Pre-Treatment of Pterostilbene Enhances H$_2$O$_2$-induced Cell Apoptosis Through Caspase-dependent Pathway in Human Keratinocyte Cells

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Abstract. Background/Aim: Hydrogen peroxide (H$_2$O$_2$) is one of the reactive oxygen species (ROS), which can induce apoptotic cell death in numerous cancer cells. Pterostilbene (PTE), a natural polyphenolic compound, induces cell apoptosis in many human cancer cells. Materials and Methods: We investigated whether PTE could enhance H$_2$O$_2$-induced cell apoptosis in human keratinocyte HaCaT cells in vitro. The morphological change of HaCaT cells was observed and photographed under a contrast-phase microscope. The percentage of cell viability was measured by propidium iodide exclusion assay. Cell apoptosis was performed by Annexin V/PI double staining and assayed by flow cytometer. DNA condensation was measured by DAPI staining. The protein expression was determined by western blotting. ROS production-associated proteins were also assayed by confocal laser scanning microscopy. Results: PTE pre-treatment enhanced H$_2$O$_2$ (600 μM)-induced cell morphological changes and reduced the total cell number (cell viability). The decreased cell viability in HaCaT cells was through induction of apoptotic cell death, which was confirmed by Annexin V/PI double staining and DAPI staining. Western blotting studies indicated that HaCaT cells which were pre-treated with PTE (100 μM) and then co-treated with H$_2$O$_2$ (600 μM) for 12 h showed significantly increased levels of SOD (Cu/Zn), SOD (Mn), Bax, caspase-3, caspase-8, caspase-9, PARP, p53, p-p53, and p-H2AX but decreased levels Bcl-2 and catalase. Results also showed that HaCaT cells pre-treated with PTE and then co-treated with H$_2$O$_2$ had increased expression of SOD (Cu/Zn) and glutathione but decreased catalase. Conclusion: These observations suggest that PTE pre-treatment can enhance the H$_2$O$_2$-induced apoptotic cell death in keratinocyte cells and may be an effective candidate for the treatment of proliferative keratinocytes.

Human skin covers the human body, and is exposed to various stressors (1, 2), including environmental factors such as UV-light (3, 4). Actually, skin plays a vital role in the body against exposure to environmental factors (temperature, humidity, and sun exposure) (5). Skin aging is the most common human aging aspect and one of its main reasons is oxidation injury of the skin (6). Reactive oxygen species...
(ROS) caused by UV light plays an essential role in skin aging (7). Furthermore, UVB radiation-induced DNA lesions may lead to DNA mutation during cell division and then induce the initiation of carcinogenesis (7). Keratinocyte hyperproliferation is highly associated with skin pathogenesis, such as skin cancer, psoriasis, keratosis, and verrucae (8, 9). Therefore, dynamic regulation of apoptosis and cell proliferation in keratinocytes is important for the function of the skin.

Cellular ROS, primarily arising from cell oxidative metabolism, plays an important role in cell signaling and homeostasis and in both chronological aging and photoaging of skin (7). Ozone (O$_3$), hydroxy radical (OH$^\bullet$), singlet oxygen ($^1$O$_2$), hydrogen peroxide (H$_2$O$_2$), and superoxide anion radical (O$_2^{•−}$) belong to ROS (10). Hydrogen peroxide (H$_2$O$_2$), one type of ROS, is produced under normal circumstances and is increased due to external stressors. In cells, the formation of H$_2$O$_2$ is linked to other forms of ROS and has been associated with cellular senescence (11). H$_2$O$_2$ has been reported to induce ROS in cells, which caused oxidative stress (12) and can directly or indirectly damage cells for inducing apoptosis and necrosis (13, 14). The majority of the epidermis consists of keratinocytes (15), which are very susceptible to oxidative stress (16, 17). Earlier reports have shown the importance of antioxidants on attenuating oxidative stress, which is associated with chronic diseases (18, 19). Thus, H$_2$O$_2$ was recognized to be used to induce oxidative stress in vitro models.

Natural compounds can be effective candidates for various skin diseases. Pterostilbene (PTE; trans-3,5-dimethoxy-4-hydroxystilbene), a natural polyphenolic compound, is also a natural dimethylated analog of resveratrol found in Pterocarpus marsupium Roxb. (Fabaceae) (20) and is also present in fruits such as blueberries, grapes, and tree wood (21). Numerous studies have shown that PTE has anticancer effects on a wide range of human cancers in vitro, including breast (22), cervical (23), leukemic cells (24), colorectal cancer (25), lung squamous cell carcinoma cells (26), hepatocellular carcinoma cells (27), multiple myeloma (28), and oral cancer (29). PTE has been the focus of several studies due to its potent antioxidant and anti-inflammatory properties (30), particularly in neuronal cells. PTE exerts neuroprotection against oxidative toxicity via oestrogen receptor α signaling pathways in human neuronal cells (31) and in hypoxia-induced brain injuries (32). It also inhibits ROS production and apoptosis by the rapamycin signaling pathway in primary spinal cord neurons (33). Besides, PTE also attenuates mitochondrial oxidative damage induced by cerebral ischemia-reperfusion injury (34).

Apoptosis, a type of programmed cell death, which constitutes multiple molecular mechanisms to maintain tissue homeostasis (35), is classified into the extrinsic and intrinsic apoptosis pathways (36). The extrinsic apoptosis pathway is triggered by apoptotic ligands binding to death receptors and TRAIL receptors (37). The intrinsic apoptosis pathway which is the response to cellular stress (38), leads to a decrease in mitochondrial membrane potential and cytochrome c release from mitochondria. Then activated caspase-9 and caspase-3 induce cell apoptosis (39). Thus, the induction of cancer or abnormal cell apoptosis is one of the strategies for cancer therapy.

PTE has been demonstrated to present cell apoptotic biological activity. No reports have shown results of PTE co-treated with H$_2$O$_2$ in skin cells; thus, herein, PTE was evaluated as to whether or not it can enhance H$_2$O$_2$-induced cell apoptosis in human keratinocyte HaCaT cells in vitro. We investigated the molecular mechanisms in pre-treatment with PTE and H$_2$O$_2$ treatment in HaCaT cells.

Materials and Methods

Chemicals and reagents. Pterostilbene (PTE), dimethyl sulfoxide (DMSO), propidium iodide (PI), and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). For HaCaT cell culture, Dulbecco’s Modified Eagle Medium (DMEM) medium, fetal bovine serum (FBS), L-glutamine, and antibiotic (penicillin/streptomycin) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies included anti-SOD (Cu/Zn), -SOD (Mn), -catalase, -β-actin, -p53, and -p-p53 were obtained from Santa Cruz Biotechnology, and anti-Bax, -Bcl-2, -caspase-3, -caspase-8, and -caspase-9 from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-MGMT, -PARP, -p-ATM, and -p-ATR antibodies were purchased from Calbiochem (San Diego, CA, USA), and anti-p-H2A.X from GeneTex Inc. (Irvine, CA, USA). The HRP-conjugated secondary antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). DMSO was used to dissolve PTE for further diluted in the culture medium.

Cell culture. Human keratinocyte HaCaT cells were kindly provided by Professor Huey-Chun Huang (China Medical University, Taiwan). HaCaT cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium supplemented with 1% antibiotic (100 units/ml penicillin and 100 μg/ml streptomycin), 10% fetal bovine serum (FBS), and 2 mM L-glutamine at 37°C in a 5% CO$_2$ humidified incubator as described previously (40).

Cell viability and morphology observation. HaCaT cells (1×10$^5$ cells/well) were cultured in 12-well plates with DMEM medium overnight and cells were treated with H$_2$O$_2$ (600 μM), PTE (100 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H$_2$O$_2$ (600 μM) for 3, 6, and 12 h. After treatment, cells were observed and photographed under contrast phase microscopy and then cells were mixed with PI solution (4 μg/ml) and analyzed by flow cytometry as described previously (41).

Annexin V/PI staining for apoptotic cell death. Apoptotic cell death was performed by using Annexin V/PI double staining assay according to the manufacturer’s instructions, as described previously (41). In brief, HaCaT cells (1×10$^5$ cells/well) in 12-well plates were treated with H$_2$O$_2$ (600 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H$_2$O$_2$ (600 μM) for 12
At the end of incubation, cells were harvested and resuspended in Annexin V binding buffer and incubated with Annexin V/PI in the dark for 15 min. After incubation, cells were analyzed using BD FACSCalibur (BD Biosciences) for apoptotic cell numbers. Experiments were performed in triplicate.

**DAPI assay.** HaCaT cells (1×10^5 cells/well) were seeded on the 12-well plates and treated with H_2O_2 (600 μM), or pre-treated with pterostilbene at 100 μM for 1 h and then co-treated with H_2O_2 (600 μM) for 12 h, and cells were examined and photographed under fluorescent microscopy at ×200. Cells were collected and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were stained with DAPI solution (2 μg/ml) and examined and photographed using a fluorescence microscope as described previously (42).

**Western blotting analysis.** HaCaT cells (2×10^6 cells) were cultured onto 10-cm dishes overnight and treated with H_2O_2 (600 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H_2O_2 (600 μM) for 12 h. Cells were harvested and were lysed using a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), and 0.1% NP-40] on ice. Each protein concentration was detected and quantified by the Protein Assay Reagent Kit (Bio-Rad, Hercules, CA, USA). Aliquots of the protein solutions (30 μg) from each treatment were electrophoresed on a 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Belford, MA, USA). The blot was then incubated overnight at 4°C with a primary antibody [anti-SOD (Cu/Zn), -SOD (Mn), -catalase, -β-actin, -caspase-3, -caspase-8, -caspase-9, -Bax, -Bcl-2, -MGMT, -PARP, -p-ATM, -p-ATR, -p-H2A.X, -p53, and -p-p53] diluted in PBST buffer (phosphate-buffered saline with 0.1% of Tween-20) and 1% BSA. The membranes were washed with PBST, incubated with peroxidase-conjugated secondary antibodies diluted in PBST buffer for 1 h at room temperature. The immune-labeled bands (protein bands) were then detected chemiluminescence signals using ECL detection kit (Amersham Biosciences ECLTM) as described previously (42, 43).

**Observation of confocal laser scanning microscopy.** HaCaT cells (5×10^4 cells/well) were maintained on coverslips and treated with...
H₂O₂ (600 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H₂O₂ (600 μM) for 12 h. At the end of treatment, cells were fixed with 4% formaldehyde in PBS and were permeabilized by using 0.2% Triton-X 100 in PBS for 15 min. Subsequently, cells were washed with PBS and probed with anti-SOD (Cu/Zn), -catalase, and -glutathione primary antibody. After washed with PBS, cells were stained by FITC-conjugated goat anti-mouse IgG (green fluorescence), and the nucleus was stained by PI (red fluorescence). All samples were examined and photographed under a Leica TCS SP2 Confocal Spectral Microscope, as described previously (44, 45).

**Statistical analysis.** All data are presented as mean±S.D. Statistical analysis were performed by one-way analysis of variance followed by Dunnett’s or Tukey test for comparing with the control group (***p<0.001) or determining significant differences among group means (p<0.05), respectively, with the Sigma Plot 12 software (Systat Software, Inc., San Jose, CA, USA).

**Results**

**PTE affects morphology and total cell viability in HaCaT cells.** HaCaT cells were treated with H₂O₂ (600 μM), PTE (100 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H₂O₂ (600 μM) for 3, 6, and 12 h, and cell morphology was monitored and photographed under phase-contrast microscopy (Figure 1A). Subsequently, the total cell viability was calculated by PI exclusion assay and results were presented in Figure 1B. As shown in Figure 1A and B, PTE significantly increased H₂O₂-induced cell morphological change and cell death at 6 and 12 h treatment based on cell morphological change and reduced total cell number (cell viability).

**PTE induces apoptotic cell death in HaCaT cells.** HaCaT cells were placed on 12-well plates and treated with H₂O₂ (600 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H₂O₂ (600 μM) for 12 h. Cells were analyzed by Annexin V/PI kit as described in Materials and Methods. (A) Representative profiles of apoptotic cell death. (B) Percentage of apoptotic cell death. ***p<0.001, significant difference between experimental groups and the control as analyzed by Dunnett’s test.
μM), or pre-treated with PTE at 100 μM for 1 h and then co-
treated with H2O2 (600 μM) for 12 h. After treatment, cells
were doubly stained with Annexin V and PI and examined
under microscopy, and total apoptotic cell death was measured
by flow cytometric assay. As shown in Figure 2A and B,
results indicated that H2O2 (600 μM) treatment alone induced
72% of early- and late-stage apoptotic cells; however, after
pre-treatment with 100 μM of PTE led to 88%. These results
indicated that HaCaT cells pre-treated with PTE induced
higher number of H2O2 (600 μM)-induced apoptotic cells than
that of H2O2 (600 μM)-treated alone.

PTE enhances chromatin condensation (apoptotic cell death)
in HaCaT cells. HaCaT cells were placed on 12-well plates
and treated with H2O2 (600 μM), or pre-treated with PTE at
100 μM for 1 h and then co-treated with H2O2 (600 μM) for
12 h. Cells were stained with DAPI solution and results are
shown in Figure 3. Results indicated that HaCaT cells pre-
treated with 100 μM of PTE and treated with H2O2 (600 μM)
led to an increase in DNA condensation (apoptotic
cells) compared to cells with H2O2 (600 μM) treatment
alone. These results indicated that cells pre-treated with PTE
enhanced the effects of H2O2 treatment on the increase in the
number of apoptotic cell death.

PTE affects ROS production, DNA damage, and apoptosis-
associated proteins in HaCaT cells. HaCaT cells were treated
with H2O2 (600 μM), or pre-treated with PTE at 100 μM for
1 h and then co-treated with H2O2 (600 μM) and protein
expression was examined by western blotting. As presented
in Figure 4A, B and C, pre-treatment with PTE and then
treatment with H2O2 increased the expressions of SOD

Figure 3. Pterostilbene pre-treatment induced DNA condensation (apoptotic cell death) in HaCaT cells. HaCaT cells (1×10^5 cells/well) were seeded in
the 12-well plates and treated with H2O2 (600 μM), or pre-treated with pterostilbene at 100 μM for 1 h and then co-treated with H2O2 (600 μM) for
12 h and cells were examined and photographed under fluorescent microscopy at ×200, as described in Materials and Methods. (A) Representative
photographs of cell DNA condensation. (B) Fluorescent intensity of DNA staining. ***p<0.001, significant difference between experimental groups and
the control as analyzed by Dunnett’s test.
Figure 4. Pterostilbene enhanced ROS, DNA damage, and cell apoptosis associated proteins in HaCaT cells. HaCaT cells were treated with H$_2$O$_2$ (600 μM), or pre-treated with pterostilbene at 100 μM for 1 h and then co-treated with H$_2$O$_2$ (600 μM) for 12 h, and cells from each treatment were harvested for protein expression estimation by western blotting, as described in Materials and Methods. (A) SOD (Cu/Zn), SOD (Mn), and catalase; (B) p-ATM, p-ATR, p53, p-p53, p-H2A.X, and MGMT; (C) Bax, Bcl-2, caspase-9, caspase-8, caspase-3, and PARP.
treatment with PTE (100 μM) and treatment with H₂O₂ (600 μM) for 12 h significantly increased the expressions of Bax, cleaved caspase-9, cleaved caspase-8, cleaved caspase-3 and PARP but decreased that of Bcl-2 in HaCaT cells (Figure 4C).

**PTE affects the expression of SOD (Cu/Zn), catalase, and glutathione.** We further investigated the expression of oxidation-association protein that is involved in the oxidative stress in HaCaT cells. Cells were exposed to H₂O₂ (600 μM), PTE (100 μM), or pre-treated with PTE at 100 μM for 1 h and then co-treated with H₂O₂ (600 μM) for 12 h and probed with SOD (Cu/Zn), catalase, and glutathione antibodies, and observed under confocal laser microscopy system. As shown in Figure 5, PTE promoted the expressions of SOD (Cu/Zn) and glutathione but decreased that of catalase in HaCaT cells.
Discussion

It is well documented that ROS plays a prominent role in cell metabolism, which regulates cell proliferation, differentiation, and death (38); however, excessive production of ROS and overall cell antioxidant capacity may induce irreversibly severe oxidative damage to the cells (46, 47). Antioxidants reduced skin injury, aging, and cancer risk and some of them have been extracted from natural products to present antioxidant activities (48, 49). The study’s major reason is that there is no available information showing the influence of PTE at a high dose which enhanced H2O2-induced cell apoptosis through oxidative stress in human skin cells. Therefore, we investigated the effects of PTE pre-treatment at a high dose on the increase of cell apoptosis induced by H2O2 in human epidermal keratinocytes (HaCaT) cells in vitro. HaCaT cells are non-tumor epidermal keratinocytes and these cells bear the mutant p53; thus, numerous studies already use it (50-52).

H2O2 is a type of ROS and can also be used as an inducer of oxidative stress in in vitro and in vivo studies. Herein, we found that H2O2 reduced viable cell numbers in HaCaT cells; however, cells that were pre-treated with PTE (100 μM) and then co-treated with H2O2 (600 μM) had significantly decreased total cell viability (Figure 1B) when compared to H2O2 treatment alone. Cells loss of their original morphology may be caused by exposure to the chemical agents. Herein, we observed cell morphological changes accompanied by reduced cell numbers after exposure to PTE and H2O2. It was reported that intracellular redox disrupted ROS balance, if excessive ROS accumulation may lead to oxidative stress and induced oxidative damage (53, 54). ROS, causing most free radicals, can damage cell proteins, lipids, and DNA (55). In cells, ROS keeps at low levels to avoid extracellular invaders and to maintain cellular signaling. Furthermore, ROS is involved in cell migration, proliferation (56), and differentiation (57) in keratinocytes. The doses of H2O2 may lead cells to survival or death. Here, the dose of H2O2 used, was of high amount, that caused cell death.

Cell death can be divided into cell necrosis and apoptosis; the former leads to damaged cell membranes and death; the latter is involved in programmed cell death called cell apoptosis. Therefore, to further investigate whether combined treatment (PTE and H2O2) led to enhancing cell apoptotic death, Annexin V/PI reagents were used to stain cells, then cells were assayed by flow cytometry, and results indicated that HaCaT cells pre-treated with PTE (100 μM) and co-treated with H2O2 (600 μM) for 12 h had increased cell morphological changes (Figure 1A) and decreased total viable cell number (Figure 1B). Furthermore, this increased apoptotic cell death compared to H2O2-alone-treated cells (Figure 2A and B) by Annexin V/PI double staining assay, a well-documented method for cell apoptosis assay. For further confirming these observations, we used the DAPI staining assay, a well-known protocol for examining cell apoptosis. Results indicated that HaCaT cells pre-treated with PTE and co-treated with H2O2 had increased apoptotic cell death based on increased DAPI intensity (Figure 3).

It is well known that H2O2, a type of ROS, is one of the factors involved in oxidative stress. However, epidermal keratinocytes are very susceptible to oxidative stress in the skin (16, 17). To understand the effects of PTE on the expression of endogenous antioxidant enzymes, HaCaT cells were pre-treated with and without PTE (100 μM) and then treated with H2O2 for 12 h and then cells were harvested for western blotting. Results from Figure 4C indicate that PTE pre-treatment increased SOD (Cu/Zn) and SOD (Mn), two superoxide dismutases, but decreased catalase when compared to H2O2 treatment alone in HaCaT cells. These results were also confirmed by confocal laser microscopy examination (Figure 5A, B, and C). The enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase, and thioredoxin reductase (TRX) are the components of the skin defense system against ROS-mediated damages and they played crucial roles for the protection of skin (58). Based on this observation, pre-treatment of PTE (100 μM) and then co-treated with H2O2 for 12 h did not exert protection or antioxidant activity in HaCaT cells because of the increased number of apoptotic cell death.

Diminished apoptosis and excessive proliferation of keratinocytes in skin diseases have been reported in psoriatic lesions. Psoriasis is a common chronic inflammatory skin disease characterized by hyperproliferation with incomplete differentiation of epidermal keratinocytes and decreased keratinocyte apoptosis (59). Induction of apoptosis may be a potent therapy for psoriasis. Results from Figure 4C indicated...
that PTE pretreatment enhanced H$_2$O$_2$-induced apoptosis and resulted in elevated Bax, PARP, active caspase-9, caspase-8, and caspase-3 but decreased anti-apoptotic protein Bcl-2 in HaCaT cells. It was reported that H$_2$O$_2$ induced apoptosis via the production of ROS (60). Cancer cells also have been shown to evade apoptosis to maintain their survival and metastasis (61, 62). It was reported that several molecular mechanisms are involved in the apoptosis suppression of tumor cells. Tumor cells avoid apoptosis through the expression of anti-apoptotic proteins (Bcl-2) or through down-regulation or mutation of pro-apoptotic proteins (Bax). The expressions of Bcl-2 and Bax are regulated by the p53 tumor suppressor gene (63). Bcl-2 and Bax are Bcl-2 members who are localized in the mitochondria and the ratio of Bax and Bcl-2 are involved in cell apoptosis. Herein, results from Figure 4C indicated PTE pretreatment enhanced the expressions of caspase-8 and caspase-3. Thus, both expressions may lead to cell apoptosis via the extrinsic signaling pathway. Furthermore, caspase-9 also was activated, indicating the involvement of the intrinsic signaling pathway in HaCaT cells in vitro.

In conclusion, in the present study, we explored the mechanism of PTE on enhancing the apoptotic effects of H$_2$O$_2$ on HaCaT cells. Our results indicated that PTE enhanced the effects of H$_2$O$_2$ on apoptosis induction via extrinsic and/or intrinsic signaling pathways in HaCaT cells, as shown in Figure 6.

Conflicts of Interest

The Authors confirm that there are no conflicts of interest.

Authors’ Contributions

Study conception and design: YCC, YCC and SFP; Acquisition of data: YCC, YPH, TCH and SFP; Analysis and interpretation of data: YCC, YPH, TCH and SFP; Drafting of manuscript: YCC, YCC and SFP; Critical revision: YCC and SFP. All Authors discussed the results and commented on the article.

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References

1 Di Meglio P, Duarte JH, Ahlfors H, Owens ND, Li Y, Villanova F, Tosi I, Hirota K, Nestle FO, Mrowietz U, Gilchrist MJ and Stockinger B: Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions. Immunity 40(6): 989-1001, 2014. PMID: 24909886. DOI: 10.1016/j.immuni.2014.04.019
2 Fureure M, Tsuji G, Mitoma C, Nakahara T, Chiba T, Morino-Koga S and Uchi H: Gene regulation of filaggrin and other skin barrier proteins via aryl hydrocarbon receptor. J Dermatol Sci 80(2): 83-88, 2015. PMID: 26276439. DOI: 10.1016/j.jdermsci.2015.07.011
3 Kageyama H and Waditte-Sirisaththa R: Antioxidative, anti-inflammatory, and anti-aging properties of mycosporine-like amino acids: Molecular and cellular mechanisms in the protection of skin-aging. Mar Drugs 17(4): 2019. PMID: 31013795. DOI: 10.3390/md17040222
4 Puizina-Ivić N: Skin aging. Acta Dermatovenerol Alp Pannonica Adriat 17(2): 47-54, 2008. PMID: 18709289.
5 Boireau-Adamczyk E, Baillet-Guffroy A and Stamatas GN: Age-dependent changes in stratum corneum barrier function. Skin Res Technol 20(4): 409-415, 2014. PMID: 24517174. DOI: 10.1111/srt.12132
6 Noh EM, Park J, Song HR, Kim JM, Lee M, Song HK, Hong OY, Whang PH, Han MK, Kwon KB, Kim JS and Lee YR: Skin aging-dependent activation of the PI3K signaling pathway via downregulation of PTEN increases intracellular ROS in human dermal fibroblasts. Oxid Med Cell Longev 2016: 6354261, 2016. PMID: 28003865. DOI: 10.1155/2016/6354261
7 Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S and Voorhees JJ: Mechanisms of photoaging and chronological skin aging. Arch Dermatol 138(11): 1462-1470, 2002. PMID: 12437452. DOI: 10.1001/archderm.138.11.1462
8 Han YH, Zhang YQ, Jin MH, Jin YH, Qiu MY, Li WL, He C, Yu LY, Hyun JW, Lee J, Yoon DY, Sun HN and Kwon T: Peroxiredoxin I deficiency increases keratinocyte apoptosis in a skin tumor model via the ROS-p38 MAPK pathway. Biochem Biophys Res Commun 529(3): 635-641, 2020. PMID: 32736685. DOI: 10.1016/j.bbr.2020.06.047
9 Raj D, Brash DE and Grossman D: Keratinocyte apoptosis in epidermal development and disease. J Invest Dermatol 126(2): 243-257, 2006. PMID: 16418733. DOI: 10.1038/sj.jid.5700008
10 Sosa V, Molîne T, Somoza R, Paciucci R, Kondoh H and ME LL: Oxidative stress and cancer: an overview. Ageing Res Rev 12(1): 376-390, 2013. PMID: 23123177. DOI: 10.1016/j.arr.2012.10.004
11 Finkel T and Holbrook NJ: Oxidants, oxidative stress and the biology of ageing. Nature 408(6809): 239-247, 2000. PMID: 11089981. DOI: 10.1038/35041687
12 Park C, Cha HJ, Hong SH, Kim GY, Kim S, Kim HS, Kim BW, Jeon YJ and Choi YH: Protective effect of phloroglucinol on oxidative stress-induced DNA damage and apoptosis through activation of the Nrf2/HO-1 signaling pathway in HaCaT human keratinocytes. Mar Drugs 17(4): 2019. PMID: 31013932. DOI: 10.3390/md17040225
13 Bedard K and Krause KH: The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87(1): 245-313, 2007. PMID: 17237347. DOI: 10.1152/physrev.00044.2005
14 Morry J, Ngamcherdrakul W and Yantasee W: Oxidative stress in cancer and fibrosis: Opportunity for therapeutic intervention with antioxidant compounds, enzymes, and nanoparticles. Redox Biol 11: 240-253, 2017. PMID: 28012439. DOI: 10.1016/j.redox.2016.12.011
15 Lewis DA, Yi Q, Travers JB and Spandau DF: UVB-induced senescence in human keratinocytes requires a functional insulin-like growth factor-1 receptor and p53. Mol Biol Cell 19(4): 1346-1353, 2008. PMID: 18216278. DOI: 10.1091/mbc.e07-10-1041
16 Kim KE, Cho D and Park HJ: Air pollution and skin diseases: Adverse effects of airborne particulate matter on various skin diseases. Life Sci 152: 126-134, 2016. PMID: 27018067. DOI: 10.1016/j.lfs.2016.03.039

17 Nakashima Y, Ohta S and Wolf AM: Blue light-induced oxidative stress in live skin. Free Radic Biol Med 108: 300-310, 2017. PMID: 28315451. DOI: 10.1016/j.freeradbiomed.2017.03.010

18 Baynes JW: Role of oxidative stress in development of complications in diabetes. Diabetes 40(4): 405-412, 1991. PMID: 2100404. DOI: 10.2334/diaab.t40.4.405

19 Osawa T and Kato Y: Protective role of antioxidative food factors in oxidative stress caused by hyperglycemia. Ann N Y Acad Sci 1043: 440-451, 2005. PMID: 16037265. DOI: 10.1196/annals.1333.050

20 Ahmad H and Rajagopal K: Pharmacology of Pterocarpus marsupium Roxib. Med Plant Res 55(53): 1-6, 2015. DOI: 10.5376/mpr.2015.05.0003

21 Dvorakova M and Landa P: Anti-inflammatory activity of natural stilbenoids: A review. Pharmacol Res 124: 126-145, 2017. PMID: 28803136. DOI: 10.1016/j.phrs.2017.08.002

22 Huang Y, Du J, Mi Y, Li T, Gong Y, Ouyang H and Hou Y: Long non-coding RNAs contribute to the inhibition of proliferation and EMT by pterostilbene in human breast cancer. Front Oncol 8: 629, 2018. PMID: 30619763. DOI: 10.3389/fonc.2018.00629

23 Chatterjee K, Mukherjee S, Vanmanen J, Banerjee P and Fata JE: Dietary polyphenols, resveratrol and pterostilbene exhibit antitumor activity on an HPV E6-positive cervical cancer model: An in vitro and in vivo analysis. Front Oncol 9: 352, 2019. PMID: 31143704. DOI: 10.3389/fonc.2019.00352

24 Czop M, Bogucka-Kocka A, Kubrak T, Knop-Czop K, Makuch-Kocka A, Galkowski D, Wawer J, Kocki T and Kocki J: Imaging flow cytometric analysis of stilbene-dependent apoptosis in drug resistant human leukemia cell lines. Molecules 24(10): 2019. PMID: 31108853. DOI: 10.3390/molecules24101896

25 Hsiao YH, Chen NC, Koh YC, Nagabushanam K, Ho CT and Pan MH: Pterostilbene inhibits adipocyte conditioned-medium-induced colorectal cancer cell migration through targeting FABP5-related signaling pathway. J Agric Food Chem 67(37): 10321-10329, 2019. PMID: 31419115. DOI: 10.1021/acs.jafc.9b03997

26 Tan KT, Chen PW, Li S, Ke TM, Lin SH and Yang CC: Pterostilbene inhibits lung squamous carcinoma growth in vitro and in vivo by inducing S phase arrest and apoptosis. Oncol Lett 18(2): 1631-1640, 2019. PMID: 31423230. DOI: 10.3892/ol.2019.10499

27 Yu CL, Yang SF, Hung TW, Lin CL, Hsieh YH and Chiou HL: Inhibition of eIF2α dephosphorylation accelerates pterostilbene-induced cell death in human hepatocellular carcinoma cells in an ER stress and autophagy-dependent manner. Cell Death Dis 10(6): 418, 2019. PMID: 31138785. DOI: 10.1038/s41419-019-1639-5

28 Zhang T, Li B, Feng Q, Xu Z, Huang C, Wu H, Chen Z, Hu L, Gao L, Liu P, Yang G, Zhang H, Lu K, Li T, Tao Y, Wu X, Shi J and Zhu W: DCZ0801, a novel compound, induces cell apoptosis and cell cycle arrest via MAPK pathway in multiple myeloma. Acta Biochim Biophys Sin (Shanghai) 51(5): 517-523, 2019. PMID: 30947332. DOI: 10.1093/abbs/gmz033

29 Chang HP, Lu CC, Chiang JH, Tsai FJ, Juan YN, Tsao JW, Chiu HY and Yang JS: Pterostilbene modulates the suppression of multidrug resistance protein 1 and triggers autophagic and apoptotic mechanisms in cisplatin-resistant human oral cancer CAR cells via AKT signaling. Int J Oncol 52(5): 1504-1514, 2018. PMID: 29512708. DOI: 10.3892/ijo.2018.4298

30 Kosuru R, Rai U, Prakash S, Singh A and Singh S: Promising therapeutic potential of pterostilbene and its mechanistic insight based on preclinical evidence. Eur J Pharmacol 789: 229-243, 2016. PMID: 27475678. DOI: 10.1016/j.ejphar.2016.07.046

31 Song Z, Han S, Pan X, Gong Y and Wang M: Pterostilbene mediates neuroprotection against oxidative toxicity via oestrogen receptor α signalling pathways. J Pharm Pharmacol 67(5): 720-730, 2015. PMID: 25644078. DOI: 10.1111/jphp.12360

32 Li D, Song T, Yang L, Wang X, Yang C and Jiang Y: Neuroprotective actions of pterostilbene on hypoxic-ischemic brain damage in neonatal rats through upregulation of heme oxygenase-1. Int J Dev Neurosci 54: 22-31, 2016. PMID: 27576146. DOI: 10.1016/j.jidnev.2016.08.005

33 He JL, Dong XH, Li ZH, Wang XY, Fu ZA and Shen N: Pterostilbene inhibits reactive oxygen species production and apoptosis in primary spinal cord neurons by activating autophagy via the mechanistic target of rapamycin signaling pathway. Mol Med Rep 17(3): 4406-4414, 2018. PMID: 29328494. DOI: 10.3892/mmr.2018.8412

34 Yang Y, Wang J, Li Y, Fan C, Jiang S, Zhao L, Di S, Xin Z, Wang B, Wu G, Li X, Li Z, Gao X, Dong Y and Qu Y: HO-1 signaling activation by pterostilbene treatment attenuates mitochondrial oxidative damage induced by cerebral ischemia reperfusion injury. Mol Neurobiol 53(4): 2339-2353, 2016. PMID: 25983033. DOI: 10.1007/s12035-015-9194-2

35 De Felici M and Picentini M: Programmed cell death in development and tumors. Int J Dev Biol 59(1-3): 1-3, 2015. PMID: 26374519. DOI: 10.1387/ijdb.150168md

36 Hengartner MO: The biochemistry of apoptosis. Nature 407(6805): 770-776, 2000. PMID: 11048727. DOI: 10.1038/35037710

37 Walczak H and Krammer PH: The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 256(1): 58-66, 2000. PMID: 10739652. DOI: 10.1006/excr.2000.4840

38 Fulda S: Evasion of apoptosis as a cellular stress response in cancer. Int J Cell Biol 2010: 770-776, 2000. PMID: 11048727. DOI: 10.1387/ijdb.150168md

39 Schafer ZT and Kornbluth S: The apoptosome: physiological, developmental, and pathological modes of regulation. Dev Cell 10(5): 549-561, 2006. PMID: 16678772. DOI: 10.1016/j.devcel.2006.04.008

40 Ying TH, Chen CW, Hsiao YP, Hung SJ, Chung JG and Yang JH: Citric acid induces cell-cycle arrest and apoptosis of human immortalized keratinocyte cell line (HaCaT) via caspase- and mitochondrion-dependent signaling pathways. Anticancer Res 33(10): 4411-4420, 2013. PMID: 24123010.

41 Chang YM, Shih YL, Chen CP, Liu KL, Lee MH, Lee MZ, Hou HT, Huang HC, Lu HF, Peng SF, Chen KW, Yeh MY and Chung JG: Ouabain induces apoptotic cell death in human prostate DU 145 cancer cells through DNA damage and TRAIL pathways. Environ Toxicol 34(12): 1329-1339, 2019. PMID: 31436044. DOI: 10.1002/tox.22834

42 Huang TY, Peng SF, Huang YP, Tsai FJ, Tsai FJ, Huang CY, Tang CH, Yang JS, Hsue YM, Yin MC, Huang WW and Chung JG: Combinational treatment of all-trans retinoic acid (ATRA) and bisdemethoxycurcumin (BDMC)-induced apoptosis in liver...
cancer Hep3B cells. J Food Biochem 44(2): e13122, 2020. PMID: 31837044. DOI: 10.1111/jfbc.13122

43 2016 Statistics of causes of death. Available at: http://www.mohw.gov.tw/cp-3327-33592-2.html [Last accessed on December 16 2020]

44 Tsai YH, Lin JJ, Ma YS, Peng SF, Huang AC, Huang YP, Fan MJ, Lien JC and Chung JG: Fisetin inhibits cell proliferation through the induction of G0/G(1) phase arrest and caspase-3-mediated apoptosis in mouse leukemia cells. Am J Chin Med 47(4): 841-863, 2019. PMID: 31096772. DOI: 10.1142/s0192415x19500447

45 Liu S-P, Hsu C-Y, Fu R-H, Huang Y-C, Chen S-Y, Lin S-Z and Shyu W-C: Sambucus williamsii induced embryonic stem cells differentiated into neurons. BioMedicine 5(1): 1-5, 2015. PMID: 25705583. DOI: 10.7603/s40681-015-0003-z

46 Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcaroci V, Squadrato F, Altavilla D and Bitto A: Oxidative stress: Harms and benefits for human health. Oxid Med Cell Longev 2017: 8416763. PMID: 28819546. DOI: 10.1155/2017/8416763

47 Moloney JN and Cotter TG: ROS signalling in the biology of cancer. Semin Cell Dev Biol 80: 50-64, 2018. PMID: 28587975. DOI: 10.1016/j.semcdb.2017.05.023

48 Zhou Y, Zheng J, Li Y, Xu DP, Li S, Chen YM and Li HB: Natural polyphenols for prevention and treatment of cancer. Nutrients 8(8): 2016. PMID: 27556486. DOI: 10.3390/nu8080515

49 Sawicka D, Car H, Borawska MH and Nikliński J: The anticancer activity of propolis. Folia Histochem Cytobiol 50(1): 25-37, 2012. PMID: 22532133. DOI: 10.2478/18693

50 Boukamp P, Popp S, Altmeyer S, Hülsen A, Fasching C, Cremer T and Fusenig NE: Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. Genes Chromosomes Cancer 19(4): 201-214, 1997. PMID: 9258654. DOI: 10.1002/(sici)1098-2264(199708)19:4<201::aid-gcc1>3.0.co;2-0

51 Yang X, Liu J, He H, Zhou L, Gong C, Wang X, Yang L, Yuan J, Huang H, He L, Zhang B and Zhuang Z: SiO2 nanoparticles induce cytotoxicity and protein expression alteration in HaCaT cells. Part Fibre Toxicol 7: 1, 2010. PMID: 20180970. DOI: 10.1186/1743-8977-7-1

52 Gius DR, Ezhevsky SA, Becker-Hapak M, Nagahara H, Wei MC and Dowdy SF: Transduced p16INK4a peptides inhibit hypophosphorylation of the retinoblastoma protein and cell cycle progression prior to activation of Cdk2 complexes in late G1. Cancer Res 59(11): 2577-2580, 1999. PMID: 10363976.

53 Fleming JE and Bensch KG: Oxidative stress as a causal factor in differentiation and aging: a unifying hypothesis. Exp Gerontol 26(5): 511-517, 1991. PMID: 1756782. DOI: 10.1016/0531-5565(91)90039-o

54 Sarkar D and Fisher PB: Molecular mechanisms of aging-associated inflammation. Cancer Lett 236(1): 13-23, 2006. PMID: 15978720. DOI: 10.1016/j.canlet.2005.04.009

55 Bottai G, Mancina R, Muratori M, Di Gennaro P and Lotti T: 17β-estradiol protects human skin fibroblasts and keratinocytes against oxidative damage. J Eur Acad Dermatol Venereol 27(10): 1236-1243, 2013. PMID: 22988828. DOI: 10.1111/j.1468-3033.2012.04697.x

56 Raja, Sivamani K, Garcia MS and Isseroff RR: Wound re-epithelialization: modulating keratinocyte migration in wound healing. Front Biosci 12: 2849-2868, 2007. PMID: 17485264. DOI: 10.2741/2277

57 Stojadinovic O, Pastar I, Vukelic S, Mahoney MG, Brennan D, Krzyzanowska A, Golinko M, Brem H and Tomic-Canic M: Deregulation of keratinocyte differentiation and activation: a hallmark of venous ulcers. J Cell Mol Med 12(6b): 2675-2690, 2008. PMID: 18373736. DOI: 10.1111/j.1582-4934.2008.00321.x

58 Jang J, Ye BR, Heo SJ, Oh C, Kang DH, Kim JH, Affan A, Yoon KT, Choi YU, Park SC, Han S, Qian ZJ, Jung WK and Choi IW: Photo-oxidative stress by ultraviolet-B radiation and antioxidative defense of eckstolonol in human keratinocytes. Environ Toxicol Pharmacol 34(3): 926-934, 2012. PMID: 22990963. DOI: 10.1016/j.etap.2012.08.003

59 Kastelan M, Pripić-Massari L and Brajac I: Apoptosis in psoriasis. Acta Dermatovenerol Croat 17(3): 182-186, 2009. PMID: 19818217.

60 Pervaiz S and Clément MV: Hydrogen peroxide-induced apoptosis: oxidative or reductive stress? Methods Enzymol 352: 150-159, 2002. PMID: 12125343. DOI: 10.1016/s0076-6879(02)52015-2

61 Plati J, Bucur O and Khosravi-Far R: Dysregulation of apoptotic signaling in cancer: molecular mechanisms and therapeutic opportunities. J Cell Biochem 104(4): 1124-1149, 2008. PMID: 18459149. DOI: 10.1002/jcb.21707

62 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100(1): 57-70, 2000. PMID: 10647931. DOI: 10.1016/s0092-8674(00)81683-9

63 Miyashita T, Krajewska S, Krajewska M, Wang HG, Lin HK, Lieberman DA, Hoffman B and Reed JC: Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9(6): 1799-1805, 1994. PMID: 8183579.