Anti-rheumatic effects of *Aconitum leucostomum* Worosch. on human fibroblast-like synoviocyte rheumatoid arthritis cells

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**Abstract.** The aim of the present study was to investigate the effects of *Aconitum leucostomum* Worosch. crude drug, processed products and monomer components on human fibroblast-like synoviocyte rheumatoid arthritis (HFLS-RA) cells, and its associated mechanisms. Following drug treatment, cell proliferation was assessed using a Cell Counting Kit-8 assay. Cellular apoptosis and cell cycle were evaluated using flow cytometry. Levels of hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF) and toll-like receptor 4 (TLR4) mRNA and protein were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. Levels of pro-inflammatory cytokines were evaluated using ELISA. Analysis of cell proliferation indicated that crude drug and processed products markedly inhibited the cell proliferation. Compared with the control group, the apoptosis rates were significantly elevated in all treatment groups (all P<0.05). Furthermore, the proportion of cells in G0/G1 phase was significantly decreased in all treatment groups compared with the control group (all P<0.05). RT-qPCR and western blotting indicated that, compared with the control group, mRNA and protein expression levels of HIF-1α, and TLR4 were significantly downregulated in all treatment groups (P<0.05). The mRNA and protein expression levels of VEGF in all treatment groups were decreased compared with those in the control group, but the difference was not significant. Results from ELISA demonstrated that the levels of interleukin (IL)-6, IL-1β and tumor necrosis factor-α in the cell culture supernatant were all significantly decreased following drug treatment in HFLS-RA cells (all P<0.05). Therefore, *A. leucostomum* Worosch. crude drug, processed products and monomer components may exert anti-rheumatic effects on HFLS-RA cells, inhibiting cell proliferation and enhancing cellular apoptosis. These effects may be attributable to the downregulated expression of HIF-1α and TLR4, as well as decreased levels of pro-inflammatory cytokines.

**Introduction**

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by synovitis and pannus formation, which causes severe pain and seriously affects patient quality of life (1). Synovial tissue is principally composed of fibroblast-like synovial cells, which are closely associated with the pathogenesis and development of RA, particularly joint damage. Fibroblast-like synovial cells may secrete pro-inflammatory cytokines, chemokines and matrix protein-degrading enzymes (2,3). These factors may cause an imbalance in the proliferation and apoptosis of synovial cells, and induce abnormalities in signal transduction in synovial tissue (4), resulting in the inflammation and destruction of joints.

It has been reported that RA-related joints are associated with a hypoxic microenvironment (5), which regulates angiogenesis, induces inflammatory cell infiltration and elevates pro-inflammatory factor production (6,7). Hypoxia-inducible factor-1α (HIF-1α) is an important regulatory factor for the hypoxic response. Under hypoxic conditions, HIF-1α may activate and upregulate the expression of hypoxic adaptation-related genes, which are involved in energy metabolism, intracellular signal transduction and angiogenesis processes in RA fibroblasts (8). Furthermore, vascular endothelial growth factor (VEGF) is a potent angiogenesis-stimulating factor, which serves key roles in angiogenesis and pathogenesis of RA (9). Toll-like receptors (TLRs) are regulators of adaptive immune responses (10). Activated TLRs may induce the antimicrobial defense system to produce interleukin (IL)-6, IL-1β, tumor necrosis factor-α (TNF-α) and other chemokines, thought to be involved in the pathogenesis of RA (11). TNF-α is an inflammatory mediator with multiple biological roles. It is primarily produced by mononuclear macrophages and serves a critical function in the pathogenesis and development of RA. Furthermore, it may stimulate the proliferation of synovial fibroblasts, as well as the secretion of IL-6, granulocyte-macrophage colony stimulating factor, chemokines,
matrix metalloproteinases and prostaglandin (12). At present, there are a number of commercially available TNF-α antagonists that may be used to treat RA (13,14); however, long-term usage of TNF-α antagonists may induce drug resistance and cause infection (15).

*Aconitum leucostomum* Worosch. is a perennial herb belonging to the family Ranunculaceae and is primarily found in Gansu, Xinjiang and northeastern areas of China (16). The root of *A. leucostomum* Worosch. is commonly used in Kazak medicine for the treatment of indigestion and pain (17,18). In particular, *A. leucostomum* Worosch. root has been reported to be an effective treatment of rheumatic diseases (19,20). A previous study by the current authors demonstrated that *A. leucostomum* Worosch. could alleviate the inflammatory response in the joints of rats with adjuvant arthritis (21). However, the detailed mechanism of this effect has not yet been fully elucidated. In the current study, the effects of *A. leucostomum* Worosch. crude drug, processed products and monomer components on *in vitro* human rheumatoid fibroblast-like synoviocyte RA (HFLS-RA) cells were investigated. The levels of HIF-1α, VEGF and TLR4, as well as the related pro-inflammatory cytokines, were analyzed and discussed.

Materials and methods

Cell line and cell culture. Human fibroblast-like synoviocyte rheumatoid arthritis (HFLS-RA) cells were purchased from the European Collection of Authenticated Cell Cultures (Porton Down, UK). These cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an incubator containing 25% CO₂.

Drug preparation and administration. *A. leucostomum* Worosch. (~30 kg) was obtained from Nilka County (Yili, Xinjiang, China) and identified by pharmacist Professor Yonghe Li at the Traditional Chinese Medicine Hospital Affiliated to Xinjiang Medical University (Urumqi, China). Monomer components were isolated and purified according to our previously published procedures, and identified to be delvestidine (WF-1) and anthranoyll cystocorine (WF-4), according to spectral analysis (22). Three kinds of processed products were prepared using water-boiling, high-pressure steaming and excipient co-boiling methods, respectively (23).

Decoction solution of the crude drug and processed products was prepared as previously reported (24). Briefly, 200 g crude drug or processed product was immersed in water (w/v=1/8) for 1 h, then boiled for 30 min. Following filtration with 4 layers of bandage, the solution was collected and subjected to another decoction. Three decoctions were filtrated with 4 layers of bandage, the solution was collected and subjected to another decoction. Three decoctions were obtained using a FastQuant RT kit with gDNAse (cat. no. KR106-02; Tiangen Biotech Co., Ltd., Beijing, China). Quantitative PCR was performed with the SYBR Select Master mix (cat. no. 4472920; Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 7500 qPCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: HIF-1α forward, 5'-TTTGCGCAAACGAACAGACA-3' and reverse, 5'-TTTTCACGGTGTTTG-3'; VEGF forward, 5'-GCCCTCAGAAGCACCATTGAA-3' and reverse, 5'-CTCATGAACTTCACTTTTCG-3'; TLR4 forward, 5'-GCCCTCAGAAGCACCATTGAA-3' and reverse, 5'-TTTGCGCAAACGAACAGACA-3'; GAPDH forward, 5'-GACGAACTTCACTTTTCG-3' and reverse, 5'-GCCCTCAGAAGCACCATTGAA-3'. Amplification conditions were as follows: Denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by extension at 50°C for 2 min. The relative expression levels of target genes were calculated using the 2^ΔΔCq method (26).
Western blot analysis. The expression of HIF-1α, VEGF and TLR4 proteins were evaluated using western blot analysis. Cells were collected and lysed with radioimmunoprecipitation lysis buffer (Tiangen Biotech Co., Ltd., Beijing, China). After vortexing for 20 sec, the lysis was incubated at 4°C for 30 min, followed by centrifugation at 20,000 x g at 4°C for 15 min, and then the supernatant was harvested. Protein concentrations were determined using the BCA protein assay kit (Tiangen Biotech Co., Ltd.). Protein samples were subjected to 10% SDS-PAGE (15 µg in each lane), then electrophoretically transferred onto a polyvinylidene difluoride membrane. Following blocking with 5% bovine serum albumin (Tiangen Biotech Co., Ltd.) at room temperature for 1 h, the membrane was incubated with mouse anti-human anti-HIF-1α primary antibody (1:400 dilution; cat. no. ab463; Abcam, Cambridge, MA, USA), mouse anti-human anti-VEGF primary antibody (1:500 dilution; cat. no. ab1316; Abcam, Cambridge, MA, USA), mouse anti-human anti-TLR4 primary antibody (1:250 dilution; cat. no. ab13867; Abcam) or rabbit anti-human anti-β-actin primary antibody (1:500 dilution; cat. no. BA2305; BOSTER, Wuhan, Hubei, China), at 4°C overnight. The membrane was then incubated with goat anti-rabbit immunoglobulin G (Pierce; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Following treatment with 1 ml SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Inc., Inc.), protein bands were detected and analyzed with the ChemiScope mini chemiluminescent analyzer (Chemiscope 3000; CLINX, Shanghai, China). β-actin was used as an internal reference.

Enzyme-linked immunosorbent assay (ELISA). Levels of pro-inflammatory cytokines IL-6, IL-1β and TNF-α were evaluated using ELISA kits (EH004-96, EH001-96 and EH009-96, respectively; Shanghai ExCell Biology, Inc., Shanghai, China), according to the manufacturer's instructions.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Student's t-test was performed for group comparisons. P<0.05 was considered to indicate a statistically significant result.

### Results

**Effect of A. leucostomum Worosch. on the proliferation of HFLS-RA cells.** HFLS-RA cells were treated with the crude drug, processed products (water-boiling, high-pressure steaming and excipient co-boiling processed products) and monomer components (WF-1 and WF-4), for 24 h, then cell proliferation was assessed with a CCK-8 assay. The IC$_{50}$ values for these treatments are presented in Table I. Considering that the amount of the extracted monomer was limited and high intervention concentrations (e.g., IC$_{50}$) may be toxic, the concentrations of 1/2 IC$_{50}$ were used. Subsequently, the cells were treated with the crude drug, processed products and monomer components at the concentrations of 1/2 IC$_{50}$ for 24, 48 and 72 h. According to the results in Table I, the optimal inhibition effect was observed at 48 h. Thus, in the following experiments, the drug treatment concentrations were set as 1/2 IC$_{50}$ values, and the treatment duration was set as 48 h.

Analysis of cell proliferation inhibition rates indicated that, crude drug and processed products notably inhibited the cell proliferation, and the highest inhibition rate was observed in the high-pressure steaming processed product group (Table I). These results suggested that the crude drug, processed products and monomer components of A. leucostomum Worosch. may inhibit the proliferation of HFLS-RA cells, with more potent inhibition effects observed in the crude drug and processed products.

**Effect of A. leucostomum Worosch. on the apoptosis rate of HFLS-RA cells.** The effects of A. leucostomum Worosch. crude drug, processed products and monomer components on the apoptosis of HFLS-RA cells were evaluated using flow cytometry. The results indicated that, compared with the control group, apoptosis rates were significantly elevated in the positive control and all treatment groups (all P<0.05; Fig. 1). Among the treatment groups, the highest apoptosis rate was observed for the water-boiling processed product group, which was significantly higher than the other treatment groups (all P<0.05) and comparable to that of the positive control group. These results suggest that the crude drug, processed products

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/ml)</th>
<th>Inhibition rate (%)</th>
<th>IC$_{50}$ (mg/ml)</th>
<th>1/2 IC$_{50}$ (mg/ml)</th>
<th>Inhibition rate at 1/2 IC$_{50}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leflunomide (positive control)</td>
<td>0.4</td>
<td>52.11</td>
<td>0.33</td>
<td>0.15</td>
<td>23.99</td>
</tr>
<tr>
<td>Crude drug</td>
<td>6.0</td>
<td>51.74</td>
<td>5.32</td>
<td>2.00</td>
<td>17.01</td>
</tr>
<tr>
<td>Water-boiling processed product</td>
<td>3.0</td>
<td>51.83</td>
<td>2.50</td>
<td>1.50</td>
<td>22.42</td>
</tr>
<tr>
<td>High-pressure steaming processed product</td>
<td>3.0</td>
<td>64.82</td>
<td>2.52</td>
<td>1.80</td>
<td>21.50</td>
</tr>
<tr>
<td>Excipient co-boiling processed product</td>
<td>2.5</td>
<td>62.30</td>
<td>1.85</td>
<td>1.20</td>
<td>21.67</td>
</tr>
<tr>
<td>WF-1 monomer component</td>
<td>0.2</td>
<td>52.88</td>
<td>0.12</td>
<td>0.04</td>
<td>18.23</td>
</tr>
<tr>
<td>WF-4 monomer component</td>
<td>0.2</td>
<td>62.55</td>
<td>0.17</td>
<td>0.10</td>
<td>19.80</td>
</tr>
</tbody>
</table>

WF-1, delvestidine; WF-4, anthranoylycottonine.
and monomer components of *A. leucostomum* Worosch. may significantly promote the apoptosis of HFLS-RA cells, and the most potent effect was observed for the water-boiling processed product.

**Effect of *A. leucostomum* worosch. on HFLS-RA cell cycle.** The effects of *A. leucostomum* Worosch. crude drug, processed products and monomer components on the HFLS-RA cell cycle were also evaluated using flow cytometry. These results demonstrated that, compared with the control group, the proportion of cells in the G0/G1 phase was significantly decreased in the positive control group and all treatment groups (all P<0.05; Table II). Among the treatment groups, the lowest G0/G1 phase percentage was observed in the high-pressure steaming processed product group, which was significantly lower than the other treatment groups (P<0.05; Table II). These results suggest that the crude drug, processed products and monomer components of *A. leucostomum* Worosch. may induce cell cycle arrest in HFLS-RA cells; the most potent effect was observed for the high-pressure steaming processed product.

**Effect of *A. leucostomum* Worosch. on HIF-1α, VEGF and TLR4 expression in HFLS-RA cells.** To investigate the
The effects of *A. leucostomum* Worosch. crude drug, processed products and monomer components on the expression of HIF-1α, VEGF and TLR4 in HFLS-RA cells. mRNA and protein levels were measured using RT-qPCR and western blot analysis, respectively. Results from RT-qPCR indicated that, compared with the control group, levels of HIF-1α and TLR4 mRNA were significantly downregulated in the positive control group and all treatment groups (all P<0.05; Fig. 2A and C). Compared with the control group, only slight changes in VEGF mRNA levels were observed in the water boiling and excipient co-boiling processed product groups, as well as in the monomer component (WF-1 and WF-4) groups (P>0.05; Fig. 2B). Results from western blot analysis indicated that, compared with the control group, there were decreases in the expression of HIF-1α, VEGF and TLR4 proteins in the crude drug, processed product and monomer component groups (Fig. 3). These results suggest that the crude drug, processed products and monomer components of *A. leucostomum* Worosch. may downregulate the expression of HIF-1α, VEGF and TLR4 in HFLS-RA cells.

### Effect of *A. leucostomum* Worosch. on pro-inflammatory cytokine levels in cell culture supernatant.

The effects of *A. leucostomum* Worosch. crude drug, processed products and monomer components on the pro-inflammatory cytokine levels in the cell culture supernatant of HFLS-RA cells were detected with ELISA. The results indicated that, compared with the control group, levels of IL-6, IL-1β and TNF-α in the cell culture supernatant were all significantly decreased in the treatment groups (all P<0.05; Fig. 4). These results suggest that the crude drug, processed products and monomer components of *A. leucostomum* Worosch. may decrease pro-inflammatory cytokine levels in HFLS-RA cells.

### Discussion

RA is pathologically characterized by synovitis, in which the pathologically altered synovial membrane enhances inflammatory cell infiltration and increased pro-inflammatory cytokine release causes hypoxia in the joint microenvironment and leads to angiogenesis and pannus formation (27). Pannus has been associated with erosion, which may eventually result in joint cartilage and bone destruction (28). Pannus formation serves a key role in the occurrence and development of RA, and HIF-1α is a key regulator of the hypoxia response in the body. Brouwer et al (29) demonstrated that HIF-1α expression is upregulated in the synovial tissue in patients with RA and that the number of HIF-1α+ cells in the RA synovial tissue is positively correlated with the blood vessel number and inflammation cell infiltration. Another study indicated that synovial hyperblastosis and vascular density was increased in RA patients (30). Furthermore, it has been reported that the serum level of VEGF is increased in patients with early-stage RA and is even higher than in patients with advanced-stage or stable RA (7). In addition, it has been determined that the serum level of VEGF is increased in patients with RA (31).

TLRs are involved in the regulation of adaptive immune responses in the body, and they serve key roles in the pathogenesis and development of various autoimmune diseases, particularly RA (32). Most activated TLRs can induce the antimicrobial defense system to produce IL-6, IL-1β and TNF-α. Therefore, the innate immune response may be involved in the pathogenesis of RA.

In the present study, HFLS-RA cells were treated with *A. leucostomum* Worosch. crude drug, processed products and monomer components. Cell proliferation was assessed using a CCK-8 assay, and apoptosis and the cell cycle were evaluated with flow cytometry. The results demonstrated that drug treatments could markedly inhibit the proliferation of HFLS-RA cells. Furthermore, the drug treatments significantly elevated the apoptosis rates of these cells, and decreased the proportion of cells in G0/G1 phase. These results suggested that *A. leucostomum* Worosch. may induce apoptosis and inhibit proliferation of HFLS-RA cells. The expression of HIF-1α, VEGF and TLR4 mRNA and protein in the HFLS-RA subjected to drug treatments were evaluated using RT-qPCR and western blot analysis, and levels of IL-6, IL-1β and TNF-α in the cell culture supernatant were detected with ELISA. A previous study demonstrated that hypoxic conditions are closely associated with upregulated levels of HIF-1α and VEGF (33). However, the results of the present study demonstrated that, compared with the control group, the drug treatments significantly decreased the mRNA and protein levels of HIF-1α, VEGF and TLR4.

### Table II. Effects of *Aconitum leucostomum* Worosch. on the human fibroblast-like synoviocyte rheumatoid arthritis cell cycle.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1 phase (%)</th>
<th>S-phase (%)</th>
<th>G2/M phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>72.03±1.10</td>
<td>14.50±0.92</td>
<td>13.47±0.46</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>56.83±0.67a</td>
<td>24.73±1.25a</td>
<td>18.43±1.76a</td>
</tr>
<tr>
<td>Crude drug</td>
<td>55.17±3.98a</td>
<td>27.77±3.86a</td>
<td>17.7±2.83</td>
</tr>
<tr>
<td>Water-boiling processed product</td>
<td>54.60±2.47a</td>
<td>25.13±2.58a</td>
<td>20.27±4.24a</td>
</tr>
<tr>
<td>High-pressure steaming processed product</td>
<td>52.57±2.14a</td>
<td>25.57±3.81a</td>
<td>21.87±2.71a</td>
</tr>
<tr>
<td>Excipient co-boiling processed product</td>
<td>56.77±1.30ae</td>
<td>25.73±0.87a</td>
<td>17.5±0.98ae</td>
</tr>
<tr>
<td>WF-1 monomer component</td>
<td>59.33±1.75abcde</td>
<td>25.50±1.76a</td>
<td>15.17±0.90ae</td>
</tr>
<tr>
<td>WF-4 monomer component</td>
<td>59.10±2.11abcde</td>
<td>26.27±1.66a</td>
<td>14.63±.55ae</td>
</tr>
</tbody>
</table>

*a,b,c,e P<0.05 vs. control group; ^a P<0.05 vs. crude drug group; ^b P<0.05 vs. water-boiling processed product group; ^c P<0.05 vs. excipient co-boiling processed product; ^d P<0.05 vs. high-pressure steaming processed product group. WF-1, delvestidine; WF-4, anthranoyllycoctonine.*
protein levels of HIF-1α and TLR4 in HFLS-RA cells, but not VEGF. Furthermore, it was demonstrated that, compared with the control group, levels of IL-6, IL-1β and TNF-α in the cell culture supernatant were significantly decreased, which was in accordance with previous results (4).

In the present study, our results showed that, persistent hypoxia may induce the upregulated expression of HIF-1α and TLR4, which, together with the enhanced release of pro-inflammatory cytokines, may contribute to enhanced synovial inflammation. Ca2+/calmodulin-dependent protein kinase II (CaMKII) is a member of the CaMK family and is expressed in the fibroblast-like synovial cells in RA. It has been determined that CaMKII may regulate the transcription and activation of HIF-1α and other factors, and inhibiting CaMKII may downregulate the expression of HIF-1α and VEGF in RA synovial cells, potentially by suppressing the pI3K/Akt signaling pathway (34). The pI3K/Akt and mitogen-activated protein kinase signaling pathways mediate the activation of HIF-1α in RA synovial cells under hypoxic conditions (35). It has been demonstrated that treatment with artesunate may inhibit the pI3K/Akt signaling pathway and downregulate the expression of HIF-1α and VEGF in RA fibroblast-like synovial cells (36). Another study indicated that inhibition of the pI3 K/Akt signaling pathway reduced HIF-1α expression in rat models of collagen-induced arthritis, which significantly alleviated arthritis clinical symptoms, imaging alterations, synovial hyperplasia and inflammatory cell infiltration (37). Therefore, HIF-1α may be activated via various pathways by receptors in the RA fibroblast-like synovial cells in a hypoxic microenvironment (38).

In conclusion, the current results indicated that the crude drug, processed products and monomer components of A. leucostomum Worosch., significantly enhanced the apoptosis of HFLS-RA cells, with the most potent effect observed for water-boiling processed products. The A. leucostomum treatments induced cell cycle arrest in the HFLS-RA cells, with the most potent effect observed for high-pressure steaming processed products. Furthermore, the A. leucostomum treatments downregulated the expression levels of HIF-1α and TLR4 in HFLS-RA cells, and decreased the pro-inflammatory cytokine levels in the culture supernatant. These findings may
contribute to understanding of the pathogenesis and development of RA and the development of novel therapeutic strategies to clinically treat the disease.

Acknowledgements

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References


