Anti-cancer activity of Carnosic acid against cervical carcinoma cell line through Catalase activity and *TGF-β* expression

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Received:9.3.2024 **Accepted: 5.5.2024**

Abstract

Background: Nowadays, the use of herbal medicines is very substantial in the treatment of cancer. One of the most common cancers among women is Cervical cancer. Due to the side effects of some chemical drugs, the use of medicinal plants with minimum side effects and interference has been considered. Rosemary (R. Official L.), a member of the Lamiaceae family, is known for its antioxidant properties. One of the main compounds for antioxidant activity is Carnosic acid (CA), a polyphenol with antitumor biological activities.

Method: This study investigated the effect of Carnosic acid at different concentrations (2.5, 5, 10, 15, 20, 25, and 50) mg/ml on a Cervical cancer cell line (Hela). Cytotoxicity and growth inhibitory power (IC50) were calculated using the MTT test, Prism software. The real-time PCR method investigated *TGF-β1* gene expression on Hela cancer cell lines and cells treated with Carnosic acid. To assess and quantify oxidative stress and apoptotic behavior, we utilized a plate reader and the appropriate kit to measure catalase activity (CAT) level by detecting 570 nm light absorption.

Results: Cytotoxicity assay (MTT) showed the antitumor activity of the group treated with CA compared to the control group (Hela cells without treatment). Gene expression analysis based on RT-PCR showed downregulation of the *TGF-β* gene and catalase activity reduction in cells treated with Carnosic acid compared to untreated cells.

Conclusion: Carnosic acid has been introduced as a promising formula for treating cervical cancer.

Keywords: Carnosic acid, Catalase, Cervical Cancer, HeLa Cell, Oxidative Stress, *TGF-β*

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Introduction

Cervical cancer is the leading cause of death in women worldwide, and researchers have exclusively focused on active and passive therapeutic delivery with little or no side effects on normal cells. (1) In 2020, cervical cancer was the most common cancer among women in the world and the fourth leading cause of death. (2) In 2021, there were approximately 528,000 cases of cervical cancer, with 266,000 deaths (almost 8% of all cancer deaths), which usually develop from precancerous changes within 10–20 years. (3) About 90% of cervical cancer cases are squamous cell carcinoma, 10% adenocarcinoma, and some other cancer types. (4) Infection with certain types of HPV is the major risk factor for this cancer. (5) The risk of contracting this virus is higher in women who have sex with men who have multiple sexual partners and in women who have multiple sexual partners. (6) Other risk factors include HIV infection,(5) smoking, a weak immune system

(7), use of contraceptive pills, and early sexual initiation. (4).

Various therapeutics, including peptides(8,9), proteins (10), and stem cell therapies11, have been developed for the treatment of cancers. Today, medicinal plants and secondary metabolites extracted from them play a role as supplements or chemotherapy agents in cancer treatment. (12) The plants Rosmarinus and Salvia, belonging to the Lamiaceae family, contain Carnosic acid (CA) and other natural compounds, which are currently being widely studied for their potential therapeutic properties against several diseases. (13) CA, found in rosemary and common sage, is a natural benzenediol-beta-ethane diterpene with strong phenolic (catechol) diterpene properties. It is known for its antioxidant and antimicrobial properties. (14)

Antioxidants are crucial for safeguarding our cells against the harmful effects of free radicals. These substances act as protectors, preventing molecules from being compromised or losing their effectiveness. The massive role of antioxidants in combating oxidative damage is a fundamental line of defence for maintaining overall health. (15)

TGF-β (Transforming growth factor-beta) is a dynamic cytokine that plays a multifaceted role in cancer. This intricate involvement is not without controversy, as TGF-β has been shown to act as a tumor suppressor in the early stages but can harm tumor progression as the disease progresses.16 The implications of TGF-β signalling in the development and advancement of cervical cancer are substantial, with its influence on critical factors such as metastasis, stemness, drug resistance, and immune evasion. (17,18)

Catalase, a highly efficient enzyme, plays a multifaceted role in cancer. On one hand, it shields cells from damaging reactive oxygen species (ROS) produced by either internal processes or external factors. On the other hand, it has been found to enhance the growth of cancer cells by reducing catalase activity in various cancer cell types. (19)

For this purpose, CA was used to treat cervical cancer cells (HELA). Antitumor activity was followed by MTT assay and cell cycle analysis. In addition, TGF-β gene expression and catalase activity levels were evaluated.

Material and Methods

chemicals

MTT, RPMI 1640 culture medium, PBS, Trizol solution and chloroform, DMEM, DMSO, and trypan blues dye were purchased from Sigma Aldrich. The catalase activity measurement kit (KCAT96) was purchased from Kiazist, and the cDNA synthesis kit was purchased from ZistVirayesh. Other chemicals and reagents used in this study were of analytical grade.

Cell culture

To culture Hela cells, we utilized Dulbecco's Modified Eagle's medium (DMEM) and RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The incubation conditions were maintained at 37°C with 5% CO2 in a humidified environment (95%). Once the cells reached 85% confluence, they were detached using trypsin (0.25% w/v) and grown in 96-well plates.(20)

To create the initial stock solution of 0.5 mg/ml, Carnosic acid (CAS number: 3650-09-7) was acquired from Sigma-Aldrich Company (Sigma-Aldrich, USA). It was then dissolved in a 1:1 solution of ethanol: PBS (pH=7.2).

Cell Viability Test

MTT assay was used to evaluate the effects of CA on the viability of HeLa cells. (21) Cells were seeded in 96-well plates at a density of 1×10^6 cells/well and then exposed to different concentrations of CA (50 µg/mL, 25 µg/mL, 20 µg/mL, 15 µg/mL, 10 µg/mL, 5 µg/mL, and 2.5 µg/mL) were placed. After 24 hours of exposure, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS) at pH 7.2 ± 0.2. Then, treated and untreated cells were incubated with MTT solution (0.5 mg/ml in

PBS) at 37°C for 4 hours. After that, ten µL of MTT and 100 µL of DMSO were added to each well, and a plate reader was used to measure the absorbance at 570 nm.

Gene Expression by Real-Time PCR

Using the Trizol and phenol-chloroform method, total RNA was extracted from untreated cells (control) and cells treated with CA. The extracted RNA was treated with DNase I enzyme to remove the genomic DNA. After ensuring the quality of the extracted RNA by spectrophotometry and checking the optical absorption at 260/280 nm, the reverse transcription reaction was performed using one microgram of total RNA for cDNA synthesis (Reagent Prime Script TM RT reagent kit, Takara, Japan) in a volume of 20 microliters according to the manufacturer's instructions. To optimize the expression levels of both the receptor gene and internal control gene, we conducted a Real-time PCR reaction using two microliters of cDNA product and customized primers for each gene. To ensure accuracy and precision, we also utilized a SYBR green master mix kit for optimal results.

Gene expression was calculated by relative quantitative method and determining ΔΔCT and using formula $2^{-\Delta\Delta CT}$. GAPDH was used as an internal control gene. Specific primers were designed with OLIGO7 software. To ensure the precision and validity of the created primers (refer to Table 1), we conducted a blast analysis on their sequences through NCBI and Gene Runner. Furthermore, we utilized REST software to accurately gauge their relative expression levels.

Table 1: Primers designed to measure *TGF-β* gene expression in RT-PCR reaction.

Gene name	Forward sequence	Reverse sequence
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
TGF-β	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA

Oxidative Stress Marker Estimation

Twenty microliters of the cell sample were lysed and prepared to measure catalase enzyme activity. Methanol and H2O2 were poured into the microtube and shaken gently. The standard solution was added and incubated for 20 minutes away from light. The reaction was stopped using potassium hydroxide, and the chromogen reagent was added and vortexed. After 10 minutes, Potassium iodate was added and centrifuged at 10,000g for 10 minutes, and at the end, the absorbance of the samples was read at 540 nm.

To determine lipid peroxidation levels, researchers utilize a standard solution that is carefully prepared using varying concentrations. It was calculated using the standard absorption curve of the samples. Using Excel software and based on the absorption of standards, the standard curve was drawn, and the concentration of the samples was calculated using the following formula.

Statistical Analysis

The statistical analysis of the results was done using GraphPad Prism software, one-way ANOVA, and Tukey tests as well as the non-parametric Mann-Whitney test. A significant level of was considered $P<0.05$. Pearson's correlation test was used to check the correlation between these two factors.

Results

Cell Viability

The phase contrast images of the cells were taken with a microscope and subjected to precise and detailed morphological analysis. Our analysis revealed a distinctive elongated appearance, characteristic of cells from epithelial tissue (Figure 1).

The IC50 values of CA against Hela cells were 9.85 µg/ml, and the MTT assay showed a significant decrease in the viability of Hela cancer cells after CA treatment (Figure 2). The obtained results compellingly demonstrate that CA is toxic for cervical cancer cells.

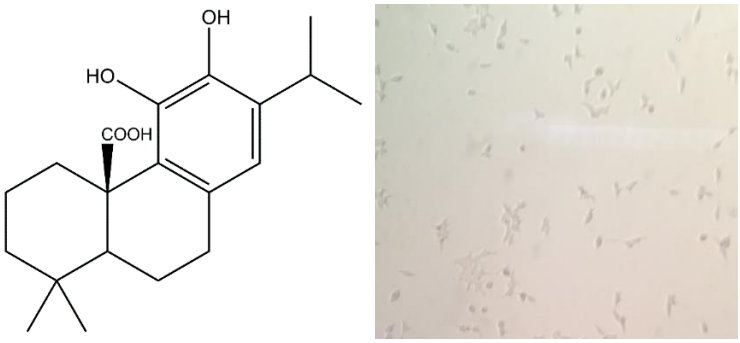

Figure 1: On the left, is the chemical structure of CA, and on the right is the phase-contrast Image of HeLa cells.

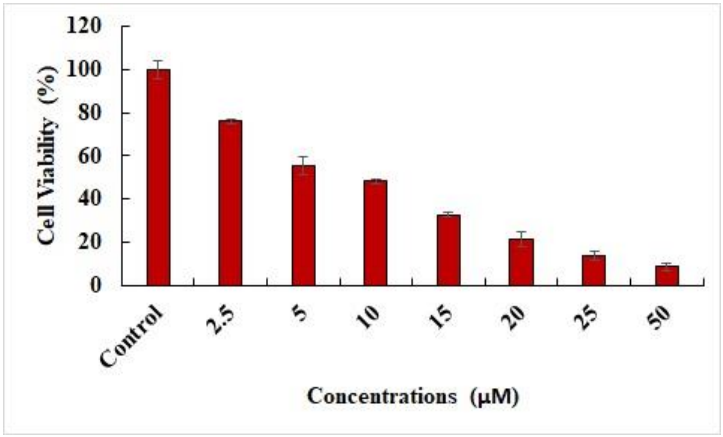

Figure 2: Viability of Hela cells after treatment with CA with p<0.05

Gene Expression

Evaluating the expression of the *TGF-β* gene in Hela cells revealed that the level of *TGF-β* gene expression in the cells treated with 10 µg/mL of CA has decreased compared to the control group (Hela cancer cells by 40%. Statistically, significance is as high as P<0.05) (Figure 3).

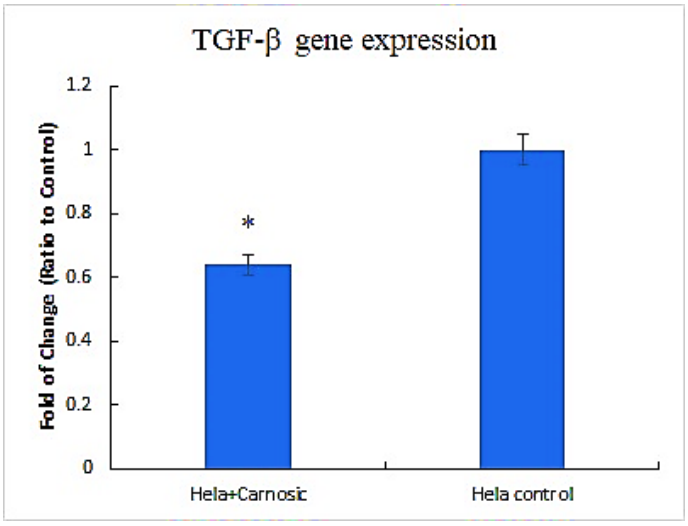

Figure 3: Quantitative expression of the TGF-β gene in a cervical cancer cell line (Hela) in the absence and presence of X mg/ml of Carnosic acid. (* has a significant difference at P<0.05).

RT-PCR response and efficiency were determined by examining melting curve plots. The distinct, singular peaks observed in the reaction for both GAPDH and TGF-β genes provided compelling proof of the reaction's targeting of the chosen genes (Figure 4).

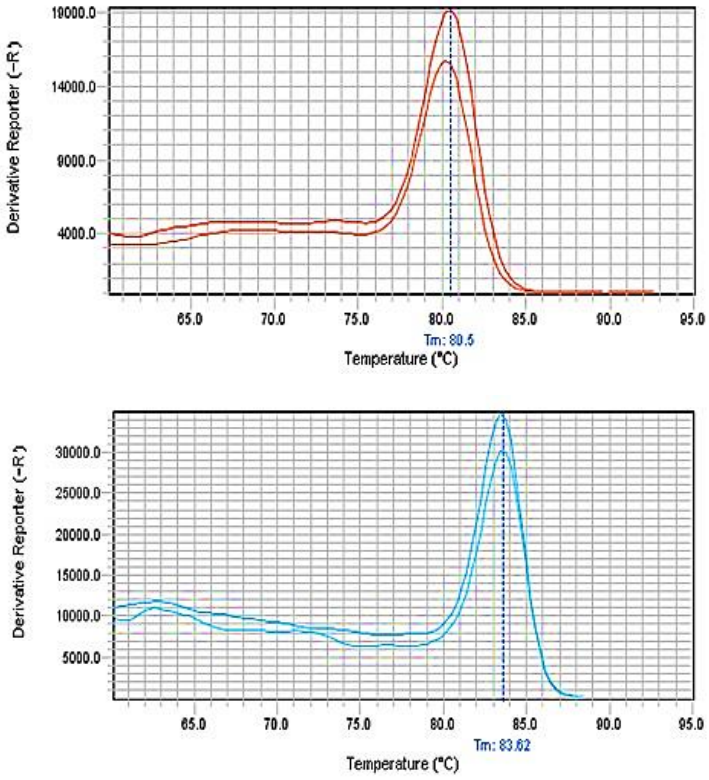

Figure 4: A) The melting curve of the GAPDH gene at 80.5°C is shown. B) The melting curve of the TGF-β gene at 83.62°C is shown.

(CAT) activity assay

The analysis showed that the CAT level of cells treated with CA was significantly decreased at the level of P<0.01 (Figure 5).

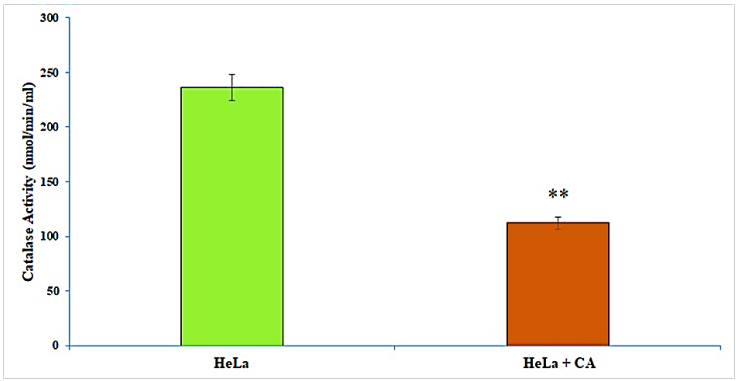

Figure 5: Investigating CAT changes. (* has a significant difference at P<0.05 ** has a significant difference at P<0.01 and * has a significant difference at P<0.001).**

Discussion

In today's society, the prevalence of cancer, particularly in developing nations, is on a troubling rise, resulting in a sharp increase in mortality rates. Therefore, more and more attention is being paid to alternative research treatments and medicines to provide more effective treatment for patients suffering from cancer, and this is essential as many of the current treatments have severe and detrimental side effects consequences. (22)

The rosemary anticancer properties have been studied in several types of cancers. (23) CA, the major derivative of rosemary, is associated with various biological effects, including antioxidant effects, epigenetic modification, anti-inflammatory effects, and immune system regulation. (13) Our study revealed that CA possesses potent anti-cancer properties, resulting in a noteworthy decrease in the growth of Hela cancer cells which aligns with previous research.

Wang et al.'s (2012) studied antibacterial and anticancer features of Rosmarinus officinalis L. essential oil and its three main components, specifically 1,8-cineole (27.23%), α-pinene (19.43%), and β-pinene (6.71%), They showed that the oil exhibited remarkable cytotoxic effects against three human cancer cell lines, namely SK-OV-3, HO-8910, and Bel-7402, with 50% inhibitory concentrations (IC50) of 0.025%, 0.076%, and 0.13% (v/v) respectively. These findings illustrate the promising

medicinal properties of Rosmarinus officinalis L. and its potential for further research in the field of cancer research. (24) Cell-based analysis showed a significant effect of CA on HeLa cells. IC50 of CA significantly induced apoptosis in cancer cells and showed a considerable decrease in cell viability after treatment with CA. It was found that the essential oil of Rosmarinus officinalis exhibited efficacy in anticancer tests compared. The potency of an essential oil is directly related to its concentration, suggesting a possible synergistic effect of the ingredients. This is consistent with our obtained results, in which of particular relevance is the characteristic elongated appearance of Hela cells. The distinct appearance of this tissue type aligns with that of epithelial tissue, the source of Hela cells. The concentration at which CA inhibits cell growth by 50% is indicated by the computed IC50 value of 9.85 µg/ml for CA against Hela cells. This value gives substantial information about how well CA suppresses the capacity of Hela cancer cells to proliferate. CA may be an effective therapeutic agent against Hela cancer and has a considerable antiproliferative impact.

Rosmarinus Officinalis essential oils (EO), including CA, tested by Contini *et al*, showed weak or no cytotoxic and genotoxic effects. This study showed that all EOs tested had little or no estrogenic activity, while some EOs had significant antiestrogenic effects. (25) On the other hand, our study suggests that CA has antiproliferative activity on Hela cancer cells; this discrepancy could be because CA concentration in our experiments is much higher than in their EO. CA may have regulatory effects on the expression of hormone receptors in cancer cells.

Remarkable progress has been made in treating patients with cervical cancer as well. This specific form of cancer is caused by the human papillomavirus (HPV) and can be successfully avoided by addressing precancerous growths and vaccinations against HPV. The essential oil of Rosmarinus officinalis has shown great potential in inhibiting the growth of HeLa cells with an impressive IC50 of 0.011. (26) Our investigation revealed compelling evidence that CA has a potent inhibitory effect on Hela cancer cell growth, with an IC50 of 9.85 µg/ml (Figure 2).

We have shown that CA suppresses the HeLa's TGF-b TGF-expression by 40 % (Figure 3). This observation could be one of the reasons for the antiproliferation effect of CA on the cells (Figure 2). The results of the present study show that CA in Hela cancer cell lines reduces the expression level of the *TGF-β* gene in the HeLa cell line treated with CA compared to cancer cells, which indicates that microRNAs may be able to regulate *TGF-β* signalling pathways, including targeting Smad proteins and other signalling molecules.

Pockel *et al.* (2008) demonstrated the other facet of CA; its anti-inflammatory feature. powerful inhibitory effects of CA and carnosol (CS) on human 5-lipoxygenase, a key enzyme involved in the production of pro-inflammatory substances. Their study also revealed that these compounds effectively suppress inflammation in stimulated human polymorphonuclear leukocytes, implying their potential as promising anti-inflammatory agents. Moreover, the authors discovered that CA and CS activate the peroxisome proliferator-activated receptor-gamma (PPAR-γ), indicating their ability to regulate inflammation at the genetic level. These findings provide conclusive evidence for the anti-inflammatory properties of CA and CS and suggest their potential use in treating inflammatory conditions. (27)

Recent studies have demonstrated that CA, has the potential to impede catalase function, resulting in an accumulation of hydrogen peroxide and ultimately increasing oxidative stress and causing cell death in different cancer cell types, such as Hela cells. (28)

The level of CAT activity, which designates an oxidative stress marker, also decreased in the Hela cell line treated with CA. The results of the present study show that CA in Hela cancer cell lines reduces the expression level of the *TGF-β* gene in the HeLa cell line treated with CA compared to cancer cells, which indicates that microRNAs may be able to regulate *TGF-β* signalling pathways, including targeting Smad proteins and other signalling molecules. The level of CAT activity, which recommends oxidative stress, also decreased in the Hela cell line treated with CA.

Furthermore, research has demonstrated the ability of CA to impact the function of enzymes involved in protecting against oxidative damage and eliminating toxins, such as NQO1.(29)

Xiang, et al, studies have demonstrated the ability of CA to trigger apoptosis in several types of cancer cells, such as HepG2 cells, by activating the mitochondrial pathway through the production of reactive oxygen species (ROS). (30)

The notable decline in CAT levels found in Hela cells treated with CA suggests a decrease in the antioxidant ability of the cells. The enzyme known as catalase, or CAT, is in charge of detoxifying reactive oxygen species (ROS) like hydrogen peroxide. The drop in CAT levels raises the possibility that CA therapy may worsen oxidative stress in the cells by upsetting the equilibrium between the generation of ROS and antioxidant defence systems. Increased cellular damage and decreased cell survival could result from this disruption.

Conclusion

Our study suggests that CA affects various cellular processes by influencing Hela cells. The significant decrease in cell viability and alterations in *TGF-β* gene expression and CAT levels indicate that CA could potentially act through various pathways, such as inducing apoptosis (programmed cell death), inhibiting cell proliferation, and affecting oxidative stress and antioxidant defence mechanisms. Further investigations could delve into the precise molecular mechanisms underlying these effects.

Assessing the efficacy and potency of CA about existing therapies can provide insights into its potential as a standalone treatment or as a complementary approach to existing treatment modalities for Hela cancer.

The research findings presented the IC50 value of CA against Hela cells as 9.85 µg/ml. A range of CA concentrations and evaluating the effects on cell viability, gene expression, and antioxidant capacity can help determine the optimal dosage and potential side effects or cytotoxicity associated with CA treatment.

Authors contribution

YBM: Investigation, Validation, Writing – original draft. AN: Data curation, Investigation, Validation, funding acquisition, Writing & Editing. NZ: Formal analysis, Writing, Investigation & review.

Funding

The Science and Research Branch of Islamic Azad University supported this work. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data Availability

All data generated or analyzed during this study are included in this manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement and disclaimers

The authors wish to thank the technical staff of the Zakaria-e-Razi Laboratory Complex, Science and Research Branch, Islamic Azad University for their cooperation and assistance throughout the research.

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