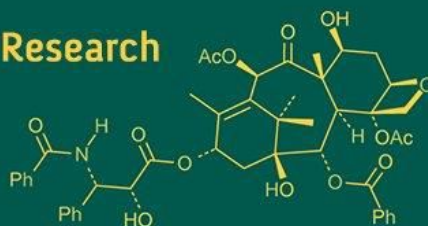


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Investigating the effect of carnosic acid on VEGF gene expression and lipid peroxidation in cervical cancer cell lines

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Abstract

Carnosic acid is a triterpene natural polyphenol found in various plant extracts, mostly in rosemary, displaying various types of bioactivities. The present paper was conducted to explore the anticancer activity of carnosic acid on the HeLa cervical carcinoma cell line and to analyze the possible mechanisms involved in the activity. The cytotoxicity of carnosic acid against HeLa cells was examined in this experiment using the MTT assay. Real-time PCR was used to analyze the expression level of the vascular endothelial growth factor gene, a potent mediator in the pathogenesis of angiogenesis. Further, a lipid peroxidation assay was conducted to investigate how carnosic acid acts in the modulation of oxidative stress levels as represented by the amount of malondialdehyde expressed. In the MTT assay, carnosic acid exhibited an IC_{50} value of 9.85 μ M in HeLa cells, reflecting a moderate cytotoxic potency. Real-time PCR analysis showed a significant 50% decrease in VEGF gene expression following carnosic acid treatment, suggests that the cancer cells' angiogenic signaling may be modulated. Furthermore, the lipid peroxidation assay demonstrated a significant reduction in MDA levels in cells treated with carnosic acid (47.2 nmol/ml) compared to untreated controls (66.06 nmol/ml), indicating modulation antioxidant properties and a protective effect against oxidative stress. This study contributes to the anticancer potential of carnosic acid on HeLa cervical cancer cells, which seems to manifest through a complex strategy that encompasses direct cytotoxic effects, inhibition of the crucial angiogenic factor, and redox regulation within cancer cells.

Keywords: Carnosic acid, Hela cells, cervical cancer, cytotoxicity, angiogenesis, oxidative stress, VEGF, Malondialdehyde (MDA), natural compounds, anticancer agents

Introduction

Cervical cancer: Cervical cancer refers to a malignancy that occurs in the cervix, the lower part of the female reproductive organ called the uterus, which connects the upper part to the vagina. Cervical cancer constitutes a major public health problem, especially in the developing world, as it ranks as one of the most prevalent lethal cancers among women ^[1]. Human Papillomavirus (HPV) is the main causative agent of cervical cancer, and it is a sexually transmitted virus that infects the epithelial cells lining the cervix. There have been identified well over 100 different versions of the Human Papillomavirus, some of which have been labeled high risk or oncogenic because they have been observed to cause epithelial cell changes which, should the infection lead to cancer, might result in the development of cervical cancer. Of these, HPVs-16 and-18 have been identified as the most prevalent high risk versions and are responsible for up to an estimated 70% of the world's total incidents of cervical cancer ^[2]. Persistent infection by high risk versions of the Human Papillomavirus leads to the integration of the DNA virus into the host cell's genetic pool. This upsets the regular regulation and normal gene expression of the Human Papillomavirus and leads to a persistent production of the virus's E6 and E7 proteins, both of which act to disrupt tumor suppressor proteins p53 and the Retinoblastoma protein (pRb). E6 increases the turnover of p53, suppressing cell cycle arrest and apoptosis, while E7 transcriptionally blocks the action of the Retinoblastoma protein, causing cells to aggressively proliferate and develop into cancer cells ^[3]. In addition to infection by the Human Papillomavirus, other risk factors for the development of cervical cancer include factors of an economic and healthcare-related nature.

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Those women who belong to the lower economic strata also have fewer opportunities and access to the regular screening tests and human vaccinations offered to protect women's populations against the threat of cervical cancer development [3]. Furthermore, reproductive factors including young maternal age at the time of first pregnancy, parity, and hormonal imbalances have been shown to influence the development of cervical cancer. During pregnancy, the body undergoes changes that may affect the vulnerability of cells to HPV infection or the development of lesions [4].

Materials and Methods

Field experiments

Cell Line and Culture Conditions

The HeLa line of cells, which is the first immortal line of human cells ever grown, was used as the model system within this experiment. Initially derived from the biopsy of cervical adenocarcinoma within the year 1951, the fact that these cells are robust and well characterized indicates that HeLa cells are the model of choice within experiments conducted on cervical carcinoma [4, 5, 6]. Table (1) summarizes key materials.

Table 1: Key Materials and Equipment Used in the Study

Item	Company	Country
HeLa Cell Line	National Center for Genetic Resources of Iran	Iran
DMEM Culture Medium	Gibco™ (Thermo Fisher Scientific)	USA
Fetal Bovine Serum (FBS)	Gibco™ (Thermo Fisher Scientific)	USA
Penicillin-Streptomycin	Sigma-Aldrich (Merck)	Germany
Trypsin-EDTA	Gibco™ (Thermo Fisher Scientific)	USA
Carnosic Acid	Sigma-Aldrich (Merck)	Germany
MTT Reagent	Sigma-Aldrich (Merck)	Germany
TRIzol Reagent	GeneAll Biotechnology Co., Ltd	South Korea
cDNA Synthesis Kit	SinaClon Bioscience	Iran
SYBR Green Master Mix	Ampliqon A/S	Denmark
CO ₂ Incubator	Memmert GmbH + Co. KG	Germany

The MTT assay is a well-recognized colorimetric technique used to measure the viability of cells. The technique works based on the activity of mitochondrial dehydrogenases, which are specific to living cells. The enzyme preferentially breaks the tetrazolium ring of the yellow-colored reagent, resulting in the formation of a purple, insoluble formazan compound. The amount formed is directly proportional to the number of viable cells. Methodology: Cells were plated at a density of 1×10^4 cells per well in a 96-well plate. Cells were allowed 24 hours for attachment before being treated with the serially diluted dose of carnosic acid. After 48 hours, the treatment medium was replaced by the MTT solution. After 4 hours, the formed formazan crystals were dissolved in DMSO, and the absorbency was read at 570 nm. The IC₅₀ concentration curve was obtained by plotting the cell viability versus the logarithm of the concentration of the drug.

Specific Techniques for Gene Expression Analysis Using Real-Time PCR. The choice of real-time PCR was made due to its high sensitivity and specificity for gene expression analysis, particularly for mRNA measurement [9, 10, 11]. Isolation and Purification of RNA. The isolation of the total RNA was performed for both untreated and carnosic acid-treated cells using the phenol-chloroform-based technique TRIzol, which is a technique with high specificity for the

isolation of total RNA with the highest purity [5, 12]. The purity of the RNA sample was measured by spectrophotometry, A260/A280. cDNA Synthesis. The synthesis of cDNA was performed using one microgram of purified RNA. The reaction was carried out using oligo(dT) primers that selectively hybridize to the poly(A) stretches located on the tails of mRNA, thus creating a virtually full-length cDNA library that preferentially represents protein-coding gene transcripts [5, 13]. qPCR and Data Analysis. Real-Time PCR was carried out using SYBR Green I, which selectively intercalates and stains double-stranded DNA. As the reaction proceeds, the fluorescence signal, which increases proportionally to the amount of amplified DNA, was generated [9]. The expression ratio of the target gene (VEGF) was assessed by referring the expression to the expression ratio of the endogenous control gene (GAPDH). The relative gene expression measurement was further analyzed using the $2^{-(\Delta\Delta Ct)}$ model, comparing the Ct values of the target gene in the sample and the standardized Ct value from the untreated sample, which has been normalized using the housekeeping gene. A definitive quality assessment step involved the melt curve analysis, which was accomplished after the completion of the experiment to ensure product specificity [9, 14]. The gene-specific primers used are listed in Table (2).

Table 2: The gene-specific primers used in this research

Gene name	Forward sequence	Reverse sequence
VEGF	TTGCCTTGCTGCTCTACCTCCA	GATGGCAGTAGCTGCGCTGATA
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

The results obtained from the $2^{-(\Delta\Delta Ct)}$ analysis are generally accepted to be used in the level of gene expression. The study was done according to the manufacturer's guidelines, using the reaction mix of the following reagents: MDA Lysis Buffer, BHT (100X), TBA Solution, Phosphotungstic Acid, MDA Standard (6.08 M), Clean 96-well plates [5, 12, 13]. Reaction reagents enabled the

measurement of oxidative stress parameters and gene expression in the HeLa cells.

Statistical Analysis

All quantitative data were derived from three independent experiments and presented as the mean $\pm$ standard deviation. The statistical significance of the differences between the

control and treated groups was determined using a one-way analysis of variance (ANOVA). A p-value < 0.05 was set as the threshold for statistical significance.

Results

The cytotoxicity effect of carnosic acid on the HeLa cell line of cervical cancer was determined using the MTT assay. As can be noted in Figure 41, the treatment of the cells with carnosic acid at different concentrations led to the reduction of their viability in a dose-dependent manner. The IC₅₀ concentration calculated was 9.85 µM, which was effective in inhibiting 50% of the viability of the cells used in the experiment [4, 15].

Real-time PCR results showed that HeLa cells treated with carnosic acid had significantly lowered vascular endothelial growth factor (VEGF) gene expression compared to the

untreated cells. Based on the graph shown in Figure 42 above, the decrease in VEGF gene expression was approximately 50% compared to the control and this was statistically significant at <0.05 significance level [15, 9, 16].

The levels of lipid peroxide were determined by the concentration of malondialdehyde (MDA). The concentration of MDA was estimated both before and following treatment of the HeLa cells with carnosic acid. The study indicated that the concentration of MDA for the HeLa cells prior to the treatment and the concentration following the usage of carnosic acid was 66.06 nmol/ml and 47.2 nmol/ml, respectively. The difference was significant because the significance level was less than 0.05 [12, 5, 13] as show in (Table 3).

Table 3: Consolidated Summary of Key Quantitative Results

Experimental Aim	Parameter Measured	Key Result	Statistical Significance
Cytotoxicity	Cell Viability (MTT Assay)	IC ₅₀ = 9.85 µM	N/A (Calculated value)
Anti-Angiogenesis	VEGF Gene Expression (qPCR)	~50% Decrease vs. Control	p<0.01
Antioxidant Effect	Lipid Peroxidation (MDA Assay)	Control: 66.06 nmol/ml Treated: 47.2 nmol/ml	p < 0.05

Discussion

The challenge facing treatments of cervical cancer is a number that needs to be answered as it is a major global health issue. Currently, cervical cancer remains a feared disease even though technological advancements have improved methods used in its detection and treatment, particularly in developing countries where treatment or preventive programs are not readily available. The major challenges that make it hard to treat cervical cancer include those that follow, according to [1]. Late diagnosis, Delayed Diagnosis Delayed diagnosis is one of the most significant hurdles in the treatment of cervical cancer. Many cervical cancer diagnoses are made when the cancer has metastasized from the cervix to the neighboring or distant organs. Late diagnosis occurs for various reasons such as the absence of knowledge or access to cancer-screening programs. Additionally, the absence of symptoms in the initial stages of the cancer can lead to a late diagnosis. Advanced cancer requires severe treatment strategies, leading to mortality rates and morbidity. This is the case in the treatment of cervical cancer [17].

Restricted accessibility of cervical screening/diagnostic facilities: Access to effective screening/diagnostic facilities is essential in the early diagnosis and prevention of cervical cancer. Unfortunately, accessibility of treatments such as the Pap smear and HPV test may be restricted or unavailable to the poorer countries of the world. It is thus important to note that the lack of accessibility of these facilities may result in the high incidence of cervical cancer being rampant within these countries [18]. Socioeconomic and cultural constraints. It is believed that there may be significant influence within the treatment of cervical cancer among these populations. Stigma or lack of information about cervical cancer may be prevalent within these areas. Access to health facilities may also be rife within these areas [19].

Conclusion

The current study offers the first in-vitro proof supporting the prominent anticancer properties of carnosic acid against cervical cancer. Carnosic acid possesses measurable cytotoxicity by inhibiting the following: direct inhibition of

the viability of cervical cancer cells by IC₅₀ = 9.85 µM; carnosic acid reduced the expression of VEGF mRNA, implying the modulation of angiogenic pathway. Carnosic acid is a potent antioxidant and profoundly decreases lipid peroxidation. The identification of this integrated, tri-modal mechanism of action qualifies carnosic acid as one of the most promising natural compounds for development into a novel therapeutic approach against cervical cancer.

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