Journal of Research in Pharmacy

Research Article

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Properties of Chlorogenic Acid as a Skin Fibroblast Antiaging Agent with Apoptosis Inhibition and Free Radical Scavenging Activities

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Received: 20 December 2022 / Revised: 14 March 2023 / Accepted: 06 April 2023

ABSTRACT: Aging is a skin phenomenon that is caused intrinsically by tissue degeneration and extrinsically by environmental toxicity including ultraviolet (UV) exposure, manifested as wrinkles and skin damage respectively. Chlorogenic acid (CA) possesses roles as antiinflammation and antioxidation by reducing reactive oxygen species (ROS). The present study examined CA anti-aging activities toward skin fibroblast (BJ) cells induced by UV. Antiaging properties of CA were analyzed by assessing the levels of collagen-1α1 (COL-1α1), elastin (ELN), 8-hydroxy 2 deoxyguanosine (8-OHdG), melatonin (MT), and hyaluronidase (HAase), and by quantifying the percentages of living cells, dead cells. CA treatment on aging cells diminished the 8-OHdG, HAase, and a percentage of apoptotic cells. It also improved the levels of COL-1α1, ELN, MT, and the proportion of living cells. This study proved CA ability as an antiaging agent through its roles as a free radical scavenger and anti-apoptotic agent.

KEYWORDS: antiaging; chlorogenic acid; fibroblast; skin; UV.

1. INTRODUCTION

Aging is a natural phenomenon of normal skin, indicated by loss of physiological ability, structure and matrix complexity, and morphological performance [1]. It is intrinsically caused by physiological impairment and extrinsically stimulated by hazardous agents, mainly ultraviolet (UV) ray. UV stimulated aging is indicated by the overproduction of reactive oxygen species (ROS) that causes oxidative stress [2]. Besides, skin aging involves other mechanisms such as mitochondrial dysfunction, disruption of circadian rhythms, inflammation, epigenetic alteration, and decreased capacity for tissue repair [1]. Moreover, UV exposure exacerbates skin degeneration through impairing connective tissue [3].

UV-induced skin aging is initialized by free radical formation and followed by the rise of ROS level, then leads to inflammation and cellular damage, and subsequently causes cell death [4]. ROS stimulates mitogen-activated protein kinase (MAPK) pathway, then increases matrix metalloproteinase (MMP) production, then ultimately reduces collagen (including collagen α type 1/COL-1α1) synthesis, causes collagen and elastin (ELN) fragmentation, and decreases hyaluronic acid (HA) level [2, 5]. Furthermore, the HA reduction involves the hyaluronidase enzyme (HAase) [6]. Moreover, the presence of ROS causes photooxidation reactions which lead to oxidation of the basic groups of skin cell DNA, and result in 8-hydroxydeoxyguanosine (8-OHdG) compound [7]. On the other hand, UV exposure directly damages mitochondria, then stimulates Caspase 9 (Casp-9) activation, activates Casp-3, and ultimately causes apoptosis and leads to skin aging [8]. Inflammation and aging resulted by this occurrence are countered by melatonin hormone (MT) which scavenges ROS and inhibits its derivation [1].

Natural topical skincare generated by phytochemical compounds is on the rise as an antiaging agent through their abilities as antioxidants, skin moisturizers, antiinflammation agents, skin barrier agents, skin

How to cite this article: Girsang E, Lister INE, Ginting CN, Widowati W, Yati A, Kusuma HS, Rizal R. Properties of Chlorogenic Acid as a Skin Fibroblast Antiaging Agent with Apoptosis Inhibition and Free Radical Scavenging Activities. J Res Pharm. 2025; 29(1): 159-167.

ISSN: 2630-6344

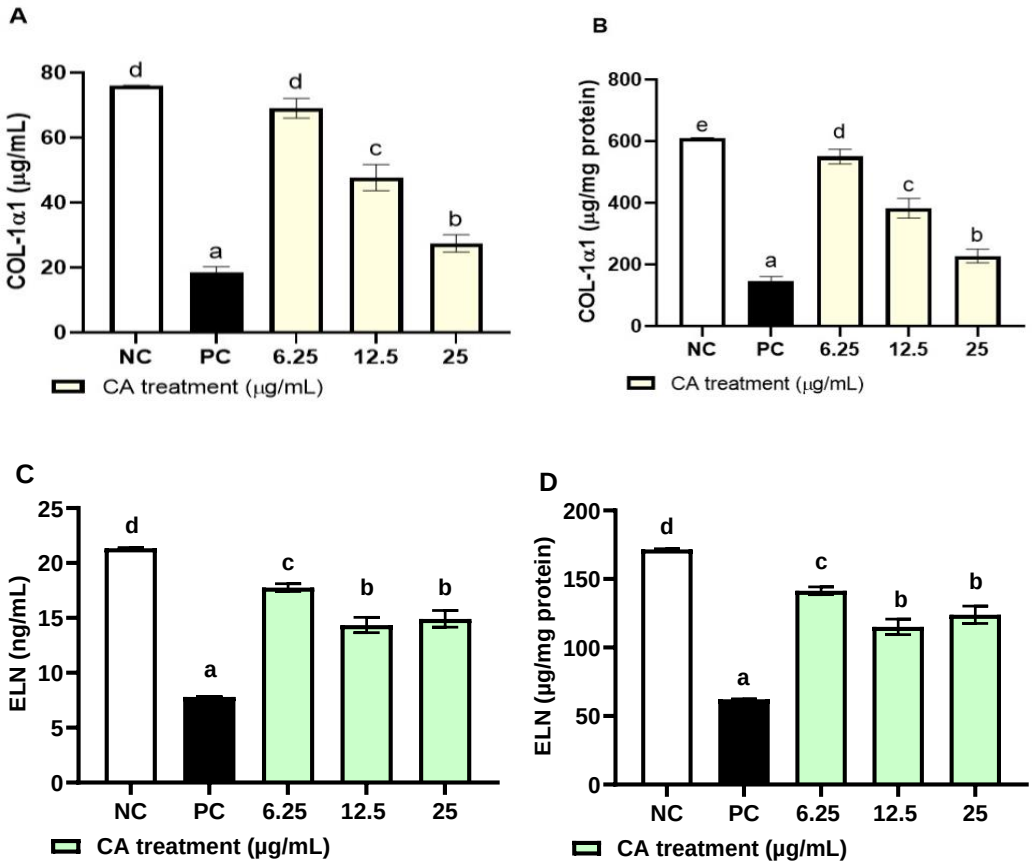
https://doi.org/10.12991/jrespharm.1628594

lightening agents, sunblock agents, and in providing vitamin and hydroxy acids [9]. Girsang et al. (2019) discovered peel extract of snake fruit (*Salacca zalacca* Gaert.) (SPE) potency as an antiaging agent through its chlorogenic acid [10]. Chlorogenic acid (CA) is a hydrocinnamic family with one of the water-soluble phenolic compounds acknowledged as an antioxidant to fight free radicals [11]. CA is reported to mitigate inflammation and counter oxidative stress in both in vivo and in vitro studies [8, 12]. Consequently, the present study was conducted to examine CA anti-aging properties, including COL-1α1, ELN, HAase, 8-OhdG, MT, and apoptosis, in UV-induced human fibroblast cell lines (BJ cells).

2. RESULTS

2.1. The effect of CA treatment on COL-1a1, ELN, 8-OhdG, MT, and HAase levels

The levels of collagen (COL-1α1), elastin (ELN), 8-hydroxydeoxyguanosine (8-OhdG), melatonin (MT), and hyaluronidase (HAase) activities in the conditioned media from the CA-treated skin aging cell models were assessed using ELISA. UV exposure significantly decreased COL-1α1, ELN, and MT levels; and increased 8-OHdG level and HAase activity (Figure 1A-J). Treatment with CA significantly increased COL-1α1, ELN, and MT levels (Figure 1A-D and Figure 1I-J). CA treatment also significantly decreased 8-OHdG level and HAase activity (Figure 1E-H).



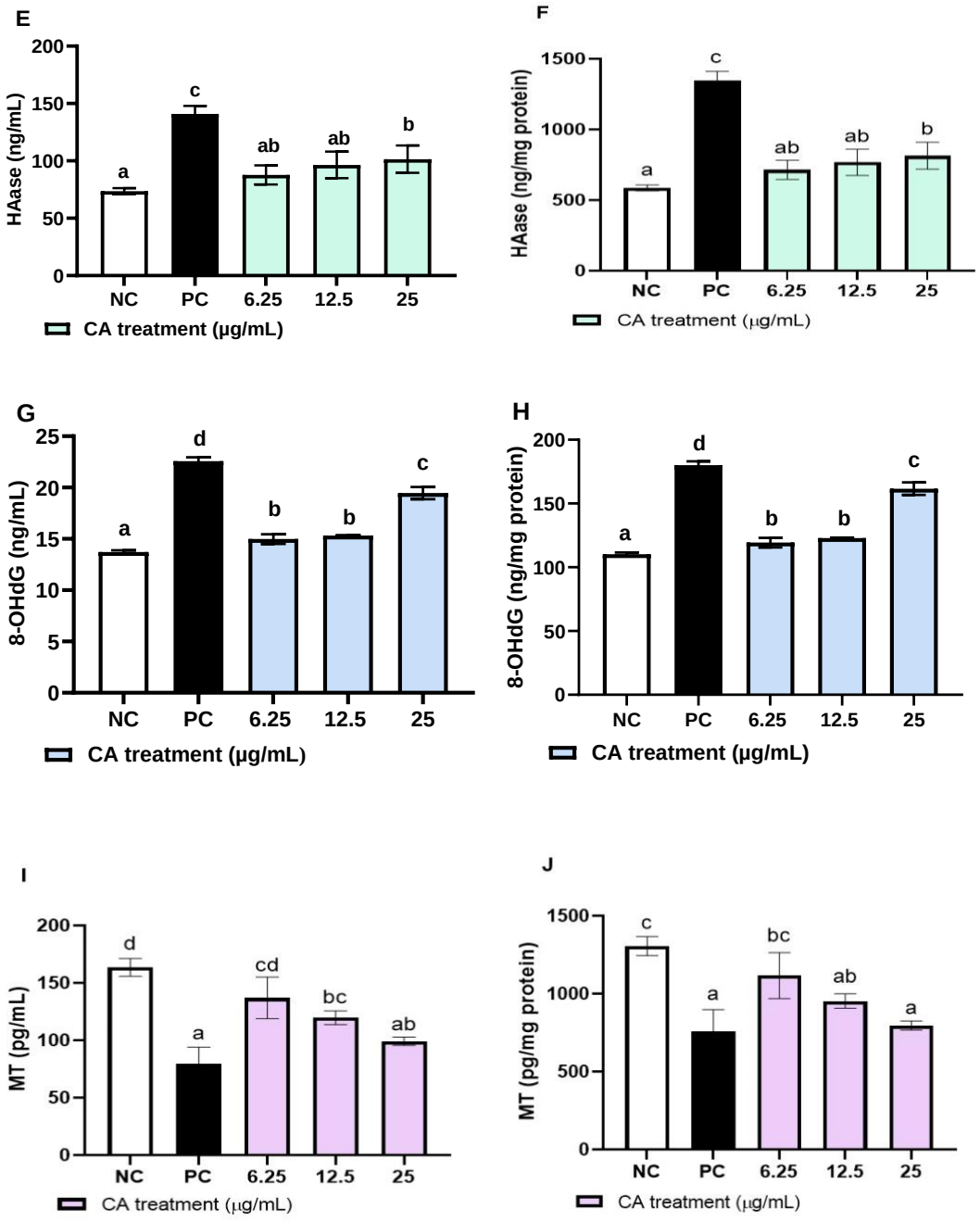


Figure 1. The effects of chlorogenic acid towards COL-1α1, ELN, 8-OhdG, MT, and HAase levels on aging cell models

The graphs are displayed as mean ± standard deviation.

NC: BJ cells (negative control/untreated cells); PC: UV-induced fibroblast cells (positive control); 6.25: UV-induced fibroblast cells + CA 6.25 µg/mL; 12.5: UV-induced fibroblast cells + CA 12.5 µg/mL; 25: UV-induced fibroblast cells + CA 25 µg/mL.

*Different letters (a, b, c, and d in figure 1A, 1C-D, and 1G-H; a, b, c, d, and e in figure 1B; a, ab, b, and c in figure 1E-F; a, ab, bc, cd, and d in figure 1I; and a, ab, bc, and c in figure 1J) indicate significance in accordance with Tukey HSD post hoc test ($p \leq 0.05$).

2.2. The effect of CA treatment on apoptotic, living, and necrotic cells in aging cells model

Necrotic cells, living cells, and apoptotic cells of CA treated skin aging cell models were quantified using flow cytometer. The dot blots represented the population of the analyzed cells (Figure 2); while the graphs displayed the percentages. UV exposure significantly led to apoptosis and decreased living cells. CA treatment as much as 6.25, 12.5, and 25 µg/mL significantly ameliorated the proportion of living cells without diminishing necrotic cells and declined the number of apoptotic cells (Figure 3).

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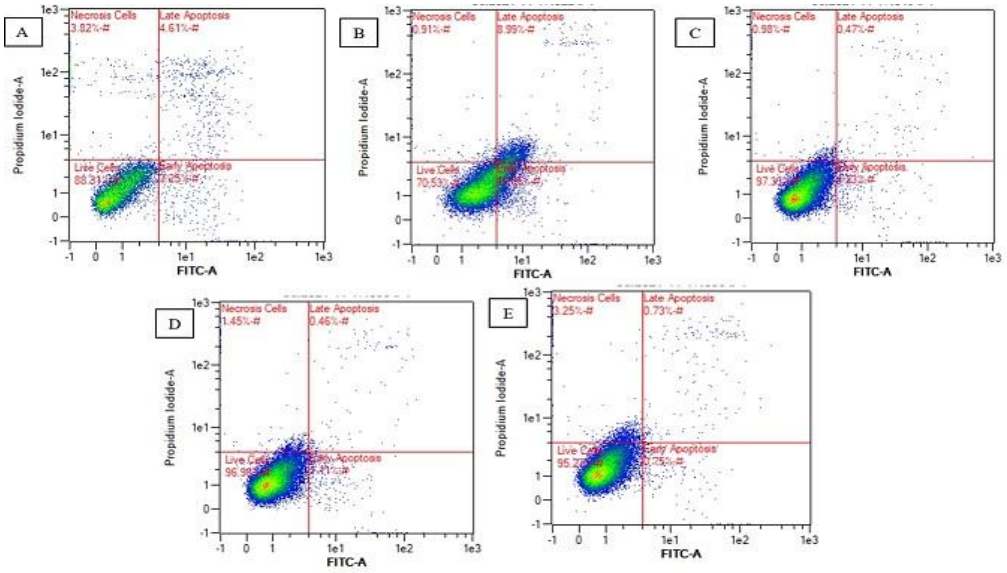

Figure 2. The dot blots of chlorogenic acid concentrations on aging cell models toward apoptotic cells by flowcytometry
(A) Fibroblast cells (negative control/ untreated cells): 88.32% ± 0.52% living cells, 3.67% ± 0.15% necrotic cells, 3.37% ± 0.11% early apoptotic cells, and 4.63% ± 0.6% late apoptotic cells;
(B) UV-induced fibroblast cells (positive control): 70.33% ± 1.46% living cells, 1.01% ± 0.17% necrotic cells, 19.26% ± 0.27% early apoptotic cells, and 9.40% ± 1.41% late apoptotic cells;
(C) UV-induced fibroblast cells + CA 25 µg/mL: 97.26% ± 0.10% living cells, 1.00% ± 0.05% necrotic cells, 1.23% ± 0.05% early apoptotic cells, and 0.51% ± 0.04% late apoptotic cells;
(D) UV-induced fibroblast cells + CA 12.5 µg/mL: 96.92% ± 0.06% living cells, 1.48% ± 0.09% necrotic cells, 1.12% 0.09% early apoptotic cells, and 0.49% ± 0.03% late apoptotic cells;
(E) UV-induced fibroblast cells + CA 6.25 µg/mL: 95.45% ± 0.37% living cells, 3.10% ± 0.34% necrotic cells, 0.79% ± 0.04% early apoptotic cells, and 0.66% ± 0.08% late apoptotic cells.

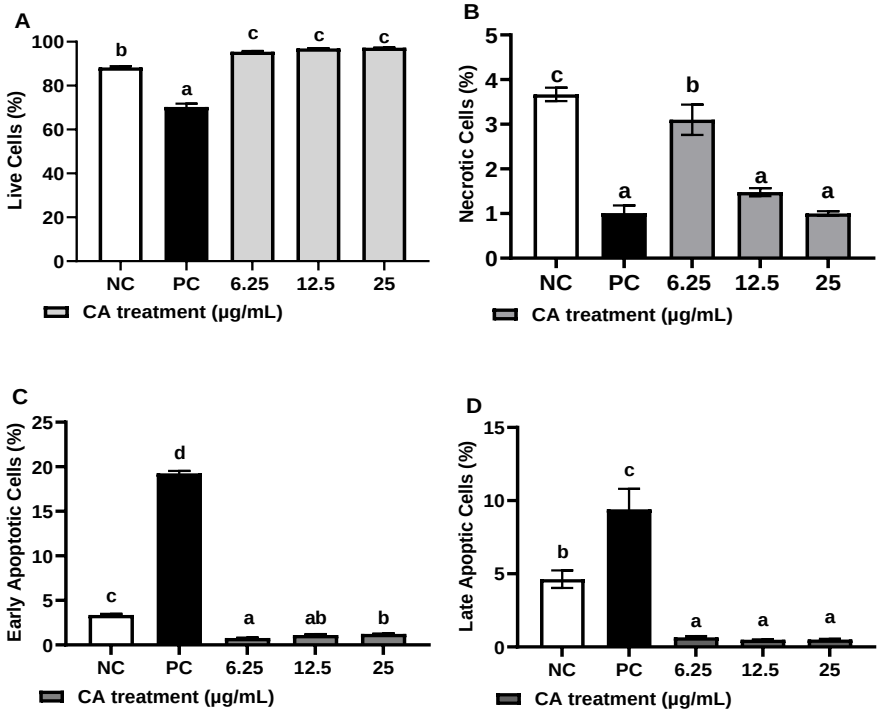

Figure 3. The effects of chlorogenic acid on aging cell models toward apoptotic cells
The graphs are displayed as mean ± standard deviation.
NC: BJ cells (negative control/untreated cells); PC: UV-induced fibroblast cells (positive control); 6.25: UV-induced fibroblast cells + CA 6.25 µg/mL; 12.5: UV-induced fibroblast cells + CA 12.5 µg/mL; 25: UV-induced fibroblast cells + CA 25 µg/mL.
Different letters (a, b, and c in figure 3A-B and 3D and a, b, ab, c, and d in figure 3C) indicate significance in accordance with Tukey HSD post hoc test ($p \leq 0.05$).

3. DISCUSSION

Fibroblast cells produce and regulate extracellular matrix (ECM) in dermal tissues consisting of COL-1α1 and ELN, which play a substantial role in tensile strength of human skin and play a role in tissue granulation and scar tissue formation [13-14]. UV exposure is one of the main environmental factors that can degrade the tissue formation, resulting in skin aging [15]. Mainly, there are three concomitated mechanisms in UV-stimulated aging, oxidative stress and inflammation, tissue fibers deformation [15-16], and cell death [17]. UV exposure stimulates an excessive production of ROS [18]. UV penetration triggers cellular oxidation, then elicits DNA base modification, manifested as an abundant level of 8-OHdG [18]. ROS promotes the increase of inflammatory mediators, both cytokines and chemokines, and exacerbates T cell infiltration (4). ROS influences another inflammatory pathway, MAPK/Activator Protein 1 (AP-1), which also contributes in Matrix Metalloproteinases (MMPs) activation [19]. The active MMPs degrade COL-1α1, ELN, and HA [18] and suppress Transforming Growth Factor β (TGF- β) signaling [11]. Altogether, these activities devastate tissue integrity [20]. Nevertheless, this condition is aggravated by the increase in cell death. UV irradiation directly shortens telomere which arrests cell cycle, then causes DNA damage, and ends with apoptosis [21]. It indirectly generates apoptotic cells by activating cell death pathway, decreasing mitochondria permeability, causing cytochrome C release, and inducing p53 activation [22]. The molecular knowledge of these biological processes allows the discovered strategy to promote an antiaging therapy.

This study examined CA activities in mitigating UV-stimulated skin aging. The UV exposure remarkably increased the levels of 8-OHdG, HAase and percentages of necrotic cells and apoptotic cells and lowered the levels of COL-1α1, ELN, MT, and percentage of living cells. The treatment using CA as much as 6.25, 12.5, and 25 µg/mL decreased the levels of 8-OHdG, HAase, and a percentage of apoptotic cells and improved the levels of COL-1α1, ELN, MT, and a percentage of living cells. These result data indicate that CA have the ability to counter cellular oxidation, improve ECM fibers, and lower cell death in ameliorating skin aging. This study revealed that CA treatment declined 8-OHdG level of UV-induced BJ cells. Accordingly, the attenuate level of 8-OHdG represents CA's ability to reduce cellular oxidation. Similarly, Girsang et al. (2021) reported that CA treatment on UV-induced BJ (fibroblast) cells lowered ROS level and CA at a dose of 6.5 µg/mL exhibited ROS level the lowest among the treatments [2]. Another tested parameter, MT, is a free radical scavenger that neutralizes the reactive substance, binds to ROS, and inhibits their generation [23]. The CA treatment ameliorated MT level in UV-exposed fibroblast cells as an indication of CA's ability to inhibit ROS activity and its generation.

The inhibition of ROS and inflammation are associated with the suppression of collagenase or MMPs and the stimulation of Tissue Inhibitor of Metalloproteinase (TIMP), hence it allows collagen regeneration to restore skin integrity [24-25]. In this study, the decline of 8-OHdG as an effect of the CA treatment was followed by the increases of COL-1α1 and ELN as well as the reduction of HAase. Moreover, a study by Xue et al. (2022) reported the increases of COL-1α1 mRNA and protein as well as the decreases of MMP-1 and MMP-3 on CA-treated UV-exposed human dermal fibroblasts [26]. These results signified the CA suppression against ROS in concomitant with skin fiber regeneration.

CA possesses abilities in attenuating apoptosis and ameliorates living cells toward fibroblast cells [12]. However, the anti-apoptotic mechanism is not clearly elucidated. This study proves CA's role in decreasing cell death caused by UV. The flow cytometry data exhibited the increase of live cells in correspond to the decrease of apoptotic cells. Chen et al. (2018) reported that CA diet for pig downregulated the intestines Casp-9 and Casp-3 [27]. CA downregulation effect on Casp-9 and Casp-3 is also reported in CA treated rat's hepatocyte [28].

Besides, the percentage of necrosis in UV-induced fibroblast cells cannot be diminished with CA treatment. This result might be related to the presence of necrosis as a double-edged sword, either as the beginning of tissue degeneration or innate immune activation. A relatively high level of necrosis might be unrepairable; hence it leads to tissue collapse. On the other side, the presence of a certain level of necrotic cells might stimulate innate immune system to produce cytokines and to attenuate pathogenesis to protect the body [29]. Once necrosis data are compared to living cell data, it can be found that the rise of necrotic cells is followed by the rise of living cells as effects of the CA treatment. This evidence indicates that CA treatment ameliorates skin tissue through increasing the number of living cells without declining the number of necrosis, and the tissue injuries resulted by necrosis might be repairable.

In summary, UV-induced fibroblast cells undergo oxidative stress and apoptosis. UV exposure triggers oxidation reaction in the cells that interferes DNA base groups and releases 8-OHdG. İt diminishes COL-1α1 and ELN levels, and increases HAase. Furthermore, it causes cell damage via mitochondria

intervention, hence it activates Casp-9 and Casp-3 to cause apoptosis. This occurrence is associated with the decline of MT level. CA treatment subsequently rises the levels of COL-1α1, ELN, MT, and a percentage of living cells and reduces a percentage of apoptosis, HAase, 8-OhdG levels. These properties exhibit CA's ability as an anti-aging agent which is illustrated in Figure 4.

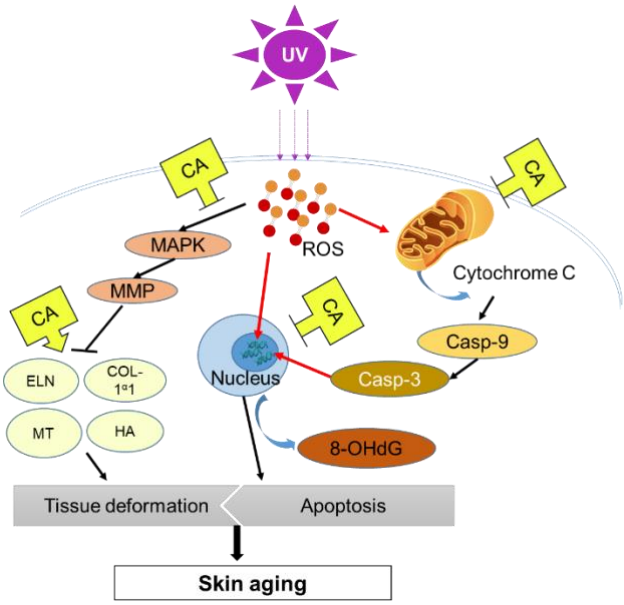

Figure 4. The proposed chlorogenic acid's action against skin aging
CA: Chlorogenic Acid, Casp-9: Caspase 9, Casp-3: Caspase 3, COL-1α1: Collagen, ELN: Elastin, HA: Hyaluronic acid, MAPK: Mitogen-Activation Protein Kinase, MMP: Matrix Metalloproteinase, MT: Melatonin, ROS: Reactive Oxigen Species, UV: Ultraviolet, 8-OHdG: 8-Hydroxydeoxyguanosine.
UV exposure causes an excessive level of ROS, then damages the cells and matrix disintegrity, and eventually leads to skin aging [13-14]. As obtained in this study, CA treatment ameliorates skin aging by lowering the oxidative stress, blocking mitochondria to activate Casp-9 and Casp-3, inhibiting MAPK pathway, decreasing 8-OHdG, and inhibiting MAPK pathway. Down regulation of MAPK pathway inhibits MMP release, then increases COL-1α1, ELN, MT, and HA productions [24-25].
→ damage, → activate, ⊣ inhibit.

4. CONCLUSION

Chlorogenic acid is a natural phenolic compound and has a great potential as a cosmeceutical to decrease 8-OHdG and HAase levels, and also improve the levels of COL-1α1, ELN, and MT. The abilities of chlorogenic acid may effectively enhance skin repaired by apoptosis inhibition through increasing the percentage of living cells and reducing the proportion of apoptotic cells and also by acting as a free radical scavenger for antiaging purposes. Further in vivo studies and clinical tests are required to validate this new strategy therapy.

5. MATERIALS AND METHODS

5.1. Skin fibroblast cell culture, UV induction, and CA treatment

Human skin fibroblast cells (BJ cell line, CRL-2522, ATCC) were provided by Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. Cell culture was conducted in Medium Essential Media (MEM) (L0416-500, Biowest, France), 10% fetal bovine serum (FBS) (S1810-500, Biowest, France), 1% antibiotics/antimycotics (ABAM) (L001010, Biowest, France), 1% nanomycopulitine (Biowest, L-X16-100), 1% amphotericin B (L0009-050, Biowest), and 0.1% gentamicin (15750060, Gibco, USA). The cells were incubated at 37°C in a humidified atmosphere with 5% CO2 (IH3543, Thermo, USA). After that, 80% of the cell's confluency, as many as $5x10^5$ cells were growth in each well of a 6-well plate. The aging cell

models were generated using a 300 J/cm2 UV exposure for 75 minutes. The determination of CA concentrations referred to cytotoxicity results in our previous study, in which 25 µg/mL was the maximum tolerated concentration [4]. Therefore, this study treated the UV-induced cells with CA 6.25, 12.5, and 25 µg/mL for 4 days (BP0345, Chengdu Biopurify Phytochemical, China) [12-30]. Subsequently, the media were harvested for Enzyme Linked Immunosorbent Assay (ELISA) and the cells were harvested for apoptotic assay.

5.2. Measurements of COL-1α1, ELN, 8-OhdG, MT contents, and HAase activity

The levels of COL-1α1, ELN, 8-OhdG, MT, HAase were measured from the conditioned media by employing the ELISA kits for Human Collagen Type Alpha 1 (COL-1α1)(E-EL-H0869, Elabscience, China), Human Hyaluronidase (HAase) (E-EL-H2201, Elabscience, China), Human Elastin (E-EL-H1163 Elabscience, China), 8-Hydroxydeoxyguanosine (8-OhdG) (E -EL-0028, Elabscience, China), and Human Melatonin (E-EL-H2016, Elabscience, China) according to the manufacturer's manuals. The absorbances were read using a microplate reader (Multiskan Go, Thermo Scientific, USA) at a 450 nm wavelength [12-30].

5.3. Measurements of apoptotic, living, and necrotic cells

The measurements of apoptotic, living, and necrotic cells were assessed using flow cytometry. The cells were seeded in 6-well plates (n = $5x10^5$), then incubated for 24 hours. Subsequently, the cells were treated with CA 6.25, 12.5, and 25 µg/mL, and then were incubated for 4 days. Afterward, treated cells were harvested and centrifuged at 1,600 rpm for 5 minutes. The pellets were added with 500 µL of Fluorescence-Activated Cell Sorting (FACS) buffer, then centrifuged. The pellets were added to 100 µL of FACS buffer and stained with annexin and Propidium Iodide (PI), incubated for 1 hour, and then analyzed using MACSQuant Analyzer 10 flowcytometer (130-096-343, Miltenyi Biotec, Singapore) [31-32].

5.4. Statistical analysis

The presented data were generated by three replication treatments. The data were analyzed using ANOVA and continued by Tukey's HSD post hoc test with $p \leq 0.05$ using SPSS software (version 20.0).

Acknowledgements: The authors acknowledge the financial support from the Ministry of Research and Technology/National Research and Innovation Agency of the Republic of Indonesia (Penelitian Dasar Unggulan Perguruan Tinggi 2022), and also to Universitas Prima Indonesia, Medan, North Sumatera, Indonesia, Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, West Java, Indonesia for facilitating this study. We thank Agung Novianto, Viranda Andria Yuninda, Anis Syahbani Muthmainnah, and Nindia Salsabila Mia Dewi from Aretha Medika Utama Biomolecular and Biomedical Research Center for supporting this study.

Author contributions: Concept – E.G., I.N.E.L., C.N.G., W.W.; Design – E.G., I.N.E.L., C.N.G., W.W.; Supervision – W.W., H.S.W.K., R.R.; Resources – W.W., A.Y., H.S.W.; Materials – W.W., H.S.W.K., E.G.; Data Collection and/or Processing – A.Y., R.R., H.S.W.K., W.W.; Analysis and/or Interpretation – A.Y., R.R., H.S.W.K., W.W.; Literature Search – E.G., I.N.E.L., C.N.G., W.W., A.Y.; Writing – W.W., A.Y., H.S.W.K, E.G., I.N.E.L., C.N.G.; Critical Reviews – W.W., H.S.W.K.

Conflict of interest statement: The author states that there is no conflict of interest in this research.

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