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RESEARCH

Effects of *artemisinin* on proliferation, oxidative damage, and apoptosis in Ishikawa endometrial cancer cells

Ishikawa endometrial kanser hücrelerinde *artemisinin* proliferasyon, oksidatif hasar ve apoptoz üzerindeki etkileri

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Abstract

Purpose: The aim of this study was to determine the anticancer effects of *artemisinin* (ART), an active ingredient, on Ishikawa endometrial cancer cells.

Materials and Methods: The antiproliferative effect of ART was evaluated using the XTT assay. The effect of ART on oxidative damage was assessed using the 8-OHdG ELISA assay. Its effect on apoptosis was evaluated using caspase-3 ELISA, TUNEL, and RT-PCR analysis of changes in mRNA expression of apoptosis-related genes.

Results: Depending on time and dose, the viability of Ishikawa cells decreased. The IC_{50} dose of ART in Ishikawa cells was determined to be 57.59 μM after 72 hours. There were no statistically significant differences in 8-OHdG and caspase-3 levels between groups. The apoptosis rate was 1.7% in the control group and 9.02% in the ART-treated group. No statistically significant changes in the gene expression levels of *Bax, Bcl-2*, and apoptotic *caspases* were observed in the treatment group compared to the control group after 72 hours of exposure to 57.59 μM ART.

Conclusion: ART shows promise as an anticancer agent. However, more comprehensive toxicological, pharmacokinetic, and in vitro and in vivo studies on its safety, bioavailability, and therapeutic efficacy are needed.

Keywords:. Apoptosis, artemisinin, cell proliferation, endometrial cancer, Ishikawa cell line.

Öz

Amaç: *Artemisinin* (ART) etken maddesinin Ishikawa endometriyal kanser hücreleri üzerindeki antikanser etkilerini belirlemektir.

Gereç ve Yöntem: ART'ın antiproliferatif etkisi, XTT testi kullanılarak değerlendirilmiştir. Oksidatif hasar üzerindeki etkisi 8-OHdG ELISA testi ile apoptoz üzerindeki etkisi ise kaspaz-3 ELISA, TUNEL ve apoptoz ilişkili genlerin mRNA ekspresyon değişiklikleri RT-PCR analizi ile değerlendirilmiştir.

Bulgular: Ishikawa hücrelerinin canlılığındaki azalma, zamana ve doza bağlı olarak gözlendi. Ishikawa hücrelerinde ART' ın IC_{50} dozu 72 saatte 57,59 μM olarak belirlendi. Gruplar arasında 8-OHdG ve kaspaz-3 düzeylerindeki farklılıklar istatistiksel olarak anlamlı değildi. Kontrol grubunda apoptoz oranı %1,7 iken, ART ile tedavi edilen grupta hücrelerin %9,02' si apoptoza uğradı. Kontrol grubuyla karşılaştırıldığında, 72 saat boyunca 57,59 μM ART' ye maruz kalan tedavi grubunda *Bax, Bcl-2* ve apoptotik *kaspazların* gen ekspresyon düzeylerinde istatistiksel olarak anlamlı bir değişiklik gözlenmedi.

Sonuç: ART, umut vaat eden bir antikanser ajan olarak potansiyel göstermektedir. Güvenliği, biyoyararlanımı ve terapötik etkinliği üzerine daha kapsamlı toksikolojik, farmakokinetik, in vitro ve in vivo çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Apoptozis, artemisinin, hücre çoğalması, endometrial kanser, Ishikawa hücre hattı.

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INTRODUCTION

Endometrial carcinoma (EC) is one of the leading cancers among women worldwide[1]. Since 2008, the global incidence of EC has increased by 21%, alongside increased life expectancy[3]. Each year, 417 thousand women worldwide are diagnosed with EC. While early-stage EC generally has a favorable prognosis, aggressive types often exhibit treatment resistance and poor outcomes[4]. The high lethality rate is due to late-stage diagnosis, high metastatic potential, and the lack of effective treatments[5,6]. This clinical heterogeneity, caused by factors such as molecular diversity, obesity, and hormonal effects, requires innovative management approaches[7]. Radiation therapy and chemotherapy are the main methods used in the treatment process. Despite technological and scientific advancements, chemotherapy and radiation therapy continue to be toxic to healthy tissues. These effects negatively impact patients' quality of life[8,9]. For this reason, research on herbal treatments continues to increase in order to find options that are more effective against malignant cells with a very low risk of toxicity[10].

Artemisinin (ART) is obtained from the plant Artemisia annua L., also known as sweet wormwood. It is a sesquiterpene characterized by a reactive 1,2,4-trioxane ring and a lactone moiety as a pharmacophore. This chemical compound is used to treat malaria. Studies have shown that artemisinin compounds regulate oxidative stress[13], arrest the cell cycle[14,15], prevent cell division[16], and increase apoptotic activity[14-17]. The anticancer properties of artemisinin and its derivatives, such as artesunate, dihydroartemisinin, and anhydrodihydroartemisinin, have been investigated in various studies, revealing their potential as therapeutic agents against different types of cancer[18-22]. ART has been reported to exhibit antiproliferative effects in endometrial cancer cell cultures by downregulating cyclin-dependent kinase 2 and 4 receptors. However, its specific role and efficacy in the context of endometrial cancer are unclear and undefined[23].

Increased proliferation due to increased oxidative stress and the suppression of apoptosis mechanisms are fundamental biological processes in carcinogenesis. The interaction between these processes is complex and constitutes an important area of research. However, the effects of the active ingredient, ART, on proliferation, apoptosis, and oxidative damage in endometrial cancer cells have not yet been clearly established. In this study, we used Ishikawa cells, a well-characterized human endometrial cell line, as an experimental model[10]. These cells originate from a well-differentiated human endometrial adenocarcinoma and express estrogen and progesterone receptors[25,26]. Through a series of experimental tests and analyses, we evaluated how ART affects fundamental aspects of cancer cell behavior, including growth, proliferation, and apoptosis. By investigating these effects, we hope to shed light on the potential of ART as a therapeutic agent for endometrial cancer and lay the groundwork for future research in this area. Ultimately, this research could contribute to the development of novel treatment strategies and improve outcomes for patients with endometrial cancer.

MATERIAL AND METHODS

Cell culture

Artemisinin was purchased from Sigma-Aldrich in the United States. Ishikawa endometrial cancer cells were grown in RPMI 1640 (Sigma) and cultured at 37 °C with 5% CO_2. The antiproliferative activity of artemisinin was evaluated in Ishikawa cells treated with different concentrations, including 18.75 μM, 37.5 μM, 75 μM, 150 μM, and 300 μM, at 24, 48, and 72 hours according to a time- and dose-dependent manner.

This research did not involve human participants, laboratory animals, or clinical data sets. All experiments were conducted on commercially sourced Ishikawa cell lines only. Accordingly, ethical approval was not required under institutional and national regulations. The experimental applications and studies were conducted at the Medical Biology and Research Laboratory of the Faculty of Medicine at Ordu University in Ordu, Turkey, a facility that complies with established institutional quality protocols. The authors performed all cell culture and molecular analyses.

Cell viability assay

We assessed the antiproliferative effect of artemisinin by seeding cells at a density of $3x10^3$ cells per well in 96-well plates. The XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) cell proliferation assay kit was used. Formazan formation was measured using an absorbance of 450 nm following the relevant kit protocols.

Cell Viability (%) = [(Treatment group OD_{450} - Blank well OD_{450}) / (Untreated group OD_{450} - Blank well OD_{450})] ×100% [27].

The IC_{50} dosage of artemisinin on Ishikawa cells was assessed using the AAT Bioquest online tool (https://www.aatbio.com/tools/ic50-calculator). This dosage was used as the dose group in the other molecular studies of this research project.

8-hydroxy-2' -deoxyguanosine assay

We investigated the protein level of 8-OHdG in Ishikawa cells using an enzyme-linked immunosorbent assay kit. The assay was performed using the kit and protocols provided by BT Lab in Shanghai, China.

The control and dose group Ishikawa cells were homogenized in a lysis buffer that contained a pre-prepared protease inhibitor cocktail. The supernatant was collected by centrifugation at 13.000 g for 30 minutes. The concentration of 8-OHdG was compared to a pre-established standard curve and expressed in nanograms per milliliter (ng/mL).

Caspase-3 assay

We measured the concentration of the caspase-3 protein using a BT Lab (Shanghai, China) ELISA kit according to the manufacturer's protocols.

The control and ART-treated groups of Ishikawa endometrial cancer cells were homogenized in a lysis buffer containing a pre-prepared protease inhibitor cocktail. The supernatant was collected by centrifugation at 13.000 g for 30 minutes. The concentration of the caspase-3 protein was compared to a pre-established standard curve and expressed in nanograms per milliliter (ng/mL).

TUNEL assay

We evaluated the apoptotic effect of artemisinin on Ishikawa endometrial cancer cells using a colorimetric TUNEL assay (ScienCell Colorimetric TUNEL Apoptosis Assay Kit), following the manufacturer's instructions. The cells were seeded in eight-well glass chamber slides at a density of $3x10^3$ cells/well. After incubating the cells for 24 hours at 37°C, they were treated with media only (control) or the IC_{50} dose of ART for an additional 72 hours. Following this treatment, the cells were fixed and processed for TUNEL labeling according to the manufacturer's general workflow. Hematoxylin and eosin were used as stains. Imaging was performed under a light microscope (Olympus BX53F). "Percent" apoptosis was determined by comparing the number of viable cells to the number of apoptotic cells at 40x magnification in at least ten randomly selected areas from the control and treatment groups.

Total RNA isolation, cDNA synthesis, and RT-PCR analysis

Total RNA was isolated from cells exposed to the IC_{50} using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The NanoDrop was used to measure the quantity and quality of the RNA (BioSpec-Nano, Shimadzu, Japan). Reverse transcription was used to synthesize cDNA from the RNA template according to the manufacturer's protocol using the Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany). Real-time PCR (RT-PCR; Rotor-Gene Q, Hilden, Germany) was used to evaluate changes in the expression of mRNA for the *Bax* (BCL2-related X), *Bcl-2* (BCL2 apoptosis regulator), and *caspase* genes. Normalization was accomplished using *Beta-Actin* (*ACTB*). The primer sequences used in this study were given in Table 1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.4.1 (San Diego, CA). Gene expression data obtained from real-time PCR were quantified using the ΔΔCT method and analyzed with the RT² Profiler PCR Array Data Analysis Tool (Qiagen). Data are presented as the mean ± standard deviation (SD). The normality of the data distribution was assessed using the Shapiro–Wilk test. For comparisons between two independent groups, an unpaired Student's t-test was applied to data showing a normal distribution, and a Mann–Whitney U test was applied to data not showing a normal distribution. Differences were considered statistically significant at $P < 0.05$ (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$).

Table 1. Real Time PCR forward and reverse primer sequences

Gene symbol	Forward and Reverse Primers
ACTB	F 5'-CACCATTGGCAATGAGCGGTTC-3' R 5'-AGGTCTTTGCGGATGTCCACGT-3'
BAX	F 5'-AGAGGATGATTGCCGCCGT-3' R 5'-CAACCACCCTGGTCTTGGA-3'
BCL2	F 5'-CAGGGCGATGTTGTCCACC-3' R 5'-GGGGAGGATTGTGGCCTTC-3'
CASP3	F 5'-CATGGAAGCGAATCAATGGACT-3' R 5'-CTGTACCAGACCGAGATGTCA-3'
CASP7	F 5'-AGTGACAGGTATGGGCGTTC-3' R 5'-CGGCATTTGTATGGTCCTCTT-3'
CASP8	F 5'-ACACCAGGCAGGGCTCAAAT-3' R 5'-GCAGGTTCATGTCATCATCCAGTT-3'
CASP9	F 5'-CTTCGTTTCTGCGAACTAACAGG-3' R 5'-GCACCACTGGGGTAAGGTTT-3'
CASP10	F 5'-TAGGATTGGTCCCCAACAAGA-3' R 5'-GAGAAACCCTTTGTCGGGTGG-3'

RESULTS

Following ART treatment, a time- and dose-dependent decrease in the viability of Ishikawa cells was observed. Viability decreased slightly at 24 and 48 hours with increasing doses of ART, but a more pronounced effect was observed at 72 hours. The half-maximal inhibitory concentration (IC_{50}) of ART was determined to be 57.59 µM after 72 hours in the Ishikawa cell line (Figure 1).

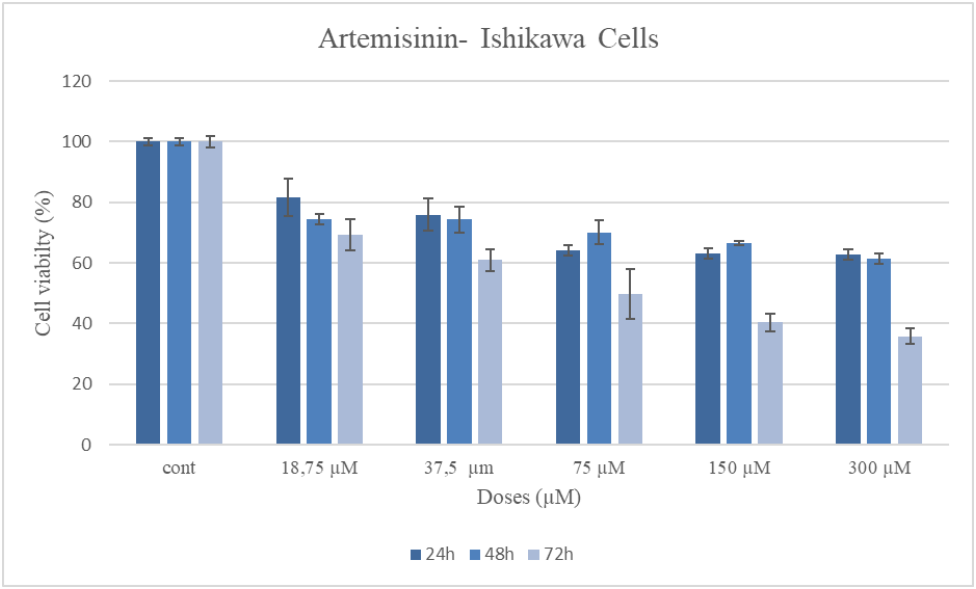


Figure 1. Ishikawa cells were treated with Artemisinin at different concentrations and time intervals, and their viability was assessed by XTT assay. Data shows the average results of three independent experiments. IC_{50} doses of Artemisinin in Ishikawa cells were detected as 57.59 µM at the 72 hours.

According to ELISA data, the average 8-OHdG concentration was found to be 44.96 ± 3.44 ng/mL in the Ishikawa control group and 42.59 ± 9.68 ng/mL in cells treated with ART. No significant difference was found when the results were statistically compared (P=1.000, Figure 2).

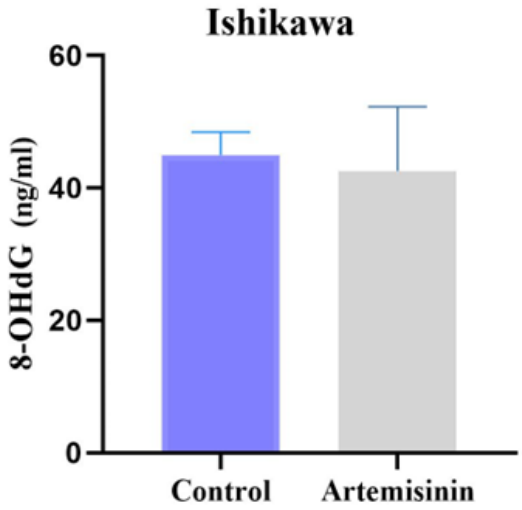


Figure 2. 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration levels of Artemisinin-treated Ishikawa endometrial cancer cells and controls.

According to ELISA data, the mean caspase-3 level in the supernatant was determined to be 2.271 (±0.346) for the control group and 2.195 (±1.071) for Ishikawa cells treated with ART. No significant difference was found when the results were statistically compared (P=1.000, Figure 3).

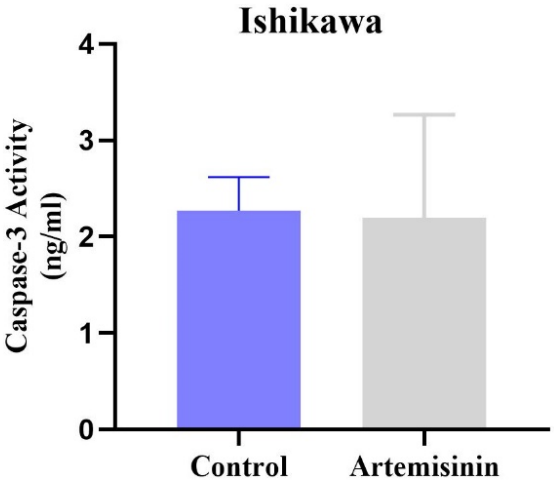


Figure 3. Caspase-3 concentration levels of Artemisinin-treated Ishikawa endometrial cancer cells and controls.

The study investigated the apoptotic effects of ART on Ishikawa endometrial cancer cells. It did so by comparing the percentage of apoptotic cells in a control group with the percentage in a group treated with ART. A statistically significant increase in apoptosis was observed at the 72-hour mark in cells exposed to 57.59 ART, a concentration identified as the IC_{50} from previous XTT assays. The apoptosis rate was 1.7% in the control group and 9.02% in the ART-treated group. This difference was statistically significant (p=0.026). These results indicate that ART induces apoptosis in Ishikawa endometrial cancer cells at this concentration (Figure 4).

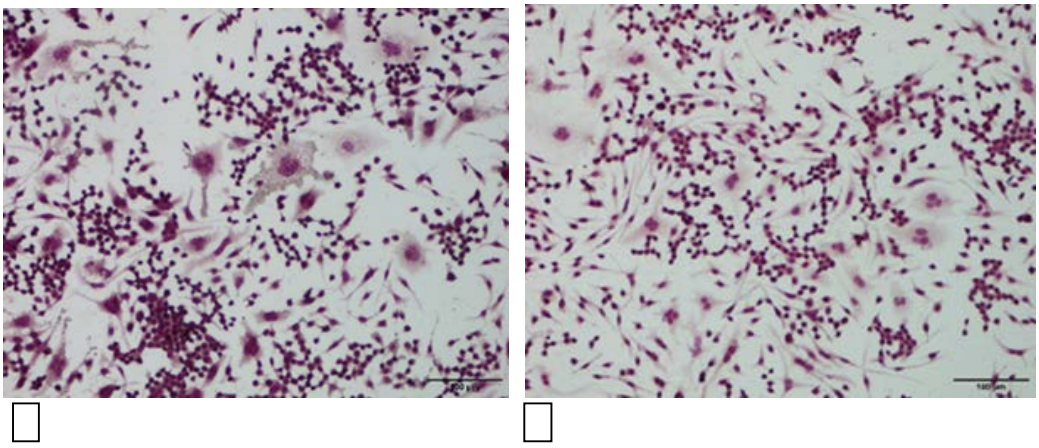


Figure 4. Quantitative evaluation of the TUNEL signal. Imaging was performed under a light microscope with 40X magnification. A. Control group, apoptosis rate 1.7%, B. Artemisinin-treated group, apoptosis rate 9.02% (p=0.026).

After isolating the RNA, we performed an expression analysis of the *Bax, Bcl-2*, and *caspase* genes using the RT-PCR method.

In the group treated with 57.59 μM of ART for 72 hours, no significant changes in gene expression were observed for *Bax* (1.93, p=0.56), *Bcl-2* (0.61, p=0.95), *Casp-3* (0.76, p=0.68), *Casp-7* (1.27, p=0.37), *Casp-8* (1.58, p=0.51), *Casp-9* (5.17, p=0.66), and *Casp-10* (8.38, p=0.32) compared to the control group.

DISCUSSION

Endometrial carcinoma (EC) is a common gynecological cancer with high incidence and mortality rates[28,29]. Although molecular-targeted drugs are increasingly used to treat EC and other cancers, the side effects of chemotherapy can complicate treatment. Consequently, ongoing research aims to develop drugs based on specific target genes or proteins[30]. For this reason, the therapeutic potential of herbal products has been studied for many years. However, due to the extreme toxicity of some herbal substances, only a few can be developed into viable drugs[5,31]. Although various studies have explored the anticancer properties of ART, its role in endometrial cancer remains unclear. This study provides the first evidence of the effects of ART on cytotoxicity, DNA damage, and the apoptotic pathway in Ishikawa endometrial cancer cells.

Oxidative stress plays a critical role in the development of many diseases, including cancer. It causes oxidative damage to lipids, proteins, and DNA and is often associated with inflammation. For this reason, markers of oxidative damage are frequently studied in in vitro and in vivo experiments[32]. One such study was conducted by Yan et al., who investigated the protective effects of ART against oxidative stress in light-exposed rats. The study found that ART reduced intracellular reactive oxygen species in the retinal neurons of the rats at

appropriate dosages[33]. Ho et al. investigated the potential antioxidant effects of artesunate in comparison with the potent corticosteroid dexamethasone using a mouse model of allergic asthma. According to their findings, levels of oxidative damage markers such as 8-isoprostane, 8-hydroxy-2-deoxyguanosine, and 3-nitrotyrosine were significantly reduced by artesunate[32]. Kim et al. studied a dietary model involving Artemisia annua L. plant extract in mice. This dietary model significantly reduced serum levels of biomarkers for lipid peroxidation and DNA damage, namely MDA and 8-OHdG. However, nutrition with Artemisia annua L. plant extract has also increased antioxidant enzyme activities in tissues such as the kidneys, stomach, small intestine, and colon[34]. El-Kareem et al showed that the active ingredient artemisinin increased total antioxidant status in Swiss albino mice with breast cancer[35]. In this study, 8-OHdG levels were not significant between groups.

Cell viability and antiproliferative effects of ART and its derivatives have been mostly demonstrated in cell culture studies. Shi et al investigated the multiple biological activities of Dihydroartemisinin in human tongue squamous cell carcinoma cells. According to their study, Dihydroartemisinin inhibited cell growth in a time-dependent manner. However, they discovered that it inhibited the nuclear localization of phosphorylated signal transducer and activator of transcription 3 (p-STAT3), ultimately leading to autophagic cell death[36]. Tang et al determined the antiproliferative effects of Dihydroartemisinin (DHA) on Ishikawa cells using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium] assay. According to this study, treatment of Ishikawa cells with DHA at doses of 20 μM, 40 μM or 80 μM for 96 hours showed a strong antiproliferative effect. Specifically, it was shown that the 40 μM (48 hours) and 80 μM (24 hours) doses inhibited cancer cells[37]. Zhang et al investigated the antiproliferative effects of Artesunate in two different endometrial cancer cell lines, Ishikawa and AN3CA. They demonstrated that ART significantly inhibited cell viability in both cell lines in a dose- and time-dependent manner. In this study, the IC_{50} values for ART treatment were determined to be 48.3 μM for Ishikawa and 104.8 μM for AN3CA, 24 hours after treatment[38]. Time and dose applications of artemisinin vary in various studies. In our study, using the XTT test, the viability of Ishikawa cells was determined as 57.59 μM at IC_{50} at the 72 hours dose. In the literature, it has been observed that ART has effects on cell viability in endometrial cancer cell lines at different doses and time intervals. This study is consistent with other results.

The effects of ART have been studied in cell lines other than those of endometrial cancer. For example, Liao et al. demonstrated that dihydroartemisinin (DHA) inhibits the proliferation of A549 lung cancer cells by suppressing the AKT/GSK-3β/Cyclin D1 signaling pathway. The same study found that DHA significantly inhibited the proliferation of A549 cells in a concentration- and time-dependent manner, as determined by the MTS test. The MTS assay revealed that DHA significantly inhibited the proliferation of A549 cells in a concentration- and time-dependent manner[39]. Ahmad et al. reported the anticancer properties of anhydroartemisinin (ADHA), a semi-synthetic derivative of artemisinin, against the prostate cancer cell line PC-3. In this study, it was found that ADHA inhibited the growth and migration of PC-3 cells. At the molecular level, ADHA was shown to suppress the expression of c-Jun, p-c-Jun, p-Akt, and NF-κB, and activated caspases 3 and 7 [40]. Huang et al. investigated the effects of artesunate, a derivative of artemisinin, on SW480 and HCT116 colorectal cancer cells. The results of this study indicated that artesunate treatment led to mitochondrial dysfunction. It promoted mitochondrial ROS formation, which caused cell cycle arrest in the G0/G1 phase. This subsequently led to cell senescence mediated by p16 and p21, thereby inhibiting cell proliferation[20].

Apoptosis and autophagy are forms of programmed cell death. Both processes are essential for maintaining tissue homeostasis in response to various stimuli and are distinguished by unique biochemical and morphological changes. Apoptosis is characterized by the formation of apoptotic bodies, and changes in the expression of Bax and Bcl-2 proteins are significant indicators[41]. Casp-3 and casp-9 are the main effector caspase for the apoptotic cascade within cells[42]. In this study, although no significant difference was found between the treatment and control groups regarding casp-3 levels, TUNEL analysis showed a significant increase in the number of apoptotic cells in the ART-treated group. Tang et al demonstrated that after DHA treatment, Caspase-3 mRNA and cleaved caspase-3 protein levels were significantly elevated in Ishikawa endometrial cancer cells. This indicates that DHA plays a role in the activation of the apoptotic cell death pathway[37]. Xing Guan and Yan Guan

elucidated the apoptosis-inducing effect of artemisinin in MDA-MB-123 cells using DAPI[41]. Chen et al. investigated the effects of artesunate in an animal model and found that Bax, an apoptosis-related protein, was upregulated while Bcl-2 expression was downregulated. They also observed an increase in the Bax/Bcl-2 ratio in a concentration-dependent manner[43]. At the 72-hour mark, no significant changes in the gene expressions of *Bax, Bcl-2, Casp-3, Casp-7, Casp-8, Casp-9*, and *Casp-10* were observed in the group treated with 57.59 μM ART compared to the control group. No expression changes were detected in genes associated with apoptotic pathways in this study. However, the increase in TUNEL-positive cells in the ART group and the absence of significant differences in caspase-3 protein levels suggest that cell death occurred through mechanisms independent of transcriptional activation. The regulation of many apoptosis steps at the post-translational level, particularly the ability of caspase-independent mitochondrial pathways to cause DNA fragmentation, may explain the inconsistency between these findings. Additionally, since the TUNEL method can demonstrate late-stage cell death and advanced DNA damage, ART may have induced alternative cell death mechanisms rather than classical, caspase-3-dependent apoptotic pathways.

This study has certain limitations. First, the findings were examined in a single cell line. Therefore, potential differences between subtypes of the same cancer could not be fully elucidated. Additionally, the inability to analyze the protein-level counterparts of the results may limit functional validation. Including experimental validation approaches in different endometrial cancer cell lines in future studies would strengthen the biological significance and reliability of the findings.

Fundamental research currently focuses on the effects of artemisinin (ART) and its derivatives in cancer treatment. This research investigates various cellular aspects, such as apoptosis, autophagy, cell cycle checkpoints, and damage repair. Because artemisinin and its derivatives affect various cellular mechanisms differently, small changes in their chemical structures may alter their effects on cells. These changes can affect molecular interactions and activities in target cells. Different derivatives may target different biological sites. While some derivatives may be effective on a specific cellular pathway, others may influence alternative pathways. Additionally, different cell types may respond differently to various dosages of artemisinin and its derivatives.

Triggering apoptosis is a complex process. Some derivatives promote apoptosis by affecting the cell cycle, oxidative stress, or enzymatic pathways, while others inhibit these processes. These factors may explain the inconsistent results observed when studying the effects of artemisinin and its derivatives on apoptosis in endometrial cancer cells and other cancer cells. Comprehensive preclinical and clinical studies are needed to determine the effects of artemisinin and its derivatives on DNA damage and apoptosis in endometrial cancer cells. Such extensive research could enhance our understanding of the potential role of artemisinin and its derivatives in endometrial cancer treatment and inform future therapeutic approaches.

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Peer-review: Externally peer-reviewed.
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