IJBPAS, August, 2019, 8(8): 1385-1392

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

THE EFFECT OF NARINGENIN IN INHIBITION OF INFLAMMATORY CYTOKINES AND CONTROLLING OXIDATIVE STRESS IN PATIENTS WITH ALOPECIA AREATA

TAHEREH REZAZADEH[1,5], ALI KARIMI BAKHSHANDI[2,5], NILOOFAR AKHAVAN[3], NILOUFAR SADATMA SHHADI MAHALLATI[4]

[1]Department of Biotechnology, M.Sc. of Biotechnology, Isfahan University of Technology, Isfahan, Iran

[2]Department of Biology, M.Sc. of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran

[3]Department of Biotechnology, M.Sc. of Biotechnology, pharmaceutical Science Branch, Azad University, Tehran, Iran

[4]Department of Biology, B.Sc. of Microbiology, Pharmaceutical Science Branch, Tehran, Iran

[5]R&D Department, Pars Azmaye Teb Co. (Cerita), Tehran, Iran

***Corresponding Author: Ali Karimi Bakhshandi, Pars Azmaye Teb Co. (Cerita), lower of Hemmat highway Bridge, Satari highway, in front of Kourosh complex, Tehran, Iran; Email: a.karimi@cerita.it; Phone: +982145857000, Fax: +982145857000**

Received 15th Jan. 2019; Revised 7th Feb. 2019; Accepted 14th March 2019; Available online 1st Aug. 2019

https://doi.org/10.31032/IJBPAS/2019/8.8.4624

ABSTRACT

Interleukin-1 alpha, TNF-α and IFN-ϒ are the most important inflammatory cytokines that contribute to alopecia areata. The aim of this study was to assess the effect of naringenin in inhibition of inflammatory cytokines and controlling oxidative stress in patients with alopecia areata. Different concentrations of naringenin were added into keratinocyte cell culture medium, and the viability of keratinocyte cells was evaluated by MTT assay. RNA was extracted from treated keratinocyte cells and control, and cDNA was made. Next, RT-PCR and real time PCR were used for the detection of target genes and measuring gene expression levels, respectively.

Moreover, DPPH method and H2O2-induced oxidative cell damage were used for assessing free radical scavenging activity of naringenin. The result showed that there was a significant difference in the level of gene expression of Interleukin-1 alpha, TNF-α and IFN-ϒ in treated samples and control ($p \leq 0.05$). In vitro free radical scavenging activity of naringenin was also confirmed. The result of in vivo test indicates that a gel containing naringenin is effective for the treatment of alopecia areata. This study provides scientific insights about the potential anti-inflammatory and anti-oxidant activities of naringenin which can be used as effective flavanone for the treatment of inflammation indifferent types of autoimmune diseases.

Keywords: Naringenin, Cytokines, Alopecia Areata

INTRODUCTION

Alopecia areata is an autoimmune disorder. Hair falls out in small patches on the scalp, beard, and eyebrows. Alopecia areata afflicts approximately 2% of the world's population (1, 2). Alopecia areata can occur at any age but it most appears in adolescence (2). It shows a potential association between hormonal activity and disorder. Alopecia may not be life threatening but it is psychologically and socially uncomfortable. Genetic and environmental factors like infections, trauma and stress are responsible for triggering the disease (3).Hair follicles to be mistakenly attack by the body's own immune system, and it leads to hair loss. Both CD4 and CD8 T cells are abundant in patient with alopecia areata (4). There has also been an increase in the production of inflammatory cytokines such as interferon gamma, interleukin 1 alpha and TNF-α (5).It leads toextreme inflammation in hair follicles in infected individuals (5). Moreover, the excess production of free radicals can damagehair follicles (6). Since citrus has been recognized as anti-inflammatory and anti-oxidant compounds in the distant past, and flavonoids found in citrus have anti-inflammatory and anti-oxidant properties (7). Naringenin is one of the most important flavanone in citrus, and its anti-inflammatory and anti-oxidant activities have been reported in previous studies for the treatment of inflammation and oxidative stress in different diseases (7, 8, 9, 10). The aim of this study was to assess the effect of naringenin in inhibition of inflammatory cytokines and controlling oxidative stress in patients with alopecia areata.

MATERIALS AND METHODS

Naringenin preparation

Naringenin was purchased from Sigma-Aldrich, and 50 mg of naringenin was

dissolved in 1 ml of distilled water for making a stock solution.

Human keratinocyte cell culture

Human keratinocyte cells was cultured in Dulbecco's Modified Eagle's medium (DMEM), and supplemented with 10% heat-inactivated fetal bovine serum and 100 u/ml gentamicin. Cultures were kept at 37°C in a humidified atmosphere containing 5% CO2.

Cell Viability and Cytotoxicity Assay

3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used for the determination of cell viability. 2500 cells were placed in 96 well plates. Then, different concentration of narengnin (0, 0.1, 1, 10, 50, 100 µg/ml) were added to cells at 24 hours. 25 µl of MTT solution (5 mg/ml) was added each well. After 4 hours incubation and dissolving of formazan crystals in dimethylsulfoxide (DMSO), the absorbance has been read at the wavelength 570 nm by using a plate reading spectrophotometer. The percentage of cell viability was calculated as the following formula:

Cell viability (%) = (A570 of treated cells)/(A570 of control cells)×100

Cell proliferation assessment

Cells were calculated by trypan blue. Next, 5000 cells were placed in each well in a 6 well plates, and allowed them to adhere overnight at 37°C in 5% CO2. After incubation, cells were stimulated by LPS (10 ng/ml; sigma).

Keratinocyte Cells Treatment

Keratinocyte cells were treated by naringenin based on optimal MTT concentration at 24, 48 and 72 hours. The first well of 6 well plates was considered as control sample void of naringenin.

RNA Extraction

First, keratinocyte culture medium was discharged by serological pipette. Next, cells were washed twice in phosphate buffered saline (PBS). Then, the cells were separated from the bottom of culture dishes by trypsin/EDTA solution, and added into falcon tubes, and centrifuged at 10000 rpm for 5 minutes. Next, RNA was extracted in treated sample and control by RNA extraction kit (Qiagen Rneasy Plus Mini Kit 50, USA). The quantity of RNA was measured by a spectrophotometer at the wavelength of 260/280 nm.

cDNA Construction

cDNA construction have been done by Revert Aid H Minus first strand cDNA synthesis kit, K1631. Reaction contains 4 µ 5x buffer, 1 µg RNA, A µloiligodt primer, 2 µldNTP (10 Mm), 1 µ RNase inhibitor (20 u/µl), 1 µl Reverse Transciptase enzyme (200 u/µl), and the volume was brought to 20

ml by sterile H2O. Program was implemented as follows: 5 minutes at 25 °C for the annealing of primers, 60 minutes at 42°C for cDNA synthesis.

Primer Designing

Primers were designed based on the exonof human Interleukin-1 alpha, TNF-α and IFN-ϒ genes available from DDBJ/GenBank with Oligo ver-7 software. Then, the specificity of the primers was checked out (see table 1).

RT-PCR

cDNA was used as a template for target region amplification by using PCR method. This reaction contains 2 µl cDNA, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP , 0,4 µM of each primer, 0.2 u/µlTaq DNA polymerase, and the volume was brought to 25 ml by sterile H2O. Program was implemented as follows: 5 minutes for initial strand separation at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 45 seconds at 57 °C, 30 seconds at 72 °C, and a final 7 minutes step at 72 °C.

Real time PCR

The quantification of Interleukin-1 alpha ، TNF-α and IFN-ϒ genes expression has been assessed by Real time PCR method. This reaction contains 12.5 µ 2x SYBR Green Master Mix, dNTP, 0.5 µ of each primer (20 nmol), 2 µ cDNA (50 ng/µl), and the volume was brought to 25 ml by sterile H2O. Program was implemented as follows: 5 minutes for initial strand separation at 94 °C, followed by 50 cycles of 15 seconds at 95 °C, 1 minute at 57 °C as annealing temperature. Also, Beta-2- macroglobulin was used as internal control.

Primer	Sequence 5´-3´	Specificity	Amplicon (bp)
Interleukin-1 alpha	CACTGACTTTCAGATACTGG	Interleukin-1 alpha/sense	90
	ACATGGTACATATGAACTGTC	Interleukin-1 alpha/anti-sense	
TNF-α	CCAATGGCGTGGAGCTGAG	TNF-α/sense	90
	CTTGGCCCTTGAAGAGGACC	TNF-α/anti-sense	
IFN-ϒ	AGATCCCATGGGTTGTGTG	IFN-ϒ / sense	101
	TGCAGGCATATTTTCAAACCGG	IFN-ϒ /anti-sense	

DPPH assay

α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method was used to evaluate the antioxidant potential of a naringenin.100 µ of naringenin stock (10 µg/ml) was mixed with 100 µ of 0.01 % methanolic DPPH solution. This was kept in the dark for 30 minutes. Then, the absorbance was determined by spectrophotometer at 517 nm against a blank sample. The anti-oxidant activity of naringenin was calculated as percentage inhibition by following equation:

$$\% \text{ Inhibition} = \frac{OD\ blank - OD\ sample}{OD\ blank} \times 100$$

OD_{blank}: the absorbance of the control

OD_{sample}: the absorbance of test sample

H2O2-induced oxidative cell damage in keratinocytes

Keratinocyte cells were treated by different concentrations of H2O2 (100, 150, 200, 250 µM) for 2 hour, then cell viability was calculated by MTT assay.

Scavenging activity of naringenin in H_2O_2-induced oxidative cell damage

5000 cells were placed in each well in a 6 well plates, and allowed them to adhere overnight at 37°C in 5% CO2. Then, optimum concentration of naringenin was added simultaneously with 150 µM H2O2 to human keratinocyte cells, and kept 37°C in 5% CO2 for 2 hours. Control sample was void of naringenin. Cell viability was determined by MTT assay.

In vivo assay

A total of 20infected individuals with alopecia areata (patchy) in their scalp, aged from 17 to 32 years, were treated with a gel formulation containing naringenin for 3 month (see figure 1).

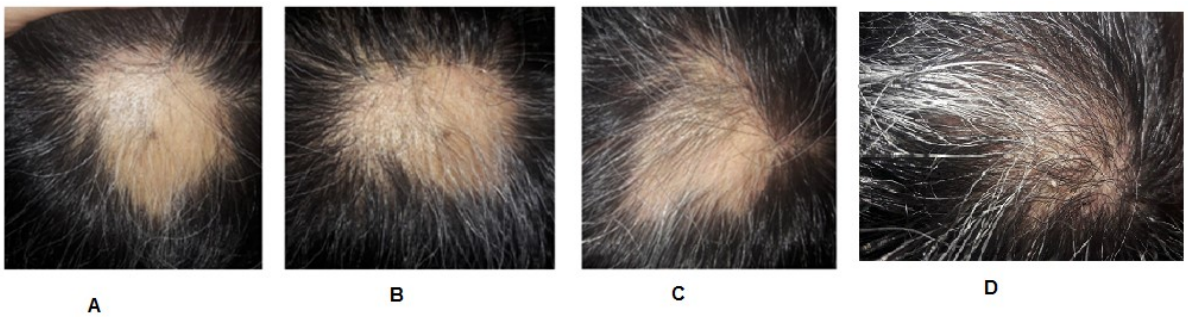

Figure 1: Hair regrowth after treatment with a gel formulation containing naringenin in a patient with alopecia areata. A-Before, B- after 3 week, C-after 6 week, D- after 3 month

RESULT

The optimum concentration of naringenin was determined by MTT assay, and was 10µg/ml. the result of RT-PCR showed that 90, 90 and 101 bp of target sequence amplified in Interleukin-1 alpha, TNF-α andIFN-ϒ, respectively (see figure 2). The real time PCR was used for the evaluation of cytokine genes expression in treated keratinocyte cells and control. Raw data obtained from this technique after amplification the form of CT. the amount of CT in treated keratinocyte cells was more than control. Significant difference between ΔΔCt of samples was measured by Student´s t-Test in the level of α: 0.05. The result illustrated that there was a significant difference in the level of protein expression

of treated keratinocyte cells and control ($p \leq 0.05$), and the level of cytokine genes expression in treated keratinocyte cells was less than control. When naringenin (10 µg/ml)was added simultaneously with H2O2 to human keratinocyte cells, this significantly increased the viability of keratinocyte cells in compared with H2O2 treatment alone, and cell viability was about 65 % in comparison with control. In DPPH assay, absorbance more rapidly decreased, and the more potent the antioxidant activity of naringenin was confirmed by this method. The result of in vivo assay also demonstrated that a gel formulation containing naringenin provided effective treatment for alopecia areata.

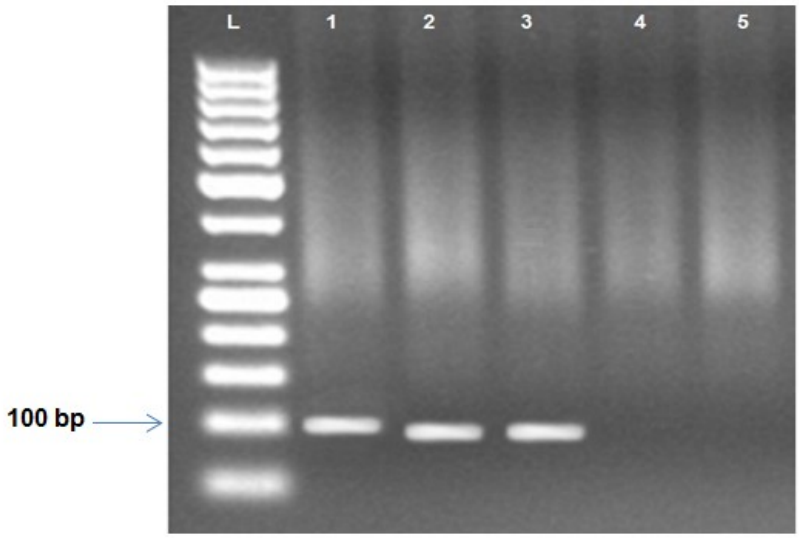

Figure 2: Electrophoretic agarose gel, L: Ladder 100, 1: the PCR product of IFN-ϒ (101 bp), 2: the PCR product of Interleukin-1 alpha (90 bp), 3:the PCR product ofTNF-α (90 bp), 4- Negative control, 5: Environmental control.

DISCUSSION

There are many different types of autoimmune diseases, and these disorders are rapidly increasing worldwide. There are different types of autoimmune diseases in the skin and hair. One of them is alopecia areata which is strongly influenced by the increased production of inflammatory cytokines such as interferon gamma, interleukin-1-alpha and TNF-α (5). Consequently, these cytokines cause severe inflammation in hair follicles of affected individuals. High concentration ofIL-1 α inhibits growth of hair and follicle, and this inhibition leads to a secondary reaction (alopecia areata). Therefore, in order to reduce inflammation, pro inflammatory and inflammatory cytokines should be controlled. Different herbs have anti-

inflammatory properties, and could be effective for the treatment of alopecia areata. Citrus have different types of flavones like naringenin, naringin, hesperetin, narirutin, eriocitrin, didymin and so on, and these compounds have anti-inflammatory and anti-oxidant effects (7). Besides, naringenin is one of the most important flavanone in citrus and its property has been reported in various studies for the treatment of inflammation and oxidative stress in different diseases likeendotoxemia and fulminant hepatitis (8). Naringenin inhibits acute inflammation by regulating cytokines secretion in mentioned disorders. Naringenin also inhibits UVB irradiation-induced inflammation and oxidative stress in the skin of hairless mice by inhibiting different kinds of pro-inflammatory and inflammatory cytokines (9). Moreover, naringenin is as an effective inducer of SOCS3 protein, mRNA and promoter activity (10). This protein plays key roles in inhibiting inflammatory cytokines. Besides, IL-6 induces pro inflammatory gene expression by stimulating STAT3 phosphorylation on tyrosine. Naringenin inhibits tyrosine phosphorylation of STAT3 (10). Naringenin also reduce skin inflammation by inhibiting inflammatory cytokines (TGF-β and IL-10) and pro inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, IL-22, and IL-23) (10, 11).

REFERENCES

[1] Amin SS, Sachdeva S. Alopecia areata: A review. *Journal of the Saudi Society of Dermatology & Dermatologic Surgery*, 2013; 17: 37-45.

[2] Herbert Pratt C, KingL EJ, Messenger AG, Christiano A M, Sundberg J P. Alopecia areata. *Nat Rev Dis Primers*, 2017; 16; 3: 17011.

[3] Bhat YJ, Sajad P, Hassan I. Etiopathogenesis of Alopecia Areata. *Hair Ther Transplant*, 2014; 4: 123. doi: 10.4172/2167-0951.1000123.

[4] Hongwei G, Yabin C, Jerry S, Kevin M. The Role of Lymphocytes in the Development and Treatment of Alopecia Areata. *Expert Rev ClinImmunol*; 2015; 11: 1335–1351.

[5] Gregoriou S, Papafragkaki D, Kontochristopoulos G, Rallis E, Kalogeromitros D, Rigopoulos D. Cytokines and other mediators in alopecia areata. *Mediators Inflamm*, 2010; 2010: 1-5.

[6] Öztürk P, Arıcan Ö, Kurutaş EB, Mülayim K. Oxidative Stress Biomarkers and Adenosine Deaminase over the Alopecic Area of

the Patients with Alopecia Areata. *Balkan Med J*, 2016; 33: 188-92.

[7] Ashraful AM, Nusrat S, Mahbubur Rahman M, Shaikh JU, Hasan MR, Satyajit DS. Effect of Citrus Flavonoids, Naringin and Naringenin, on Metabolic Syndrome and Their Mechanisms of Action. *AdvNutr*, 2014; 5: 404–417.

[8] Jin L, Zeng W, Zhang F, Zhang C, Liang W. Naringenin Ameliorates Acute Inflammation by Regulating Intracellular Cytokine Degradation. *J Immunol*, 2017; 199: 3466-3477.

[9] Renata MM, Felipe AP, Vinicius SS, Carla V C, Josiane AV, Décio SB, Marcela MB, Sandra RG, Waldiceu AV, Rubia C. Naringenin Inhibits UVB Irradiation-Induced Inflammation and Oxidative Stress in the Skin of Hairless Mice. *J. Nat. Prod*, 2015; 78: 1647–1655.

[10] Wiejak J, Dunlop J, Mackay SP, Yarwood SJ. Flavanoids induce expression of the suppressor of cytokine signalling 3 (SOCS3) gene and suppress IL-6-activated signal transducer and activator of transcription 3 (STAT3) activation in vascular endothelial cells. *Biochem J*, 2013; 454: 283-93.

[11] Marília FM, Rubia C, Waldiceu AVJ. Naringenin: an analgesic and anti-inflammatory citrus flavanone. *Oncotarget*, 2017; 8: 3766–3767.