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RESEARCH

Carnosol and carnosic acid may be a promising anticancer agent in non-small cell lung cancer treatment

Karnosol ve karnosik asit küçük hücreli dışı akciğer kanseri tedavisinde umut verici bir antikanser ajanı olabilir

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Abstract

Purpose: This study aimed to evaluate the possible anticancer and apoptotic effect of carnosol and carnosic acid on non-small cell lung cancer (NSCLC) cell lines *in vitro*.

Materials and Methods: Three different NSCLC cell lines (H441, H520, and H661) were used in the study and treatment (carnosol and carnosic acid treatment) and control (no treatment) groups were established in all cell lines. The IC_{50} doses were determined by MTT method on NSCLC cell lines and the changes in expression levels of apoptosis-related genes (*Bax*, *Bak*, *caspase-3*, *p53*, *c-Myc*, *Bcl-2*, and *Bcl-XL*) were demonstrated by real-time polymerase chain reaction (qPCR), and apoptosis was confirmed by flow cytometry. The treatment group and control group results were compared.

Results: The IC_{50} doses of carnosol and carnosic acid were determined; 60 μM for the H441, 20 μM for the H661, 40 μM for the H520, 20 μM for the H441, 40 μM for the H661, 40 μM for the H520, respectively. Carnosol treatment increased *Bax*, *Bak*, *caspase-3*, and *p53* expression but decreased *Bcl-XL* expression in studied lung cancer cell lines. Carnosic acid treatment increased apoptotic *Bax*, *Bak*, *caspase-3*, and *p53* expression but decreased antiapoptotic *Bcl-2* and *Bcl-XL* expression ($p<0.05$).

Conclusion: In this study showed that carnosol and carnosic acid have apoptotic effects on NSCLC cells. With more detailed studies on these agents, these come become a promising agent that can be used in NSCLC cancer treatment in the future.

Keywords:. Apoptosis, carnosol, carnosic acid, rosemary, non-small lung cancer

Öz

Amaç: Bu çalışma, karnosol ve karnosik asidin küçük hücreli dışı akciğer kanseri (KHDAK) hücre dizileri üzerindeki olası antikanser ve apoptotik etkisini *in vitro* olarak değerlendirmeyi amaçladı.

Gereç ve Yöntem: Çalışmada üç farklı KHDAK hücre hattı (H441, H520 ve H661) kullanılmış olup, tüm hücre hatlarında uygulama (karnosol ve karnosik asit uygulanmış) ve kontrol (uygulama yapılmayan) grupları oluşturulmuştur. IC_{50} dozları KHDAK hücre hatlarında MTT yöntemiyle belirlendi ve apoptozla ilişkili genlerin (*Bax*, *Bak*, *kaspaz-3*, *p53*, *c-Myc*, *Bcl-2* ve *Bcl-XL*) ekspresyon seviyelerindeki değişiklikler gerçek zamanlı polimeraz zincir reaksiyonu (qPCR) ile gösterildi ve apoptoz akış sitometrisi ile doğrulandı. Tedavi grubu ve kontrol grubu sonuçları karşılaştırıldı.

Bulgular: Karnosol ve karnosik asidin IC_{50} dozları sırasıyla belirlendi; H441 için 60 μM, H661 için 20 μM, H520 için 40 μM, H441 için 20 μM, H661 için 40 μM, H520 için 40 μM. Karnosol uygulaması, çalışılan akciğer kanseri hücre hatlarında *Bax*, *Bak*, *kaspaz-3* ve *p53* ekspresyonunu arttırdı ancak *Bcl-XL* ekspresyonunu azalttı. Karnosik asit tedavisi apoptotik *Bax*, *Bak*, *kaspaz-3* ve *p53* ekspresyonunu arttırdı ancak antiapoptotik *Bcl-2* ve *Bcl-XL* ekspresyonunu azalttı ($p<0.05$).

Sonuç: Bu çalışmada karnosol ve karnosik asidin KHDAK hücreleri üzerinde apoptotik etkileri olduğu gösterilmiştir. Bu ajanlar haklarında daha detaylı çalışmalar yapılmasıyla gelecekte KHDAK tedavisinde kullanılabilecek umut verici bir ajanlar olabilecektir.

Anahtar kelimeler: Apoptoz, karnosol, karnosik asit, biberiye, küçük olmayan akciğer kanseri

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INTRODUCTION

Lung cancer is a major cause of death worldwide and is the second most common form of cancer after prostate cancer in men and breast cancer in women[1]. Lung cancer is equally common between sexes (11.6% of total cases), and its mortality rate is approximately 18.4%; indeed, lung cancer-related death rates are followed by those of female breast cancer (11.6%), prostate cancer (7.1%) and colorectal cancer (6.1%)[2].

Diet is an important factor in reducing the risk of cancer, and a number of plant constituents are known to play an important role in good health. The rosemary plant (*Rosmarinus officinalis* L.) is grown in many parts of the world and has received strong interest for its potential applications in cancer prevention[3]. Besides the extract of rosemary, other active substances, such as ursolic acid, carnosic acid, carnosol, and rosmarinic acid, have been reported to have anticarcinogenic activity[3,4,5]. Rosemary can inhibit tumor growth in many organs, including the colorectum, breast, stomach, and liver. Rosemary extract has antioxidant, anti-inflammatory, antidiabetic, and anticancer properties[4,6,7].

The constituents of rosemary, especially carnosic acid, carnosol, and rosmarinic acid[5,8,9,10,11], have shown antiproliferative effects on several tumor cell lines[12,13]. Carnosic acid inhibits the proliferation of human myeloid leukemia cells without inducing apoptosis[14]. In a previous experimental study, carnosic acid-induced apoptosis in IMR-32 human neuroblastoma cells through the activation of P38 using reactive oxygen species[15]. Vergara et al. reported that carnosol reduces cell viability in human breast, ovarian, and colon tumour cell lines and inhibits cancer cell adhesion to fibronectin[16]. Although there are few studies investigating the effect of carnosol and carnosic acid on lung cancer, it has been shown that they induce apoptosis in cancer cells by causing the activation of various signaling pathways[17,18].

The mechanism of the anti-tumor activity of rosemary is still not clear. This study hypothesizes that the active ingredients of rosemary (carnosol and carnosic acid) have a lethal effect on lung cancer cells. The aim of this study is therefore to investigate the apoptotic effects of two components of rosemary, namely, carnosol and carnosic acid, on H441 (lung adenocarcinoma), H520 (lung squamous cell carcinoma), H661 (large-cell lung cancer) cells. In addition, we aimed to compare the apoptotic effects of these compounds on different types of small non-lung cancers (H441, H520, H661). At the end of the study, the apoptotic effects of carnasol and carnosic acid on different lung cancer cells were revealed.

MATERIALS AND METHODS

Cell culture and drugs

Experimental applications on cell lines were completed by researchers specialized in the field at KTO Karatay University Faculty of Medicine Central Research Laboratory and Selçuk University Faculty of Medicine Laboratories. Also, this study was conducted on cell lines that do not require ethical approval. In this study, carnosol and carnosic acid, one of the active ingredients of rosemary, were commercially obtained from ChromaDex (Irvine, CA, USA). The H441, H520, and H661 cell lines were obtained from ATCC (American Type Culture Collection; Rockville, MD, USA). Cells were cultured as previously described[19].

Cell viability assay

Trypsinized cells were seeded into a 96-well plate (~$5x10^3$ cells per well). Approximately one day later, a fresh medium containing carnosol and carnosic acid (at concentrations of 5, 20, 40, or 60 μM) was introduced to the cells (for 24, 48, and 72 hours). At the end of the period, the medium was quickly removed and treated with MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) dye for ~4 hours (in the culture incubator). Formazans were fixed with DMSO (in a dark room) and final measurements were taken in a microplate reader (Biotek Instruments, USA, 490 nm)

Apoptosis analysis by flow cytometry

Annexin V–FITC apoptosis detection kit (BD Biosciences, CA, USA) was used to determine the percentage of treated and untreated H441, H661, and H520 lung cancer cells actively undergoing apoptosis after treatment with IC_{50} concentrations of carnosol or carnosic acid. Carnosol and carnosic acid-treated and untreated cells were harvested from the culture dishes and transferred to FACS tubes. After washing with 1× PBS (pH 7.4), annexin V/propodium iodide mixture solution (1:1, v:v) and incubated for 20-30 min at 25°C. After the binding buffer was diluted 10-fold with 1xPBS, it was transferred to a FACS tube.

Cells were assessed by flow cytometry (BD FACSAriaTM III Cell Sorter).

Total RNA isolation and cDNA synthesis

Total cell RNA isolated was carried out by using the phenol-chloroform extraction method with TRIzol (Sigma, ABD) (As previously described[19]). After the removal of ethanol, the RNA pellet was dissolved in Ambion nuclease-free water (Life Technologies Corporation) and stored at –80 °C. cDNAs were synthesized by using a RevertAid First Strand cDNA synthesis kit according to the protocol (Thermo Scientific, ABD).

qPCR reaction

Gene expression of *Bax*, *Bak*, *caspase-3*, *p53*, *c-Myc*, *Bcl-2*, *Bcl-XL*, and *GAPDH* were analyzed by using specific primers from Macrogene (Seoul, Korea). The primer sequences used in this procedure are summarized in Table 1. qPCR was performed in triplicate using a LightCycler 480 instrument (Roche Diagnostics). The steps of the qPCR reaction are shown in Table 2.

Table 1. Apoptotic and antiapoptotic primer sequences used in this study.

Primer	Oligonucleotide sequence (5'-3')	Location	Amplicon size (bp)	Tm (°C)
Bak F	5'-CAT CAA CCG ACG CTA TGA CTC-3'	252-272	192	60.6
Bak R	5'-GTC AGG CCA TGC TGG TAG AC-3'	443-424		62.3
Bax F	5'- CCC GAG AGG TCT TTT TCC GAG-3'	116-136	155	62.1
Bax R	5'- CCA GCC CAT GAT GGT TCT GAT-3'	270-250		61.9
Bcl-2 F	5'-GGT GGG GTC ATG TGT GTG G-3'	460-478	89	62.6
Bcl-2 R	5'-CGG TTC AGG TAC TCA GTC ATC C-3'	548-527		61.8
p53 F	5'-CAG CAC ATG ACG GAG GTT GT-3'	382-401	125	62.4
p53 R	5'-TCA TCC AAA TAC TCC ACA CGC-3'	506-486		60.1
caspase-3 F	5'- CAT GGA AGC GAA TCA ATG GAC T-3'	64-85	139	60.7
caspase-3 R	5'- CTG TAC CAG ACC GAG ATG TCA-3'	202-182		60.6
c-Myc F	5'- GGC TCC TGG CAA AAG GTC A -3'	858-876	119	62.2
c-Myc R	5'- CTG CGT AGT TGT GCT GAT GT -3'	976-957		60.4
Bcl-XL F	5'- GAGCTGGTGGTTGACTTTCTC -3'	19-39	119	60.6
Bcl-XL R	5'- TCCATCTCCGATTCAGTCCCT -3'	137-117		61.9
GAPDH F	5'- GGAGCGAGATCCCTCCAAAAT-3'	108-128	197	61.6
GAPDH R	5'-GGCTGTTGTCATACTTCTCATGG -3'	304-282		60.9

F: Forward, R: Reverse

Table 2. qPCR steps and applied temperature protocol.

Stage	Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)
Pre-incubation	95	00:02:00	4.4
Amplification	95	00:00:10	4.4
	56	00:00:30	2.2
	72	00:01:00	4.4

h: hour, m: minute, s: second

Statistical analysis

The comparative Livak's ΔΔCT method was used to determine relative gene expression[20]. SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis (housekeeping gene: *GAPDH*). In this study, according to the Ct values obtained for each gene, a 2-fold or more increase or decrease in gene expression was considered significant ($p<0.05$).

RESULTS

Carnosol and carnosic acid could reduce the viability of lung cancer cells

The optimal application times and IC_{50} doses of carnosol were 24 h and 60 μM for the H441 cell line, 72 h and 20 μM for the H661 cell line, and 48 h and 40 μM for the H520 cell line. Similarly, the optimal application times and IC_{50} doses of carnosic acid were 24 h and 20 μM for the H441 cell line, 24 h and 40 μM for the H661 cell line, and 72 h and 40 μM for the H520 cell line (Figure 1).

Carnosol and carnosic acid could enhance apoptosis in lung cancer cell lines

The annexin V results were consistent with the MTT analysis. The carnosol dose determined by MTT assay to induce apoptosis in the H441, H661, and H520 cell lines was statistically significant ($p<0.05$). Carnosic acid significantly induced apoptosis in H441 and H661 cells ($p<0.05$) but not in H520 cells (Figure 2). Only PI^+ cells indicate necrosis, only annexin V^+ cells indicate early apoptosis, and PI^+annexin V^+ cells indicate late apoptosis.

Carnosol and carnosic acid could induce the expression of apoptosis-initiating genes in NSCLC cells

Analysis of the expression of genes associated with apoptosis is shown in Figure 3. Carnosol treatment increased *Bax*, *Bak*, *caspase-3*, and *p53* expression but decreased *Bcl-XL* in all NSCLC cell lines examined in this study. Carnosic acid treatment yielded results very similar to those obtained from carnosol treatment. Treatment with carnosic acid increased the expression of apoptotic genes *Bax*, *Bak*, *caspase-3*, and *p53* but decreased the expression of antiapoptotic genes *Bcl-XL and Bcl-2*. It is possible to generalize to increase or decrease results for almost all genes evaluated in this study except *c-Myc* gene. *c-Myc* gene expression increased in the carnosol-treated H661 cell line and the carnosic acid-treated H520 cells. However, the expression of this gene decreased in all other cell lines.

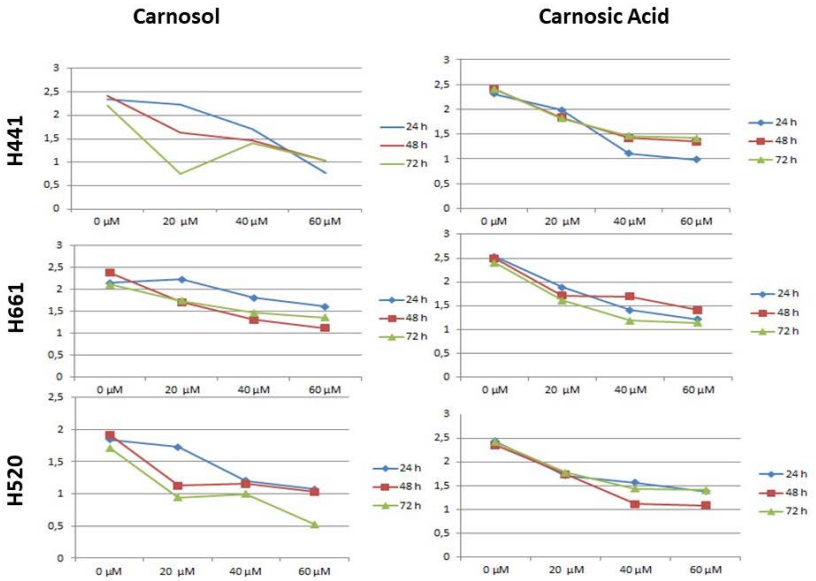

Figure 1. MTT cell viability assay results of carnosol and carnosic acid.

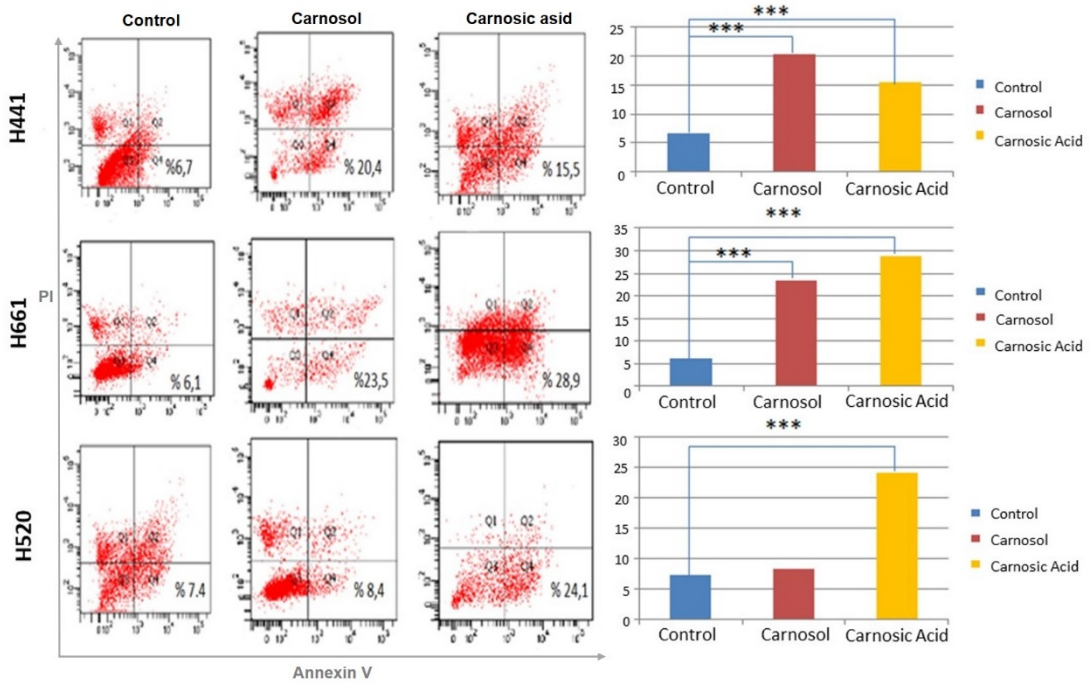

Figure 2. Apoptosis analysis by flow cytometry results.

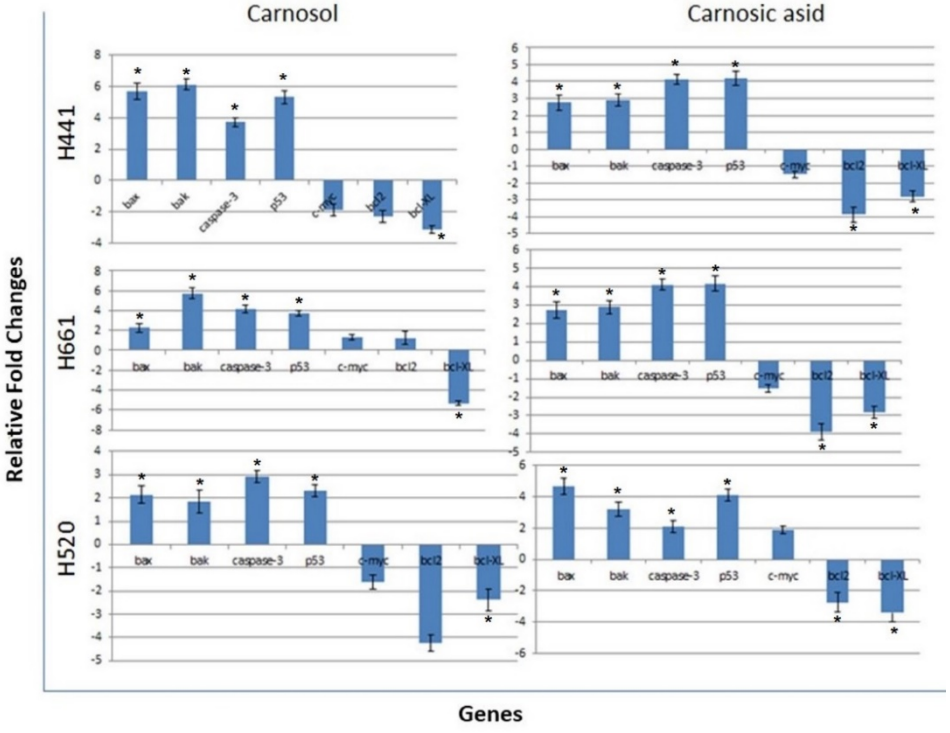

Figure 3. Analysis of expression of apoptosis-related genes (*p<0.05).

DISCUSSION

The American Cancer Society's estimates for lung cancer in the United States for 2021 included about 235,760 new cases of lung cancer (119,100 in men; 116,660 in women) and approximately 131,880 deaths from lung cancer (69,410 in men; 62,470 in women). In addition, the COVID-19 pandemic outbreak in 2020 has led to delays in the diagnosis and treatment of almost all types of cancer. For example, there may appear to be a decrease in cancer incidence, with cancer patients restricting their access to the hospital and healthcare setting[21]. Also, the fact that some cancer patients get COVID-19 disease can also show as COVID-19 even if the cause of death of the patients is cancer. This may have shown the number of cancer deaths to be even lower.

Apoptosis is a major component of the pathogenesis of cancer. During carcinogenesis, cells exhibit irregular cell proliferation due to the suppression of apoptotic mechanisms. Such proliferation could lead to the development and growth of tumors[22]. In addition, some structural changes occur in the plasma membrane when cell death is imminent. When phosphatidylserine (PS) passes to the outer surface of the plasma membrane, the membrane integrity remains complete. During the early apoptosis, PS is bound to annexin V, which is a phospholipid-binding protein bound to Ca^{2+} [23]. We used annexin V analysis to confirm our MTT results on early-apoptotic cells and found that carnosol and carnosic acid generally have a statistically significant apoptotic effect in all cell lines studied; however, carnosic acid did not affect H520 cells.

We have found that carnosol and carnosic acid can reduce cell viability in several lung cancer cell lines. Danilenko et al. reported that carnosic acid shows better inhibition effects on the growth of cancer cells compared with other substances, such as 1α, 25(OH)$_2$D$_3$, and ATRA, and induces the differentiation of HL60 cells[24]. Our results are consistent with previous findings indicating that carnosic acid inhibits cell proliferation. Barni et al. found that carnosic acid induces apoptosis and decreases cell migration in the human Caco-2 cell line[25]. Huang et al. also found that carnosol significantly reduces the migration and invasion of mouse melanoma B16/F10 cells by suppressing metalloproteinase-9 through the AP-1 and NF-κB pathways[26]. In another study, carnosol and carnosic acid limited angiogenesis by reducing the migration of endothelial cells[27].

When the effects of carnosic acid and carnosol on the expression of genes associated with apoptosis were examined, both compounds significantly increased the expression of apoptosis-efficient genes in the studied cell lines and inhibited the expression of genes with antiapoptotic effects. Park et al. showed that carnosol is induced by p53-induced reactive oxygen species (ROS) and inhibits STAT3 in the colon cancer cell line HCT116[28]; by decreasing the expression of *Bcl-2* and increasing the expression of *Bax, STAT3* induces apoptosis. Antioxidant, antibacterial, and biological activities of rosemary extracts have been previously investigated[29]. Additionally, rosemary extract has been reported to inhibit *in vivo* tumor growth by causing ROS-induced necrotic cell death[30] and inhibit proliferation, survival, Akt, and mTOR signaling in triple-negative breast cancer cells[31]. Also, a significant reduction in apoptosis has been reported after the treatment of carnosine-treated renal carcinoma cells with a pan-caspase inhibitor benzyloxycarbonylvalyl-alanyl-aspartyl fluoromethyl ketone. Carnosic acid induces the expression of endoplasmic reticulum stress marker proteins, including transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein-homologous protein (CHOP), and increases intracellular ROS production[32]. These reports show that carnosic acid-induced endoplasmic reticulum stress plays a role in apoptosis and that carnosic acid is a potent apoptosis inducer. When these findings and our results are evaluated together, carnosol and carnosic acid appear to have an apoptotic effect on cancer cells.

In conclusion, our study supports the antitumor and anticancer roles of carnosic acid and carnosol in inducing apoptosis in the human NSCLC cell lines aH441, H520, and H661. The limitations of the study are that the study is in vitro, only at the gene expression level, and does not include expression at the protein level. The results suggest that these two compounds may have chemotherapeutic agents and contribute to the literature on lung cancer prevention. In future studies, the anticancer effects of carnosol and carnosic acid should be investigated on different pathways and the study should be transferred to the *in vivo* platform. In addition, expression changes at the protein level should be supported by investigating methods such as Western blot.

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