A set of plant essential oils have antioxidant activity in ultraviolet B-irradiated epidermal keratinocytes by upregulating antioxidant and detoxifying enzymes

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ABSTRACT

Plant-derived extracts including essential oils have long been used as important ingredients of cosmetics. In recent years, there has been a demand for safe cosmeceuticals with excellent anti-aging activity. This study aimed to develop candidate plant extracts with excellent antioxidant and detoxifying activities in keratinocytes with a view to protecting photo-aging of the skin. The ROS-scavenging activities of candidate plant essential oils were determined in ultraviolet B (UVB)-irradiated normal human epidermal keratinocytes (NHEKs). Their antioxidant and detoxifying activities were determined by measuring enzymatic antioxidants, phase 2 enzymes and total polyphenol content. In DCF-DA and confocal microscopy studies, the plant essential oils including Lavender oil, Lemongrass oil, Rosemary oil, Chamomile oil and Peppermint oil showed excellent ROS-scavenging activity in UVB-irradiated NHEKs. In RT-PCR studies, all these oils upregulated the expression of enzymatic antioxidants (CuSOD, GPx II and Prx I) and phase 2 detoxifying enzymes (HO-1, NQO-1, GSTpi, GSTA4, and GCLM) via Nrf2 in NHEKs. All of the five essential oils can be regarded as good candidate natural products with potential for use as new cosmeceuticals.

Keywords: Plant-derived extracts, cosmeceuticals, keratinocytes, photo-aging, skin.

INTRODUCTION

Essential oils are complex mixtures of low molecular weight compounds, which are extracted by steam distillation, hydrodistillation, or solvent extraction (Nakatsu et al., 2000). Approximately, 3000 essential oils were reported from at least 2000 plant species and at least 300 of these are regarded as important sources of industrial products (Raut et al., 2014). As plant essential oils contain a variety of plant secondary metabolites, they can be applied to health, agriculture, cosmetics and food products (Clardy and Walsh, 2004).

Essential oils have been reported to have various pharmaceutical and biological activities including antimicrobial, anticancer, anti-inflammatory and antioxidant efficacies (Raut et al., 2014; Ali et al., 2015). The beneficial effects of essential oils are widely used in the production of various cosmetic products to be applied to the skin. Fortunately, essential oils are generally safe, with minimum adverse reactions in the skin, such as irritating and allergic reactions and photocotoxicity (Ali et al., 2015).

The skin is directly exposed to a wide variety of chemical and physical attacks such as ultraviolet (UV) irradiation, which produce a variety of reactive oxygen species (ROS). Therefore, keratinocytes (KCs), as the major compound cells in the epidermis are equipped with a variety of antioxidants that can be classified into non-enzymatic and enzymatic, which protect the skin from ROS-induced oxidative damage. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are well-known...
antioxidant enzymes in the skin. Peroxiredoxins (Prxs) are a family of peroxidases that are widely expressed in various tissues including the skin (Lee SC et al., 2000) and are also important antioxidant enzymes in the protection of cells from oxidative damage by reducing peroxides and scavenging radicals (Fujii and Ikeda, 2002; Rhee et al., 2005).

Another group of enzymes called phase 2 enzymes, including heme oxygenase-1 (HO-1), NADP(H): quinone oxidoreductase-1 (NQO-1), glutathione-S-transferase (GST) and glutamate-cysteine ligase (GCLM) functions as an important cellular defense mechanism by detoxifying xenobiotic or electrophilic carcinogens and promoting antioxidant activity against pro-oxidants (Zhang et al., 2013). These phase 2 enzymes share common transcriptional regulation by redox-sensitive transcription factor, NF-E2-related factor-2 (Nrf2). In the skin, they have been reported to be induced in response to environmental stressors such as UV irradiation, hydrogen peroxide ($\text{H}_2\text{O}_2$) and lipopolysaccharides (Marrot et al., 2008, Wojas-Pelc et al., 2007), as well as cell differentiation (Piao et al., 2011).

In our previous studies, NQO-1 was found to be upregulated in differentiated KCs and HO-1, NQO-1 and GST were upregulated by UVB irradiation of dermal fibroblasts (Piao et al., 2011; Choi et al., 2013).

We are interested in the antioxidant activities of plant essential oils that can be used as beneficial ingredients in cosmeceuticals. The present study was performed to determine the antioxidant activity of plant essential oils by studying their modulatory activity of some antioxidant (SOD, GPx, Prx) and phase 2 (HO-1, NQO-1, GST, GCLM) enzymes in NHEKs. Their ROS-scavenging activity was evaluated in primary cultured normal human epidermal keratinocytes (NHEKs), which were irradiated with ultraviolet B (UVB) to induce oxidative stress.

**MATERIALS AND METHODS**

**Cells**

NHEKs were purchased from EpiLife (EpiLife; Cascade Biologics, Portland, OR). Cells were cultured in basal keratinocyte growth media (EpiLife) supplemented with human keratinocyte growth supplement (0.06 mM calcium) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a 5% CO$_2$ incubator. Passages 2 to 5 were used for all experiments.

**MTT assay**

To determine cell viability, NHEKs were seeded onto 96-well plates and treated with different concentrations of herb extracts for 24 h.

Cell viability was assayed using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) kit (Chemicon International Inc., Billerica, MA, USA) according to the manufacturer’s instructions. Briefly, 10 μl MTT solution was added to each well (0.5 mg/ml) and plates incubated at 37°C for 2 h. The produced formazan crystals were dissolved in 100 μl dimethylsulfoxide and the absorbance was read at 570 nm using a plate reader (Molecular Devices, Sunnyvale, CA).

**Measurement of intracellular ROS levels**

Intracellular ROS levels were measured using fluorogenic 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen, Carlsbad, CA), which was detected by fluorescence-activated cell sorting (FACS) analysis. Briefly, NHEKs were seeded on each well of a 60-mm culture dish at a density of 5 × 10$^6$ cells per well and treated with plant extracts for 1 h. In our experimental system to produce ROS by UVB irradiation, cells were pretreated with plant extracts, since ROS rapidly produced and disappeared during UVB irradiation. After washing with PBS, NHEKs were labeled with 10 μM DCF-DA at 37°C for 1 h, followed by washing with PBS again, and were then exposed to UVB irradiation at a dose of 50 mJ/cm$^2$. After further washing, NHEKs suspended in 500 μl PBS were analyzed using a FACSC alibur flow cytometer (FACSC alibur; Becton Dickinson, CA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Images were analyzed using the Cell Quest software. At the same time, UVB-induced intracellular ROS was detected using confocal microscopy with DCF-DA dye. Counter staining was performed with 4', 6-diamidino-2-phenylindole and fluorescent signals detected at excitation wavelengths of 492 to 495 nm and emission wavelengths of 517 to 527 nm. Images were visualized using confocal microscopy with a 40x objective lens (LSM 510; Carl Zeiss, Jena, Germany), and analyzed using the LSM 5 browser imaging software.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) for antioxidant and detoxifying enzymes**

Total RNA was isolated using an RNeasy mini kit (Qiagen, Fremont, CA) according to the manufacturer’s instructions. cDNA was generated from 1 μg total RNA using an Omniscript RT kit (Qiagen). A polymerase chain reaction (PCR) pre-mixture kit (ELPIS, Daejeon, Korea) was used for
Table 1: Primer sequences for RT-PCR.

<table>
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<th>Primer sequences (Forward/Reverse)</th>
<th>Product size (bp)</th>
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<td>360</td>
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<tr>
<td>Human Gpx II</td>
<td>5’-tgaggtgaaattggccagaag-3’/5’-ttttgccgtttccacactg-3’</td>
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<tr>
<td>Human Prx I</td>
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<tr>
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<td>5’-caggccagagaagctggagtcggtc-3’/5’-gatgttgacgagaggcaggt-3’</td>
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<tr>
<td>Human NQO-1</td>
<td>5’-cagcgccccggacgacgagcc-3’/5’-ggaagcttgaaagataccaga-3’</td>
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<tr>
<td>Human GSTpi</td>
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Determination of total phenol content

The content of total phenolic compounds (TPC) in the extracts was determined using Folin-Ciocalteu's phenol reagent (Singleton and Rossi). The absorbance was measured at 750 nm with an ELISA (Molecular Devices, Menlo Park, CA) and the TPC concentration of the samples is expressed in mg of gallic acid equivalents (GAE)/kg extract.

Statistical analysis

All experiments were performed in triplicate and the results expressed as the mean ± standard deviation. Comparisons between samples were carried out using a
Table 1: Primer sequences for RT-PCR.

<table>
<thead>
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<th>Genes</th>
<th>Primer sequences (Forward/Reverse)</th>
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<td>Human GCLM</td>
<td>5'-actagaagtgcagttgacatgg -3'/5'-agctgaatgcctcaagg - 3'</td>
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<tr>
<td>Human Nrf-2</td>
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Results

MTT assay of plant essential oil extracts in NHEKs

For the present study, a group of plant essential oils that are widely used in cosmetics or aromatherapy were selected. In the MTT cell viability assay, the following concentrations of plant essential oils were found to be optimal with no cytotoxicity: >0.01% Lavender oil; >0.005% Lemongrass oil; >0.13% Rosemary oil; >0.005% Chamomile oil and >0.08% Peppermint oil (Figure 1).

Total polyphenol content of plant essential oil extracts

The total polyphenol content of the tested essential oils were 1.0 GAE mg/kg Lavender oil, 11.9 GAE mg/kg Lemongrass oil, 0.5 GAE mg/kg Rosemary oil, 17.9 GAE mg/kg Chamomile oil and 0.4 GAE mg/kg Peppermint oil.

ROS-scavenging activity of plant essential oils in UVB-irradiated NHEKs

In humans, life-long exposure to UV irradiation inevitably causes photoaging due to ROS-induced oxidative damage. Safe cosmetics with efficient antioxidant and detoxifying activities are highly sought after in skin research. As a functional assay to evaluate the ROS-scavenging activity of candidate essential oils, treated NHEKs were irradiated with 50 mJ/cm² UVB to produce a large amount of ROS. In FACS analysis, the DCF-DA signal was increased to 811.1% in UVB-irradiated NHEKs from the baseline value in non-irradiated NHEKs (negative control, 100%). As a positive control experiment, NHEKs were irradiated with UVB in the presence of 0.1% NAC (a known ROS scavenger) and the DCF-DA-positive signal was markedly decreased to 378.5% (Figure 2A). Under the same conditions, the DCF-DA signal

Student's t-test, with statistical significance considered to be p < 0.05.
Figure 1: Cytotoxicity of various essential oils in NHEKs. To determine the non-toxic concentrations of plant essential oils including Lavender oil, Lemongrass oil, Rosemary oil, Chamomile oil and Peppermint oil, an MTT assay was performed in NHEKs treated with different doses of these oils for 24 h.

![Graph showing cell survival percentage for different essential oils at various concentrations.]

Figure 2A: ROS-scavenging activity of plant essential oils in UVB-irradiated NHEKs. A) FACS analysis of DCF-DA signals in NHEKs. After NHEKs were treated with UVB at a dose of 50 mJ/cm² and DCF-DA fluorescent signals were measured by FACS in (a) non-UVB-irradiated, (b) UVB-irradiated and (c) NAC-treated UVB-irradiated cells (positive control). Under the same conditions, DCF-DA signals were also measured in essential oil-treated UVB-irradiated cells.

![Histograms showing DCF-DA signals in UVB-irradiated NHEKs treated with different essential oils.]

was markedly attenuated in the UVB-irradiated NHEKs pre-treated with essential oils: to 321.93% by 0.005% Lavender
Figure 2B: ROS-scavenging activity of plant essential oils in UVB-irradiated NHEKs. The relative fluorescence intensity of the tested samples was analyzed by densitometry.

Antioxidant activity of plant essential oils due to the upregulation of a set of antioxidant enzymes in NHEKs

An accumulation of studies showed that various plants are a good source of antioxidants for scavenging ROS (Khan et al., 1992). To evaluate the antioxidant activity, NHEKs were treated with the essential oils at different concentrations for 24 h. In RT-PCR, maximal mRNA expression of CuSOD was induced by 0.01% Lavender oil, 0.0006% Lemongrass oil, 0.01% Rosemary oil, 0.01% Chamomile oil and 0.0012% Peppermint oil (Figure 4). Maximal mRNA expression of GPx II was induced by 0.01% Lavender oil, 0.005% Lemongrass oil, 0.04% Rosemary oil, 0.04% Chamomile oil and 0.005% Peppermint oil (Figure 4). Maximal mRNA expression of Prx I was induced by 0.01% Lavender oil, 0.0006% Lemongrass oil, 0.01% Rosemary oil, 0.01% Chamomile oil and 0.0012% Peppermint oil (Figure 4). Collectively, these plant essential oils were found to be a good natural source for the induction of the antioxidant enzymes (CuSOD, GPx II, and Prx I) in NHEKs.

Detoxifying activity of plant essential oils due to the upregulation of a set of Nrf2-dependent phase 2 enzymes in NHEKs

Next, we measured the mRNA expression levels of phase 2 enzymes in the same samples in order to test the detoxifying activity of the essential oils. In RT-PCR, maximal mRNA expression of HO-1 was induced by 0.01% Lavender oil, 0.0006% Lemongrass oil, 0.04% Rosemary oil, 0.005%
Figure 3: Confocal microscopy analysis to detect DCF-DA signals in NHEKs. DCF-DA fluorescent signals from control (non-UVB-irradiated NHEKs), UVB-irradiated NHEKs and essential oil-treated UVB-irradiated NHEKs are depicted. DAPI indicates the staining of cell nuclei and DCF-DA images are overlapped with DAPI images of the same samples (merge).

Figure 4: Effect of plant essential oils on the expression of enzymatic antioxidants in NHEKs. To evaluate the effect of plant essential oils on the expression levels of a set of enzymatic antioxidants including CuSOD, Gpx II and Prx I, NHEKs were treated with different doses of the oils for 24 h and mRNA levels of the enzymatic antioxidants were then measured using RT-PCR.
Figure 5A: Effect of plant essential oils on the expression of phase 2 enzymes in NHEKs. A) To evaluate the effect of plant essential oils on the expression levels of a set of phase 2 enzymes including HO-1, NQO-1, GSTpi, GSTA4, GCNM, NHEKs were treated with different doses of the oils for 24 h and mRNA levels of phase 2 enzymes were then measured using RT-PCR.

Chamomile oil, and 0.01% Peppermint oil (Figure 5). Maximal mRNA expression of NQO-1 was induced by 0.01% Lavender oil, 0.005% Lemongrass oil, 0.04% Rosemary oil, 0.005% Chamomile oil and 0.02% Peppermint oil (Figure 5A). Maximal mRNA expression of GSTpi was induced by 0.0025% Lavender oil, 0.0012% Lemongrass oil, 0.01% Rosemary oil, 0.0012% Chamomile oil and 0.02% Peppermint oil (Figure 5A). Maximal mRNA expression of GSTA4 was induced by 0.01% Lavender oil, 0.005% Lemongrass oil, 0.04% Rosemary oil, 0.0025% Chamomile oil and 0.01% Peppermint oil (Figure 5A). Maximal mRNA expression of GCNM was induced by 0.01% Lavender oil, 0.005% Lemongrass oil, 0.04% Rosemary oil, 0.0006% Chamomile oil and 0.02% Peppermint oil (Figure 5A). As a transcription factor for the modulation of phase 2 enzymes, maximal mRNA induction of Nrf-2 expression was observed in 0.0025% Lavender oil, 0.005% Lemongrass oil, 0.04% Rosemary oil, 0.02% Chamomile oil and 0.005% Peppermint oil (Figure 5B). Collectively, the plant essential oils were found to be a good natural source for the induction of the phase 2 enzymes (HO-1, NQO-1, GSTs, and GCLM) in NHEKs.

**DISCUSSION**

Development of novel cosmeceuticals is of importance in skin research due to their new therapeutic modalities in addition to the original concept of use as cosmetics. All five plant essential oils tested (Lavender oil, Lemongrass oil, Rosemary oil, Chamomile oil and Peppermint oil) were found to have potent antioxidant and ROS-scavenging activity, since they upregulate both enzymatic antioxidant and phase 2 enzymes in KCs. In our experience, there is no concern with respect to the safety of plant essential oils, as they have been widely used for a long time in cosmetics and
enzymes is challenging due to the complicated barrier transdermal delivery of large-molecular weight antioxidant (Rinnerthal er et al., 2015). On the other hand, topical or ROS effectively by forming intramolecular disulfide bonds disproportionately enriched with cysteine, which quenches the SPRP2 subfamily, are not only rich in proline but are skin with a protective barrier against the external and small proline-rich proteins (SPRPs), etc provides the involucrin, loricrin, elafin, cystatin A, S100 family proteins (Kalinin et al., 2001). This complex structure, consisting of involucrin, loricrin, elafin, cystatin A, S100 family proteins and small proline-rich proteins (SPRPs), etc provides the skin with a protective barrier against the external environment including UV irradiation. SPRPs, in particular the SPRP2 subfamily, are not only rich in proline but are disproportionately enriched with cysteine, which quenches ROS effectively by forming intramolecular disulfide bonds (Rinnerthaler et al., 2015). On the other hand, topical or transdermal delivery of large-molecular weight antioxidant enzymes is challenging due to the complicated barrier system of the skin.

To circumvent such problems in the delivery of antioxidants, it is more desirable to stimulate KCs to produce endogenous antioxidant enzymes by permeable materials such as low-molecular weight essential oils (usually less than 500 Daltons) (Natatsu et al., 2000). A group of natural oils extracted from flowers and various parts of plants have been used for aromatherapy to enhance psychological and physical well-being. Aromatherapy as a form of alternative medicine is gaining attention for use as an adjuvant therapy to improve various functions of the body. Aromas can be absorbed into our body through inhalation and skin application and stimulate various cells and organs including the brain and skin (Ali et al., 2015). From the results of this study, we suggest that plant essential oils are ideal materials to penetrate the skin, which in turn will stimulate KCs to produce antioxidant enzymes. Considering that the total polyphenol content of our tested enzymes varies, a mixture of candidate oils may yield more promising anti-photoaging activity in the skin.

Currently, novel cosmeceutical products are expected to not only improve cosmetic outcomes but also to possess pharmacological actions such as antioxidant activity, in order to improve skin inflammation and skin aging (Baumann, 2007). In this respect, our results conform to this ideal by showing potent antioxidant and detoxifying actions of the tested plant essential oils, which are good candidate ingredients for the development of novel cosmeceuticals in the future. Clinical studies regarding their beneficial effect in the skin are required for further development of new skin products containing these plant extracts. We expect that a combination of essential oils with good biological efficacy is likely to create a synergistic effect in the production of antioxidant and detoxifying activities in the skin.

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