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Biological Effects of Vinca minor extract; Tyrosinase inhibition, stimulation of ROS generation and increasement of cell migration activity in keratinocytes

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Abstract : Vinca alkaloids from plant Vinca minor have been investigated for their effects of tyrosinase inhibition, stimulation of ROS generation and increasement of cell migration activity. The methanolic crude extract and the water-soluble fraction exhibited IC_{50} value of 3.1 mg/mL and 2.1 mg/mL. *Vinca minor* extract treatment significantly increased ROS levels in HaCaT cells, in a concentration-dependent manner. Treatments of *Vinca minor* extract led to increase wound closure when compared with non-treatment. Low dose (0.1% or 0.3%) of extracts have not significantly affected, compared with that in controls. By contrast, 0.5% extract have dramatic effect on wound healing activity of keratinocytes. Effects of *Vinca minor* extract in a filter-based cell mobility assay appear similar to that of wound closure assay, which suggests that the *Vinca minor* extract have wound healing effects on skin.

Keywords : Vinca minor extract, Vincamine, Tyrosinase inhibition, ROS generation, Wound Healing

1. Introduction

Less Periwinle (Vinca minor L.) is a medicinal plant producing an important alkaloid, vincamine, found in leaves, which shows a celebrovasodilatiory and neuroprotective activity. So far, alkaloids of *Vinca minor* L. have been characterized more than 50 compounds belong to various classes such as kempferols, quercetins and vincamine. Some biological activities of Vinca minor extract have been studies previously[1-3]. Vinca minor Extracts showed antioxidant activity by testing against the DPPH (2,2-diphenyl-1- picrylhydrazyl) free-radical [4]. Antimicrobial properties of those extracts have been investigated against dandruff causing microorganism *Malassezia furfur*, *yeast,* Gram positive and negative bacteria. Vincamine, one of the major natural products of Vinca minor,

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showed no sign of antimicrobial activity, whereas Vinca minor hot-water extract had the activity against *M. furfur*[5-7]. Furthermore hot water extract had antimicrobial activity against Gram positive *Bacillus* sp. and Gram negative *Escherichia coli*. Cytotoxic properties of those extracts have also been investigated with HaCaT cell (human keratinocyte), HT-29 cell (human colorectal adenocarcinoma cell) and Raw cell, showing no significant cytotoxic effects.

In the present research, the effects of tyrosinase inhibition, stimulation of ROS generation and increasement of cell migration activity of the Vinca minor extract have been investigated. Reactive oxygen species (ROS) function as signaling molecules to mediate various biological responses, including cell growth and gene expression, host defense, and even cell migration [8]. ROS are short-lived, diffusible molecules. Therefore, the localization of the ROS signal at specific subcellular compartments may be essential for initiating redox-dependent, spatially restricted signaling events after receptor activation [9]. Especially, ROS have pivotal roles differentiation, proliferation, and cell migration of keratinocytes for skin reconstruction [10-12]. Numerous studies have been reported that many natural products and their extracts can induce ROS formation in mammalian cells. Thus, we checked whether *Vinca minor* can induce ROS formation in keratinocytes. ROS levels in control and *Vinca minor* extract-stimulated HaCaT cells were measured by fluorescence spectroscopy using DCFDA. We also checked the potential of Vinca minor extracts as whitening agents because these extracts had antioxidant potency which could contribute to prevent pigmentation resulting from non-enzymatic oxidation from our previous study.

2. Materials and Method

2.1. Isolation procedure of Vinca minor extract

50g of Vinca minor leaf was extracted with 95% ethanol using mixer at room temperature and concentrated to remove EtOH. The crude extract was treated with EtOAc and distilled water (50:50). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give green oil. The water layer was lyophilized to give brownish powder.

2.2. High resolution UPLC/MS/QTOF analysis

Ultra performance LC analysis was performed on Ultra high resolution Q-TOF LC MS/MS system (MicroQTOF III, Bruker co.) and C18 column with a 1.7 mm particle size, 2.1 x 100 mm dimension, and a flow rate of 0.6 ml min^{-1} with an ESI source.

2.3. Measurement of Reactive Oxygen Species

Intracellular ROS generation were performed by measuring the fluorescence intensity of the 2′ 7′ -dichlorofluroescein diacetate (DCF-DA) probe, which was proportional to the amount of ROS formed. HaCaT cells pre-treated with and without 0-0.5% Vinca minor extract for 24 h or 48 h and then were mixed with DCF-DA solution and incubated at 37° C for 1 h. Fluorescence intensity was measured with excitation of 485 nm and emission at 530 nm.

2.4. In vitro wound healing assay

The wound healing assay was performed using a scratch assay format; to increase the reproducibility of the assay, we used IBIDI cell culture inserts (Integrated BioDiagnostics, Munich, Germany). Using a six-well plate, the cell culture inserts were placed aseptically into each well and HaCaT cells (4×10^4) were seeded into each chamber of the cell culture insert and incubated overnight. The following day, the medium was removed and replaced with medium containing 10 μg/ml

mitomycin-C (Sigma) for 2 h to inhibit cell proliferation. Then, the cell culture insert was removed carefully, leaving a 500 μm cell-free gap and then the cells were treated with or without (0-0.5%) Vinca *minor* extract for 12 h or 24 h. To quantify migration, the cells were examined with an Olympus inverted microscope under dark-field optics and the percentage of each field occupied by migration tracks was calculated using the Image software (http://rsb.info.nih.gov/ij/). Each experiment was performed in triplicate.

2.5. In vitro Transwell migration assay

HaCaT cell in vitro Transwell (Millipore; Millicell 8 μm) migration assay was as described (5). Type IV collagen was used to coat the membrane for 3 h and dried. Six hundred microliters serum-free DMEM with 0.1% BSA (wt/vol) and 0-0.5% Vinca minor extract was added to the lower chamber, and cells in 200 μl serum-free DMEM were added to the upper chamber. After 24 h of migration, cells remaining on the upper membrane were scraped off, and cells that had migrated to the lower membrane were stained with 0.5% crystal violet/20% methanol. The crystal violet dye retained on the filters was resolved in 10% acetic acid and absorbance was colorimetrically measured at 595 nm.

2.6. Assay for mushroom tyrosinase activity

Tyrosinase inhibitory activity was measured spectrophotometrically according to the method of Han et al with some modifications[14]. First, 20 μL of mushroom tyrosinase was added to 240 μL solutions of Vinca minor extract (0.0125 ~ 0.3wt%) in phosphate buffer (0.05M, pH 6.8). Then, 40 μL of tyrosine (1.5mM) in phosphate buffer was added and the assay mixture was then incubated at 37° C for 15 min. Following incubation, the amount of dopachrome production in the reaction mixture was determined spectrophotometrically at 490 nm in a microplate reader. Arbutin (0.0125 ~ 0.3 wt%) in phosphate buffer (0.05M, pH 6.8) was used as a positive control.

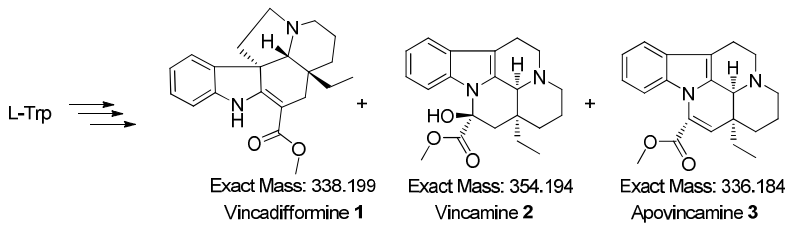
3. Results and Discussion

3.1. Characterization of Vinca alkaloids in Vinca *minor*.

First, alkaloid-enriched extract was characterized by means of UPLC/MS/QTOF. Vincadifformine 1, vincamine 2 and apovincamine 3 have been found in crude extract. Its main alkaloid, vincamine is a recognized neuroprotector.

The alkaloid content of the aerial parts of V. minor was about 1%±0.2. Among them, vincamine was found to be the dominant alkaloid in the aerial parts of Vinca minor with the content of about 0.05%±0.01.

From the crude extracts, vincamine **2** as the major alkaloid and two other derivatives, Vincadifformine **1** and apovicamine **3**, were identified from HPLC and LC/MS/QTOF analysis as shown in Fig 1 and Fig 2. The ESI-MS spectra showed molecular ions of vincadiffornine **1** at m/z 339.207 [MH+, theoretical exact mass 339.199], vincamine **2**



Scheme 1. Vinca alkaloids in vincamine biosynthesis

at m/z 355.203 [MH+, theoretical exact mass 355.194] and apovincamine **3** at m/z 337.191 [MH+, theoretical exact mass 337.184] respectively.

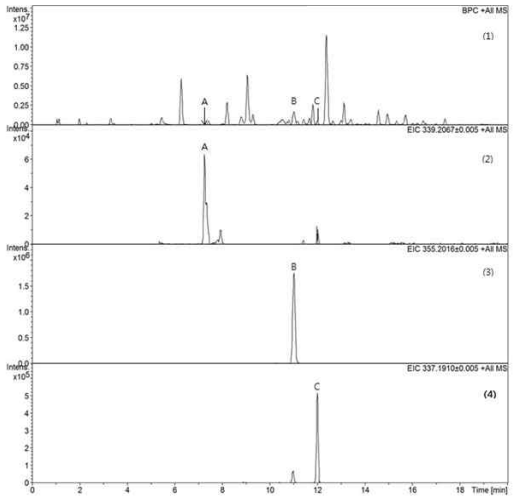


Fig. 1. UPLC/MS/QTOF chromatograms of Vinca minor extract. (A): LC/MS/QTOF chromatogram of crude extract, (B): Vincadifformine peak **A** eluted at 7.3min, (C): Vincamine peak **B** eluted at 11.0min, (D): Apovincamine peak **C** eluted at 12.0min.

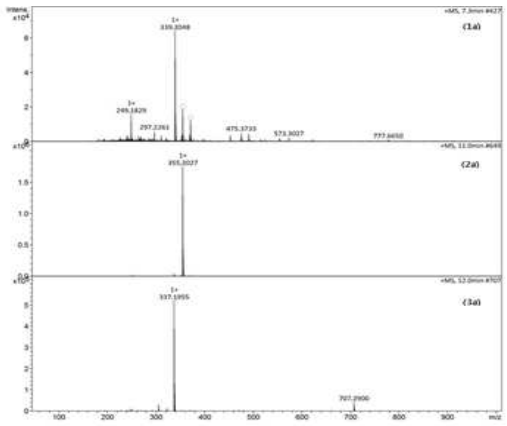


Fig. 2. UPLC/MS/QTOF spectra of Vinca alkaloids 1~3. (1a): Vincadifformine peak eluted at 7.3min, (2a): Vincamine peak eluted at 11.0min, (3a): Apovincamine peak eluted at 12.0min.

3.2. Biological activities of Vinca alkaloids

It has been known that many natural products and their extracts can induce ROS formation in mammalian cells. Thus, we checked whether Vinca minor extract can induce ROS formation in keratinocytes. ROS levels in control and Vinca minor extract-stimulated HaCaT cells were measured by fluorescence spectroscopy using DCFDA. As a result, Vinca minor extract treatment significantly increased ROS levels in HaCaT cells, in a concentration-dependent manner (Fig 3. A and B).

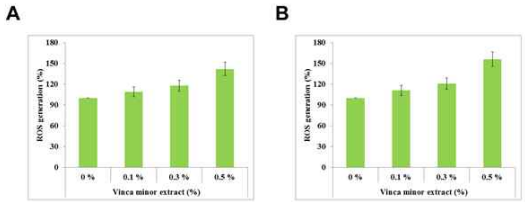


Fig. 3. Vinca minor extract stimulates ROS formation in HaCaT cells. HaCaT cells were treated with or without 0-0.5% Vinca minor extract for 24 (A) or 48 h (B). ROS generation was determined by measuring the fluorescence intensity of the oxidation-sensitive fluorescein DCFDA. Non-treated cells were defined as showing 100% ROS generation and the histogram shows the mean values ± SD of ROS generation obtained by analysis of three separate experiments. * $P < 0.05$

Since Vinca minor extract induce cellular ROS generation, we hypothesized that Vinca minor extract can induce keratinocyte migration. As expected, treatment of Vinca minor extract led to increased wound closure compared with non-treatment (Fig. 4A and 4B). Although low dose (0.1% or 0.3%) of extracts have not significantly effect, compared with that in controls, 0.5% extract have dramatic effect on wound healing activity of

keratinocytes. Also, effects of Vinca minor extract in a filter-based cell motility assay appear similar to that of wound closure assay (Fig. 5A and 5B). These data suggest the Vinca minor extract have beneficial effects on skin environment through the ROS- dependent regulation.

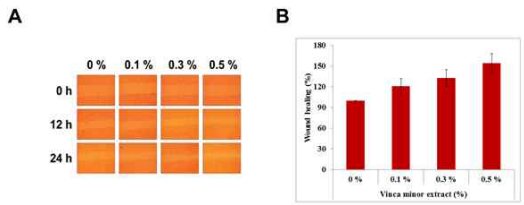


Fig. 4. Vinca minor extract induce wound healing of keratinocytes. (A) HaCaT cells were cultured and scratched as described in the Materials and Methods. The initial scratch (0h) was observed to have almost the same area in all conditions. Phase contrast images were taken 12 h or 24h after wounding and treatment of Vinca minor extract. (B) Quantitative evaluation of cell migration. Non-treated cells were defined as showing 100% scratch closure. The average ± SD of three experiments is shown. *,p < 0.05

Table 1. Tyrosinase inhibitory activity of crude Vinca minor extract

Extract concentration (mg/mL)	Inhibition (%)		
	arbutin	Crude extract (A)	water -soluble fraction (B)
0	0	0	0
0.25	33.71	17.74	30.82
0.50	42.01	23.40	33.08
1.00	52.94	28.30	39.62
2.00	59.12	38.24	47.67
3.00	68.30	48.05	58.34

Water-soluble fraction of the Vinca minor extract was subjected to phase partitioning. Of the hexane, ethyl acetate and water fractions, the water-soluble fraction exhibited the highest anti-tyrosinase activity. The result of tyrosinase inhibitory activity by the crude extract and the water-soluble fraction of Vinca minor extract was similar showing significant inhibition of tyrosinase activity. The methanolic crude extract and the water-soluble fraction exhibited IC_{50} value of 3.1 mg/mL and 2.1 mg/mL. In comparison, arbutin as a control showed tyrosinase activity at 0.9 mg/mL. Those similar tyrosinase inhibitory activities indicate that the water-soluble fraction may also contain natural antioxidants.

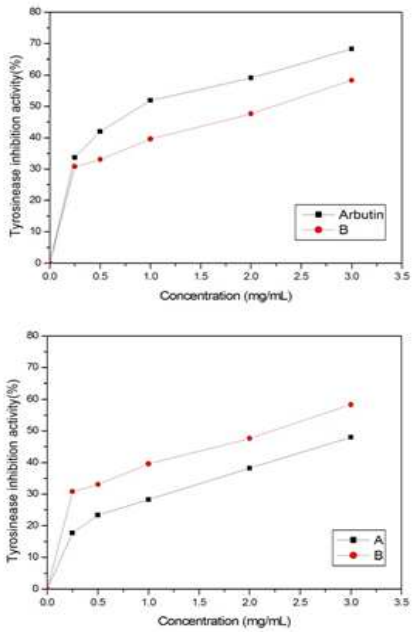


Fig. 5. Inhibitory effects of Vinca minor crude extract (A) and water-soluble fraction (B). Arbutin used as a standard.

ROS levels in control and Vinca minor extract-stimulated HaCaT cells were measured by fluorescence spectroscopy using DCFDA. Vinca minor treatment significantly increased ROS levels in HaCaT cells, in a concentration-dependent manner. Because

Vinca *minor* extract induce cellular ROS generation, we hypothesized that Vinca minor extract can induce keratinocyte migration. Treatment of Vinca minor extract led to increased wound closure compared with non-treatment. 0.5% of Vinca minor extract have significant effect on wound healing activity of keratinocytes. Effects of Vinca minor extract in a filter-based cell motility assay appear similar to that of wound closure assay. These data suggest the Vinca minor extract have beneficial effects on skin environment through the ROS-dependent regulation.

4. conclusion

Very few studies have been reported about the biological properties of plant Vinca minor alkaloids. In this current study, we revealed that Vinca minor alkaloids have the effects of tyrosinase inhibition, stimulation of ROS generation and increasement of cell migration activity. The methanolic crude extract and the water-soluble fraction exhibited IC_{50} value of 3.1 mg/mL and 2.1 mg/mL. We also checked whether Vinca minor can induce ROS formation in keratinocytes. As a result, Vinca minor extract treatment significantly increased ROS levels in HaCaT cells, in a concentration-dependent manner. Treatments of Vinca minor extract led to increase wound closure, compared with non-treatment. Although low dose (0.1% or 0.3%) of extracts have not significantly affected, compared with that in controls, 0.5% extract have dramatic effect on wound healing activity of keratinocytes. Effects of Vinca minor extract in a filter-based cell mobility assay appear similar to that of wound closure assay, which suggests that the Vinca *minor* extract has beneficial effects on skin environment through the ROS- dependent regulation. In future, we will isolate the Vinca alkaloids from plant Vinca minor and characterize the structures of the active compounds.

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