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Selective Immune Modulating Activities of *Viscum album* and Its Components; A Possibility of Therapeutics on Skin Rash Induced by EGFR Inhibitors

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Abstract

Viscum album var. *coloratum* (Kom.) Ohwi is a traditional herbal medicine used in East Asia to treat hypertension, skeletal muscle disorders, and cancer. The inhibitory effects of *Viscum album* (VA) extract on chemokines and its therapeutic potential in erlotinib-induced skin rash were investigated in this study. ELISA was used to measure the levels of chemokines, MCP-1 and RANTES, which are thought to be mediators of erlotinib-induced skin rash in RAW264.7 cells. Western blot analysis was used to look into the activation of signaling pathways like AKT, MAPK, and EGF. In order to investigate the active compounds in VA extract, solvent fractionation and preparative HPLC were performed sequentially. VA extract significantly reduced the production of TNF-α, MCP-1, and RANTES but not IL-1. Furthermore, macrophage transmigration was inhibited without causing cell toxicity. VA extract had no effect on the phosphorylation of EGF receptors stimulated by EGF or suppressed by erlotinib in both A549, a non-small cell lung cancer cells, and Hacat, a human skin keratinocyte. The isolated viscumneoside III and viscumneoside V from VA extract significantly suppressed the expression of MCP-1, according to activity guided fractionation with organic solvent fractionation and preparative HPLC. These findings suggest that VA extract and its active compounds, viscumneoside III and viscumneoside V, regulate MCP-1 production and may have the potential to suppress erlotinib-induced skin toxicity by modulating macrophage activity without neutralizing anti-cancer efficacy.

Keywords

Viscum album, anti-inflammation, erlotinib, MCP-1, RANTES, skin rash, viscumneoside, mistletoe

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Introduction

Lung cancer is the most common type of cancer with the highest mortality rate, and it is classified into several subtypes.[1] Adenocarcinoma has the highest incidence at around 40%, followed by squamous cell carcinoma, large cell carcinoma, small cell lung cancer, and so on.[2] Recently, the incidence of adenocarcinomas in nonsmoking women has been increasing as a result of increased exposure to passive smoking and indoor air pollution.[3] Furthermore, genetic factors play an important role in the pathogenesis of lung adenocarcinoma.[4] Approximately 59% of east Asian female lung adenocarcinoma patients had epidermal growth factor receptor (EGFR) mutations.[5] Erlotinib is one of the approved EGFR targeted anti-cancer therapies. It is a drug

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that is taken orally and has a high selectivity for the epidermal growth factor receptor (HER1/EGFR) tyrosine kinase.[6]

Although erlotinib treatment significantly increases progression-free survival in NSCLC patients, patients often suffer from its side effects. Skin rash is the most common of the side effects, and it affects approximately 75% of patients who are prescribed erlotinib.[7] Despite the fact that skin rash is thought to have a positive correlation with anti-cancer therapy responses, patients complain of skin discomfort and demand treatment for the cutaneous toxic effect.[8] Above all, the dermatological side effects of the drugs must be controlled because they reduce the patient's quality of life.[9]

Although the pathophysiology of EGFR therapy's toxic effect on skin is not well understood, abnormal inflammatory responses in macrophages and keratinocytes are proposed as the main molecular mechanisms of erlotinib-induced skin rash. Erlotinib treatment appears to stimulate fibroblasts to bring various types of inflammatory cells to the skin lesion, such as dendritic cells, macrophages, granulocytes, and mast cells.[10] The infiltrated macrophages in the skin lesion activate and produce the chemokines such as CCL2, CCL5, and CCL27.[10] Keratinocytes with impaired EGFR signaling also produce chemokines such as CCL2, CCL5, and CXCL10, which causes skin inflammation in vivo.[11] Since macrophage depletion can reverse EGFR-induced skin inflammation, the immune cell has been proposed as a treatment target.[12]

Viscum album var. *coloratum* (Kom.) Ohwi is a type of Korean mistletoe in the Viscaceae family. *Viscum album* (VA) is a semi-parasitic plant that grows on mulberry, pine, poplar, oak, maple, birch, hawthorn, ash, and willow.[13] VA was traditionally used in many parts of the world. In Japan, it was used to treat cardiovascular diseases such as hypertension and heart spasms. Mistletoe leaves were used in India to treat diabetes. *Viscum album* was used to treat gastrointestinal disorders such as diarrhea, indigestion, and constipation in Africa and Israel.[14] Korean traditional medicine characterizes VA as follows; Temperature is neutral. Taste is bitter sweet. It tonifies liver and kidney, strengthens the sinews and bones, expels wind-dampness, prevents miscarriage, and cures arthralgia, lower back and leg soreness and pain, joint problems, numbness, weakness, uterine bleeding, blood leaks in pregnancy, and hypertension.[15] Among those, VA's anticancer pharmacological activity has received the most attention. The primary antitumor compounds in *Viscum* species are thought to be lectins and viscotoxins.[16,17]

A case study published recently found that Nobongsangki-Jeong treatment reduced olmutinib-induced skin rash in a patient with non-small cell lung cancer.[18] We hypothesized that because VA is the main component of Nobongsangki-Jeong, the medicinal herb could modulate the drug-induced cutaneous toxic effect. The anti-inflammatory activity of VA on macrophages was investigated in this study, as well as the herb's therapeutic potential in the management of Epidermal Growth Factor Receptor-induced dermatological toxic effects.

Materials and Methods

Preparation of Viscum album *Extraction*

In order to prepare the extract of *Viscum album*, we purchased VA from Cho Won Hanbang Plus (Seoul, Korea). Chopped *Viscum album* (3 kg) was immersed in 10 L of 70% ethanol for 3 days followed by filtration with Whatman No. 2 filter (Maidstone, UK), and only the components extracted into the solution were separated. From filtered *Viscum album*, another 10 L of 70% ethanol was added and it was subjected to cold extraction for 3 days again. Only the components extracted into the solution were separated through Whatman No. 2 filter. A total of 20 L extract solution was evaporated under reduced pressure at 40°C in a rotary evaporator (Büchi, Switzerland) and then lyophilized to obtain a total of 584.78 g. The yield was 19.5%. Powder was stored at −80°C, and it was diluted with distilled water or saline solution and filtered using a 0.45 μm filter for use in experiments.

Fractionation of Viscum album *Extract*

Lyophilized powder of *Viscum album* extract (544.92 g) was suspended in 1 L of distilled water and then solvent fractionated as shown in Supplemental Figure 1 to give each fraction. To suspended *Viscum album* extract in distilled water, the equal volume of hexane was added, and the 2 solvents were stirred well and then it was left to sit still so that the 2 solvent layers were separated from each other. We retrieved the bottom water layer; then the upper layer, a hexane layer, was obtained. The hexane layer was then lyophilized to give 46.16 g of dry powder. The separated aqueous layer was again mixed with 1 L of methylene chloride. After sitting still while the 2 layers are well separated, the methylene chloride layer bottom layer was retrieved. The retrieved solution was lyophilized to obtain 3.89 g of methylene chloride fraction. With this method, solvent fractionation was carried out utilizing ethyl acetate and butanol. This gave 9.54 g of the ethyl acetate fraction, 68.68 g of the butanol fraction and 341.24 g of the final water fraction. For cell culture experiments, the fractions were dissolved in DMSO and diluted in phosphate-buffered saline (PBS). The cells were exposed to DMSO less than 0.1% without any cytotoxicity.

Isolation of Single Compounds From Fraction

The hexane-soluble layer (1.86 g) was purified by preparative HPLC (Capcell Pak C18 MG column, 5 μm, 250 × 20 mm I.D.) using acetonitrile (solvent A) and water in a 0.05%

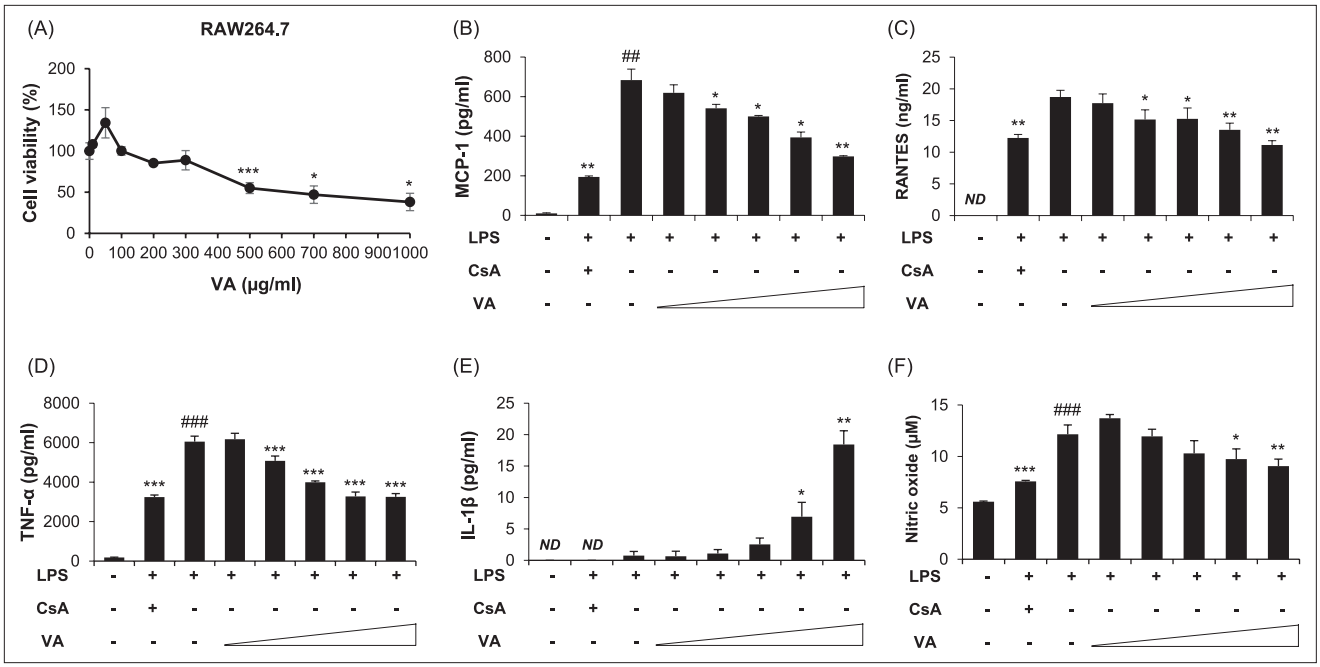

Figure 1. Effects of *Viscum album* extract on the production of inflammatory mediators in LPS-stimulated RAW264.7 cells. (A) Cytotoxicity of VA extract was performed in RAW264.7 cells by MTT assay. The RAW264.7 cells were incubated with VA extract (10, 50, 100, 200, and 300 μg/mL) for 1 hour and treated with LPS (0.5 ng/mL) for 24 hours. The cell culture supernatants were harvest, then the levels of (B) MCP-1, (C) RANTES, (D) TNF-α, and (E) IL-1β were measured by ELISA and (F) the level of nitric oxide was analyzed by using NO detection kit. *$P < .05$, **$P < .01$, ***$P < .001$ versus 0 μg/mL of VA extract or LPS only. ##$P < .01$, ###$P < .001$ versus non-treated group.
Abbreviations: VA, *Viscum album* extract; CsA, cyclosporine (1 μM).

TFA (solvent B) gradient: 0 to 30 minutes, 20% to 40% of A; 30 to 50 minutes, 40% to 100% of A at a flow rate of 15 mL/min as a mobile phase to yield compound 1 (6.3 mg, t_R = 21.8 minutes) and compound 2 (5.7 mg, t_R = 25.0 minutes). The structures of isolated compounds were identified by comparison of their spectral data with those reported in the literature.[19]

Identification of the Single Compounds

NMR spectra were acquired on a Bruker Ascend III 700 spectrometer (Rheinstetten, Germany) using CD_3OD as solvent. HRESIMS was performed on Triple TOF 5600 + mass spectrometer (AB SCIX, USA). HPLC was performed on a Agilent 1100 using a Capcell pak UG80 column (5 μm, 250 × 4.6 mm I.D.), with a mixed MeCN/H_2O solvent system at a flow rate of 1 mL/min. All other chemicals and reagents were of analytical grade.

Viscumnesoside V (**1**). HR-ESI-MS *m/z* 729.2243 $[M+H]^+$. 1H NMR (700 MHz, MeOD, δ, ppm, *J* Hz): 7.01 (1H, s, H-2′), 6.86 (1H, dt, *J*=7.7, H-6′), 6.74 (1H, dd, *J*=7.7, H-5′), 6.10 (1H, dd, *J*=2.1, H-8), 6.07 (1H, dd, *J*=2.1, H-6), 5.33 (2H, m, H-2, 1‴), 4.99 (1H, dd, *J* = 7.0, H-1″), 4.72 (1H, dd, *J* = 9.8, H-1⁗), 3.93 (1H, dd, *J* = 9.8, H-4‴a), 3.86 (1H, dd, *J* = 3.5, H-2⁗), 3.81 (3H, s, OMe), 3.784-3.765 (2H, m, H-6″a, 4⁗a), 3.73 (1H, t, *J* = 2.1, H-3″), 3.69 (1H, d, *J* = 9.8, H-4‴b), 3.62-3.58 (3H, m, H-6″b, 5‴a, 4⁗b), 3.54-3.51 (2H, m, H-2″, 5″), 3.41-3.40 (1H, m, 5⁗), 3.37-3.30 (2H, m, H-5‴b, 2⁗), 3.15 (1H, m, H-3a), 2.66 (1H, ddd, *J*=17.1, H-3b). 13C NMR (175 MHz, MeOD, δ, ppm): 198.7 (C-4), 166.9 (C-7), 165.1 (C-5), 164.8 (C-9), 149.2 (C-3′), 148.3 (C-4′), 131.6 (C-1′), 120.8 (C-6′), 116.2 (C-5′), 111.6 (C-2′), 110.8 (C-1‴), 110.8 (C-1⁗), 105.1 (C-10), 99.8 (C-1″), 97.9 (C-6), 96.9 (C-8), 81.1 (C-3⁗), 80.6 (C-2), 79.7 (C-3‴), 78.7 (C-5″), 78.6 (C-2″), 78.3 (C-2⁗), 77.9 (C-3″), 75.6 (C-4‴), 75.2 (C-4⁗), 71.8 (C-4″), 71.3 (C-5‴), 65.7 (C-5⁗), 62.4 (C-6″), 56.6 (OMe), 44.2 (C-3).

Viscumnesoside III (**2**). HR-ESI-MS *m/z* 597.1816 $[M+H]^+$. 1H NMR (700 MHz, MeOD, δ, ppm, *J* Hz): 7.00 (1H, s, H-2′), 6.84 (1H, dt, *J*= 8.4, H-6′), 6.73 (1H, d, *J*=7.7, H-5′), 6.10 (1H, t, *J*=2.5, H-8), 6.07 (1H, d, *J*=2.1, H-6), 5.33 (1H, dt, *J*=2.1, H-1‴), 5.30 (1H, dt, *J*=3.3, 12.6, H-2), 4.97 (1H, dd, *J*=7.0, 10.5, H-1″), 3.89 (1H, dd, *J*=6.6, 9.4, H-4‴a), 3.85 (1H, s, H-2‴), 3.79 (3H, d, *J*=0.7, OMe), 3.77 (1H, m, H-6″a), 3.69(1H, d, *J*=9.8, H-4‴b), 3.61-3.55(1H, m, H-6″b), 3.55-3.49 (2H, m, H-3″, 5″), 3.43 (1H, s, H-5‴), 3.37-3.34 (1H, m, H-2″), 3.14 (1H, dd, *J*=7, 10.5, H-1″). 13C NMR (175 MHz, MeOD, δ, ppm): 198.6 (C-4), 166.9 (C-7), 165.1 (C-5), 164.7 (C-9), 149.2 (C-3′), 148.3 (C-4′), 131.6 (C-1′), 120.8 (C-6′), 116.2 (C-5′), 111.5 (C-2′), 111.0 (C-1‴), 105.0 (C-10), 99.9 (C-1″), 98.0 (C-6), 96.9

(C-8), 81.0 (C-3‴), 80.8 (C-2), 78.7 (C-5″), 78.5 (C-2″), 78.3 (C-2‴), 78.2 (C-3″), 75.5 (C-4‴), 71.3 (C-4″), 66.0 (C-5‴), 62.4 (C-6″), 56.6 (OMe), 44.4 (C-3).

Cell Viability Test

In order to investigate the cytotoxicity of *Viscum album*, A549, Hacat and RAW264.7 cells were cultured in a 96-well plate. In each well, 100 μL of 2×10^5 cells/mL were dispensed and cultured for a day and incubated with *Viscum album* extract for 24 hours. After removing cell culture medium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/mL, Sigma-Aldrich, MO, USA) solution was added to the cell and incubated for 4 hours in the incubator. When a purple crystal was generated, MTT reagent was removed, and 100 μL of dimethyl sulfoxide was added to dissolve the formazan crystal. The cell viability was calculated by measuring the absorbance of 570 nm using microplate reader (Molecular Devices, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

RAW264.7 cells were inoculated into a 24-well plate (2×10^5 cells/mL, 500 μL/well), then pretreated with *Viscum album* extract and cyclosporine A (CsA) 1 μM for 1 hour. After the pretreatment was completed, the cells were stimulated with lipopolysaccharide (LPS, Sigma-Aldrich, 0.5 ng/mL) for 24 hours, and ELISA kit (R&D Systems, MN, USA) was used to measure the amount of MCP-1, RANTES, TNF-α, and IL-1β according to the manufacturer's instructions. The concentration was calculated by measuring the absorbance at 450 nm using a microplate reader (Molecular Devices, CA, USA).

Western Blot Analysis

RAW264.7, Hacat, and A549 cells were dispensed in 6-well plates (2×10^5 cells/mL, 2 mL/well), and then, cultured at 37°C, 5% CO_2. Next day, the cells were preprocessed with 300 μg/mL of *Viscum album* extract for 1 hour, and treated with 1 μM erlotinib. After culturing for 16 hours, 100 ng/mL of EGF was treated for 10 minutes and then washed twice with cold PBS. The cells were harvested with 120 μL of RIPA buffer supplemented with protease inhibitor cocktail and phosphate inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice, and centrifuged at 13 000 rpm at 4°C for 20 minutes to obtain a supernatant. The obtained protein was quantified by the method of BCA (INTRON, Korea). Proteins of 30 μg were electrophoresed on 10% SDS-PAGE and transferred to PVDF membrane. TBST added with 5% skim milk was left blocking in room temperature for 1 hour with antibody. After washing 3 times with TBST for 10 minutes each, it was allowed to react with a secondary antibody with horseradish peroxidase (HRP). Anti-phosphorylated EGFR and anti-EGFR antibodies were purchased from (Cell Signaling Technology Inc., MA, USA) and β-actin antibodies were purchased from Sigma-Aldrich. For ECL system, Amersham™ ECL Select™ Western Blotting Detection Reagent from GE Healthcare (IL, USA) was used.

Transwell Migration Assay

The upper chamber's membrane of TransWell (Corning Inc., NY, USA) was coated with collagen and dried thoroughly. RAW264.7 cells contained in serum free medium were dispensed with an amount of 150 μL per 6×10^5 cells/mL. After placing 500 μL of DMEM medium containing 10% FBS in the lower chamber, the upper chamber was treated with 100, 200, and 300 μg/mL of *Viscum album* extract for each concentration, and 100 ng/mL MCP-1 (R&D systems) was treated in lower chamber. After culturing in a cell incubator at 37°C and 5% CO_2 for 24 hours, the cells on the top of the insert were carefully removed with a cotton bud. After fixing for 10 minutes with 70% ethanol, it was dyed with 0.1% crystal violet for 10 minutes. It was washed thoroughly with distilled water until clean, then dried thoroughly. The dried membrane was taken with a microscope, then the cells were dissolved using 33% acetic acid, transferring 200 μL each to a 96 well plate, and read at the wavelength of 570 nm with the microplate reader (Molecular Device). Then the relative absorbance values were calculated.

Nitric Oxide (NO) Analysis

RAW264.7 cells were seeded in 24-well plates and were incubated with the CsA (1 μM) or *Viscum album* extract (10, 50, 100, 200, and 300 μg/mL) pretreated for 1 hour, after treating with LPS (0.5 ng/mL) for 24 hours. And then NO levels were quantified in the supernatants using the NO detection kit (iNtRON BioTechnology, Korea) according to the manufacturer's instructions.

Statistical Analysis

Results were analyzed using GraphPad Prism 4 software (Version 4.03; GraphPad Software, Inc., San Diego,CA, USA). Data are presented as mean ± standard error. Group comparisons were performed using one-way analysis of variance, followed by a post hoc Tukey's test. Differences with $P < .05$ were considered to be statistically significant.

Results

Viscum album *Suppressed Production of Inflammatory Mediators Involved in Erlotinib-Induced Skin Rash*

The main complaint of EGFR-targeted anti-cancer therapy is cutaneous toxicity, such as skin rash, which is thought to

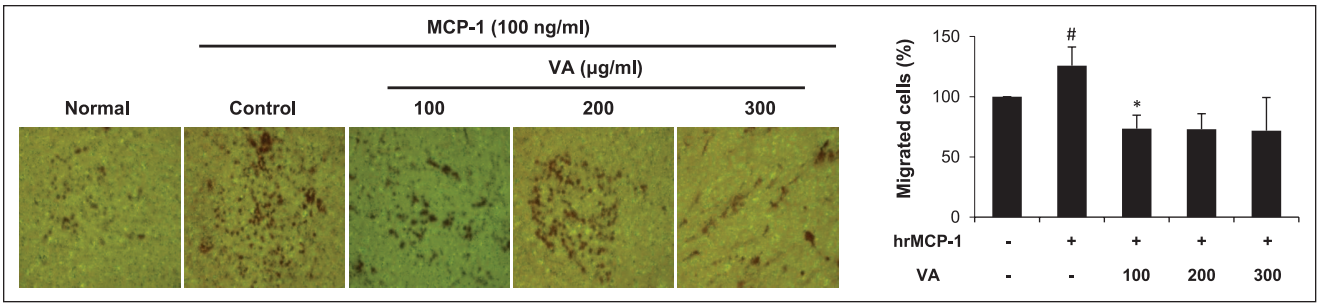

Figure 2. Effects of *Viscum album* extract on the transmigration of MCP-1-stimulated RAW264.7 cells. RAW264.7 cells were placed on upper chamber of TransWell containing VA extract and induced transmigration to lower chamber by adding MCP-1 (100 ng/mL). The transmigrated cells were stained with crystal violet and dissolved in acetic acid. The optical density was measured by microplate reader. *$P < .05$ MCP-1 only group. #$P < .05$ versus non-treated group.
Abbreviation: VA, *Viscum album* extract.

be caused by abnormal chemokine production in macrophages. Therefore, we investigated the inhibitory effects of *Viscum album* (VA) extract on MCP-1 and RANTES in RAW264.7 cells. First, the cytotoxicity of VA extract was determined using the MTT assay. The cell viability was greater than 88% up to a concentration of 300 μg/mL (Figure 1A), so we performed the in vitro experiment at that concentration.

To investigate the effects of VA extract on the production of inflammatory mediators involved in skin rash caused by EGFR-I treatment in macrophages, the extract was treated in LPS-stimulated Raw 264.7 cells, and the levels of MCP-1, RANTES, TNF-α, and IL-1β were measured using ELISA. VA extract significantly reduced MCP-1, RANTES, and TNF-α production in a concentration-dependent manner (Figure 1B-D), whereas VA extract treatment increased the level of IL-1β (Figure 1E). VA extract treatment also significantly reduced NO production in LPS-stimulated RAW264.7 cells (Figure 1F).

Raw264.7 cells were stimulated by MCP-1 for transmigration in Transwell in the presence or absence of VA extract to investigate the effects of VA extract on immune cell migration. MCP-1-induced macrophage transmigration was significantly reduced by VA extract treatment (Figure 2). These findings suggested that VA extract has the potential to suppress macrophage activity, which is linked to skin rash caused by an EGFR-targeted anti-cancer reagent.

Immune Suppressive Effects of Viscum album *Did Not Affect the Pharmacological Activity of Erlotinib*

To investigate the effects of VA extract on efficacies of erlotinib, we examined the phosphorylation of the EGF receptor in the presence or absence of erlotinib in EGF-stimulated RAW264.7 cells. However, we could not detect the phosphorylation of EGF receptor due to the lack of expression in RAW264.7 cells (Figure 3A). To explore the effects of VA extract on the pharmaceutical activity of erlotinib, we performed the same experiment in A549, a non-small cell lung cancer cell, and HaCat, a keratinocyte, which express EGFR and respond to EGF and target cells of erlotinib. Cytotoxicity test showed that there was no toxicity up to 300 μg/mL in both cell types (Figure 3B and C). EGF treatment highly increased the phosphorylation of EGF receptor in A549 cells and erlotinib dramatically suppressed the EGF-induced phosphorylation (Figure 3D). The VA extract treatment did not change either the level of EGF-induced phosphorylation of EGFR, or return the suppressive effect of erlotinib on EGF-induced phosphorylation of EGFR in A549 cells (Figure 3D). The Hacat cells showed the same responses to the combined treatments of EGF, erlotinib, and VA extract (Figure 3E). These results proposed that VA extract did not affect the pharmaceutical efficacies of erlotinib in lung cancer cells or keratinocytes.

Viscum album *Inhibited AKT Signaling Pathway But Not MAPK Pathways*

To investigate the signaling pathways involved in the suppressive effects of VA extract on macrophage activation, we examined the phosphorylation of AKT and MAPKs, JNK, ERK, and p38 by Western blot analysis in RAW264.7 cells. The VA extract treatment decreased the level of phosphorylated AKT in a dose-dependent manner (Figure 4A). Meanwhile, the levels of phosphorylated MAPKs were not altered by VA extract except the marginal decrease of phosphorylated ERK and JNK at the highest dose, 300 μg/mL (Figure 4B). These results suggest that VA extract inhibits the production of inflammatory mediators by suppressing the AKT signaling pathway but not MAPKs.

Identification of Active Compounds in Viscum album *by Fractionation*

To identify the active compound(s) of VA extract on modulating the activity of macrophages, we performed an activity guided fractionation. First we conducted a serial organic

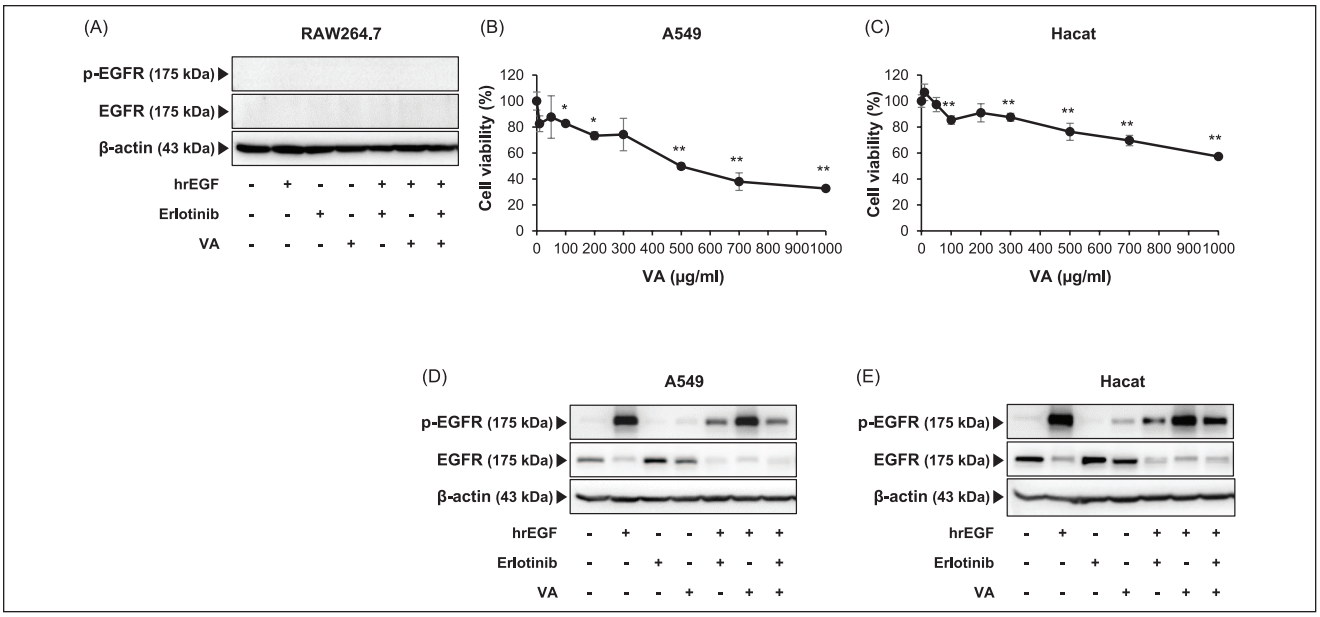

Figure 3. Effects of *Viscum album* extract on EGF signaling interfered by erlotinib. (A) RAW264.7, (D) A549, and (E) Hacat cells were treated with VA extract (300 μg/mL) and erlotinib (1 μM) for 16 hours. After stimulation with EGF (100 ng/L) for 10 minutes, the levels of phosphorylated EGFR, total EGFR and β-actin were measured by western blot analysis. (B) A549 and (C) Hacat cells were incubated with VA extract for 24 hours and cytotoxicity was measured by MTT assay. *$P < .05$, **$P < .01$ versus 0 μg/mL of VA extract.

Abbreviations: VA, *Viscum album* extract; CsA, cyclosporine (1 μM).

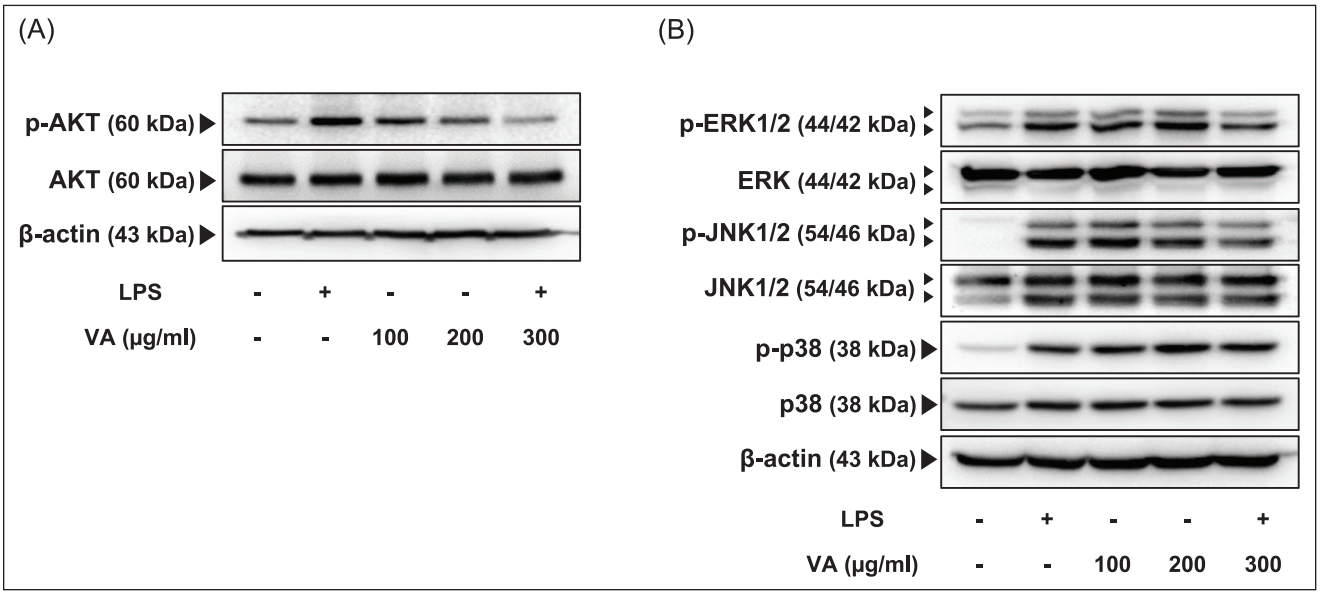

Figure 4. Effects of *Viscum album* extract on phosphorylation of AKT and MAPKs. The RAW264.7 cells were incubated with VA extract (100, 200, and 300 μg/mL) for 1 hour and treated with LPS (0.5 ng/mL) for 30 minutes. The cell lysates were harvested, then the phosphorylated levels of (A) AKT and (B) ERK, JNK, and p38 were analyzed by western blot.

Abbreviation: VA, *Viscum album* extract.

solvent fractionation from n-hexane (Hex), methylene chloride (MC), ethyl acetate (EA), and butanol (BuOH). We tested the suppressive effect of each fraction on MCP-1 production. The cytotoxicity test of each fraction showed that there was no cytotoxicity up to a concentration of 100 μg/mL except for the MC fraction (Figure 5A-B). MC

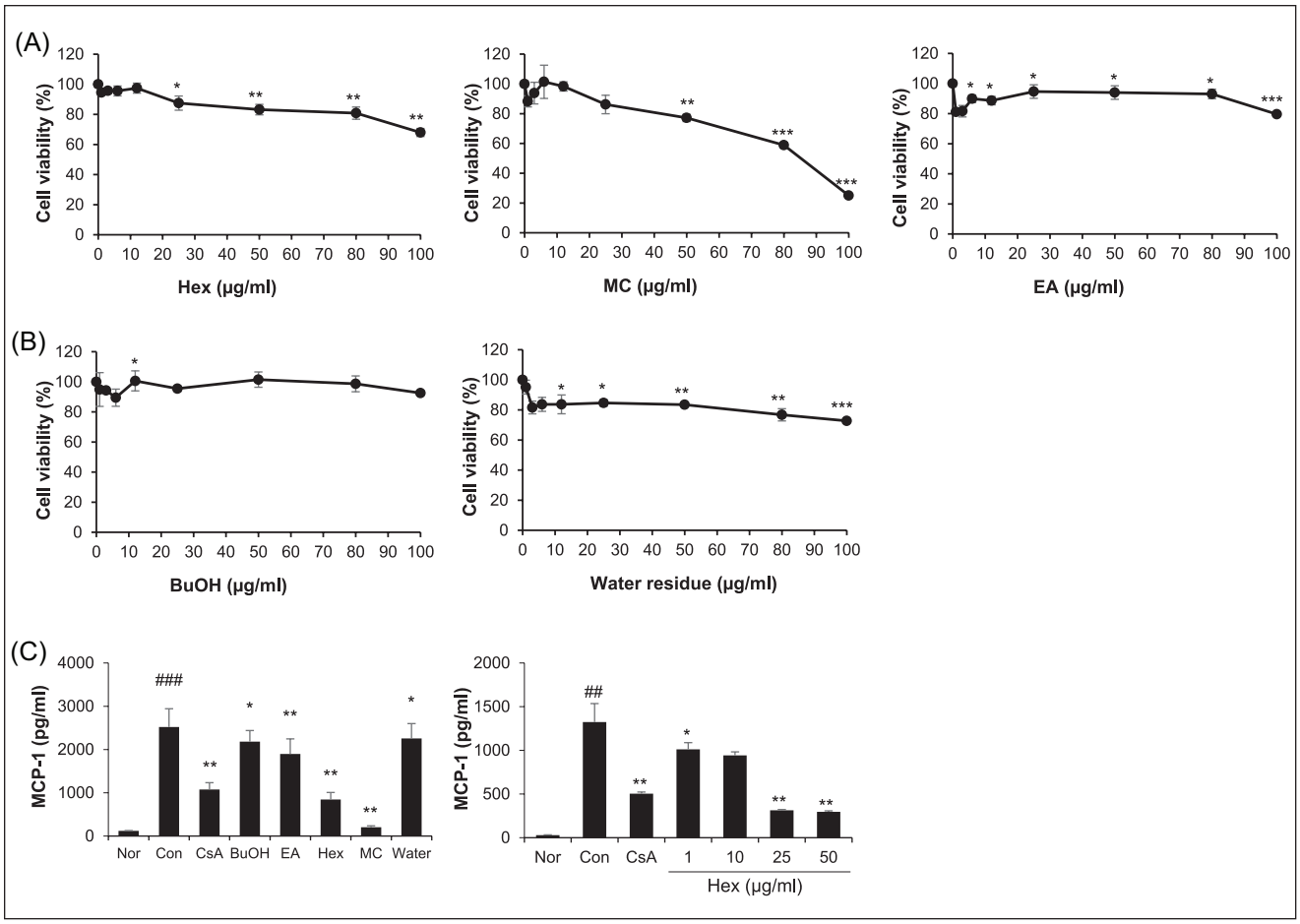

Figure 5. Activity guided-organic solvent fractionation of *Viscum album* extract. (A) The RAW264.7 cells were incubated with each organic solvent fraction for 24 hours and the cytotoxicity was measured by MTT assay. (B) The cells were incubated with each organic solvent fraction (each 50 μg/mL) for 1 hour and treated with LPS (0.5 ng/mL) for 24 hours. The cell culture supernatants were harvested, then the level of MCP-1 was measured by ELISA. (C) The cells were incubated with hexane fraction (1, 10, 25, and 50 μg/mL) for 1 hour and treated with LPS for 24 hours. The level of MCP-1 in cell culture supernatant was measured by ELISA. . $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus 0 μg/mL of fraction or Con. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ versus Nor.
Abbreviations: VA, *Viscum album* extract; CsA, cyclosporine (1 μM); Hex, n-hexane fraction; Mc, methylene chloride fraction; EA, ethyl acetate fraction; BuOH, butanol fraction; Nor, normal with non-treatment; Con, control with LPS stimulation.

fraction showed cytotoxicity at higher concentrations than 50 μg/mL (Figure 5A). MC and Hex fractions showed the most prominent suppressive effects on the production of MCP-1 (Figure 5C). Although the MC fraction dramatically suppressed the production of MCP-1 at 10 μg/mL, we could not exclude the effect of cytotoxicity. Hex fraction had suppressive effect on MCP-1 production in a dose-dependent manner with low cytotoxicity (Figure 5C).

Next, we subject the EA fraction to preparative HPLC and found that there were 3 major peaks, viscumneoside V, viscumneoside III, and cinnamic acid. We isolated viscumneoside V and viscumneoside III from the fraction and analyzed the suppressive effect on the production of the chemokines, MCP-1 and RANTES. Each compound did not have cytotoxicity up to 100 μM in RAW264.7 cells (Figure 6A and B). Thereafter, the cells were treated with each compound, viscumneoside V and viscumneoside III, and the levels of MCP-1 and RANTES were measured by ELISA. The 2 compounds suppressed the production of MCP-1 in RAW264.7 cells (Figure 6C). However, they increased the production of RANTES in macrophages. Taken together, viscumneoside V and viscumneoside III from VA extract may have selective anti-inflammatory effects on the production of chemokines in macrophages.

Discussion

In this study, we looked into the potential of *Viscum album* extract as a treatment for EGFR-I-induced skin rash. We investigated the production of inflammatory mediators because EGF signaling modulates the homeostasis of the skin barrier and the production of cytokines and chemokines.

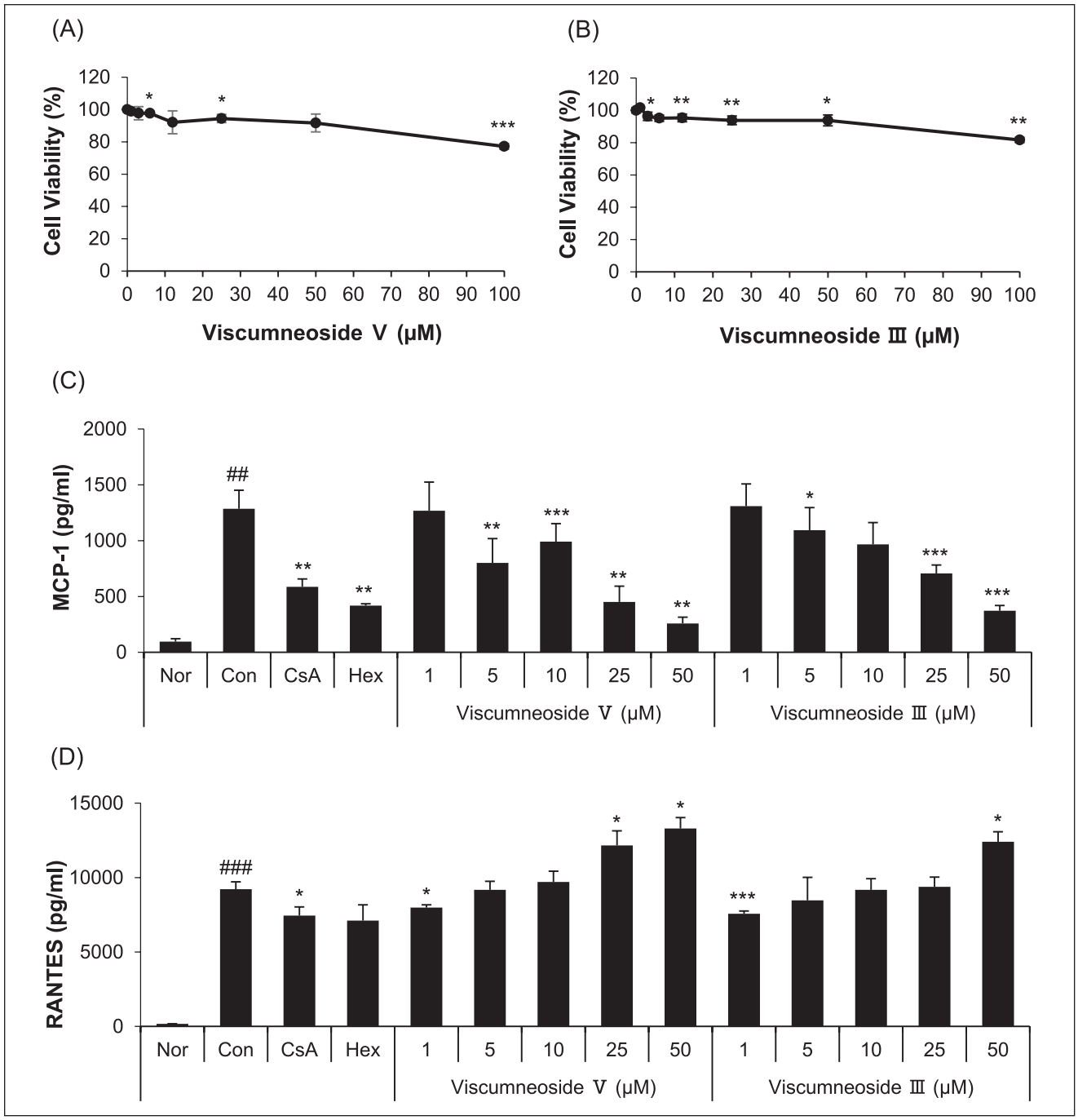

Figure 6. Effects of single compounds isolated from *Viscum album* extract on chemokine production. RAW264.7 cells were incubated with (A) viscumneoside V and (B) viscumneoside III for 24 hours and the cytotoxicity was measured by MTT assay. The RAW264.7 cells were incubated with viscumneoside V and viscumneoside III for 1 hour and treated with LPS (0.5 ng/mL) for 24 hours. The cell culture supernatants were harvest, then the levels of (C) MCP-1 and (D) RANTES were measured by ELISA. *$P < .05$, **$P < .01$, ***$P < .001$ versus control. ##$P < .01$, ###$P < .001$ versus normal.

Abbreviations: VA, *Viscum album* extract; CsA, cyclosporine (1 μM); Hex, n-hexane fraction (25 μg/mL); Nor, normal with non-treatment; Con, control with LPS stimulation.

We investigated whether *Viscum album* extract and its components, viscumneoside III and V, could down-regulate macrophage activities by suppressing the production of specific immune mediators such as MCP-1, RANTES, and TNF-α, which have been proposed as pathologic factors involved in the pathogenesis of skin rash by EGFR-I

treatment. *Viscum album* extract did not interfere with erlotinib's suppressive effect on EGF signaling, which is the drug's main anti-cancer mechanism.

In Europe, *Viscum album* is known as mistletoe, and numerous studies on its anticancer properties have been published. *Viscum album* extract is thought to have anticancer properties by inhibiting the cell cycle and inducing apoptosis in cancer cells.[20] In addition to cytotoxicity, it has been proposed that the anticancer effect of *Viscum album* extract is manifested via immunomodulation. *Viscum* species' immunomodulatory activities also contributed to anticancer activity.[21]

Viscum album extract appears to have unique immune modulating properties. Chemokines, MCP-1, RANTES, and TNF-α were all suppressed by the extract, but not IL-1β. TNF-α is thought to aggravate skin rash caused by EGFR-I in combination with MCP-1 and RANTES in keratinocytes.[11] *Viscum album* extract suppressed TNF-α production in LPS-activated macrophages in this study. It is worth noting that *Viscum album* extract increased the level of IL-1β while decreasing the level of TNF-α. It appears to respond differently to *Viscum album* extract depending on the type of immune cell, cell activation state, and cytokine. *Viscum album*'s immunomodulatory activity appears to be produced in a variety of ways. TNF-α and IL-1β production by human peripheral blood mononuclear cells is stimulated by lectin isolated from *Viscum album*.[22] Yoo et al discovered that heparin-binding protein (HBP) isolated from *Viscum album* stimulates TNF-α production in macrophages.[23] These findings contradict the findings of this study, which show that *Viscum album* has a suppressive effect on TNF-α production. We did, however, test *Viscum album*'s immunomodulatory effects in LPS-stimulated macrophages. This study differs from the previous studies in that LPS activates the immune system, which appears to be similar to the cutaneous microenvironment caused by EGFR suppression. *Viscum album*, on the other hand, has been linked to immune suppression in several studies. *Viscum album* suppressed the production of prostaglandin E2 and the expression of COX-2 in IL-1β-stimulated A549 cells, which are human lung cancer cells.[24] A diarylheptanoid, (3S,5R)−3-hydroxy-5-methoxy-1,7-bis(4-hydroxyphenyl)−6E-heptene and a flavonoid glycoside, 3,7,3′-tri-O-methylquercetin-4′-O-β-d-apiofuranosyl-(1→2)-O-β-d-glucopyranoside, which are isolated from *Viscum*, were reported to inhibit the production of TNF-α, IL-6 and IL-12 in bone-marrow-derived dendritic cells.[25] In the presence of LPS, the Korean mistletoe lectin synergistically activates the expression of TNF-α, according to Lee et al.[26] Interestingly, the Korean mistletoe lectin suppressed the basal expression of TNF-α in the absence of LPS, and LPS-induced NO production was reduced. Whereas, *Viscum album* agglutinin-I did not affect the production of cytokines in LPS-stimulated neutrophils.[27] As a result, it appears that the response to *Viscum album* differs between activated and inactivated immune cells. In addition, it is believed that the various compositional substances included in *Viscum album* have various immune-modulating activities depending on the state of the immune system.

In this study, we demonstrated that *Viscum album* extract could suppress the levels of chemokines, MCP-1 and RANTES, which are produced under inhibition of EGFR activities.[28] Keratinocytes and macrophages are thought to be the major cells producing the chemokines in responding to blockage of EGFR signaling. However, these chemokines are considered difficult to be used as a sole target for skin lesion healing. Even though the expression of MCP-1 or RANTES was inhibited, EGFR-I treatment- or EGFR knockout-induced skin rash did not improve.[10] On the other hand, clodronate-induced macrophage depletion could reverse the inflammatory skin lesion provoked by anti-EGFR drugs.[12] These studies suggest that the regulation of single inflammatory factors may not be sufficient to manage EGFR-I-induced skin toxicity. Rather, modulating the activity of macrophages could be the target of the adverse cutaneous side effects. It is expected that the *Viscum album* extract may have the effect of inhibiting the activity of macrophages as a whole, and thus could exhibit efficacy in skin rash. *Viscum album* extract had the effect of inhibiting not only the chemokine but also the cytokine TNF-α, and also reduced the production of NO. In addition, it suppressed the chemokine-induced transmigration, thereby inhibiting macrophage activity. Therefore, it is considered that *Viscum album* extract has the effect of regulating the activity of macrophage itself. Therefore, it is considered that skin rash caused by EGFR-I can be suppressed.

Here, we isolated viscumneoside III and V from *Viscum album* and investigated their biological activities on the immune system. Viscumneoside III was reported to have anti-tyrosinase activity.[29] And it is proposed to be effective on osteoporosis since it suppresses the formation of osteoclast-like multinucleated cells.[30] However, it is not yet reported whether the viscumneoside has an immune modulating activity. This study showed that viscumenoside III and V from *Viscum album* could effectively suppress the production of MCP-1. In the case of RANTES, the single compounds stimulated the production of the chemokine in macrophages. While the whole *Viscum album* extract showed the efficacy of inhibiting both MCP-1 and RANTES production, viscumneoside selectively inhibited only MCP-1 and, rather, promoted RANTES production. Although the exact mechanism remained to be investigated, the substance that inhibits the production of RANTES is likely to be a component other than viscumneoside V or III. The physiological activity of *Viscum album* is expected to show more effect when used as a whole rather than as individual compounds. Felenda et al[31]

reported that complete mistletoe extract showed the strongest antiproliferative activity on tumor cells compared with the isolated compounds, and combination of mistletoe compounds demonstrated a synergistic effect on tumor cell apoptosis compared to each single compound.[32]

The purpose of this study was to investigate the efficacy of Nobongsangki-Jeong, which contains *Viscum album*, based on clinical cases on the efficacy of inhibiting the side effects of skin rash in the EGFR-I treatment of patients with non-small cell lung cancer.[18] Nobongsangki-Jeong is a Korean herbal medicine composed of *Viscum album* and propolis. The patient in the case study also had acupuncture treatment as well as the herbal medicine. Therefore, it is difficult to say that the pharmacological action of inhibiting skin rash caused by olmutinib was shown by *Viscum album* alone. Nevertheless, it is considered that the anti-cancer and immune-regulating effects of the *Viscum album* were clinically effective.

The limitation of this study is that the efficacy of inhibiting skin rashes of *Viscum album* was presented only through cell culture experiments and was not clinically proven. However, the immunomodulatory substances suppressed by *Viscum album* in activated macrophages are expressed when the activity of EGFR is inhibited, and it has been suggested that they may affect the skin rash caused by EGFR-Is. Therefore, it is supposed that *Viscum album* can act on the cutaneous side effects related to the target anti-cancer drugs, and the clinical case study is expected to support our hypothesis.

Hydrocortisone is currently widely used in clinical practice to control the skin side effects of EGFR-I drugs.[33] However, there are limitations, such as not recommending use for more than 14 days due to toxicity, so a safe treatment must be developed. *Viscum album* extract had no effect on EGF signaling, implying that it may be able to improve skin lesions without impairing the anticancer efficacy of EGFR-I's. As a result, this study established a scientific foundation for the future use of *Viscum album* as a pharmacological treatment for EGFR-I-caused skin rash.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

References

1. Boolell V, Alamgeer M, Watkins DN, Ganju V. The evolution of therapies in non-small cell lung cancer. *Cancers*. 2015;7:1815-1846.
2. Ettinger DS, Wood DE, Akerley W, et al. NCCN guidelines insights: non-small cell lung cancer, version 4.2016. *J Natl Compr Cancer Netw*. 2016;14:255-264.
3. Lo YL, Hsiao CF, Jou YS, et al. Polymorphisms of MLH1 and MSH2 genes and the risk of lung cancer among never smokers. *Lung Cancer*. 2011;72:280-286.
4. Matakidou A, Eisen T, Houlston RS. Systematic review of the relationship between family history and lung cancer risk. *Br J Cancer*. 2005;93:825-833.
5. Patel JD. Lung cancer in women. *J Clin Oncol*. 2005;23:3212-3218.
6. Rudin CM, Liu W, Desai A, et al. Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity. *J Clin Oncol*. 2008;26:1119-1127.
7. Agero AL, Dusza SW, Benvenuto-Andrade C, Busam KJ, Myskowski P, Halpern AC. Dermatologic side effects associated with the epidermal growth factor receptor inhibitors. *J Am Acad Dermatol*. 2006;55:657-670.
8. Wacker B, Nagrani T, Weinberg J, Witt K, Clark G, Cagnoni PJ. Correlation between development of rash and efficacy in patients treated with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib in two large phase III studies. *Clin Cancer Res*. 2007;13:3913-3921.
9. Sun PL, Seol H, Lee HJ, et al. High incidence of EGFR mutations in Korean men smokers with no intratumoral heterogeneity of lung adenocarcinomas: correlation with histologic subtypes, EGFR/TTF-1 expressions, and clinical features. *J Thorac Oncol*. 2012;7:323-330.
10. Lichtenberger BM, Gerber PA, Holcmann M, et al. Epidermal EGFR controls cutaneous host defense and prevents inflammation. *Sci Transl Med*. 2013;5:199ra111.
11. Mascia F, Mariani V, Girolomoni G, Pastore S. Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. *Am J Pathol*. 2003;163:303-312.
12. Mascia F, Lam G, Keith C, et al. Genetic ablation of epidermal EGFR reveals the dynamic origin of adverse effects of anti-EGFR therapy. *Sci Transl Med*. 2013;5:199ra110.
13. Bussing A. *Mistletoe: The Genus Viscum*. CRC Press; 2000.
14. Lev E, Ephraim M, Ben-Arye E. European and oriental mistletoe: from mythology to contemporary integrative cancer care. *Eur J Integr Med*. 2011;3:e133-e137.
15. National College of Oriental Medicine Collaborative Textbook Compilation Committee. *Herbal Medicine*. Young Lim Company; 2016.
16. Thies A, Nugel D, Pfüller U, Moll I, Schumacher U. Influence of mistletoe lectins and cytokines induced by them on cell proliferation of human melanoma cells in vitro. *Toxicology*. 2005;207:105-116.
17. Schaller G, Urech K, Giannattasio M. Cytotoxicity of different viscotoxins and extracts from the European subspecies of *Viscum album* L. *Phytother Res*. 1996;10:473-477.

18. Choi Y, Kim J, Bae K, Cho C, Yoo H. A case of non-small cell lung carcinoma patient who improved skin rash due to olmutinib by administration of Nobongsangki-Jeong. *Korean J Clin Oncol*. 2017;22:13-22.
19. Park B, Matsuta T, Samejima H, et al. Chemical constituents of mistletoe (*Viscum album* L. var. *coloratum* Ohwi). *IOSR J Pharm Biol Sci*. 2017;12:19-23.
20. Harmsma M, Ummelen M, Dignef W, Tusenius KJ, Ramaekers FC. Effects of mistletoe (*Viscum album* L.) extracts iscador on cell cycle and survival of tumor cells. *Arzneimittelforsch*. 2006;56:474-482.
21. Oei SL, Thronicke A, Schad F. Mistletoe and immunomodulation: insights and implications for anticancer therapies. *Evid Based Complement Alternat Med*. 2019;2019:1-6.
22. Hostanska K, Hajto T, Spagnoli GC, Fischer J, Lentzen H, Herrmann R. A plant lectin derived from viscum album induces cytokine gene expression and protein production in cultures of human peripheral blood mononuclear cells. *Nat Immun*. 1995;14:295-304.
23. Yoo JY, An H, Kim IB, et al. Isolation and partial characterization of heparin-binding protein from Korean mistletoe (*Viscum album* coloratum). *J Med Plan Res*. 2013;7: 234-242.
24. Hegde P, Maddur MS, Friboulet A, Bayry J, Kaveri SV. *Viscum album* exerts anti-inflammatory effect by selectively inhibiting cytokine-induced expression of cyclooxygenase-2. *PLoS One*. 2011;6:e26312.
25. Nhiem NX, Kiem PV, Minh CV, et al. Diarylheptanoids and flavonoids from viscum album inhibit LPS-stimulated production of pro-inflammatory cytokines in bone marrow-derived dendritic cells. *J Nat Prod*. 2013;76:495-502.
26. Lee JY, Kim JY, Lee YG, et al. In vitro immunoregulatory effects of korean mistletoe lectin on functional activation of monocytic and macrophage-like cells. *Biol Pharm Bull*. 2007;30:2043-2051.
27. Lavastre V, Cavalli H, Ratthe C, Girard D. Anti-inflammatory effect of viscum album agglutinin-I (VAA-I): Induction of apoptosis in activated neutrophils and inhibition of lipopolysaccharide-induced neutrophilic inflammation in vivo. *Clin Exp Immunol*. 2004;137:272-278.
28. Holcmann M, Sibilia M. Mechanisms underlying skin disorders induced by EGFR inhibitors. *Mol Cell Oncol*. 2015;2:e1004969.
29. Park C, Kim J, Hwang W, Lee BD, Lee K. In vitro anti-tyrosinase activity of viscumneoside III and homoflavoyadorinin B isolated from Korean mistletoe (*Viscum album*). *Korean J Plant Resour*. 2016;29:690-698.
30. Han N, Huang T, Wang YC, Yin J, Kadota S. Flavanone glycosides from viscum coloratum and their inhibitory effects on osteoclast formation. *Chem Biodivers*. 2011;8:1682-1688.
31. Felenda JE, Turek C, Stintzing FC. Antiproliferative potential from aqueous *Viscum album* L. preparations and their main constituents in comparison with ricin and purothionin on human cancer cells. *J Ethnopharmacol*. 2019;236:100-107.
32. Twardziok M, Kleinsimon S, Rolff J, et al. Multiple active compounds from *Viscum album* L. synergistically converge to promote apoptosis in Ewing sarcoma. *PLoS One*. 2016;11:e0159749.
33. Lynch TJ Jr, Kim ES, Eaby B, Garey J, West DP, Lacouture ME. Epidermal growth factor receptor inhibitor-associated cutaneous toxicities: an evolving paradigm in clinical management. *Oncologist*. 2007;12:610-621.