UVC-Protective Activity of Lemongrass Among 12 Fat-soluble Herbal Extracts: Rapid Decay Due to Cytotoxicity

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Abstract. Background/Aim: The COVID-19 pandemic led to the rapid spread of the use of ultraviolet C (UVC) sterilizers in many public facilities. Considering the harmful effects of prolonged exposure to UVC, manufacturing of safe skin care products is an important countermeasure. In continuation of our recent study of water-soluble herbal extracts, the present study aimed at searching for anti-UVC components from fat-soluble herbal extracts. Materials and Methods: Human dermal fibroblast and melanoma cells were exposed to UVC (1.193 W/m²) for 3 min. Viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell-cycle analysis was performed using a cell sorter. UVC-protective activity was quantified by the selective index (SI), i.e., the ratio of the 50% cytotoxic concentration for unirradiated cells to the concentration that restored viability of UVC-treated cells by 50%. Results: Only lemongrass extract, among 12 fat-soluble herbal extracts, showed significant anti-UVC activity, comparable to that of lignified materials and tannins, but exceeding that of N-acetyl-L-cysteine and resveratrol. Lemongrass extract was highly cytotoxic, producing a subG1 cell population. During prolonged incubation in culture medium, the anti-UVC activity of lemongrass extract, sodium ascorbate and vanillic acid declined with an approximate half-life of <0.7, 5.4-21.6, and 27.8-87.0 h, respectively. Conclusion: Removal of cytotoxic principle(s) from lemongrass extract is crucial to producing long-lasting UVC-protective effects.

Infection by severe acute respiratory syndrome coronavirus 2 has caused a pandemic with serious morbidity and mortality (1). This led to the dissemination of ultraviolet C (UVC) sterilization apparatuses in commercial, public, transportation, and health care facilities as a measure to prevent the virus from spreading and to protect public health (2). Although UVC has strong bactericidal (3) and viricidal (4) activities, prolonged exposure to UVC is harmful to humans (5, 6). Therefore, it is important to search for substances that reduce the toxicity of UVC for users of such devices. Sakagami et al. recently reported that human non-malignant cells are generally resistant to UVC irradiation, as compared with human malignant cell lines (7), and that
components (12). It is thus necessary to investigate not only their toxicity. In the present study, the UVC-protective irradiation, but declined thereafter to a basal level, possibly due to their cytotoxicity or the coexistence of cytotoxic compared with vitamin C and vanillin (12). Their potent willow) showed several-fold lower UVC protection melanoma (COLO679) cell lines. Selected herbs (butterbur, Izawa et al. recently investigated the anti-UVC activity of a total of 108 hot-water-soluble herbal extracts (including 59 herbal extracts recommended by the Japan Medical Herb Association) against human dermal fibroblast (HDFa) and melanoma (COLO679) cell lines. Selected herbs (butterbur, clove, curry tree, evening primrose, rooibos, stevia and willow) showed several-fold lower UVC protection compared with vitamin C and vanillin (12). Their potent UVC-protective activity was maintained for at least 6 h post irradiation, but declined thereafter to a basal level, possibly due to their cytotoxicity or the coexistence of cytotoxic components (12). It is thus necessary to investigate not only the UVC-protective activity of candidate substances but also their toxicity. In the present study, the UVC-protective activity of 12 fat-soluble herbal extracts was quantified by the selectivity index (SI) (also known as chemotherapeutic index), using regular culture medium instead of phosphate buffer as irradiation medium, in order to prevent cell detachment during and after irradiation (7).

Table I. Selectivity index (SI) values representing the anti-ultraviolet C (UVC) activity of 12 fat-soluble herbal extracts, sodium ascorbate and vanillic acid. SI=CC_{50}/EC_{50} where CC_{50} was the 50% cytotoxic concentration and EC_{50} the concentration that restored half of the UV-induced decrease in viability.

<table>
<thead>
<tr>
<th>SI</th>
<th>HDFa</th>
<th></th>
<th>COLO679</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>48 h</td>
<td>0 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Herbal extract (Latin name): Extracted part</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bergamot (Citrus aurantium bergamia): Fruit peel</td>
<td>0.76</td>
<td>0.46</td>
<td>0.92</td>
<td>0.36</td>
</tr>
<tr>
<td>Clary sage (Salvia sclarea): Flowers and leaves</td>
<td>0.75</td>
<td>0.6</td>
<td>0.86</td>
<td>0.33</td>
</tr>
<tr>
<td>Eucalyptus (Eucalyptus globulus): Leaves</td>
<td>1.0</td>
<td>1.0</td>
<td>1.08</td>
<td>0.86</td>
</tr>
<tr>
<td>Geranium (Pelargonium graveolens): Leaves</td>
<td>0.66</td>
<td>0.56</td>
<td>0.71</td>
<td>0.46</td>
</tr>
<tr>
<td>Lavender (Lavandula officinalis): Flowers</td>
<td>0.86</td>
<td>0.69</td>
<td>0.83</td>
<td>0.99</td>
</tr>
<tr>
<td>Lemongrass (Cymbopogon flexuosus): Leaves</td>
<td>57.3</td>
<td>0.01</td>
<td>28.4</td>
<td>0.01</td>
</tr>
<tr>
<td>Neroli (Citrus aurantium): Flowers</td>
<td>0.97</td>
<td>0.71</td>
<td>1.0</td>
<td>0.55</td>
</tr>
<tr>
<td>Peppermint (Mentha piperita): Leaves</td>
<td>0.97</td>
<td>0.71</td>
<td>0.86</td>
<td>0.34</td>
</tr>
<tr>
<td>Rosemary (Rosmarinus officinalis): Flowers</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Tea tree (Melaleuca alternifolia): Leaves</td>
<td>1.0</td>
<td>0.92</td>
<td>1.0</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Cells were UVC irradiated or not in triplicate for 3 min without (control), or with 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 or 1000 μg/ml of each herbal extract in culture medium. Data are derived from Figure 3.

Flavonoids that are widely distributed in nature also show UV-protective effects (9) and are used as plant-derived medicinal ingredients (10), however, as far as we are aware, anti-UVC activity and cytotoxicity have not been compared using the same cells.

Herbs are used for cooking, deodorization, and in maintenance and improvement of health in daily life. The Japan Medical Herb Association (11) has engaged in disseminating useful information of their medicinal efficacy. Degradation products of lignin showed prominent anti-UVC activity, comparable with that of sodium ascorbate (8).

Materials and Methods

Materials. Dulbecco’s modified Eagle’s medium, fetal bovine serum, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); dimethyl sulfoxide (DMSO) was from Wako Pure Chemical Ind., (Osaka, Japan); sodium ascorbate and vanillic acid were from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan); and 96-microwell plates were from Techno Plastic Products AG (Trasadingen, Switzerland). Twelve fat-soluble herbal extracts (Table I) were provided by Green Flask Laboratory (Tokyo, Japan) and diluted with DMSO.

Cell culture. Human dermal fibroblasts adult (HDFa) from adult skin (C0135C; Thermo Fisher Scientific, Waltham, MA, USA) and human melanoma cells (COLO679) (R21-0267; Riken Cell Bank, Tsukuba, Japan) were cultured at 37°C in regular culture medium [Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate] in a humidified atmosphere with 5% CO₂ (7). HDFa cells were harvested by
treatment with 0.25% trypsin-EDTA and seeded at a 1:4 ratio, with an intermediate medium change, to increase the population doubling level (PDL) by two in 1 week. HDFa cells at 16-34 PDL, showing comparable UVC sensitivity (12), were used in this study. COLO679 cells were similarly detached by trypsinization and seeded at a 1:8 ratio to allow their logarithmic growth, when they became near-confluent.

UVC irradiation. The 96-microwell plates on which cells were growing were placed at 555 mm from the center of a UVC lamp (254 nm, germicidal lamp GL15; Toshiba Co. Ltd., Tokyo, Japan) set within an MCV-B131F BioClean Bench (Panasonic Healthcare Co., Ltd., Tokyo, Japan) (Figure 1A). The radiation intensity at the center below the UVC lamp as measured by a UVC radiometer (Gigahertz Optik GmbH, Tuerkenfeld, Germany) was 1.193 W/m² (12). In experiments, cells were irradiated for 3 min.

UVC protection assay. Cells (3×10⁴/ml, 0.1 ml) were inoculated in the inner 60 wells of a 96-microwell plate. The surrounding 36 exterior wells were filled with 150 μl of sterile distilled water to minimize the evaporation of water from the culture medium (Figure 1A), as described previously (12). Cells were incubated for 48 h to achieve complete attachment to the plate. After replacing the medium with fresh culture medium containing different concentrations [0 (control), 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 or 1000 μg/ml] of test extracts, cells were irradiated for 3 min by UVC as described above. After changing the medium (Method I) or without changing it (Method II) (Figure 1B), cells were incubated for up to 48 h to determine the viable cell number by MTT method (7, 8). From the dose–response curve, the 50% cytotoxic concentration (CC₅₀) and the concentration that restored UVC-induced loss of viability by 50% (EC₅₀) were determined in triplicate. The selectivity index (SI) was then determined using the following equation: SI=CC₅₀/EC₅₀ (Figure 2).

Calculation of half-life of UVC-protective activity during culture. If the remaining UVC-protective activity at time t is N, N can be calculated by the following equation:

\[ N = N_0 \times (1/2)^{t/t_{1/2}} \]

where \( t_{1/2} \) is the half-life, and \( N_0 \) is the initial UVC-protective activity.

Cell sorter analysis. COLO679 cells (3×10⁴/ml, 10 ml) were inoculated in a 10-cm dish. Cells were incubated for 48 h to achieve complete attachment to the dish. The culture medium was then replaced with fresh containing different concentrations of test extracts and cells were irradiated for 3 min with UVC as described above. Cells were incubated for 24 or 48 h without medium change (Method II) (Figure 1B). Both non-adherent and adherent cells were harvested and combined. Cells were then fixed, digested with RNase A, stained with propidium iodide, filtered through Falcon ® 40 μm cell strainer (Corning, Inc., Corning, NY, USA) and then subjected to cell sorting based on the DNA content. The samples were analyzed using an SH800 cell sorter (Sony, Tokyo, Japan) (7, 8).

Statistical analysis. Experimental data are expressed as the mean±standard deviation of triplicate determinations. Student’s t-
Results

Prominent UVC-protective activity of lemongrass extract among 12 fat-soluble herbal extracts. Relative UVC-protective activity of 12 fat-soluble herbs was investigated in HDFa and COLO679 cells, using Method I (Figure 3, upper panel) and Method II (Figure 3, lower panel). From the dose–response curves, the CC50, EC50 and SI values were determined (Table I). Among the 12 herbal extracts, only lemongrass (Cymbopogon flexuosus) showed significant UVC-protective activity (SI=57.3 for HDFa; and 28.4 for COLO679). However, when cells were incubated for 48 h at 37°C with medium containing lemongrass extract, the cytotoxicity of lemongrass extract became apparent, and UVC-protective activity (SI) of lemongrass diminished to the baseline (lower panel in Figure 3; Table I).

Rapid decay of UVC-protective activity of lemongrass. The stability of UVC-protective activity of lemongrass extract, sodium ascorbate and vanillic acid during long-term culture was next investigated. HGFa cells were exposed to UVC irradiation for 3 min in the presence of various concentrations of lemongrass, and incubated for a further 6, 24 or 48 h without medium change (Method II), followed by further incubation for 0, 24 or 48 h in fresh culture medium (Method I) (Figure 1B). With longer exposure to lemongrass extract, cytotoxicity became more pronounced and the dose–response curve of cell viability shifted to the left. The SI of lemongrass was drastically reduced after 6 h incubation in both HGFa and COLO679 cells (Figure 4 A), with an approximate half-life (t1/2) of <0.7 h (Figure 4B). This was not due to any cytotoxic effects of DMSO, in which lemongrass extract was dissolved, since exposure to 1% DMSO for 6 h did not affect the viability of cells (12). Therefore, the rapid decay of UVC-protective activity is due to cytotoxicity of components of lemongrass extract itself. It should be noted that the cytotoxicity of lemongrass measured at 48 h (CC50=11 μg/ml for both cell lines) was much greater than those of the other 11 fat-soluble herbal extracts (CC50>464 μg/ml for HDFa; CC50>332 μg/ml for COLO679) (Figure 3).

Vanillic acid had high UVC-protective activity (SI=92.0 and 82.8, respectively), confirming findings in our previous report (12). Furthermore, the UVC-protective activity of vanillic acid was more stable (Figure 4A). After 48 h, 68% (62.7/92.0) (HDFa) and 30% (24.7/81.8) (COLO679) of initial UVC-protective activity remained, yielding t1/2 of 87 and 27.8 h, respectively (Figure 4B).

Sodium ascorbate showed comparable or slightly less UVC-protective activity (SI=69.5 and 47.2, respectively) (Figure 4A) than vanillic acid. However, only 21%
(14.9/69.5) for HDFa and 0.2% (0.1/47.2) for COLO679 of initial UVC-protective activity remained after 48 h, yielding $t_{1/2}$ of 21.6 and 5.4 h, respectively (Figure 4B).

**Induction of subG1 population by lemongrass extract.** Microscopical observation (Figure 5A) revealed that treatment of COLO679 with actinomycin D (1 μM, 24 h) and UVC irradiation for 3 min (following 24 h incubation) induced cell shrinkage, a characteristic of apoptosis induction (13, 14). In contrast, lemongrass extract at 100 μg/ml induced cell enlargement at 24 h.

UVC irradiation for 3 min following 24 and 48 h incubation time-dependently increased the subG1 population (to 22.1% and 60.0%, respectively (Figure 5B). Lemongrass extract at 10 μg/ml only slightly, not significantly, reduced the production of the subG1 population, in contrast to water-soluble rooibos extract (375 μg/ml), which significantly reduced the UVC-induced production of subG1 population ($p<0.05$) (Figure 5B).

**Discussion**

The present study investigated the UVC-protective activity of a total of 12 fat-soluble herbal extracts. We demonstrated that only lemongrass extract showed UVC-protective activity [SI=57.3 (for HDFa); 28.4 (COLO679)], while the other 11 fat-soluble herbal extracts were inactive (SI<1) (Table I). Taking into consideration our previous reports (7, 8, 12),
lemongrass extract was found to show UVC-protective activity comparable with that of lignified materials and tannins, and much higher than water-soluble herbal extract \(^{(8)}\), N-acetyl-L-cysteine and resveratrol \(^{(7)}\) (Figure 6). However, the UVC-protective activity of lemongrass extract disappeared within 6 h, with a short half-life \((t_{1/2} < 0.7\ h)\) (Figure 4B). Since the addition of 1% DMSO (a solvent used for dissolving the samples) to culture medium for 6 h did not reduce cell viability, the rapid decline of SI (which represents ant-UVC activity) by prolonged incubation with lemongrass extract for more than 6 h may be due to the cytotoxic action of lemongrass extract. We found that lemongrass extract at 100 μg/ml produced subG1 population (containing various sizes of DNA fragments) of 38.0% and 67.3% after 24 and 48 h, respectively, suggesting the possible activation of DNases \(^{(14)}\) that may lead to the potent cytotoxicity. However, if such cytotoxic compounds could be removed, for example by washing out with organic solvent, the UVC-protective activity of lemongrass extract should be increased and maintained for much longer. We previously reported that

Figure 4. Time course of anti-ultraviolet C (UVC) activity of lemongrass extract, sodium ascorbate and vanillic acid during prolonged incubation in culture medium. A: HDFa cells (upper panel) and COLO679 cells (lower panel) were irradiated with UVC or not in triplicate for 3 min, and incubated for 0, 6, 24 or 48 h without medium change, and then the medium was replaced with fresh medium and cells were incubated for a further 48, 42, 24 or 0 h, respectively (total incubation time kept to 48 h). The viable cell number was then determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Each value represents the mean±SD. From the dose–response curve, the 50% cytotoxic concentration \((IC_{50})\) and the concentration that restored half of the UV-induced decrease in viability \((EC_{50})\), and SI value \((SI=CC_{50}/EC_{50})\) were determined. B: Calculation of the half-life \((t_{1/2})\) of anti-UVC activity of lemongrass extract, sodium ascorbate and vanillic acid, using the data of 6 h and 24 h \((A)\), according to the formula described in the Materials and Methods. SI values determined in A were plotted vs. incubation time. The concentration axis uses a log scale.
Figure 5. Induction of morphological changes and accumulation of a subG1 population by lemongrass extract, rooibos and actinomycin D. COLO679 cells were irradiated for 3 min without (control), or with the indicated concentrations of actinomycin D (Act D), lemongrass extract (10 or 100 μg/ml), or rooibos (125 or 375 μg/ml), and then incubated for 24 or 48 h without medium change (Method I, Figure 1B). Cells were harvested and then subjected to morphological examination under light microscopy (A) and cell-cycle analysis (B), as described in the Materials and Methods.
n-hexane extraction enhanced the antimicrobial activity of mastic (15). We are planning to isolate anti-UVC components from lemongrass extract and monitor the removal of cytotoxic compounds using gas chromatography-mass spectrometry.

Rao et al. reported that hydroalcoholic extract of *Cymbopogon citratus* (lemongrass also belongs to the *Cymbopogon* genus) scavenged 2,2-diphenyl-2-picryl hydrazyl, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), hydroxyl, superoxide, and nitric oxide free radicals in vitro, and showed moderate anti-lipid peroxidative and antigenotoxic effects (16). Quintans-Júnior et al. reported that citronellal, one of the components of lemongrass extract, exhibited antinociceptive and antioxidant activity, scavenging superoxide and nitric oxide radicals, but not hydroxyl radical (17). However, they did not describe cytotoxicity data that are needed for calculating SI (chemotherapeutic index), thus this makes it difficult to compare our data with theirs.

The present study confirmed our previous finding that sodium ascorbate (SI=69.5 for HDFa; 47.2 for COLO6-79) and vanillic acid (SI=92.0 for HDFa; 81.8 for COLO679) showed prominent UVC-protective activity. However, the UVC-protective activity of sodium ascorbate decayed with t1/2 of 21.6 h for HDFa, and 5.4 h for COLO679 cells (Figure 4B), possibly due to the oxidation of sodium ascorbate and concomitant production of hydrogen peroxide in the presence of oxygen (18). On the other hand, the UVC-protective activity of vanillic acid was much more stable as compared with sodium ascorbate, having much longer t1/2 of 87.0 h for HDFa and 27.8 h for COLO679 (Figure 4B). This suggests that vanillic acid may be suitable for manufacturing skin care products with much less cytotoxicity to skin cells for a longer period compared with sodium ascorbate. Comprehensive studies of the UVC-protective activity of natural and synthetic lignified materials are under way.

Whether lemongrass components and lignin degradation products are also effective against damage induced by mechanical stresses (all types of radiation, and hyperthermia) is also to be pursued.

We reported recently that (i) the UVC-protective activity of phenylpropanoids (8) correlated well with their hydroxyl radical-scavenging activity, as measured by deoxyribose degradation (19), and (ii) N-acetyl-L-cysteine, a supplier of glutathione (20), and resveratrol, which exhibits antioxidant activity (21), did not show any detectable UVC-protective activity (8). This indicates that UVC-protective activity at the initial stage (during 3 min irradiation) may mostly reflect hydroxyl radical-scavenging activity. However, with prolonged incubation, UVC-protective activity declines, depending on the strength of cytotoxicity. The present measurement system using regular culture medium rather than phosphate buffer is suitable for searching for skin care (anti-UVC) substances.

In conclusion, this study demonstrated for the first time that among 12 fat-soluble herbal extracts, only lemongrass exhibited significant UVC-protective effects on cells, which, however declined rapidly due to its potent cytotoxicity. The following aspects remain to be investigated: (i) Identification of the active component(s) in lemongrass; (ii) verification of the UVC-protective effects of current skin care substances; and (iii) confirmation of the correlation between anti-UVC activity and hydroxyl radical-scavenging ability.

**Conflicts of Interest**

S.H. is a representative director of Green Flask Laboratory and provided fat-soluble herbal extracts. However, it was confirmed that such support did not influence the outcome of the experimental study. The Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
Authors’ Contributions

YO, MI and HS performed the experiments of the present study. HS and YO wrote the article. NS, NT, ST, SA, SU, KS, YK, GN, SH and SK reviewed the article. HS, MI and SY designed and interpreted the experimental results. HS provided interpretation of experimental results and edited the article. All Authors read and approved the final version of the article.

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