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Article

The Human Host Defense Peptide LL-37 Overexpressed in Lung Cell Lines by Methanolic Extract of Valeriana officinalis

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The present study investigated the effects of valerian methanolic extract and valerenic acid on the expression of LL-37 gene and protein in A549 and MRC5 line cells. After preparing Valerian seeds, sowing them in March 2020, and harvesting the rhizome in October 2020, the extract was prepared from the valerian rhizome by maceration method. Valerian acid content was determined using high performance liquid chromatography (HPLC). Two cell lines (A549 and MRC-5) were used to study the effects of valerian extract, and the MTT test was used to evaluate cell viability. The expression of LL-37 mRNA and protein was assessed by Real-Time PCR and western blot, respectively. In vivo safety assessments and histopathological analysis were also conducted. Data was analyzed by Graphpad Prism 8 software. Valerian methanolic extract and valerenic acid upregulated the LL-37 mRNA and protein expression in both treated cell lines. Valerenic acid showed a greater effect on upregulating LL-37 expression than valerian methanolic extract. A549 cells were more sensitive to valerian methanolic extract compared to MRC5 cells, and its cell viability was reduced. Furthermore, liver and kidney-related safety assessments showed that valerian methanolic extract had no toxic effects. In general, it was concluded that the methanolic extract of valerian as well as the resulting valerenic acid as the most important component of the extract has the ability to upregulate LL-37expression. Therefore, methanolic extract of valerian and valerenic acid can be considered for improving the immune system.

Keywords: Valerenic acid. Overexpression. Gene. Protein. Upregulation.

INTRODUCTION

Respiratory diseases are among the most common disorders and causes of death worldwide. Most prevalent are diseases such as asthma, bronchitis, chronic obstructive pulmonary disease (COPD), cystic fibrosis, pneumonia, and lung cancer (Seiler, Bals, Beisswenger, 2016). Death rates from infectious diseases of the respiratory tract, usually viral or bacterial infections, are very high (Pinner *et al*., 1996). One important mechanism of innate immunity against invasion by infectious agents is the overexpression of antimicrobial peptides (AMPs) (Stotz, Waller, Wang, 2013).

Antimicrobial peptides (AMPs) are derived from animals and plants and have antibacterial, antiviral, and antifungal activities (Wang, Shih, Chang, 2017). AMPs have a promising future because of their wide range of activities, lower toxicity, and reduced resistance to

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microbial agents (Nguyen, Haney, Vogel, 2011). Most of these peptides are cations that together with their amphipathic properties, give them the ability to react with cell membranes (Bahar, Ren, 2013). Antimicrobial peptides typically contain less than 100 amino acids (Zasloff, 2019). More than 2300 antimicrobial peptides have been collected (Piotto *et al*., 2012). Antimicrobial peptides produced in humans include cathelicidins, thrombocidins, and Defensins (Wang, 2014).

Cathelicidins are supplied by the innate immune system (Zanetti, 2004). The antimicrobial peptide LL-37 is derived from human cathelicidins protein and is structurally cationic, amphipathic, and α-helical (Fabisiak, Murawska, Fichna, 2016). LL-37 peptide is composed of 37 amino acids weighing between 4-5 kDa (Oren *et al*., 1999) and is derived from in-body fluids and various cells such as mast cells, natural killer cells (NK), B cells, and immature neutrophils; it kills bacterial, viral, and fungal pathogens (Yang, Chertov, Oppenheim, 2001).

Valerian, *Valeriana officinalis* L., is an herbaceous plant from the Valerianaceae family that grows in temperate regions of Asia, Europe, and the Americas. Its rhizome and sprouting roots are used as medicine (Nandhini, Narayanan, Ilango, 2018). The most important active ingredient in valerian is valerenic acid, which is extracted from the roots and rhizomes of the plant. There are three groups of chemical compounds in this plant, namely mono-sesquiterpenes, iridoid triesters, and pyridine alkaloids (Nandhini, Narayanan, Ilango, 2018). Today, the sedative effects of valerian are attributed to its volatile oils, including valerenal and valerenic acid (Rezaie *et al*., 2010). This plant has been used in traditional medicine to relieve fatigue, for its sedative effects, as well as to treat seizures, nerve pain, and muscle cramps (Ekhteraei et al., 2010; Duke, 2002).

To the best of our knowledge, no study has examined the effects of valerian extract on LL-37 gene and protein expression in lung cells. Because improving the immune system can lead to a better response to respiratory infections, including Covid-19, the present study aimed to investigate the effects of valerian extract on respiratory cells as well as the expression of LL-37. The toxicity of this extract was also investigated.

MATERIAL AND METHODS

Plant materials

Valerian seeds prepared from the herbarium of Shahid Beheshti University (Deposit number: 55822, confirmed by: Dr. Ahmad Reza Mehrabian), Tehran, Iran, were planted in March 2020, and the rhizomes were transferred to the laboratory for extraction in October 2020. The rhizomes were first washed with water, then sliced, and dried at room temperature. After drying, the prepared rhizome pieces were kept in a closed, completely dark container to protect them from the light.

Plant Extraction

To separate the active constituents from the valerian rhizomes, the dried rhizomes were fully ground to a fine powder in a blender. Then, 10 g rhizome powder was mixed with 10 mL methanol 60%. The sample was sonicated for about 35 min to break the cell wall and then homogenized by a magnetic stirrer at 1000 rpm overnight. Afterward, the sample was centrifuged at 4000 rpm for 10 min, and the supernatant was collected for extraction.

The obtained supernatant was transferred to another sterile tube and centrifuged at 3000 rpm for 5 min, then collected and filtered by a 0.45 µm membrane filter to remove particles and impurities. Next, the filtered solution was transferred to a rotary evaporator for concentration for about one hour. Then 1 mg of the dry powder of the extract was dissolved in 1 mL of methanol and water solution to obtain stock at a concentration of 1 mg/mL.

Determination of Valerenic acid

The Valerenic acid in the extract were determined by HPLC using a column packed with C18 (Waters, 2695) and a UV detector (Waters, 996) at a wavelength of 220 nm. The mobile phase was 20:80 mixture of acetonitrile (HPLC grade, Merck) and water. The elution rate was 0.5 mL/min and the column temperature was 25 °C. Valerenic acid (HPLC grade, Extrasyntese) was used as an external standard to confirm the reported coefficients

for HPLC area ratios between the internal standard and the corresponding external valerenic acid.

Lung cell culture

Two lung cell lines (A549 and MRC-5) were purchased from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, and cultured in 24-well plates containing DMEM medium, 10% FBS, and 1% gentamicin (Sigma-Aldrich, USA). The prepared plates were incubated for 48 h at 37 °C, 5% CO2. To assess the impacts of the crude extract of *V. Officinalis* on human lung cells, both A549 and MRC-5 cultures were optimized in a range of 3×10^5 total viable cells, and the formation of monolayer cells was visually verified using an inverted phase-contrast microscope (Leica, DMi1). After appropriate density was reached, cell cultures were divided into three groups, i.e. the control group, the methanolic extract-receiving group, and the valerenic acid-receiving group. The cultures were then incubated for 72 h.

MTT

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide test (MTT) is one of the methods to study the cytotoxicity in vitro. This method is based on mitochondrial activity. Mitochondrial activity in living cells is stable and therefore an increase or decrease in the number of living cells is linearly related to mitochondrial activity. MTT tetrazolium dye is reduced in active metabolically cells. Mitochondrial dehydrogenases in living cells break the tetrazolium ring and produce NADH and NADPH, leading to the formation of a purple insoluble deposit called formazan. This precipitate can be dissolved by isopropanol or dimethyl sulfoxide. Dead cells, on the other hand, do not have this ability and therefore do not show a signal. High safety and providing a colorimetric and non-radioactive system are important advantages of this method. Cells were cultured in 96 cells plate at 3×10^5 cells/well density and incubated for 72h. Then 100 ml MTT was added to each well. The plates were then incubated at 37 °C for 3 to 4 h and after that the color of the solution turns to blue due to the production of formazan. Next, the plates were removed from the incubator and the surface solution on the cells was removed and 70 µl DMSO was added to each well to dissolve the crystals produced. They were incubated in a shaker incubator for 20 min and after this time, all plate cells were pipetted and the absorbance was read at 595 nm using a spectrophotometer (Sunrise) and the IC_{50} of the cells was calculated.

LL-37 Gene eexpression

Real-time PCR was used to evaluate the expression of *LL-37* in the two cell lines. A pair of specific primers was designed for assessing the expression of *LL-37*, using the Primer3 program and BLAST (Table I). The expression ratio of the studied genes in this study was evaluated by Threshold Cycle (CT) method.

The one-step real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Sequences Detection Systems. Foster City, CA) according to the manufacturer's protocol. For each reaction, 5 µL SYBR® Green real-time PCR master mix, 0.5 µL forward primer and 0.5 µL reverse primer, 2 µL cDNA, and 2 µL sterile water were added to reach 10 µL PCR amplification was performed in 40 cycles using the following program:

Initial denaturation at 95°C for 15 minutes; 40 cycles of denaturation at 95°C for 15 minutes, annealing at 60°C for 30 minutes, and extension at 72°C for 30 minutes; and final extension at 72 °C for 5 minutes. In all cases, the expression levels were compared to that of the GAPDH as the house-keeping gene. Extracted RNA was run on a 1% agarose gel and their concentration and purity was measured using a UV-Vis spectrophotometer. The light absorption of the sample was evaluated at 260/280 nm, and its concentration was obtained based on the dilution coefficient in ng /µL.

TABLE I - PCR primers designed by Primer3 program

Gene	Primer sequence	Length (bp)	Tm (°C)	Gene bank Code
LL-37	F: 5' – TGCTAACCTCTACCTCCTCCT - 3'	165	62.43	NM_004345.5
	R:5' – CGCTTCACCAGCCCGTCCTTC - 3'		65.85	
GAPDH	F: 5' – GCAGGGATGATGTTCTGG - 3'	126	55.07	NM_001357943.2
	R: 5' – CTTTGGTATCGTGGAAGGAC - 3'		55.86	

Western blot

The cells were washed and homogenized with cold PBS, lysed with the protease-inhibiting Radioimmunoprecipitation assay (RIPA) buffer, and then centrifuged at 10000 rpm, at 4°C, for 15 minutes. The superior phase was then collected for the total protein content defined by Lowry method. Homogeneous samples (50 µg) were detected by electrophoresis in 12.5% sodium dodecyl sulfate-polyacrylamide (Bio-Rad Laboratories, Hercules, CA, USA), and subsequently the proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane. The membranes were incubated for 60 minutes at room temperature by blocking solution (5% non-fat dry milk). Afterward, the unique primary LL-37 antibodies (Sc-166770, Santa Cruz) were used on a shaker for 120 minutes. During the next stage, the membranes were washed with Tris-buffered saline with Tween 20 detergent (TBST) for three times. Secondary antibody (Sc-2005, Santa Cruz), a monoclonal antibody from the rodent, was used and then applied to the shaker at room temperature for 90 minutes, and protein expression was confirmed. The protein bands obtained from each extract were normalized against the corresponding values for GAPDH (house-keeping protein) bands and the density of protein bands was quantified using Image J technology (National Health Institute, USA).

Toxicity assay

Outbred SW1 mice were prepared from the Faculty of Veterinary Medicine, Tehran University of Medical Sciences (n = 10) and kept in animal house with 25°C temperature, 50% relative humidity and 12/12 light/dark cycle. All animals had free access to water and food. After 1 week, they were divided into two groups: control (saline) (n=5) and treatment (receiving valerian extract) (n=5). The valerian total extract and normal saline were injected intravenously (50 mg/Kg) and after 60 minutes, blood samples were collected via cardiac puncture under general anesthesia induced by Ketamine (100 mg/kg) and Xylazine (18 mg/Kg) and the serums were isolated.

Glomerular filtration rate (GFR) was assessed using the serum creatinine and liver enzyme levels in outbred SW1 mice. The GFR was estimated by the single-blood-sample method (Al-Mousawi *et al.*, 2010). The association between the GFR values from the single-blood-sample method and serum creatinine concentrations was assessed in blood samples from the SW1 mice. The levels of serum glutamic oxaloacetic transaminase (SGOT) and alanine aminotransferase (SGPT) enzymes were measured using spectrophotometry and laboratory kit (ALAT, 119395H917).

At the end of the study, mice were euthanized in a CO_2 chamber based on an approved protocol, and the liver and kidneys were isolated and evaluated for further histopathological evaluations.

The fixed kidney and liver samples were dehydrated in graded alcohols, cleared in xylene (Sigma Aldrich, Cat No # 534056), and embedded in paraffin. The samples were then serially sliced into 4-6 µm sections. These prepared slides were stained with Hematoxylin- Eosin (H&E) (code no: ab245880). Horizontal orientation was made on thin sections to obtain the largest cut surface. Then images were captured by light microscope with 4X, 10X, and 40x magnification (Zeiss, Germany).

All procedures in this research were carried out by the rules of the Iran National Research Ethics Committee (IR.IAU.PS.REC.1398.328), Tehran, Iran.

Statistical analysis

Cell viability assay was performed based on the ratio of the live and dead cells, and the graphs concerning different concentrations of the extract were drawn using the GraphPad Prism 8.0 software. Millennium 32 software was used to analyze the HPLC data. Gene and protein expression levels were compared by One-Way ANOVA, followed by Post-hoc multiple Tukey test. Data were expressed as the mean ± SE.

RESULTS

HPLC analysis

The prepared samples were analyzed by HPLC and evaluated based on retention time and area under the curve in comparison to external standard using Millennium32 software. 0.5 mg/gr valerenic acid was obtained in total crude extract (Figure 1a, b).

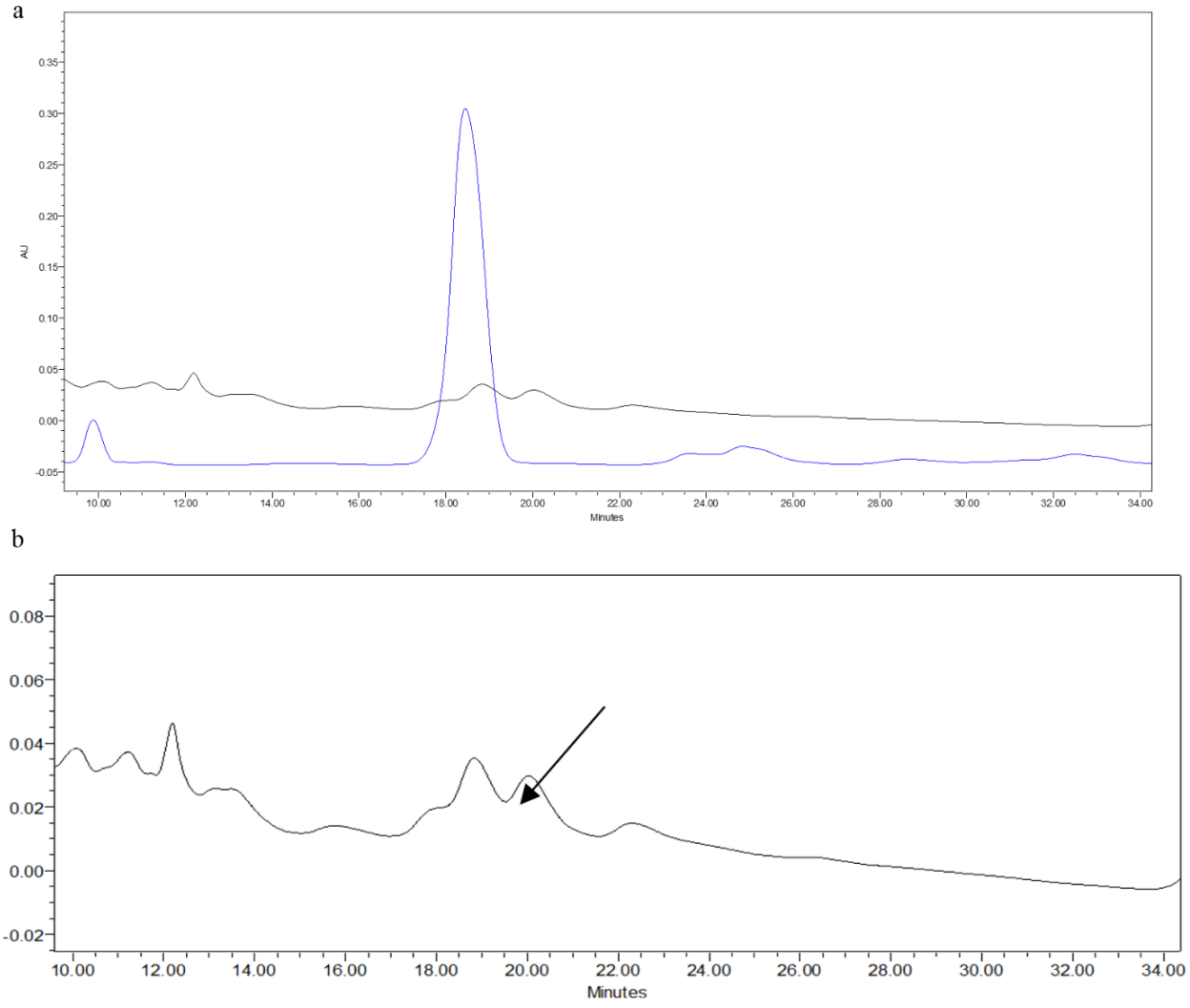

FIGURE 1 - (a): HPLC chromatogram of *Valeriana officinalis* methanolic extract. Blue graph and black graph represent standard Valerenic acid and prepared sample, respectively. (b): HPLC chromatogram of the total extract. The black arrow shows the Valerenic acid peak based on retention time.

MTT Assay

In A549 cell line treated with valerenic acid, IC_{50} (15.35 ± 3.98 µg/mL) was much lower than MRC-5 fibroblast cell line (52.44 ± 4.08 µg/mL). The reduction of cell viability in the presence of valerenic acid was dose-dependent, by increasing concentration up to 100 µg/mL, the viability was reduced to 10%. In valerenic acid-treated A549 cells, the viability was reduced from 90% in 5 µg/mL to 5% at 50 µg/mL. Also, the IC_{50} obtained for valerenic acid in both tested cell lines was lower than the IC_{50} obtained for methanolic extract (Figure 2a,b).

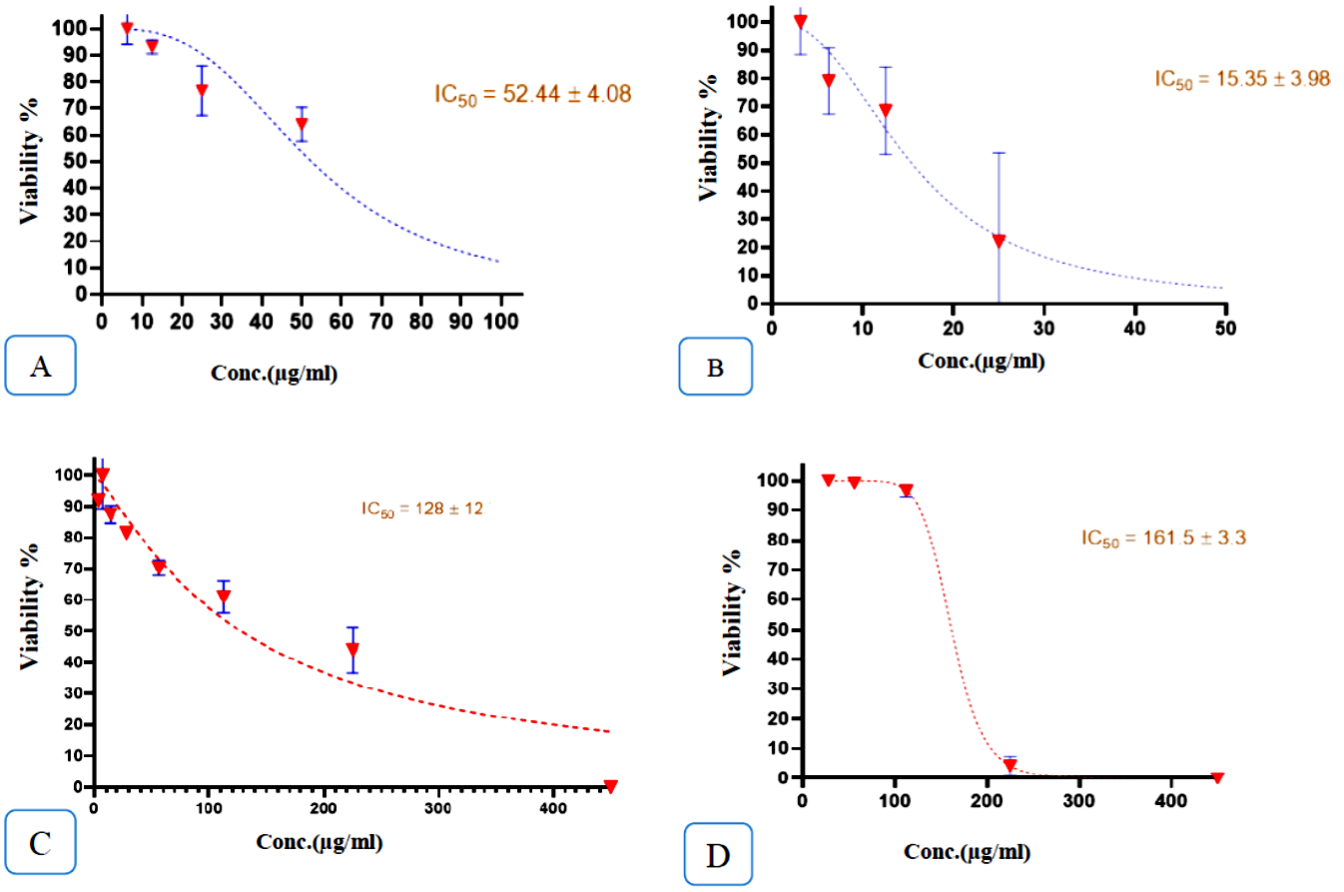

FIGURE 2 - Cytotoxicity effect of Valeronic acid on MRC-5 (A) and A549 (B) cell lines and total Valerian extract on A549 (C) and MRC-5 (D) cell line.

The dose-dependent reduction in A549 cells viability treated with different concentrations of methanolic Valerian extract (10-500 µg/ml) was shown (IC_{50}= 128±12 µg/ml). An accentuated decrease in the viability of MRC-5 cells during treatment with methanolic Valerian extract was observed in concentrations above 100 µg/ml and IC_{50}=161.5 ± 3.3 µg/ml was calculated (Figure 2c,d).

LL-37 Gene Expression

Valerian acid and valerian methanolic extract in both A549 and MRC5 cell lines overexpressed LL-37 gene compared to controls (Figure 3). In A549 cells, treatment with valerenic acid resulted in an almost twofold upregulation in LL-37 expression compared to controls (Figure 3a), while treatment of A549 cells with valerian methanolic extract caused a slight increase in

LL-37 expression compared to controls. (Figure 3c). Also, a marked overexpression of LL-37 was observed in MRC5 cells treated with valerenic acid compared to controls (Figure 3b). It seems that the effect of valerenic acid treatment on the overexpression of LL-37 gene is more than valerian methanolic extract.

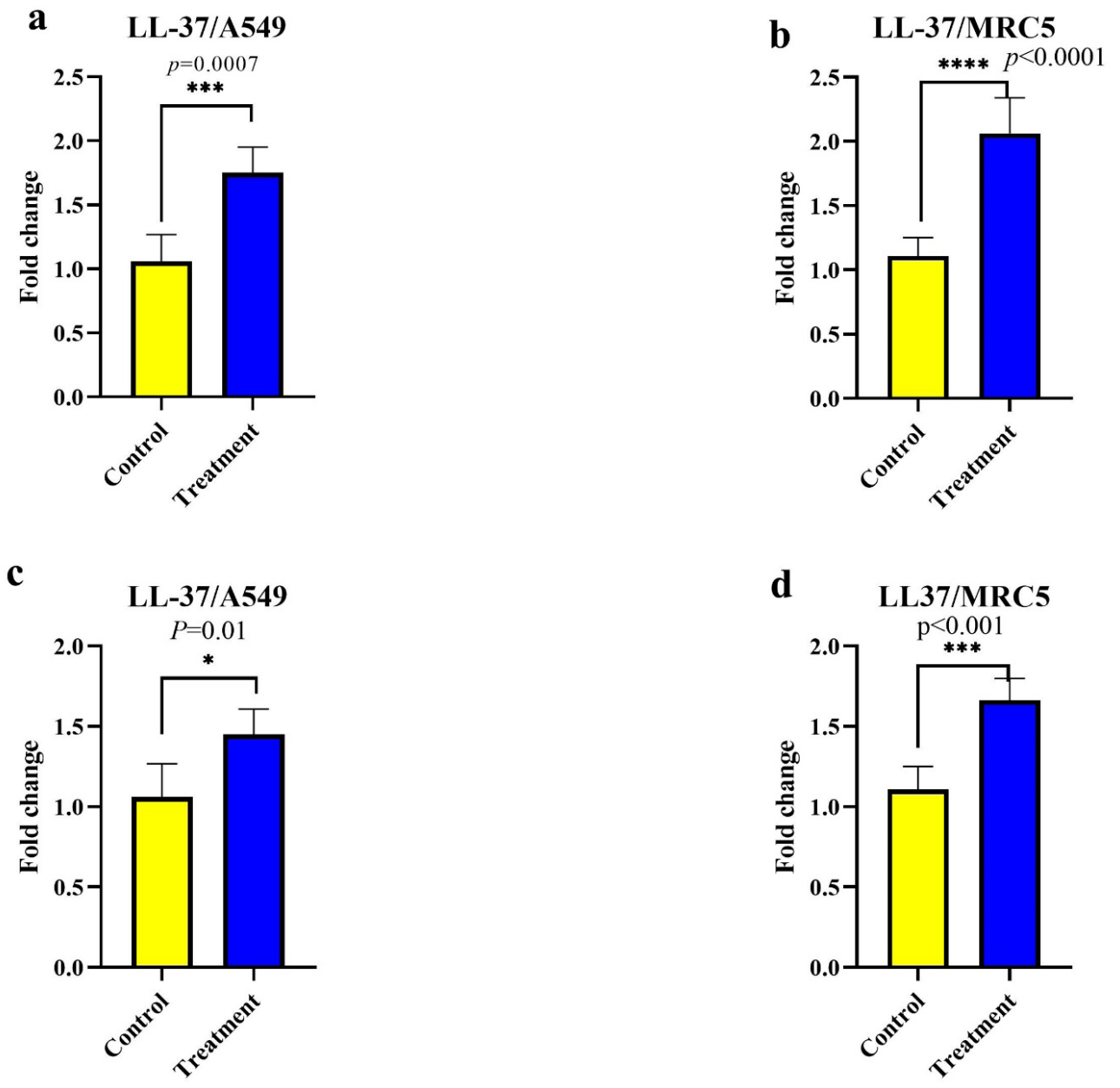

FIGURE 3 - LL-37 gene expression in A549 cell line and MRC5 cell line treated with valerenic acid (a, b) and Valerian methanolic extract (c, d).

Western blot

Significant differences were observed in the expression of LL-37 protein in two cell lines A549 and MRC5 treated with valerian methanolic extract. The expression of LL-37 protein in A549 and MRC5 cells treated with methanolic extract was significantly upregulated compared to the control group (Figure 4).

Also, the expression of LL-37 protein in MRC5 cell lines treated with valerenic acid upregulated significantly compared with the control group (Figure 4). It seems that treatment of both cell lines with methanolic extract or valerenic acid upregulated LL-37 expression and this was stronger during treatment with valerenic acid.

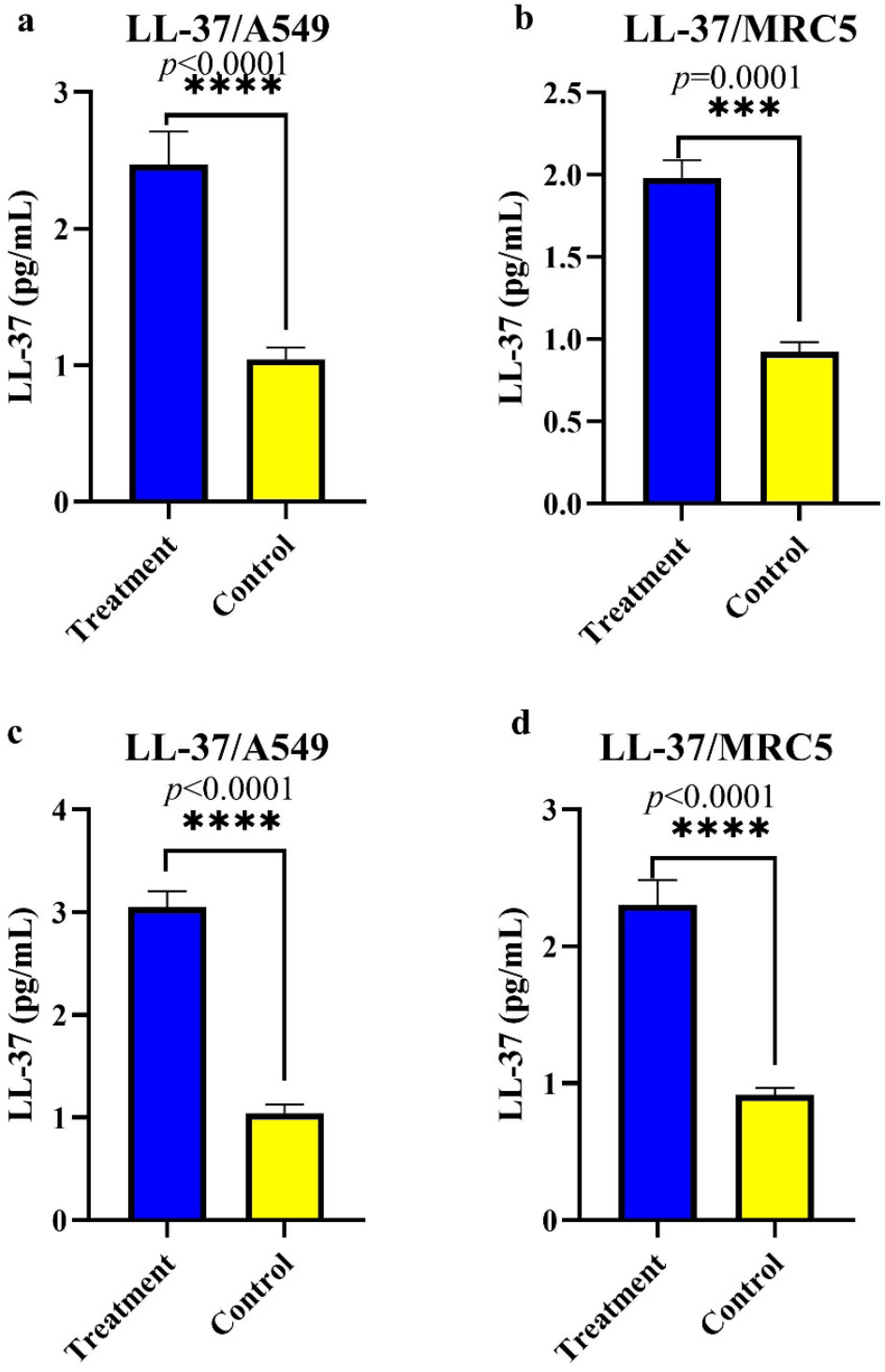

FIGURE 4 - LL-37 protein expression in A549 cell line and MRC5 cell line treated with valerenic acid (a, b) and Valerian methanolic extract (c, d).

The LL-37 protein expression in A549 and MRC5 cells treated with valerian methanolic extract and valerenic acid was compared and the results showed significant differences (Figure 5). Valerinic acid and valerian methanolic extract significantly upregulated LL-37 protein in both cell lines. Comparison of the mean LL-37 expression of in A549 cells treated with valerenic acid with A549 cells treated with valerian methanolic extract showed significant differences ($P < 0.05$). However, MRC5 cells treated with valerenic acid did not show any significant difference compared to cells treated with valerian methanolic extract (Figure 5c).

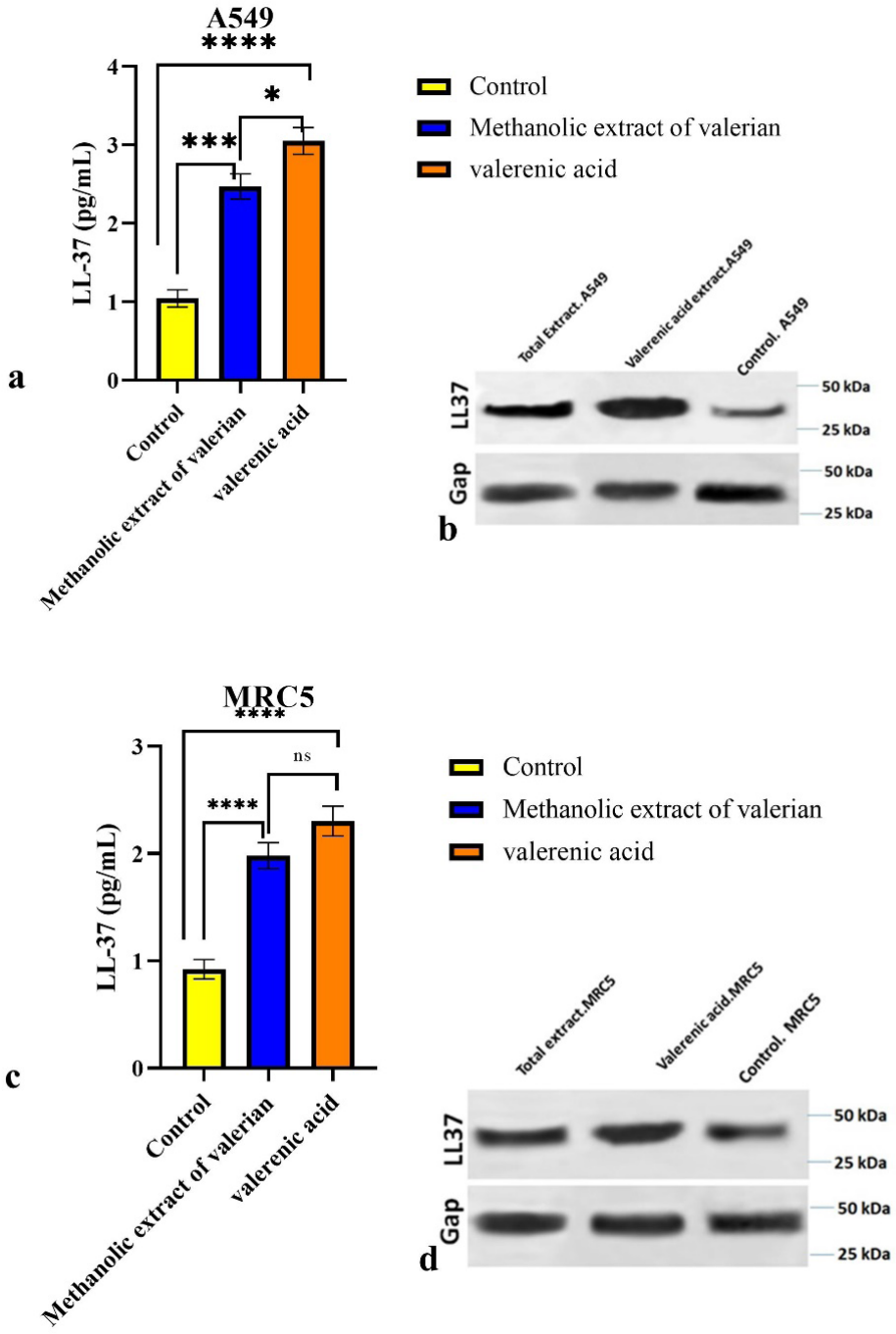

FIGURE 5 - The comparison of LL37 peptide expression of in A549 (a, b) and MRC5 (c, d) cell lines treated with methanolic extract of valerian and valerenic acid

Toxicity assays

The results showed that the glomerular filtration rate (GFR), glutamic pyruvate transaminase (GPT) and glutamic oxalic acid transaminase (GOAT) of the control mice was lower than mice treated with methanolic extract of valerian, but these differences was not statistically significant (P>0.05) (Figure 6).

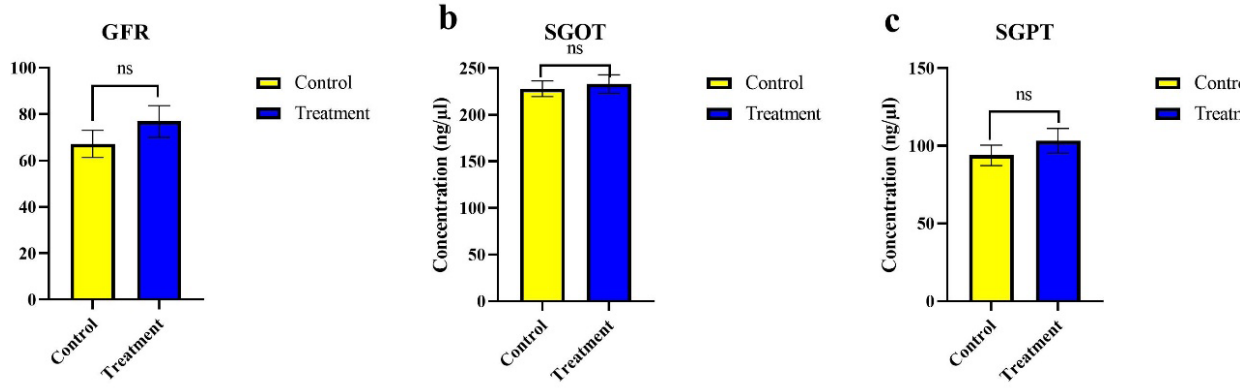

FIGURE 6 - Glomerular filtration rate (GFR) (a), serum glutamic-oxaloacetic transaminase (SGOT) (b) and serum glutamic pyruvic transaminase (SGPT) (c) levels in control mice and treated with methanolic extract of valerian.

The results of the histological analysis revealed that the animals in the control and treatment group showed normal histological features. The histopathological evaluation of kidney revealed that the epithelial cells of the bowman capsule in the glomerulus had a regular appearance in the control and treatment group. The infiltration rate of lymphocytes was in a normal range, and tissue congestion and hyperemia not observed afterward extraction and normal saline injection. In both groups, adjacent epithelial cells in the cortex were detected in all the proximal and distal tubules without any significant differences (Figure 7a, b).

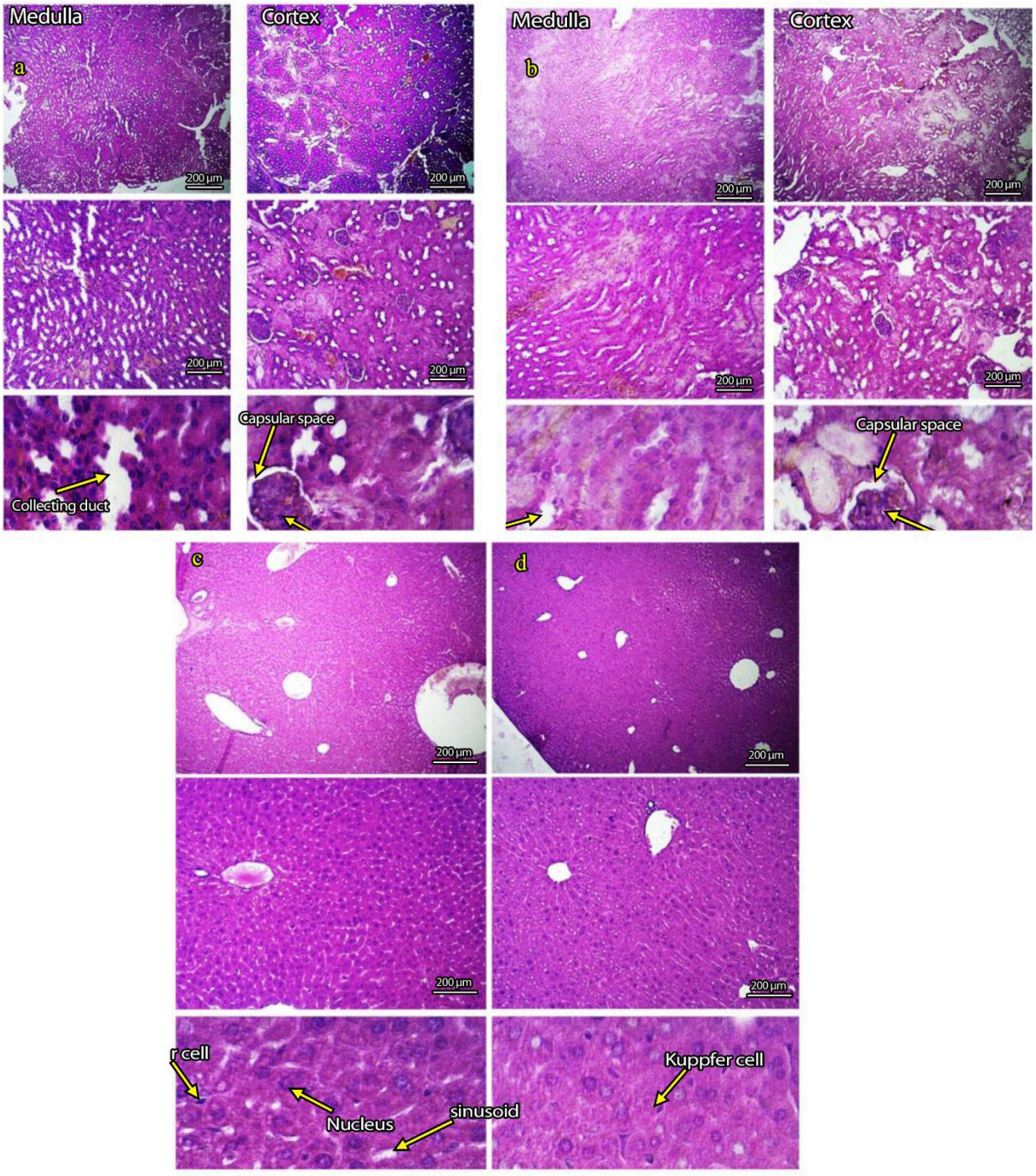

FIGURE 7 - Longitudinal section of mice kidney tissue in control (a) and treated with methanolic extract of valerian (b). Cross-section of mice liver tissue in control (c) and treated with methanolic extract of valerian (d). In the group treated with methanolic extract of valerian, the epithelial cells of the Bowman capsule wall in the renal glomerulus had a normal appearance. Lymphocytes in tissue, and tissue bleeding were not observed. In the renal cortex, all the proximal and distal tubes with the epithelial wall and cells were observed. Epithelial cells were seen normally in the proximal and distal tubes. According to the results, liver tissue in both control and treated with methanolic extract of valerian hepatic cells had a normal appearance. In the space between the lobules, the hepatic triad and between each lobule, a central vein with a normal appearance was observed. Kupffer cells were observed around the sinusoidal vessels. Hepatocytes were observed in small numbers without nuclei and with a similar appearance to apoptotic cells; Scale: 200µm.

The hepatic parenchyma with hepatocytes and sinusoids ('lobular' region), which were interspersed with central venules and portal tracts, were normal in control and treatment groups. Portal tracts which contain a portal vein, a branch of the hepatic artery and a bile duct remained intact followed extract injection. The hepatic cells patterns and infiltration in both groups were in normal condition (Figure 7c, d).

DISCUSSION

Improving the immune system can lead to resistance to various infectious diseases including Covid-19. Due to the side effects of chemical drugs, a lot of attention is paid to medicinal plants today. Therefore, it is important to find compounds or extracts of medicinal plants that have the ability to strengthen the immune system. The results of the present study indicated the positive effect of valerian methanolic extract and valerenic acid on upregulating the expression of LL-37 gene and protein in both A549 and MRC5 cell lines. To the best of our knowledge, the present study is the first to investigate the effects of valerian extracts and valerenic acid on the expression of human host defense peptide LL-37. Also, the resulting extract had no toxicity on vital organs such as liver and kidney. Therefore, it can be considered as a booster of the immune system, especially in the Covid-19 pandemic condition.

LL-37 gene expression has been reported at the epithelial surface of lung cells and its role in bactericidal activity has been shown (Jha, Shah, Ks, 2012). It has been suggested that LL-37 may play a role in the host innate defense in the lung (Lau *et al*., 2005). It has been suggested that LL-37 cationic AMP may play an important role in early invasion and the spread of microbial infection (Fabisiak, Murawska, Fichna, 2016). The membrane attack (de Miguel, *et al*., 2019) and host immune regulation (Zsila, Kohut, Beke-Somfai, 2019) are two well-known LL-37 action mechanisms. Therefore, the overexpression of LL-37 observed in the present study due to the treatment of lung cells with valerenic acid or valerian methanolic extract can increase the resistance of lung cells to pathogens, which is of great importance.

In this study, the effect of valerian extract and its active ingredient, valerenic acid, on viability of both normal (MRC5) and tumor (A549) cell lines was studied. The results showed that the IC_{50} of valerenic acid in both cells was lower than the IC_{50} of the extract. In other words, valerenic acid has a more lethal effect on cells. Also, the IC_{50} of both methanolic extract and valerenic acid was lower for tumor cells than normal cells. This means that cancer cells are more sensitive to the methanolic extract or its active ingredient valerenic acid. These results indicate the cytotoxic effects of methanolic extract of valerian and valerenic acid on cancer cells, which can also be considered in the treatment of cancer. However, more studies are needed in this area.

The LL37 gene expression in cancer cells was higher than normal cells, i.e. cancer cells in the presence of the extract or active substance show more antimicrobial properties, in other words, in a tumor, the probability of developing infection in the presence of this substance is lower. Cells have a better ability to kill bacteria. On the other hand, they are sensitive to the extract, which means that this extract not only kills tumor cells but also reduces the involvement of cancer cells with bacterial infection.

Histological results of mice treated with methanolic extract showed that the methanolic extract did not have any toxic effect on lung tissue. These observations well confirm that the methanolic extract extracted from this plant has no toxic effect on vital organs. Therefore, consumption of this plant root does not seem to have side effects. However, a study has shown that valerian is harmful to the kidneys and it is better to eat with tragacanth (Diaper, Hindmarch, 2004). Its harmful effect on kidney function has been due to long-term use or high doses.

In summary, our results confirmed that *Valeriana officinalis* extract, upregulated LL-37 expression in human respiratory cells, A549 and MRC5, thereby contributing to AMP-mediated antibacterial action without in vivo general toxicity effects on vital organs. Because of traditional plant beneficial characteristics such as improving the immune system due to infectious diseases and improve human health, present outcomes encourage scientists to consider indigenous herbal medicine.

As the numerous side effects of chemical drugs used in the care of respiratory infectious diseases, the use of

biological agents will improve the quality of patient's life. The *Valeriana officinalis* properties make it ideal for herbal therapy due to enhancement natural antimicrobial peptides and based on obtained results herbal medicine can be suggested as a new therapeutic component in the treatment of patients with respiratory disorders which recently increased due to Covid-19 pandemic.

CONCLUSION

In conclusion, the obtained results revealed that both methanolic extract and valerenic acid could upregulate the innate cellular immune peptide LL-37 in A549 and MRC5 cell lines and Valerian methanolic extract had any toxic effects on liver and kidneys function, which might helpful and effective to improve the respiratory infections treatments.

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CONFLICTS OF INTEREST STATEMENT

The researchers declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Eftekhari.Z and Mohammadi.Z engendered the study design, performed the study, analyzed the data, drafted and revised the manuscript. Pishkar.L, Barzin.G and Babaeekhou.L revised the manuscript. All authors discussed the results and approved the final manuscript.

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