

Curcuma kwangsiensis Extracts Produced Antioxidant Effects Against Injury Induced by H₂O₂ on PC12 Cells

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Abstract: The radix of *Curcuma kwangsiensis* S. G. Lee & C. F. Liang (Zingiberaceae) is a traditional medicine in China, and used for treating qi stagnation and blood stasis. Previous chemical study showed it is rich in sesquiterpenoids and diarylheptanoids, similar to those in *Curcuma longa* L., which showed obviously protective effects against oxidative damage. However, the antioxidant and underlying mechanism of *C. kwangsiensis* has not been studied yet. In current study, the antioxidant activities of *C. kwangsiensis* extracts (ECWs), including 95 % EtOH extract (HCECW), 75 % EtOH extract (MCECW), methanol extract (MECW), dichloromethane extract (DECW) and petroleum ether extract (PECW), and their possible mechanisms, were studied on the model of H₂O₂-induced PC12 cell damage *in vitro*. The results showed different concentrations (1, 10, 50 µg/mL) of HCECW, MCECW and DECW could increase damaged PC12 cell viability significantly. In the extract-treated groups, the release rate of LDH significantly decreased, while SOD, CAT, and GSH markedly increased. In the meantime, the intracellular Ca²⁺ and cell apoptosis decreased significantly, while MMP increased and apoptosis morphology was clearly improved. Compared with the model group, they produced effects on up-regulating of Bcl-2, down-regulating of Bax and Caspase-3. Further, the chemical analysis of those five extracts by UPLC-DAD-Q-TOF-MS showed their major constituents were sesquiterpenoids and diarylheptanoids, indicating both of them presented in ECWs are antioxidant substances in this plant. This study provided an experimental basis for the future development of antioxidants from the genus *Curcuma*.

Keywords: *Curcuma kwangsiensis*; antioxidant effect; PC12 cell; oxidative injury; chemical analysis. © 2019 ACG Publications. All rights reserved.

1. Introduction

Neurodegenerative diseases related to the central nervous system (CNS) have been becoming a class of the major diseases that are serious harmful to people's physical and mental health, and the harmony of the society, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). As reports, the functional diversity of CNS leads to the complexity of

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causes for those diseases [1-2]. However, the nerve system injury, caused by oxidative stress, accumulation reactive oxygen species (ROS) and the imbalance between oxidant and antioxidant occurred in intracellular systems, was considered to play a crucial role in neurodegenerative disease [3,4]. Based on this point, the usage of an inhibitor of oxidative stress is a potential therapeutics to prevent the occurrence of those diseases.

Considering that typical neurons characteristics, the rat adrenal pheochromocytoma PC12 has been a common cell applied to studies of neurodegenerative diseases [5]. Under the normal operating condition, the oxidative damage on PC12 will be induced easily by exogenous H₂O₂ stimulation for some time [6-7]. Thus, the PC12 cell model induced by H₂O₂ has been regarded as a cell model *in vitro* for developing of neuroprotective materials and antioxidants.

In recent years, natural medicine, especially traditional Chinese medicine, has been a hot focus in the field of discovery of inhibitor against oxidative stress. Previous studies indicated that many extracts and constituents from Chinese medicines, such as catalpol from *Rehmannia glutinosa* [8], ginsenoside Rg1 from *Panax ginseng* [9], extract from *Terminalia chebula* [10], have markedly antioxidative and neuroprotective effects, which prevent the accumulation of active oxides in CNS, as to produce effects of oxidation resistance, inhibiting cytotoxicity and antiapoptosis.

The radix of *Curcuma kwangsiensis* G. Lee et C. F. Liang (CW), also called 'Guangxi Ezhu' in China, is a traditional medicine and used to treat qi stagnation and blood stasis. Previous reports have found it is rich in sesquiterpenes and curcuminoids [11-13], similar to those in *Curcuma longa* L., which has been confirmed to have obviously protective effects against oxidative damage [14]. It provides us an evidence that *C. kwangsiensis* is another potentially antioxidant source and need to further evaluation. In the present work, extracts of radix of *C. kwangsiensis* (ECWs), including 95 % EtOH extract (high concentration of EtOH extract, HCECW), 75 % EtOH extract (moderate concentration of EtOH extract, MCECW), methanol extract (MECW), dichloromethane extract (DECW) and petroleum ether extract (PECW), and their underlying mechanisms were investigated on H₂O₂-induced the damage of PC12 cells. In addition, the chemicals of those five extracts were analyzed by the method of UPLC-DAD-Q-TOF-MS to clarify the difference of chemicals among those extracts preliminarily.

2. Materials and Methods

2.1. Preparation of Extracts

The radix of *C. kwangsiensis*, purchased from the Traditional Chinese Medicine market in Kunming, China, was identified by Prof. Shiming Guo (Yunan Institute of Traditional Chinese Medicine and Material Medica, China). Its voucher specimen (No. CW-2014-05) was deposited in our laboratory.

Two of powdered *C. kwangsiensis* (500 g per sample) were extracted with 95 % ethanol (high concentration of EtOH) and 75 % ethanol (moderate concentration of EtOH) at 80°C for three times (3 × 1L × 6 h), separately. The other three (500 g per sample) were soaked with methanol, dichloromethane, and petroleum ether for three times at room temperature (3 × 1 L × 24 h), respectively. The organic solvent was removed from each extract at vacuum to get the 95 % EtOH extract (high concentration of EtOH extract, HCECW), 75 % EtOH extract (moderate concentration of EtOH extract, MCECW), methanol extract (MECW), dichloromethane extract (DECW) and petroleum ether extract (PECW), respectively. Further, each extract was dissolved in DMSO to get tested sample at different concentrations for bioassays. For chemical analysis, each extract was dissolved in MeOH to prepare sample at 1 mg/mL.

2.2. Drugs and Reagents

PC12 cells (undifferentiated, product ID: KCB200735YJ) were obtained from Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. Vitamin E, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl (MTT) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fetal bovine serum and Hoechst 33342 nuclear dye were purchased from Solar Bioscience & Technology Co., Ltd. (Beijing, China), and Dulbecco's modified Eagle's medium (DMEM, high

glucose) culture medium was obtained from Gibco Inc. (Grand Island, NY, USA). All antibodies used in our study were purchased from Abcam Inc. (Cambridge, MA, USA).

2.3. Cell Culture

Cells were cultured in DMEM, further supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 $\mu\text{g/ml}$ streptomycin, and incubated in the humidified atmosphere of 5% CO_2 air at 37 °C.

2.4. Assay of Cell Viability

Cell viability was determined by MTT assay. PC12 cells (1×10^4 cells/well) were incubated for 24 h, then treated with three dosages of ECWs or Vit E, and 400 μM H_2O_2 for 24 h at 37 °C. Then, 5 mg/mL MTT was added and continued incubation was carried out for 4 h. At last, after culture medium was removed, 150 μL DMSO was added to each well, followed by violently shaking the plate until formazan dissolving completely. The absorbance of each well was detected at 490 nm with a microplate reader (Molecular Devices, CA, USA). The results were expressed as a percent of the non-treated control.

2.5. Measurement of ROS

Overproduction of intracellular ROS is a key reason to cause the neuro cell damage. The intracellular ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, MO, USA) according to the previous report [15]. PC12 cells (1×10^4 cells/well) were seeded in 96-well plates for 24 h, then incubated with ECWs or VitE, and 400 μM H_2O_2 for 24 h at 37 °C. The old medium was wiped out and cells were washed one time with PBS, finally 100 μM DCFH-DA was added in dark and incubated at 37 °C for 30 min. After DCFH-DA contained culture medium was removed, the cells were washed once with PBS, DCFH-DA-loaded cells were detected using fluorescence plate reader (excitation, 485 ± 12 nm; emission, 530 ± 12 nm). The percentage increase in fluorescence per well was calculated by the formula $[(F_{t30} - F_{t0}) / F_{t0} \times 100]$, where F_{t0} is the fluorescence at 0 min and F_{t30} is the fluorescence at 30 min in PC12, presented in H_2O_2 .

2.6. Measurement of Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

ROS could provoke Ca^{2+} -dependent enzymes via increasing intracellular Ca^{2+} concentration, which will trigger the cell death by mode of necrosis or apoptosis. $[\text{Ca}^{2+}]_i$ was determined by the fluorescent Ca^{2+} -sensitive dye, Fura 2-acetoxymethyl ester (Fura-2 AM) (Kumamoto, Japan) [16]. PC12 cells were treated with ECWs at the dosages of 1, 10, 50 $\mu\text{g/ml}$, respectively, in the presence of 400 μM H_2O_2 . After removing the culture medium, cells were harvested and slowly washed three times with PBS solution, then resuspended in PBS solution containing 0.2 % BSA at 1×10^6 cell/ml. The Fura-2AM (5 μM) was added to cells and incubated at 37 °C for 30 min. The fluorescence of the microplate was measured at 510 nm (emission wavelength) in response to 340 and 380nm (excitation wavelengths) with a microplate reader (Molecular Devices, CA, USA). The fluorescence ratio (F_{340}/F_{380}) was calculated as an indicator of $[\text{Ca}^{2+}]_i$.

2.7. Analysis of LDH, SOD, CAT, and GSH

The degree of cell damage can be evaluated by the leakage level of LDH, which is an important index related to the integrity of the cell membrane. SOD, CAT, and GSH, as the key antioxidant enzymes, could prevent oxidative injury from free radical to cells. After PC12 cells were exposed to 400 μM H_2O_2 and three doses of HCECW, MCECW, DECW or Vit E for 24 h, the culture medium was collected and LDH leakage rate in the cell was measured using a commercial LDH assay kit (A020-1, Jiancheng Bioengineering, Nanjing, China). The absorbance of samples was read at 440 nm. Superoxide dismutase (SOD) activity, catalase (CAT) and intracellular level of glutathione (GSH) were also measured using commercial assay kits (A001-1-1, A007-1-1, A005, Jiancheng

Bioengineering, Nanjing, China), respectively. Above procedures were performed in accordance with the manufacturer's instructions.

2.8. Detection of Apoptotic Cells with Flow Cytometry

H₂O₂ causes oxidative injury and eventually leads the cells to apoptosis. The procedures for the study of cell apoptosis were performed by the instruction described in the annexin V - fluorescein isothiocyanate annexin/propidium iodide (annexinV-FITC/PI) detection kit (KeyGEN, Nanjing, China). PC12 cells were incubated with H₂O₂ in the presence of HCECW, MECCW and DECCW at the concentrations of 1, 10 and 50 µg/mL or VitE for 24h at 37 °C. Cells were washed immediately with cold PBS once and then stained with 5 µL annexin V-FITC and 5 µL PI for 30 min at room temperature in the dark. Then, 500 µL PBS was added to resuspend cells. Finally, the cells were analyzed by flow cytometry (FACS, Becton Dickinson, USA).

2.9. Measurement of Mitochondrial Membrane Potential (MPP)

MPP is an important index to indicate cell health, mitochondrial membrane permeability and apoptosis. Rhodamine 123 (Rh123) (St. Louis, MO, USA), a fluorescence dye, was used to assay the mitochondrial membrane potential. Cells were collected and poured into the centrifuge tubes. After washed with PBS twice, cells were incubated in PBS containing 10 µM Rh123 for 30 min at 37 °C in the dark. Unbounded dye was removed by washing the cells twice with pre-warmed PBS. Cells (1×10⁶/tube) were analyzed with flow cytometry. The state of depolarization of MMP was represented as the mean fluorescence intensity in the cells.

2.10. Fluorescence Microscopic Assay of Apoptotic Cells

Chromatin condensation and nuclear fragmentation are some characteristics of apoptosis, which can be observed by fluorescence microscopic assay. PC12 cells (10⁴/well) were grown on 24-well plates. After exposed to 400 µM H₂O₂ and three concentrations of HCECW, MCECW, DCEW or vitE for 24 h, the cells were fixed for 30 min with 4 % paraformaldehyde at room temperature. Then washed with cool PBS three times, cells were stained with 10 µg/mL Hoechst 33342 at 37 °C in the dark for 30 min. Samples were observed under Olympus IX70 inverted fluorescence microscope

2.11. Western Blot

Apoptosis is an active process, which was associated with activation, expression, and regulation of a series of proteins. Treated PC12 cells were gathered and washed three times with PBS, then lysed with cell lysis buffer containing 1 % phenyl methyl sulfonyl fluoride. The lysates of whole cells were centrifuged, and the protein contents in samples were measured using bicinchoninic acid assay. Equal amounts of 10 µg protein were separated by electrophoresis on 12 % sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with 5 % (w/v) non-fat milk powder in Tris-buffered saline containing 0.1 % (v/v) Tween-20 (TBST) for 40 min. Then, the membranes were incubated with the primary antibodies overnight at 4 °C. Finally, membranes were washed with TBST and incubated with the secondary antibodies at room temperature for 1 h. After rewashed with TBST, the bands were developed by enhanced chemiluminescence.

2.12. Chemical Analysis by UPLC-DAD-Q-TOF-MS

MS data were determined by Agilent UPLC-Q-TOF-MS (6530B) with an electrospray ionization (ESI) source in positive ion mode on split injection mode (4:1). The parameters of ESI-MS were set as follows: capillary voltage, + 4000 V; nebulizer gas pressure, 30 psi; dry gas flow rate, 8.0 L/min; and temperature, 350 °C. The data were analyzed using Agilent MassHunter Data Analysis 5.0 software.

Chromatographic analysis was performed on an Agilent Technologies 1200 Series System (Agilent, USA), equipped with ZORBAX SB-C₁₈ column (4.6 × 200 mm, 5 μm, Agilent, USA) at 30 °C of column temperature. The mobile phases were composed of water (A) and methanol (B) with the flow

rate at 1 mL/min. The sample volume injected was set at 10 μL. The detailed gradient program of chromatographic analysis for each sample was listed in Table S1.

2.13. Statistical Analysis

The data are expressed as means ± standard deviation (SD) and statistical comparisons were made by means of a one-way ANOVA test followed by student's t-test. Differences were accepted as statistically significant at P<0.05. Each experiment was performed in triplicate at least.

3. Results

3.1. ECWs Protected PC12 Cells Against Oxidative Damage Induced by H₂O₂

The conventional MTT reduction assay was applied to the determination of cell viability. The viability of PC12 cells induced with H₂O₂ at 400 μM for 24 h was 66.7 % of the control value, whereas the viabilities of cells treated with vitamin E (Vit E, as reference) or HCECW, MCECW, MECW, DECW and PECW increased obviously to 83.4 % (at 100 μM), 91.9 % (at 5 μg/ml), 102.1 % (at 5 μg/mL), 76.9 % (at 10 μg/mL), 93.8 % (at 5 μg/mL) and 85.9 % (at 10 μg/mL), respectively (Figure 1). The results revealed that all extracts could produce a protective effect against H₂O₂-induced injury at varying degrees.

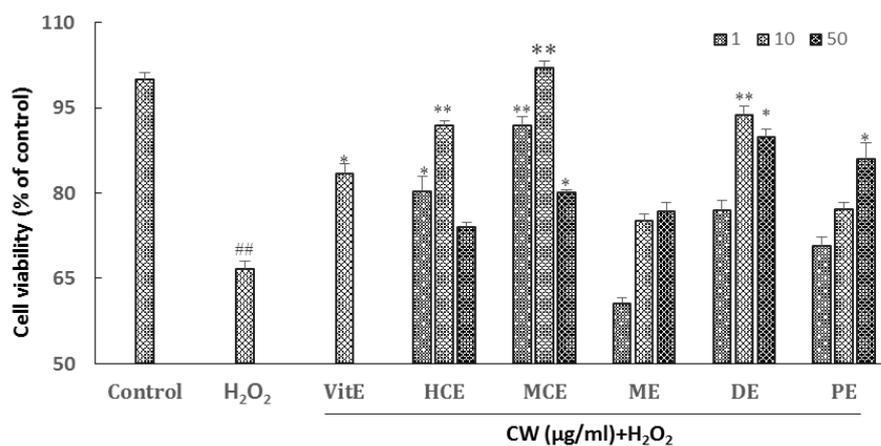


Figure 1. Effects of ECWs or Vit E on cell viability using MTT assay on PC12 cells treated by H₂O₂. The data are expressed as mean ± SD (n=3). ##P<0.01 as compared with the control group; *P<0.05 and **P<0.01 by compared with the H₂O₂ group

3.2. ECWs Inhibited the Intracellular Accumulation of ROS induced by H₂O₂

The results showed PC12 cells exposed to H₂O₂ for 24 h displayed a significant increase of ROS level in the intracellular, relative to 186.7 in control cells. In the presence of H₂O₂, the ROS levels markedly reduced in ECWs or Vit E -treated PC12 cells, of which the ROS levels with Vit E, HCECW, MCECW, MECW, DECW, PECW at 100 μM, 10 μg/mL, 10 μg/mL, 50 μg/mL, 10 μg/mL and 50 μg/mL were 108.2, 130.3, 111.6, 170.3, 135.3 and 169.6, respectively (Figure 2). The results were in accordance with the MTT results.

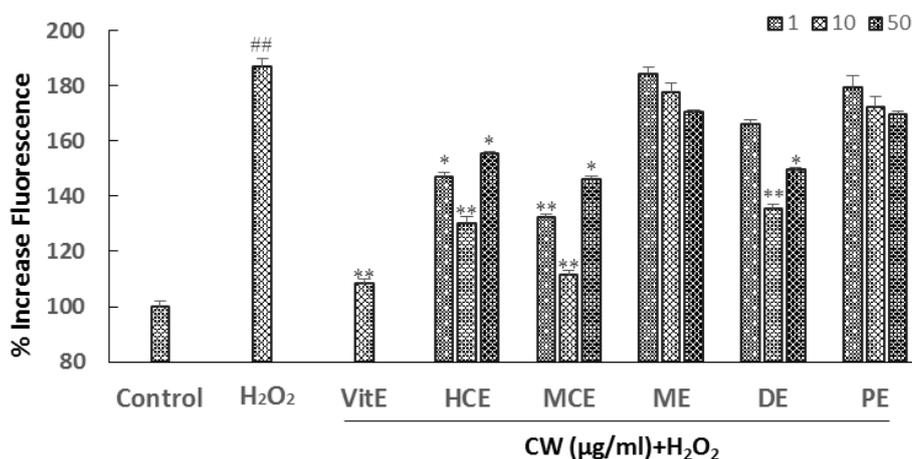


Figure 2. Inhibitory effects of ECWs or VitE on the H₂O₂-induced intracellular accumulation of ROS. Intracellular ROS levels were assayed based on DCFH-DA fluorescence. The data were expressed as % increase fluorescence by means ± SD (n=3). A difference was considered statistically significant when **p < 0.01 or *p < 0.05 vs. H₂O₂ group, ##p < 0.01 vs. control

3.3. ECWs Inhibited the Increase of [Ca²⁺]_i in H₂O₂-treated PC12 Cells

As shown in Figure 3, the fluorescence ratio (F₃₄₀/F₃₈₀) in PC12 cells can effectively increase to 188.3 after its treatment by H₂O₂ for 24 h, compared with those in untreated control culture. However, the fluorescence ratio (F₃₄₀/F₃₈₀) significant decreased to 127.5, 140.4, 128.4, 169.6, 156.3 and 177.5, when it was treated with 100 μM Vit E, 10 μg/mL HCECW, 10 μg/mL MCECW, 50 μg/mL MECW, 10 μg/mL DECW and 50 μg/mL PECW, respectively, for 24h at exposure of H₂O₂.

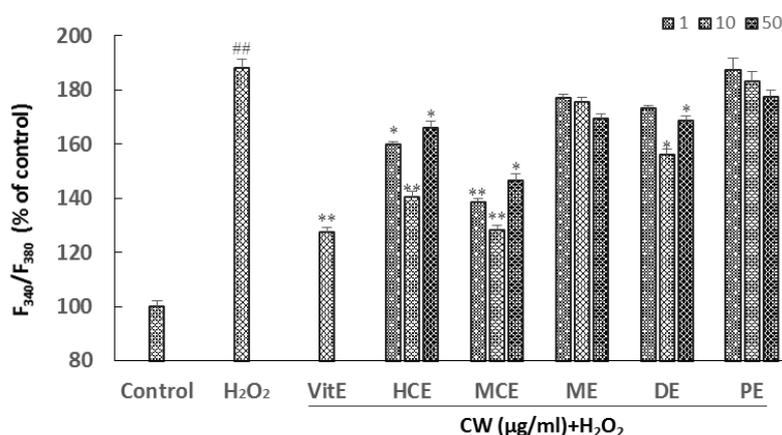


Figure 3. Effects of ECWs or VitE on intracellular [Ca²⁺]_i in H₂O₂-induced PC12 cells. Intracellular [Ca²⁺]_i were measured by Fura-2/AM 340/380nm fluorescence ratio (F₃₄₀/F₃₈₀). Each value represents the means ± SD (n=4). ##p < 0.05 vs. control, *p < 0.05 or **p < 0.01 vs. H₂O₂ group

3.4. Effects of HCECW, MCECW, and DECW on LDH, SOD, CAT, and GSH in H₂O₂-induced PC12 cells

To further investigate the protective effects of HCECW, MCECW and DECW, the released LDH and GSH contents, and SOD and CAT activities were detected. Treatment of PC12 cells with 400 μ M H₂O₂ for 24 h caused the increase of LDH leakage rate, the activities of SOD and CAT, and the content of GSH, at 67.8 %, 34.4 %, 47.5 %, and 52.5 %, respectively (Table 1). Compared with model group, LDH leakage rates in treated groups of Vit E at 100 μ M, HCECW, MCECW, and DECW at 10 μ g/mL markedly attenuated to 27.1 %, 16.6 % and 29.3 %, respectively (Table 2). Meantime, SOD activities were observed as 88.2 %, 85.4 %, 93.3 % and 77.3 %, when 100 μ M Vit E, 10 μ g/mL HCECW, MCECW and DECW were treated on the damaged cells, respectively. In addition, treatments of HCECW, MCECW and DECW at 10 μ g/mL dosage can increase of CAT activities to 85.6 %, 89.3 % and 76.4 %, and GSH contents to 87.7 %, 101.9 % and 83.9 %, respectively. Totally, treatment with VitE, HCECW, MCECW, and DECW can significantly attenuate the changes of the contents of SOD, CAT and GSH in a dose-dependent manner (Table 1). That change trends in leakage rate of cellular LDH, activities of SOD and CAT, and contents of GSH, were similar to that in cell viability, intracellular accumulation of ROS and [Ca²⁺]_i.

Table 1. Protective effect of extracts of CW on H₂O₂-induced PC12 cell injury on LDH, SOD, CAT and GSH*

Treatment	LDH leakage (% of control)	SOD activity (% of control)	CAT activity (% of control)	GSH content (% of control)
Control	37.74±6.6	100.0±1.7	100.0±1.89	100±0.816
H ₂ O ₂	67.76±3.56 ^{##}	34.41±4.70 ^{##}	47.49±1.27 ^{##}	52.48±0.47 ^{##}
VitE (100 μ M) + H ₂ O ₂	32.60±4.50 ^{**}	88.18±2.05 ^{**}	77.77±1.81 ^{**}	81.58±1.25 ^{**}
HCECW (1 μ g/mL) + H ₂ O ₂	42.85±2.55 [*]	56.18±2.05 [*]	65.93±6.68 [*]	76.66±2.05 ^{**}
HCECW (10 μ g/mL) + H ₂ O ₂	27.01±5.90 ^{**}	85.39±1.27 ^{**}	85.58±1.94 ^{**}	87.66±1.63 ^{**}
HCECW (50 μ g/mL) + H ₂ O ₂	49.20±2.22 [*]	47.46±2.45 [*]	52.62±2.12	62.65±3.30
MCECW (1 μ g/mL) + H ₂ O ₂	22.61±1.03 ^{**}	83.11±1.25 ^{**}	63.59±1.41 [*]	89.68±1.41 ^{**}
MCECW (10 μ g/mL) + H ₂ O ₂	16.53±0.97 ^{**}	93.33±8.16 ^{**}	89.25±5.19 ^{**}	101.9±2.05 ^{**}
MCECW (50 μ g/mL) + H ₂ O ₂	37.26±1.00 [*]	75.65±2.36 ^{**}	58.26±4.71 [*]	75.31±1.70 [*]
DECW (1 μ g/mL) + H ₂ O ₂	51.73±0.59 [*]	44.14±1.11	51.20±2.36	60.69±2.62
DECW (10 μ g/mL) + H ₂ O	29.33±6.01 [*]	77.32±4.71 [*]	76.41±5.58 [*]	83.92±2.76 [*]
DECW (50 μ g/mL) + H ₂ O ₂	38.90±4.35 ^{**}	63.22±2.45 ^{**}	63.65±2.62 ^{**}	75.85±1.70 ^{**}

The cells were exposed to 400 μ M H₂O₂ in the presence of Vit E and different levels of HCECW, MCECW and DECW for 24 h for LDH, SOD, CAT, and GSH assay. Each value was represented as the means \pm SD (n=3). ^{##}P<0.01 as compared with the control group; ^{}P<0.05 and ^{**}P<0.01 as compared with the H₂O₂ group.

3.5. Effects of HCECW, MCECW, and DECW on H₂O₂-Induced Apoptosis of PC12 Cells

Figure 4 showed that apoptotic rate of PC12 cells increased to 43.2 % from 6.5 % in control group, after exposure of the cells to 400 μ M H₂O₂ for 24 h (p<0.01 vs. H₂O₂ group). Incubation of 100 μ M Vit E, 10 μ g/mL of HCECW, MCECW and DECW in the presence of H₂O₂ reduced the cellular apoptosis to 14.2 %, 23.4 %, 17.9 % and 27.5 %, respectively (p<0.01 vs. H₂O₂ group). The results indicated that HCECW, MCECW, and DECW revealed inhibition against H₂O₂-induced apoptosis of PC12 cells.

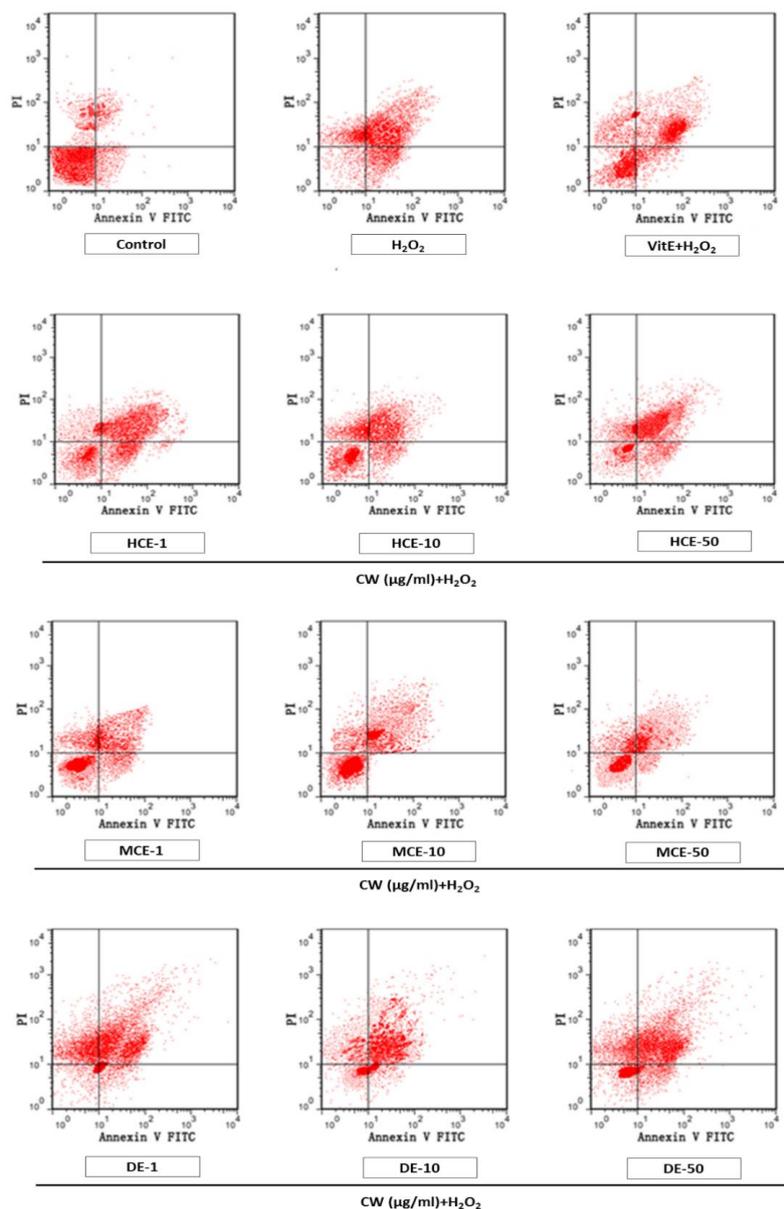


Figure 4. Effects of HCECW, MCECW, and DECW or Vit E against H_2O_2 -induced apoptosis of PC12 cells. PC12 cells were treated with 1, 10, 50 $\mu\text{g}/\text{mL}$ HCECW, MCECW and DECW and exposed to 400 μM H_2O_2 for 24 h. Then, cellular apoptosis was assayed by annexin V-FITC and PI staining and analyzed by flow cytometry with fluorescence-activated cell sorting. Data were presented as means \pm SD ($n=3$)

3.6. Effects of HCECW, MCECW, and DECW on the Mitochondrial Membrane Potential (MMP) of PC12 Cells

As shown in Figure 5, the effects of ECWs on MMP of PC12 cells was detected by the weakening of the fluorescence intensity of Rh123 treated with 400 μM H_2O_2 for 24 h. Compared with the control group, a significant increase of Rh123 from 10.2 % to 28.7 % was observed in the model group ($p<0.01$). Treatment of the cells with 100 μM Vit E, or 10 $\mu\text{g}/\text{mL}$ of HCECW, MCECW, and DECW, can protect PC12 cells against the rise of MMP induced by H_2O_2 via decreasing Rh123 to 19.0 %, 11.6 %, 11.2 and 17.1 %, respectively.

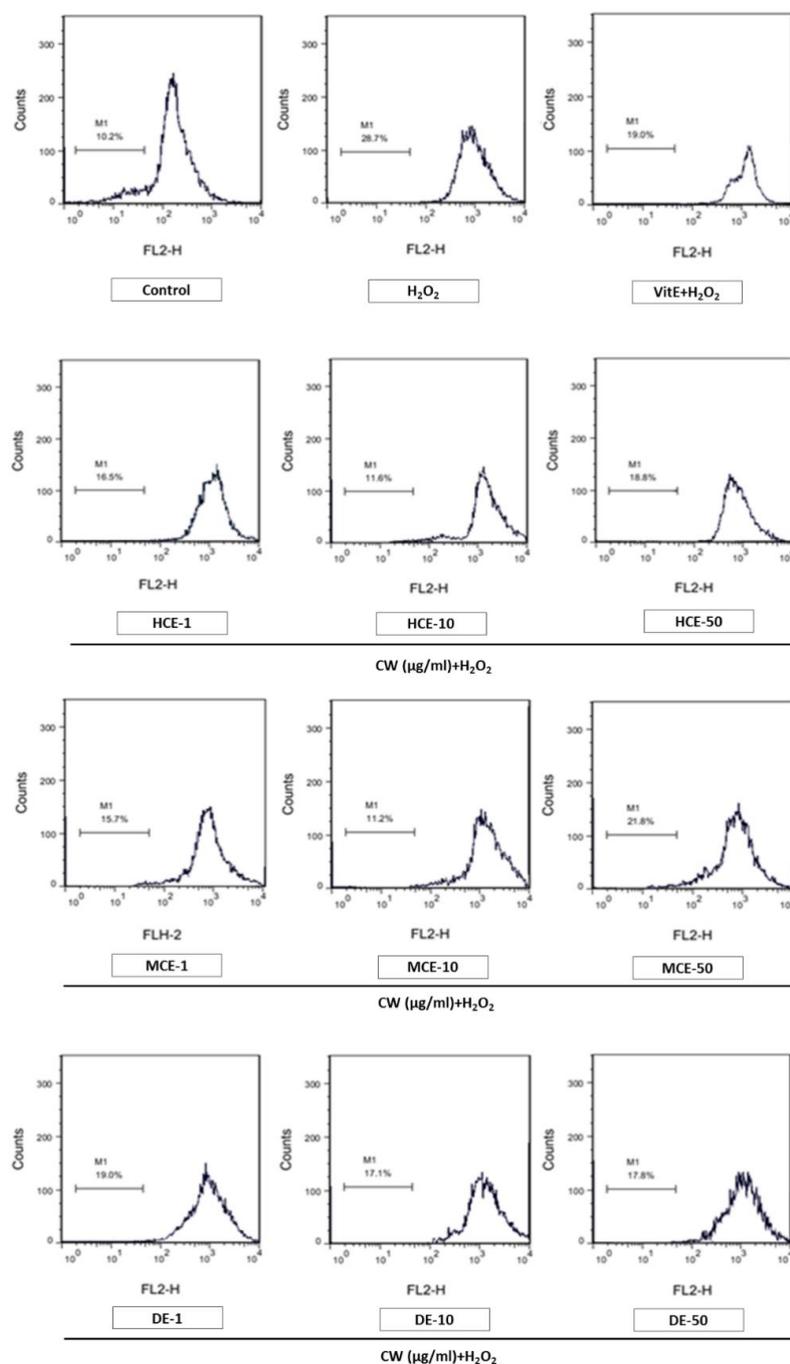


Figure 5. Effects of HCECW, MCECW, and DECW or Vit E on the loss of MMP in H_2O_2 -induced PC12 cells. MMP were determined based on the Rh123-associated MF1. Each value was represented as the means \pm SD (n=3)

3.7. Effects of HCECW, MCECW, and DECW on the Morphology of H_2O_2 -treated PC12 Cells

DNA fluorescent dye Hoechst 33342 was used in this study for investigation of the effects of treatments with HCECW, MCECW or DECW on the DNA and nuclear structure of PC12 cells. As illustrated in Fig.6, compared with the control group, cells treated with H_2O_2 revealed lots of small bright blue dots, representing chromatin condensation or nuclear fragmentation in the model group. However, treatment with $100 \mu M$ Vit E, or $10 \mu g/mL$ HCECW, MCECW and DECW, decreased the nuclear condensation and fragmentation significantly (Figure 6), indicating above-mentioned extracts can prevent neuronal cell death induced by H_2O_2 on the other hand.

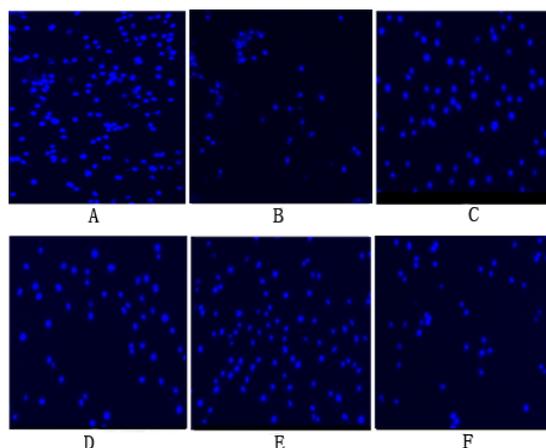


Figure 6. Effects of HCECW, MCECW and DECW or VitE on the cell survival in H_2O_2 -treated PC12 cells by Hoechst 33342 staining method. A: Control conditions. B: After exposure to $400 \mu M$ H_2O_2 for 24 h. C: Vit E ($100 \mu M$) + H_2O_2 ; D: HCECW ($10 \mu g/mL$) + H_2O_2 ; E: MCECW ($10 \mu g/mL$) + H_2O_2 ; F: DECW ($10 \mu g/mL$) + H_2O_2

3.8. Effects of HCECW, MCECW, and DECW on Bcl-2/Bax Ratio in H_2O_2 -treated PC12 Cells

As shown in Figure 7, the significant decrease of Bax expression and increase of Bcl-2 expression were found in treatment groups with $100 \mu M$ Vit E, or $10 \mu g/mL$ HCECW, MCECW and DECW, respectively, indicating those extracts can decrease apoptosis of damaged cells by H_2O_2 . Accordingly, the ratio of Bcl-2/Bax decreased to 1.0 in the model group, but increased to 2.7, 4.4, 9.5 and 4.1, respectively, in the above-mentioned treatment groups.

3.9. Inhibition of Caspase-3 Expression by HCECW, MCECW, and DECW in Damaged PC12 Cells

The protective effects of HCECW, MCECW, and DECW were also evaluated by Western blot analysis of caspase-3. In Figure 7, the level of caspase-3 was increased to 20.3 in model group, whereas it decreased to 8.5, 10.1, 5.0, 7.3 by treatment with $100 \mu M$ Vit E, or $10 \mu g/mL$ HCECW, MCECW and DECW, respectively, demonstrating that HCECW, MCECW, and DECW can suppress H_2O_2 -induced apoptosis in PC12 cells on this assay.

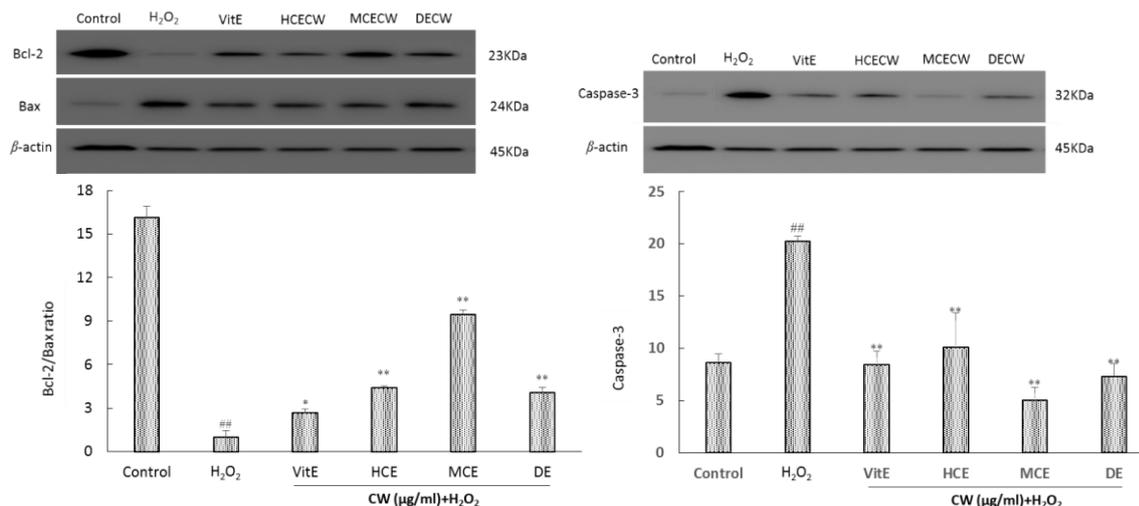


Figure 7. Effects of $100 \mu M$ Vit E, $10 \mu g/mL$ HCECW, $10 \mu g/mL$ MCECW and $10 \mu g/mL$ DECW on the expression of Bax, Bcl-2 and Caspase-3 in H_2O_2 -induced PC12 cells by western blot analysis. The data are expressed as the means \pm SD ($n=3$). Densitometric analyses of protein bands were normalized to a loading control β -actin. ## $P<0.01$ as compared with the control group; * $P<0.05$ and ** $P<0.01$ as compared with the H_2O_2 group. All experiments using β -actin as the loading control

3.10 Major Constituents in ECWs

15, 11, 16, 13 and 12 constituents were determined and identified by the of the UPLC-DAD-Q-TOF-MS analysis method in HCECW, MCECW, MECW, DECW, and PECW, respectively (Tables S2-S6, Figure 8). Those determined and identified compounds were almost sesquiterpenoids and diarylheptanoids. And the number and proportion of diarylheptanoids in HCECW, MCECW, and DECW were more than that in other extracts.

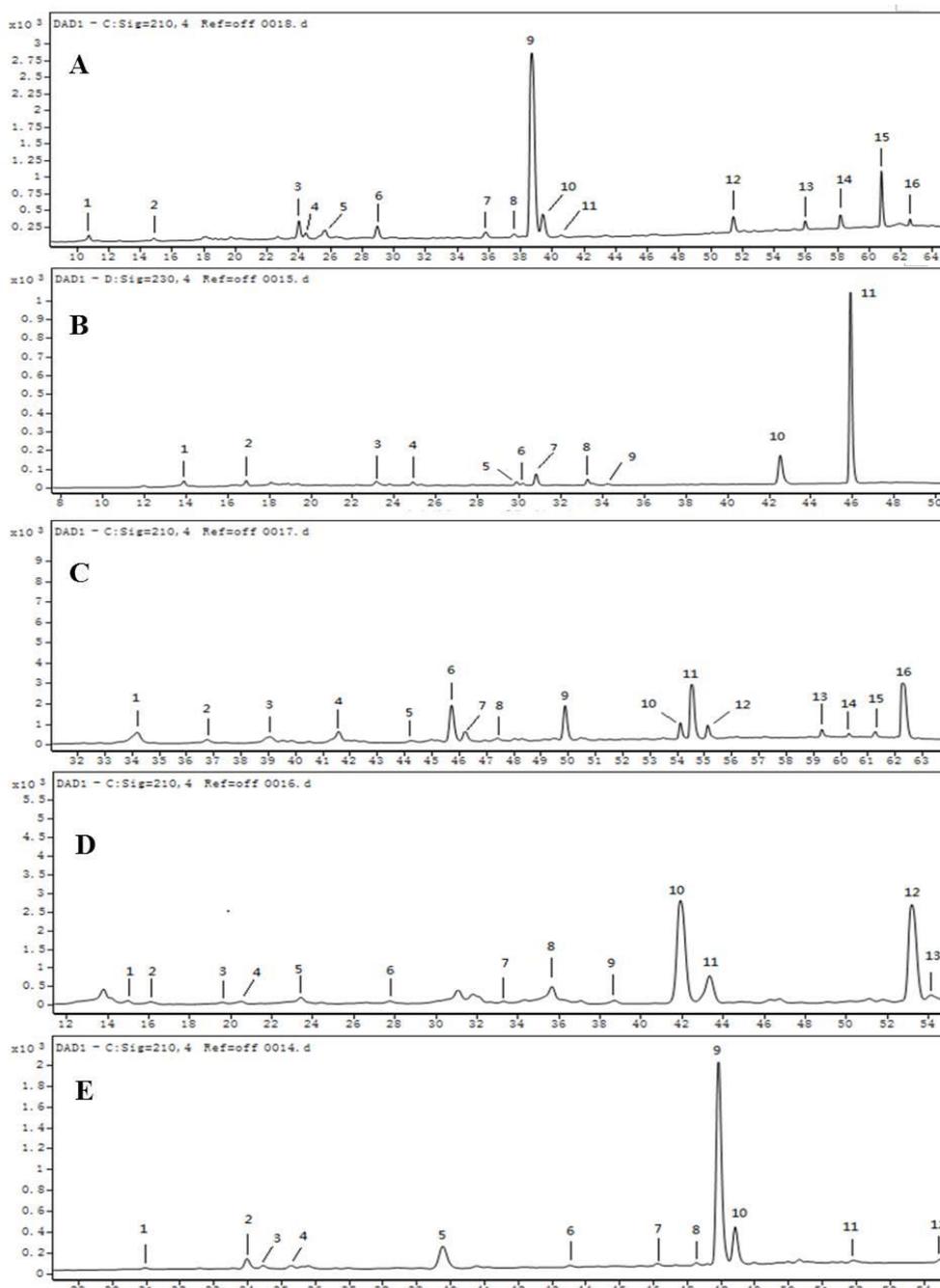


Figure 8. UPLC chromatograms for ECWs. A: 95 % EtOH extract (HCECW). B: 75 % EtOH extract (MCECW). C: Methanol extract (MECW). D: Dichloromethane extract (DECW). E: Petroleum ether extract (PECW).

4. Discussion

Overproduction of intracellular ROS can cause injury on nerve cells, such as PC12 [17]. So far, H₂O₂, as one of the major ROS, has been found to give rise to oxidative stress and subsequently lead to cell death via apoptosis [18]. In the current study, the H₂O₂-treated group showed a marked reduction of the cell viability, a significant increase of ROS level and obvious cell apoptosis of PC12. Also, an obvious increase of the number of dead cells, over-accumulation of LDH, exhaustion of antioxidant enzymes and abnormal expression of Bax, Bcl-2 and Caspase-3, were observed in PC12 cells of model group, confirming that H₂O₂ can induce obvious neurotoxicity to PC12 cells. However, H₂O₂-induced cell damage can be inhibited by antioxidants via a variety of pathways [19]. Our experimental results showed treating with HCECW, MCECW and DECW at a dosage of 10 µg/mL in presence of 400 µM H₂O₂ for 24 h, the damage induced by H₂O₂ on PC12 cells were partly reversed, demonstrating that anti-oxidative effect of these extracts may be associated with its neuroprotective effects.

On the other hand, cells are often equipped with several endogenous antioxidants, such as SOD, CAT, and GSH, etc., which usually act as a detoxifying system to prevent injury caused by ROS [20]. But when oxidative stress occurred in this system, the intracellular accumulation of ROS will rapidly exhaust those antioxidants and lead to LDH leakage in damaged cells. Our present study revealed that HCECW, MCECW, and DECW produced remarkable inhibition of LDH leakage in H₂O₂-induced PC12 cells, and the increase of the activities of SOD and CAT, and the contents of GSH. Notably, the effects of those three extracts working on those indicators are better than that of Vit E.

The possibly active mechanisms of HCECW, MCECW, and DECW were also investigated in our study. As reports [21], the large generation of intracellular Ca²⁺ revealed a detrimental insult from oxidative stress imposed by ROS in the cells. With increasing of intracellular ROS, intracellular Ca²⁺ levels will increase and mitochondrial membrane potential (MMP) will lessen. Continuously, enhanced Ca²⁺ levels in cells may injure function of mitochondria, activate phospholipase, protease and endonucleases, trigger damages of the irreversible membrane, organelles and chromatin, and eventually induce death of cell [22,23]. Therefore, it considered that the increase of intracellular Ca²⁺ levels and lessening of MMP are mostly related to apoptosis. Furthermore, the mitochondria-dependent pathway of apoptosis is adjusted by Caspase-3 [24] and members of the Bcl-2 family of proteins [25]. It has been revealed that expressions of decreased Bcl-2 and increased Caspase-3 and Bax play a critical role in reducing MMP and increasing ROS production in neurons [26]. Currently, it showed that 10 µg/mL of HCECW, MCECW, and DECW decreased the intracellular Ca²⁺ and cell apoptosis, and increased MMP significantly, whereas apoptosis morphology was clearly improved. Meantime, they performed up-regulated the Bcl-2 protein level, down-regulated the Bax and Caspase-3 protein expression, compared with the model group. Notably, their antioxidant effects of HCECW, MCECW and DECW (10 µg/mL) were similar to, or even better than, that of the positive control, Vit E.

Previously chemical investigations indicated that sesquiterpenes and curcumins are the main chemicals in *Curcuma* plants [27,28] and they are also rich in radix of *C. kwangsiensis* [11-13]. As far as we know, curcumins from *C. longa* L. have been confirmed they possessed obviously protective effects against oxidative damage, although they have poor solubility in water and low bioavailability [29,30]. And some sesquiterpenes in *C. aromatica* Salisb. have been found to display antioxidant activities [31]. The chemical analysis by UPLC-Q-TOF-MS revealed the major constituents in those active extracts are sesquiterpenoids and diarylheptanoids, which may act together as the antioxidants and are worthy of further study based on our current study.

In total, five extracts from the radix of *C. kwangsiensis* were evaluated their antioxidant effects on the model of H₂O₂-induced PC12 cells *in vitro*, and three of them, HCECW, MCECW and DECW, showed better activities, including the decrease of the release rate of LDH, the increase of SOD, CAT and GSH. Their possibly active mechanisms might be relative to inhibition of the mitochondrial apoptosis pathway via stabilizing intracellular Ca²⁺ and the regulation of related-protein expression. This study provided an experimental basis for the future development of antioxidants from the genus *Curcuma*.

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Supporting Information

Supporting information, including Tables S1-S6, accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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