The anticancer molecular mechanism of Carnosol in human cervical cancer cells: An *in vitro* study

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Abstract. Carnosol, a phenolic diterpene, is one of the effective anticancer agents naturally occurring in rosemary, sage, parsley, and oregano. The chemoresistance problem increased with the routinely used chemotherapy. Therefore, the efforts to find a substitute with safe and low cost have become crucial worldwide. The current study attempts to inspect the anticancer molecular mechanisms of Carnosol on modulating up- and down- regulation of multiple genetic carcinogenesis pathways. The cytotoxicity of Carnosol on Hela cells was evaluated by MTS assay. Flow cytometry was used to assess apoptosis and cell cycle arrest. The apoptotic morphological changes were obvious by dual apoptosis assay. The differential gene expression after treatment with Carnosol was investigated by qRT-PCR. Up to 80% of the treated cells with Carnosol IC₅₀ underwent apoptosis. Apoptosis together with cell cycle arrest in G₀/G₁ phase were induced significantly after treatment with Carnosol IC₅₀. Fifteen out of nineteen genes studied were found to be remarkably up- or down- regulated after treatment with Carnosol. Six up-regulated genes (EREG, FOS-2, ID2, CRYAB, DUSP5, and TICAM2) and nine down-regulated genes (FN1, KRAS2, CCNB1-1, FEN1, MCM4, MCM5, GTSE1, CXCL1, and RALA) were recorded. These genes are candidates for future research for elucidating anticancer molecular targeted therapies, cancerous signaling and cancer development pathways in cervical cancer; moreover, elucidating the role of apoptosis, inflammation, cell proliferation, and cell differentiation in the development of cervical cancer.

Keywords: Carnosol, Hela cells, anticancer molecular therapy, phenolic diterpene, carcinogenesis pathways

INTRODUCTION

The fourth most common type of cancer among the female population is cervical cancer. It has become a health problem in term of morbidity and mortality worldwide (Pfaffenzeller *et al.*, 2020, Keinan Boker *et al.*, 2020). A recent epidemiological study by the American Cancer Society estimated that mortality rates in low- and middle-income countries were 2 fold higher for cervical cancer during 2012-2016 (Siegel *et al.*, 2019, Keinan Boker *et al.*, 2020).

In Iraq, 244 women are diagnosed with cervical cancer every year and about 159 die from the disease. Moreover, cervical cancer is the 10th most common cancer ranks among women between the age of 15 and 44 years in Iraq (Al-Mussawy, 2019; Alwan, 2017). In contrast, the 3rd most common cancer in Malaysian women is cervical cancer with more than 2,000 records every year and over 600 deaths annually (Seng *et al.*, 2018). The increased incidence of resistant cancer cells along with the high cost of the routinely used

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therapies upsurge the need for a cheap source of therapy. The anticancer activity of different compounds isolated from plants was highlighted by several studies (Hafidh *et al.*, 2018, Hafidh, 2017). Carnosol is a naturally occurring phenolic diterpene, obtained from rosemary, sage, parsley, and oregano; it was shown to have antioxidant and anticarcinogenic properties (Johnson, 2011, de Oliveira *et al.*, 2019). The anticancer property of Carnosol has been evaluated in diverse types of cancers like prostate, breast, skin, leukemia, and colon cancer (Johnson, 2011). The molecular mechanism of the anticancer potential of this phytochemical compound has not yet been fully clarified. For cervical cancer, although many studies were conducted on the gene expression profiling and some of these studies ended with fruitful therapies, yet many molecular target therapies are still in need for this highly malignant cancer. Nineteen genes (*IL6*, *TNF- α* , *DDIT3*, *MKI67-2*, *EREG*, *FOS-2*, *ID2*, *CRYAB*, *DUSP5*, *TICAM2*, *FN1*, *KRAS2*, *CCNB1-1*, *FEN1*, *MCM4*, *MCM5*, *GTSE1*, *CXCL1*, and *RALA*) were screened to test Carnosol effect on their expression. The chosen genes were shown by previous studies to be related to the development of cervical cancer and other types of cancer (Hao *et al.*, 2020; Babapour *et al.*, 2019; Lin *et al.*, 2018; Clarke *et al.*, 2019; Stintzing *et al.*, 2018; Wang *et al.*, 2019; Mirzaei *et al.*, 2020; Roszik *et al.*, 2018; Ponzio *et al.*, 2017; Kori & Yalcin Arga, 2018; Slattery *et al.*, 2018; Dun & Gao, 2019; Li *et al.*, 2019; Kim & Park, 2014; Zago *et al.*, 2018).

MATERIALS AND METHODS

The compound and Hela cells preparation

An absolute dimethyl sulfoxide (DMSO), (BIO BASIC INC., NY, USA) was used to dissolve Carnosol, C9617-5MG, (Sigma, Germany). The stock concentration, 5mg/ml, was incubated at -20°C for further needed analyses. RPMI-1640 medium/ L- glutamine (biowest, Florida, USA) with 10% fetal bovine serum (Sigma, Germany), 2.5 µg/ml amphotericin B (biowest, Florida, USA), and 50 U/ml penicillin-streptomycin (biowest, Florida, USA) were used to cultivate Human cervix adenocarcinoma cells (Hela cells, ATCC: CCL-2) at 37°C with 5% CO₂.

MTS cell viability assay

The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA) was used to measure Carnosol-treated cells viability. The viability was measured by MTS [3-(4, 5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium] metabolism in active cells. Freshly prepared Carnosol working concentrations of (5, 10, 20, 40, 50, and 80 µg/ml) were diluted in 10% RPMI-1640 medium. Each concentration was used to treat Hela cells (1×10^5 cell/well) with 200 µl/well as a final volume. The plates were incubated for (24, 48, and 72 h) in 5% CO₂ at 37°C. DMSO in 10% RPMI-1640 medium was used as negative control with the same volume. At the end of the incubation times the wells' contents were replaced with a solution composed of 10% RPMI-1640 medium (100 µl) and MTS (20µl). An ELISA reader (Sunrise Basic Tecan, Grödig, Austria) was used to read the absorbance at 490 nm, after 4 h.

The amount of 50% killed cells (IC₅₀) by the compound concentration was calculated (Cory *et al.*, 1991).

Cell treatment

Based on the IC₅₀ value (50 µg/ml) obtained from the MTS assay, 1×10^5 Hela cell/well were treated with 10% RPMI-1640 medium with or without Carnosol for 48 h. DMSO in the medium was used as a negative control. After the incubation time was ended, Carnosol-treated Hela cells and untreated control were harvested and washed. The cells were subjected to the following bioassays.

Flow cytometry analysis

The apoptosis level of cells and cell cycle arrest was analyzed. Ice-cold ethanol 70% with PBS was used for the fixation of treated and untreated cells (1:10 v/v). Incubation was for 2 h at -20°C. Fixed cells were washed by PBS. The following staining DNA solution (500 µl) composed of propidium iodide (25 µl) as 1 mg/ml (MP Biomedicals, LLC, IIIKrick, France) and Ribonuclease A (50 µl) as 1 mg/ml (Sigma, Germany) in PBS was prepared (Siddik, 2010). Measurement was done using CyAn ADP apparatus (BECKMAN COULTER, USA) as triplicates of each sample and control

(untreated cells). Analysis of the results was completed by the software Summit (V4.3).

A real-time apoptosis detection

Apoptosis Assay Kit (Dual), NucView™ 488 (Biotium Inc, USA) was used along with caspase-3 substrate and sulforhodamine 101 (Texas Red) conjugated Annexin V. It provides two apoptosis events in intact cells in real-time, caspase-3 activity and phosphatidylserine translocation. The procedure followed the manufacturer's instructions and it was as follows: treated and untreated cells were washed with Annexin V-binding buffer after an aspiration of the wells' contents. Then the following materials were added: 0.2 mM NucView™ 488 caspase-3-substrate (5 µL), Annexin-binding buffer (100 µL), and sulforhodamine 101-annexin V (5 µL) to each well and incubated for 45 minutes. The treated and untreated cells were run as triplicates. Fluorescence microscope (Nikon eclipse 80 i) using FITC and Texas-Red filters, was used to observe the apoptotic events in the stained cells. The apoptotic index has resulted from apoptotic cells' number relative to the total number of cells.

RNA extraction

Total RNA from the harvested cells (treated and untreated) was isolated according to GF-1 kit (Vivantis Technologies, Malaysia). A concentration of RNA (546 ng/ul) was measured by Life Science UV/Vis Spectrophotometer (BECKMAN COULTER, USA). The total RNA quality, concentration, purity, and integrity were checked by Agilent 2100 Bioanalyzer (Agilent, USA). The integrity number of total RNA was 8.5. Later, -80°C temperature was used to store the isolated and checked RNA.

Gene expression profile using real-time semi-quantitative RT-PCR

The cDNA Synthesis Kit (BIO-RAD, Hercules, Canada) was used to convert the isolated RNA from Hela cells (0.5 µg) to cDNA. Briefly, a final volume of 20 µl per reaction was prepared by mixing 5× iScript reaction mix (4 µl) with iScript reverse transcriptase (1 µl) and RNA template (15 µl). Thermo Bath (FINEPCR, Seoul, Korea) was used to incubate the prepared mixture for 5 min at 25°C then 30 min at 42°C. Later, the temperature of incubation was raised to 85°C for

5 min. A temperature -80°C was adjusted to store the prepared cDNA.

The differential expression of the targeted genes was completed using Syber green RT-PCR master mixes (Life Technologies, USA) on the iQ5 (Bio-Rad, USA) between treated and untreated Hela cells. Forward and reverse primers' sequence (F: 5-AGAGCTACGAGCTGCCTGAC-3, R: 5-AGCACTGTGTTGGCGTACAG-3) of the housekeeping gene β -actin (ACTB) was used for normalization. The Nineteen primers used to specifically amplify target gene cDNA are shown in (Table 1). The sequence of primers and the pursued cycling regimen for the designed primers was carried out using Primer software (Primer Blast, NIH, USA). According to the specifications entered to the designing utilities software and after in-lab verification of the PCR amplification, the thermal cycling conducted was as follows: one cycle (95°C for 180 sec) followed with 40 cycles (95°C for 60 sec, and 60°C for 70 sec). Quadruplicates of all samples were assayed. Preparation of the negative controls (none-template controls) was done. The differential expression of the housekeeping gene ACTB along with the target genes was measured in folds according to delta-delta Ct method, known as the $E^{-\Delta\Delta C_t}$ method, where E stands for the value of PCR efficiency calculated out of the efficiency curve. In general, all runs of PCR turned out efficiency >90% with $R^2=0.99$. A comparison between the expression levels of untreated controls with treated cells was performed. PCR efficiency for all primers was measured after low-efficiency wells turned out to be zero and calculation of the fold changes in genes expression was carried out.

Analysis of data

The data was analyzed by using SPSS software version (16.0.0.2) and data were shown as (mean $\pm$ SD). The influence of the compound on cell growth inhibition was appraised by using 95% confidence intervals. Linear regression index equations were used to calculate IC50 value. Student *t-test* was carried out to compare the mean of each cell cycle phase, in triplicates, of treated vs untreated cells in flow cytometric analysis. The calculation of the fold change of genes expression is based on the comparative Ct method in real-time qPCR. The equation for comparative Ct

method which is also known as the $2^{-\Delta\Delta Ct}$ method was as follows: $\Delta\Delta Ct = \Delta Ct \text{ treated} - \Delta Ct \text{ untreated}$, where: $\Delta Ct \text{ treated} = Ct \text{ treated} - all$

mean treated and $\Delta Ct \text{ untreated} = \Delta Ct \text{ untreated} - all \text{ mean untreated}$. Less than 0.05 of p values were reflected as significant.

Table 1. Genes names along with their primer sequences used in Real-Time qPCR.

Gene Name	Sequence	Avg. Tm	Amplicon size
IL-6 Interleukin 6 (human)	F: 5'-CCAGCTATGAACTCCTTCTC-3' R: 5'-GCTTGTTCCTCACATCTCTC-3'	60	413
DDIT3 DNA damage-inducible transcript 3 (human)	F: 5'-GAACGGCTCAAGCAGGAAATC-3' R: 5'-TTCACCATTCGGTCAATCAGAG-3'	59.85	80
MKI67-2 marker of proliferation Ki-67 (human)	F: 5'-AATCCAACTCAAGTAAACGGGG-3' R: 5'-TTGGCTTGCTTCCATCCTCA-3'	60	245
TNFA Tumor necrosis factor (human)	F: 5'-CTCTTCTGCCTGCTGCACTTTG-3' R: 5'-ATGGGCTACAGGCTTGTCACTC-3'	61.1	135
EREG epiregulin (human)	F: 5'-GCAGGGATAACAGTGTGCCT-3' R: 5'-GACCATAGACTCCCCAGGGT-3'	60	547
FOS-2 Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (human)	F: 5'-CCCGGACTTGCACCTTACTT-3' R: 5'-ACTGATCTGTCTCCGTCTCCT-3'	59.72	553
DUSP5 dual specificity phosphatase 5 (human)	F: 5'-GTGCCTACTGCACATTCCCT-3' R: 5'-TCCCAGAACCTACCCTGAG-3'	60	288
ID2 inhibitor of DNA binding 2 (human)	F: 5'-ATCCTGTCTTGCAGGCTTC-3' R: 5'-ACCGCTATTTCAGCCACACA-3'	60.35	81
TICAM2 toll like receptor adaptor molecule 2 (human)	F: 5'-ACATTAACCCCTGACTCACAGC-3' R: 5'-CTCGGGGAAGGGGATTGTTC-3'	60.1	989
CRYAB Crystallin Alpha B (human)	F: 5'-ACTGCAAGCACCAAGTGTGT-3' R: 5'-GACTCCAACAGGTGCTCTCC-3'	60.15	328
FN1 Fibronectin 1 (human)	F: 5'-AGCAGACCCAGCTTAGAGTT-3' R: 5'-GCAGAAAGTGTTTGGGTGACT-3'	59	310
CCNB1 cyclin B1 (human)	F: 5'-CCTACTGGGTGCGGAAGTCA-3' R: 5'-GACTACATTCTTAGCCAGGTGCT-3'	60.3	592
FEN1 Flap structure-specific endonuclease 1 (human)	F: 5'-CAACCCCGAACCAAGCTTTAG-3' R: 5'-ACACGACCCCATAGGTGGA-3'	60	634
KRAS proto-oncogene, GTPase (human)	F: 5'-CCCAGGTGCGGGAGAGA-3' R: 5'-CCCTCCCAGTCCTCATGTA-3'	60.2	259
GTSE1 G2 and S-phase expressed 1 (human)	F: 5'-ACCAGCAATCTCGCAAGGAA-3' R: 5'-CGCGAAAGCTTTGGTGTGTA-3'	59.9	503
CXCL1-2 C-X-C Motif Chemokine Ligand 12 (human)	F: 5'-CTACAGATGCCCATGCCGAT-3' R: 5'-GTGGGTCTAGCGGAAAGTCC-3'	60	226
MCM5 Minichromosome maintenance complex component 5 (human)	F: 5'-GCAAGGTTCTCTACCGCCTC-3' R: 5'-CAGACCCTGTTCCCTCACAC-3'	60	352
MCM4 Minichromosome Maintenance Complex Component 4 (human)	F: 5'-CTTTCACGGTACTCCGAGC-3' R: 5'-AGTGCCGTATGTCTAGTGGTG-3'	60.1	305
RALA RAS like proto-oncogene A (human)	F: 5'-GATCTCCCCAGAGCAAAGCGT-3' R: 5'-TAAGAAGAATCTGCACCGCGA-3'	60.3	229

RESULTS

Carnosol cytotoxic effect

The anti-proliferative effect of Carnosol on human cancer cells was investigated by previous studies and it is in a concentration-dependent manner (Bourhia *et al.*, 2019, Amar *et al.*, 2017).

In the current study, Hela cell viability was reduced by Carnosol treatment in a time- and concentration-dependent manner (Figure 1). The concentration of Carnosol that killed 50% of Hela cells, or IC₅₀, was (50 µg/ml). This concentration was used in the downstream tests to study the anticancer molecular mechanism of the compound.

Figure 1. The mean percentages of Hela cells viability after treatment with Carnosol for (24, 48, and 72 h). The viability reduced in a time- and concentration-dependent manner.

Cell-cycle arrest and apoptosis induced by Carnosol

Carnosol IC₅₀ induced apoptosis and cell-cycle arrest in the treated Hela cells compared to untreated one (Figure 2). Carnosol was observed to promote apoptosis in the treated cells 48 h after treatment (24.81% ± 1.26) in comparison with untreated cells (9.61% ± 3.82), ($p = 0.002$). The treated cells were arrested significantly in G₀/G₁ phase (62.12% ± 5.83) while untreated cells were (73.75% ± 2.21), ($p = 0.032$). On the other hand, the treatment showed non-significant effect on both S phase and G₂/M phase, (6.5% ± 1.9) in contrast to untreated one (8.81% ± 3.01), ($p = 0.32$) and (7.58% ± 3.01) in contrast to untreated one (8.96% ± 1.7), ($p = 0.52$), respectively.

Detection of real-time apoptosis in intact cells

The morphological changes of apoptosis were investigated by fluorescence microscopy to detect

the apoptosis induction by Carnosol (Figure 3). The two important apoptosis markers were simultaneously monitored by the kit. The monitored markers were the translocation of phosphatidylserine on the cell membrane (identified by sulforhodamine 101-annexin V) and caspase-3 activation (identified by NucView™ 488 caspase-3 substrate activity) within individual whole cells (Balijepalli *et al.*, 2010). The nucleus stained as a bright green after the cleavage of NucView™ 488 caspase-3 substrate by activated caspase-3 (Figure 3C). Besides, phosphatidylserine that had translocated from the inner to the outer leaflet of the plasma membrane, produced a red border around the cell under a fluorescent microscope using a red filter (Figure 3D).

The morphological changes that could be detected after treatment like detachment, rounding of cells, and cell shrinkage were considered as apoptosis-related events (Desjardins & MacManus, 1995). A comparison between the treated cells and untreated cells in the negative control plates were done. The Carnosol IC₅₀ (50 µg/ml) induced apoptosis in 80% of Hela cells after 48 h of treatment.

Figure 2. Hela cells DNA content after 48 h was represented by this histogram of the flow cytometry analysis in untreated cells and treated cells' cultures with Carnosol IC₅₀. Treatment with Carnosol stimulated cell-cycle arrest in G₀/G₁ phase and apoptosis. The cells' percentages with fractional DNA content was detected by software CyAn ADP apparatus and Summit (V4.3) program: apoptotic cells (R2), G₀/G₁ (R3), S (R4), and G₂/M (R5) phases. The p value of less than 0.05 was considered as significant.

Figure 3. A) The photomicrograph of HeLa negative control cell lines showed no morphological changes in these cells. B) The photomicrograph of HeLa cell lines treated with Carnosol IC50 (50 µg/ml) for 48 h. The cells viewed under a fluorescent microscope (Nikon eclipse 80 i). The morphological changes were manifested after 48 h of treatment with Carnosol IC50. C) The photomicrograph of HeLa cell lines treated with Carnosol IC50 (50 µg/ml) for 48 h and stained with caspases-3 substrate. Caspase-3 activation was indicated by green nuclei in HeLa cells. D) The photomicrograph of HeLa cell lines treated with Carnosol IC50 (50 µg/ml) for 48 h and stained with Annexin-V substrate. Phosphatidylserine translocation from inner to the outer leaflet of the cell membrane was indicated as red borders around HeLa cells. E) The merged photomicrograph of stained HeLa cell lines treated with Carnosol IC50 (50 µg/ml) for 48 h. The apoptotic HeLa cells were characterized by green nuclei surrounded by red borders.

The up- and down-regulation of target genes after Carnosol treatment

The profile of the gene expression of the treated Hela cells with IC50 of Carnosol for 48h was assessed by the semi-quantitative analysis of real-time qPCR. Four genes (*IL6*, *TNF- α* , *DDIT3*, and *MKI67-2*) gave no results neither up-regulated nor down-regulated after treatment.

Fifteen genes belong to different carcinogenesis pathways that attributed to cancer progress in human normal cells were shown to be up- or down-regulated after treatment (Table 2).

An immense up-regulation was manifested with *EREG* gene (13.04 ± 4.34 folds). On the other hand, immense down-regulation was manifested with *FN1* gene (14.05 ± 4.91 folds). In addition, up-regulation of the following genes was recorded in folds: *FOS-2* (9.34 ± 2.45); *ID2* (6.73 ± 1.65); *CRYAB* (5.15 ± 1.04). The rest genes with a significant up-regulation were: *DUSP5* (7.51 ± 1.98); *TICAM2* (5.57 ± 1.54). In contrast, the genes with a remarkable down-regulation in folds were: *KRAS2* gene (9.89 ± 2.52); *CCNB1-1* gene (12.37 ± 3.02); *FEN1* gene (10.84 ± 2.56); *MCM5* gene (9.42 ± 2.42); *GTSE1* gene (8.87 ± 1.59); *MCM4* gene (7.86 ± 1.43) and *RALA* gene (7.41 ± 1.15).

Table 2. The up- and down-regulation of Hela cells' genes after 48 h treatment with Carnosol.

Expressed genes ^{fold change}	
Up-regulated	Down-regulated
<i>EREG</i> ^{13.04}	<i>FN1</i> ^{14.05}
<i>FOS-2</i> ^{9.34}	<i>CXCL1-2</i> ^{9.11}
<i>CRYAB</i> ^{5.15}	<i>CCNB1-1</i> ^{12.37}
<i>ID2</i> ^{6.73}	<i>KRAS2</i> ^{9.82}
<i>TICAM2</i> ^{5.57}	<i>MCM4</i> ^{7.68}
<i>DUSP5</i> ^{7.51}	<i>RALA</i> ^{7.41}
	<i>GTSE1</i> ^{9.24}
	<i>MCM5</i> ^{8.87}
	<i>FEN1</i> ^{10.84}

DISCUSSION

Carnosol anticancer effect has been studied on different cancer cell types (Aliebrahimi *et al.*, 2018; de Oliveira *et al.*, 2019); however, so far, no studies were found on human cervical cancer cell

lines. The flow cytometry and the real-time apoptosis assay confirmed the effect of Carnosol IC50 to stimulate apoptosis in 80% of the treated cells in addition to significant morphological changes. Furthermore, a momentous cell cycle arrest was evident at G0/G1 phase in the treated cells. RT-qPCR analysis was integrated to confirm the anticancer effect of Carnosol IC50 treatment on Hela cells after 48 h.

Treatment didn't show any effect on four genes, namely (*IL6*, *TNF- α* , *DDIT3*, and *MKI67-2*) which found to be correlated to cervical cancer development by previous studies. Studies find that the development of different cancers including cervical cancer is related to the pro-inflammatory cytokines expression like *IL-6* (Hao *et al.*, 2020) and *TNF- α* (Babapour *et al.*, 2019). The overexpression of *DDIT3* can act as a promoter for apoptosis in cervical cancer (Lin *et al.*, 2018). The gene *MKI67* is considered as a biomarker that could be used to test the susceptibility to develop cervical cancer in human papilloma positive patients (Clarke *et al.*, 2019). The reason behind this unresponsiveness was not clear and need to be evaluated by another study in the future.

Epiregulin (EREG) was highly up-regulated after treatment. Many studies found that recurrent/metastatic head and neck squamous cell carcinoma and colorectal cancer patients with high EREG gene expression, look to benefit more from chemotherapy than those with lower expression (Kogashiwa *et al.*, 2018, Bormann *et al.*, 2019). Unlike high significant down-regulation was manifested with Fibronectin 1 gene (FN1) in the current study. This gene has been established to stimulate cell proliferation and migration in different tumors and is up-regulated in numerous cancer types (Sun *et al.*, 2020; Song *et al.*, 2020). Additionally, FN1 is up-regulated in cervical cancer tissues as detected by Wang *et al.* (2019). Thus, Carnosol could be used as a therapy to inhibit the development of different cancer types.

Different pathways have been affected by Carnosol treatment in this study. FOS-2 gene also known as activating protein-1 (AP-1) which is responsible for a pathway where deregulation makes the host more vulnerable to cancer development (Mirzaei *et al.*, 2020). This signaling pathway gene was found to be up-regulated by

the treatment with Carnosol. Similarly, inhibitor of DNA binding 2 (ID2), known as a dominant anti-retinoblastoma (Ponzio *et al.*, 2017) helix-loop-helix protein, was found to be up-regulated by the treatment. A previous study found that the down-regulation of this gene is linked with reduced apoptosis and increased cancer cell survival (Luo *et al.*, 2010). Similarly, a study focuses on the fact that multiple epithelial-mesenchymal transition-related genes, like ID2 may be potential targets for cervical cancer gene-targeted therapy (Roszik *et al.*, 2018).

Recent studies found that alpha B-crystallin (CRYAB), a small heat shock protein that can reduce neuroinflammatory responses, exerts neuroinflammatory mechanisms and plays crucial roles in bone cancer pain (Hang *et al.*, 2020). Moreover, the overexpression of CRYAB in cancer cell lines which was also seen in this study inhibits tumor cell migration and invasion (Ruan *et al.*, 2020) and could be a novel biomarker candidate for a therapy in cervical carcinoma (Kori & Yalcin Arga, 2018).

Dual specificity phosphatase 5 gene (DUSP5) negatively regulates members of genes that are related to cellular proliferation and differentiation (Youns *et al.*, 2018; Kidger & Keyse, 2016). The current study was in agreement with other studies that the up-regulation of this anticancer gene has attributed to the anticancer molecular effect of the tested natural product on cancer cells (Ponzio *et al.*, 2017).

One of the nuclear factor-kappa B (NF-kappaB) signaling pathway genes is the toll-like receptor adaptor molecule 2 (TICAM2) which was shown to be up-regulated after Carnosol treatment. This gene is one of the regulators of an inflammation in the host immune response and that has been associated with the carcinogenic process (Slattery *et al.*, 2018). As several factors are involved in cervical cancer development, including intrinsic factors related to the inflammatory process (Pfaffenzeller *et al.*, 2020) the up-regulation to related genes seems crucial.

KRAS is the most regularly mutated oncogene in 30% of human neoplasia with many studies tried to develop molecular-targeted therapies for KRAS mutant cancer (Dai *et al.*, 2020; Liu *et al.*, 2019). Carnosol treatment

reduced KRAS expression which opens the gate to use it as a potential treatment option for cancer patients with KRAS mutations and blocking KRAS function as highly encouraged by other findings (Chen *et al.*, 2019). A previous study finds that the down-regulation of KRAS can subsequently suppress the proliferation and chemotherapy resistance of cervical cancer cells (Dun & Gao, 2019).

The down-regulation effect of the investigated genes was in coordination with the arrested cell cycle phase recovered by the analysis of the flow cytometry and apoptosis morphological changes shown by the dual apoptosis assay after treatment with Carnosol. The cyclin B1 gene (CCNB1-1) is a regulatory protein involved in mitosis (Kabir *et al.*, 2018). This gene has been down-regulated markedly after treatment. The down-regulation of this gene inhibits cell line proliferation and growth and induced apoptosis as proofed by another study (Awasthi *et al.*, 2018). Additional genetic down-regulation was recorded after treatment with Carnosol; one of the DNA damage repair genes, flap endonuclease 1 (FEN1) was found to be down-regulated. This was a mirror image to the cell cycle arrest at the apoptosis phase induced after treatment with Carnosol; this observation is reported by another study as well (Dong *et al.*, 2019). A study disclosed the fact FEN1 inhibition could increase the sensitivity of cervical cancer cells to radiotherapy (Li *et al.*, 2019).

Moreover, the enhanced cell cycle arrest found in this study after treatment with Carnosol was backed by the down-regulation of the replicative helicase proteins (MCM4 and MCM5) which have found to be as enhancers for DNA replication in cervical cancer (Cheng *et al.*, 2017) and cell proliferation promoter in other cancer types (Mio *et al.*, 2016). Similarly, GTSE1 (G2 and S phase-expressed-1 gene) which is a key regulator of tumor cell migration and metastasis (Penna *et al.*, 2017), was down-regulated in this study. The targeting of this gene in the cervical cancer cell line inhibits culture proliferation as seen by the previous study (Khokhlova *et al.*, 2010).

Moreover, the Carnosol treatment was shown to be effective to reduce the role of the chemokine (C-X-C motif) ligand 1 (CXCL1) in

the current cancer cells. This inflammatory protein has been stated to be up-regulated in several human cancers (Miyake *et al.*, 2019). This protein is associated with metastasis of cervical cancer cells, thus could be considered as a target therapy of this process (Kim & Park, 2014).

RALA gene has a crucial role downstream to Ras oncoproteins in human cancers and found to be activated in different cancer types. It is used to be the basis of a novel class of anticancer therapy (Moghadam *et al.*, 2017; Zago *et al.*, 2018). In the current study, the down-regulation of this gene comes in harmony and back up previous studies findings that consider the RALA gene as a target for anticancer therapy.

CONCLUSION

The anticancer molecular mechanisms of Carnosol have not fully studied so far. Additionally, its anticancer effect on human cervical cancer has not investigated yet. On the other hand, there is an increased incidence of resistance to the most regularly used chemotherapy. This lays urgency to the need for discovering novel anticancer molecular target therapy, taking into consideration the cost and the availability of such therapy. The current study revealed that different genes associated with different signaling pathways were shown to be related to apoptosis, cell differentiation, proliferation, migration, metastasis, regulation of the immune response, and inflammation. These genes were all remarkably affected by the treatment with Carnosol. Moreover, Carnosol was shown to shift gene expression toward the protection against carcinogenesis. This was shown by the collective effect of the up- or down-regulated genes studied in this study. Therefore, we recommend further studies to be done on Carnosol effect on the carcinogenesis of the cervical cancer cells and other types of cancer cells more deliberately.

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