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Original Research Article

Artemisia anomala extracts enhance the viability and anti-oxidation capacity of human keratinocytes

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Abstract

Purpose: *To investigate the effect of extracts of Artemisia anomala S. Moore tissues on viability, apoptosis and antioxidant capacity of human keratinocytes.*
Methods: *Human keratinocyte cell line HaCaT were treated with extracts of A. anomala for 12 h or 24 h. Cell viability, level of reactive oxygen species (ROS), and incidence of apoptosis were measured by flow cytometry. Levels of mRNA and key proteins in the mitogen-activated protein kinase (MAPK) pathway were determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Key proteins of caspase pathways were assessed by western blot. The influence of the extract on the MAPK pathway was further probed by treating cells with MAPK activator in the presence and absence of the extract.*
Results: *Treatment of cells with extracts of A. anomala enhanced viability and reduced apoptosis in a time-dependent manner, and increased ROS level, compared with control. mRNA and protein expressions of c-Jun N-terminal kinase (JNK), extracellular regulated protein kinase (ERK), and p38 MAPK decreased in extract-treated cells. The extracts also reversed the inhibitory effects of the MAPK pathway activator, actinomycin, on cell viability and ROS, and inhibited protein-cleaved caspase-8 and cleaved caspase-3.*
Conclusion: *A. anomala extract increases cell viability and antioxidant capacity via inactivation of MAPK pathway, and also inhibits cell apoptosis via inactivation of caspase pathways. Hence, the extract may serve as a promising drug for the treatment of psoriasis.*

Keywords: *Artemisia anomala, MAPK pathway, Anti-oxidation, Keratinocyte, Psoriasis*

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INTRODUCTION

Artemisia anomala S. Moore is a perennial herb in the family Compositae [1]. In Chinese medicine, it has traditionally been used as an analgesic, antibiotic, and wound-curing agent. Chemical analysis has revealed that their tissues are rich in phenolic compounds and others also are biologically active [2]. Recent studies confirm that extracts of *A. anomala* have antibacterial and antioxidant properties. These extracts are promising drugs available for bacterial or inflammatory diseases treatment [1] and may serve as a novel therapeutic strategy.

Psoriasis is a chronic inflammatory dermatopathy that affects 1 – 3 % of individuals worldwide [3-8]. Typically, psoriasis patients have well-defined scaly, erythematous patches on the skin, which can seriously reduce the quality of life of afflicted patients [9,10]. The key physiological mechanism of psoriasis is the activation of the innate and adaptive immune systems, causing rapid division and differentiation of epidermal keratinocytes, generating hyperplasia finally [11]. Given the potential therapeutic role of extracts of *A. anomala* in inflammation, its effect on psoriasis is of interest. Xiong et al. showed that the viability of human keratinocytes was enhanced during the treatment of psoriasis [12]. Thus, the keratinocyte viability may be a key index of psoriasis. However, the effect of *A. anomala* extract on keratinocyte viability remains unknown.

The present study was undertaken to determine the effect of *A. anomala* extracts on cell viability and apoptosis of keratinocytes, as well as the antioxidant properties of keratinocytes treated with these extracts. In this study, *A. anomala* extracts may have potential as therapy for psoriasis. They may modulate the mitogen-activated protein kinase (MAPK) and caspase signaling pathways.

EXPERIMENTAL

Cell culture

The keratinocyte cell line HaCaT (American Type Culture Collection, Manassas, VA, USA) was used. It was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, UK) supplemented with 1.4 mM L-glutamine, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), and 10 % fetal bovine serum (FBS; Gibco, Invitrogen, Paisley, UK). Cells were cultured in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere for 2 days until they reached 70 - 80 % confluency, and then were photographed with a phase contrast microscopy (Olympus Optical Co., Tokyo, Japan).

Plant material and *A. anomala* extracts

A. anomala material was acquired from Ruixing Drugstore (Hangzhou, China) as the Chinese herbal medicine. A total of 400 kg dried aerial parts were chopped, suspended in acetone: (15:1), and sonicated with ultrasound for 30 min to yield the extracts.

The flavonoid content of the extracts was assessed with $NaNO_2$-$Al(NO_3)_3$ colorimetry, using an ultraviolet spectrophotometer [13]. The flavonoid content of a water extract was calculated, and was normalized to the level of the plant flavonoid pigment, rutin. The flavonoid content of A. anomala extracts (3.1 µg/mL) was higher than the other plant tissues.

A. anomala extract treatment

The effect of the extract on the HaCaT keratinocytes in media (DMEM) was evaluated. For this experiment, HaCaT cells were incubated in *A. anomala* extract for 12 h or24 h, respectively. The control group cells were incubated in DMEM without the extract.

The effect of *A. anomala* extract on MAPK signaling pathway was also examined. Cells received the MAPK signaling pathway activator anisomycin at 10 µM.

Cell viability assay

The viability of HaCaT cells treated with different extracts was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [14]. HaCaT cells (5×10^4 cells/mL) were seeded into 96-well culture plates for 4 days, and 200 µL MTT (Sigma, USA) was added to each well for 4 h at 37 °C. The blue formazan crystals were dissolved by adding 0.5 mL dimethylsulfoxide (DMSO; Lonza, Allendale, NJ, USA) for 1 h. The percentage of living HaCaT cells was determined daily for 4 days at 570 nm with a SpectraMax 190 automated plate reader (Thermo Scientific, West Palm Beach, FL, USA).

Reactive oxygen species (ROS) assay

The ROS content in HaCaT cells treated with different extracts was quantified by an OxiSelect ROS assay kit (Cell Biolabs Inc, San Diego, CA, USA). HaCaT cells (1×10^5 cells/well) were inoculated in 6-well culture plates, washed three times with cold phosphate buffered saline (PBS), and co-incubated with 100 µL of 2, 7-dichlorofluorescein diacetate (DCFH-DA; Shanghai, China) for 45 min. Then, samples were collected by a trypsin digestion approach, centrifuged, and re-suspended in PBS. ROS level was analyzed on a flow cytometer (Cytomics FC 500; Beckman Coulter, USA).

Apoptosis assay

Apoptosis was quantified with an Annexin V-FITC/PI apoptosis detection kit (Invitrogen, USA). Keratinocytes treated with different extracts were placed in 6-well culture plates, fixed with 2% paraformaldehyde, washed with cold PBS, and

re-suspended in 100 µL annexin-binding buffer. At this point, cell counting was carried out. Apoptotic HaCaT cells were assessed by flow cytometry, based on the manufacturer's protocol.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

When the cultured HaCaT keratinocytes had reached 70-80 % confluency, total RNA in the cells that had received different extract treatments was extracted with TRIzol reagent (Life Technologies, USA), according to the manufacturer protocol. Reverse transcription was performed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) using 2 µg of RNA. qRT-PCR for c-Jun N-terminal kinase (JNK), extracellular regulated protein kinase (ERK), and MAPK was carried out *via* FastStart Universal SYBR Green Master (Roche Diagnostics, Tokyo, Japan). The amplification parameters were: 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C.

Western blot

Standard western blotting was carried out for the protein expression assay from HaCaT cells treated with different extracts. In brief, cells were washed two or three times with cold PBS, and the proteins were isolated with 10-times radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Biomiga Inc., San Diego, CA, USA). Then the proteins were evaluated with the bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, USA). Primary antibodies to c-Jun N-terminal kinase (ab124956; 1:1,000), ERK (ab196883; 1:1,000), MAPK (ab197348; 1:1,000), caspase-9 (ab32539), caspase-8 (ab25901), caspase-3 (ab2171), Bcl-2 (ab32124), B-cell lymphoma-2 associated X (Bax; ab32503), and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Abcam (Cambridge, United Kingdom). Thereafter, secondary antibodies (1:1,000) were marked by horseradish peroxidase for 2 h at 37 °C. Samples were dissolved on a polyvinylidene fluoride (PVDF) microporous membrane. Finally, membranes were scanned by a fluorescence imager (Odyssey CLX, LI-COR Biosciences-Biotechnology, Lincoln, NE, USA) to detect bands.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical differences were evaluated by SPSS 21.0 software (SPSS Inc, USA). Comparison between different groups were performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of *A. anomala* extracts on cell viability

The effect of *A. anomala* extracts on HaCaT cell growth were assessed 12 or 24 h after treatment by MTT assay. The results were presented in Figure 1, which showed that cell viability was increased in the presence of the extract compared with control. *A. anomala* extracts treatment for four days increased cell viability by about 3-fold ($p < 0.05$). The difference increased in a time-dependent manner ($P < 0.01$). Cells treated with extract for 12 h were slightly less viable than cells treated with the extract for 24 h, but this was not significant ($p > 0.05$). Thus, extracts of *A. anomala* promoted viability of HaCaT cells.

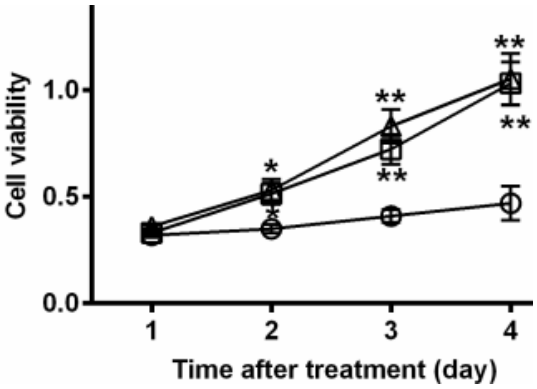

Figure 1: Effect of extract of *A. anomala* (Aa) on viability of HaCaT cells. ○:Control;□:Aa 12 h; △:Aa 24 h; *, $p < 0.05$; **, $p < 0.01$, both compared with control

Effect of extracts of *A. anomala* on the ROS content of HaCaT cells

The effect of extracts of *A. anomala* on the ROS content of HaCaT cells was also investigated by flow cytometry. The ROS were measured to assess the antioxidant capacity of the cells. As shown in Figure 2, treatment with the *A. anomala* extracts for either 12 or 24 h resulted in cells that scavenged approximately twice the number of ROS as the control. Cells were capable of scavenging and the difference was significant compared with control ($P < 0.01$). The level of ROS was similar in HaCaT cells treated with *A. anomala* for 12 h compared with those treated with *A. anomala* for 24 h (32.15 vs. 28.73, respectively; $P > 0.05$). Treatment with the extract effectively reduced ROS content in HaCaT cells, and increased their antioxidant capacity, compare with control.

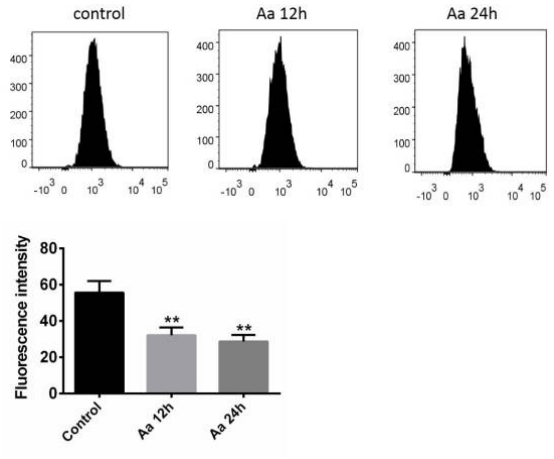

Figure 2: Effect of extracts of *A. anomala* extracts on reactive oxygen species (ROS) content of HaCaT cells. Cells were treated with the extract for 12 or 24 h (*p < 0.05; **p < 0.01, both vs. control)

Apoptosis in HaCaT cells treated with *A. anomala* extracts

Apoptosis in HaCaT cells treated with *A. anomala* extracts were also measured. Figure 3 showed the percentage of apoptotic cells were decreased by about half in extract-treated cells compared with the control (p < 0.05). There was no significant difference between the cells treated for 12 or 24 h: values were 22.71 and 16.92, respectively (p > 0.05). These findings indicated that *A. anomala* extracts inhibited apoptosis in HaCaT cells.

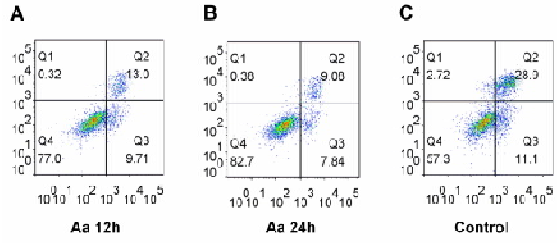

Figure 3: Effect of extracts of *A. anomala* (Aa) on apoptosis of HaCaT cells. Cells were treated with extract for 12 or 24 h

Mechanism of action of *A. anomala* extracts on HaCaT cells

To further understand the mechanism by which the extract of *A. anomala* act on HaCaT cells, the key proteins in the MAPK signaling pathway, specifically JNK, ERK, and MAPK were measured in untreated cells or cells treated with extract. First, the mRNA for these proteins with qRT-PCR was quantified. The results were shown in Figure 4 A. The mRNA for ERK, JNK, and p38 MAPK were decreased of 49, 49 and 78 %, respectively, after 24 h of treatment with extract compared with control (p < 0.01).

For western blot assay, treatment of the keratinocytes with *A. anomala* extracts for 12 and 24 h decreased expression of pro-inflammatory cytokines (Figure 4 B). There was no detectable difference between cells treated with extract for 12 or 24 h in either mRNA or protein expression of JNK, ERK, and MAPK. Therefore, the addition of extracts of *A. anomala* suppressed the MAPK signaling pathway in HaCaT cells.

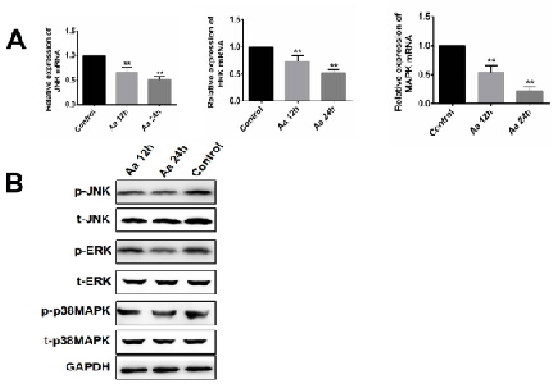

Figure 4: Effect of extracts of *A. anomala*(Aa) on mRNA expression and protein expression in mitogen-activated protein kinase (MAPK) pathway in HaCaT cells. Cells were treated with the extract for 12 or 24 h**. A**. Relative mRNA expression of JNK, ERK, and p38MAPK from qRT-PCR. **B.** Results of western blot analysis of proteins in the MAPK signaling pathway. Note that inclusion of the extract in the media decreased the protein expression of p-JNK, p-ERK, and p-p38MAPK. (JNK, c-Jun N-terminal kinase; ERK, extracellular regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *p < 0.05; **p < 0.01, both vs. control)

Cell viability and ROS content of HaCaT cells

Cell viability was reduced relative to the control when the cells were treated with anisomycin alone for 48 h (Figure 5A; p < 0.05 on Day 2, p < 0.01 Days 3 and 4). Specifically, it was decreased 17.22 % on Day 2, 35.01 % on Day 3, and 63.18 % on Day 4. When cells were treated with anisomycin and the extract, viability was reduced 31.17% on Day 2, but was greater than Day 4, which was still lower than only *A. anomala* extracts treatment (31.82 %, p < 0.01).

The ROS content of cells is shown in Figure 5B. The ROS content increased in response to 48 h of anisomycin treatment relative to the control (P < 0.05 on Day 2 and P < 0.01 on Days 3 and 4). It increased 15.04% over the control on Day 2, 27.31 % on Day 3, and 52.25 % on Day 4. When cells were treated with both anisomycin and the extract, the ROS content was enhanced 39.9 %

over the control on Day 2, but decreased by Day 4 ($P < 0.01$). So, treatment with the extract neutralized the effects of MAPK activator, which is consistent with the proposal that the extract acted *via* the MAPK signaling pathway in the HaCaT cells.

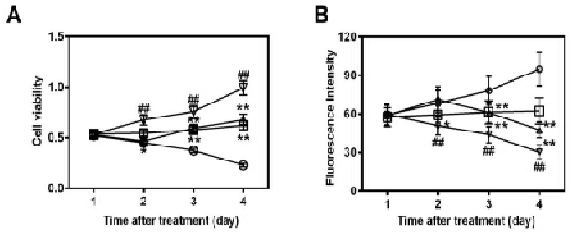

Figure 5: Viability and reactive oxygen species (ROS) content of HaCaT cells treated with both *A. anomala* (Aa) extracts and the MAPK signaling pathway activator anisomycin. The effect of the extract was to inhibit the MAPK pathway. **A.** Cell viability. **B.** ROS content. (○: anisomycin 48h; □: Control; △: anisomycin 48h + Aa 24h; ▽: Aa 24h; *, compared with 48 h anisomycin treatment, **$p < 0.05$, vs anisomycin 48h; #$p < 0.01$, vs anisomycin 48 h + Aa 24 h, ##$p < 0.05$, compared with 48 h anisomycin treatment + 24 h Aa treatment, $p < 0.01$

A. anomala extract inhibited cell apoptosis in HaCaT cells

Previous findings indicate that the *A. anomala* extract inhibited cell apoptosis in HaCaT cells. To explore the mechanism and evaluate the effect, the extract on key proteins of apoptosis pathways was measured by western blot. The results are shown in Figure 6. Treatment with the extract for 12 h and 24 h slightly suppressed expression of cleaved caspase-9 protein and markedly inhibited expression of cleaved caspase-8 and cleaved caspase-3 proteins, compared with the control. The ratio of Bcl-2/Bax was enhanced by *A. anomala* extracts. The extract inactivated the caspase pathways in HaCaT cells.

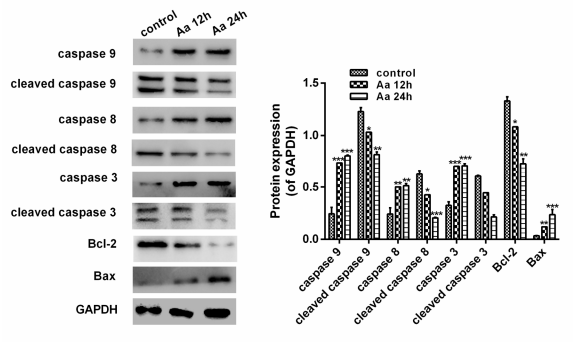

Figure 6: Effect of *A. anomala* (Aa) extract on proteins in the caspase pathway. HaCaT cells for 12 or 24 h

DISCUSSION

In this study, the effect *A. anomala* extracts on HaCaT cell growth was investigated. Treatment of cells with extracts increased their viability and the production of ROS, consistent with enhancement of the antioxidant capability of the cells by the extract. On the other hand, *A. anomala* extracts inhibited the apoptosis of HaCaT cells. In experiments of qRT-PCR and western blot assay, treatment with extracts from *A. anomala* suppressed the MAPK signaling pathway and caspase pathway in HaCaT cells was carried out. The present study indicated that the extracts exerted their effects on cell growth and anti-oxidation capacity by modulating the MAPK and caspase pathways. It was tested with respect to the MAPK pathway with experiments in which the MAPK pathway activator, anisomycin. Therefore, the ability of the extract overcame the effects of MAPK pathway activation by anisomycin, which the extract acted on these cells through the MAPK pathway.

This study provided a foundation for understanding the role of the extract in growth of keratinocytes, as the extract has potential for clinical application in psoriasis therapy. In the past two decades, dramatic advances have been achieved in the discovery and application of plant extracts as sources of natural medicinal products. Many of them have anti-oxidative activity, which has formed the foundation for a variety of applications in pharmaceuticals, alternative medicine, and naturopathy [15,16]. It has been noted that methanol extracts of *A. anomala* possessed potent antioxidant and antibacterial features, and might serve as a natural herbal source used in the food and pharmaceutical industries [17].

The properties of keratinocytes, specifically their growth and antioxidant capability, are important in psoriasis. Moreover, keratinocytes can affect the pathogenesis of psoriasis by interplaying with various innate immune cells, such as dendritic cells and macrophages [18]. Xiong *et al* noted that the viability of keratinocytes could guide the therapy of psoriasis [12]. The level of ROS was enhanced in lesional and non-lesional skin of psoriasis patients, and the oxidant-antioxidant imbalance was of great significance in psoriasis [18]. In fact, the ROS content of keratinocytes has been used as a marker of oxidative damage in the tissue. As result, extracts of *A. anomala* act on two critical factors in psoriasis.

Proteins in the MAPK signaling pathway, specifically JNK, ERK, ERK5, and p38 MAPK, are involved in a host of critical cellular

processes and activate mitochondrial pathways [19,20]. It is clear that the ERK signaling pathway regulates cell proliferation, apoptosis, and differentiation [21-23]. The current study revealed that the phosphorylation of JNK, ERK, and p38MAPK in HaCaT cells were inhibited by treatment with extracts of *A. anomala.* The results suggest that these extracts aberrantly inactivated the MAPK signaling pathway.

The effect of the extract on the MAPK pathway by examining the effect of an activator of the MAPK pathway, anisomycin, and the extract in tandem was further assessed. If the extract inhibits the MAPK pathway, it should reverse the effects of the activator on cell viability and the accumulation of ROS in the keratinocytes. The experiment was consistent with this prediction, and *A. anomala* extracts regulating the MAPK signaling pathway in keratinocytes.

To further investigate the mechanism of the extract's inhibitory effect on apoptosis, signaling cascades involving the caspase pathway, specifically caspase-3, caspase-8, caspase-9, Bax, and Bcl-2 were measured [11]. It has been proposed that the activating caspases, caspase-3, -8, and -9, serve as biochemical hallmarks of apoptosis [24]. The extract inactivated the expression of caspase-3 and 8, markedly at 24 h after treatment. *A. anomala* extracts might affect apoptosis of HaCaT cells by inhibiting the initiation as well as execution of caspase pathways.

CONCLUSION

A. anomala extract enhances cell viability and the production of ROS in human keratinocytes via dampening the activation of MAPK signalings. On the other hand, the extract also reduces the apoptosis of keratinocytes by suppressing caspase-dependent signaling pathway. These findings suggest that *A. anomala* extracts may serve as a potentially effective drug for the treatment of psoriasis.

DECLARATIONS

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. Ying Gao and Jing Yuan designed all the experiments and revised the paper. Xiaofang Liang, Yimin He and Peng Li performed the experiments, and Ming Yang wrote the paper.

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