

Neuroprotective effect of *Citrus unshiu* immature peel and nobiletin inhibiting hydrogen peroxide-induced oxidative stress in HT22 murine hippocampal neuronal cells

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ABSTRACT

Background: Oxidative stress-induced cell damage is common in the etiology of several neurobiological disorders, including Alzheimer's disease and Parkinson's disease. In a case study, nobiletin-rich *Citrus reticulata* peels could prevent the progression of cognitive impairment in donepezil-preadministered Alzheimer's disease patients. **Objective:** In this study, we investigated the effects and underlying mechanism of nobiletin and *Citrus unshiu* immature peel (CUIP) water extract, which contains nobiletin as a major compound, on hydrogen peroxide-induced oxidative stress in HT22 cells, a murine hippocampal neuronal model. **Materials and Methods:** HT22 cells were treated with hydrogen peroxide in the presence or absence of various concentrations of CUIP and nobiletin. Cytotoxicity and apoptotic protein levels were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and Western blotting. **Results:** Pretreatment with CUIP and nobiletin inhibited cell death due to hydrogen peroxide. Hydrogen peroxide-induced the expression of phospho-Jun N-terminal kinases (p-JNK) and p-p38 proteins in HT22 cells; however CUIP and nobiletin suppressed p-JNK and p-p38 without changing JNK or p38. Regarding apoptosis, caspase 3, B-cell lymphoma 2 (Bcl-2), and Bax protein expression was determined. CUIP and nobiletin suppressed caspase 3 and Bax expression, but they induced Bcl-2 expression in HT22 cells. **Conclusion:** These results show that CUIP and nobiletin can protect against hydrogen peroxide-induced cell death in HT22 neurons via mitogen-activated protein kinases and apoptotic pathways.

Key words: *Citrus unshiu* immature peel, neuroprotective effect, nobiletin, oxidative stress

INTRODUCTION

Oxidative stress-induced cell damage is common in the etiology of several neurobiological disorders, such as ischemia, Alzheimer's disease, and Parkinson's disease.^[1-3] In these pathological processes, oxidative stress leads to many biological consequences, including cell death, which involves the mitogen-activated protein kinases (MAPKs) and apoptotic signals.^[4] H₂O₂ has also been shown to activate several intracellular signaling pathways, including poly (adenosine diphosphate-ribose) polymerase in response

to DNA damage,^[5] nuclear factor-κB,^[6] activator protein 1.^[7] HT22 cells, an immortalized mouse hippocampal cell line, have been widely used as *in vitro* model for studying the mechanism of oxidative stress-induced neuronal cell death.^[8,9]

Nobiletin is a polymethoxyflavone. It is well-known that nobiletin has biological activities such as anti-inflammatory effects, anti-carcinoma effects, and ameliorating scratching behavior in mice.^[10-12] In a recently report, nobiletin rescued memory deterioration in Alzheimer's disease model rats by restoring β-amyloid-impaired cAMP response element binding protein phosphorylation.^[13] *Citrus unshiu* peel is the dried peel of *C. unshiu* Markovich or *C. reticulata* Blanco. It has been used as traditional medicine in Korea, China, and Japan and also divided as immature peel or mature peel.

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C. unshiu immature peel (CUIP) (Cheong pi or Chung-pi in Korea, Qing pi in China, Jyōhi in Japan) has been used for relief of pleuralgia caused by liver disease and remedy of indigestion. On the other hand, *C. unshiu* mature peel (Jin pi in Korea, Chen pi in china, Chinpi in Japan) has been used for improving bronchial, asthmatic conditions, cardiac and blood circulation in these countries.^[14,15] CUIP and mature peel contains flavonoids including hesperidin, rutin, nobiletin, and naringenin.^[16] In a case study, nobiletin-rich *C. reticulata* peels could prevent the progression of cognitive impairment in donepezil-preadministered Alzheimer's disease patients.^[17] Moreover, there are evidences that nobiletin content in CUIP is higher than *C. unshiu* mature peel.^[18,19] However, the mechanisms of action of CUIP and nobiletin's neuroprotective efficacy against oxidative stress in HT22 cells have not been investigated. These encouraged us to focus on the relationship between CUIP and neuroprotective effect of nobiletin. In this study, we investigated how CUIP and nobiletin inhibited hydrogen peroxide-induced cell death in HT22 cells through MAPK activity, and the Bax, and Bcl-2 pathway.

MATERIALS AND METHODS

Materials

Nobiletin, hesperidin, and H₂O₂ (30%) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) ⇒ erase, penicillin/streptomycin were obtained from Gibco (NY, USA). Cell proliferation assay solution was obtained from Promega (Madison, USA). phospho-Jun N-terminal kinases (p-JNK), JNK, p-p38, p38, caspase-3, and horseradish peroxidase-linked anti-mouse and rabbit antibodies were purchased from cell signaling technology (Danvers, MA, USA). Bax, Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Assay Designs (MI, USA).

Preparation of *Citrus unshiu* immature peel

Dried CUIP was provided by JBT Co. Ltd., in Jangheung, Republic of Korea. It was identified by Dr. Yeo (Medicinal Crop Seed Supply Center, Jangheung, Republic of Korea). The quality CUIP (100g) was boiled in 1 L distilled water for 3 h. The water extract was filtered and then removed under reduced pressure and lyophilized. In total, 12.4 g of extract powder of CUIP was recovered and kept at -20°C (JKTM-13-02). The powder was redissolved in distilled water (200 mg/mL) and stored in a freezer.

Cell line and culture

The HT22 cells, a murine hippocampal cell line, were provided by Dr. Soo Hyun Park (Jeonnam National University, Republic of Korea). HT22 cells were cultured

in DMEM, supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and were maintained in a humidified incubator with 5% CO₂. In all experiments, cells were incubated in the presence of the indicated concentrations of CUIP or nobiletin for 1 h before the addition of H₂O₂ (250 µM).

Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (CellTiter96 Aqueous One Solution, Promega) according to the manufacturer's protocol. Briefly, HT22 cells were plated in 96-well culture plates (1 × 10⁴ cells/well). After 24 h, the medium was changed, and the cells were treated with CUIP or nobiletin at various concentrations for 1 h. The plates were then cultured for 24 h with 250 µM H₂O₂ or not. Then, 20 µL MTS (5.0 µg/µL) was added to each well for an additional 4 h of incubation at 37°C. Absorbance was measured at 490 nm with a microplate reader (infinite M200 Pro, Tecan Group Ltd. Salzburg, Austria).

Western blot analysis

Cells were lysed with radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1.2% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl, pH 7.4) with protease inhibitor cocktail (Sigma). Total protein (40 µg) was separated by 10% or 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Western blot procedures were performed routinely. pJNK, JNK, p-p38, p38, caspase3, Bax, Bcl-2, and GAPDH were detected with the respective antibodies and a chemiluminescence detector (LiCor 2600, Lincoln, NE, USA) after enhanced chemiluminescence treatment.

High-performance liquid chromatography analysis of nobiletin in *Citrus unshiu* immature peel

To assay nobiletin and hesperidin in CUIP, we performed high-performance liquid chromatography (HPLC) (Shimadzu, Japan) by using a diode array detector. Analytical HPLC was run on a Luna C-18 reverse phase column, 100A, 25 cm × 4.6 mm, 5 µm. The mobile phase consisted of two solvents, water (0.1% formic acid) and acetonitrile. Table 1. shows the ratio of mobile phases for 40 min; the flow rate was 1 mL/min and the injection volume was 10 µL. 10 mg/mL CUIP, 100 µg/mL nobiletin, and hesperidin were used for HPLC.

Statistics

The data are expressed as means ± standard deviation. Statistical analyses used student's *t*-test, assuming unequal

variances. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Cell viability with *Citrus unshiu* immature peel and nobiletin

HT22 cells were treated for 24 h with different concentrations of CUIP or nobiletin. Neither CUIP nor nobiletin showed cytotoxicity at the concentrations tested. When HT22 cells were treated with $250 \mu\text{M H}_2\text{O}_2$, cell viability was decreased by 84% ($P < 0.05$). However, cell viability with 1 mg/mL CUIP or 50 μM nobiletin

pretreatment were 95.8% and 97.02%, respectively. Thus, pretreatment with CUIP and nobiletin could protect against cell death induced by oxidative stress in HT22 cells [Figure 1].

Inhibitory effects of *Citrus unshiu* immature peel and nobiletin on phosphorylation of Jun N-terminal kinases and p38

In the previous studies, H_2O_2 induced phosphorylation of MAPKs.^[4] In this study, pJNK and p-p38 were overexpressed with $250 \mu\text{M H}_2\text{O}_2$ treatment for 6 h. However, CUIP or nobiletin pretreatment inhibited the expression of phosphorylation of JNK and p38 without changing total JNK and p38 expression. These result showed that CUIP and nobiletin could affect the MAPKs pathway in protecting HT22 cells against death [Figure 2].

Inhibitory effects of *Citrus unshiu* immature peel and nobiletin on apoptosis

To investigate mitochondrial dysfunction associated apoptosis in HT22 cells due to CUIP and nobiletin, cells were pretreated with CUIP (1 mg/mL) or nobiletin (50 μM) for 1 h and then $250 \mu\text{M H}_2\text{O}_2$ was added for 24 h to activate the caspase-3, Bax, and Bcl-2 pathway.

Table 1: The ratio of mobile phases for 40 min for HPLC

Time (min)	0.1% formic acid in water (%)	0.1% formic acid in acetonitrile (%)
0	80	20
5	80	20
32	30	70
34	30	70
35	80	20
40	80	20

HPLC: High-performance liquid chromatography

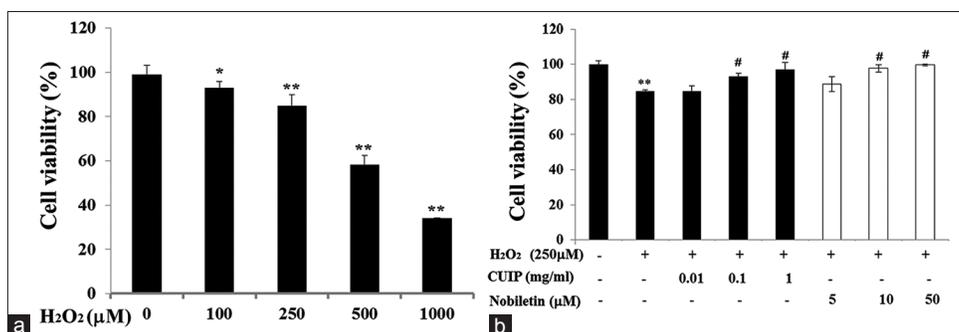


Figure 1: Effects of *Citrus unshiu* immature peel and nobiletin on H_2O_2 -induced cytotoxicity in HT22 cells. (a) Cell viability was reduced by H_2O_2 , dose-dependently. (b) *C. unshiu* immature peel and nobiletin protected the cell death by $250 \mu\text{M H}_2\text{O}_2$. Data are presented as means \pm S.D. from three experiments. * $P < 0.05$, ** $P < 0.005$; Blank vs. H_2O_2 treated group, # $P < 0.05$; H_2O_2 treated group vs. CUIP or nobiletin treated group with H_2O_2

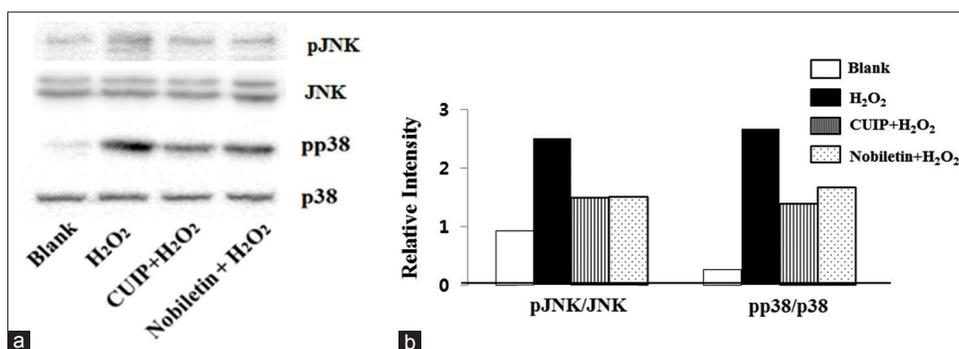


Figure 2: Effects of *Citrus unshiu* immature peel and nobiletin on H_2O_2 -induced phosphorylation of Jun N-terminal kinases and p38 in HT22 cells. (a) Protein bands and (b) relative intensities of protein bands. Cells were treated with *C. unshiu* immature peel and nobiletin 1 h after $250 \mu\text{M H}_2\text{O}_2$ was added to the cells and incubated for 6 h. Then, 30 μg cell lysates were subjected to Western blot analysis for phospho-Jun N-terminal kinases, Jun N-terminal kinases, pp38, and p38 with monoclonal antibodies

H₂O₂ activated caspase-3 and Bax but suppressed Bcl-2 expression. However, CUIP and nobiletin inhibited caspase-3 and Bax expression but activated Bcl-2 expression [Figure 3]. These results indicated that CUIP and nobiletin can protect the cells against death through mitochondria-associated apoptosis after oxidative stress.

Identification of nobiletin and hesperidin in *Citrus unshiu* immature peel by high-performance liquid chromatography

To identify nobiletin and hesperidin in CUIP, we performed HPLC analysis using a C18 cartridge column and eluted with increasing concentrations of acetonitrile. HPLC revealed that the yield of nobiletin

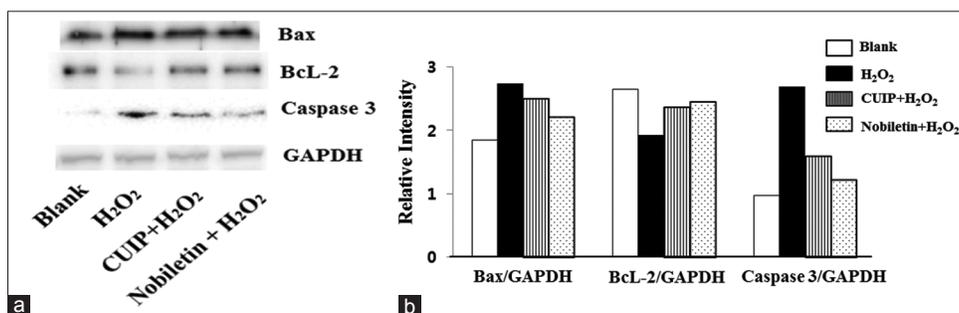


Figure 3: Effects of *Citrus unshiu* immature peel and nobiletin on H₂O₂-induced Bax, Bcl-2, and caspase-3 in HT22 cells. (a) Protein bands and (b) relative intensities of protein bands. Cells were treated with *C. unshiu* immature peel and nobiletin 1 h after 250 μM H₂O₂ was added to the cells and incubated for 24 h. Then, 30 μg cell lysates were subjected to Western blot analysis for Bax, Bcl-2, and caspase-3 with monoclonal antibodies. glyceraldehyde 3-phosphate dehydrogenase was used as a control

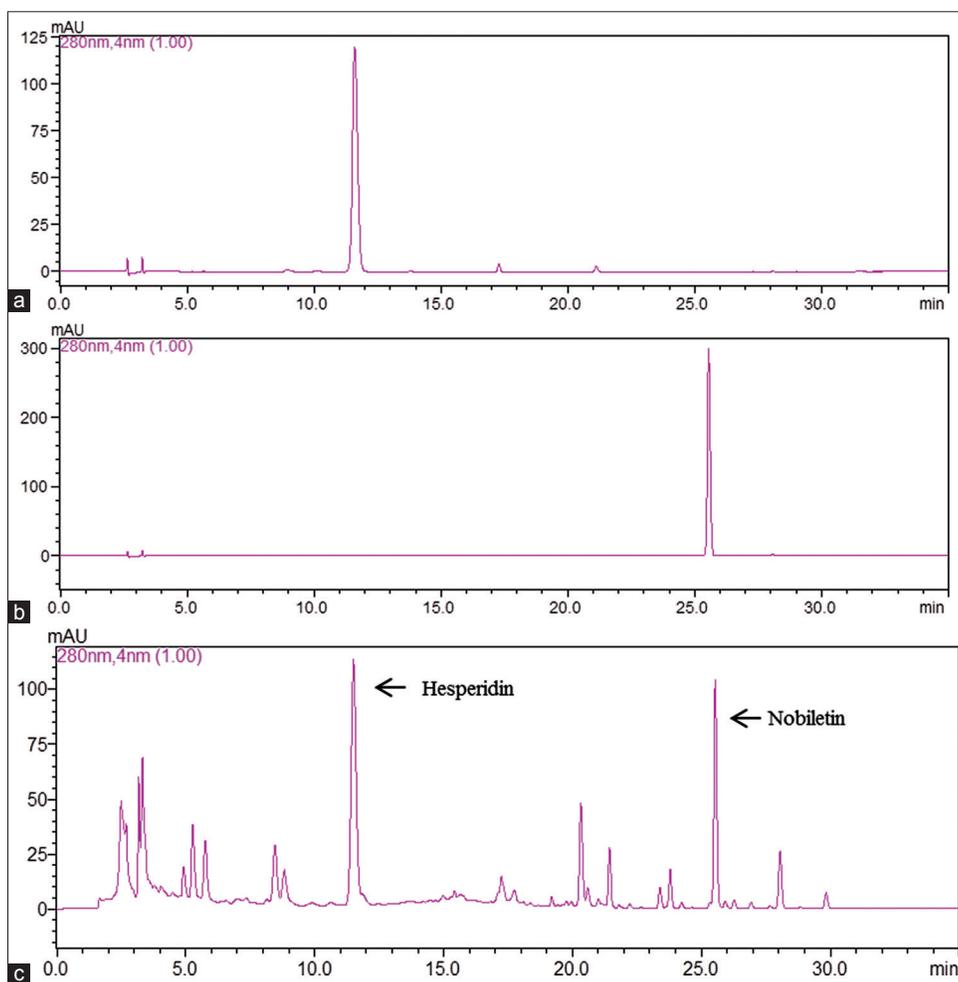


Figure 4: Analysis of nobiletin and hesperidin in *Citrus unshiu* immature peel by high-performance liquid chromatography at 280 nm. (a) Standard of hesperidin; (b) standard of nobiletin; (c) *C. unshiu* immature peel in methanol

and hesperidin from CUIP was 0.64% and 1.41%, respectively [Figure 4].

DISCUSSION

In the present study, we found that CUIP and nobiletin prevent the H_2O_2 -induced cell death through inhibiting JNK and p38 phosphorylation, caspase-3 and Bax activation, and activating Bcl-2 suppression in HT22 cells.

Jun N-terminal kinases and p38 are major members of the MAPKs, a family of serine/threonine protein kinases, and involved in many cellular processes, including cell growth, differentiation, inflammation, and cell death.^[20] Inhibition of pJNK and p-p38 pathway by diverse stimulators such as glutamate, amyloid beta, and H_2O_2 is involved in neuroprotective effects.^[4,21,22] In this study, we found that H_2O_2 induced phosphorylation of JNK and p38 in HT22 cells; however, pretreatment with CUIP and nobiletin inhibited the phosphorylation of JNK and p38. These results accorded with the previous study, which showed nobiletin modulated p38 and JNK in PC12 cells challenged by H_2O_2 .^[23] In this study, we reconfirmed that the neuroprotective effect of nobiletin involved in MAPKs pathway also CUIP protected against cell death by oxidative stress through inhibiting the phosphorylation of p38 and JNK.

Because, apoptosis is known to be one the most sensitive biological markers for evaluating oxidative stress, representing an imbalance between free radical generation and the efficacy of the antioxidant system,^[24,25] we determined whether effects on apoptosis-related proteins could be seen in H_2O_2 -induced apoptosis in HT22 cells. H_2O_2 activates the “intrinsic apoptosis” or mitochondrial apoptotic pathway. When Bcl-2 family proteins translocate to mitochondria, intrinsic apoptosis are initiated. Bax and Bcl-2 belong to the Bcl-2 family. Bax plays as a pro-apoptotic protein. On the other hand, Bcl2 plays as anti-apoptotic protein.^[26] Bax translocate to the mitochondrial outer membrane, where it oligomerizes or forms complexes with Bid or Bad. Bax oligomers facilitate the release of proapoptotic molecules such as cytochrome c or Smac from mitochondria membrane. Cytochrome c leads caspase-9 activation result in caspase-3 activation.^[27] In this study, we observed CUIP and nobiletin activated Bcl-2 expression but inhibited caspase-3 and Bax expression against H_2O_2 stimulation in HT22 cells. In the previous studies, nobiletin also regulated caspase-3 levels in PC12 cells stimulated by H_2O_2 .^[23,28] In this study, we showed that CUIP and nobiletin can protect against H_2O_2 -induced oxidative stress in HT22 cells by upregulating Bcl-2, and an anti-apoptotic protein downregulating cleaved caspase-3 and Bax.

Hesperidin and nobiletin are well known main flavonoids of Citrus species. Zhang *et al.*, reported 95% ethanol extract of citrus peel, purchased from a local factory in Jinhua, Zhejiang, China in November 2004, contained $2.81 \pm 0.09\%$ hesperidin and $0.27 \pm 0.03\%$ nobiletin.^[29] In another report, Lu *et al.*, analyzed flavonoids in fresh fruit and traditional chinese medicine ingredients of 27 citrus and three not-citrus cultivars from Zhejiang, Fujian and Jiangxi Province in China. According to the report, fresh peel of *C. unshiu* contained 5.86% and 6.25% hesperidin and 0.01% and 0.02% nobiletin from *C. unshiu* peel.^[18] Choi *et al.*, analyzed flavonoids in immature peel and mature of *C. unshiu* grown on Jeju Island in Korea. They reported that CUIP ethanol extract contained $17.23 \pm 0.34\%$ hesperidin and $1.21 \pm 0.48\%$ nobiletin. However, its mature peel ethanol extract had $4.47 \pm 1.29\%$ hesperidin and $0.75 \pm 0.00\%$ nobiletin.^[19] These reports showed that the flavonoid in *C. unshiu* were different by collecting time or growing place. Also immature peel of *C. unshiu* contained hesperidin and nobiletin more than mature peel. In our study, we detected 1.41% hesperidin and 0.64% nobiletin in CUIP water extract. Because hesperidin and nobiletin are hard to be extracted by hot water, contents of these compounds were detected lower in other reports.

CONCLUSION

In conclusion, CUIP and nobiletin protected against cell death induced by H_2O_2 in HT22 cells through inhibiting the activation of JNK, p38 MAPKs, caspase-3, and Bax but upregulation of Bcl-2. These results suggest that CUIP and nobiletin may be useful to protect against neurodegenerative disorders involving oxidative stress. Moreover nobiletin may play an active compound of CUIP for preventing the cell death in HT22 by H_2O_2 . However, further studies are required to clarify the anti-apoptotic mechanisms of CUIP and nobiletin.

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