

In vitro Anti-inflammatory Effects of *Satureja Khuzestanica* Essential Oil Compared to Carvacrol

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Abstract

Introduction: *Satureja khuzestanica* grows mainly in the southwest part of Iran as a native plant. This edible herb contains various compounds including the *S. Khuzestanica* essential oil (SKEO) and monoterpene known as Carvacrol. Previous studies have shown the anti-inflammatory effects of *S. Khuzestanica* without mentioning the exact mechanism of its function. Given that prostaglandin synthesis, which is one of the main mediators of inflammation, is regulated by the cyclooxygenase-2 (COX2) gene, the present study investigated the effects of SKEO and Carvacrol on the expression of the COX2 gene in the stimulated-J774A.1 macrophage cell line.

Methods: To this end, fresh aerial parts of the plant were processed to prepare SKEO. Then, different doses of SKEO and Carvacrol (i.e., 0.004%, 0.008%, and 0.016% v/v) were used to treat with the lipopolysaccharides (LPS)-stimulated cell line for eight hours. After RNA extraction, the real-time polymerase chain reaction technique was applied for gene expression analysis.

Results: In the LPS-stimulated J774A.1 macrophage cell line, COX2 gene expression reduced significantly in a dose-dependent manner (0.004%, 0.008%, and 0.016%, $P=0.024$, $P=0.021$, and $P=0.013$ v/v of SKEO, respectively) by SKEO, and the effect of Carvacrol was less powerful (0.008% and 0.016%, $P=0.027$ and $P=0.038$ v/v, respectively) compared to SKEO. Finally, the comparison between SKEO and Carvacrol showed higher significant inhibitory effects of SKEO on COX2 gene expression in comparison with Carvacrol in 0.004% v/v concentration ($P=0.037$).

Conclusion: In general, SKEO significantly reduced COX2 gene expression, thus it can be suggested that its anti-inflammatory effect may result from the inhibition of the synthesis of this pro-inflammatory gene.

Keywords: Carvacrol, *Satureja khuzestanica*, Cyclooxygenase-2 gene

Introduction

Satureja khuzestanica (*S. Khuzestanica*), as an herbaceous and aromatic medicinal plant, grows in the southwest part (i.e., Lorestan and Khuzestan provinces) of Iran. It was first identified and named by Jamzad in 1994.¹ Recently, different studies have proven the anti-inflammatory and anti-analgesic effects of *S. Khuzestanica*.²

S. khuzestanica contains the *S. Khuzestanica* essential oil (SKEO). The gas chromatography-mass spectrometry analysis of SKEO shows that flavonoids include Carvacrol (2-methyl-5-Isopropyl phenol, 86.29%) and Paracemin (3.35%) as their main components.³ Carvacrol is a monoterpene compound with anti-

inflammatory properties.⁴ Many mediators such as prostaglandins can modulate inflammatory processes. One of the key enzymes, which is involved in prostaglandin synthesis, is cyclooxygenase (COX) that has three isoforms of COX1, COX2, and COX3.⁵ COX2 is rapidly expressed by extracellular stimuli as part of the inflammatory reaction and is undetectable in many normal tissues. In addition, it is actually associated with inflammation situations like pain, fever, and atherosclerosis through prostaglandin synthesis.⁶

The long-term consumption of COX2 suppressors such as nonsteroidal anti-inflammatory drugs (NSAIDs) can boost the risk of cardiovascular diseases with side



effects in the digestive system and kidneys.^{7,8} Therefore, medicinal plants with fewer side effects could play critical roles in treating inflammation. Hence, the present study evaluated the effect of SKEO on COX2 gene expression, as one of the enzymes involved in inflammation regulation.

Materials and Methods

SKEO Preparation

S. Khuzestanica, which was grown in Khorramabad (Lorestan province in the west of Iran), identified by Medicinal Herbs Research Center of National Forestry Organization, and finally used for SKEO preparation based on previous protocols.^{9,10} Carvacrol was also purchased from Sigma Company (Sigma, MO, USA).

Macrophage Cell Line Treatment

The J774A.1 murine macrophage cell line was purchased from the Pasteur Institute of Iran (Code No; NCBI-C483). The cells were cultured in the RPMI-1640 medium (Gibco, NY, USA) supplemented with a 10% fetal bovine serum (Gibco, NY, USA), 100 µg/mL streptomycin (Sigma, MO, USA), and 100 µg/mL penicillin (Sigma, MO, USA) under 5% CO₂ and 95% humidity at 37 °C. The cultured cells at the density of 5×10⁵ cells/well were stimulated with 1 µg/mL of lipopolysaccharides (LPS, Sigma, MO, USA) in parallel with the control (without LPS stimulation). Both LPS-stimulated and non-stimulated samples were treated with different concentrations of Carvacrol and SKEO (i.e., 0.00%, 0.004%, 0.008%, and 0.016% v/v) in separate wells for eight hours to be used in the evaluation of COX2 gene expression.

Gene Expression Analysis

The total RNA was extracted from harvested cells using the Promega RNA extraction kit (Promega, Madison, USA) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed into cDNA (Fermentas, USA) according to the manufacturer's instructions. Then, the synthesized cDNA was amplified in duplicates using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) by the real-time polymerase chain reaction (Applied Biosystems, CA, USA). In addition, the forward and reverse primers (Table 1) of COX2 (accession number: NM_011198) and beta-actin (Actb; accession number: NM_007393) as target and housekeeping genes

Table 1. Sequences of the Designed Primers

Name	Accession Number	Primers 5'→3'	Amplicon Length (bp)
β-actin	NM_007393	F: AGCTTCTTTGCAGCTCCTTC R: GCTTTGCACATGCCGGA	107
COX2	NM_011198	F: TGCACTATGGTTACAAAAGCTGG R: TCAGGAAGCTCCTTATTCCCTT	124

Note. COX2: Cyclooxygenase-2; F: Forward; R: Reverse.

were designed, respectively, using Allele ID software (version 7.5, Premier Biosoft, CA, USA) and confirmed by PubMed/Blast. Finally, the data were analyzed by the 2^{-ΔΔCT} method.

Statistical analysis

SPSS software version 16.0 (SPSS, Inc., Chicago, Illinois, USA) was used for statistical analysis. Independent sample t-test was used to explore meaningful differences between variables in each group. Mann-Whitney U test was used for comparisons the results between the studied groups. P value < 0.05 was considered as statistically significant.

Results

Carvacrol effect on COX2 gene expression

The Carvacrol effect on the expression of COX2 in the J774A.1 cell line was measured in the absence and presence of LPS. The effects of different doses (0.00%, 0.004%, 0.008%, and 0.016% v/v) of Carvacrol on control and test samples are shown in Figure 1. LPS induced COX2 gene expression in all treated samples, but Carvacrol inhibits significantly gene expression dose-dependently (0.008%; $P=0.027$ and 0.016%; $P=0.038$ v/v) in LPS-treated test sample (Figure 1).

SKEO Effect on COX2 Gene Expression

The SKEO effect on COX2 gene expression in the J774A.1 cell line was measured in the absence and presence of LPS. The samples were treated by different doses of SKEO (i.e., 0.00%, 0.004%, 0.008%, and 0.016% v/v) to verify the effect of this compound on COX2 gene expression. As shown in Figure 2, the COX2 gene expression reduced significantly (0.004%, 0.008%, and 0.016%, $P=0.024$, $P=0.021$, and $P=0.013$ v/v of SKEO, respectively) in LPS-stimulated cells.

Comparison of the Effect of Carvacrol and SKEO on COX2 Gene Expression

Carvacrol and SKEO inhibited the expression of COX2 gene expression in the LPS-induced J774A.1 macrophage cell line. Figure 3 shows higher significant inhibitory effects of SKEO on COX2 gene expression compared to Carvacrol in 0.004% v/v concentration ($P=0.037$).

Discussion

Inflammation, as a central constituent of many pathological conditions, has serious consequences for patients with a chronic form of inflammation and is considered as one of the important factors of tumor development.¹¹ In this regard, inflammatory mediators may play some roles in CNS disorders such as Alzheimer's, autoimmunity-like rheumatoid arthritis, and cardiovascular diseases.¹²⁻¹⁴

Some mediators of inflammation are derived from the endogenous substances of the body like the arachidonic acid which is a normal constituent of cellular membranes.¹⁵

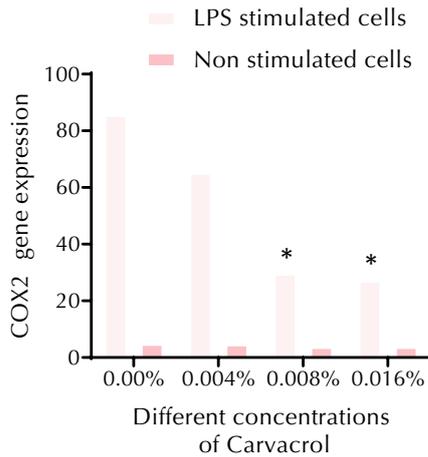


Figure 1. COX2 Expression in J774A.1 Macrophage Cell Line Treated With Carvacrol

Note. COX2: Cyclooxygenase-2; LPS: Lipopolysaccharides; The results indicate that the level of COX2 gene expression in LPS-stimulated cells reduced significantly in higher Carvacrol concentrations (0.008% and 0.016%, $P=0.015$ and $P=0.019$ v/v, respectively) in comparison with the absence of Carvacrol stimulation (0.00% v/v; $*P<0.05$).

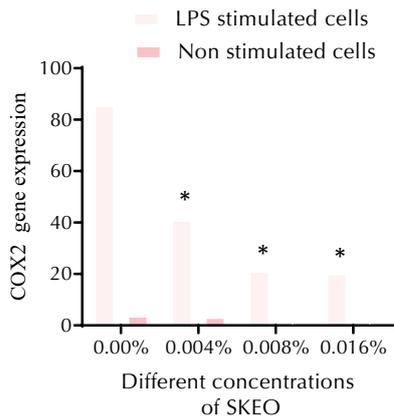


Figure 2. COX2 Gene Expression in J774A.1 Macrophage Cell Line Exposed to SKEO

Note. COX2: Cyclooxygenase-2; LPS: Lipopolysaccharides; SKEO: *Satureja khuzestanica* essential oil; The results demonstrate that the level of COX2 gene expression in LPS-stimulated cells reduced significantly in higher SKEO concentrations (i.e., 0.004%, 0.008%, and 0.016% v/v) compared with the absence of SKEO stimulation (0.00% v/v; $*P<0.05$).

Further, arachidonic acid is a fatty acid that is a precursor of prostaglandins which are one of the important mediators of inflammation. Furthermore, the conversion of this precursor to prostaglandins is catalyzed by a key enzyme which is called COX2. Moreover, the lipopolysaccharide of Gram-negative bacteria and some interleukins induce prostaglandin production¹⁶ which could be suppressed by the inhibition of COX2 and inducible nitric oxide (iNOS) synthesis. Previous research has confirmed the beneficial effect of iNOS and COX2 inhibition.¹⁷

COX2 activity has been studied in many diseases

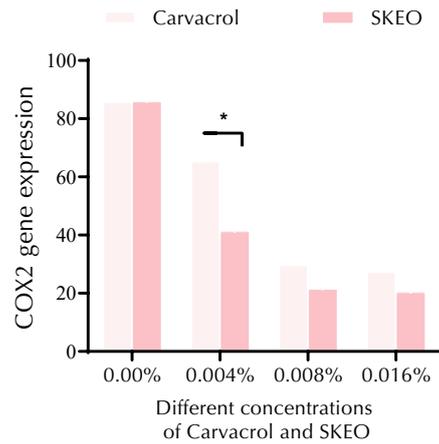


Figure 3. Comparison Between the Effect of Carvacrol and SKEO on COX2 Gene Expression in LPS-induced J774A.1 Macrophage Cell Line

Note. SKEO: *Satureja khuzestanica* essential oil; COX2: Cyclooxygenase-2; LPS: Lipopolysaccharides; $*P<0.05$.

including Parkinson’s disease in which the pathogenesis role of COX2 is noticeable. The result of a study showed that there is an association between COX2 activity and disease progression since COX2 suppression led to disease amelioration.¹⁸ Based on the findings of another study, the suppression of COX2 activity by nonsteroidal anti-inflammatory drugs (NSAIDs) was associated with a reduction in intestine cancer incidence in human and animal models.¹⁹ Additionally, the inhibition of COX2 enzyme production was associated with anti-inflammatory effects. However, the long-term consumption of selective COX2 inhibitory compounds such as NSAIDs has unwanted effects on cardiovascular, gut, and renal systems.^{7,8,20} Accordingly, more studies should be conducted on the treatment of inflammation in elderly patients in order to develop a better substituent for NSAIDs because of their dangerous side effects.²¹ To this end, the current study investigated the effect of two natural products of Carvacrol and SKEO on COX2 gene expression in the J774A.1 macrophage cell line. Based on the results, both of these compounds reduced COX2 gene expression dose-dependently in the absence and presence of LPS. Similarly, the inhibitory effect of SKEO was more potent than Carvacrol which is the main constituent of the *S. Khuzestanica* herb. This enhanced inhibitory effect could be due to other components of the herb other than Carvacrol.

Amanlou et al showed that *S. Khuzestanica* has anti-inflammatory effects They also investigated the anti-inflammatory effects of *S. Khuzestanica* in comparison with indomethacin and concluded that it is as potent as this standard drug.² In a similar study, Ghazanfari et al found that the anti-inflammatory effects of the plant were comparable with prednisolone.²²

Other properties of *S. Khuzestanica*, along with its anti-

inflammatory activity have also received attention. One of these studies evaluated the antioxidant activity of the plant and reported that *S. Khuzestanica* can inhibit the oxidation of low-density lipoprotein (LDL) by copper sulfate in a dose-dependent manner (50-200 µg/mL). The researches further claimed that *S. Khuzestanica* could be a good candidate for reducing the risk of cardiovascular and atherosclerotic disease development.²³

Another previous research evaluated the effect of *S. Khuzestanica* on lipid profile, atherogenic indices, alanine aminotransferase, aspartate aminotransferase (AST), and alkaline phosphatase. It was shown that *S. Khuzestanica* can reduce the level of fasting blood sugar, triglyceride, LDL, and high-density lipoprotein in diabetic mice. The level of hepatic enzymes except for AST also reduced by the extract of *S. Khuzestanica*. Thus, the consumption of this plant may reduce the risk of atherosclerosis and hepatic disease in diabetic patients.²⁴

Eventually, Ahmadvand et al studied the effect of *S. Khuzestanica* on lipid peroxidation in diabetic mice in vivo and in-vitro studies. To this end, they removed the livers and kidneys of diabetic mice just after treatment with the *S. Khuzestanica* extract and measured lipid peroxidation by the thiobarbituric acid method. It was observed that could remarkably *S. Khuzestanica* reduce LDL oxidation in-vitro. The findings also revealed that *S. Khuzestanica* reduced lipid peroxidation, glomerular hypertrophy, and glomerulosclerosis in diabetic mice. Therefore, a protective role against peroxidation may prevent the adverse effects of diabetes, including nephropathy in diabetic patients.²⁵

Conclusion

In summary, the result of this study showed that SKEO and its main component, namely, Carvacrol could inhibit COX2 gene expression thus and *S. Khuzestanica* may be useful in the treatment of inflammatory diseases.

Ethical Approval

The study protocol was approved by the Ethics Committee of Arak University of Medical Sciences, Arak, Iran (Code No: 90-119-6).

Conflict of Interest Disclosure

The authors declare no competing interests.

Authors' Contributions

Masumeh Jalalvand, Ali Sheikhan, and Gholamreza Shahsavari extracted the Satureja oil and performed all experiments. In addition, Ali Ganji analysed the results and then wrote and revised the paper. Finally, Ghasem Mosayebi designed and supervised the project.

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