Journal of Cell and Molecular Research (2011-2012) 3 (2), 51-56

Cytotoxic effect of essential oils from *Salvia leriifolia* Benth. on human Transitional Cell Carcinoma (TCC) and mouse fibroblast

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Received 2 September 2011 Accepted 15 November 2011

Abstract

Essential oils, with plant origin, have been of special attention in cancer research during recent years. Despite many reports on cytotoxic effects of plants from genus salvia, the potential application of their extracts in cancer therapy remains to be assessed in more precise and detailed examinations on the main cause of such effects. In this research, the cytotoxic effect and anticancer activity of essential oils from *S. leriifolia* on human Transitional Cell Carcinomaa (TCC) were studied *in vitro*. The antiproliferative activity of essential oils on TCC and L929 (control) cells was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay, by which the mitochondrial dehydrogenase enzyme activity is assessed based on reduction of the MTT to purple. The amount of essential oils to induce 50% of cells to die, designated as IC_{50}, was determined by repeated experiments and application of different doses of the essence. The established IC_{50} on TCC cells for the essences extracted in two different years of 2006 and 2008 and from two locations of Bajestan and Neyshabour was respectively as: 466 and 250 µg/ml, and 233 and 212 µg/ml. *S. leriifolia* essential oil did not show any detectable effect on L929 cells in this range of concentration. *S. leriifolia* essential oil has inhibitory effects on the growth of both TCC and normal L929 cell lines, although the effective concentrations were significantly different in these cell lines. This effect was dose dependent.

Keywords: *S. leriifolia,* essential oil, cytotoxic, MTT

Introduction

Essential oils are traditionally known for their antiviral, antibacterial, and antifungal properties. In recent years, however, a great deal of attention has been directed to their capacity in the field of cancer research (Jalili et al., 1999; Young, 2005).

In search for better sources of essential oils, *S. leriifolia,* growing mainly in south and hot regions of Khorassan and Semnan provinces, I. R. Iran (Jones, 2009) seems to be a good candidate, due to its valuable pharmacological significance as anti hyperglycemia, anti-inflammatory, analgesic, muscle relaxation, anti-oxidant and sedative effects (Feizzadeh, 2008; Hosseinzadeh and Arabsanavi, 2001; Hosseinzadeh et al., 2009; Jackson, 2009).

S. leriifolia leaves are rich for a special chalcon, butein, with known inhibitory effects on protein kinases. Butein is a very important component in controlling of cell division and differentiation process, and induces apoptosis in human leukemic HL-60 cells. The caspase-3 activity was increased significantly following butein-induced apoptosis (Russin et al., 1989). Despite convincing evidences of considerable content of butein in *S. leriifolia,* there is not enough investigation on antitumor, anticancer, cytotoxic and antiproliferative properties of this plant. In this project the cytotoxic activities of essential oils from *S. leriifolia*, collected from different locations (Bajestan and Neyshabour) and time periods (2006, 2008) were examined on TCC cells in comparison with L929 as control cells.

Materials and Methods

Essential oil extraction and preparation

Essential oils were extracted from *S. leriifolia* leaves by hydrodistilation as previously described and preserved in freezing temperature of about -20°C (Rustaiyan et al., 2007)

Cell culture

The extracts of essential oils were dissolved in dimethyl sulfoxide (DMSO), (Merk, Germany) and diluted in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal calf serum

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(FCS) to reach different working concentrations. Two cell lines, TCC and L929, were cultured in DMEM, supplemented with 10% fetal calf serum (FCS), (Gibco, Scottland) at 37°C in a humidified atmosphere containing 90% air and 10% CO_2.

Preparation of solutions with different concentrations of essential oils

In order to prepare solutions with different concentrations of essential oils, 50 µl (based on the calculated density of the solution, 1 µl equals to 1 µg) of the essences were dissolved in 950 µl DMSO and culture medium, and used as stock solution.

Different concentrations (80, 160, 320, 640 and 1300 µg/ml) were prepared by diluting the stock solution in proper volumes of the culture medium.

Cytotoxicity assay

Assessment of the cell viability was carried out by the MTT assay (Mossman, 1983) using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (sigma, Deisenhofen, Germany). This assay is based on the metabolic reduction of soluble colored formazan product, which can be determined spectrophotometrically after dissolving in DMSO. Briefly, the cells were cultured in T_{25} and T_{75} flasks, and after 90% confluency, they were removed from the culture dishes by trypsinization and suspended in 10 ml culture medium, and seeded with a cell counts of 8×10^3 for TCC and 5×10^3 for L929 cells per well in 96-well plates. The final volume of each well reached to 200 µl by adding the culture medium. The cells were left to grow for 24 to 48 h, and they were then exposed to different concentrations of essential oils (0, 80, 160, 320, 640 and 1300 µg/ml). The MTT assay was performed 24, 48 and 72 h after the treatment. For this, 5 mg/ml of fresh and sterilized MTT dye, in Phosphate-buffered saline (PBS), was prepared. 20 µl of the MTT solution was added to each well and the plates were incubated at 37°C for 4 h. During this period, the living cells produced a blue, insoluble formazan from the yellow, soluble MTT. The remaining MTT solution was removed and 200 µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance for each well was measured at 570 nm (single wavelength) using an ELIZA plate reader. All experiments were performed in triplicates. Cells were treated with various concentrations of essential oils observed under a light-inverted microscope for morphological alterations after 48 h.

Statistical analysis

The data were analyzed statistically using PRISM, INSTAT and SPSS software's. The significant level was ascertained by one way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. Results were expressed as the mean ± SD. P values of <0.05, <0.01, <0.001 in the Tukey test were considered as significant.

Results

The microscopic observations indicated that the TCC cells altered their morphology 48 h after treatment with the essential oils (figure 1).They turned to round shape and nuclear pigmentations happened. These changes were intensified by the time and increasing the concentrations of the extracts. The percentage of living cells in the treated cultures against the control ones was calculated using the following formula:

Living cells (%): (absorbance of the treated cells in each well / mean absorbance of the control cells) × 100.

The dose-responsive curves were calculated at different concentrations of the essential oils and expressed as the mean percentage fraction of control ± standard division (SD). The amount of essential oils to induce 50% of the cells to die, called IC_{50}, was determined by repeated experiments and the application of different doses of the essence.

The most significant effect of apoptotic induction occurred at 48 h after the essential oils administration. The established IC_{50} values, after this period of time, in TCC cells treated with extracts from plants collected in two different years of 2006 and 2008 and from two locations of Bajestan and Neyshabour were respectively as: 466 and 233 µg/ml; and 250 and 212 µg/ml. The essential oil from plants of Neyshabour (2008) showed the highest antiproliferative activity. There was a significant difference between cell viability of TCC and L929 in all examined concentrations (figure 2):

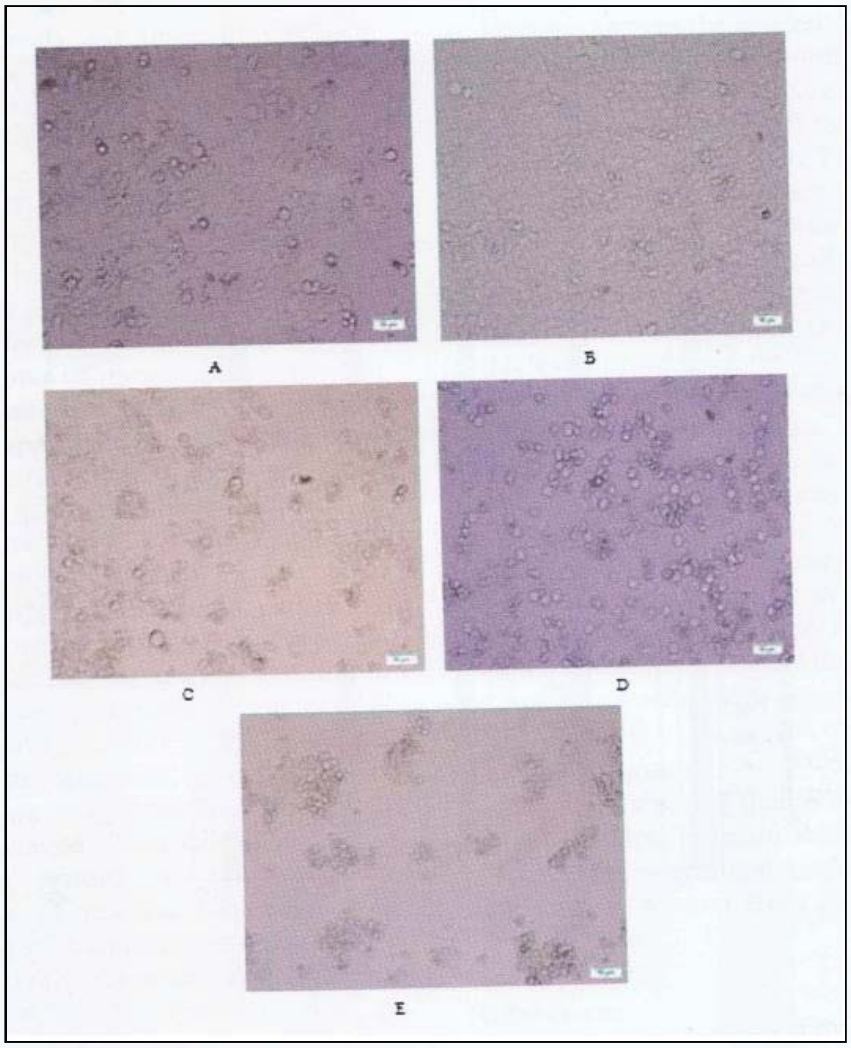
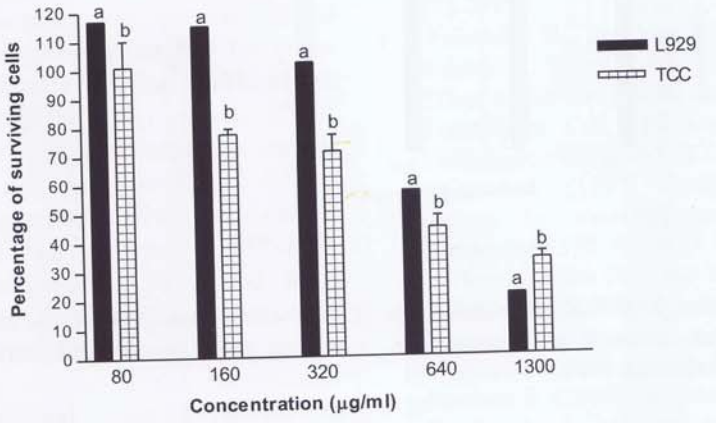
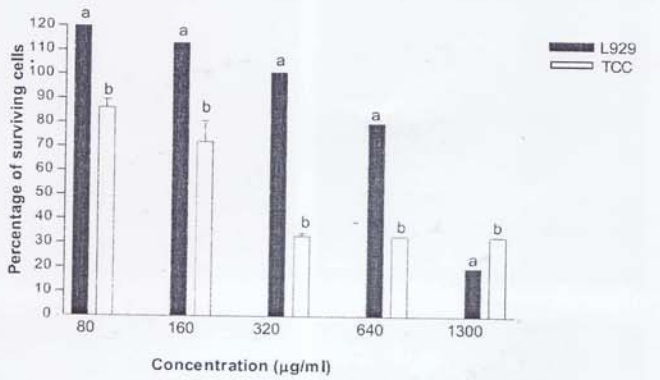


Figure 1. Micrographs of the TCC cells cultured for 48 h and under conditions of (A) without any treatment, and treated with (B) 160 µg/ml, (C) 320 µg/ml, (D) 640 µg/ml, and (E) 1300 µg/ml of essential oil which extracted from plants of Neyshabour (2008) collections.



A



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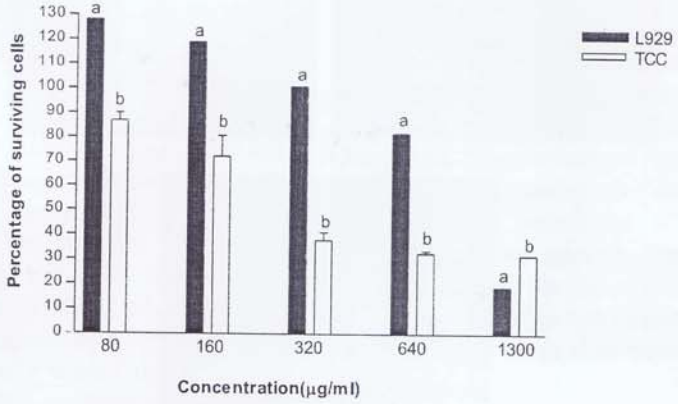

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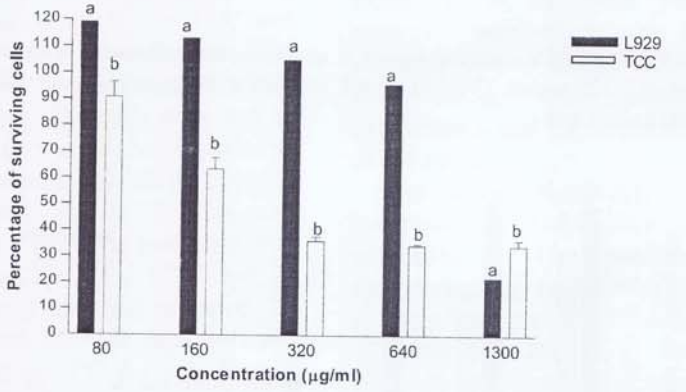

D

Figure 2. Surviving percentage of TCC and L929 cells at 48 h after treatment with the essential oil extracts. The extracts were obtained from different plant collections, including (A) Bajestan 2006, (B) Neyshabour 2006, (C) Bajestan 2008, and (D) Neyshabour 2008.

Discussion

Essential oils, the highly concentrated volatile, aromatic essences of plants, are of special interest for their antiproliferative effects on mammalian cells. Most essential oils contain monoterpenes, in their molecular structures. Monoterpenes are formed in the mevalonic acid pathway in plants. This is the same pathway that makes cholesterol in animals and humans. Early on, cancer researchers realized that some aspects of cholesterol metabolism are involved in cancer growth. They then discovered that plant monoterpenes could interfere with animal cholesterol synthesis, thereby

reducing cholesterol levels and tumor formation in animals (Karlsson and Alexandria, 1997). Some monoterpenes, including limonene and menthol, inhibit hepatitis 3-hydroxy 3-methylglutaryl (HMG) COA-reductase activity and reduce serum cholesterol (Gould, 1997).

Monoterpenes prevent the process of carcinogenesis at both the initiation and promotion/progression stages. In addition, monoterpenes are effective in treating early and advanced cancers. Some of monoterpenes may be chemo-preventive agents with possible cancer blocking and / or suppressing activity. Blocking monoterpenes act during the initiation phase of carcinogenesis. This prevents the interaction of chemical carcinogens with DNA, e.g., by modulating carcinogen metabolism to less toxic forms. The cancer suppressing chemo preventive activity of monoterpenes during the promotion phase of carcinogenesis may be due to inhibition of tumor cell death and/or induction of tumor cell differentiation (Gould, 1997). In addition, monoterpens stimulate apoptosis, a cellular self-destruction mechanism triggered when the DNA content is badly damaged. This safety feature is generally activated before a cell becomes cancerous. Increasing of reactive oxygen species (ROS) is another act of monoterpens, causing the death of the cancer cells (Crowell, 1999; Young, 2005). Leaf extracts of *S. leriifolia*, rich in monoterpen components, are shown here to exhibit different levels of toxicity in cancer cell line of TCC compared to the normal cells of L929.

Probably existence of α-pinen, β-pinen and eucalyptol, as chief members of the *S. leriifolia* essential oils, with about 50% of the essential oils composition, as well as other rare components and the synergic effects of these parts are the main cause of such cytotoxic effects. The profile of the rare components seems different in extracts of this plant, collected from different locations and at different times. For example, samples collected from Neyshabour on year 2008 include components such as: nopinene (0.1%), verbenon (0.09%), β-copaen (0.11%), α-calacoren (0.23%) and neryl isovalerat (17%), which do not exist in detectable amounts in samples from Bajestan. This is in line with higher cytotoxic effects of the Neyshabour extracts. Another pivotal role attributed to monoterpens is their inhibitory effects on isoprenylation. Protein isoprenylation involves the post – translational modification of certain proteins by the covalent attachment of a lipophilic farnesyl or geranyl geranyl isoprenoid group to a cystein residue at or near the carboxyl terminus and inhibition of this process alter the protein activation. Among the crucial proteins subjected to such inhibition are small G-proteins with molecular weight of 20-26 KD, such as rac, ras and rho. Such inhibitions could alter signal transduction and result in altered gene expression. The alterations in the gene expression of mammary carcinomas lead to a G1 cell cycle block, followed by apoptosis, redifferentiation, and finally complete tumor regression in which the tumor cells is replaced by normal cells (Hosseinzadeh et al., 2000; Karlsson J. and Alexandria, 1997).

Cells use the isoprenylation process to help a protein, find its proper location within the cell. In case of ras protein, if it is not in the right place, it becomes over active and can spur cancerous cell growth (Kim et al., 2001; Yoo et al., 2005). In summary, a variety of dietary monoterpenes have been shown to be effective in the chemoprevention and chemotherapy of cancer. A cytotoxic activity of essential oils from *S. leriifolia* is being considered for further investigations *in vivo*.

Acknowledgment

The authors would like to thank Dr. Moghaddam Matin and Mrs. Behnam Rasuli for their excellent support and technical help. This work was supported by a grant from Ferdowsi University of Mashhad, Iran.

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