

Supplementary data

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Title; Walnuts (seeds of *Juglandis sinensis* L.) protect human epidermal keratinocytes against UVB-induced mitochondria-mediated apoptosis through upregulation of ROS-elimination pathways.

Materials and methods

Measuring NADH dehydrogenase

NADH dehydrogenase was measured using the MTT assay. HaCaT cells were seeded on 96-well plates and pretreated with JSE at doses of 10–100 µg/mL for 1 h, and then stimulated with 1 mM H₂O₂ for an additional 6 h. The treated cells were incubated with 1 mg/mL MTT for 2 h. The MTT medium was aspirated carefully from the wells, and the formazan dye was eluted using DMSO. The absorbance was measured using a spectrophotometer (Versamax microplate reader; Molecular Device, Sunnyvale, USA) at a wavelength of 570 nm and was expressed as a percent of the value for the control.

Measuring intracellular ROS

Intracellular ROS generation was measured using DCFH-DA fluorescence dye. DCFH-DA enters cells passively and is converted into non-fluorescent DCFH, which reacts with ROS to form the fluorescent product DCF. HaCaT cells were seeded on black 96-well plates and treated with JSE at doses of 10 and 100 µg/mL for 1 h. Then, they were stimulated with 1 mM H₂O₂ for an additional 30 min. The cells were incubated with 25 µM DCFH-DA for 30 min. The fluorescence intensity was determined at 485 nm excitation and 535 nm emission using a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Device; Sunnyvale, CA, USA).

Measurement of SOD activities

The SOD activities were detected using kits according to the manufacturers' protocol (SOD kit: Dojindo molecular technologies, Japan).

Measuring the total GSH level

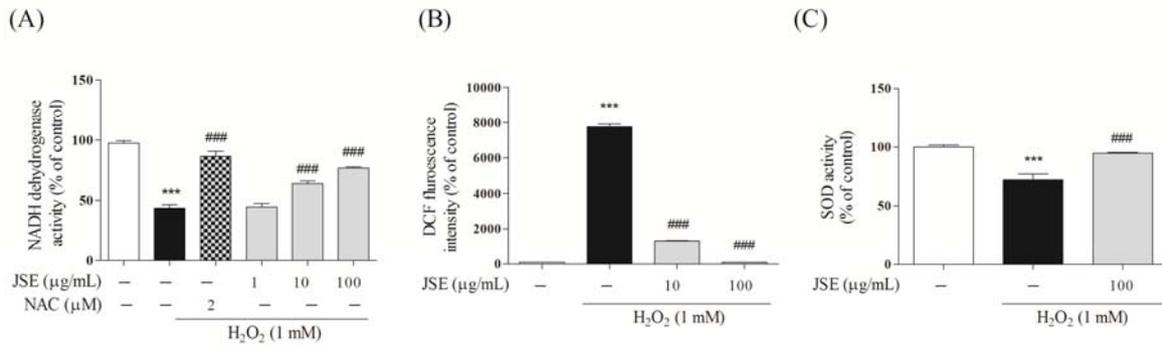
Total GSH levels were determined using a total glutathione quantification kit, according to

the instruction manual (Dojindo molecular technologies, Japan).

Immunofluorescence

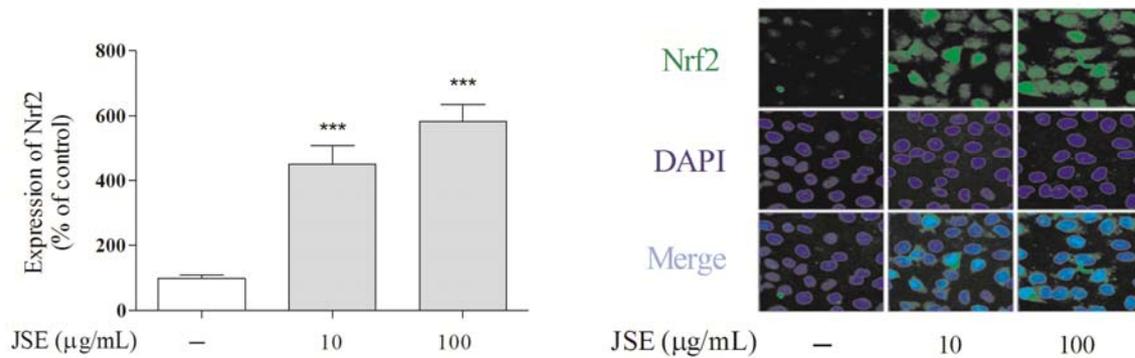
Cells were fixed through incubation with 4% PFA at room temperature for 30 min. Fixed cells were rinsed in PBS and then incubated overnight at 4°C with rabbit anti-Nrf2 and HO-1 antibody (dilution 1:500). They were then incubated with a biotinylated anti-rabbit IgG (1:200 dilutions) for 90 min, followed by incubation in ABC solution for 1 h at room temperature or incubated for 2 h with an Alexa Fluor 594-conjugated horse anti-rabbit IgG (dilution 1:500). Cells were finally washed in PBS and mounted using Vectashield Mounting Medium containing DAPI. Confocal immunofluorescent images were captured using an LSM 700 confocal microscope (Carl Zeiss, Thornwood, USA) and the fluorescence intensity was measured using Axio Vision 4.4 (Carl Zeiss, Oberkochen, Germany). Data are presented as percentages of control values. For the assessment of intra-experimental variability, three independent experiments were carried out in triplicate.

Supplementary figure legends

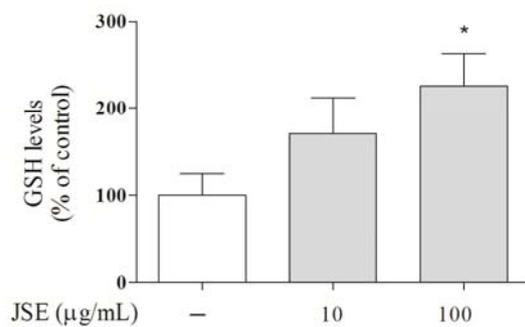


Supplementary figure. 1. Effects of JSE on H₂O₂-induced mitochondrial dysfunction, ROS generation, and SOD levels in human epidermal keratinocytes. In mitochondrial dysfunction and SOD levels, after cells were confluent, they were treated with JSE or NAC for 1 h and incubated with 1 mM H₂O₂ for further 6 h. NADH dehydrogenase activity (A) and SOD levels (C) is expressed as a percentage of the control. In ROS generation, the cells were treated with JSE for 1 h before 1 mM H₂O₂ stimulation. ROS generation was measured by the fluorescence intensity of DCF-DA after H₂O₂ stimulation for 30 min (B). Values are means ± SEM. ***p < 0.001, vs. the control; ###p < 0.001, vs. the H₂O₂-alone group.

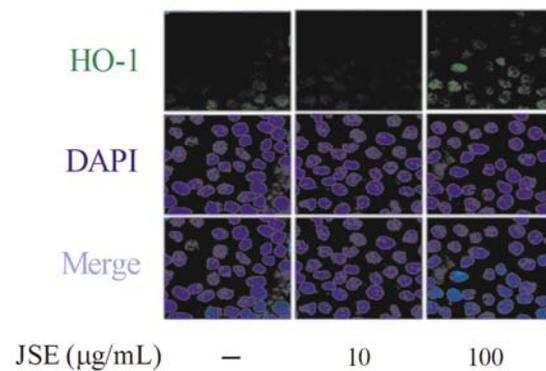
(A)



(B)

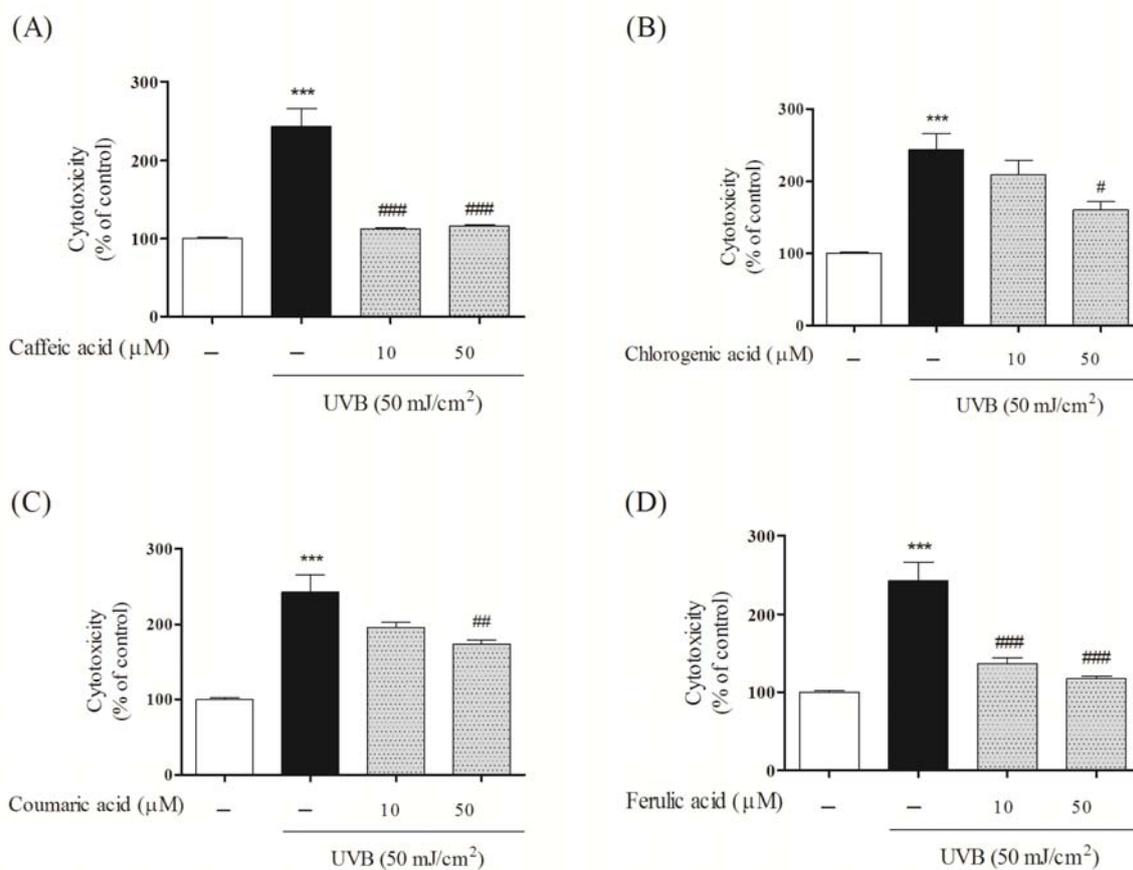


(C)



Supplementary figure. 2.

Effect of JSE on Nrf2 and HO-1 levels in human epidermal keratinocytes. Nrf2 or HO-1 (green) in cells incubated with or without 10 or 100 µg/mL JSE was detected by immunofluorescence using an anti-Nrf2 or HO-1 antibody. Nuclei were visualized by DAPI staining (blue). The fluorescence intensity due to Nrf2 and HO-1 expression was measured (A and C). Then, Effect of JSE on GSH levels in human epidermal keratinocytes. GSH levels in cells incubated with or without 10 or 100 µg/mL JSE was detected by kit assay. Values are means \pm SEM. * $p < 0.05$ and *** $p < 0.01$, vs. the control.



Supplementary figure. 3. Effects of different ingredients of JSE such as caffeic acid, chlorogenic acid, coumaric acid, and ferulic acid on UVB-induced apoptotic damage in human epidermal keratinocytes. After cells reached confluence, they were treated with different ingredients of JSE such as caffeic acid (A), chlorogenic acid (B), coumaric acid (C), and ferulic acid (D) for 1 h and then incubated with UVB irradiation (50 mJ/cm²) for a further 23 h. Cytotoxicity is expressed as a percentage of the control. Values are means ± standard error of the mean (SEM). *** $p < 0.001$, vs. the control; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, vs. the UVB-alone group.