Histol Histopathol (2025) 40: 327-334

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Inhibitory effect of Curcumin on a cervical cancer cell line via the RAS/RAF signaling pathway

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Summary. Objective. Cervical cancer has a very important place in female infertility and ranks fourth among cancers affecting women. Curcumin (CUR) is closely associated with the expression and activity of various regulatory proteins. It is also known that curcumin has preventive and therapeutic effects on various types of cancer. In this study, the anticancer activities of curcumin were demonstrated in the human cervical cancer cell line (HeLa). Methods. qRT-PCR and western blot analyses were used to evaluate mRNA and protein expression of curcumin in HeLa and immortalized human skin keratinocyte cell lines (HaCaT) (proliferation and apoptosis regulatory markers of the RAS/RAF signaling pathway). MTT analysis was performed, showing HeLa and HaCaT cell proliferation depending on the dose and duration of curcumin and doxorubicin. A wound scratch healing assay was applied to examine cell migration and invasion of HeLa after curcumin application. To determine the role of curcumin and doxorubicin in the apoptosis of HeLa cells, the mRNA levels of caspase-3 were examined by qRT-PCR. The results were analyzed with a one-way ANOVA SPSS 20.0 program. Results. CUR (IC50: 242.8 μM) and DOX (IC50: 92.1 μM) were determined to have the ability to inhibit the proliferation of HeLa cells and induce apoptosis over a 72-hour period and dose-dependently. Moreover, the results revealed that the mRNA and protein expression levels of RAF and RAS in HeLa cells were downregulated by CUR and DOX. Conclusions. The findings show that an alternative treatment method for cervical cancer can be developed with the application of CUR and DOX. Alternative methods for cervical cancer treatment may be developed using different methods in future studies.

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www.hh.um.es. DOI: 10.14670/HH-18-797

Key words: Curcumin, HeLa cells, Cervical cancer, RAF/RAS signaling pathway

Introduction

Cervical cancer remains a significant global health concern, particularly in developing countries where access to early detection and treatment is limited. Advanced screening programs, different treatment approaches, and vaccines have not reduced the death rate. It is thought that the reason for the increased death rate may be due to increasing drug resistance in cancer cells (Emran et al., 2022). Although methods such as chemotherapy, radiotherapy, and surgery were used in its treatment, the desired results were not achieved (Dröge et al., 2021). Although Pap smear is the most commonly used method, especially in the detection of positive lesions in the area surrounding cervical cancer and in the early screening of many pre-cancerous lesions (Twu et al., 2007), this method also has limitations, such as a high false positivity and low sensitivity. Recently, complementary uses, defined as human papillomavirus (HPV) DNA chip testing, have been proposed to eliminate these problems. With this method combined with Pap smear, early diagnosis of cervical cancer can be facilitated (Lee et al., 2023). However, drug resistance in the treatment of cervical cancer poses a major obstacle to clinical treatment. Despite advancements in therapeutic approaches, the need for novel, effective treatments persists, especially considering the limitations and adverse effects associated with current therapies.

In recent years, the molecular components of signaling pathways have been investigated more extensively in cancer treatment. RAS/RAF/MEK/ERK signal transduction pathways are also important targets in cancer treatment. The RAF/MEK/ERK pathway is frequently activated by mutations in growth factor receptors (Bahar et al., 2023). This pathway is responsible for 20-30% of human cancers. Agents that

inhibit the molecules of this pathway have been developed and are partially used in clinical studies (Friday and Adjei, 2008). The development of therapies targeting this pathway may induce cell growth suppression and apoptosis. Different targets and techniques have been used in signaling pathway studies on cervical cancer. It has been reported that downregulation of miR-26a-5p suppresses the proliferation, migration, and invasion of cancer cells in cervical cancer patients, and also alleviates cancer progression by promoting apoptosis (Li et al., 2022). Another group of researchers reported that the expression of genes related to apoptosis (Bax, Bcl2) and genes related to the cell cycle increased by downregulating the Notch-1 and Hes-1 signaling pathways in a cervical cancer cell line (Khan et al., 2021). Chang et al. reported that lncRNA plasmacytoma variant translocation 1 (PVT1) promotes the progression of cervical cancer by sponging miR-140 and increasing Smad3 expression (Chang et al., 2019).

Recently, interest in anti-cancer drugs has increased, and different herbal flavonoids and polyphenolic compounds are being investigated, especially for the discovery of new drugs with minimal or no side effects. There is great interest in investigating signaling pathways that will reveal the protective and preventive potential of phytochemicals against different types of cancers and other diseases (Khan et al., 2021; Gao et al., 2022). Curcumin (CUR), a polyphenolic compound derived from turmeric (*Curcuma longa*), has garnered immense attention in recent years due to its multifaceted pharmacological properties, including anti-inflammatory, antioxidant, and anticancer effects (Zhao et al., 2023; Sun et al., 2022). Numerous studies have demonstrated curcumin's potential to inhibit proliferation and induce apoptosis in various cancer cell lines, making it a promising candidate for cancer therapy (Ashrafizadeh et al., 2020). Additionally, studies show that Curcumin is used for the treatment of cervical cancer. Curcumin application (13 μM) induces DNA damage in HeLa cells and promotes the translocation of p53 and H2A. Studies have suggested that it increases reactive oxygen species (ROS) formation to stimulate curcumin (Wang et al., 2020). To potentiate the anti-tumor activity of curcumin, its combination with emodin has been applied to inhibit Wnt/β-catenin signaling via TGF-β downregulation, thus reducing cervical cancer progression (Thacker and Karunagaran, 2015). In addition, the clinical application of curcumin suffers from limitations due to its poor water solubility and instability, leading to low bioavailability of curcumin in cancer cells. Efforts to increase the therapeutic effectiveness of curcumin have been carried out with different methods and have led to many studies (Feng et al., 2017). This investigation studied the effect of curcumin on the biological behavior of cervical cancer cells and explored the RAS/RAF signaling pathway.

Materials and methods

Cell culture

This study was performed on the cervical adenocarcinoma cell line HeLa (CCL-2™) and the human skin keratinocyte cell line HaCat (RRID: CVCL_0038). The HeLa cell line was cultured in EMEM medium containing 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin, and the HaCaT cell line was cultured in DMEM medium with a similar additives content and grown in a sterile environment at 37°C and in an incubator containing 5% CO_2. The cells used throughout the study were obtained after the 5th passage, and the study was terminated at the latest in at the 15th passage.

MTT assay

One of the enzymatic test methods commonly used to evaluate cytotoxicity is the MTT [3-(4,5-dimethylthiazol–2-yl)-2,5-diphentyltetrazoliumbromide] test. This test measures dehydrogenase enzyme activity, which can convert MTT to the insoluble blue formazan compound. The cell lines were cultivated in 96-well culture dishes with automatic pipettes at an average of 5000/well, and the IC50 doses of DOX and CUR were determined. After one night, DOX was applied at 10-1000 nM and CUR at 10-1000 μM dose ranges at nine different concentrations obtained by serial dilution and incubated for 24, 48, and 72h. In the MTT analysis, the wells on the outside of the plate were excluded to reduce trial error. The MTT test was performed for viability analysis after incubation (Khan et al., 2021).

Wound healing assay

In the study, HeLa cells were seeded in 6-well cell culture plates at approximately 400,000 cells/well. When the cells completely covered the petri dish, a wound was made on the well using a sterile 200 μl volume micropipette tip. Then, serum-free medium containing vehicle, DOX, CUR, and DOX+CUR agents was added to these wells. Cells were imaged at hour 0 using the Thermo EVOS® FL Imaging System. Since the study determined that the wound was closed in 90-100% of the control group, the trial was terminated at the 36th hour and wound healing (cell migration) was photographed in the control and application groups. The value obtained from the vehicle-treated control group was determined as the comparative cell migration rate based on 100% wound healing (cell migration).

Total RNA isolation and cDNA synthesis

HeLa and HaCaT cells were treated with a 242.8 μM CUR active IC50 dose in a cell culture medium and retreated 48 hours later. All cell groups, including the

control group, were subjected to the classic RNA isolation procedure using trizol (Sigma-Aldrich), chloroform, and isoamyl alcohol. The RNA pellet was precipitated with 75% ethanol (96%, v/v) and dissolved in nuclease-free water. cDNAs were synthesized from total RNAs equalized to 1 µg using the cDNA Synthesis Kit (Thermo Fisher Scientific) (Chang et al., 2019).

Quantitative real-time PCR (qRT-PCR)

A qRT-PCR analysis was performed for all genes in triplicate with the QuantStudio™3 Real-Time PCR system (Applied Biosystems). RAF, RAS, and Caspase 3 were evaluated using qRT-PCR (Table 1). A thermal profile followed by melting curve analysis steps were performed at 95°C for 15 min, 40 cycles of 95°C for 15 s, 56-60°C for 30 s, and 72°C for 15 s. The comparative ΔCT method and glyceraldehyde 3-phosphate dehydrogenase (GAPHD) as a housekeeping gene were used to normalize gene expressions (Chang et al., 2019).

Western blotting assay

In the study, HeLa cells were planted in 75 cm^2 culture flasks. When the cells reached the logarithmic phase, vehicle control, DOX IC50: 92.1 nM, and CUR IC50: 242.8 µM were applied individually and in combination. Protein was isolated from the samples 48 hours after the application. Proteins after blotting: B-RAF Antibody (OTI5A9) (Novus bio, CAT no: NBP1-47668), RAS Antibody (JF10-11) (Novusbio, CAT no: NBP2-67097) Beta-actin Antibody (Invitrogen, CAT no: MA1- 140) were treated with specific primary antibodies, then the antibodies were labeled with appropriate secondaries and observed with the Micro ChemiDoc (DNR Bio-Imaging Systems Ltd, USA) gel imaging system. Band intensities were calculated using GelQuant software (Pan et al., 2022).

Statistical analysis

IBM SPSS version 20.0 was used for statistical analysis. Based on delta Ct data, a Student's t-test was performed between control and treatment groups of RAF and RAS expression primers for the analyzed genes.

Results

The cell viability obtained as a result of the MTT test and IC50 values calculated using probit analysis in the HeLa cell line after DOX application are given in Figure 1. In the data obtained, the IC50 value was not found in 24h DOX application in the HeLa cell line. With 48h of DOX application, the IC50 value was found to be 141.2 nM. It was observed that cell proliferation decreased as the dose increased (Fig. 1). IC50 values were not determined by DOX application to HaCaT cells taken as healthy cells for 24, 48, and 72 hours. Decreases in cell proliferation were detected in parallel with the increase in dose (Fig. 2).

MTT, cell viability, and calculated IC50 values in HeLa cells after CUR application are given in Figure 2. As a result of 24, 48, and 72 hours of CUR application, IC50 values were found to be 294.6, 230.2, and 242.8 µM, respectively. Statistical significance between the control group and CUR was calculated using one-way ANOVA. It was observed that with 48-hour CUR application, cell viability decreased significantly after

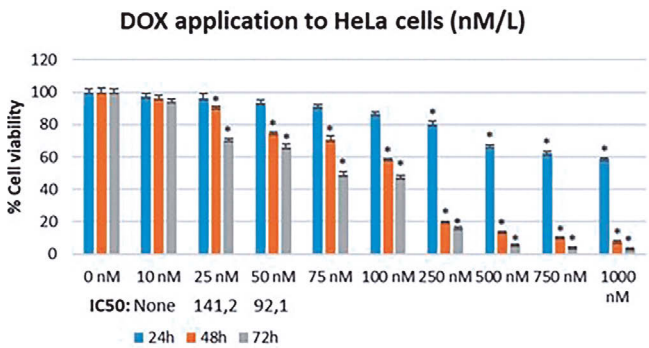

Fig. 1. Effect of DOX at different times and concentrations on the survival rate of HeLa cells.

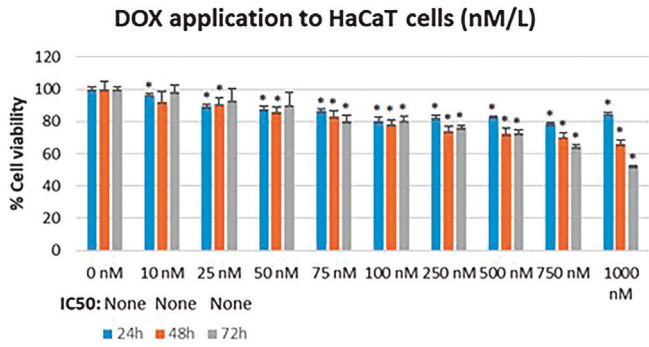

Fig. 2. Effect of DOX at different times and concentrations on the survival rate of HaCaT cells.

Table 1. The expression levels of RAF/RAS genes responsible for cell proliferation and the expression levels of CASP3 genes responsible for the apoptosis pathway in the control and treatment groups of HeLa cells were analyzed by qRT-PCR method. Primers are given in the order 5'-3'.

RAF:	F: CACGAGCGCTGCTCAGATAGC,	R: ACAGGCACAAACACGCACAAA
RAS:	F: TTCATCCAGGATCGAGCAGA,	R: GCAAAGTAGAAGGCAACG
CASP3:	F: GGTATTGAGACAGACAGTGG,	R: CATGGGATCTGTTTCTTTGC
β-Actin:	F: CCTCTGAACCCTAAGGCCAAC,	R: TGCCACAGGATTCCATACCC
GAPDH;	F: CGGAGTCAACGGATTTGGTCGTAT,	R: GCCTTCTCCATGGTGGTGAAGAC

the 100 µM dose (Fig. 3). As a result of 24-hour CUR application, no IC50 value was found in the HaCaT cell series. At the end of 48 and 72 hours, the CUR IC50 value was determined as 584.9 and 280.9 µM, respectively. A significant decrease in the viability of the cells was observed as the dose increased (Fig. 4).

Additionally, a wound scratch healing assay was performed to detect the effect of CUR and DOX on the migration of cervical cancer cells. It was determined that the motility of HeLa cells in the control group covered the area almost completely. While the migration rate of cells was completed at 60% in the DOX-applied group, it was observed that cell migration could occur at around 30% in the CUR and DOX+CUR-applied groups. It was determined that CUR significantly reduced cell migration (Fig. 5).

RAS/RAF/CASP3 expression

In the continuation of the study, 230.2 µM CUR (IC50), 141.2 nM DOX (IC50), and CUR + DOX combined were applied to the experimental groups, determined by MTT analyses and NucBlue staining in Quantitative Real-Time PCR analyses for 48 hours. qRT-PCR experiments were performed on a total of nine samples from all experimental groups. Gene expressions of *RAS/RAF*, which activate important signaling pathways, such as proliferation and cell proliferation, and *CASP3*, which plays a central role in the execution of apoptosis, were normalized according to the expression of β-actin, the internal control gene used in the same sample. *RAS/RAF*, *CASP3*, and *β-actin* gene expression were determined at detectable levels, and amplification curves were created. The amplification curves of these genes were determined with the number of cycles on the x-axis and the Rn value on the y-axis.

Calculations showed a decrease in the amount of *RAS* in the 48-hour control group. *RAS* gene expression was determined at a detectable level only in the group administered DOX for 48 hours. However, no significant difference was detected between DOX and the control group ($p>0.05$). A significant decrease in RAS gene expression was observed in the CUR-treated group compared with the control and DOX-treated groups ($p<0.0001$). A statistically significant difference was detected in RAS gene expression between the control and DOX+CUR and DOX and DOX+CUR groups ($p<0.0001$). No significant difference was detected in RAF gene expression between the control group and other groups ($p>0.05$). It was determined that RAF gene expression showed a significant increase in the DOX

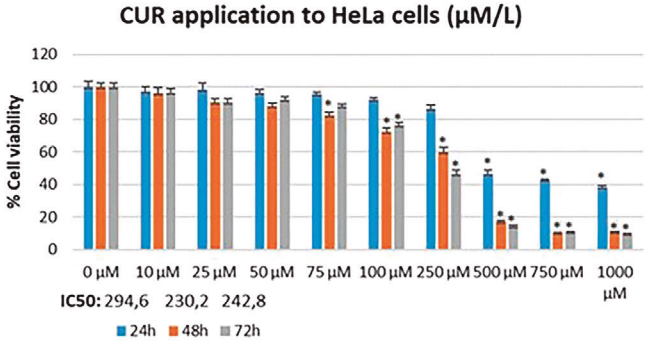

Fig. 3. Effect of CUR at different times and concentrations on the survival rate of HeLa cells.

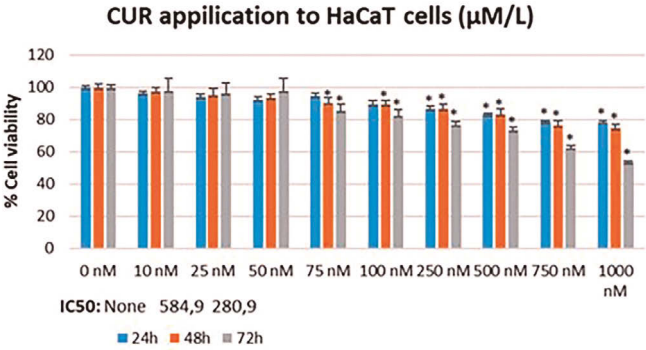

Fig. 4. Effect of CUR at different times and concentrations on the survival rate of HaCat cells.

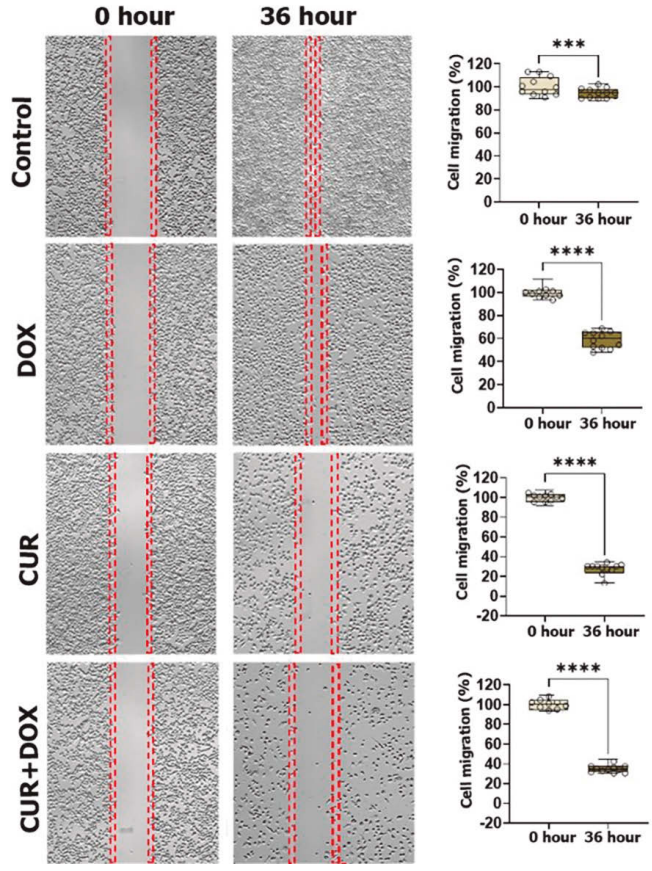

Fig. 5. Cell migration rate and % wound closure determined by the wound healing assay (over 36 hours) in HeLa cervix adenocarcinoma cells. N=12, *$p\leq 0.001$.

group compared to the CUR-only and DOX+CUR groups (p<0.001). CAS3 gene expression was found to be quite low in the control group. A statistically significant increase was observed between the control and experimental groups (p<0.001). Significance was determined as p<0.001 between the control and DOX and CUR groups, and p<0.0001 with the DOX+CUR group. Among the experimental groups, significance was detected as p<0.03 only between the CUR group and the DOX+CUR group. No statistically significant difference was detected in *CAS3* gene expression between other groups (Fig. 6).

Subsequently, we investigated possible mechanisms underlying the inhibitory effect of curcumin on apoptosis and cell proliferation. It was determined that RAS and RAF protein expression, which provide control of cell proliferation and growth factors, was significantly inhibited by 72-hour CUR and DOX application (Fig. 7).

Discussion

Our findings delve into the intricate molecular mechanisms underlying the inhibitory effect of curcumin on cervical cancer cells, focusing particularly on its modulation of the RAS/RAF signaling pathway. Through a comprehensive analysis of our experimental findings, we aimed to elucidate the potential therapeutic implications of targeting this pathway with curcumin in the context of cervical cancer treatment. By dissecting the interplay between curcumin and key components of the RAS/RAF pathway, we can discern how curcumin disrupts critical signaling cascades implicated in cancer cell proliferation, survival, and metastasis. Furthermore, we explore the broader implications of our results, considering the multifaceted pharmacological properties of curcumin and its potential for combination therapy approaches. Ultimately, this discussion serves to contextualize our findings within the broader landscape of cervical cancer research and provides insights into the therapeutic potential of curcumin as a promising candidate for further clinical investigation.

Currently, methods for treating cervical cancer are delayed by numerous problems, such as chemo-resistance, serious side effects, and possible recurrence, as well as a poor therapeutic index. In many malignant tumors, including cervical cancer, the search for effective drugs through phytotherapy has accelerated to discover complementary and alternative medicines (Sun et al., 2022; Zhao et al., 2023). Our findings delve into the intricate molecular mechanisms underlying the inhibitory effect of curcumin on cervical cancer cells, focusing particularly on its modulation of the RAS/RAF signaling pathway.

Cervical cancer is the third most common type of cancer in women worldwide, with increasing prevalence and limitations in treatment (McGraw and Ferrante, 2014). Invasive-type cervical cancer and its associated precursor lesions develop as a result of persistent infections caused by oncogenic HPVs (Arbyn et al., 2014). It is not enough to immortalize and cancerize the host's epithelial cells as a result of HPV infection. Additional cofactors are essential to achieve an immortal phenotype and transform into a malignant or invasive species. Smoking along with HPV increases the risk

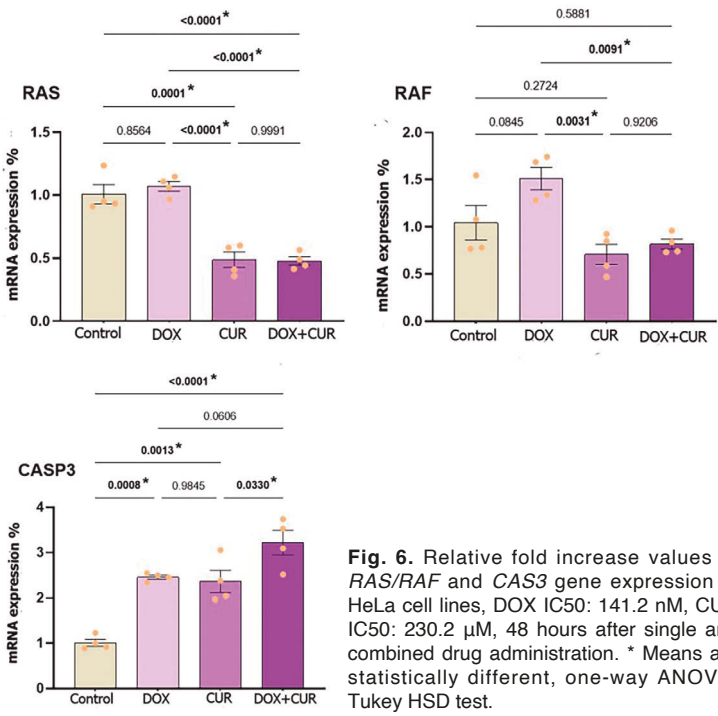

Fig. 6. Relative fold increase values of *RAS/RAF* and *CAS3* gene expression in HeLa cell lines, DOX IC50: 141.2 nM, CUR IC50: 230.2 µM, 48 hours after single and combined drug administration. * Means are statistically different, one-way ANOVA, Tukey HSD test.

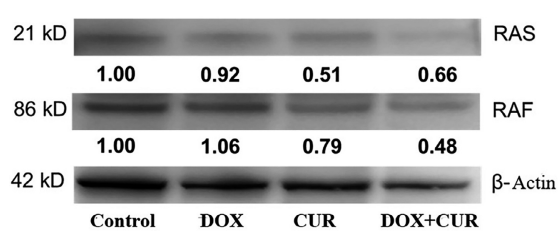

Fig. 7. RAS and RAF protein levels were determined 72 hours after applying single and combination doses of DOX IC50: 92.1 µM and CUR IC50: 242.8 µM to HeLa cells.

factor. It has been reported that the level of carcinogenic substances increases in the cervical mucus of women who smoke (Koliopoulos et al., 2017). There are limitations in traditional treatments (chemotherapy/radiation, drug resistance, and systemic toxicity) in patients with recurrence and metastatic cervical cancer despite treatment (Dueñas-González and Campbell, 2016). Therefore, it is imperative to develop safe and effective natural compounds that are widely used in humans and have fewer systemic side effects than chemopreventive drugs.

It has been reported that natural compounds exhibit anticancer potential against many types of carcinomas with the bioactive substances they contain. Among these, plant-derived bioactive compounds have revealed remarkable effects both pre-clinically and clinically (Gali-Muhtasib et al., 2015). The scientific world accepts flavonoids in plants as a different group of therapeutic substances due to their various pharmacological properties (Jucá et al., 2020). Among these, curcumin stands out with its antimicrobial, antioxidant, anticancer, anti-diabetic activities, and cardiovascular protective properties (Gao et al., 2022). Curcumin has a superior inhibitory effect against cervical cancer cells. It has been observed that this effect is due to the inhibition of RAS and ERK signaling pathways in telomerase activity, cyclin D1, COX-2, iNOS activity, and the mitochondrial pathway (Zaman et al., 2016). In previous studies, they examined the anticancer properties of nano-curcumin made by curcumin and the OA400 nano-carrier applied in various cancers (Montazeri et al., 2016). Thus, studies have shown that curcumin nanoformulation can affect many cellular pathways, including differentiation and cell proliferation (Mortazavi Farsani et al., 2020). Particularly in the context of *in vivo* studies, they revealed that when BALB/c tumor-bearing mice were treated with nano-curcumin, it could reduce the existing tumor size more effectively and efficiently than curcumin (Babaei et al., 2012). Due to the high drug resistance of cervical cancer and the high prevalence of systemic toxicity in traditional chemotherapy and radiation therapy, it is necessary to identify safe, effective, and natural chemopreventive anticancer compounds. For this reason, we planned to reveal how curcumin affects proliferation by revealing its antioxidant properties in the HELA cell line. It should be known that curcumin is a safe and effective natural chemopreventive anticancer compound due to the high prevalence of systemic toxicity to counteract drug resistance in the traditional chemotherapy and radiation treatment of cervical cancer. Therefore, in this study, we investigated how the new curcumin would reveal its potential therapeutic effects on cervical cancer. Curcumin has also been shown in many studies to have target molecules that are more common in cancer cells (Sordillo and Helson, 2015). Through analysis of previous studies, the ability of nano-curcumin to induce cancerous and undifferentiated cell apoptosis was determined in a time- and dose-dependent manner; however, no significant effect on normal cells was observed (Javidi et al., 2019). HeLa cells are derived from a woman's aggressive glandular cervical cancer and have been used in many studies to evaluate cervical cancer treatments (Manzo-Merino et al., 2013). Western blotting from another study revealed that the expression level of cleaved caspase-3 was significantly increased in cells treated with curcumin and paclitaxel compared with that in the paclitaxel ($p<0.01$) and control groups ($p<0.001$) (Dang et al., 2015). In considering a brief definition of apoptosis, it is a genetically determined programmed cell death and has an important place in the development and treatment of cervical cancer (Yu et al., 2018). Apoptosis occurs through two main pathways, the first of which is defined as caspase-dependent (extrinsic) and caspase-independent (intrinsic) pathways (Wu et al., 2018).

Cell signal transduction has a very complex structure and can modulate intracellular signals according to changes in the expression of target genes in the nucleus. Abnormal activations of these signal transduction pathways, which are normally necessary and regulate cellular differentiation, growth, and apoptosis, trigger a series of abnormal biological modifications that lead to malignancy (Shi et al., 2022). Recent research has focused on the molecular biology of cancer cells by targeting signaling pathways. Numerous reports suggest that dysfunction of the RAS/RAF signaling pathway is frequently associated with the occurrence and progression of more than one type of carcinoma. The RAF/MEK/ERK signaling pathway is the best-defined MAPK cascade involved in cell proliferation, differentiation, and survival. Abnormal regulation in this pathway plays a very important role in most cancers (Ullah et al., 2022; Barbosa et al., 2021). This signaling pathway controls cell proliferation and, when activated, accelerates migration, invasion, and metastasis (Pearlman et al., 2017). In our study, CUR and DOX significantly reduced the phosphorylation levels of RAF-1 in the treated HeLa cell line, thus inactivating the RAF/RAS signaling pathway. In contrast, the effectiveness of this pathway was seen in the control group. The activity of the RAF/MEK/ERK signaling pathway plays a redundant role in regulating basic biological processes such as proliferation and survival in many types of cancer, including cervical cancer (Yang et al., 2018; Brandt et al., 2019). However, potential connections between CUR and the RAF/RAS signaling pathway, which mediates the initiation and growth of cervical carcinoma, are unknown. Findings that will contribute to elucidating these connections were obtained.

In conclusion, the results of this study underscore the therapeutic promise of curcumin in cervical cancer management. Importantly, the use of curcumin as a therapeutic agent offers several advantages, including its favorable safety profile, low cost, and wide availability. Unlike conventional chemotherapeutic agents, curcumin

exhibits minimal toxicity towards normal cells, making it an attractive candidate for combination therapy or adjuvant treatment regimens. By targeting the RAS/RAF signaling pathway and other molecular pathways involved in cancer progression, curcumin holds great potential as a novel and effective therapeutic agent for cervical cancer. Future research endeavors aimed at harnessing the full therapeutic potential of curcumin will undoubtedly contribute to the advancement of cancer treatment and improve patient outcomes. It also suggested that curcumin may have a preventive role against doxorubicin toxicity. Curcumin promotes the process of accelerating apoptosis and inhibiting cell proliferation by activating the RAF/RAS signaling pathway in cervical cancer cells.

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Accepted July 17, 2024