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Anti-Inflammatory Effects of Usnic Acid in Breast Cancer

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Abstract—Breast cancer is the most common type of cancer among women. Usnic acid has anticancer and anti-inflammatory potential. In this study, we evaluated the effects of usnic acid on inflammation and oxidative stress in the breast cancer cell line (MCF-7). Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) gene expression, cytokines, nitric oxide (NO), prostaglandin E2 (PGE_2) levels and oxidative stress were measured in all groups. Our study showed that usnic acid was decreased NO, VEGF, PGE_2 levels, gene expression levels of COX-2 and iNOS in the meantime cytokines (IL 2, CXCL 10, CXCL8, CCL2 (MCP-1), TNF-α, IL-6), glutathione levels, moreover decreased GSH and increased MDA levels in dose depended manner. According to our evidence usnic acid was showed potential cytotoxic, anti-inflammatory and pro-oxidant role.

Keywords: usnic acid, breast cancer, anti-inflammatory, MCF-7, oxidative stress

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INTRODUCTION

Cancer is defined as uncontrolled and irregular proliferation of cancer cells. Breast cancer is the most common type of cancer in women [1]. Inflammation is known to be associated with 3 stages of tumor development (initiation, progression and metastasis) [2].

Cyclooxygenase-2 (COX-2) is an important enzyme that converts arachidonic acid into prostaglandins. COX-2 induces tumor proliferation, carcinogenesis. Over-expression of COX-2 associated with cell proliferation, invasion, and apoptotic resistance of breast cancer. According to epidemiological studies, COX targeting drugs are reported to be protective against breast cancer [3].

Nitric oxide (NO) is a free radical with many biological activities in the body synthesized by the organism. Under normal physiological conditions, NO is produced in the presence of nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline. The production of excessive and unregulated NO can lead to proliferation of tumor cells. It is known that excessive NO production is related to various inflammatory diseases [4].

Oxidative stress is defined as the shift of balance between Reactive oxygen species (ROS) and antioxidant defense system on ROS side. ROS and reactive nitrogen species are involved in the pathophysiology of various diseases such as neurodegenerative disorders and cancer [5]. Cytokines are known as proteins that enable intercellular interactions. Analysis of cytokine interaction network in breast cancer patients may help early detection of this disease and develop new therapeutic [6].

Usnic acid is lichen secondary metabolite which was firstly isolated by W. knop in 1844. It has become popular in pharmacology and clinic due to its biological activities. Usnic acid has anti-proliferative, antioxidant, pro-oxidant, anti-viral, antimicrobial, antiprotozoal and anti-inflammatory activity [6].

The aim of this study was evaluate possible role of usnic acid on inflammation and oxidative stress in MCF-7 breast cancer cell line.

RESULTS AND DISCUSSION

Biological Screening

Biochemical findings. Cancer is second most common disease which resulting with death [7]. While designing new treatment strategies, targeting various

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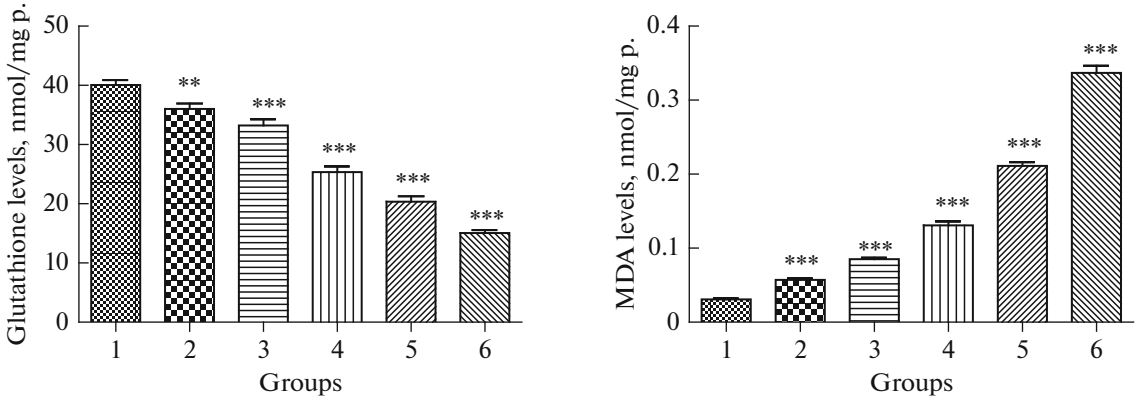

Fig. 1. Glutathione and MDA levels evaluated in MCF-7 cells before and after treatment with different concentrations of usnic acid. We indicated with *** the comparisons statistically significant ($p < 0.05$) between the treated and untreated cells.

molecular pathways should destroy cancer cells without damaging healthy cells or at least help preventing the proliferation of these cells [8]. Natural products are a potential source of new drug formulations in cancer treatment [9]. Lichen species and their related metabolites can be used in the pharmaceutical industry. One of the secondary metabolites from lichen is usnic acid [10, 11]. It has been showed that usnic acid has anti-proliferative activity in several cancer cell lines. Kılıç et al. found LD50 value of usnic acid as 13.11 μM [12]. In our study, this value was determined as 15 638 μM. In another study by Venkata Mallavadhani et al. showed that usnic acid inhibited proliferation in HeLa, MDA-MB-231, A549, and MiaPaca cell lines [13].

ROS has a critical role for regulating life cycle of cells. In this study, MDA levels, a marker for lipid peroxidation, and glutathione levels were measured which has major role in antioxidant defense system. Previous research has established that usnic acid has antioxidant and pro-oxidant activities under different conditions. Polat et al. reported that usnic acid has antioxidant and pro-oxidant effects depending on concentration [14]. In our study, usnic acid increased MDA level and decreased GSH level. Therefore, in these concentrations, it has a pro-oxidant effect in MCF-7 cell line.

A study demonstrated that usnic acid-induced oxidative stress is likely initiated by glutathione depletion in HEPG2 cells [15].

In this study, GSH and MDA levels were determined (Fig. 1). In group 2, group 3, group 4, group 5 and group 6, MDA levels were increased 1.9, 1.49, 1.53, 1.61, 1.62 fold, respectively when compared to control group. GSH levels were decreased 1.11, 1.09, 1.32, 1.25, 1.33 fold respectively compared to control group ($p < 0.05$).

TNF-α and NO may activate the regulatory mechanisms of cancer, causing over-expression of bcl-2 and sFAS in breast cancer [16].

There is evidence that usnic acid play crucial role in regulating inflammatory response on breast cancer. TNF-α has an important role in viral replication, proliferation, apoptosis. Moreover, increased level of TNF-α induces tumor progression via activation of NF-kB [17]. It is highly expressed in breast cancer. TNF levels are lower in bening tissue than invasive breast cancer tissue [18]. Both ER+ and ER– breast cancer cell lines produce IL-6 which induces growth and invasiveness in MCF-7 cells. Moreover, IL-6 is the most important acute phase response and it performances as anti and pro inflammatory cytokine. Methanol Extract from the Marine Sponge *Geodia cydonium* was able to inhibit cytokines such as IL-6, TNF-α, IL-1β in MCF-7 cell lines [19].

PGE_2 levels and NO production decreased significantly ($p < 0.05$) after treatment with at different concentration usnic acid for 48 h. There was a significant decrease in VEGF at 0.623 μM usnic acid concentrations ($p < 0.05$) (Table 1).

Several studies have reported that usnic acid show anti-inflammatory effects, we evaluated the concentrations of 6 cytokines (expressed in pg/mL) in MCF-7 supernatants after treatment with usnic acid at different concentrations by multiplex biometric ELISA-based immunoassay. IL-6 levels were observed to be significantly variable among groups ($p < 0.05$). TNF-α levels decreased by 1.31 fold in group 2 compared to control group ($p < 0.05$). Moreover, in the group 6, increased usnic acid treatment increased this ratio to 2.53 fold. IL-2 levels were observed to be decreased in all usnic acid treatment groups compared to control group ($p < 0.05$) (Table 1).

Recent studies suggest that NF-κB activate MCP-1 (CCL2) expression. Inhibition of MCP-1 in immunodeficient mice with breast cancer caused an increase in the survival of these animals [20]. CXCL8 promoters contain different recognition sites for NF-κB.

IL-2 is one of pro-inflammatory cytokines which has role for cell proliferation, growth and T cell differ-

Table 1. NO, PGE_2, VEGF and cytokine levels (expressed in pg/mL) measured in MCF-7 cells before and after treatment with different concentrations of usnic acid. Data are presented as mean ± standard deviation. We indicated with * the comparisons statistically significant ($p < 0.05$) between the treated and untreated cells

Parameters/Grous	1	2	3	4	5	6
NO, pg/mL	80.3 ± 0.9	76.3 ± 0.9*	43.2 ± 2.0*	40.7 ± 2.6*	32.8 ± 1.6*	28.6 ± 2.6*
PGE_2, pg/mL	73.7 ± 4.8	69.4 ± 1.3*	57.4 ± 1.1*	50.4 ± 1.6*	42.5 ± 0.9*	30.5 ± 1.1*
CXCL10, pg/mL	41.1 ± 1.5	38.2 ± 0.8*	36.0 ± 0.7*	33.0 ± 0.7*	31.3 ± 1*	16.8 ± 0.6*
CXCL8, pg/mL	365.4 ± 1.9	330.2 ± 3.6*	320.1 ± 8.7*	230.3 ± 2.5*	160.4 ± 0.9*	130.5 ± 3.9*
CCL2, pg/mL	238.0 ± 3.5	214.0 ± 3.8*	199.9 ± 2.4*	185.3 ± 2.4*	163 ± 1.2*	125.6 ± 2.43*
IL2, pg/mL	119.4 ± 3.8	105.7 ± 3.2*	99.8 ± 2.5*	89.8 ± 1.3*	76.2 ± 1.7*	68.1 ± 3*
IL6, pg/mL	391.7 ± 7.8	332.3 ± 14.5*	306 ± 15. 1*	284.3 ± 7.4*	280 ± 8*	222 ± 8*
TNF-α, pg/mL	3.3 ± 0.07	2.5 ± 0.06*	2.2 ± 0.08*	2.1 ± 0.06*	1.8 ± 0.03*	1.3 ± 0.01*
VEGF, pg/mL	37066.7 ± 492.2	34800 ± 355.9*	27010.7 ± 396.9*	24606.7 ± 287.7*	23330.7 ± 241.9*	22360 ± 151.2*

entiation. According to our results IL-2 levels were down-regulated [21]. Hence this down-regulation can reduce cancer progression. CXCL10 shows tumor-promoting ability. IFN-γ depend upon NF-κB for induce expression of the CXCL10 gene. Over-expression of CXCL10 binds to CXCR3 and down-regulates CXCR3-B, induce breast cancer growth [22].

CXCL8 levels were decreased in group 2, group 3, group 4, group 5 and group 6 1.1, 1.14, 1.58, 2.28, 2.8 fold in dose depended manner statistically significant when compared to control group ($p < 0.05$). CXCL10 levels decreased by 59.15% in group 6 to compared control group but lowest concentration of usnic acid treatment decreased this ratio to 7.13% (Table 1). Usnic acid decreased VEGF levels according to our study.

COX-2 and iNOS gene expression levels. Targeting of important genes such as COX-2 and iNOS in inflammation pathways is important both in early detection of cancer and in the development of new strategies for treatment. The inhibition of COX-2 by usnic acid reduced the invasion and metastasis of cancer [23]. Previous study has investigated the effect of usnic acid isolated from lichen parmelia saxatilis on COX-2 and iNOS gene in RAW-264.7 murine macrophages cells [24]. Their study showed correlation with current study. Our study examined the effect of usnic acid anti-inflammatory and oxidative stress properties in breast cancer cell.

RT-PCR was performed to evaluate usnic acid effect on expression of COX-2 and iNOS genes. COX-2 is known to be a key enzyme mediating prostaglandin synthesis and according to our results, its expression levels were significantly decreased in treated groups compared to the control group.

iNOS is one of the reactive oxygen and nitrogen metabolite-metabolizing enzymes. INOS gene expression was decreased 12 to 81% percent respectively compared to control group after usnic acid treatment (Fig. 2).

EXPERIMENTAL

Cell Culture

MCF-7 cell breast cancer cell line was purchased from HUKUK-Ankara. The cells were grown in

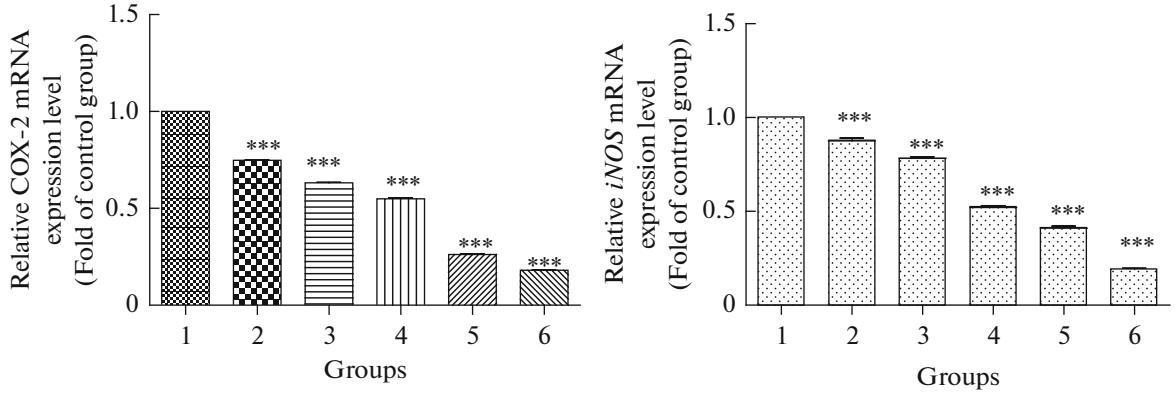

Fig. 2. COX-2, iNOS gene expression levels measured in MCF-7 cells before and after treatment with different concentrations of usnic acid. We indicated with *** the comparisons statistically significant ($p < 0.05$) between the treated and untreated cells.

RPMI which was supplemented with 10% FBS and 1% penicillin/streptomycin and 2 mM L-glutamine. Cells were incubated at 37°C with humidified atmosphere of CO_2 5%. Cells were plated at 5×10^5 cells/well in ACEA plate. After 24 h, cells were reached to 90% confluency. Usnic asid was obtained from Sigma aldrich. Usnic acid was prepared in 14 mM DMSO. Then cells were treated with different concentration with usnic acid 48 h. Untreated cells were used as a control. To determine LD_{50} value of usnic acid, stock solution of was diluted as 40, 20, 10, 5, 1 μg/mL. Cell cytotoxicity test was performed with ACEA's xCELLigence system (ACEA Biosciences, San Diego, CA, USA). 90 μL medium 10 μL cell suspend (MCF-7 cells were suspended appropriate medium) totally 100 μL added into well in E-plate 16 and incubated overnight. LD_{50} value was determined 15,638 μM. Cells were treated with different concentration of usnic acid (group 2: 0.623 μM, group 3: 4.376 μM, group 4: 8.130 μM, group 5: 11.884 μM, group 6: 15.638 μM).

Effect of Usnic acid on gene expression (COX-2 and iNOS) by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) of MCF-7 cells.

After 48 h, treatment with usnic acid, mRNA was isolated by using Roche High Pure RNA Isolation Kit from MCF-7 cells. cDNA was synthesized with Thermo Fisher Scientific according to company instruction. COX-2 (Hs00153133_mL), iNOS (Hs00167257_mL) genes expression levels detected with TaqMan Gene Expression Assays. The PCR protocol consisted of 42 cycles for 15 s at 95°C and 30 s at 60°C each sample was tested triplets. β-actin was used as a housekeeping gene.

ELISA Assay

Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and prostaglandin E2 (PGE_2) levels were measured for each 100 μL cell sample by using ELISA kit (MyBioSource, San Diego, CA) according to manufacturer's instructions. 100 μL sample added to sample wells. Then, covered the plate with a sealer and incubated 90 min at 37°C. The reaction mixture was removed from each well and washed two times with washing buffer. Subsequently, Biotinylated Antibody solution was added and incubated 60 min at 37°C. 100 μL Enzyme Conjugate was added and incubated for 30 min at 37°C in the dark. After washing 5 times, 100 μL of color reagent was added, the optical density was measured at 450 nm using Microplate Reader.

Bio-Plex Assay

CCL2, CXCL8, CXCL10, VEGF, IL-2, levels were measured at the same time with The Bio-Plex assay, according to Manufacturer's instructions. The results were expressed as pg/mL.

Biochemical Analysis

Malondialdehyde (MDA) levels were measured according to the method developed by Yagi [25]. This method is based on the principle that the pink color formed during the reaction between lipid peroxidation products and thiobarbituric acid is measured at 532 nm by UV–VIS spectrophotometer. MDA levels are expressed as nmol/mg protein. NO levels were determined according to the method reported by Griess et al. [26]. Cell supernatant were measured spectrophotometrically at 540 nm via ELISA reader. Sodium nitrate was used as a standard. GSH analysis was performed according to Fairbanks and Klee method which was measured colorimetrically at a wavelength of 412 nm via spectrophotometer [27]. Protein content of cells was assayed using the method of Lowry [28]. All experiments were performed in triplicate.

Statistical Analysis

Statistical analyses were performed using Statistica for windows version 13. Difference testing between groups was performed using analysis of variance. The assumption of normality was tested with Shapiro Wilks test; all variables provided assumption of normality for all groups ($p > 0.05$). All statistics were two-tailed, and a $p < 0.05$ was considered to be statistically significant.

CONCLUSIONS

Usnic acid reduced COX-2, iNOS gene expression, PGE_2, VEGF, NO and 6 cytokine levels in a dose-depended way in MCF-7 cell line. In conclusion, our findings suggested that usnic acid had antiproliferative, anti-inflammatory activity on MCF-7 cell line. According to our results usnic acid may use for decreasing inflammatory response in breast cancer. However further research is needed to completely for clinical use of usnic acid.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any studies involving animals or human participants performed by any of the authors.

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