Satureja Khozestanica essential oil (SKEO) inhibits iNOS gene expression in Lipopolysaccharide-stimulated J774A.1 macrophage cell line

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Abstract: Background: Satureja Khozestanica is a medicinal herb indigenous to Iran which grows mainly in Lorestan and Khuzestan Provinces.The main component of this herb is a monoterpene named Carvacrol. Previous studies have shown that this herb has anti-inflammatory properties, so we aimed to investigate the effect of its essential oil (SKEO) and Carvacrol on iNOS gene expression in LPS-stimulated J774A.1macrophage cell line. **Materials and Methods**: Essential oil was prepared from fresh aerial parts of the plant. 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. **Results:** 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**: Anti-inflammatory property of Satureja khozestanica may be due to its effect on iNOS gene expression and reduction of NO as one of the mediator of inflammation.
[Jalalvand M, Shahsavari R, Sheikhian A, Mosayebi G. **Satureja Khozestanica essential oil (SKEO) inhibits iNOS gene expression in Lipopolysaccharide stimulated J774A.1 macrophage cell line**. *Life Sci J* 2013;10(2s):230-235] (ISSN:1097-8135). http://www.lifesciencesite.com. 40

Keywords: Satureja khozestanica, Essential oil, Carvacrol, Inflammation, iNOS

1. Introduction

Satureja Khozestanica is a medicinal herb which grows in northern Khuzestan and southern Lorestan provinces of Iran. Recently, many studies have been undertaken to investigate the effects of this herb extract to discover therapeutic potentials of its essential oil.It has been shown that this herb has antimicrobial, anti-inflammatory and antioxidant properties (1-4). GC-Mass analysis of essential oil of endemic Satureja Khozestanica have shown that flavonoids, mainly Carvacrol (86.29%) and Paracymene (3.35%), are the main components of its essential oil (5). Carvacrol (2-methyle-5-isopropylphenol) is a monoterpene with anti-inflammatory properties (6, 7).

Nitric Oxide (NO) is produced by many cell types and has diverse biological effects such as antimicrobial and tumoricidal activities (8). It has a prominent role not only in adaptive but also in innate immunity (9). It is derived from L-arginine by an enzyme called Nitric Oxide Synthase (NOS). There are three isoforms of NOS, two of them (nNOS and eNOS) are constitutively expressed in cells (neuronal and endothelial cells, respectively) and the third one (iNOS) is induced in response to activating agents such as Lipopolysaccharide (LPS) which binds to the toll-like receptor 4 on the cell surface of macrophages (10). In contrary to old views, all isoforms of NOS function in the immune system. Currently, it is believed that iNOS helps to control detrimental immune reactions and protects us to some degree against pathophysiological conditions such as autoimmunity (11). It is also one of the main effector molecules in disease conditions such as septic shock (12). Researchers are recently interested in investigation and research into the effect of natural products from medicinal herbs to ameliorate human diseases. Since Satureja Khozestanica affects biological processes such as inflammation (2) as a critical player in many disease processes- and NO is one of the molecules involved in inflammation and immunity, we aimed to investigate its effect on the iNOS gene expression in simulated J774A.1 macrophage cell line by Real-time PCR method.

2. Materials and Methods

2.1. Primary culture of J774A.1 macrophage cell line

J774A.1 macrophage-like cell line (NCBI-C483) was purchased from Pasteur Institute of Iran.The cells (1-2×10^6) were cultured in complete RPMI-1640 medium, supplemented with 10% fetal bovine serum, containing 100µg/mL Streptomycin and100µg/mL Penicillin and stimulated with LPS (1µg/mL).The

cultures were maintained in the presence of 5% CO_2 and 95% relative humidity at 37°C.

2.2. Isolation of the essential oil from Satureja khozestanica

Satureja khozestanica essential oil(SKEO) was prepared from Satureja khozestanica which was grown (wild and cultivated types) in Khorram Abad (Lorestan province, western Iran) and was identified by Medicinal Herbs Research Center of National Forestry Organization. he aerial parts (shoots) of the plant were collected and were air-dried at ambient temperature in the shade and completely powdered by hand. The distilled, dried powder from plant shoots was mixed and boiled in a Clevenger apparatus for 5h. Mixture of water and essential oil was evaporated and was entered into a condenser by a connector tube. The two main components were separated because of the difference between essential oil and water densities. The essential oil was yellowish in color and soluble in ether, chloroform and alcohol. Then, suspended water drops were absorbed by Sodium Sulphate and the final product was saved at 4°C.

2.3. Effect of Satureja khozestanica essential oil (SKEO) on proliferation of LPS-stimulated cell line

Cell proliferation was considered as an indicator of cytotoxic effect of SKEO on the J774A.1 macrophage cell line. The LPS- stimulated cells were treated by different concentrations of Carvacrol and Satureja Khozestanica essential oil (0.001%, 0.002%, 0.004%, 0.008%, and 0.016%) for 12, 24 and 48 hours. The cells cultured without any additive were used as the control. We used MTT test to measure the quantity of cell proliferation according to Carmichael *et al* protocol (13).Each test was repeated three times. Optical density was measured at 570nm.

2.4. The effect of essential oil and Carvacrol on the iNOS gene expression

Cultured cells were treated with 0.004%, 0.008%, 0.016% doses of Carvacrol and SKEO for 8 hours with 1µg/mL lipopolysaccharide and iNOS gene expression was quantified by Real-time PCR method(14, 15). Total RNA was extracted from cell line using Promega kit. Electrophoresis of the extracted RNA was done in 1% agarose gel, 80-90 volts for 45min and it was quantitatively measured by Nano-drop Spectrometry device. Reverse transcription was performed using Fermentas cDNA synthesis kit. Real-time PCR was done by (Applied Biosystems, ABI) and Light Cycler Fast Start DNA Master SYBR Green I kit (Roche, Germany)and conditions according to the manufacturer's protocol were applied.

2.5. Properties of iNOS gene Primers and Conditions of RT-PCR Reaction

In order to do Real-time PCR for iNOS and β-actin gene, we used specific primers which are shown in table 1.The reaction for cDNA synthesis was carried out for 40 cycles. The polymerase chain reaction profile was 95°C for 7s,60°C for 15 s and72°C for 15s.

3. Results

3.1. Cytotoxicity Assay

The quantity of cell proliferation (as a criteria of possible cytotoxic effect of SKEO) under different doses (0.001%, 0.002%, 0.004%, 0.008%, 0.016 %.) of SKEO and Carvacrol for 12 hours ,is shown in figure 1.All doses of both agents increased cell proliferation ,and did not any inhibitory effect on the proliferative activity of the macrophage cell line .The same results were obtained when the treatment period was increased to 24 and 48 hours (data not shown). So, both substances had no cytotoxic effect on this cell line as assayed by proliferation activity.

3.2. Quality of extracted RNA

Extracted RNA was electrophoresed on 1%-agarose gel .Absorption ratio at 260 nm was 1.8 to 2 in 260nm which shows that RNA is not contaminated with DNA and protein.

3.3. Effect of Carvacrol on iNOS gene expression in macrophagecell line

The effect of Carvacrol on iNOS gene expression was assayed in the presence and the absence of LPS. As it can be seen in figure 2(the red line), LPS stimulate iNOS gene expression in macrophage cell line, but its stimulatory effect is gradually suppressed by increasing doses of Carvacrol. The greatest inhibitory effect was exerted by 0.008% and 0.016% doses. A little difference can be observed between these two doses, but it is not significantas analyzed by correlation curve and Error Bars.

3.4. The effect of SKEO on iNOS gene expression in macrophage cell line

The effect of SKEOon iNOS gene expression was also assayed in the presence and the absence of LPS. As it can be seen in figure 3 (the blue line), LPS stimulate iNOS gene expression in macrophage cell line in comparison with control (red line), but its stimulatory effect is gradually suppressed by increasing doses of SKEO. The greatest inhibitory effect was exerted by 0.016% dose.

3.5. Comparison of the effect of Carvacrol and SKEO on iNOS gene expression in macrophage cell line.

The inhibitory effect of SKEO on iNOS gene expression was stronger than that of Carvacrol .Of course, both were effective in modulating basic gene

expression .It was shown that the inhibitory effect of 0.004%, 0.008% and 0.016% doses of SKEO on iNOS gene expression were more potent than the same doses of Carvacrol (figure 4).The 0.008% dose of SKEO had the maximum and 0.004% dose of Carvacrol had the minimum effect on the iNOS gene expression inhibition (Figure 5).

3.6. Conformity of RT-PCR products with iNOS gene amplicon

In order to be confident about the specificity of RT-PCR products, electrophoretic profile of them was compared with the length of iNOS gene amplicon, as it can be seen in figure 6, their length are same and there is a complete conformity between them.

4. Discussion

Inflammation, specifically the chronic type, can lead to many clinical problems for patients. In spite of modern medical treatment for inflammation-driven diseases, an extensive research has been launched to evaluate traditional therapeutic modalities based on medicinal herbs. The aim is to find better and more effective therapies with fewer side effects. In this study we aimed to test the effect of Satureja Khozestanica essential oil and Carvacrol on the expression of iNOS gene as a main player of many inflammatory conditions. Previous studies have shown that iNOS is one of the factors involved in inflammatory process(7). Inflammatory agents such as LPS stimulate iNOS gene expression in macrophages(16). Although NO is required biologically and performs many physiological functions in the body, in inflammatory chronic diseases, its quantity will be increased and can lead to harmful septic shock (12).So, many studies have so far been conducted to inhibit iNOS gene expression, but there are no studies conducted on the effect of Satureja Khozestanica on iNOS gene inhibition.

We showed that essential oil and Carvacrol can decrease iNOS expression in a dose-dependent manner. Effects of Satureja Khozestanica were stronger than Carvacrol in iNOS gene expression inhibition by lipopolysaccharide; however, other components of Satureja Khozestanica could amplify the effect of Carvacrol on iNOS gene expression inhibition under the treatment of LPS.

In previous studies, it has been observed that Satureja Khozestanica has anti-inflammatory properties (2).In this study, Amanlou and coworkers compared the effect of Satureja Khozestanica with that of indomethacin and found that its anti-inflammatory effect is comparable with indomethacin. In another study, effect of this herb and that of prednisolone on the inflammatory bowel disease (IBD) was compared. It was found that Satureja Khozestanica essential oil protects subjects against IBD(17).

Since Carvacrol is the main component of this herb, in this study a comparison was made between the effect of Carvacrol and that of Satureja Khozestanica on iNOS gene expression in LPS-stimulated J774A.1 macrophage cell line. The results showed that SKEO is more potent than Carvacrol in their inhibitory effects on the expression of this gene (the percent of iNOS gene expression in LPS-stimulated macrophage by SKEO was 70%). This study illustrated that anti-inflammatory properties of the SKEO could be exerted by iNOS gene expression inhibition.

Whereas iNOS is produced in activated cells, it is not expressed in macrophages naturally and its production needs an inducer . Therefore, we investigated the effect of SKEO and Carvacrol on iNOS gene expression in the presence and the absence of bacterial LPS as one of the most important inducers of iNOS. The philosophy behind this idea was to verify gene expression changes by SKEO in LPS-activated cell not in resting normal cell. As it can be seen in Figure 5, the percentage of gene expression inhibition by the SKEO is higher than Carvacrol and is about 70%. iNOS gene expression inhibition by SKEO and Carvacrol can be investigated in more detail and this will require more molecular and cellular studies. For example, expression of each gene will be regulated in different levels, for example in transcription and in translation levels. In order to regulate gene expression in transcriptional level, respective gene transcription factors and regulation should be verified and at translation level, the factors affecting mRNA stability should be examined. Some evidence indicates that this gene is expressed in normal non-stimulated cells, and IRF1 and NF-κB, work as enhancer for the promoter of this gene(18).

LPS will cause iNOS gene expression through NF-κB pathway (19), but on the other hand it should be noted that IFN γ and TNF α induce IRF1 production by STATα which operates as a transcription co-factor and regulates iNOS gene expression by this pathway. (18) iNOS gene expression regulation is very complicated and is affected by different cytokines (20)Many studies have shown that its expression is regulated in transcriptional level, by different cytokines (21, 22) and through a promoter which is located in upstream -4.7 kb area of gene within enhancer region. In murine iNOS gene promoter, about 1kb of 5'-flanking sequence is required for LPS and cytokines to exert their effects(23). Therefore, more than one mechanism may be exploited to negatively regulate iNOS gene expression. So, different intervening strategies can be implemented to overcome inflammation. Our results have shown that Satureja khozestanica have probably positive medical potentials to inhibit inflammation.

Table 1: The forward and reverse primers used for Real time -PCR of β-actin and iNOS genes.

Gene	**Accession number**	**Forward & Reverse** Primers	**Primer length(bp)**	**Amplicon(bp)**
ACTB	NM_007393	F: 5'-AGCTTCTTTGCAGCTCCTTC-3' R: 5'-GCTTTGCACATGCCGGA-3'	20 17	107
NOS2	NM_010927	F: 5'-CCTGGAGGTTCTGGATGAG-3' R: 5'-CTGAGGGCTGACACAAGG-3'	19 18	194

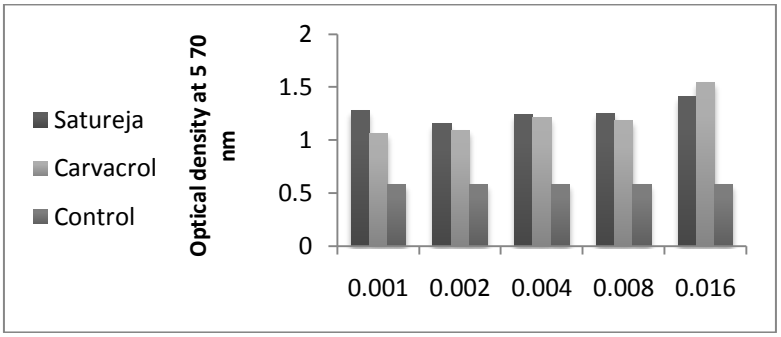

Figure 1: Effect of different doses of SKEO essential oil and Carvacrol on proliferation of J774A.1 macrophage cell line.The vertical axis shows the amount of optical density which measured in MTT test and the horizontal axis shows different doses of SKEO and Carvacrol (percentage of the stock solution). Greater OD is indicative of more proliferation. All doses had not any harmful effect on the cell proliferation.

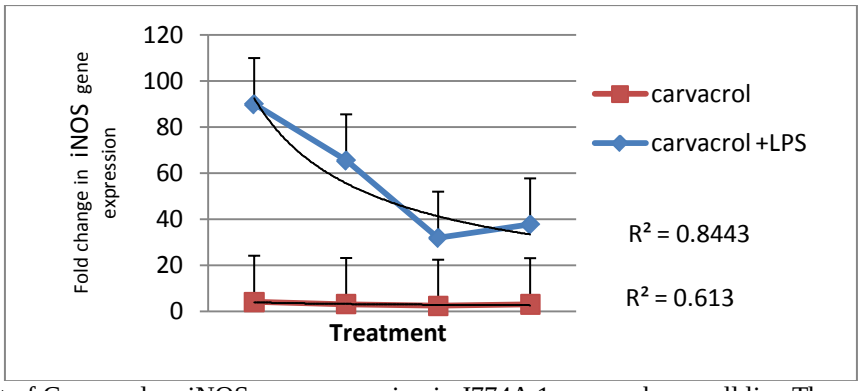

Figure2: The effect of Carvacrol on iNOS gene expression in J774A.1 macrophage cell line.The red line indicates the level of gene expression in control samples (treated with Carvacrol in the absence of LPS) and the blue line indicates the level of gene expression in LPS-stimulated samples treated with different doses of Carvacrol (From left to right 0.00%, 0.004%, 0.008% and 0.016% doses respectively).

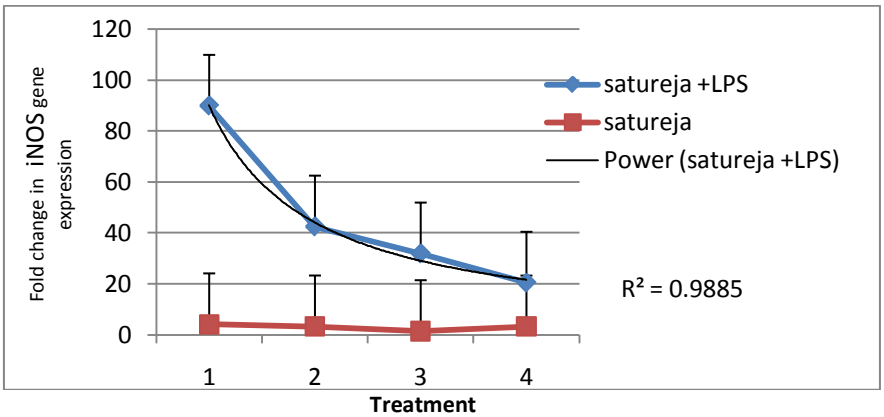

Figure 3:The effect of SKEO on iNOS gene expression in J774A.1 macrophage cell line .The red line indicates the level of iNOS gene expression in SKEO-treated samples(in the absence of LPS) and the blue line indicates the level of gene expression in LPS-stimulated samples treated with different doses of SKEO (From left to right 0.00%, 0.004%, 0.008% and 0.016% doses respectively).

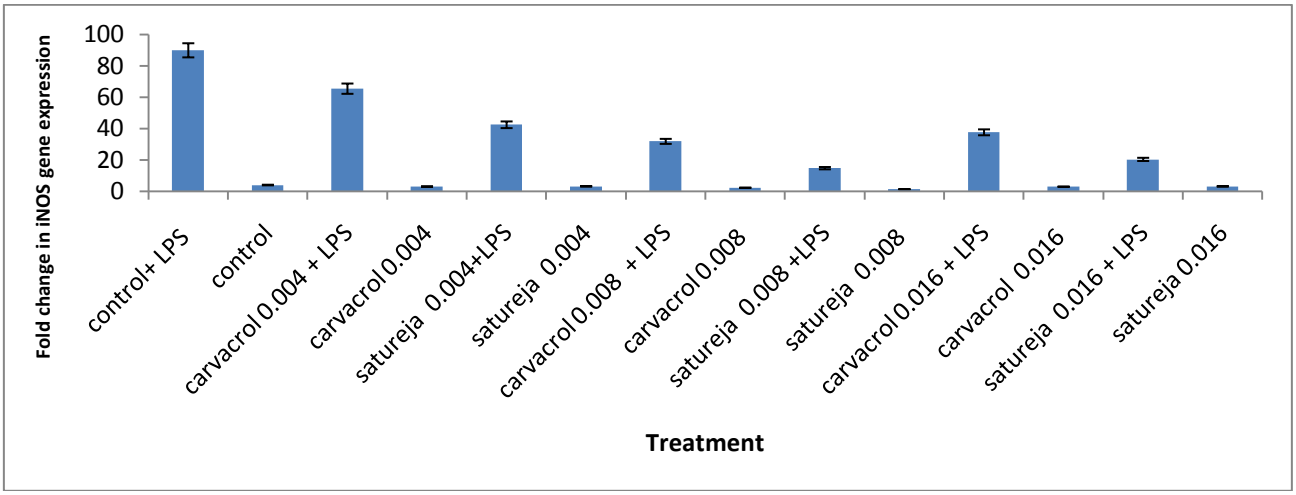

Figure 4: A comparison between the effect of Carvacrol and SKEO on iNOS gene expression in J774A.1 macrophage cell line. All doses of SKEO are more potent than the equal doses of Carvacrol.

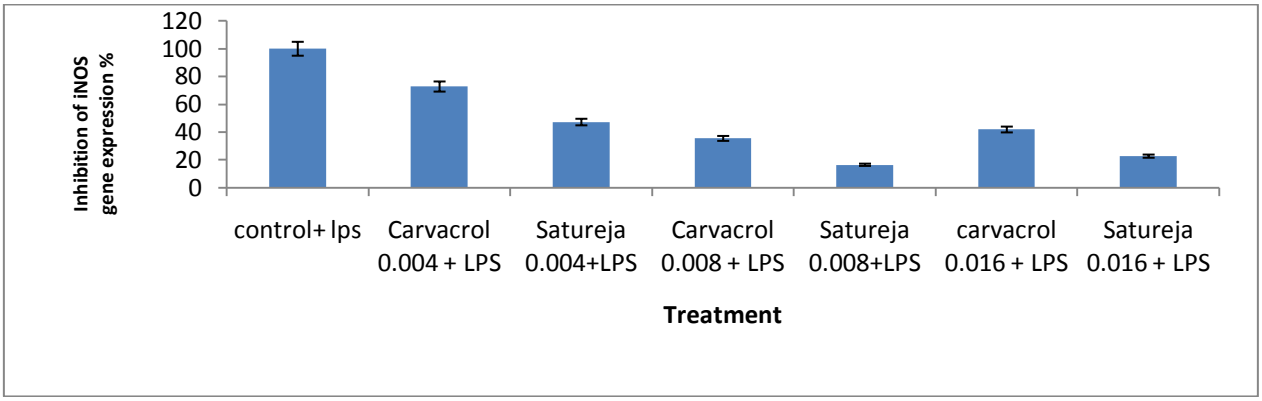

Figure5**:** Comparison between the effect of Carvacrol and SKEO on iNOS gene expression in J774A.1 macrophage cell line. All doses of SKEO are more potent than the equal doses of Carvacrol.

Figure 6: Conformity between RT-PCR products of iNOS gene and iNOS gene amplicon. Ladder 100 bp Fermentase.iNOS PCR product:197bp

Acknowledgement
This work was supported by a grant from the Research deputy of Arak University of Medical Sciences. We express our special thanks to all of the members of the research committee.

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1/18/2013