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THE EFFECT OF *SYRINGE VULGARIS* EXTRACT ON THE EXPRESSION OF CATHELICIDIN ANTIMICROBIAL PEPTIDE

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ABSTRACT

Anti-bacterialpeptides (AMPs) are a part of the innate immune system, and play an important role in protecting of skin against pathogens like bacteria, fungi, parasites and viruses. Therefore, by using the antimicrobial peptides ability can inhibit skin pathogens. The aim of this study is to investigate the effect of *syringe vulgaris* leaf extract on the expression of cathelicidin antimicrobial peptide in skin. In this research, the leaves of *s. vulgaris* were extracted by Maceration method and analyzed by gas chromatography. Keratinocyte cells isolated from

human neonatal foreskin. Next, different concentrations of *S. vulgaris* extracts were added into the keratinocyte medium, and the viability of keratinocyte cells was evaluated by MTT assay, and concentration 200 µg/ml of *S. vulgaris* extract was confirmed in comparison with other concentrations.RNA was extracted from keratinocyte cells in treated sample and control, and cDNA was made. Then, RT-PCR and real time PCR were used for the detection of target gene and measuring the level of protein expression, respectively. Also, DAPI based staining and ICC methods were used to confirm the protein expression. The result showed that there was a significant difference in the level of protein expression of treated samples and control sample ($p \leq 0/05$).

Keywords: AMPs, cathelicidin, *Syringa vulgaris*, keratinocyte cells.

Abbreviations: AMPs (Anti-bacterial peptides), *S. vulgaris* (*Syringa vulgaris)*

INTRODUCTION

Anti-microbial peptides (AMPs) are main component of the innate immune system to defend against pathogenic agents such as bacteria, fungi, parasites and viruses [1, 2, 3, 4]. These small peptides kill directly microorganisms through the induction of microbial cell death [5, 6]. Also, these peptides present at epithelial and non-epithelial surfaces of mammalian, and act as a barrier preventing invasion by microbes. These peptides penetrate into cell membrane of microbes, and inhibit the enzyme activity and destroy intracellular reaction of microbes [7, 8, 9]. AMPs are introduced as new antibiotics [10, 11]. There are many proteins and peptides in the skin with antimicrobial activity. Some peptides produce permanent, while others synthesis during microbial invasion [12]. Hence, quick access to AMPs is vital for innate immune system. Cathelicidin is as AMP in mammalian, and was detected in different species. In fact, this peptide has considerable variation in different creatures. But, one gene is characterized for human cathelicidin [13]. This peptide has anti-microbial property against pathogens like bacteria, fungi, parasites and viruses. Human cathelicidin gene is located on chromosome 3 at location: 3p21.3 [14, 15]. It contains 4 exons and 5 introns, and codes a protein 18 kDa (human cationic antimicrobial protein 18 kDa) [16, 17, 18]. This peptide has a conserved cathelin domain and C-terminal domain. C-terminal domain has anti-bacterial activity [19, 20, 21]. The expression of peptide is significantly increased in response to inflammatory stimulus. The widespread use

of antibiotics for the treatment of infection promotes the development of antibiotic-resistant bacteria, and it leads to serious problems in the field of medical science in the whole world [22]. Therefore, AMPs have attracted the attention of many research groups due to have a broad efficiency against pathogens [23, 24]. These features make them good candidates for medical applications and researches [25, 26]. Many studies showed that some herbal extracts increase the expression a number of genes [27, 28, 29, 30]. In this study is to investigate the effect of *S. vulgaris* leaf extract on the expression of AMP in skin.

MATERIALS AND METHODS

Plant material and extract preparation

S. vulgaris has been bought from National Botanical Garden of Iran. First, *S. vulgaris* leaves were converted into powder by mechanical milling, and prepared for ethanolic extraction. Next, 100 g of powder was extracted with ethanol 70 % (400 ml) in Soxhlet apparatus for 1.5 hours. Next, extract were condensed with a rotary machine for 1 hour at 60°C. Gas chromatography was used to identify the types of compounds in this extract.

Isolation and culture of human epidermal keratinocytes

Human neonatal foreskin was used to isolate keratinocyte cells. First, the skin was washed with PBS, and divided into small segments. Next, these segments were placed in Hanks' Buffer containing 25 u/ml Dispase enzyme for 12 hours at 4°C. It leads to separate the epidermis from the dermis. Then, Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) were used for the separation of keratinocytes for 15 minutes at 37 °C. Trypsin enzyme was deactivated by using 10 % fetal bovine serum (FBS). Cells were precipitated by centrifuge at 10,000 rpm for 5 minutes at 4°C. Next, keratinocyte serum free medium (K-SFM) was used for the culture of keratinocyte cells. Cells were suspended in K-SFM medium containing 0.1-0.2 ng/ml of epidermal growth factor (EGF) and 25 -30µg/ml of bovine pituitary extract (BPE). Finally, cells were incubated at 37 °C in 5 % CO2 with 90 % humidity, and allowed to adhere overnight, then were washed by PBS.

In vitro assay for Cytotoxicity activity (MTT assay)

3-(4, 5-dimethyl thiazol- 2yl)- 2,5-diphenyl tetrazolium (MTT) assay was used for the determination of cell viability. 2500 cells were placed in 96 well plates. Then, different concentrations of extracts (0, 200, 300, 400 µg/ml) were added to cells at 24, 48 and 72 hours, and investigated by MTT solution (5

mg/ml in PBS). After 4 hours incubation and dissolving of formazan crystals in dimethylsulfoxide (DMSO), the absorbance have been read at the wavelength 570 nm by using a plate reading spectrophotometer . The fluorescence intensity is directly proportional to the number of viable cells. The percentage of cell viability was calculated as the following formula:

$$\text{Cell viability (\%)} = \frac{A570\ of\ treated\ cells}{A570\ of\ control\ cells} \times 100$$

Adding *S. vulgaris* extract into the keratinocyte medium

Isolated cells were calculated by trypan blue, and placed 5000 cells in each well in a 6 well plates, and allowed to adhere overnight at 37 °C in 5 % CO2. After incubation of cells were stimulated with LPS (10 ng/ml; Sigma). Then, the cells were treated by *S. vulgaris* extract 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 *S. vulgaris* extract.

RNA extraction

First, keratinocyte 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 (QiagenRNeasyPlus 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 RevertAid H Minus First Strand cDNA Synthesis Kit, K1631. Reaction contains 4 µ 5x buffer, 1 µg RNA, 1 µloligodt primer, 2 µldNTP (10 mM), 1 µ RNase Inhibitor (20 U/ µl), 1 µl Reverse Transcriptase enzyme (200 u/ µl),.and the volume was brought to 20 ml by sterile H2O. Program was implemented as follows: 5 min at 25 °C for the annealing of primers, 60 minutes at 42 °C for cDNA synthesis.

Primer designing

Primers were designed based on the exon 4 of cathelicidin gene available from DDBJ/GenBank with Oligo ver-5 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 µlcDNA, 1.5 mM MgCl2, 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 minute for initial strand separation at 94 °C; followed by 35 cycles of 30 seconds at 94 °C, 45 seconds at 59 °C, 30 seconds at 72 °C, and a final 7 minute step at 72 °C.

Real time PCR

The quantification of cathelicidin gene expression has been assessed by Real Time PCR method. This reaction contains 12.5 µ 2 x SYBR Green Master Mix, dNTP, 0.5 µ of each primer (20nmol), 2 µ cDNA (50 ng/µl), and the volume was brought to 25 ml by sterile H2O.Program was implemented as follows: 5 minute for initial strand separation at 94 °C; followed by 50 cycles of 15 seconds at 95 °C, 1 minute at 60 °C as annealing temperature. Also, Beta-2-microglobulin was used as internal control.

DAPI staining and Immuno cytochemistry (ICC)

The supernatant was discarded. The cells were washed by PBS. Next, 300 µl of 0.4% paraform- aldehyde was added into each well, and placed into the fridge for 20 minutes. Then, it was kept at room temperature for 5 minutes. The cells were washed with PBS again. Next, 200 µl Triton X-100 was added into cells, and kept at room temperature for 5 minutes. The cells were washed with PBS again. Finally, 1µg/ml DAPI (4',6-diamidino-2-phenylindole) was added, and the images has been assessed by fluorescence microscopy. Immuno-cytochemistry was used to confirm the protein expression. First, the supernatant was discarded, and the cells were washed by PBS. Next, 300 µl of 0.4% paraform- aldehyde was added into each well, and placed into the fridge for 20 minutes. Then, it was kept at room temperature for 5 minutes. The cells were washed with PBS again. Next, 200 µl Triton X-100 was added into cells, and kept at room temperature for 5 minutes. The cells were washed with PBS again, and added goat serum, and kept at room temperature for 45 minutes. The supernatant was discarded. Next, primary antibody was added to a ratio of 1 to 200, and incubated overnight at 2-8 °C. The supernatant was discarded, and the cells were washed by PBS containing Tween, and added PBS/BSA. Next, secondary antibody was added to a ratio of 1 to 200, and kept 1 hour at room temperature. Finally, this was washed 3 times by PBS containing Tween. PE color was used for the detection of protein at treated sample and control.

RESULTS & DISCUSSION

The result of gas chromatography has been shown that the *S. vulgaris* leaf extract contains a variety of compounds (see table 2). MTT assay showed that cell viability decreased in dose- and time-dependent.

Therefore, 200 µg/ml of*S. vulgaris* extract concentration was determined as optimal concentration for treating keratinocyte cells. The purity of RNA was determined by the ratio of the absorbanceat260 and 280 nm (A260/280) which was 1.9. The result of RT-PCR showed that 123 bp segment of target sequence amplified in treated samples and control (see figure1). The real time PCR was used to evaluate the level of cathelicidin protein expression. Raw data obtained from relative quantitative real time PCR after amplificationin the form of CT (see table 3). The level of cathelicidin protein expression was evaluated by Pfaffl method. Significant difference between ΔΔ CT of samples were tested by Student's t-Test in the level of α: 0/05. The result showed that there was a significant difference in the level of protein expression of treated samples and control sample ($p \leq 0/05$). Also, the concentration of 200 µg/ml of *S. vulgaris* extract at 48 hours showed large amounts of cathelicidin protein expression in comparison with 24 and 72 hours. In control sample also has been seen that the level of cathelicidin protein expression was lower than treated samples (see figure. 2), and there was a significant difference between them. It result showed that *S. vulgaris* extract increases the level of mRNA expression of cathelicidin gene. The result of DAPI staining and ICC method also confirmed the cathelicidin protein expression in treated samples and control (see figure. 3).

Table 1: Sequences of Primers are used in this study

Primer	Sequence 5´-3´	Specificity	Amplicon (bp)
CAMP cathelicidin antimicrobial peptide (human)	CCTgCTgggTgATTTCTTCC	cathelicidin /sense	123 bp
	GgCACACACTAggACTCTgTCC	cathelicidin /anti-sense	

Table 2: Compounds of *S. vulgaris* leaf extract analyzed by GC

	Ingredients
1	Echinacoside
2	Rutin
3	Jasminidin
4	Jasminin
5	Isooleoacteoside
6	Isoligustroside
7	Isooleuropein
8	Neooleuropein
9	Lilacoside

10	**Fliederoside**
11	**Syringopicroside B**

Table 3: Real-time PCR results (negative control, internal control, treated samples and control sample)

Colour	Name	Ct	Rep. Ct
	Control sample	**40.22**	**40.22**
	Beta-2-M (internal control)	**30.10**	**30.10**
	Treated sample at 24 h	**35.13**	**35.13**
	Treated sample at 72 h	**36.06**	**36.06**
	Treated sample at 48 h	**33.22**	**33.22**

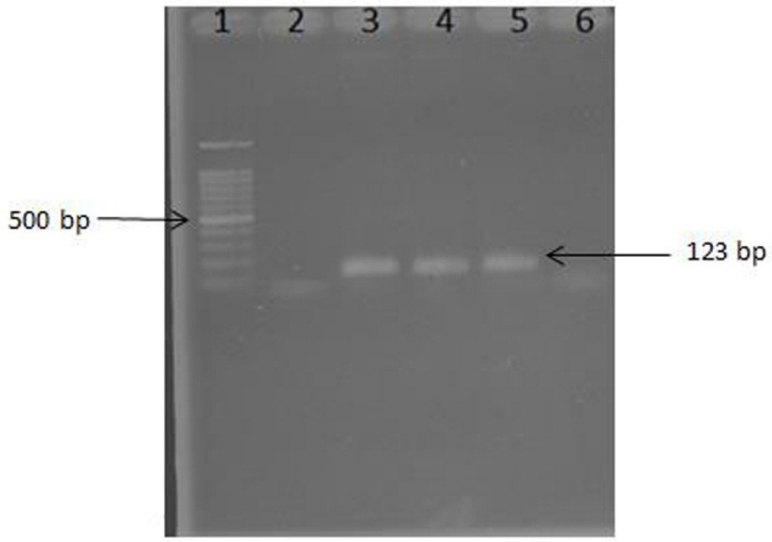

Figure 1: Electrophoretic Agarose gel, stained with ethidium bromide, of the PCR products of cathelicidin gene.1-Molecular marker (100 bp ladder), 2-negative control, 3-cathelicidin gene in treated sample at 48 hours (123 bp), 4-cathelicidin gene in treated sample at 24 hours (123 bp), 5-cathelicidin gene in control sample (123 bp) and 6-environmental control

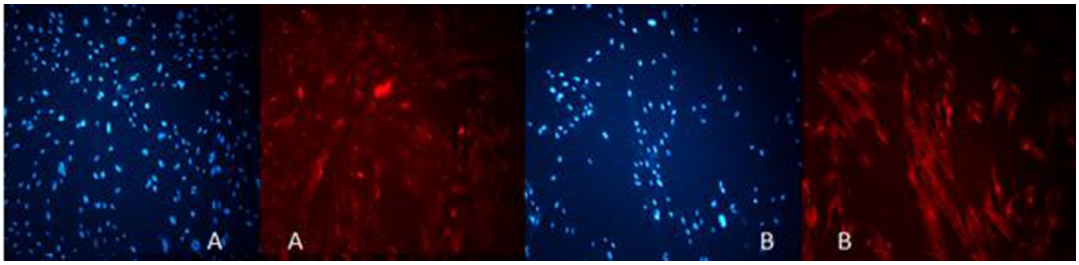

Figure 2: DAPI based staining and ICC method with scale bar 100: A: control sample, B: treated sample.Nuclei are stained with DAPI and cathelicidin protein stained by Immunocytochemistry

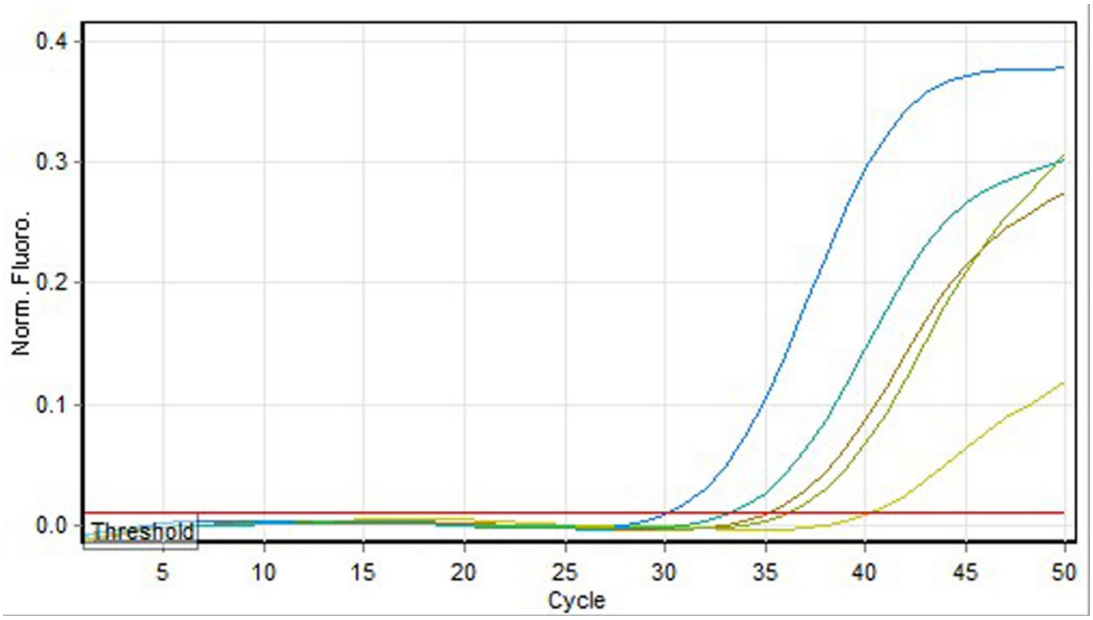

Figure 3: Real time PCR curves

CONCLUSION

Different ways could be used for increasing the level of AMPs in skin because of the importance of these peptides to inhibit skin pathogens. These peptides are biologically active molecules, and the cost of treatment with AMPs is very low. Generally, the importance of AMPs has greatly increased in recent years. These properties make them good candidates for medical applications and researches, and AMPs could be used as alternative remedies to treat skin infections instead of using synthetic drugs. Many studies showed that some herbal extracts could increase the expression a number of genes. For example, potential mechanism of different herbal extracts increased antimicrobial peptide expression [27]. Besides, Chinese herbal medicine (CHM) has been increased both epidermal permeability barrier homeostasis and antimicrobial peptide expression in normal epidermis [28]. Houttuynia cordata extract increased the expression of human β-defensin 2 (hBD2), which play a significant role in the mucosal innate immunity in the female [29]. Cinnamon extract also increased the expression of Phosphoenol Pyruvate Carboxy Kinase (PPECK) mRNA in diabetic rats compared to control rats [30]. According to modern pharmacological studies, different kinds of compounds are present at *S. vulgaris* which play an important role in defense mechanism, and have antitumor, antihypertensive, anti-inflammatory, anti-oxidant, antifungal activities, and also have a

strong anti-bacterial property which could be used in the pharmaceutical industry and medical. It implies that *S. vulgaris* could be considerably different from other plants in terms of its special components. All above mentioned properties lead to further research on this genus, and its effect on the expression of cathelicidin antimicrobial peptide. On the basis of this study, *S. vulgaris* extract increased the synthesis of cathelicidin, and its efficacy was confirmed. Therefore, the concentration 200 µg/ml of *S. vulgaris* extract could be more effective and safer rather than synthetic drugs which have severe side effects.

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Conflict of interest statement

The authors have declared no conflict of interest.

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