to 1600 nL/mL. Untreated cells cultured in DMEM were used as the control group. The concentrations selected for downstream mechanistic experiments (400, 800, and 1600 nL/mL) were chosen based on the MTT dose-response profile because they represented a graded range from moderate to pronounced growth inhibition while preserving sufficient viable cells for functional and molecular analyses.

Cell Viability Assay (MTT Assay)

Cell viability was assessed using the MTT assay. Hep-2 cells were seeded into 96-well plates and treated with increasing concentrations of ABS (50-1600 nL/mL). After incubation for 24, 48, and 72 h, 10 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated at 37 °C for an additional 3 h. The culture medium was then removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed. Absorbance was measured at 570 nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as a percentage relative to untreated control cells.

Colony Formation Assay

The clonogenic potential of Hep-2 cells following ABS treatment was evaluated using a colony formation assay. Briefly, 500 cells were seeded into each well of a 6-well plate and allowed to adhere for 24 h. The cells were then treated with ABS at concentrations ranging from 50 to 1600 nL/mL for an additional 24 h. After treatment, the cells were washed with phosphate-buffered saline (PBS) and cultured in fresh medium containing 10% FBS for 8-10 days to allow colony formation.

Colonies were fixed with 100% methanol and stained with crystal violet. Colonies consisting of at least 50 cells were counted under a light microscope (Euromex, Arnhem, Netherlands) and photographed with a digital camera (Nikon, Tokyo, Japan). Colony formation efficiency was calculated relative to the untreated control group.

Apoptosis Assay (Annexin V-FITC/PI)

Apoptosis was quantified using Annexin V-FITC/propidium iodide (PI) double staining, followed by flow cytometry. Hep-2 cells were treated with ABS (400-1600 nL/mL) for 48 h. Both floating and adherent cells were collected, washed with PBS, and resuspended in $1 \times$ binding buffer. The cells were stained with Annexin V-FITC (5 μ L) and PI (5 μ L) and incubated for 15 min at room temperature in the dark. Subsequently, 400 μ L of $1 \times$ binding buffer was added, and the samples were analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, USA). A minimum of 10,000 events was acquired per sample. Data were analyzed using FlowJo software. All experiments were performed in independent replicates.

Cell Migration Assay (Wound Healing Assay)

The migratory capacity of Hep-2 cells following ABS treatment was evaluated using a wound-healing assay. Hep-2 cells were seeded into 6-well plates and allowed to reach approximately 80-90% confluence before treatment. A linear wound was created using a sterile 10 μ L pipette tip, and detached cells were removed by washing with PBS. The cells were then incubated with serum-free DMEM containing ABS at concentrations of 400-1600 nL/mL for 24 h. Images of the wound area were captured at 0 and 24 h using an inverted microscope. Wound width was measured using ImageJ software, and the migration rate was calculated as follows:

Cell Invasion Assay

The invasive potential of Hep-2 cells following ABS treatment was assessed using a Matrigel-coated Transwell

invasion assay. Briefly, 5×10^4 Hep-2 cells were treated with ABS (400-1600 nL/mL) for 24 h. Cells were then resuspended in serum-free DMEM and seeded into the upper chambers of Millicell inserts with an 8 μ m pore size. The lower chambers were filled with culture medium containing 10% FBS as a chemoattractant, and the plates were incubated at 37 °C for 16 h.

After incubation, non-invading cells on the upper surface of the membrane were removed, and the invading cells on the lower surface were fixed with 3.7% formaldehyde, permeabilized with methanol, and stained with 0.5% crystal violet. Invaded cells were counted in five randomly selected microscopic fields under a light microscope, and the average number of invaded cells was calculated per field.

Nuclear Morphology Analysis (DAPI Staining)

Hep-2 cells (3×10^5 cells/well) were seeded onto glass coverslips in 24-well plates. Cells were synchronized in serum-free medium for 24 h and then treated with ABS (400-1600 nL/mL) for 24 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with DAPI. Nuclear morphology was examined using a confocal laser scanning microscope (Zeiss LSM 710; Carl Zeiss).

DNA Fragmentation Assay

Hep-2 cells were treated with different concentrations of ABS (400, 800, and 1600 nL/mL) for 48 h. Genomic DNA was extracted and subjected to electrophoresis on a 2.0% agarose gel containing ethidium bromide (EtBr). DNA fragments were visualized under UV illumination using a gel documentation system.

Cell Cycle Analysis

The cell cycle distribution was analyzed using flow cytometry. Hep-2 cells were treated with ABS (400, 800, and 1600 nL/mL) for 48 h, fixed with 70% cold ethanol, and stored at -20°C . The cells were then washed with PBS, treated with RNase A (100 μ g/mL), and stained with propidium iodide (50 μ g/mL). The cell cycle phases were analyzed using a FACSCalibur flow cytometer.

Intracellular ROS Measurement

Intracellular reactive oxygen species (ROS) levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Hep-2 cells were treated with ABS (400, 800, and 1600 nL/mL) for 22 h, incubated with H₂ DCFDA for 30 min in the dark, and analyzed using flow cytometry. The

fluorescence intensity was recorded and expressed in arbitrary units.

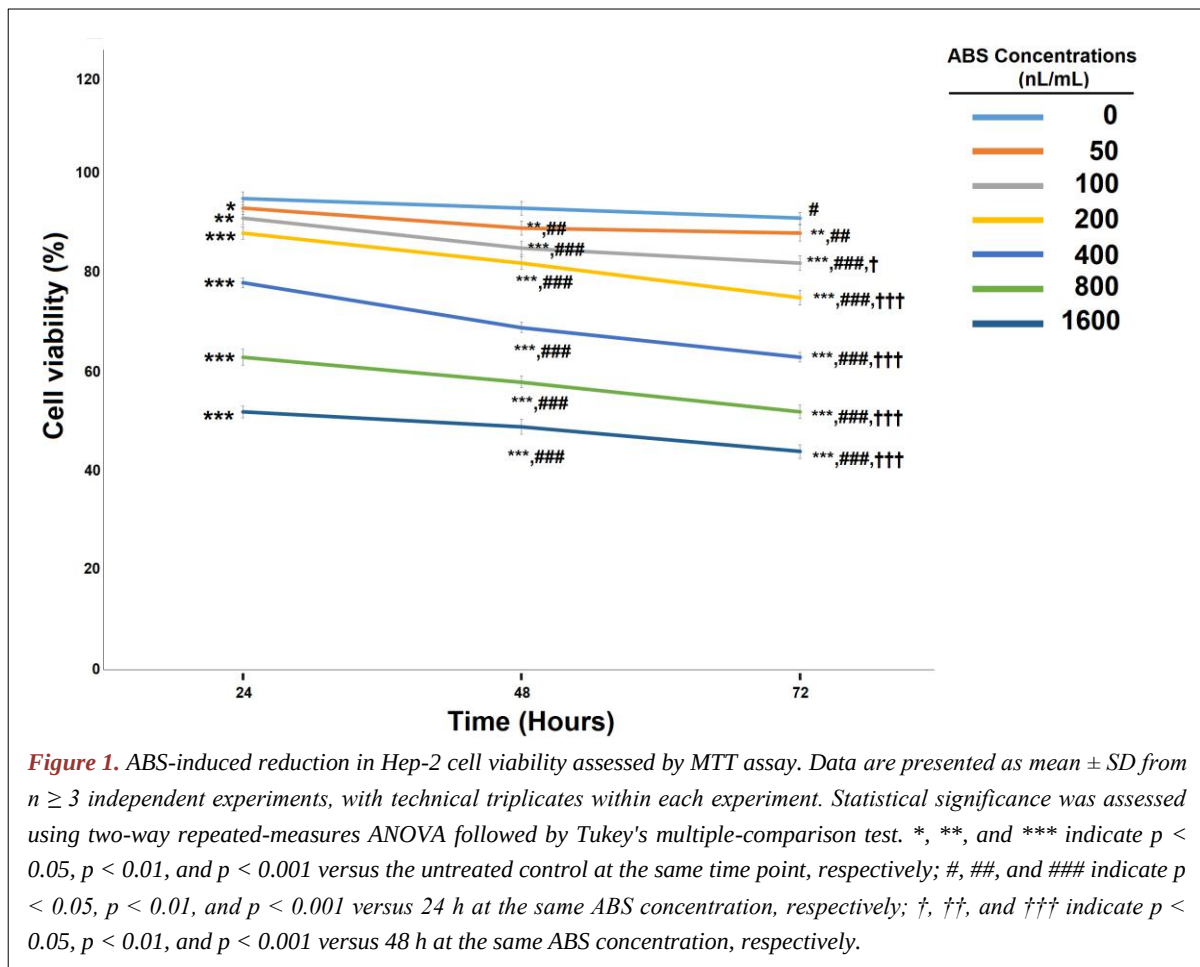
Western Blot Analysis

Total protein was extracted from Hep-2 cells using RIPA buffer, and the protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk. Primary and secondary antibodies used for western blotting were obtained from Santa Cruz Biotechnology (CA, USA), Cell Signaling Technology (MA, USA), and Invitrogen (Carlsbad, CA, USA). Primary antibodies targeted Bax, Bcl-2, cleaved caspase-3, caspase-3, cleaved PARP, PARP, phospho-PI3K, PI3K, phospho-Akt, Akt, phospho-mTOR, mTOR, MMP-2, MMP-9, E-cadherin, VEGF, and β -actin. Following incubation with the appropriate secondary antibodies, protein bands were visualized using a chemiluminescence detection system and quantified by densitometry analysis. Bax, Bcl-2, MMP-2, MMP-9, E-cadherin, and VEGF signals were normalized to β -actin; cleaved caspase-3 and cleaved PARP

were normalized to total caspase-3 and total PARP, respectively; and phospho-PI3K, phospho-Akt, and phospho-mTOR were normalized to their corresponding proteins.

Statistical Analysis

Quantitative assays were repeated independently at least three times ($n \geq 3$), with technical triplicates performed within each experiment, where applicable. The apoptosis assay was performed in five replicate experiments, and the invasion assay was performed in quintuplicates within each experiment. Data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test. For comparisons among multiple treatment groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used after verifying parametric assumptions. The MTT time-course experiment was analyzed using two-way repeated-measures ANOVA to assess the effects of ABS concentration and incubation time, followed by Tukey's multiple-



comparison procedure. All tests were two-sided, and statistical significance was set at $p < 0.05$.

RESULTS

Effects of ABS on Hep-2 Cell Viability

The effects of ABS on Hep-2 cell viability were evaluated using the MTT assay. ABS treatment significantly reduced cell viability in a dose- and time-dependent manner compared with the untreated control group (**Figure 1**). Treatment with increasing concentrations of ABS (50–1600 nL/mL) resulted in a progressive decrease in cell viability at 24, 48, and 72 h ($p < 0.05$). The most pronounced cytotoxic effects were observed at 48 and 72 h, particularly at higher ABS concentrations.

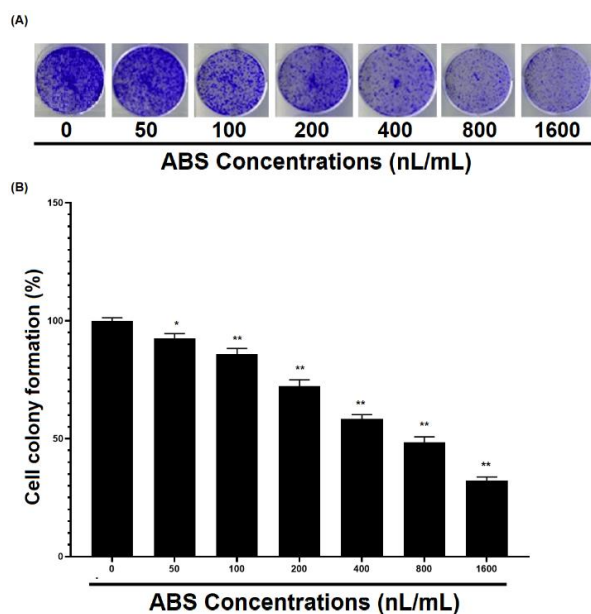


Figure 2. Effect of ABS on the clonogenic survival of Hep-2 cells. (A) Representative colony formation images. (B) Quantitative analysis of colony formation. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments, with technical triplicates where applicable. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

Effects of ABS on Colony Formation

The long-term antiproliferative effect of ABS on Hep-2 cells was assessed using a colony formation assay. ABS treatment markedly reduced the number and size of colonies compared with the control group (**Figure 2**). Quantitative analysis revealed a significant dose-dependent decrease in colony formation efficiency in ABS-treated cells ($p < 0.05$), indicating a strong inhibitory effect of ABS on the clonogenic survival.

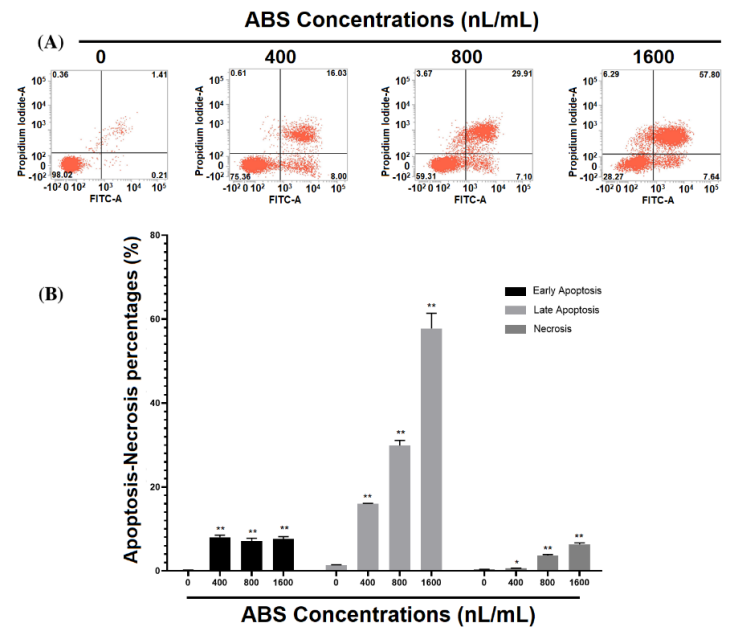


Figure 3. ABS-induced apoptosis in Hep-2 cells assessed by Annexin V-FITC/PI staining. (A) Representative flow cytometry plots. (B) Quantitative analysis of early apoptosis, late apoptosis, and necrosis. Data are presented as mean $\pm$ SD from five replicate experiments ($n = 5$). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test for each cell-death category; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the corresponding untreated control.

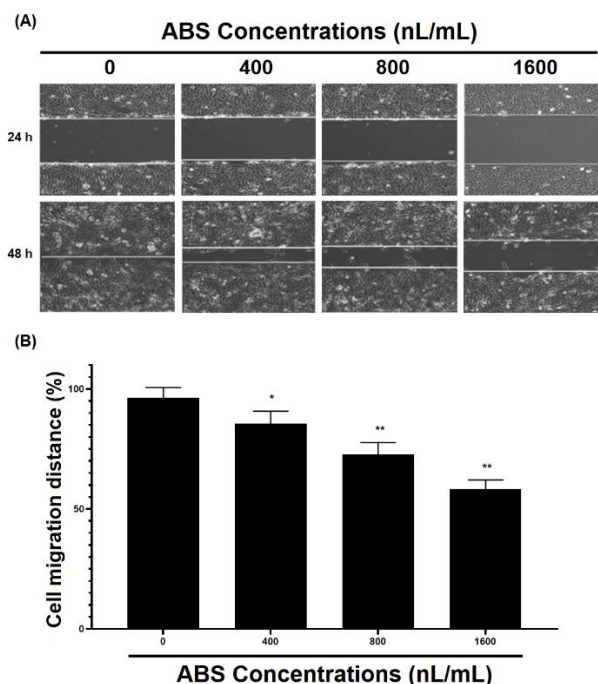


Figure 4. Effect of ABS on the migratory capacity of Hep-2 cells assessed by a wound-healing assay. (A) Representative images at 0 and 24 h. (B) Quantitative analysis of migration. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments, with technical replicates where applicable. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

ABS Induces Apoptosis in Hep-2 Cells

Apoptosis induced by ABS was analyzed using Annexin V-FITC/PI double staining and flow cytometry. As shown in **Figure 3A**, ABS treatment significantly increased the proportion of apoptotic cells compared to that in the control cells. Quantitative analysis demonstrated that the total apoptotic rate (early + late apoptosis) increased from $1.5 \pm 0.2\%$ in control cells to $24.6 \pm 1.7\%$, $38.1 \pm 3.2\%$, and $66.3 \pm 4.8\%$ in cells treated with 400, 800, and 1600 nL/mL ABS, respectively (**Figure 3B**; $p < 0.05$).

ABS Suppresses Migration of Hep-2 Cells

The effect of ABS on the migratory capacity of Hep-2 cells was examined using a wound-healing assay. Representative images showed delayed wound closure in ABS-treated cells compared with control cells (**Figure 4A**). Quantitative analysis confirmed that ABS significantly inhibited cell migration in a dose-dependent manner after 24 h (**Figure 4B**; $p < 0.05$).

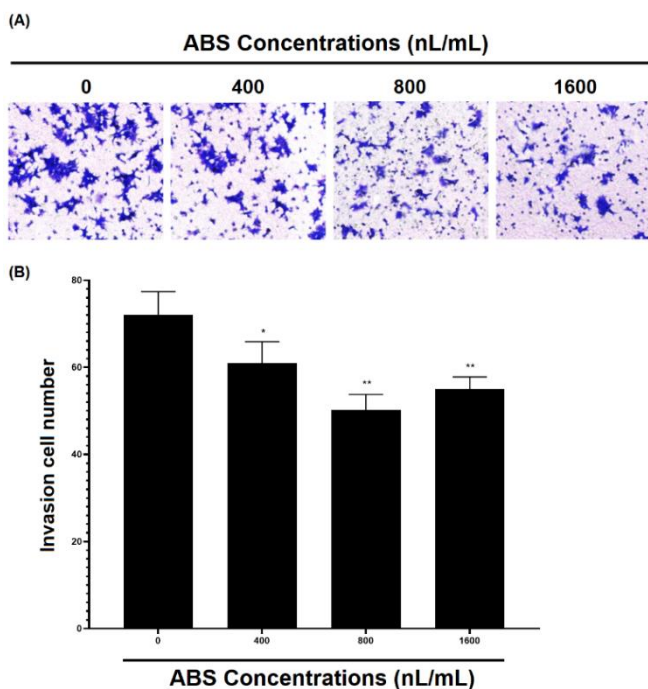


Figure 5. Effect of ABS on the invasive capacity of Hep-2 cells assessed by a Matrigel-coated Transwell assay. (A) Representative images of invading cells. (B) Quantitative analysis of invasiveness. Each condition was assayed in quintuplicate within each experiment; quantitative data represent at least three independent experiments ($n \geq 3$). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

ABS Inhibits Invasion of Hep-2 Cells

The invasive potential of Hep-2 cells following ABS treatment was assessed using a Matrigel-coated Transwell

invasion assay. As shown in **Figure 5A**, the number of cells invading the Matrigel-coated membrane was markedly reduced in the ABS-treated groups compared to that in the control group. Quantitative analysis revealed a significant dose-dependent decrease in the number of invading cells following ABS treatment (**Figure 5B**; $p < 0.05$).

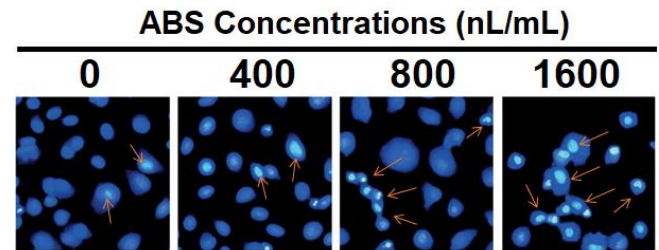


Figure 6. Nuclear morphological alterations in Hep-2 cells following ABS treatment, as visualized by DAPI staining. Images are representative of $n \geq 3$ independent biological experiments. This figure is qualitative; no inferential statistical test was applied.

ABS Alters Nuclear Morphology and Induces DNA Fragmentation

Nuclear morphology was evaluated using DAPI staining to assess apoptotic nuclear changes. Control cells exhibited uniformly stained nuclei with intact morphology, whereas ABS-treated cells showed marked nuclear condensation and fragmentation, indicative of apoptosis (**Figure 6**).

Consistent with these findings, DNA fragmentation analysis revealed a characteristic DNA ladder pattern in ABS-treated Hep-2 cells, whereas no fragmentation was observed in control cells (**Figure 7**), further confirming apoptosis induction by ABS.

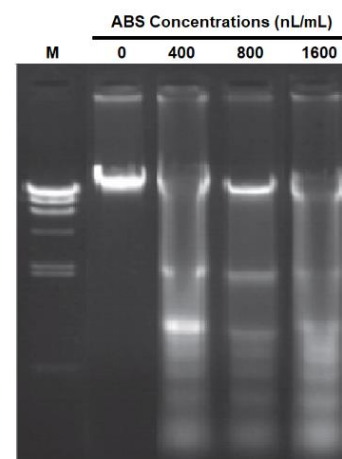


Figure 7. Dose-dependent DNA fragmentation in Hep-2 cells induced by ABS treatment. The gel image is representative of $n \geq 3$ independent biological experiments. This figure is qualitative; no inferential statistical test was applied.

Effects of ABS on Cell Cycle Distribution

The impact of ABS on cell cycle progression was evaluated using flow cytometry. ABS treatment significantly altered the cell cycle distribution compared to that in the control cells (**Figure 8A**). Quantitative analysis demonstrated a dose-dependent increase in the proportion of cells in the G₂/M phase, accompanied by a corresponding decrease in the G₀/G₁ and S phase populations (**Figure 8B**; $p < 0.05$), indicating that ABS induced G₂/M cell cycle arrest.

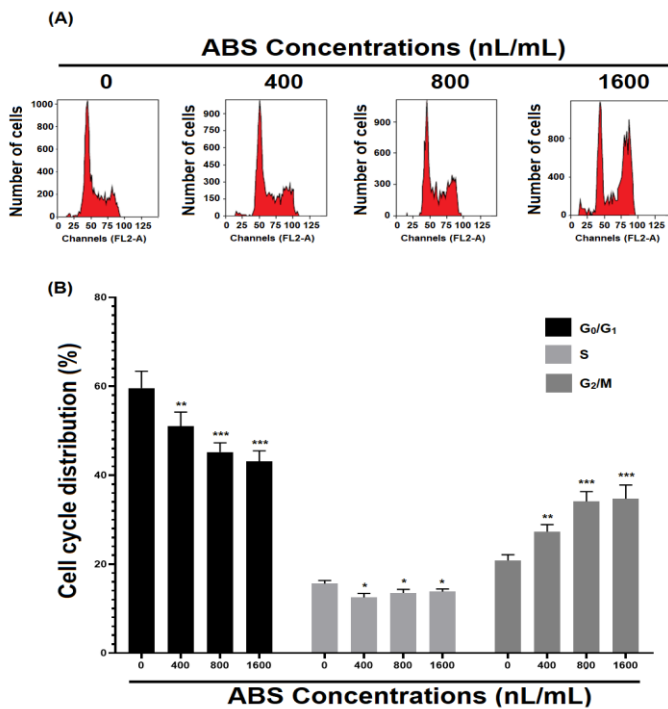


Figure 8. Effects of ABS on cell cycle distribution in Hep-2 cells. (A) Representative flow cytometry histogram. (B) Quantitative analysis of cell cycle phases. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test for each cell-cycle phase; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the corresponding untreated control.

ABS Increases Intracellular ROS Levels

Intracellular ROS production following ABS treatment was assessed using H₂DCF-DA staining and flow cytometry. ABS treatment resulted in a significant dose-dependent increase in intracellular ROS levels compared to the control group (**Figure 9A**). Quantitative analysis confirmed a marked increase in ROS fluorescence intensity in ABS-treated cells (**Figure 9B**; $p < 0.05$).

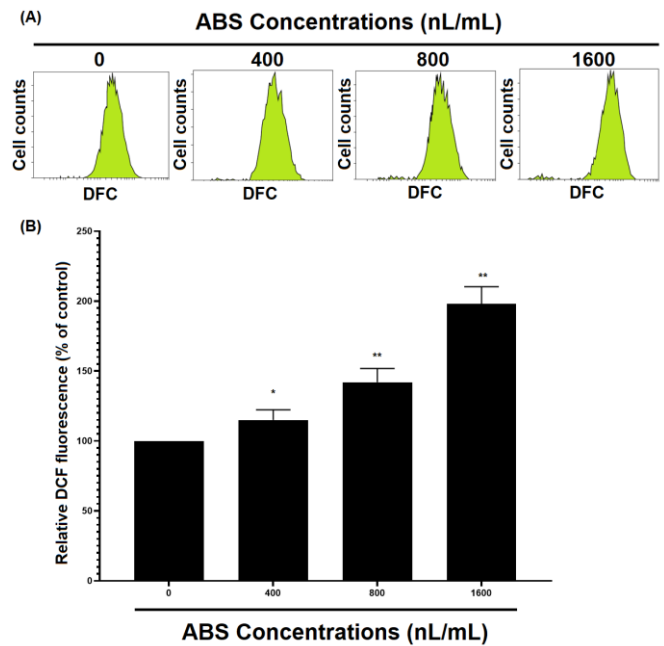


Figure 9. ABS-induced intracellular ROS production in Hep-2 cells. (A) Representative flow cytometry plots. (B) Quantitative analysis of ROS levels. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

Effects of ABS on Apoptosis- and Signaling-Related Protein Expression

The effects of ABS on apoptosis-related protein expression were examined using western blot analysis. ABS treatment significantly increased Bax expression and decreased Bcl-2 levels compared to the control group (**Figure 10A**). Moreover, ABS markedly increased the levels of cleaved caspase-3 and cleaved PARP, confirming activation of the apoptotic pathway (**Figure 10B**).

ABS Suppresses PI3K/Akt/mTOR Signaling

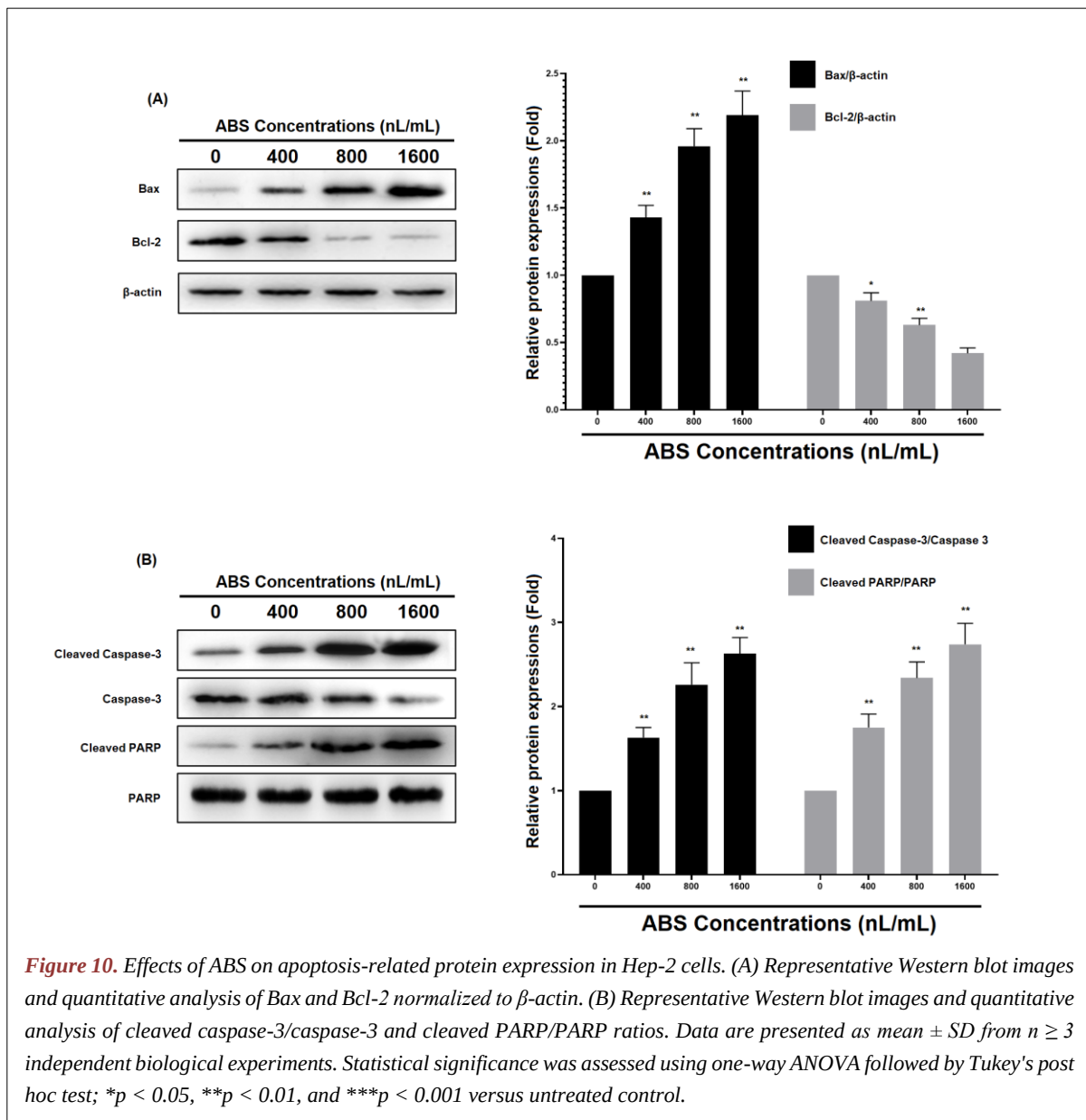
To investigate the molecular mechanisms underlying the anticancer effects of ABS, we analyzed the PI3K/Akt/mTOR signaling pathway. ABS treatment significantly reduced the phosphorylation levels of PI3K, Akt, and mTOR in a dose-dependent manner, whereas total protein levels remained largely unchanged (**Figure 11A** and **11B**; $p < 0.05$), indicating the inhibition of this key survival pathway.

Effects of ABS on Metastasis-Related Proteins

The effects of ABS on the expression of metastasis-associated proteins were further evaluated. ABS treatment significantly downregulated MMP-2, MMP-9, and VEGF expression, whereas E-cadherin expression was increased compared with that in the control cells (**Figure 12A** and **12B**;

$p < 0.05$), suggesting that ABS suppresses the metastatic potential of Hep-2 cells.

This is consistent with previous studies that reported the antiproliferative effects of ABS and its botanical components



DISCUSSION

In this in vitro study, ABS reduced Hep-2 cell viability and clonogenic survival, induced apoptosis and G2/M cell cycle arrest, increased intracellular ROS production, inhibited migration and invasion, and modulated apoptosis-, metastasis-, and survival-related proteins. These findings extend previous observations of ABS antineoplastic activity in other experimental tumor models by providing an integrated functional and pathway-level assessment using a laryngeal carcinoma cell model [4-9].

The dose- and time-dependent reduction in viability and marked suppression of colony formation indicate both acute cytotoxic stress and impaired long-term clonogenic capacity.

in several tumor models [13,14]. Importantly, the present study links these growth-inhibitory effects to multiple downstream cellular mechanisms, rather than viability alone.

Apoptosis was supported by concordant Annexin V/PI findings, nuclear condensation and fragmentation, DNA laddering, increased Bax expression, reduced Bcl-2 expression, and the activation of caspase-3 and PARP. These changes are consistent with the established Bcl-2-family and caspase-mediated apoptotic mechanisms [22]. ABS also increased intracellular ROS, consistent with prior evidence that redox perturbation can contribute to DNA damage and apoptosis in cancer and ABS-exposed cells [20,21]. Recent NCMR studies have demonstrated a close coupling between

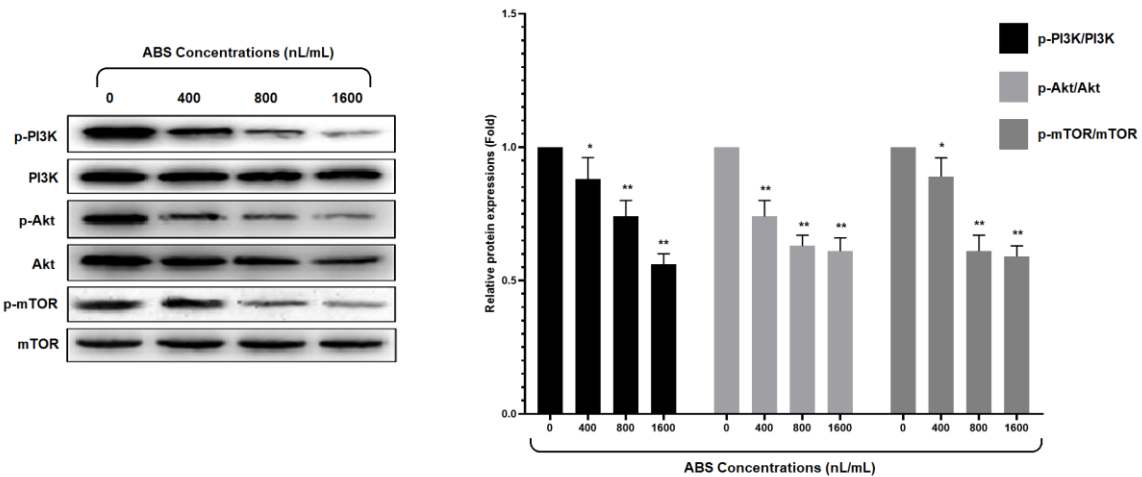


Figure 11. Inhibitory effect of ABS on PI3K/Akt/mTOR signaling pathway activation in Hep-2 cells. (A) Representative Western blot images. (B) Quantitative analysis of phospho-PI3K/PI3K, phospho-Akt/Akt, and phospho-mTOR/mTOR ratios. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

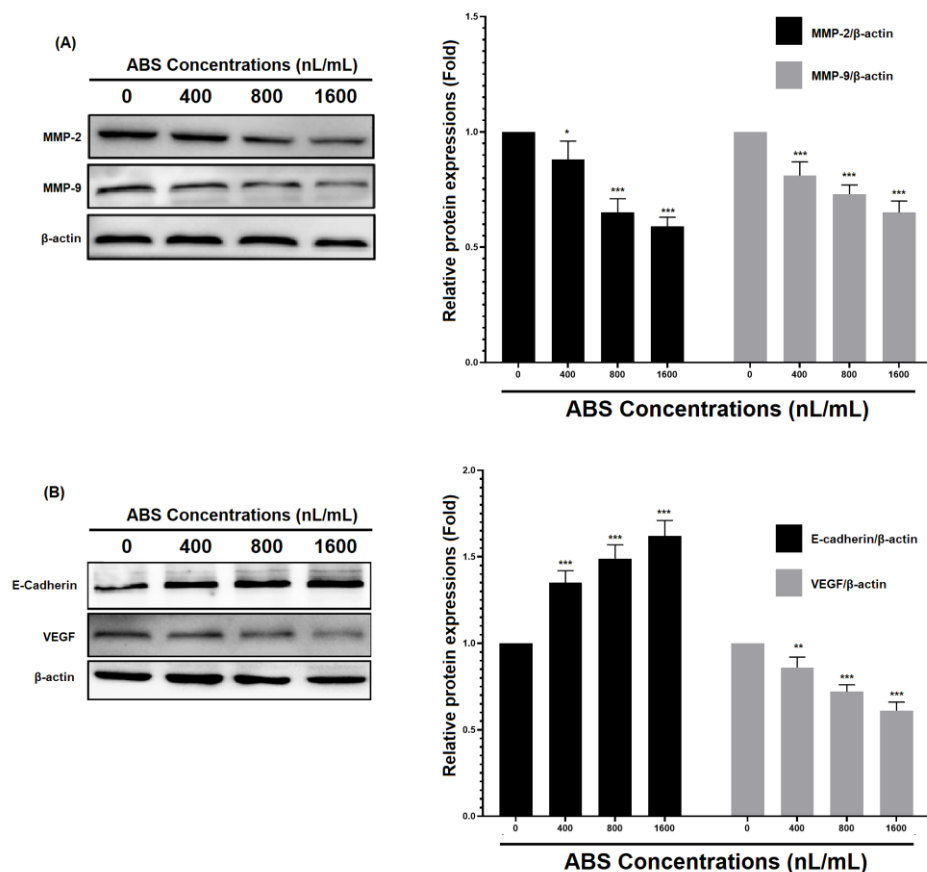


Figure 12. Effect of ABS on metastasis-related protein expression in Hep-2 cells. (A) Representative Western blot images. (B) Quantitative analysis of MMP-2, MMP-9, VEGF, and E-cadherin expression normalized to β -actin. Data are presented as mean $\pm$ SD from $n \geq 3$ independent biological experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus untreated control.

ROS/oxidative stress and apoptotic signaling. Akay et al. reported ROS-associated caspase activation and apoptosis in a gastric cancer cell model, whereas Aydoğan et al. described parallel increases in ROS, mitochondrial depolarization, and apoptosis in human cardiomyocytes [23,24]. Although these models differ from laryngeal carcinoma, they support the broader mechanistic relationship between redox imbalance and programmed cell death observed in this study.

Cell cycle analysis demonstrated a dose-dependent accumulation of cells in the G2/M phase. G2/M arrest is an established mechanism through which anticancer agents can limit cell division, and similar cell cycle effects have been reported for several plant-derived polyphenolic compounds [16-19]. Together with the clonogenic assay, these findings suggest that ABS suppresses proliferative capacity through both cell cycle disruption and induction of apoptosis.

ABS significantly inhibited cell migration and invasion. These functional effects were accompanied by the downregulation of MMP-2, MMP-9, and VEGF and the upregulation of E-cadherin, a pattern consistent with reduced extracellular matrix remodeling, angiogenic signaling, and invasive potential [6,11]. The agreement between functional migration/invasion assays and metastasis-associated protein expression strengthens the evidence for an anti-invasive effect in this in vitro model system.

At the signaling level, ABS reduced the phosphorylation of PI3K, Akt, and mTOR without a corresponding reduction in total protein abundance. Because PI3K/Akt/mTOR signaling supports tumor-cell survival, proliferation, metabolism, and resistance to apoptosis, its suppression provides a plausible mechanistic link among the observed growth inhibition, apoptosis, and altered invasive behavior [4,5,7]. In combination with increased ROS and pro-apoptotic signaling, these findings suggest a coordinated disruption of redox homeostasis and pro-survival signaling rather than a single isolated cellular effect.

This study has several limitations. All experiments were performed in a single established Hep-2 cell line under in vitro conditions; therefore, cell line-specific effects cannot be excluded, and the findings should not be extrapolated directly to clinical efficacy. The study also lacked a non-malignant laryngeal epithelial comparator, and the upstream molecular targets of ABS and its individual bioactive constituents were not manipulated directly. Confirmation in additional laryngeal squamous cell carcinoma cell lines, non-malignant controls, three-dimensional or organoid systems, targeted pathway modulation experiments, and in vivo models is required to establish reproducibility and translational relevance.

CONCLUSION

The present findings provide preclinical in vitro evidence that ABS modulates multiple tumor-related processes in Hep-2 laryngeal carcinoma cells, including cell viability and clonogenic survival, apoptosis, G2/M cell cycle progression, intracellular ROS generation, migration and invasion, metastasis-associated proteins, and the PI3K/Akt/mTOR signaling pathway. These results identify biologically relevant mechanisms but do not establish their therapeutic efficacy. Validation in additional laryngeal cancer cell lines, non-malignant controls, and in vivo experimental models is required before the translational potential of ABS can be assessed.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

Disclosure

The authors have reported no conflicts of interest in preparing and publishing this article. This study has not been presented as an oral or a poster presentation in any scientific meeting.

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Referee Evaluation Process

Externally peer-reviewed

REFERENCES

1. Weir HK, Thun MJ, Hankey BF, Ries LAG, Howe HL, Wingo PA, et al. Annual report to the nation on the status of cancer, 1975-2000, featuring the uses of surveillance data for cancer prevention and control. *J Natl Cancer Inst.* 2003;95(17):1276-1299. <https://doi.org/10.1093/jnci/djg040>
2. Li X, Gao L, Li H, Gao J, Yang Y, Zhou F, et al. Human papillomavirus infection and laryngeal cancer risk: a systematic review and meta-analysis. *J Infect Dis.* 2013;207(3):479-488. <https://doi.org/10.1093/infdis/jis692>
3. Huang J, Chan SC, Ko S, Lok V, Zhang L, Lin X, et al. Updated disease distributions, risk factors, and trends of laryngeal cancer: a global analysis of cancer registries. *Int J Surg.* 2024;110(2):810-819. <https://doi.org/10.1016/j.ijssu.2024.03.012>
4. Malkan UY, Haznedaroğlu IC. Antineoplastic effects of Ankaferd hemostat. *Biomed Res Int.* 2022;2022:2665903. <https://doi.org/10.1155/2022/2665903>
5. Koçak E, Çelebier M, Haznedaroğlu IC, Altınöz S. Analysis of antiproliferative effect of Ankaferd hemostat on Caco-2 colon cancer

- cells by LC/MS shotgun proteomic approach. *Biomed Res Int*. 2019;2019:5268031. <https://doi.org/10.1155/2019/5268031>
6. Sarı H, Çelik S, Çağlar F, Aktaş S, Bozkurt O, Yörükoğlu K, et al. The Ankaferd Blood Stopper is a candidate antineoplastic botanical agent for bladder cancer. *Int J Clin Pract*. 2021;75(11):e14789. <https://doi.org/10.1111/ijcp.14789>
 7. Demiralp DÖ, Haznedaroğlu IC, Akar N. Functional proteomic analysis of Ankaferd blood stopper. *Türk J Hematol*. 2010;27(2):72-79. <https://doi.org/10.5152/tjh.2010.12>
 8. Özle M, Uğar Çankal DA, İlhan M, Keleş H, Küpeli AE. Evaluation of the chemopreventive effects of the Ankaferd Blood Stopper in 7,12-dimethylbenz[a]anthracene-induced oral epithelial dysplasia. *Clin Oral Investig*. 2018;22(9):3091-3096. <https://doi.org/10.1007/s00784-018-2381-0>
 9. Göker H, Çetinkaya D, Kılıç E, Haznedaroğlu İ, Kirazlı S, Fırat H. In vitro anti-cancer activity of Ankaferd Blood Stopper on osteosarcoma (SAOS-2) cell lines. In: *Ankaferd: Scientific Perspectives and Basic–Clinical Data*. İstanbul: Naviga Yayınları; 2008. p. 109.
 10. Nirmala JG, Narendhirakannan RT. Vitis vinifera peel and seed gold nanoparticles exhibit chemopreventive potential and antioxidant activity and induce apoptosis through mutant p53, Bcl-2, and pan-cytokeratin downregulation in experimental animals. *Biomed Pharmacother*. 2017;89:902-917. <https://doi.org/10.1016/j.biopha.2017.02.101>
 11. Yang Y, Zheng H, Zhan Y, Fan S. Collective cell migration is an emerging mechanism of tumor invasion. *Am J Transl Res*. 2019;11(9):5301-5312. <http://www.ajtr.org/files/ajtr0095447.pdf>
 12. Piccolella S, Crescente G, Pacifico S, Fiorentino A. Antioxidant and cytotoxic potential of polyphenolic extracts from Mediterranean plants. *Food Chem*. 2019;277:351-360. <https://doi.org/10.1016/j.foodchem.2018.10.106>
 13. Ghil S. Antiproliferative activity of *Alpinia officinarum* extract in the human breast cancer cell line MCF-7. *Mol Med Rep*. 2013;7(4):1288-1292. <https://doi.org/10.3892/mmr.2013.1322>
 14. Abaza MS, Orabi KY, Al-Quattan E, Al-Attayah RJ. Growth inhibitory and chemosensitization effects of naringenin, a natural flavanone purified from *Thymus vulgaris*, on human breast and colorectal cancer. *Cancer Cell Int*. 2015;15:46. <https://doi.org/10.1186/s12935-015-0194-4>
 15. Wang M, Nie Y, Peng Y, He F, Yang J, Wu C, et al. Purification, characterization, and antitumor activities of a new protein from *Syngnathus acus*, an official marine fish. *Mar Drugs*. 2012;10(1):35-50. <https://doi.org/10.3390/md10010035>
 16. Dickson MA, Schwartz GK. Development of cell-cycle inhibitors for cancer therapy. *Curr Oncol*. 2009;16(2):36-43. <https://doi.org/10.3747/co.v16i2.428>
 17. Li Y, Yang F, Zheng W, Hu M, Wang J, Ma S, et al. *Punica granatum* leaves extract induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in non-small cell lung cancer in vitro. *Biomed Pharmacother*. 2016;80:227-235. <https://doi.org/10.1016/j.biopha.2016.03.023>
 18. Gonzalez-Sarrias A, Espin JC, Tomas-Barberan FA, Garcia-Conesa MT. Gene expression, cell cycle arrest, and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolite, urolithins. *Mol Nutr Food Res*. 2009;53(6):686-698. <https://doi.org/10.1002/mnfr.200800150>
 19. Rafi MM, Vastano BC, Zhu N, Ho CT, Ghai G, Rosen RT. A novel polyphenol molecule isolated from licorice root *Glycyrrhiza glabra* induces apoptosis, G2/M cell cycle arrest, and Bcl-2 phosphorylation in tumor cell lines. *J Agric Food Chem*. 2002;50(4):677-684. <https://doi.org/10.1021/jf010984k>
 20. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and their therapeutic implications. *Drug Resist Updat*. 2004;7(2):97-110. <https://doi.org/10.1016/j.drug.2004.01.004>
 21. Kocyigit A, Guler EM, Haznedaroglu IC, Malkan UY. Ankaferd hemostat induces DNA damage, apoptosis, and cytotoxicity by generating reactive oxygen species in melanoma and normal cell lines. *Int J Clin Exp Med*. 2017;10(2):2116-2126.
 22. Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in apoptosis regulation. *Mol Cell Biochem*. 2011;351(1-2):41-58. <https://doi.org/10.1007/s11010-011-0703-y>
 23. Akay İB, Günel MY, Övey İS. TRPV1 channel-mediated apoptotic effects of Liquidambar Orientalis Miller balsam on gastric cancer cell line: Liquidambar Orientalis Miller Gum induces apoptosis in gastric cancer cells. *Neuro-Cell Mol Res*. 2024;1(3):82-88. <https://doi.org/10.66588/NCMR.v01i03.4>
 24. Aydoğan E, Övey İS, Karahan O. Melatonin reverses bupivacaine-induced cardiotoxicity in human cardiomyocytes by attenuating TRPV1-mediated apoptosis, ROS production, and mitochondrial depolarization. *Neuro-Cell Mol Res*. 2026;3(1):13-19. <https://doi.org/10.66588/NCMR.v3i1.2>