Rhus verniciflua Stokes extract induces inhibition of cell growth and apoptosis in human chronic myelogenous leukemia K562 cells

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Abstract. Rhus verniciflua Stokes has been widely used as a traditional medicinal plant with a variety of pharmacological activities. We investigated the mechanisms involved in mediating the effects of Rhus verniciflua Stokes (R. verniciflua) extract in human chronic myelogenous leukemia K562 cells, including caspase-dependent apoptotic pathways related to cell-cycle arrest, as well as the inhibition of nuclear factor NF-κB activation and upregulation of the mitogen-activated protein kinase (MAPK) signaling pathway. R. verniciflua extract suppressed the abnormal cellular proliferation of K562 cells in a dose- and time-dependent manner and increased the quantitative proportions of cells involved in the early and late process of apoptosis. Furthermore, R. verniciflua extract significantly mediated the mRNA levels of pro-apoptotic and anti-apoptotic regulators, such as Bcl-2, Bax, Mcl-1 and survivin in apoptotic cells. Particularly, the treatment of K562 cells with R. verniciflua extract augmented the caspase-3 activity and increased the expression of caspase-3 protein, while co-treatment with R. verniciflua extract and the permeant pan-caspase inhibitor Z-VAD-FMK and caspase-3 inhibitor Z-DEVD-FMK inversely enhanced the proliferation of K562 cells. The extract of R. verniciflua activated the phosphorylation of NF-κB and ERK. Collectively, these results indicated that the extract of R. verniciflua inhibited the proliferation of human chronic myelogenous leukemia K562 cells by activating the apoptotic process via caspase-3 overexpression and the regulation of the NF-κB and MAPK signaling.

Introduction

Rhus verniciflua Stokes (R. verniciflua) has long been used as a traditional herbal medicine, mainly in East Asia, including South Korea, Japan and China. R. verniciflua Stokes is a medicinal plant used for the treatment of gastrointestinal diseases as well as for relieving symptoms possibly caused by cancer since the 15th century in the East Asian region (1). Previous research has revealed that the extract of R. verniciflua has various therapeutic effects, encompassing antioxidant, anti-proliferative, anti-inflammatory and antitumor activities (2-7). Additionally, increasing evidence from experimental studies indicates that the R. verniciflua extract decreases oxidative stress and prevents tumor progression, although the molecular mechanisms of these pharmacological effects remain to be determined. According to a recent study of an ethanol extract of R. verniciflua, it efficiently inhibited human lymphoma cell growth, which was evaluated by confirming the apoptotic changes based on increased nuclear fragmentation, the suppressed fluorescence intensity of nuclei stained with propidium iodide and the obvious DNA fragments visualized through the DNA laddering in human lymphoma cells (8).

Apoptosis is a form of programmed cellular death characterized by its abnormal morphological features, including cellular nuclear shrinkage, nuclear fragmentation, cytoplasmic blebbing, chromatin condensation and caspase activation (9,10). Two pathways regulate the apoptotic process: the extrinsic pathway involving the activation of death receptors and the intrinsic or mitochondrial pathway. The anti-apoptotic Bcl-2 family consists of apoptotic mediators that play important roles in the process of programmed cell death. This Bcl-2 family of proteins includes several pro-apoptotic and anti-apoptotic molecules, such as Bax and Bcl-2, which regulate the cellular commitment to apoptosis (11).

The activation of nuclear factor-κB (NF-κB) plays a pivotal role in regulating the pathological changes during tumor progression from cellular proliferation to the invasion to other organs. The suppression of NF-κB transcription factor activation is involved in inhibiting tumor cell growth by inducing apoptosis. NF-κB and extracellular signaling-regulated kinase (ERK) have long been regarded as the main signaling pathways contributing to several aspects of tumorigenesis, such as cell proliferation and cell death (12,13). Numerous antitumor drugs are known to trigger cell death by inducing apoptosis. However, the pharmacological effects of the R. verniciflua extract in inhibiting cell growth via the induction of the apoptotic process in human chronic myelogenous leukemia K562 cells remain unclear.

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In the present study, we examined the mechanism by which the effects of *R. verniciflua* activity in human chronic myelogenous leukemia K562 cells are mediated, including the induction of apoptosis via the caspase-dependent apoptotic effects, as well as the suppression of the expression of the NF-κB transcription factor and the activation of the mitogen-activated protein kinase (MAPK) pathway.

**Materials and methods**

*Extraction of R. verniciflua.* *R. verniciflua* (14,15) grown in Yeosu (Korea) was purchased from Kyung Hee Pharmaceuticals (Wonju, Korea). Firstly, 1 kg of *R. verniciflua* was roasted at 180°C for 1 h, and then extracted twice with sterile distilled water for 3 h. The supernatant was evaporated and freeze-dried and the extract was obtained as a 43 g powder (yield, 4.3%). A constituent analysis using high-performance liquid chromatography (HPLC) revealed that fisetin is one of the major components of the *R. verniciflua* extract (Fig. 1).

*Cell culture.* The human chronic myelogenous leukemia K562 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium ( Gibco; Thermo Fisher Scientific, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37°C under a humidified atmosphere of 5% CO₂ and 95% air.

*Cell viability.* CellTiter 96 AQmouse One Solution (Promega, Madison, WI, USA) was used to assess the intensity of the cellular proliferation. The cells were seeded at a density of 1x10⁴ cells/well in 96-well plates and cultivated in different concentrations of *R. verniciflua* extract at 37°C for 24, 48 and 72 h. A colorimetric assay with PMS/MTS solution determined the cell viability. The absorbance value was determined at 490 nm, with reference subtraction at 650 nm.

*Flow cytometry.* The cells were incubated under treatment with 100, 200 and 300 µg/ml of *R. verniciflua* extract for 24, 48 and 72 h. After incubation, the cells were harvested and then washed with phosphate-buffered saline (PBS). Apoptosis was detected by the FITC Annexin V apoptosis detection kit (BD Biosciences, San Diego, CA, USA). The cells were incubated in 5 µl FITC-conjugated Annexin V and 5 µl propidium iodide (PI) with 100 µl binding solution for 15 min at room temperature in the dark. Annexin V-FITC and PI fluorescence were determined by flow cytometry. Apoptosis was assessed with a FACS Calibur device using CellQuest software (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

*Apoptosis assay.* To detect apoptosis in K562 cells, apoptotic cells were quantitatively analyzed using a cell death detection ELISAplus kit (Roche Molecular Biochemicals, Mannheim, Germany). The cells (1x10⁶) were incubated with 100, 200 and 300 µg/ml *R. verniciflua* extract for 24, 48 and 72 h. Subsequently, the cells were lysed with 200 µl lysis buffer. Cell lysates were assayed to detect DNA fragments using the cell death ELISAplus kit according to the manufacturer's instructions. DNA fragmentation was estimated at 405 nm relative to the untreated control level. A caspase colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) was used to assess the enzymatic changes of caspase proteases. The K562 cells were treated with 100, 200 and 300 µg/ml of *R. verniciflua* extract for 24, 48 and 72 h. The cells were harvested and the cell pellets obtained were lysed with 50 µl lysis buffer on ice for 10 min. The concentration of protein in the supernatant (cytotoxic extract) was assessed using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Caspase-3-like protease activity was determined using methods for identifying the proteolytic cleavage of substrates, including DEVD-pNA (caspase-3 substrate). These colorimetric substrates were dissolved with an assay buffer. After incubation with the solubilized substrates at 37°C for 1 h in the dark, the intensity of color production in the lysates was assessed with a microplate reader capable of detecting the absorbance at a wavelength of 405 nm and we compared the caspase-3 activity with the level of the control. To assess the effect of co-treatment with a caspase inhibitor on cell viability, K562 cells (1x10⁶ cells) were pretreated with a pan-caspase inhibitor, Z-VAD-FMK, or a caspase-3-specific inhibitor, Z-DEVD-FMK (R&D Systems) for 2 h. Subsequently, we treated the K562 cells with 300 µg/ml *R. verniciflua* extract. Following the caspase inhibitor and the *R. verniciflua* extract treatments, the PMS and MTS reagents were added to the cells. A colorimetric assay determined the absorbance of the colored formazan product at 490 nm with background subtraction at 650 nm.

*RNA extraction and real-time PCR procedures.* Total RNA was extracted and purified from cultured cells using an RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). First-strand cDNA was then synthesized from 1 µg of RNA template using a reverse transcriptase system (Promega). Random hexamers primed the reverse transcription reaction and the primer sequences and product sizes were as follows: Bcl-2 (5′-GATTTGATGGATCT GTTGCCCTTA-3′, 5′- CCTTGGCATGAGATGCAGGA-3′; 200 bp), Bax (5′-GATTGGCATCACCAAGAAG-3′, 5′-GCT AGACCCACCAT TTCG-3′; 216 bp), Mcl-1 (5′-CTCATTT CT TTGCGTCCCTT-3′, 5′- CAGTCCGGTTTTG CTAC-3′; 117 bp), survivin (5′-GCGACATTGTTCTTCTG CT-3′, 5′-GCAACCGGACATGCTT-3′; 91 bp), β-actin (5′-GCGAGAAGATGACCCAGATC-3′, 5′-GGATAAGC AGC TTGATAG-3′; 77 bp). Real-time PCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster, CA, USA) with the Power SYBR-Green PCR Master Mix (Applied Biosystems). We performed the PCR with 1 µl cDNA in 20 µl reaction mixtures that consisted of 10 µl Power SYBR-Green PCR Master Mix, 2 µl primers and 7 µl PCR water. The amplifying reactions were processed with an initial denaturing step of the target DNA at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The formula 2⁻^[(target gene - β-actin)] was used to calculate the crossing point of target genes with β-actin and the relative expression amounts were quantified.

*Immunoblot analysis.* K562 cells were harvested and washed with cold PBS to remove the medium, and then lysed in lysis buffer containing 1 mM PMSF (Cell Signaling Technology,
Boston, MA, USA). The concentration of protein contained in the incubated cells was assessed using a BCA protein assay, according to the manufacturer's instructions. The protein sample (30 µg) was separated by 12% SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. The membranes were rewetted and blocked with 5% blocking buffer for 1 h at room temperature, and then incubated overnight with human antibodies against NF-κB p65 (cat. no. 8242), phosphorylated (p-)NF-κB p65 (cat. no. 3031), p38 MAPK (cat. no. 9228), p-p38 MAPK (cat. no. 9215), MEK (cat. no. 4694), p-MEK (cat. no. 9154), ERK (cat. no. 4696) and p-ERK1/2 (cat. no. 4376; all from Cell Signaling Technology) and β-actin (cat. no. A5441; Sigma-Aldrich Co.) diluted 1:1,000 with Tris-buffered saline containing 0.05% Tween-20 (TBS-T). After 1 h washing with TBS-T solution, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (rabbit; cat. no. 7074; mouse; cat. no. 7076; both from Cell Signaling Technology) diluted 1:2,500 in TBS-T solution for 1 h at room temperature. These incubated membranes were subsequently washed with TBS-T solution for 1 h and the proteins were determined using an Amersham ECL Prime reagent kit (GE Healthcare Life Sciences, Little Chalfont, UK). The protein expression from cultured cells was detected using a Davinch-Chemi Chemiluminescence Imaging system (Davinch-K Co., Ltd., Seoul, Korea).

**Statistical analysis.** Values are expressed as the mean ± SD. Student's t-test was used to evaluate differences between the control group and the R. verniciflua extract-treated groups. The degree of inhibition of apoptosis was determined by the differences between the R. verniciflua extract-treated sample and the samples treated with a combination of caspase inhibitor and R. verniciflua extract. The data were analyzed statistically using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). p<0.05 and p<0.01 were considered to indicate statistically significant differences.

**Results**

**R. verniciflua extract inhibits cell proliferation.** K562 cells were treated with increasing concentrations of R. verniciflua extract (0-500 µg/ml) for 24, 48 and 72 h. The effects of R. verniciflua extract on K562 cellular proliferation were evaluated using a PMS/MTS solution. In the 200 and 300 µg/ml treated groups, the IC50 values were 211 and 457 µg/ml respectively when calculated by the cell viabilities of the three time-points. The morphologies of the cells were not changed with those concentrations.

**R. verniciflua extract induces cell cycle progression.** The K562 cells were treated with increasing concentrations of R. verniciflua extract (0-500 µg/ml) for 24, 48 and 72 h. To determine whether the R. verniciflua extract induced cell cycle arrest, the apoptotic cell distribution was assessed by flow cytometry. The R. verniciflua extract increased the number of apoptotic cells in the early and late stages in a dose- and time-dependent manner when compared with the controls (Fig. 3).

**R. verniciflua extract induces cell apoptosis and caspase activation.** The K562 cells were treated with 100, 200 and 300 µg/ml R. verniciflua extract for 24, 48 and 72 h and we used a cell death detection ELISA assay to identify apoptotic cells (Fig. 4A). The numbers of apoptotic cells were significantly increased in a dose-and time-dependent manner under the treatment of R. verniciflua extract. Caspase-3 activity was assayed using a colorimetric ELISA and the level of caspase-3 protein was assessed by immunoblotting. Caspase-3 activity
and protein expression were enhanced in a dose- and time-dependent manner following treatment with \textit{R. verniciflua} extract (Fig. 4B and C). To determine whether the caspase-3 activation was associated with the \textit{R. verniciflua} extract-induced apoptosis, K562 cell proliferation was detected by a PMS/MTS solution with \textit{R. verniciflua} extract treatment. Pretreatment with the pan-caspase inhibitor Z-VAD-FMK and the caspase-3 inhibitor Z-DEVD-FMK on K562 cells increased \textit{R. verniciflua} extract-induced cell proliferation (Fig. 4D).

\textit{R. verniciflua} extract regulates mRNA transcription. The K562 cells were treated with 100, 200 and 300 µg/ml of \textit{R. verniciflua} extract for 24, 48 and 72 h. Subsequently, the mRNA levels of the apoptotic genes Bcl-2, Bax, Mcl-1 and survivin were assessed using real-time PCR. The mRNA levels of Bcl-2, Mcl-1 and survivin were decreased in a dose- and time-dependent manner, whereas the Bax level was increased (Fig. 5).

\textit{R. verniciflua} extract inactivates NF-\kappa B p65 and the activity of MAPK signaling. The K562 cells were treated with 100, 200 and 300 µg/ml of \textit{R. verniciflua} extract for 24, 48 and 72 h. The expression of NF-\kappa B and MAPK proteins were analyzed by immunoblotting. The levels of the activation and phosphorylation of important proteins in the K562 cells were inhibited significantly. The levels of NF-\kappa B p65 and of p38 MAPK, MEK and ERK1/2 phosphorylation were markedly suppressed (Fig. 6A and B).

**Discussion**

\textit{R. verniciflua} Stokes has long been used as a therapeutic medicinal herb in Asian countries. To investigate the potent pharmacological effect of \textit{R. verniciflua} extract against K562 leukemia cells, we identified the effect of \textit{R. verniciflua} extract on the viability of the K562 cells. \textit{R. verniciflua} extract inhibited K562 cell proliferation, as previously reported (16). Experimental results from the present study indicated that \textit{R. verniciflua} extract not only blocked K562 cells in early and late stages of apoptosis but also increased the apoptotic cell numbers. A recent study reported a quantitative decrease in the number of A431 cells in the G1 phase and cellular arrest in the sub-G0/G1 phase after treatment with triptolide (17).

Caspases are central components of the mechanism responsible for apoptosis (18). To determine the molecular mechanism

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**Figure 3.** Effects of \textit{R. verniciflua} extract on early and late apoptosis fractions in K562 cells. The cells were treated with \textit{R. verniciflua} extract (0, 100, 200 and 300 µg/ml) for 24, 48 and 72 h. Apoptosis was assessed by flow cytometry with Annexin V-FITC and propidium iodide (PI) fluorescence. The data are the means ± SD of three independent samples. *p<0.05 and **p<0.01 compared with the control.
Figure 4. Effects of R. verniciflua extract on the induction of apoptosis in K562 cells. The cells were cultured with various concentrations of R. verniciflua extract (0, 100, 200 and 300 µg/ml) for 24, 48 and 72 h. (A) A cell death detection ELISA was used to quantify apoptotic cells. Effects of R. verniciflua extract on caspase-3 activity and protein expression in K562 cells. (B) Caspase-3 activity was assessed with a caspase-3 colorimetric assay. (C) Levels of caspase-3 protein were examined using immunoblotting. Values are the means ± SDs of three independent experiments. *p<0.05 and **p<0.01 vs. the control. (D) K562 cells were pretreated with Z-VAD (pan-caspase inhibitor) and Z-DEVD (caspase-3-specific inhibitor) for 2 h and were then incubated with 300 µg/ml R. verniciflua extract for 72 h. The cell proliferation was determined using a PMS/MTS assay. Values are the means ± SD of three independent experiments. *p<0.05 and **p<0.01, R. verniciflua extract-treated cells vs. R. verniciflua extract and caspase inhibitor-treated cells.

Figure 5. Effects of R. verniciflua extract on mRNA expression in K562 cells. The cells were treated with R. verniciflua extract (0, 100, 200 and 300 µg/ml) for 24, 48 and 72 h. The mRNA expression was detected by real-time PCR. The crossing points of Bcl-2, Bax, Mcl-1 and survivin with β-actin were entered into the formula 2^ΔΔCt and relative amounts were quantified. Values are the means ± SD of three independent experiments. *p<0.05 and **p<0.01 vs. the control.
of apoptosis in K562 cells, we revealed that treatment with R. verniciflua extract increased intracellular caspase-3 activity and enhanced the levels of caspase-3 and cleaved caspase-3 protein. This result was confirmed by co-treatment using a pan-caspase inhibitor and caspase-3-specific inhibitor, which led to an inverse in R. verniciflua extract-mediated cell proliferation. These results indicated that R. verniciflua extract-induced apoptosis pathway was associated with caspase activation in K562 cells. We then found that the K562 cells exhibited reduced mRNA expression levels of Bcl-2, Mcl-1 and survivin with R. verniciflua extract treatment, whereas the Bax level was increased. These results indicated that R. verniciflua extract induced apoptosis through the regulation of anti- and pro-apoptotic genes and the activation of caspase-3. Tubeimoside-1 increased the apoptotic activities of caspase-3, -8 and -9, whereas a caspase-3-specific inhibitor significantly inhibited tubeimoside-1-induced apoptosis in

Figure 6. Effects of R. verniciflua extract on the NF-κB and MAPK protein expression in K562 cells. The cells were treated with R. verniciflua extract (0, 100, 200 and 300 µg/ml) for 24, 48 and 72 h and the protein expression was assessed by immunoblotting. (A) The cells were lysed and 30 µg of soluble protein mixture was separated by electrophoresis through an SDS-PAGE gel. (B) Densitometric analyses of proteins are presented as the relative ratios of NF-κB, p38 MAPK, MEK, ERK and β-actin. Values are the means ± SDs of three independent experiments. *p<0.05 and **p<0.01 vs. the control.
HepG2 cells. Tubeimoside-1-induced apoptosis also decreased Bcl-2 and increased Bak, with no change in Bax levels (19).

NF-κB is a key regulator consisting of a variety of transcription factors related to both pro- and anti-apoptotic processes. Bcl-2 and Bcl-XL are anti-apoptotic molecules belonging to the Bcl-2 family and their expression level is regulated by the activation of the NF-κB (20,21). NF-κB is a critical transcription factor that plays a pivotal role in the expression of apoptosis-related proteins (22). In the present study, R. verniciflua extract inhibited the NF-κB activity. Therefore, blocking of the NF-κB signaling pathway may be effective at inducing apoptosis in K562 cells. Triptolide induced apoptosis of human anaplastic thyroid carcinoma cells by suppressing the NF-κB expression. It also downregulated Bcl-2 and Bcl-XL, which were transcriptionally mediated by NF-κB-dependent and p53-independent mechanisms (23). Our results indicated that R. verniciflua extract induced apoptosis by the downstream inhibition of the NF-κB signaling and the expression of the gene encoding Bcl-2.

MAPK pathway is an important regulator of cell death, proliferation, differentiation and autophagy (24,25). We demonstrated that R. verniciflua extract inhibited MAPK signaling in the K562 cells. Gleditsia sinensis thorn induced p38 MAPK, ERK1/2 and JNK phosphorylation in human colon cancer cells (26). Guibi-tang (GBT) treatment upregulated the expression of p38, ERK and JNK, which wereretained during apoptosis in the A431 cells (27). Poninincell growth by arresting G1-cell cycle and inducing apoptosis in HT29 cells. The Akt and MEK signaling pathway were also blocked by ponincin, whereas the p38 MAPK signaling was activated (28). In the present study, our results revealed that R. verniciflua extract inhibited K562 cell proliferation by inducing apoptosis and suppressing the MAPK signaling pathways.

In conclusion, in the present study, we demonstrated that R. verniciflua extract induced apoptosis, contributing to the inhibition of K562 cell proliferation, which is partly mediated by caspase activation, inhibition of the NF-κB activity and suppression of MAPK signaling. These results indicate the need for further research on the in vivo effect of the R. verniciflua extract.

References