

Original Research Article

Anacardic acid inhibits the proliferation and inflammation of HaCaT cells induced by TNF- α via the regulation of NF- κ B pathway

Tao Liu*, Yuanmin He, Yongmei Liao

Department of Dermatology, Affiliated Hospital of Southwest Medical University, Luzhou City, Sichuan Province 646000, China

*For correspondence: **Email:** TaoLiu@163.com; **Tel:** +86-18982496851

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Abstract

Purpose: To determine the effect of anacardic acid on HaCaT cells in vitro and to elucidate its molecular action.

Methods: HaCaT cells were incubated in varying concentrations of anacardic acid (10 to 50 μ M). To model psoriasis, the cells were treated with tumor necrosis factor- α (TNF- α); cell viability was gauged by CCK-8 assay. Apoptosis was determined, and Bcl-2, Bax, p65, p-p65, p-I κ B α and I κ B α by Western blot. Levels of pro-inflammatory cytokines were evaluated by enzyme-linked immunosorbent assay (ELISA).

Results: Anacardic acid resulted in reduced HaCaT cell viability and increased cell apoptosis in a concentration-dependent manner ($p < 0.05$). It also curtailed TNF- α -mediated inflammatory responses and downregulated the NF- κ B signaling axis.

Conclusion: The results indicate that anacardic acid impedes HaCaT cell growth and inflammatory cytokine production by interfering with NF- κ B signal transduction, and may influence the development of AA-based therapies for psoriasis.

Keywords: Anacardic acid, Psoriasis, Keratinocytes, NF- κ B, Skin diseases

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INTRODUCTION

Psoriasis is a prevalent chronic inflammatory dermatological condition [1]. The precise etiology remains elusive, but it is widely recognized that dysfunctional interactions between keratinocytes and immune cells contribute significantly to its pathogenesis [2]. Key in the disease's development is the release of various cytokines from immune cells, which in turn provoke keratinocyte hyperproliferation. These keratinocytes then produce an array of

inflammatory cytokines, further exacerbating the condition [3]. Addressing the over-proliferation and inflammation in keratinocytes is considered a promising approach to managing psoriasis.

Among the various signaling pathways related to psoriasis, NF- κ B signaling is a pivotal pathway and is a type of TNF- α -induced inflammatory response. Activation of this pathway follows the phosphorylation and subsequent degradation of I κ B kinase, leading to an inflammatory cascade. TNF- α is a critical mediator in this process, often

associated with psoriatic inflammation and other dermatological disorders [4]. There is a large amount of evidence supporting that TNF- α targeted therapy can be used to treat various inflammations, including psoriasis [5]. However, TNF inhibitors have many side effects such as lymphoma, infection, etc. therefore, it is necessary to find a safer treatment or other auxiliary options.

Anacardic acid (AA) is an important component of cashew nuts, ginkgo biloba leaves and fruits. It has anti-inflammatory properties and anti-cancer activities against hand and foot edema and peritonitis, and also in TNF- α -induced venous endothelial cells [6]. In addition, AA inhibits the activation of NF- κ B and subsequent I κ B kinase, leading to the disappearance of kinase phosphorylation and the degradation of I κ B [7]. In view of the anti-inflammatory properties of AA and due to the advantage of natural ingredients, the aim of this study was to investigate the anti-psoriasis of AA.

EXPERIMENTAL

Anacardic acid

Anacardic acid (AA) was sourced from MedChemExpress (Shanghai, China, Catalog no. HY-N2020), RPMI-1640 medium, obtained from Gibco by Thermo Fisher Scientific, served to solubilize and dilute AA.

Cells

HaCaT cells, a line of immortalized human keratinocytes, were procured from the National Infrastructure of Cell Line Resource (NICR; Cat no. 1101HUM-PUMC000373; Beijing, China). These cells were cultured in RPMI-1640 medium enriched with 10% fetal bovine serum (FBS) from Gibco, CA, USA, and 1 % penicillin-streptomycin solution (Catalog no. 15640055; Gibco, Thermo Fisher Scientific). The cultures were maintained at 37 °C with 5 % CO₂.

To assess the effect of AA, we prepared solutions at concentrations of 10 μ M, 30 μ M, and 50 μ M for HaCaT cell treatment. Cells were incubated at 37°C for 24 hours. A psoriasis-like disease state was induced using tumor necrosis factor-alpha (TNF- α) from Solarbio (Beijing, China). Following induction, cells were categorized into groups: control, TNF- α alone, and TNF- α combined with varying concentrations of AA (10 μ M, 30 μ M, and 50 μ M). After 48 hours of treatment, cells were harvested for subsequent analyses.

CCK-8 assay

Cell viability was assessed using the CCK-8 assay (Solarbio, Beijing, China). HaCaT cells were seeded into 96-well plates at 4×10^3 cells/mL and incubated for 24 hours. Subsequently, cells were treated with TNF- α (10 ng/ml) to induce a psoriasis-like state. Anacardic Acid (AA) at concentrations of 10 μ M, 30 μ M, and 50 μ M was administered to evaluate its effect on cell viability. Post-treatment, CCK-8 reagent was added, and after 1 hour of incubation at 37 °C, absorbance readings were taken at 490 nm using a microplate reader. This process was repeated in triplicate for statistical robustness.

Enzyme-linked immunosorbent assay (ELISA)

To quantify cytokine production, ELISA kits for IL-6, IL-1 β , IL-8, and IL-22 in HaCaT cells. HaCaT cells were first treated with TNF- α for 48 hours, followed by AA treatment for an additional 24 hours. Post-treatment, supernatants were collected to measure the cytokine levels. (SEKF105, Solarbio, Beijing, China)

Flow cytometry

Flow cytometry was conducted using a BD Accuri™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine the rate of apoptosis in HaCaT cells. The cells were exposed to increasing AA concentrations and stained with an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Solarbio, Beijing, China). Following staining, flow cytometric analysis was performed, and apoptosis rates were calculated using FACS scan software (BD, San Jose, USA).

Western blot

Total cellular proteins from HaCaT cells were extracted using RIPA buffer (Cat no. 89901; Thermo Fisher, MA, USA). Centrifugation at 16,000xg for 15 minutes at 4°C was the next step for lysate collection. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Cat no. 23225; Thermo Fisher, MA, USA). The proteins underwent denaturation at 95°C for 5 minutes and separation on a 7.5% SDS-PAGE, loading 30 μ g per lane. They were then blotted onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Blocking was achieved with 5% skim milk in Tris-buffered saline containing 0.1% Tween (TBST), followed by primary antibody incubation overnight at room temperature.

The antibodies applied included Bcl-2 (1:500; Cat no. 3498), Bax (1:500; Cat no. 5023), p65 (1:500; Cat no. 8242), p-IkB α (1:500; Cat no. 3033), and I κ B α (1:1000; Cat no. 4814) all from Cell Signaling Technology, Danvers, MA, USA, and GAPDH (1:5000; Cat no. 5174; Cell Signaling Technology), serving as the loading control. Detection involved horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000; Cat no. 7074; Cell Signaling Technology, Inc.) for 2 hours at 37°C. The Enhanced Western Blot Kit (Beijing TransGen Biotech, Beijing, China) was used to visualize the proteins, with signal quantification performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were presented as mean \pm standard deviation (SD) from triplicate experiments. Differences between groups were analyzed using Student's t-test or one-way ANOVA as appropriate. Statistical significance was set at $P < 0.05$. Data processing and graphical representations were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Anacardic acid decreased the cell viability of HaCaT

Anacardic Acid (AA) reduced HaCaT cell viability in a concentration-dependent manner. HaCaT cells treated with 10 μ M, 30 μ M, and 50 μ M of AA for 24 hours showed a significant decrease in viability assessed by CCK-8 assay ($p < 0.05$), and colony formation was notably affected.

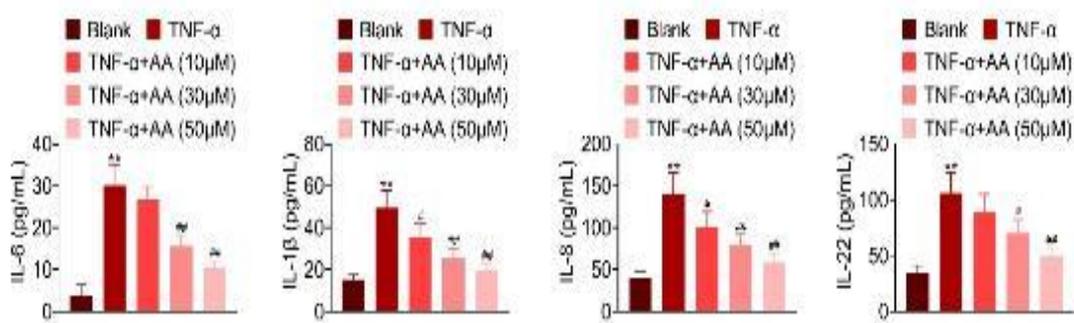


Figure 2: Dose-dependent Inhibition of Cytokine Secretion by AA in TNF- α Stimulated HaCaT Cells. ELISA analysis demonstrates that TNF- α significantly elevates the secretion levels of IL-6, IL-1 β , IL-8, and IL-22 in HaCaT cell culture medium. Treatment with AA markedly suppressed the TNF- α -induced upregulation of these cytokines in a concentration-dependent manner. Each bar graph quantifies the inhibitory effect of AA across increasing doses. Data represent mean \pm SD ($n=3$), * $p < 0.05$, ** $p < 0.01$ vs. 0 μ M of AA. # $p < 0.05$, ## $p < 0.01$ vs. TNF- α only treated group

Compared to the TNF- α group, AA treatment led to a marked reduction in colony formation, demonstrating AA's inhibitory effect on HaCaT cell proliferation ($p < 0.05$; Figure 1 A, B and C).

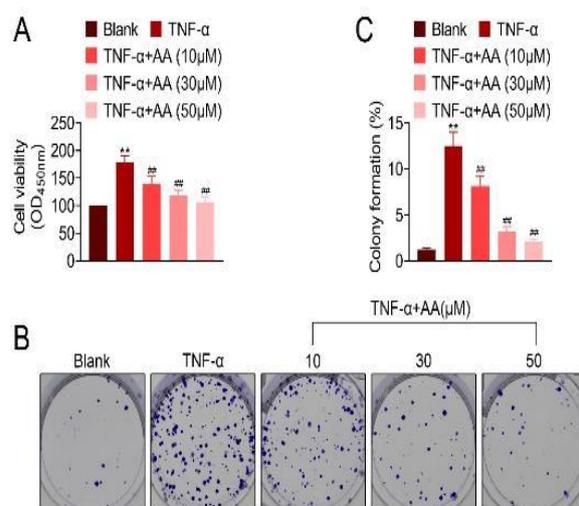


Figure 1: Effects of AA on the growth of human HaCaT keratinocytes *in vitro*. (A) Cell viability. (B) Colony formation in HaCaT cells in each group. (C) Quantification of colony formation Data are presented as the mean \pm standard deviation (SD) ($n=3$), * $P < 0.05$, ** $p < 0.01$ vs. 0 μ M of AA

Anacardic acid decreased TNF- α -induced inflammation

ELISA results showed that compared with the Blank group, the expressions of IL-6, IL-1 β , IL-8 and IL-22 in HaCaT cell culture medium were significantly enhanced by TNF- α , but were significantly inhibited following treatment with ($p < 0.05$; Figure 2).

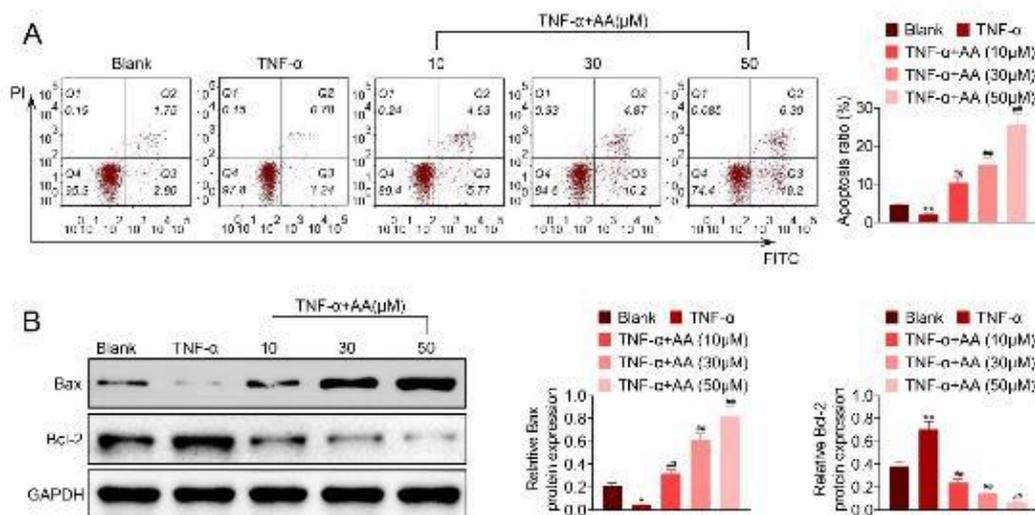


Figure 3: Effects of AA on Apoptosis and Apoptosis-Related Protein Expression in HaCaT Cells. Panel A shows apoptosis ratios, determined by flow cytometry, increase with AA concentration compared to the control. Panel B reveals changes in Bax and Bcl-2 protein levels, assessed by Western blot, with GAPDH as the loading control. Quantitative analysis demonstrates a dose-dependent augmentation in Bax expression and a decrease in Bcl-2 levels upon AA treatment. Data are mean \pm SD (n=3), * p < 0.05, ** p < 0.01 vs. 0 μ M of AA

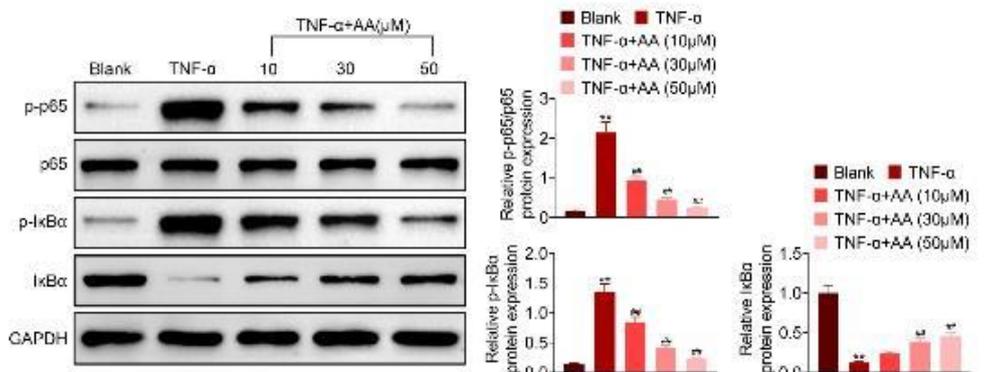


Figure 4: Impact of AA on NF- κ B signaling components in HaCaT cells. Western blot analysis shows the differential expression of p65, phosphorylated p65 (p-p65), phosphorylated I κ B α (p-I κ B α), and I κ B α across treatments. Quantification relative to the GAPDH loading control illustrates the effect of AA on TNF- α -stimulated cells. Bar graphs represent mean \pm SD of protein expression levels, normalized to the blank group. * p < 0.01 compared with the control group; # p < 0.01 compared with TNF- α treatment group

Anacardic acid promotes TNF- α -induced apoptosis

TNF- α treatment enhanced the apoptosis of cells as concentrations of AA increased. (Figure 3 A), but AA reduced the proliferation of cells. AA reduced the protein expression of Bcl-2 but increased the protein levels of Bax in HaCaT cells in a concentration-dependent manner (Figure 3 B).

Anacardic acid inhibited NF- κ B signaling pathway

TNF- α treatment enhanced the protein expression of p-I κ B α , I κ B α , and p-p65; p-p65/p65 ratio was normalized relative to the Blank group.

However, AA significantly decreased the protein level of p-I κ B α and p-p65, as well as p-p65/p65 ratio, but increased the protein level of I κ B α as AA concentration increased (p < 0.05; Figure 4). TNF- α treatment significantly stimulated NF- κ B pathway, whereas AA significantly inhibited the pathway (p < 0.05).

DISCUSSION

Psoriasis is not uncommon. It occurs at all ages worldwide, and the cost of diagnosis and treatment of the disease is tremendous. There are several causative factors of psoriasis, including depression, arthritis, and cardiometabolic syndrome [8]. The underlying

molecular mechanisms of psoriasis have still not been unravelled.

Keratinocytes, which play a key role in psoriasis, respond to cytokines produced by diseased immune cells, which in turn leads to signals crosstalk of the path [9]. Furthermore, most of the current treatments have side effects-Dithranol has a burning sensation[10], while methotrexate causes liver damage, etc [11]. A drug with higher safety and low toxicity is expected to become an effective way to treat psoriasis [12]. It is clear how keratinocytes respond to these cytokines, and the changes in signal pathways are beneficial to clinical treatment [13]. This study aimed to use TNF- α to treat HaCaT immortalized human keratinocytes to establish an *in vitro* model of psoriasis.

AA is a natural product which has shown efficacy against some diseases [14], and has anti-inflammatory, analgesic and antioxidant properties. In the present study, AA reduced HaCaT cell viability and induced apoptosis in a dose-dependent manner. It also significantly reduced the expression of Bcl2 and increased the expression of Bax, but reduced the clone formation rate of HaCaT. A previous *in vitro* work showed that AA inhibits the growth of pancreatic cancer cells and induces apoptosis in a dose-dependent manner, indicating that AA may be an effective adjuvant therapy [15]. In the present study, AA showed its inhibitory effect on the proliferation of keratinocytes in a dose-dependent manner. However, the highest concentration of AA used was 50 μ M. as it was not known whether AA would be toxic to normal cells at higher concentrations [16].

Studies have demonstrated that although TNF- α is primarily recognized as a pro-inflammatory cytokine, it exerts complex effects on keratinocytes, influencing both their survival and apoptosis [17]. TNF- α stimulates cytokine production, including IL-6, IL-1 β , IL-8, and IL-22, a finding corroborated by prior research [18-20]. Post-treatment with AA, there was a notable decrease in TNF- α -induced cell activity, following a dose-response pattern. Additionally, AA was observed to downregulate the activation of p-p65/p65 and reduce p-I κ B α levels, suggesting an inhibitory effect on the NF- κ B signaling pathway, known for its dual role in both oncogenesis and tumor suppression [21]. The data suggest that AA may have potential therapeutic implications for psoriasis by modulating this pathway [22]. However, the immediate impact of AA on psoriasis requires further exploration. The findings underscore AA's potential to suppress TNF- α -stimulated proliferation and inflammatory

cytokine production via the NF- κ B pathway *in vitro*.

Limitations of the study

This study is preliminary being an *in vitro* investigation. Therefore, the role of AA in psoriasis requires further investigation, including *in vivo* studies and clinical trials, to determine whether the effect of AA on psoriasis occurs via NF- κ B signaling pathway. In subsequent studies, pathway inhibitors may be used to establish animal psoriasis models, and determine the toxicity of AA to normal cells.

CONCLUSION

AA hampers both cell growth and the secretion of inflammatory mediators in HaCaT keratinocytes by disrupting NF- κ B pathway. These results may influence the development of AA-based therapies for psoriasis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tao Liu, Yuanmin He and Yongmei Liao designed the study and carried them out; Tao Liu, Yuanmin He and Yongmei Liao supervised the data collection, analyzed and interpreted the data; Tao Liu, Yuanmin He and Yongmei Liao prepared the manuscript for publication and reviewed the draft

of the manuscript. All authors read and approved the manuscript for publication.

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REFERENCES

- Roos AB, Berg T, Nord M. A relationship between epithelial maturation, bronchopulmonary dysplasia, and chronic obstructive pulmonary disease. *Pulm Med* 2012; 2012.
- Albanesi C, Madonna S, Gisondi P, Girolomoni G. The Interplay Between Keratinocytes and Immune Cells in the Pathogenesis of Psoriasis. *Front Immunol* 2018; 9: 1549-1549.
- Cai Y, Fleming C, Yan J. New insights of T cells in the pathogenesis of psoriasis. *Cell Mol Immunol* 2012; 9(4): 302-309.
- Qu R, Chen X, Hu J, Fu Y, Peng J, Li Y, Chen J, Li P, Liu L, Cao J, et al. Ghrelin protects against contact dermatitis and psoriasiform skin inflammation by antagonizing TNF- α /NF- κ B signaling pathways. *Sci Rep* 2019; 9(1): 1348.
- Li SJ, Perez-Chada LM, Merola JF. TNF Inhibitor-Induced Psoriasis: Proposed Algorithm for Treatment and Management. *J Psoriasis Psoriatic Arthritis* 2019; 4(2): 70-80.
- Önal B, Özen D, Demir B, Gezen Ak D, Dursun E, Demir C, Akkan AG, Özyazgan S. The Anti-Inflammatory Effects of Anacardic Acid on a TNF- α - Induced Human Saphenous Vein Endothelial Cell Culture Model. *Curr Pharm Biotechnol* 2020; 21(8): 710-719.
- Sung B, Pandey MK, Ahn KS, Yi T, Chaturvedi MM, Liu M, Aggarwal BB. Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-kappaB-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-kappaBalpha kinase, leading to potentiation of apoptosis. *Blood* 2008; 111(10): 4880-4891.
- Gelfand JM, Dommasch ED, Shin DB, Azfar RS, Kurd SK, Wang X, Troxel AB. The risk of stroke in patients with psoriasis. *J Invest Dermatol* 2009; 129(10): 2411-2418.
- Ippagunta SK, Gangwar R, Finkelstein D, Vogel P, Pelletier S, Gingras S, Redecke V, Häcker H. Keratinocytes contribute intrinsically to psoriasis upon loss of *Trip1* function. *Proc Natl Acad Sci U S A* 2016; 113(41): E6162.
- Raj DRS, Adjei B, Kamil ML, Al Nahhas O. A Rare Case of a Chemical Burn: Dithranol with Salicylic Acid. *World J Plast Surg* 2021; 11(1):138-140 .
- Cheng HS, Rademaker M. Monitoring methotrexate-induced liver fibrosis in patients with psoriasis: utility of transient elastography. *Psoriasis (Auckl)* 2018; 8: 21-29.
- El-Gammal A, Nardo VD, Daaboul F, Tchernev G, Wollina U, Lotti J, Lotti T. Is There a Place for Local Natural Treatment of Psoriasis? *Open Access Maced J Med Sci.* 2018 May 20; 6 (5): 839-842.
- Ben Abdallah H, Johansen C, Iversen L. Key Signaling Pathways in Psoriasis: Recent Insights from Antipsoriatic Therapeutics. *Psoriasis (Auckl)* 2021; 11: 83-97.
- Xiaoying Ma ZS, Qinghua Zhang, Wenbiao Ma. Oxymatrine inhibits proliferation and migration of breast cancer cells by inhibiting miRNA-188 and upregulating its target gene, PTEN. *Trop J Pharm Res* 2021; 20: 11.
- Park M, Upton D, Blackmon M, Dixon V, Craver S, Neal D, Perkins D. Anacardic acid inhibits pancreatic cancer cell growth, and potentiates chemotherapeutic effect by Chmp1A - ATM - p53 signaling pathway. *BMC Complement Altern Med* 2018; 18(1): 71.
- Natural Products as Cytotoxic Agents in Chemotherapy against Cancer. *Cytotoxicity* 2018, 10.
- Yamashita M, Passequé E. TNF- α coordinates hematopoietic stem cell survival and myeloid regeneration. *Cell stem cell* 2019; 25(3): 357-372. e357.
- Gao J, Chen F, Fang H, Mi J, Qi Q, Yang M. Daphnetin inhibits proliferation and inflammatory response in human HaCaT keratinocytes and ameliorates imiquimod-induced psoriasis-like skin lesion in mice. *Biol Res* 2020; 53(1):48.
- Siakavellas SI, Bamias G. Role of the IL-23/IL-17 axis in Crohn's disease. *Discov Med* 2012; 14(77): 253-262.
- Saito N, Honma M, Shibuya T, Iinuma S, Igawa S, Kishibe M, Ishida-Yamamoto A. RIPK1 downregulation in keratinocyte enhances TRAIL signaling in psoriasis. *J Dermatol Sci* 2018; 91(1): 79-86.
- Kim C, Pasparakis M. Epidermal p65/NF- κ B signalling is essential for skin carcinogenesis. *EMBO Mol Med* 2014; 6(7): 970-983.
- Yan S, Xu Z, Lou F, Zhang L, Ke F, Bai J, Liu Z, Liu J, Wang H, Zhu H, et al. NF- κ B-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis. *Nat Commun* 2015; 6: 7652.