Insights Into Antioxidant Activities and Anti-Skin-Aging Potential of Callus Extract From *Centella Asiatica* (L.)

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Insights into Antioxidant Activities and Anti-skin-aging Potential of Callus Extract from *Centella asiatica* (L.)

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Abstract

Formation of oxidative stress in dermal fibroblasts plays crucial roles in aging processes of skin. The use of phytochemicals that can promote capacity of fibroblasts to combat oxidative stress is an attractive strategy to prevent skin aging and promote skin beauty. Callus culture offers a powerful platform for sustainable, rapid and large-scale production of phytochemicals to serve extensive demands of pharmaceutical and cosmeceutical industries. Here, we demonstrated the application of callus culture of *Centella asiatica* to produce bioactive metabolites. The 50% ethanolic extract of callus culture has distinctive features of chemical compositions and biological profiles. Results from TLC and HPLC analysis showed that callus extract has unique chemical components, compared with those isolated from authentic plant. Moreover, callus extract possesses promising antioxidant and anti-skin-aging activities. Pre-treatment with callus extract attenuated H₂O₂-induced-cytotoxicity on human dermal fibroblasts. The results from RT-qPCR clearly suggested that the upregulation of cellular antioxidant enzymes appeared to be major contributor for the protective effects of callus extract against oxidative stress. Moreover, supplementation with callus extract inhibited induction of matrix metalloproteinase-9 following H₂O₂ exposure, suggesting its potential anti-skin-aging activity. Our results demonstrate the potential utility of *C. asiatica* callus extract as anti-skin-aging agent in cosmeceutical preparations.

Introductions

Oxidative stress is a cellular phenomenon caused by a redox imbalance between accumulation of reactive oxygen species (ROS) and capacity of cells to detoxify these harmful agents. The excessive induction of oxidative stress is one of the major driving forces of several pathological condition, including aging processes of skin [1].

Fibroblasts in dermal layer of human skin is the principal target of oxidative stress [2]. Induction of oxidative stress in fibroblasts following exposure to external inducers *e.g.* ultraviolet radiation [3], tobacco smoke [4], and air pollutants [5], causes a reduction in biosynthesis of collagen as well as a promotion of degradation of collagen. This aberrant homeostasis of collagen weakens structural integrity and mechanical properties of dermal skin, which eventually resulting in the emergence of aging characteristics, *e.g.* formation of wrinkle and loss of skin elasticity [6]. Hence, the use of compounds or extracts that can enhance the capacity of fibroblasts to neutralize the oxidative stress would be an attractive approach for preventing age-related skin disorders.

Centella asiatica (L.) is a medicinal herb with high economic value. This plant has been traditionally used for treatment of variety diseases in Chinese and Ayurvedic medicines for centuries [7]. Extracts from *C. asiatica* demonstrates a broad range of pharmacological effects through their antioxidant activities in several preclinical models. Treatment with water extract of *C. asiatica* prevented mitochondrial dysfunctions of isolated neurons through an attenuation of oxidative stress [8]. Ethanolic extract of *C. asiatica* decreased oxidative stress *via* an enhanced activities of cellular antioxidant enzymes in Parkinson's model of mice [9]. Oral administration of water extract of *C.*

asiatica suppressed lipid peroxidation in an *in vivo* model of hyperlipidemia [10]. Supplementation of standardized extract of *C. asiatica* inhibited rotenone-induced-hepatotoxicity by inhibition of lipid peroxidation *in vivo* [11]. Altogether, extract from *C. asiatica* has a potential therapeutic utility on oxidative stress-related-disorders, including skin aging.

Naturally grown plant serves as source for secondary metabolites which can be used in cosmeceutical and pharmaceutical industries. However, the major limitations for production of compounds from wild natural plant are the dependence of geographical, seasonal and environmental factors. In addition, further drawbacks for extraction and purification of secondary metabolites from intact plants include complex and time-consuming process; and low concentration of bioactive compounds [12]. To overcome these obstacles, *in vitro* callus culture has been proposed to be an alternative procedure to efficiently produce bioactive metabolites. This *in vitro* method offers a manufacturing system which ensures the continuous supply of compounds with uniform quality and high yields. With this approach, cells of any plants, even rare or endangered species, can be easily manipulated and maintained to produce compounds of interest. The active compounds can be produced independently of external factors. Moreover, plant cell culture is not threatened by micro-organisms or pests [13]. Taken together, plant cell culture represents an attractive platform for mass production of phytochemicals.

In this present study, we demonstrated the application of callus culture of *C. asiatica* to produce bioactive metabolites. The major goals of this study are (1) to evaluate the antioxidant capacities of callus extracts; (2) to investigate the beneficial activities of extracts against oxidative stress in human dermal fibroblasts. Data from our

study will provide an invaluable insight into potential application of *C. asiatica* callus extract in anti-aging cosmeceutical products.

Material and methods

Chemical and reagents

Asiaticoside (98%), asiatic acid (97%), madecassoside (95%), madecassic acid (90%), Kaempferol (97 %), Quercetin (95%), rutin (95%), hydrogen peroxide (H₂O₂), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), adenosine 5'-triphosphate (ATP) disodium salt hydrate, *p*-anisaldehyde, dimethyl sulfoxide (DMSO) and TLC silica gel 60 F₂₅₄ were purchased from MilliporeSigma (Burlington, MA, USA). HPLC-grade acetonitrile and methanol were obtained from J.T. baker (Phillipsburg, NJ, USA). Cell culture media and supplements (minimum essential medium (MEM), fetal bovine serum (FBS), pyruvate, and penicillin/streptomycin) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals in this study were analytical grade.

Plant material

The explants were obtained from in vitro germinated *C. asiatica* seedlings. *C. asiatica* is a commercially available plant and it is grown at the garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand (Latitude-13.7440° N and longitude- 100.5307° E). Experimental research on plants complies with relevant institutional, national, and international guidelines and legislation. The seed of the plant used in the study was collected under the permission of the Department of

Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The plant was identical to a voucher specimen (herbarium number 010018) deposited at Museum of Natural Medicines, Chulalongkorn University, Thailand. To remove dust particles, collected seeds were washed with detergent for 10 min. The pre-washed seeds were sterilized with 70% ethanol for 1 min, followed by 20% commercial bleach solution for 10 min. All subsequent steps were performed under a laminar flow cabinet. The seeds were washed five times with sterile distilled water to remove all traces of sterilant. The sterilized seeds were then inoculated on Murashige and Skoog medium (MS; Murashige and Skoog, 1962), supplemented with 0.5 % AgargellanTM (Phytotechnology Laboratories, USA) plus 3 % sucrose, and kept in dark at $23 \pm 2^{\circ}\text{C}$.

Callus induction

About 1 cm long stolon explants of 15 days old seedling of *C. asiatica* were cut and inoculated on MS medium (pH 5.7) supplemented with 1 mg/L of 1-naphthaleneacetic acid, 1 mg/L of 6-benzylaminopurine, 0.5 % AgargellanTM and 3% sucrose. The cultures were incubated at $23 \pm 2^{\circ}\text{C}$ under 16 h photoperiod and $30 \mu\text{E m}^{-2}\text{s}^{-1}$ irradiance provided by cool white fluorescent tubes. Callus were routinely sub-cultured onto the same medium every 30 days.

Preparation of extracts and standards

About 100 mg of ground callus and authentic plant samples were individually mixed with 1 mL of 50% ethanol (v/v); extracted for 30 minutes by using sonication at 25

°C; and then filtered through Whatman Grade 42 paper (Whatman plc, Maidstone, UK). The filtrate was concentrated to dryness by using Savant SpeedVac SC100 Centrifugal Evaporator (Canada). The dried samples were dissolved in 50% ethanol (v/v). Aliquots of samples were filtered through a 0.22-µm filter membranes for further use.

For standards (asiaticoside, asiatic acid, madecassoside, madecassic acid, kaempferol, quercetin and rutin), the working solutions were prepared in methanol at final concentration of 1 mg/mL and filtered for further use.

Thin layer chromatography (TLC)

The compounds in extracts were separated by using TLC technique. Briefly, 10 µL/band of the *Centella* extract and 5 µL/band of the standards were applied on the TLC plate by using an automatic Linomat 5 applicator (CAMAG, Switzerland) using 8 mm bands, 11 mm distance from the lower edge. The development of plate was done in a 20 cm x 10 cm saturated twin-trough chamber using mobile phase ethyl acetate:methanol:water (100:25:10) up to a migration distance of 90 mm.

For observation of flavonoids, the TLC plates were visualized under UV light at 254 nm. To investigate triterpenoids, the TLC-plate was further derivatized with anisaldehyde sulphuric acid reagent (0.5 mL *p*-anisaldehyde, 10 mL glacial acetic acid, 85 mL methanol and 5 mL concentrated sulfuric acid), following by heating the derivatized plate at 100 °C for 2 min. The chromatograms were then examined under white light. The TLC Visualizer Documentation system (CAMAG, Switzerland) were used to visualize the TLC plates.

TLC-DPPH bioautographic assay

In order to estimate the antioxidant activity of tested samples, TLC-DPPH was performed as a screening assay. After development, the TLC plates was sprayed with 0.2 % DPPH-methanol solution and incubated in dark for 2 hours. The chromatogram was then examined under white light. Generated bands with yellowish color on the purple backgrounds were considered as compounds with antioxidant activities.

DPPH assay

The radical scavenging activities of tested extracts were evaluated by traditional DPPH assay. Briefly, 100 µL of DPPH solution (0.008% w/v DPPH in methanol) was mixed with 100 µL of varying concentrations of callus extract in a 96-well microplate and incubated at 25 °C for 30 min. After incubation, the absorbance was recorded at 517 nm using CLARIOStar microplate reader (BMG Labtech, Germany). 100% methanol was used as a control.

The percentage of radical scavenging activity (%RSA) of sample was calculated using the following formula:

$$\% RSA = \frac{(OD_{517_{Control}} - OD_{517_{sample}}) \times 100}{OD_{517_{Control}}}$$

$OD_{517_{Control}}$ = Optical density at 517 nm of 100% methanol; $OD_{517_{sample}}$ = Optical density at 517 nm of samples.

The concentration of extracts that resulted in 50% RSA was estimated from dose-response curve using GraphPad Prism version 9.0 (GraphPad Software).

HPLC analysis

Seven standards, namely, asiaticoside, asiatic acid, madecassoside, madecassic acid, kaempferol, quercetin and rutin, along with the callus extract were used for the analysis. The analysis was performed using a Shimadzu HPLC (LC-20A) connected with a PDA detector. A C-18 column (100 mm x 4.6 mm, 5 µm particle size, Phenomenex, USA) was used. The HPLC analysis was conducted according to the method of Buraphaka and Putalun [14]. The mobile phase used was a gradient of 1% acetic acid (A) and acetonitrile (B) at a flow rate of 1 mL/min with the following linear gradient HPLC solvent program: solvent B was increased from 0 to 25% over 20 min and further increased to 40% over 35 min, followed by further increase to 65% till 40 min and then held at 100% A for 5 min before returning to the initial state. The column temperature was controlled at 30 °C, and chromatograms were recorded at 210 and 320 nm.

Cell culture

Human foreskin fibroblasts, BJ (ATCC® CRL-2522), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The BJ cells were maintained in MEM supplemented with 1mM pyruvate, 10% FBS, and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

Exposure of H₂O₂

H₂O₂ was used as an inducing agent for oxidative stress. A solution of H₂O₂ was freshly prepared in serum-free MEM prior each experiment. To examine the cytotoxicity of H₂O₂, the BJ cells were exposed to H₂O₂ at 200 µM for 1 h. For a control group, the medium was replaced with a fresh serum-free MEM similar to the H₂O₂-treated cells.

Centella extract treatments

Centella extracts were dissolved with DMSO to produce a 1 mg/mL stock solutions. To observe the preventive activities of *Centella* extracts against H₂O₂-induced cytotoxicity, BJ cells were pre-treated with the *Centella* extracts (final concentration, 15 - 60 µg/mL; 0.5% DMSO) for 24 h prior to H₂O₂ treatment. Control groups were incubated with an equivalent amount of DMSO (0.5% DMSO). DMSO at 0.5% is considered as the non-toxic concentration of DMSO for the BJ cells.

Cell viability assay

The cell viability was determined by using MTT assay. Briefly, BJ cells were seeded into 96-well plates at density of 20,000 cells/well. The cells were cultured for 24 h at 37 °C, 5% CO₂ before exposure to the experimental condition. After indicated treatments, exposure media were aspirated, and the cells were washed with phosphate buffer solution (PBS; pH 7.4). The washed cells were incubated in dark with MTT solution (200 µL; 1 mg/mL in serum-free MEM). After 3-h incubation, the MTT solution was removed, and the formazan crystals were dissolved with DMSO (200 µL/well). The absorbance of the formazan solution was then measured at 570 nm using the CLARIOStar microplate reader.

Measurement of an intracellular ATP

The BJ cells were seeded into multiple culture dishes (60 mm) at a density of 250,000 cells per dish in 4 mL of the MEM. Cells were cultured for 24 h at 37 °C, 5% CO₂ before exposure to experimental conditions. Following the indicated treatments, treated cells were trypsinized and resuspended with PBS. Levels of intracellular ATP were immediately measured with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) as previously described [15]. Briefly, a cell suspension (50,000 cells in 100 µL of PBS) was added into each well of opaque walled, 96-well white microplate. Subsequently, 100 µL of the CellTiter-Glo reagent was homogenously mixed with the cell suspension at room temperature to induce cell lysis and start the luminescent reaction. After a 10-min incubation, the luminescent signal was measured using the CLARIOStar microplate reader. The generated luminescent signal is directly proportional to the amount of ATP in the sample. To estimate the intracellular level of ATP, standard curves for each experiment were produced with serial dilutions of ATP solutions (0 – 1,000 µM). The amount of ATP in each sample was calculated from the corresponding standard curve and then further transformed to an intracellular concentration using the cell number.

Real time-quantitative polymerase chain reaction (RT-qPCR)

After treatments, total RNA was isolated from BJ cells by using GENEzol reagent (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to manufacturer's protocol. One microgram of extracted RNA was then reverse transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA,

USA). The RT-qPCR was carried out in CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instruction. The expression of interested genes were quantified by using $\Delta\Delta C_q$ approach with normalization to house-keeping β -Actin. The PCR conditions and sequences of primer nucleotide (Macrogen, Seoul, South Korea) are listed in Supplementary Table S1 and S2, respectively.

Statistical analysis

The unpaired *t*-test and one-way analysis of variance (ANOVA) with Tukey's *post hoc* were conducted to evaluate statistical differences between means. All means and standard errors of the mean (SEMs) were calculated from three independent experiments. Data were expressed as the mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism version 9.0.

Results

Induction of Centella callus culture

We found that there are two distinct colors of callus culture of *C. asiatica*, green and brown. These two calluses can be obtained depending upon time of collection. Green callus was harvested after 30 days, while brown callus was collected after 45 days (Supplementary Fig. 1). The difference in characteristic of these callus is possibly due to a variation on their chemical components. Hence, we evaluated the chemical profiles and evaluated antioxidant activities of 50% ethanolic extracts from both types of calluses.

Antioxidant activities of Centella extracts

Due to their abundance and pharmacological activities, asiaticoside, asiatic acid, madecassoside and madecassic acid are considered to be major active components of *C. asiatica* [7, 16]. To examine whether 50% ethanolic extracts (green callus extract, GCE; brown callus extract, BCE; and authentic plant extract, APE) contains these triterpenoids, active compounds in extracts were separated with TLC approach. Surprisingly, none of these four triterpenoids were detected in GCE, BCE as well as APE (Fig. 1A). To screen active antioxidants in extracts, TLC plates were further derivatized with DPPH reagent. A stable free radical DPPH is a widely used substrate to evaluate free radical scavenging activities of tested compounds. On TLC plates, the compounds generating yellow zones against the purple background were considered as antioxidants. As shown in Fig. 1B, all of tested triterpenoids did not show antioxidant activities. Moreover, GCE and APE exhibited highly interesting free radical scavenging activities, compared to BCE. These TLC-DPPH data suggested that asiaticoside, asiatic acid, madecassoside and madecassic acid are not contribute to antioxidant properties of extracts.

Kaempferol, quercetin and rutin reported in *C. asiatica* [17, 18] are flavonoid compounds with antioxidant activities. Chromatogram on TLC plate demonstrated that these three flavonoids were not presented in any extracts (Fig. 2A). Consistent with data on Fig. 1B, the data from TLC-DPPH showed that GCE and APE exhibited higher antioxidant activities than BCE, as evidenced by a greater intensity of yellow spots (Fig. 2B). Moreover, *Centella* extracts exhibited potent radical scavenging properties without the presence of kaempferol, quercetin and rutin (Fig. 2B). Taken together, these results

strongly suggested that antioxidant activities of extracts are not due to these three flavonoids but possibly from other flavonoids.

As shown in Fig. 1 and 2, the antioxidant properties of GCE were comparable to APE. We then further evaluate total antioxidant activity of these two 50%-ethanolic extracts by determination of IC₅₀ of %RSA with DPPH assay. The IC₅₀ of %RSA represents the concentration of extracts that is required for 50% free radical scavenging activity. We exhibited that GCE had approximately 2.5 folds greater ability to neutralize free radicals than APE (Supplementary Fig. 2; GCE, 98.6 ± 1.6 µg/mL v.s. APE, 243.3 ± 14.6 µg/mL). These results suggest that extract from callus culture possesses a strong free radical scavenging ability, which is even greater than from native plant.

HPLC analysis of Centella extracts

HPLC was conducted to analyze and compare chemical compositions in *Centella* extracts (Fig. 3) and confirmed the result of TLC technique. On comparison with retention time (RT) between standards and extracts, GCE and APE did not contain asiaticoside (RT = 30.48 min), asiatic acid (RT = 44.13 min), madecassoside (RT = 28.35 min), madecassic acid (RT = 42.21 min), kaempferol (RT = 37.68 min), quercetin (RT = 32.68 min) and rutin (RT = 22.88 min). These HPLC data are consistent with TLC results demonstrated in Fig. 1 and 2. Moreover, GCE demonstrated different chemical profiles in term of number of compounds, when compared to APE and BCE. GCE contains greater number of compounds as observed by the presence of higher number of peaks. Hence, the difference in antioxidant capacity between GCE v.s. APE could possibly be due to variation in active components.

Protective effects of Centella extracts against oxidative damage on human fibroblasts

The compounds with antioxidant capacity have been proposed as promising candidate for anti-skin-aging agents [19]. According to cell-free based antioxidant assay, GCE and APE were selected to further examine their beneficial activities on human dermal fibroblast BJ cells. Firstly, we evaluated potential toxicity of GCE and APE on dermal fibroblasts. The data from MTT assay demonstrated that 24-h exposure of GCE or APE, up to 60 $\mu\text{g/mL}$, did not cause toxicity to BJ cells (Fig. 4A, 4B). Hence, concentrations of CE and APE at 15 – 60 $\mu\text{g/mL}$ were chosen for subsequent experiments.

To investigate the preventive effects of GCE and APE against oxidative insults, BJ cells were pre-treated with GCE or APE at non-toxic concentration for 24 h prior to H_2O_2 treatment. Exposure to H_2O_2 (200 μM ; 1 h) reduced cell viability of BJ cells to approximately 25% (Fig. 4C). Pre-treatment with GCE significantly inhibited H_2O_2 -induced toxicity (Fig. 4C). Interestingly, the protective effects of GCE were comparable to APE pre-treatments (Fig. 4D). These data suggested that GCE and APE could exert their beneficial effects against H_2O_2 on dermal fibroblasts *via* their antioxidant activities.

The imbalance of cellular bioenergetics due to oxidative stress is closely linked to aging processes of several tissues, including skin [20]. Exposure to H_2O_2 caused an immediate depletion of steady-state levels of ATP in dermal fibroblasts (Fig. 4E). In contrast, treatment with non-toxic concentration of GCE (60 $\mu\text{g/mL}$; 24 h) did not alter intracellular levels of ATP, supporting the safety of GCE on the selected concentration on BJ cells (Fig. 4E). Interestingly, pre-treatment with GCE significantly inhibited

reduction of intracellular ATP pool following H₂O₂ treatment in dose-dependent fashion (Fig. 4F). The results from these bioenergetics studies were consistent with the protective effects of GCE against oxidative damage from MTT assay, highlighting the contribution of antioxidant activity of GCE on its protective effect against oxidative insults.

Effects of Centella extracts on expression of antioxidant enzymes

The beneficial effects of GCE and APE against oxidative damage as showed in Fig. 4 are possibly due to an induction of capacity of fibroblasts to detoxify reactive oxygen species (ROS). To examine this hypothesis, the mRNA expression of key antioxidant enzymes, including catalase (CAT), glutathione peroxidase 1 (GPx1), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2), were measured following 24-h treatments. Our RT-qPCR results demonstrated that GCE and APE have different profiles on the induction of antioxidant machinery of fibroblasts; GCE induced expression of *CAT* in dose-dependent fashion (Fig. 5A), while APE enhanced expression of both *SOD1* (Fig. 5C) and *SOD2* (Fig. 5D). The expression of *GPx1* did not alter with either GCE or APE treatment (Fig. 5B). These RT-qPCR data suggested that an upregulation of cellular antioxidant enzymes is the principal factor for protective effects of GCE and APE.

We also observed the effects of *Centella* extracts on expression of antioxidant enzymes after H₂O₂ treatment. Exposure to H₂O₂ leaded to dramatic decrease in *GPx1* (Fig. 5B) and *SOD1* (Fig. 5C); and significant increase in *SOD2* (Fig. 5D). The increase of *SOD2* expression is possibly due to adaptive mechanism of fibroblasts following H₂O₂

treatment. This phenomenon has been reported in several models of aged fibroblasts [21-23]. Again, CE and APE demonstrated distinctive effects on induction of antioxidant enzymes following H₂O₂ exposure. Pre-treatment with GCE induced *CAT* (Fig. 6A), *GPx1* (Fig. 6B) and *SOD1* expression (Fig. 6C), whereas APE upregulated *SOD2* expression following 1-h exposure to H₂O₂ (Fig. 6D). Altogether, these results clearly exhibited that *Centella* extracts could prevent H₂O₂ cytotoxicity by an enhanced capacity of fibroblasts to remove deleterious ROS.

Effects of Centella extracts on expression of matrix metalloprotease 9 (MMP-9)

Matrix metalloprotease-9 (MMP-9) is the gelatinase enzyme that is responsible for regulation of homeostasis of collagen. Formation of oxidative stress can lead to upregulation of MMP-9 in fibroblasts, which subsequently results in degradation of collagen. The increased breakdown of collagen by MMP-9 appears to be the major factor for aging processes of skin tissue [24, 25]. Here, we demonstrated that exposure to H₂O₂ markedly upregulated the expression level of *MMP-9* of dermal fibroblasts (Fig. 7A). Elevation of *MMP-9* expression was significantly suppressed by GCE and APE pre-treatment (Fig. 7B). These data suggested that GCE and APE may possess anti-aging activities *via* an inhibition of *MMP-9* transcription.

Discussions

C. asiatica is a medicinal plant with broad range of pharmacological effects *e.g.* antioxidant [8-11], anti-inflammation [26, 27], wound healing [28-30], neuroprotective [8, 9] and memory improvement [31, 32]. Due to its high therapeutic potential, the demand for this plant in cosmeceutical and pharmaceutical industries has exceeded the supply

from conventional cultivation. Callus culture offers a manufacturing system which ensures the continuous supply of compounds with uniform quality and high yields [13]. Here, we demonstrated that 50% ethanolic extracts from callus culture has unique chemical profiles and biological activities. Compared to APE, the data from TLC (Fig. 1,2) and HPLC (Fig. 3) suggested that (i.) GCE comprises greater number of compounds; (ii.) the types of secondary metabolites in GCE are different from APE. Interestingly, results from DPPH assay showed that the total antioxidant activity of GCE is higher than APE (Supplementary Fig. 2). The greater antioxidant activity of GCE is possibly due to the differences in active components in the extracts. We demonstrated that the major triterpenoids of *C. asiatica* (asiaticoside, asiatic acid, madecassoside and madecassic acid) are not responsible for antioxidant activities of these extracts (Fig. 1). These findings are parallel to previous observation demonstrating that triterpene-free-extract of *C. asiatica* possesses greater *in vitro* radical scavenging properties as well as *in vivo* antioxidant activities than triterpene-enriched-extract [33]. Kaempferol, quercetin and rutin have been reported to be major flavonoids with antioxidant properties of *C. asiatica* [17, 18]. Our TLC-DPPH and HPLC results demonstrated that these flavonoids did not present in 50% ethanolic extracts from both callus culture and authentic plant; then these series of flavonoids did not contribute to antioxidant activities of these extracts (Fig. 2). Hence, future studies are required to identify and characterize novel antioxidants presented in these extracts.

We further investigated biological activities of *Centella* extracts against oxidative stress on dermal fibroblasts. Our data from MTT assay as well as bioenergetic study showed that GCE significantly inhibited the cytotoxicity of H₂O₂ on dermal fibroblasts

(Fig. 4). The preventive effects of GCE were comparable to APE. Moreover, we found that the protective activities of these extracts could be due to an increase in capacity of fibroblasts to eliminate ROS. We used RT-qPCR approach to investigate the alterations of mRNA expression of key antioxidant enzymes in response to treatments. Catalase is the major enzyme involved in the elimination of high fluxes of H_2O_2 , while GPx1 is responsible for detoxification of low fluxes of H_2O_2 [34]. SOD1 is a cytosolic Cu/Zn-superoxide dismutase, whereas SOD2 is a mitochondrial Mn-superoxide dismutase. Both SOD1 and SOD2 are crucial for removal of superoxide free radical ($\text{O}_2^{\bullet-}$) during oxidative stress condition. The dismutation of $\text{O}_2^{\bullet-}$ by SOD leads to generation of H_2O_2 , which in turn eliminated by catalase and other peroxidases [35]. RT-qPCR data revealed that GCE and APE have different downstream targets on cellular antioxidant machineries. Treatment with GCE promoted mRNA expression of H_2O_2 -detoxifying enzyme, *CAT*. Supplementation with APE enhanced mRNA expression of $\text{O}_2^{\bullet-}$ -removal enzymes, *SOD1* and *SOD2* (Fig. 5). In response to H_2O_2 treatment, pre-exposure to GCE induced *CAT*, *GPx1* and *SOD1* expression, whereas pre-treatment with APE upregulated *SOD2* expression (Fig. 6). Upregulation of these antioxidant machineries due to *Centella* extracts could promote capacity of fibroblasts to eliminate harmful ROS, resulting in suppression of oxidative damage and prevention of cell death. Further studies on protein levels and enzymatic activities of antioxidant machineries following treatments of GCE and APE are necessary to better understand the protective mechanisms of these extracts on fibroblasts.

In addition to effects on cellular antioxidant enzymes, we also observed activities of *Centella* extracts on expression of *MMP-9*. MMP-9 is zinc-containing-gelatinase

which plays an important role in degradation of dermal extracellular matrix, especially collagen type IV. Environmental insults, *e.g.* UV irradiation, tobacco smoke and pollutants, have been reported to induce expression of *MMP-9* of skin cells through formation of oxidative stress [25]. Upregulation of *MMP-9* leads to fragmentation of dermal collagen; thereby diminish skin elasticity and integrity; and eventually promote wrinkle and sagging formation of skin [24, 36]. Hence, agents with *MMP-9* inhibitory activities would be an attractive candidate to combat skin aging [37]. Our RT-qPCR data demonstrated that GCE and APE significantly inhibited upregulation of *MMP9* following H_2O_2 exposure (Fig. 7), suggesting anti-skin-aging activities of these extracts. Future observations on detailed mechanisms of GCE/APE-mediated-*MMP9*-expression are required to better understand their anti-skin-aging effects. The proposed mechanisms for antioxidant and anti-skin aging activities of GCE and APE are summarized in Fig. 8.

In conclusion, our present study provides the potential application of callus culture platform to produce biomass and biochemical from *C. asiatica*. We demonstrated that the 50% ethanolic extract from callus culture has unique chemical profiles and distinct antioxidant properties. Moreover, this study provides pharmacological evidence to support the use of *Centella* extracts as anti-skin-aging agents in cosmeceutical and pharmaceutical products.

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Author contributions

V.B., D.R., A.M., K.K. and S.V. conceived and designed the experiments; D.R. and K.K. prepared extracts and performed TLC-DPPH, DPPH and HPLC; V.B. performed MTT and RT-qPCR study; A.M. conducted RT-qPCR study; V.B., D.R., A.M., and K.K. analyzed the data under supervision of S.V.; V.B. wrote main manuscript. All authors have read, revised and provided approval for final manuscript.

Competing interests

The authors declare no competing interests.

Figure Legend

Fig. 1 The antioxidant activities of *Centella* extracts are not due to asiaticoside, asiatic acid, madecassoside and madecassic acid.

TLC fingerprints of triterpene standards (1, asiaticoside; 2, asiatic acid; 3, madecassoside; 4, madecassic acid), and *Centella* extracts (5, GCE; 6, BCE; 7, APE).

A. The TLC plate was derivatized with anisaldehyde sulphuric acid reagent and visualized with white light. **B.** The TLC plate was sprayed with DPPH reagent and visualized with white light.

Fig. 2 The antioxidant activities of *Centella* extracts are not due to kaempferol, quercetin and rutin.

TLC fingerprints of flavonoid standards (1, kaempferol; 2, quercetin; 3, rutin), and *Centella* extracts (4, GCE; 5, BCE; 6, APE).

A. The TLC plate was visualized under UV light at 254 nm. **B.** The TLC plate was sprayed with DPPH reagent and visualized with white light.

Fig. 3 *Centella* extract from callus culture has unique chemical profiles.

HPLC chromatogram of **A**, standard solutions of triterpenes and flavonoids (1, rutin; 2, madecassoside; 3, asiaticoside; 4, quercetin; 5, kaempferol; 6, madecassic acid; 7, asiatic acid); **B**, GCE; **C**, BCE; and **D**, APE (GCE, green callus extract; BCE, brown callus extract; APE, authentic plant extract).

Fig. 4 *Centella* extracts prevent H₂O₂-induced cytotoxicity in dermal fibroblasts.

A and **B**, GCE and APE at 15 – 60 µg/mL did not cause toxicity in dermal fibroblasts. BJ cells were incubated with GCE or APE (15 – 60 µg/mL) for 24 h. **C** and **D**, GCE and APE inhibit oxidative damage from H₂O₂ exposure. BJ cells were pre-incubated with GCE or APE (15 – 60 µg/mL) for 24 h then exposed to H₂O₂ (200 µM) for 1 h. The cell viability was evaluated with MTT assay immediately after the treatments. **E** and **F** GCE inhibit depletion of intracellular ATP following H₂O₂ exposure. The protocol for treatment was as **A** and **C**. The amounts of ATP were measured immediately after treatments ($n = 3$; mean $\pm$ SEM; *, $P < 0.05$ v.s. untreated control; †, $P < 0.05$ v.s. H₂O₂-treated cells).

Fig. 5 *Centella* extracts elevate transcription of antioxidant enzymes.

A - D, GCE promoted *CAT* expression, while APE enhanced *SOD1* and *SOD2* expression. BJ cells were incubated with GCE (15 – 60 µg/mL; 24 h) or APE (60 µg/mL; 24 h) or H₂O₂ (200 µM; 1h). The expressions of antioxidant machineries, including *CAT*, *GPx1*, *SOD1*, and *SOD2*, were determined with RT-PCR following treatments ($n = 3$; mean $\pm$ SEM; *, $P < 0.05$ v.s. untreated control)

Fig. 6 *Centella* extracts promote expression of antioxidant enzymes following H₂O₂ treatment

A - D, In response to H₂O₂ treatment, *CAT*, *GPX1* and *SOD1* expressions were increased in GCE-treated fibroblasts, while *SOD2* transcription were elevated in APE-treated fibroblasts. BJ cells were incubated with GCE (60 µg/mL; 24 h) or APE (60 µg/mL; 24 h), then exposed to H₂O₂ (200 µM; 1h). The transcriptional levels of *CAT*, *GPx1*, *SOD1*, and *SOD2*, were evaluated with RT-PCR following treatments ($n = 3$; mean $\pm$ SEM; †, $P < 0.05$ v.s. H₂O₂-treated cells)

Fig. 7 *Centella* extracts inhibit expression of *MMP9* following H₂O₂ treatment.

A, Exposure to H₂O₂ resulted in elevation of *MMP9* expression, while GCE and APE did not affect the transcription of *MMP9*. BJ cells were treated GCE (15 –60 µg/mL; 24 h) or APE (60 µg/mL; 24 h) or H₂O₂ (200 µM; 1h). **B**, GCE and APE suppressed H₂O₂-induced *MMP9* expression. BJ cells were pre-incubated with GCE or APE (60 µg/mL) for 24 h then exposed to H₂O₂ (200 µM) for 1 h. The transcriptional levels of *MMP9* were evaluated with RT-PCR following treatments ($n = 3$; mean $\pm$ SEM; *, $P < 0.05$ v.s. untreated control; †, $P < 0.05$ v.s. H₂O₂-treated cells)

Fig. 8 Proposed mechanisms for antioxidant and anti-skin aging activities of *Centella* extract

Pre-treatment with *Centella* extracts prevent H₂O₂-induced cytotoxicity on dermal fibroblasts by upregulation of cellular antioxidant machineries. Supplementation with GCE upregulated *CAT*, *GPx1* and *SOD1* expression, whereas pre-treatment with APE

induced *SOD2* expression following H_2O_2 treatment. Moreover, pre-treatment with GCE or APE suppressed H_2O_2 -mediated-upregulation of *MMP-9*.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

A. **B.** **C.** **D.** **Fig. 6**

A. **B.** **C.** **D.** **Fig. 7**

A.

B.

Fig. 8

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Figures

Figure 1

The antioxidant activities of Centella extracts are not due to asiaticoside, asiatic acid, madecassoside and madecassic acid. TLC fingerprints of triterpene standards (1, asiaticoside; 2, asiatic acid; 3, madecassoside; 4, madecassic acid), and Centella extracts (5, GCE; 6, BCE; 7, APE). A. The TLC plate was derivatized with anisaldehyde sulphuric acid reagent and visualized with white light. B. The TLC plate was sprayed with DPPH reagent and visualized with white light.

Figure 2

The antioxidant activities of Centella extracts are not due to kaempferol, quercetin and rutin. TLC fingerprints of flavonoid standards (1, kaempferol; 2, quercetin; 3, rutin), and Centella extracts (4, GCE; 5, BCE; 6, APE). A. The TLC plate was visualized under UV light at 254 nm. B. The TLC plate was sprayed with DPPH reagent and visualized with white light.

Figure 3

Centella extract from callus culture has unique chemical profiles. HPLC chromatogram of A, standard solutions of triterpenes and flavonoids (1, rutin; 2, madecassoside; 3, asiaticoside; 4, quercetin; 5, kaempferol; 6, madecassic acid; 7, asiatic acid); B, GCE; C, BCE; and D, APE (GCE, green callus extract; BCE, brown callus extract; APE, authentic plant extract).

Figure 4

Centella extracts prevent H₂O₂-induced cytotoxicity in dermal fibroblasts. A and B, GCE and APE at 15 – 60 µg/mL did not cause toxicity in dermal fibroblasts. BJ cells were incubated with GCE or APE (15 – 60 µg/mL) for 24 h. C and D, GCE and APE inhibit oxidative damage from H₂O₂ exposure. BJ cells were pre-incubated with GCE or APE (15 – 60 µg/mL) for 24 h then exposed to H₂O₂ (200 µM) for 1 h. The cell viability was evaluated with MTT assay immediately after the treatments. E and F GCE inhibit depletion of intracellular ATP following H₂O₂ exposure. The protocol for treatment was as A and C. The amounts of ATP were measured immediately after treatments (n = 3; mean ± SEM; *, P < 0.05 v.s. untreated control; †, P < 0.05 v.s. H₂O₂-treated cells).

Figure 5

Centella extracts elevate transcription of antioxidant enzymes. A - D, GCE promoted CAT expression, while APE enhanced SOD1 and SOD2 expression. BJ cells were incubated with GCE (15 – 60 µg/mL; 24 h) or APE (60 µg/mL; 24 h) or H₂O₂ (200 µM; 1h). The expressions of antioxidant machineries, including CAT, GPx1, SOD1, and SOD2, were determined with RT-PCR following treatments (n = 3; mean ± SEM; *, P < 0.05 v.s. untreated control)

Figure 6

Centella extracts promote expression of antioxidant enzymes following H₂O₂ treatment A - D, In response to H₂O₂ treatment, CAT, GPX1 and SOD1 expressions were increased in GCE-treated fibroblasts, while SOD2 transcription were elevated in APE-treated fibroblasts. BJ cells were incubated with GCE (60 µg/mL; 24 h) or APE (60 µg/mL; 24 h), then exposed to H₂O₂ (200 µM; 1h). The transcriptional levels of CAT,

GPx1, SOD1, and SOD2, were evaluated with RT-PCR following treatments (n = 3; mean ± SEM; †, P < 0.05 v.s. H2O2-treated cells)

Figure 7

Centella extracts inhibit expression of MMP9 following H2O2 treatment. A, Exposure to H2O2 resulted in elevation of MMP9 expression, while GCE and APE did not affect the transcription of MMP9. BJ cells were treated GCE (15 – 60 µg/mL; 24 h) or APE (60 µg/mL; 24 h) or H2O2 (200 µM; 1h). B, GCE and APE suppressed H2O2-induced MMP9 expression. BJ cells were pre-incubated with GCE or APE (60 µg/mL) for 24 h then exposed to H2O2 (200 µM) for 1 h. The transcriptional levels of MMP9 were evaluated with RT-PCR following treatments (n = 3; mean ± SEM; *, P < 0.05 v.s. untreated control; †, P < 0.05 v.s. H2O2-treated cells)

Figure 8

Proposed mechanisms for antioxidant and anti-skin aging activities of Centella extract Pre-treatment with Centella extracts prevent H_2O_2 -induced cytotoxicity on dermal fibroblasts by upregulation of cellular antioxidant machineries. Supplementation with GCE upregulated CAT, GPx1 and SOD1 expression, whereas pre-treatment with APE induced SOD2 expression following H_2O_2 treatment. Moreover, pre-treatment with GCE or APE suppressed H_2O_2 -mediated-upregulation of MMP-9.

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