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The Effectiveness of *Satureja khuzistanica* Against Cancerous Cells

Davoodi M[1], Rustaiyan A[1], Ebrahimi SN[2] and Esmaeili D[3*]

[1]*Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2]*Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G. C., Evin, Tehran, Iran*

[*3]*Department of Microbiology and Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.*

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ABSTRACT

Plants play an important role in cancer prevention and treatment. *Satureja khuzistanica*, an endemic plant to Iranian flora, is widely distributed in the southern and western parts of the country and is famous for its medical applications as an analgesic and antiseptic in folk medicine. *Satureja khuzistanica* has been shown to possess inhibitory effects on the proliferation and reproduction of certain tumorigenic microorganisms and viruses such as *Heliobacter pylori*. Transcriptional regulation of some oncogene and carcinogenesis-related gene expression and interaction with both DNA and RNA is also well documented. Besides, *Satureja khuzistanica* is a spectrum enzyme inhibitor. More importantly, the suppression of tumor growth and metastasis, the beneficial application in combined medication, and the improvement of multidrug resistance both in vivo and in vitro clearly show its potentials as an alternative medicine for tumor chemotherapy.

Keywords: *Satureja khuzistanica*, Cancer chemotherapy, Plant

INTRODUCTION

Cancer is a growing health problem around the world and has been reported as the second main cause of death after heart diseases [1]. The use of effective drugs with low toxicity and minor environmental impacts is of high significance for the prevention and treatment of the disease. As such, natural products play a vital role in preventing and treating cancer. Currently, a significant number of anti-tumor agents that are used in clinics are of a natural origin [2]. Therefore, the use of herbs as natural drugs can be of great help to solve this problem.

Satureja khuzistanica is an endemic plant of Iran with a wide distribution in the southern part of the country, and is well-known for its therapeutic value as an analgesic and antiseptic in folk medicine [3,4]. The genus of Satureja belongs to the family of Lamiaceae as a subfamily of Nepetoideae and the tribe of Mentheae [5] and the composition of the essential oil of this genus have been explored in different researchers [6-11].

Given the wide application of *Satureja khuzistanica*, an exploration of chemical compounds found in extracts of this plant to identify its bioactive compounds and standardization of ducts and its quality control as a pharmaceutical raw material is high importance. The phytochemical profile of nonvolatile part of the plant extract has been explored in a few reports [12-14], where the chemical composition of the essential oil from the aerial parts of *S. khuzistanica* has been investigated very well. The main component of this plant is an oxygenated monoterpene named carvacrol (natural isopropyl cresol/5-isopropyl-2-methyl phenol [15-17]. This phenolic monoterpene is found in most of the essential oils of medicinal and aromatic plants and has received a lot of attention on account of its useful biological activities.

Koparal and Zeytinoglu [18] studied the effects of carvacrol on non-small cell carcinoma (NSCLC) A549. The effects of carvacrol on cell morphology, apoptosis and total protein amount on selected cells were evaluated by incubation of various concentrations of carvacrol in DMSO for 24 h. As carvacrol was applied to A549 cell line, an increased dose of carvacrol induced a decrease in cell number, degeneration of cell morphology, and a decrease in total protein level. Light microscopy techniques were used to examine cells induced changes in cell morphology by carvacrol. Afterwards, the

Corresponding author: Esmaeili D, Department of Microbiology and Applied Microbiology Research Center, Systems biology and poisonings institute, Baqiyatallah University of Medical Sciences, Tehran, Iran, E-mail: esm114@gmail.com

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cells were treated using carvacrol detached from the disk, with cell rounding, cytoplasmic blabbing and irregularity in shape. The results also showed that carvacrol is a very strong inhibitor of cell growth in A549 cell line [18].

Zerrin and Gülşen [19] examined the impact of apoptotic carvacrol in H-RAS and N-RAS transformed CO25 cells and carvacrol ability as a chemotherapeutic medicine. The results showed that carvacrol has cytotoxic effects on the two cell lines in terms of time and concentration.

Mehdi et al. [20] studied the carvacrol cytotoxic effects on cervical cancer (Hela & Sitla cells). Carvacrol cytotoxic effects were also studied separately using MTT and LDH techniques. Apoptosis was also measured using the DNA cleavage test. It was found that carvacrol cytotoxic effects on Hela & Sitla cells in 50 lc out of 50 mg/l. The dying cells displayed apoptotic features such as DNA cleavage. The results also illustrated carvacrol cytotoxic effects on cervical cancer cells. IC50 values were measured for 0.04 mg/mL 5RP7 and 0.1 mg/mL CO25 cell lines. Carvacrol, depending on the IC50 values, can also induce morphological changes on both cell types.

Phosphatidylserine localisation mobility was detected using flow cytometry only on 5RP7 cells. DNA laddering as the late apoptotic determinant was also found on H-ras transformed cells but not on N-ras transformed cells at a concentration of IC50 and lower values. This may show that the sensitivity of H-ras transformed a cell to carvacrol is greater than that of N-RAS-transformed cells. It was also found that carvacrol can be employed as anti-cancer medication for its apoptotic effects on cancer treatment [20].

The cytotoxic effects of six monoterpenes, carvacrol, thymol, carveol, carvone, eugenol and isopulegol plus their molecular mechanisms were compared by Jaafari et al. [21]. A comparison of the in vitro antitumor activity of the tested products against five tumor cell lines suggested that the carvacrol is the most cytotoxic monoterpene. In addition, the assessment of eventual synergistic effects of the six natural monoterpenes with two anticancer drugs indicated a significant level of synergy among them ($P<5\%$). The flow cytometry after DNA staining was used to assess the effects of the tested products on cell cycle progression with the aim of determining the molecular mechanisms behind their cytotoxic activity. It was found that carvacrol and carveol stopped the cell cycle progression in S phase, while thymol and isopulegol stopped it in G0/G1 phase. In contrast, carvone and eugenol were shown to have no impact on cell cycles when the used tumors were P-815, K-562, CEM, MCF-7 and MCF-7 gem.

Arunasree [22] explored carvacrol anti-proliferative effects on human metastatic breast cancer cell line MDA-MB 231 and determined the basic molecular mechanisms involved in its activity. In addition, carvacrol-induced apoptosis was determined using various assays like MTT assay, Annexin V, mitochondrial membrane potential assay, multi-caspase activation assay and cell cycle analysis by flow cytometer. The authors also examined the PARP cleavage, cytochrome C release and Bax modulation, and Bcl2 ratio using the Western blot analysis. Carvacrol-induced apoptosis was observed in MDA-MB 231 cells dose dependently at an IC50 of 100 mM with a decrease in the mitochondrial membrane potential of the cells, inducing the release of cytochrome C from mitochondria, caspase activation and PARP cleavage [22].

In another study, Yin et al. [23] explored the anti-proliferative and pro-apoptotic effects of carvacrol on human hepatocellular carcinoma cell line HepG-2. Carvacrol was found to inhibit HepG2 cell growth by inducing apoptosis as shown by the results from the Hoechst 33258 stain and Flow cytometric (FCM) analysis. In addition, the incubation of HepG2 cells using carvacrol for 24 h caused apoptosis by activating caspase-3, PARP cleavage and decreased Bcl-2 gene expression. It was also shown that a substantial portion of carvacrol treated cells died as a consequence of an apoptotic pathway in HepG2 cells. Besides, carvacrol selectively was shown to modify the phosphorylation state of members of the MAPK superfamily, decrease phosphorylation of ERK1/2 significantly in a dose-dependent manner, and activate phosphorylation of p38 but it had no impact on JNK MAPK phosphorylation. It was also shown that carvacrol might induce apoptosis via the direct activation of the mitochondrial pathway. The mitogen-activated protein kinase pathway may also play a significant role in the antitumor effect of carvacrol. For the first time, it was noted that the biological activity of carvacrol in HepG2 cells might contribute to the development of carvacrol for liver disease therapy.

Liang et al. [24] studied the effect of carvacrol on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]i$), cell viability, and apoptosis in OC2 human oral cancer cells. It was shown that the natural essential oil carvacrol-induced Ca^{2+} releases from the endoplasmic reticulum in a PLC- and PKC-dependent manner and also causes Ca^{2+} entry through non-store-operated Ca^{2+} channels in OC2 human oral cancer cells. In addition, it caused carvacrol-induced apoptosis that activates ROS and caspase-3 [24].

Llana-Ruiz-Cabello et al. [25] examined the cytotoxicity and morphological effects as induced by carvacrol and thymol on the human cell line Caco-2. Cytotoxicity endpoints assayed (total protein content, neutral red uptake and the tetrazolium salt reduction) and the annexin/propidium iodide staining showed that carvacrol and the mixture carvacrol/thymol induced toxic effects. A morphological study was also performed in order to determine the ultrastructural cellular damages caused by these substances. The main morphological modifications were vacuolated cytoplasm, altered organelles and finally cell death. However, no cytotoxic effects were seen for thymol at any

concentration level and exposure time. Ultrastructural changes supported cellular damages such as lipid degeneration, mitochondrial damage, nucleolar segregation, and apoptosis [25].

Cytotoxicity and pro-apoptotic activity of carvacrol on human breast cancer cell line MCF-7 were assessed by Al-Fatlawi et al. [26]. Cytotoxic effects of carvacrol were measured by MTT and LDH assays and induction of apoptotic was analyzed by expressional analysis of anti- and pro-apoptotic regulatory genes by reverse transcriptase PCR and DNA cleavage assays. The researchers showed that the carvacrol cytotoxicity against MCF-7 cancer cells was in a dose-dependent manner at 24 and 48 h time points ($p<0.05$). In addition, IC50 of carvacrol at 48 h time point was 244.7 ± 0.71 μM. It was also noted that carvacrol treated MCF-7 cells stimulated apoptosis through p53 dependent and Bcl-2/Bax pathway. The results also suggested that carvacrol treatment induces caspase-3, -9 and -6 enzymes gene expression and genomic DNA cleavages [26].

Aydin et al. [27] conducted a study to assess in vitro antiproliferative and/or cytotoxic properties (by3-(4,5 dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) test), genotoxic damage potentials (by single cell gel electrophoresis (SCGE) or Comet assay), and antioxidant activities (using total antioxidant capacity (TAC) and total oxidative stress (TOS) analysis) of CVC in vitro. Dose (0-400 mg/L) dependent effects of CVC were tested on both cultured primary rat neurons and N2a neuroblastoma cells. MTT assay results showed significant decreases ($p<0.05$) in cell proliferation rates in both cell types when treated with CVC at 200 and 400 mg/L. In contrast, the mean values of the total scores of cells supporting DNA damage (for comet assay) was not significantly different from the control values for both cells ($p<0.05$). In addition, it was noted that 10, 25 and 50 mg/L of CVC treatment increased TAC levels in cultured primary rat neurons but not in the N2a cell line. Nevertheless, CVC treatments increased TOS levels in cultured primary rat neurons at only 400 mg/L while they increased TOS levels in N2a neuroblastoma cells at 200 and 400 mg/L [27]. The present findings suggest that CVC could be a source of antioxidant and chemopreventive activities whose effects to be studied on cancer diseases.

MATERIALS AND METHODS

The data were collected by searching a number of key terms including "cancer", carvacrol" and "*Satureja khuzistanica*" in Scopus, PubMed, Web of Science, Google Scholar and IranMedex databases.

RESULTS AND DISCUSSION

Cells in the carvacrol treatment showed significant morphological variations such as cell shrinkage with rounding of cells and formation of membrane blebs characteristic of apoptosis as indicated by microscopic studies [28] in non-small lung cancer cells A549 treated by carvacrol and a study by Arunasree [22] in human metastatic breast cancer cell line MDA-MB 231. One of the biochemical characteristics of apoptotic cells is that cell surface markers achieved by flip-flop movement of the phosphatidylserine are expressed from the inner membrane to the outer membrane of the plasma membrane [29]. Annexin V as a recombinant phosphatidylserine-binding protein interacts strongly and specifically with phosphatidylserine residues and can be used for apoptosis detection [30].

Results from Annexin V assay using flow cytometer showed a dose-dependent increase in the Annexin V positive cells, which indicated the induction of apoptosis by carvacrol [22]. However, the DNA content loss is a typical distinguishing feature of apoptosis and staining of the cell with Propidium iodide and the flow cytometric analysis would be useful in evaluating the cell viability. Consequently, the flow cytometric analysis of carvacrol-treated cells was performed [31,32] and it was found the increase of sub G0/G1 phase (the apoptotic peak) of the cell cycle and a decrease of cells at S phase in a concentration-dependent manner which support the induction of apoptosis and inhibition of DNA synthesis in S phase.

Carvacrol induced cytochrome C release from mitochondria and the activation of caspases. A loss of mitochondrial membrane potential (DcM) shows the loss of cell viability as it reflects the pumping of protons across the inner membrane during the processes of electron transport and oxidative phosphorylation that drive the conversion of ADP to ATP [22]. DcM in this study was measured using the flow cytometer. The results demonstrated a dose-dependent decrease in the membrane potential and consequently, a dose-dependent increase in the percent apoptotic cells. A decrease in DcM changes the membrane stability, leading to the release of mitochondrial apoptosis initiation factors (AIFs), cytochrome c, and the apoptosis protease-activating factor (Apaf-1) into the cytosol. In the cytoplasm, cytochrome c is associated with caspase-9, Apaf-1 and dATP to form the apoptosome complex [33], which in turn activates caspase- 9, -3 and -7. To further assess the apoptotic pathway, the release of cytochrome c from mitochondria into cytosol was analyzed using the Western blot analysis in cytosolic fractions of carvacrol-treated cells. The results showed a dose-dependent increase at the levels of cytochrome c in the cytoplasm, pointing to the execution of apoptosis. In addition, the activation of caspases (multi-caspase activation) through cytochrome c was also studied by using flow cytometer and the results clearly showed the increased activity with an increase in the carvacrol concentration.

Oligonucleosomal cleavage from DNA and PARP cleavage in response to carvacrol treatment were among other effects of carvacrol on cancerous cells. Another AIF, Caspase-Activated DNase (CAD), released from mitochondria,

translocate into the nucleus, after getting cleaved by the activated caspase-3 and leads to oligonucleosomal cleavage of DNA into 180 bp fragments [30,31]. In a study conducted by Arunasree [22], the fragmentation of DNA into 180 bp ladder was found in carvacrol-treated MDAMB 231 cells confirming the apoptosis.

As carvacrol is one of the main constituents of *Satureja khuzistanica*, it can be concluded that it shows strong anti-tumor effects. In this regard, Jalavend et al. [34] used *Satureja khuzistanica* essential oil to inhibit iNOS gene expression in Lipopolysaccharide-stimulated J774A.1 macrophage cell line. For this purpose, the effect of different doses of SKEO and carvacrol (0.004%, 0.008% and 0.016%) on iNOS gene expression in normal and LPS-stimulated macrophage cell line was assessed by RT-PCR method and the results showed that both substances reduced the expression of iNOS gene in LPS-stimulated macrophage cell line in a dose and time-dependent manner, but SKEO was more potent than carvacrol.

CONCLUSION

The anti-inflammatory property of *S. khuzistanica* may be due to its effect on iNOS gene expression and reduction of NO as one of the mediators of inflammation [34]. Several reports published on the inhibitory effect of *S. khuzistanica* essential oil on cancer cell lines, lipid reduction, and healing point out that it can be effective as a combination, prevention and treatment used.

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