Journal of Medicinal Plants Research Vol. 4(14), pp. 1422-1427, 18 July, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.259
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Essential oil of *Zataria multiflora* Boiss upregulates retinoblastoma tumor suppressor in the liver of rat

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Accepted 25 June, 2010

The *Zataria multiflora* Boiss is endemic plant of Iran, Pakistan and Afghanistan. Several traditional purposes have been indicated for this medicinal plant including medical purposes, natural preservative of a wide variety of foods and as a food flavour. Currently, the correlation has been found between the essential oil of *Z. multiflora* Boiss and anti-carcinogenesis effects by regulation of two controller genes of mammalian cell cycle named ATM and MDM2. The aim of this study is to understand the molecular response of retinoblastoma tumor suppressor gene (Rb1) and protein (pRb) to the essential oil of this plant. Rb1 gene is required for inhibiting of cell cycle entering to S-phase and its inactivation contributes to tumorigenesis. In present study, liver samples of eight normal male rats were examined for expression analysis of retinoblastoma tumor suppressor gene and its product by using Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and western blotting methods, respectively. The statistical analysis was conducted using SPSS software through independent-sample T-test. The essential oil supplementation at concentration of 50 µl/kg body weight per day induced significantly upregulation in gene and protein expression of Rb1 tumor suppressor in rat liver after one week treatment (P = 0.001). Induction in level of retinoblastoma tumor suppressor by essential oil of *Z. multiflora* Boiss along with its property in regulation of ATM and MDM2 genes expression appear to play an important role in cell cycle control. This data implicated that the essential oil of *Z. multiflora* Boiss may offer new tools for therapeutic intervention in the retinoblastoma pathway for cancer prevention and treatment. This is the first report showing the potential role of essential oil of *Z. multiflora* Boiss as a new class of natural retinoblastoma tumor suppressor inducer and therefore, it could be used as a natural preservative ingredient in food and pharmaceutical industries.

Key words: *Zataria multiflora* Boiss, retinoblastoma tumor suppressor, essential oil, cancer.

INTRODUCTION

Zataria multiflora Boiss is a medicinal plant belonging to the Lamiaceae family that grows only in Iran, Pakistan and Afghanistan (Ali et al., 2000). This plant has been used for several purposes including food flavour, food preservative and has several medicinal purposes including pre-mature labour pains, rupture, anaesthetic, antispasmodic and antibacterial activity (Hossinzadeh et al., 2000; Ebrahimzadeh et al., 2003; Fazeli et al., 2007; Misaghi et al., 2007; Sharififar et al., 2007). Many of these properties are related to the main constituents that are thymol and carvacrol. These compounds have antioxidant properties and are able to inhibit linoleic oxidation (Ebrahimzadeh et al., 2003; Sharififar et al., 2007; Shaffiee et al., 1997).

There is complicated balance between growth stimulatory/inhibitory signals in the body. Perturbation of this balance can allow a cell to proliferate out of control, resulting in cancer development. The best characterized growth inhibitory signals of mammalian cell cycle are the product of tumor suppressor genes. The first recognized tumor suppressor gene is retinoblastoma gene (Rb1) that was discovered by Alfred Knudson (Knudson, 1971). The product of this critical gene is retinoblastoma suppressor protein (pRb) that is a negative regulator of cell cycle by arresting in the G1-phase through binding with E2F-1, PU.1, ATF-2, UBF, Elf-1 and c-Abl transcription factors. The presence of E2F-1 transcription factor is essential for entering to S and M phases of cell cycle. Therefore, in pRb-

deficient cells, it cannot inhibit the E2F-1 transcription factor, leading to increase in cell proliferation. Besides to this main function, pRb has different roles in development, differentiation, senescence and apoptosis (Kaelin, 1999; Brantley et al., 2001).

The loss of both alleles of Rb1 gene through mutation, loss of heterozygosity (LOH) and promoter methylation result in initiation of retinoblastoma disease and also, it is found in a variety of other human malignancies including breast, lung, colon, prostate, osteosarcomas, soft tissue sarcomas and leukemia cancers (Tamrakar et al., 2000; Harbour et al., 1999; Driscoll et al., 1999). Even, patients who develop retinoblastoma disease due to germline RB1 mutations, have a more higher risk than public population of developing epithelial carcinomas, particularly bladder and lung cancers (Fletcher et al., 2004). The goal of this work is to identify the effect of essential oil of *Z. multiflora* Boiss on gene and protein expression of retinoblastoma tumor suppressor in the liver of rat.

MATERIALS AND METHODS

Plant material

The tops at the full flowering stage (June and July) were collected from plants growing wild in the Firoozabad, Fars, Iran. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist. Voucher specimens were deposited in the Herbarium of Kerman Faculty of Pharmacy, Kerman, Iran.

Isolation of the essential oil

The air-dried and ground herbal parts of the plant collected were submitted for 4 h to water-distillation using a British-type Clevenger apparatus (yield 2.8% v/w). The obtained essential oil was dried over anhydrous sodium sulphate, and then stored at 4°C until tested and analyzed (Sharififar et al., 2007).

Animal groups

Six-week-old male Sprague-Dawley rats were obtained from animal room of Afzalipour School of Medicine, Kerman, Iran. The rats were randomly assigned to four experimental groups with eight animals maintained under standard conditions at 22°C, with a period of light/dark cycles of 12 h in store room since two weeks before treatment for adaptation. The rats had free access to synthetic diet and water in this period as well as in one-week treatment period. The groups were as follow: (a) control (400 µl DMSO/kg body weight/day); (b) low-dose essential oil-treated (50 µl/kg body weight/day); (c) medium-dose essential oil-treated (100 µl/kg body weight/day) and (d) high-dose essential oil-treated (200 µl/kg body weight/day). Body weight of rats was recorded during the experimental period.

Tests preparation

The treatment was injected intraperitoneally (IP) once daily for one week at light cycle. Then, rats were sacrificed by decapitation and afterward, liver tissue was rapidly dissected. Individual sections was placed under liquid nitrogen, and then stored at -70°C for subsequent analysis. The animal protocol was approved by Vice Chancellor for Research, Kerman University of Medical Sciences, Kerman, Iran.

RNA extraction and Semi-quantitative reverse-transcriptase polymerase chain reaction analysis

All pipette tips and tubes were treated with 0.1% diethyl pyrocarbonate (DEPC) solution at 37°C for overnight, then heated at 100°C for 30 min and finally autoclaved. Between 25 - 50 mg liver tissue was lysised by sonicator on ice. Total RNA was isolated using RNA tissue extraction kit (Roche Applied Sciences, Germany) based on manual protocol and extracted RNA solved in DEPC-treated water for further processing. In this procedure, DNase I enzyme (Roche Applied Sciences, Germany) was used to eliminate the DNA source of samples. The concentration of total RNA was measured by spectrophotometer NanoDrop ND-1000. The tubes stored at -80°C. Then, cDNA synthesis carried out by first strand cDNA synthesis (Fermentas, Litany) in 25 µl volume was utilized 1 µg total RNA, 1 µl oligo-dT(18), 4 µl buffer, 1 µl RNase inhibitor enzyme, 2 µl dNTPs mix (10 mM) and DEPC-treated water and finally 5 u of M-MuLV reverse transcriptase (Fermentas, Litany). Then, second strand was made by RNase H and DNAPI recombinant (Fermentas, Litany).

The designed primers and NCBI reference number for cDNA fragment amplification for target and control genes as follow: Rb1, 5′-CTT-GGG-TTT-GAG-TCC-TCT-GC-3′ (forward) and 5′-AAT-GGC-ATC-TCA-TCC-AGG-TC-3′ (reverse) with XM_001071121.1; Beta-actin as the control gene, 5′-TCG-TGG-GCC-GCC-CTA-GGC-AC-3′ (forward) and 5′-GGC-CTT-AGG-GTT-CAG-AGG-GGC-3′ (reverse) with NM_031144.2. All Primers were synthesized by Eurofins MWG Operon, Germany. Polymerase chain reaction (PCR) test was carried out in a final volume of 25 µl reaction mixture containing: 1 x PCR Buffer (Fermentas, Litany), 0.2 mM dNTPs mix (Fermentas, Litany), 2 mM MgCl2 (Fermentas, Litany), 1 µM forward and reverse primers, 3 u of Taq DNA polymerase (Fermentas, Litany), cDNA and RNase-free water (Fermentas, Litany). The mixture was heated at 95°C for 3 min and then subjected to 40 cycles of PCR as follows: 94°C for 40 sec, 60°C for 40 sec, 72°C for 40 sec and final extension at 72°C for 5 min. The PCR product lengths of Rb1 and beta-actin genes were 350 and 256 bp. Then, the PCR products were loaded on a 2% gel agarose electrophoresis and visualized by ethidium bromide using ultraviolet (UV) illuminator device. Densitometric analysis was applied to quantify mRNA levels. All tests were performed in at least three independent experiments.

Western blotting analysis

Total proteins were extracted from liver using complete Lysis-M, EDTA-free kit which supplemented with protease inhibitor cocktail (Roche Applied Sciences, Germany) and quantified by spectrophotometer NanoDrop ND-1000 (Pierce, Rockford, IL, USA). Equal quantities of protein (200 µg) were loaded and separated on 12.5% SDS-PAGE gels followed by transfer to PVDF Western Blotting Membranes (Roche Applied Sciences, Germany). Ponceau-S staining (0.1% in 5% acetic acid) was performed to check equal loading/transfer. Blotting carried out by Lumi-LightPLUS Western Blotting Kit (Roche Applied Sciences, Germany). Membranes were blocked with 2% blocking buffer for 2 h, probed with primary antibodies including Anti-phospho-retinoblastoma and Anti-B-actin (Sigma-Aldrich) in 1:1000 dilution with 1% blocking solution for an hour, and then washed 3 × 10 min with 1 × TBST solution (TBS-Tween 20 containing 50 mM Tris, 150 mM NaCl and 0.05% Tween-20). Then, membranes were incubated with an appropriate secondary anti-mouse antibodies conjugated

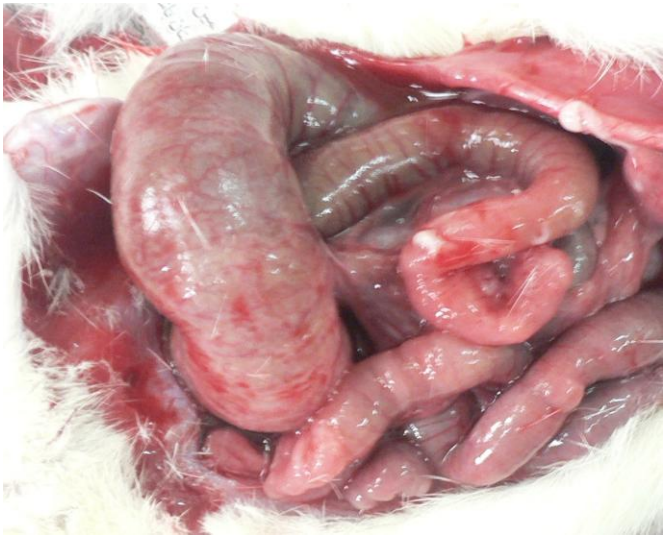

Figure 1. The viscera of dead rat after high-dose essential oil treatment period.

with horseradish peroxidase for another 1 h. Finally, membranes were washed 3 × 10 min with 1 × TBST solution, signals were detected using ECL and Lumi-Film Chemiluminescent Detection Film (Roche Applied Sciences, Germany). All stages of immunoblotting were done under constant shaking.

Statistical analyses

The results were calculated by mean ± S.E.M. Statistical analyses were conducted using SPSS with independent-sample T-test. Group differences resulting in P-values of less than 0.05 were considered to be statistically significant.

RESULTS

In the present study, rats were treated daily with essential oil of *Z. multiflora* Boiss for one week. As shown in Figure 1, following this period, in contrast to previous study (Sharififar et al., 2007) that described the dose of 200 µl/kg body weight/day of essential oil of *Z. multiflora* Boiss is the highest dose that is not lethal in rat, all of rats from the high-dose essential oil-treated group were died, and also, half of the rats from the medium-dose essential oil-treated group were died. However, the rats from the low-dose essential oil-treated group were healthy and also, there was no significant changes in body weight of low-dose essential oil-treated group compared to control group (data not shown).

Essential oil of *Z. multiflora* Boiss upregulated Rb1 gene expression level

To assess the effect of this essential oil on Rb1 gene expression, the Rb1 gene levels examined in control and low-dose essential oil-treated groups by semi-quantitative RT-PCR. As shown in Figure 1, the treatment of essential oil of *Z. multiflora* Boiss caused about 3-fold increase in Rb1 mRNA level of essential oil-treated group compared to control group (*P = 0.001).

Essential oil of *Z. multiflora* Boiss up-regulated pRb protein expression level

The striking elevation of Rb1 mRNA in essential oil-treated group compared to control group promote to measure the level of de-novo pRb protein synthesis by western blot analysis. As shown in Figure 2, the pRb protein level was significantly over-expressed approximately 2.7-fold in essential oil-treated oil compared to control group (*P = 0.001) (Figure).

DISCUSSION

Since ancient time, the medicinal plants have been used as excellent source of pharmaceutical agents for several proposes. Many medicinal plants have anticancer effects and the clinical utilization of plant-derived chemotherapeutic agents has been evident for about half a century (Pan et al., 2010; Lucas et al., 2010).

The clinical trials efforts to discover medicinal plants and also, the candidate substance derived from these plants have important role in prevention and treatment of cancer. So, the present study focused on understanding the molecular mechanism of the essential oil of *Z. multiflora* Boiss on the expression of Rb1 gene and pRb protein in liver sample of normal rat. As the results shown, the essential oil of *Z. multiflora* Boiss increased both the levels of retinoblastoma tumor suppressor gene and protein significantly, depending on the dose. Therefore, provides more evidences for its therapeutic application on cancer therapy. However, the fraction of essential oil that has these potential effects and also, the involved mechanism remain as important issue to resolve. Also, it has been shown previously that the essential oil of this medicinal plant affects on p53 protein stability through regulation of MDM2 and ATM mRNA levels (Vaziri Gohar et al., 2010a,b). Thus, the results of these studies have been shown that the essential oil of *Z. multiflora* Boiss can induce p53 and RB pathways which are so important for normal cell cycle progression. Therefore, this essential oil may be efficient for the tumors that exist concomitant dysfunction of both p53 and Rb1 tumor suppressors occurs.

The pRb tumor suppressor protein has very important role in regulation of mammalian cell cycle. In its active form (hypophosphorylated), it is able to repress transcription of genes involved in S-phase progression by interacting with heterodimeric transcription factor E2F1/DP1 and recruiting histone deacetylases. Therefore, pRb protein has important role against neoplastic

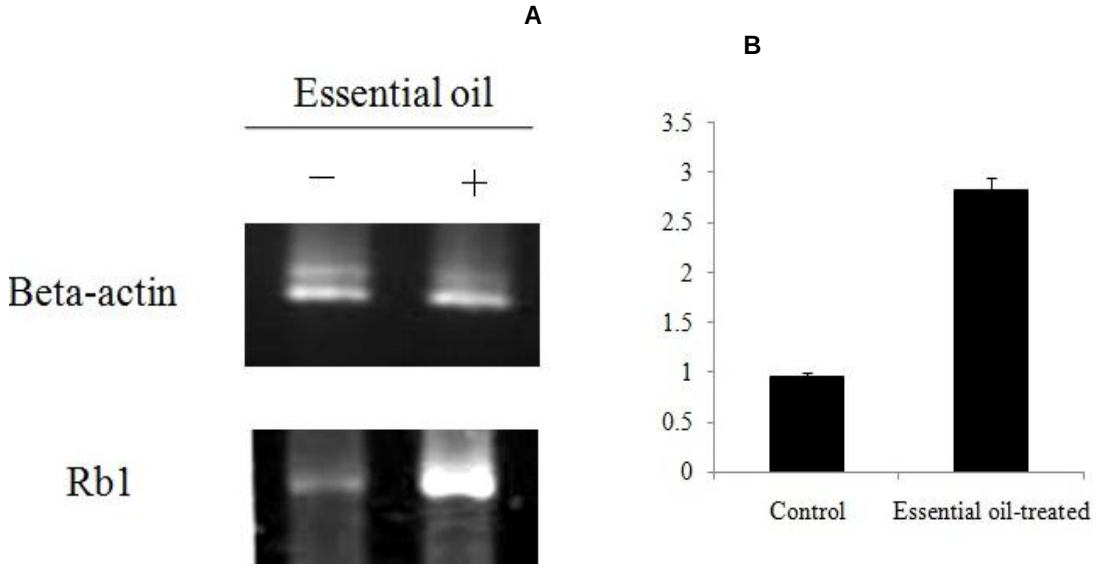

Figure 2. The Rb1 transcripts were increased in essential oil-treated cells. (A) Following seven days essential oil of *Z. multiflora* Boiss treatment, the expression of Rb1 gene was analyzed by semi-quantitative RT-PCR in two percent agarose gel electrophoresis. RT-PCR products of Rb1 gene and Beta-actin (as control gene) were shown. (B): The values presented are the means ± S.E.M for three independent experiments of control and essential oil-treated groups. Densitometric analysis was performed and normalized values to Beta-actin mRNA levels. Bars not sharing a common letter are significantly different ($^{*}P = 0.001$).

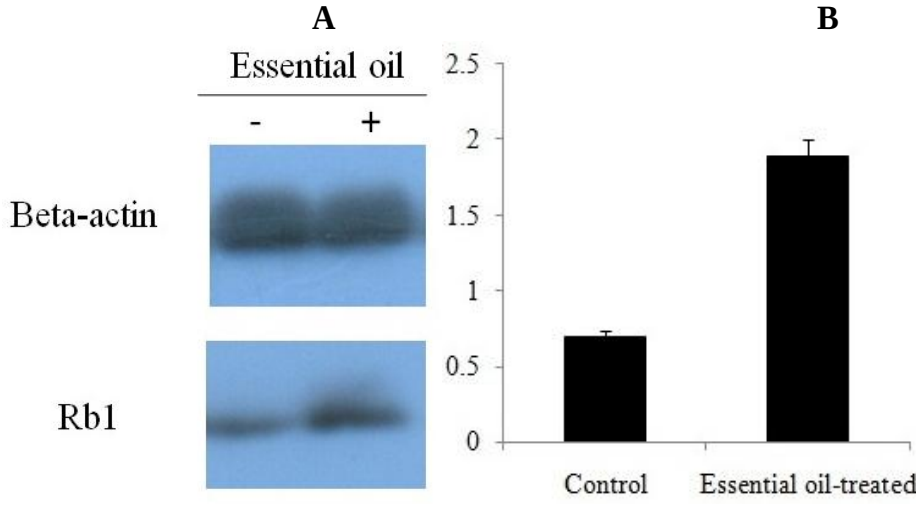

Figure 3. The pRb protein level was increased in essential oil-treated cells. (A) Following seven days essential oil of *Z. multiflora* Boiss treatment, the liver cells of rats were collected and pRb protein level was measured by western blot analysis. Beta-actin served as control. Results are expressed as compared with the control group. (B) The values presented are the means ± S.E.M for three independent experiments of control and essential oil-treated groups. Bars not sharing a common letter are significantly different ($^{*}P = 0.001$).

transformation (Chicas et al., 2010; Vaziri Gohar et al., 2007).

Loss or lowered expression of Rb1 gene through genetic or epigenetic alterations leads to inappropriate DNA synthesis result in extensive proliferation. Also, aberrant Rb1 expression has been associated as a prognostic indicator with worse outcome, less differentiated state, high proliferation rate and high metastatic potential which is seen most commonly in high-grade breast adenocarcinomas, melanoma and urothelial

carcionomas (Botos et al., 2002; Hilton et al., 2004; Halaban, 2005; Vaziri Gohar et al., 2010a,b). Also, most high-grade invasive tumors that progress to serious metastases have both structural and functional defects in the p53 and pRb proteins pathways (Wu, 2005).

The role of epigenetic in Rb1 gene inactivation is through Rb1 promoter methylation, promoter-specific E2F/Rb1 interaction and up-regulation of CDK activity results mostly from elimination of the CDK inhibitor p16INK4A (Halaban, 2005; Vaziri Gohar et al., 2007). The twenty-five compounds were identified in the essential oil of this plant. The main constituents are phenolic compounds such as thymol and carvacrol that consist of 37.7 and 33.6%, respectively. The other compounds are p-cymene, c-terpinene, b-caryophyllene, b-caryophyllene and monoterpenes (Shaffiee et al., 1997; Ebrahimzadeh et al., 2003; Sharififar et al., 2007). However, the efficacy of this plant against cancer may be related to terpenoid compounds. Natural terpenoids, including monoterpenoids, diterpenoids, triterpenoids and tetraterpenoids are able to inhibit tumor cell proliferation of human breast and prostate by inhibiting multiple cancer-specific targets including the proteasome, Nf-kB and Bcl-2 (Yang et al., 2010). Another triterpenoids that found in medicinal plants are Cucurbitacins that have anticancer effects through inhibition of JAK-STAT and MAPK pathways which are important for cancer cell proliferation and survival (Lee et al., 2010). As pRb protein and E2F1 transcription factor down-regulated by MDM2 oncoprotein, the MDM2 down-regulation by essential oil of *Z. multiflora* Boiss may be a possible mechanism for the present results (Martin et al., 1995; Xiao et al., 1995; Vaziri Gohar et al., 2010a,b). The similar result has been obtained by retinoblastoma regulator named Ad5CMV–RB94 as an adenoviral vector that it can restore p53 and RB pathways, telomere erosion, growth arrest in urothelial tumor cell lines, without affecting normal urothelium (Wu, 2005).

In summary, the results presented in this study revealed that essential oil treatment with a low-pharmacological dose may consider as novel inducer of retinoblastoma tumor suppressor which upregulated both endogenous Rb1 mRNA level and the pRb protein expression in normal male rats, although, the mechanism of this issue remains unclear. Also, there is insufficient scientific basis to set a precise figure for an intake level of essential oil usage. These results suggest that an appropriate combination of this compound with other cancer-preventive agents.

ACKNOWLEDGEMENT

The part of this project is supported by grant #86/126 of Vice Chancellor for Research, Kerman University of Medical Sciences, Kerman, Iran. Also, the author is grateful to Dr. Fariba Sharififar and Mr. Amin Pourhemati for their kindness in providing me with essential l oil isolation and removing the liver tissue of rats, respectively.

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