Abstract. Background/Aim: To analyze the apoptotic effect of Houttuynia cordata Thunb (HCT) extract on human melanoma A375 cells and its underlying mechanisms. Materials and Methods: The effects of HCT on cell death were determined using the MTT assay. Hoechst 33342 staining was conducted to confirm the detection of cell apoptosis. Caspase-3 and caspase-8 mRNA and cleaved protein levels were investigated by RT-PCR and western blotting, respectively. The release of high mobility group box 1 (HMGB1) and phosphorylation of mitogen-activated protein kinase (MAPK) were determined by ELISA. Results: Caspase-3 and caspase-8 specific inhibitors suppressed HCT-induced cell death. HCT increased caspase-3 and caspase-8 mRNA, protein levels, and caspase activities in a concentration- and time-dependent manner. HCT induced MAPK phosphorylation in a time-dependent fashion. Pretreatment of cells with a selective inhibitor of p38 MAPK reduced apoptosis and reversed the levels of HMGB1 release in response to HCT treatment. Conclusion: HCT induces A375 programmed cell death by activating the caspase-dependent pathway and by p38 phosphorylation associated with HMGB1 reduction.

Melanoma is a highly aggressive cancer that resists most conventional treatments. Worldwide in the last 30 years, the incidence and mortality rates for melanoma have continued to rise rapidly (1). Melanoma arises from abnormal proliferation of melanocytes and occurs within any anatomic territory occupied by melanocytes (2). The major risk factors for malignant melanoma are personal or family history of melanoma, exposure to intense and intermittent ultraviolet irradiation, phenotypic characteristics (fair skin or red hair), and multiple nevi (3). Cutaneous melanoma is the most common cause of mortality among skin cancers in Caucasians (4). Despite a wide variety of therapies to treat melanoma, the prognosis remains very poor (5). Therefore, novel, effective, and safe treatments are urgently needed.

Apoptosis is a programmed cell death that is necessary to maintain homeostasis in multicellular organisms. The mechanism of apoptosis is complex and involves many pathways. The two commonly described initiation pathways are the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis (6). Many gene products are critical in apoptosis regulation (7), including caspases and their upstream regulators (8). Caspase activation is often regulated by various cellular factors, including members of the Bcl-2 family and inhibitors of apoptosis family proteins (9).

Houttuynia cordata Thunb (HCT) is a member of the Saururaceae family. HCT has been traditionally used as a medicinal plant in Asian countries, including, China, Taiwan, Japan, and Thailand (10). HCT has been shown to have a wide range of biological activities, including anti-cancer (11), antiviral (12), anti-oxidant (13), anti-bacterial, and anti-inflammatory activities (14). HCT provided anti-oxidative protection in mice against frying oil- and CCl4-induced injury (15). Aqueous extracts of HCT suppressed high-fat diet-induced oxidative and inflammatory stress in heart and liver by reducing malondialdehyde levels, maintaining glutathione peroxidase activity, and reducing tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 production (16). Previous studies have suggested that HCT provides nutritional benefits against liver cancer (17). However, it remains unknown whether HCT can induce apoptosis in melanoma cells.

HMGB1 is a DNA-binding protein with many additional functions. It may support tumor growth and metastasis though its ability to act as an extracellular ligand and/or its
pro-inflammatory properties as a damage-associated molecular pattern to induce proliferation or angiogenesis (18, 19). Extracellular HMGB1 behaves as a pro-tumor protein with cytokine, chemokine and growth factor functions (20). Increased expression of HMGB1 is correlated with progression of human cutaneous melanoma and poor patient survival (21). Knockdown or knockout of HMGB1 greatly inhibited proliferation, invasion, and cell cycle G2/S transition of bladder urothelial carcinoma cells (22). The aim of this study was to elucidate the potential roles of caspase-3 and caspase-8 in HCT–mediated apoptosis and to dissect the mechanisms that regulate HMGB1 release in a malignant melanoma cell line.

Materials and Methods

Chemicals, reagents, and antibodies. Quercetin and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-caspase-3, anti-caspase-8, and horseradish peroxidase-conjugated secondary (goat anti-rabbit) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-Actin antibody (13E5) was purchased from Cell Signaling Technology (Beverly, MA). Extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) inhibitor (U0126), p38 MAPK inhibitor (SB203580), and Jun N-terminal kinase (JNK) MAPK inhibitor (SP600125) were purchased from Calbiochem (La Jolla, CA, USA). Caspase-3 inhibitor (Z-DEVD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) were purchased from R&D systems (Minneapolis, MN, USA). Hoechst dye 33342 was purchased from AnaSpec (Fremont, CA, USA). PhosphoTracer enzyme-linked immunosorbent (ELISA) kit (ab119674) was purchased from Abcam (Cambridge, MA, USA).

Plant material and extraction. HCT leaves were obtained from Community Enterprises, People of the Land, Organic Agriculture-Sufficiency King, Pa Sang District, Lamphun Province, Thailand. HCT was extracted in 95% (v/v) ethanol (yield: 3.5% of dry wt.). The ethanol extract was filtered through a 0.45-μm filter (Sorbonics, Minnetonka, MN, USA), concentrated at 40˚C using a Buchi B-490 rotary evaporator (BUCHI Laborotechnik AG, Flawil, Switzerland), lyophilized (Labconco Corp., Kansas City, MO, USA), and stored at 4˚C. The dried extract was dissolved in distilled water before use.

Phytochemical analysis. Total phenolic content was determined using Folin-Ciocalteu reagent (FCR) as previously described (23). Briefly, 2.5 ml of the extract was mixed with 0.5 ml of FCR and 1.0 ml of 20 g/100 g solution of sodium carbonate. The mixture was measured at 765 nm using a UV-Vis Genesys 10 UV spectrophotometer. The total phenolic content is expressed as gallic acid equivalents (GAE/mg/gfw). Ferric Reducing Antioxidant Power (FRAP) was measured as previously described (6, 23). Briefly, FRAP reagent, which consisted of 0.3 M acetate buffer (pH 3.6) and 10 mM TPTZ (Fluka, Switzerland) in 40 mM HCl and 20 mM FeCl₃·6H₂O at a ratio of 10:1:1 (v/v/v) was freshly prepared before each measurement. Then, 200 μl of the extract was mixed with 1.3 ml of FRAP reagent and incubated for 30 min at 37˚C. The absorption was measured at 595 nm using a spectrophotometer (Epoch; Biotek, USA). FRAP values are expressed as mmol of Fe(II) equivalents (FeFmM/gFW).

Quantification of quercetin in HCT. The quantification of quercetin in HCT was performed using high-performance liquid chromatography (HPLC) with an external standard. The analyses were performed on a Nexera/SHIMADZU, TSKgelODS-100V HPLC Column (15 cm x 4.6 mm, 5 μm) with SPD-M20A Photo Diode Array detectors. The mobile phase was methanol: 0.1% phosphoric acid (60:40), used at a flow rate of 1.5 ml/min. The column temperature was maintained at 40˚C. The injection volume for all standards and samples was 5 μl. Quercetin was monitored at 370 nm. Identification was based on comparing retention times and UV-VIS spectral data of peaks detected to those of the standards.

Cell culture and treatment. The malignant melanoma cell line (A375) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) containing 7% fetal bovine serum, penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM glutamine (HyClone Logan) at 37˚C in a humidified atmosphere of 95% air and 5% CO₂. Cells were cultured in serum-free DMEM before stimulation with HCT extract.

Cell proliferation and viability assay. Cells were cultured in the absence and presence of HCT extract (25, 50, 100, 150, and 200 μg/ml) for 12, 24 or 48 h. Cells were trypsinized, washed with PBS, and viable cells were scored using a hemocytometer via trypan blue exclusion. Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the inhibition assays, cells were pretreated with MAPK inhibitors for 1 h before the 48-h HCT treatment.

Detection of apoptotic cells. Cells were plated in 8-well Lab-Tek™ chamber slides (Nagle Nunc International, Rochester, NY, USA) at 1x10⁵ cells/well. Cells were subsequently treated with 100 μg/ml HCT for 0, 12, 24 or 48 h. Cells were fixed, washed, and stained with Hoechst dye 33342, as described previously (24). Nuclei were examined and photographed using a fluorescence microscope (BX51; OLYMPUS, Tokyo, Japan). Images were captured digitally at 40× magnification. Four images per group/time point were counted for morphological changes in the nuclear chromatin of A375 cells. (A and B) Cells were incubated with the indicated concentrations of HCT extract for 0 to 48 h, and cell viability was measured by the MTT assay. Cells were incubated with various (C) concentrations and (D) time points following HCT extract treatment, and cell proliferation was measured by hemocytometer counts. (E) Following treatment of cells with 100 μg/ml HCT extract for the indicated times, cells were stained with Hoechst dye 33342 (magnification x400). Arrows indicate apoptotic bodies. (F) Quantitative analysis of apoptotic cells. Data are expressed as means±SD of three independent experiments. *p<0.05 versus control. (G) High-performance liquid chromatography (HPLC) analysis of quercetin in HCT. Chromatogram of the extract of HCT at 370 nm. A quercetin peak was detected with a retention time of 1.504 min.
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![Graph A: % Cell viability vs. HCT (μg/ml)](image)

![Graph B: % Cell viability vs. HCT (h)](image)

![Graph C: Cell numbers vs. HCT (μg/ml)](image)

![Graph D: Cell numbers vs. HCT (h)](image)

![Graph E: Control vs. HCT images](image)

![Graph F: Apoptotic cell (%) vs. HCT (h)](image)

![Graph G: Graphical representation](image)
Figure 2. Continued
apoptotic cells, and values were calculated as percentage of apoptotic cells. For the inhibition assays, cells were pretreated with 15 μM caspase-3 or caspase-8 inhibitors for 1 h before 100 μM HCT treatment for 24-48 h.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Cells were seeded in 60-mm cell culture dishes and were cultured in the absence or presence of HCT extract (10, 50, or 100 μg/ml) for 2, 6 or 12 h. Total RNA was extracted using an RNA extraction kit, according to the manufacturer’s instructions (AXygen Bioscience, CA, USA). First-strand cDNA was synthesized by reverse transcription using a commercial kit (Bio-Rad, CA, USA). The resulting cDNA mixture was amplified with SsoFast ™ EvaGreen ® Supermix with Low Rox (Bio-Rad, CA, USA). The primer sequences were as follows: caspase-3, 5'-TTAATAAAGGTATCCATGGAG AACACT-3' and 5'-'TTAGTGATAAAAATAGAGTTCTTTTGTGAG-3' (nucleotides 32-58 and 851-880 in GenBank XM012508178.1); caspase-8, 5'-CACTAGAAAGGAGGAGA TGGAAAG-3' and 5'-CTATCCTGTTCTCTTGGAGAGTCC-3' (nucleotides 380-403 and 722-745 in GenBank XM003253917.2); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-CGGAGTCAACGGATTTGGTCGT AT-3' and 5'-'AGGCTTCT CCTGAGTGTTGAAGAC-3' (nucleotides 380-403 and 722-745 in GenBank XM003253917.2); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-COGAGTCAACGGATTTGGTCGT AT-3' and 5'-'AGGCTTCT CCTGAGTGTTGAAGAC-3'. For caspase-3 and caspase-8 PCR amplification, the following conditions were used: 94˚C for 1 min (denaturation), 54˚C for 1 min (annealing), 72˚C for 45 s (extension), and 72˚C for 5 min (final extension). There were 35 amplification cycles. GAPDH transcription was analyzed as a control and was amplified in 28 PCR cycles. The amplification products were electrophoresed through 2% agarose gels containing ethidium bromide. The densitometry readings of the bands were normalized to those of GAPDH.

Preparation of cell lysates and western blotting. Apoptosis-protein profiling by western blot was performed according to a previous study (25). Cells were treated with HCT at 0, 50, and 100 μg/ml for 12 h, and were lysed in RIPA buffer [1% (w/v) NP40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2, 2 mM EDTA, and 50 mM phosphatase inhibitor cocktail]. Lysed cells were centrifuged at 14,000 r.p.m. for 10 min to remove cell-debris. Protein concentrations were determined by Bradford protein assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg) were subjected to 12% SDS-PAGE, electrophoretic transfer, and western blotting as previously described (26). Band intensities were quantified using National Institutes of Health Image 1.63 software, and were normalized to β-actin signals.

Caspase activity assay. Enzymatic activity of caspases induced by HCT was assayed using a colorimetric assay kit according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). Briefly, the cells were lysed in a lysis buffer for 30 min. The samples were centrifuged, and 100 μg of the protein was incubated with a reaction buffer and colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) for caspase-3 and Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8, at 37˚C for 2 h. Optical density of
Figure 3. HCT suppresses HMGB1 release via p38 MAPK and JNK1/2/3 phosphorylation in A375 cells. (A) Cells were incubated with various concentrations of H$_2$O$_2$ or (B) were co-incubated with HCT extract for 24 h, and HMGB1 levels were measured in culture media by ELISA. Cells were stimulated with 100 μg/ml of HCT extract for the indicated time periods. Phosphorylated (C) ERK 1/2 (D) p38 and (E) JNK1/2/3 levels were measured by ELISA and plotted as relative fluorescence intensity unit (RFU). Data are expressed as the means±SD. *p<0.05 vs. control. BL, Blank (negative control). Posi cont, positive control (control lysates). (F) Cells were pretreated with ERK1/2 (U0126), p38 (SB203580), or JNK (SP600125) inhibitor for 1 h before H$_2$O$_2$ and HCT extract co-incubation for 24 h. HMGB1 levels were measured in culture media. The data are summarized as the means±SD. *p<0.05 vs. control cells. **p<0.05 vs. H$_2$O$_2$ + HCT group.
the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

**MAPK measurement by ELISA.** Cell lysates were prepared according to a previous method, with modifications (27). Briefly, cells were stimulated with HCT extracts for the indicated time periods, and were lysed by adding 120 μl of SDS sample buffer containing 50 mM dithiothreitol, 1 mM phenylmethane sulfonyl fluoride, and 0.5 mM Na₂VO₃. The amount of phosphorylated protein (ERK 1/2, p38, and JNK1/2/3 levels) was determined by Phosphotracer ELISA kit.

**HMGB1 measurement by ELISA.** For high mobility group box 1 (HMGB1) analysis, cells were incubated with various concentration of H₂O₂ for 24 h in the presence or absence of HCT extract (50-100 μg/ml), and HMGB1 levels were then quantified in culture media by ELISA using a commercial kit (Shino-test, Sagamihara, Kanagawa, Japan). To determine the effect of MAPK signaling inhibition, cells were pretreated with MAPK inhibitors for 1 h before HCT stimulation.

**Statistical analysis.** Data were analyzed using SPSS statistical software version 3.0 (SPSS, Inc., Chicago, IL, USA). Data are shown as the means±standard deviations. Significance differences between two groups were assessed using Student’s t-test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by Scheffé’s multiple range testing. Differences with p<0.05 were considered statistically significant.

**Results**

**Growth inhibition and apoptosis induction by HCT in A375 cells.** As assessed by MTT assay, treatment of A375 cells with HCT extract resulted in a significant reduction in cell viability in a concentration- and time-dependent manner (Figure 1). Cell death was significantly observed following 25 mg/ml exposure but a marked decrease in cell viability (62±6.7%) was observed at 50 mg/ml HCT, which in turn sharply decreased to (52±1.7%) when cells were exposed to 200 mg/ml HCT (Figure 1A). The reduction in cell viability caused by HCT (100 μg/ml) over time was not significantly observed at 12 h (92±2%) but was evident at 24 h (75±3%), and noticeably decreased to 63±2.6% at 48 h (Figure 1B). Similar results were obtained when cell numbers were used as an indicator of cell proliferation (Figures 1C and 1D). Next, experiments were performed to determine whether the...
inhibitory effects of HCT on cell viability and proliferation are the result of apoptotic cell death. Morphological analysis with Hoechst staining revealed nuclei with chromatin condensation and apoptotic bodies in cells cultured with HCT extract, which increased in a time-dependent manner (Figures 1E and 1F). In contrast, only minimal morphological changes could be observed in the control culture (Figure 1E). These results demonstrate an association between the cytotoxic effects observed in response to HCT extract and the induction of apoptosis in A375 cells. For phychochemical analysis, HCT extract revealed the presence of flavonoids and phenolic content as major components. HPLC analysis of the extract of HCT revealed a well-resolved peak of quercetin eluting at 1.504 min (Figure 1G). The concentration of quercetin in the HCT extract was 1.042 mg/g, the total phenolic content was 177.76 GAEmM/Gfw, and the FRAP was 18.62 FeFmM/gFW.

**HCT modulates apoptosis-regulatory genes in A375 cells.** To clarify the mechanism of the cytotoxic effects of HCT on A375 cells, we investigated the involvement of caspases in apoptotic cell death. Cells were treated with HCT extract at various concentrations and times, and the expression of caspase-8 and -3 mRNA was examined using RT-PCR. The increase in caspase-8 mRNA expression occurred early (by 2 h, p<0.05), whereas significant up-regulation of caspase-3 mRNA did not occur until 12 h (Figure 2A). Furthermore, compared to that of the control, the expression of caspase-8 and -3 mRNA increased with increasing HCT concentration, becoming statistically significant (p<0.05) beyond 50 μg/ml, and the peak caspase-8 and -3 mRNA expressions occurred at a HCT concentration of 100 μg/ml (data not shown). Next, we assessed protein expression by immunoblot analysis. Cleaved (i.e., activated) caspase-8 and caspase-3 were detected in the cells as protein bands with a molecular mass of 18 and 17 kDa, respectively. HCT extract significantly (p<0.05) induced caspase-8 and -3 cleavage (Figure 2B), which peaked at 12 h for caspase-8 and 12-24 h for caspase-3 (Figure 2C). In order to identify the enzymatic activity of these enzymes during HCT-induced apoptosis, *in vitro* caspase activity was measured following treatment with HCT using specific fluorogenic peptide substrates for each caspase. The activities of caspase-8 and caspase-3 were significantly (p<0.05) increased in a concentration-dependent manner (data not shown). These results suggest that caspase-8 and caspase-3 play a crucial role in HCT-induced apoptosis in A375 cells.

**HCT induces cell death and inhibits HMGB1 release via activation of p38 MAPK.** HMGB1 is overexpressed in melanoma relative to levels in normal skin and nevi (21). Hydrogen peroxide (H$_2$O$_2$) is a key contributor to cellular oxidative stress and is involved in a wide variety of pathological processes (28). After incubation with 0.125 and 0.25 mM H$_2$O$_2$, the levels of HMGB1 in the media increased to ~14 and 31 ng/ml, respectively (Figure 3A), and HMGB1 significantly decreased after exposure to 50-100 μg/ml HCT extract (Figure 3B). MAPKs are involved in cell proliferation, differentiation, and apoptosis (29). HCT extract induces the phosphorylation of ERK1/2 (Figure 3C), p38 (Figure 3D), and JNK1/2/3 (Figure 3E) time-dependently. Based on these findings, we used ERK1/2 (U0126), p38 (SB203580), and JNK (SP00125) inhibitors to determine the effect of these pathways on HCT-stimulated A374 cell death. We found that SB203580 significantly inhibited the cell death, whereas U0126 and SP600125 had no effect (data not shown). In addition, the reduction in H$_2$O$_2$-induced HMGB1 levels observed with HCT extract was significantly blocked by pretreatment with SB203580 or SP600125, but not with U0126 (Figure 3F). In conclusion, HCT-induced apoptosis might occur, at least in part, through activating p38 and suppressing HMGB1 in A375 cells. The proposed signaling pathways are shown in Figure 4. Taken together, these findings provide important possible molecular mechanisms for the activity of HCT in melanoma cells and confirm that HCT may have a promise in melanoma treatment in the future.

**Discussion**

Recent studies have reported that the extracts of HCT or its components can cause cell cycle arrest and apoptosis in various human cancer cell lines, suggesting that their growth inhibitory effects occur through G$_1$/S or G$_2$/M arrest and subsequent apoptosis (30). Our current results demonstrate that HCT exhibits significant anti-tumor properties by inducing apoptosis, at least in part through p38 MAPK in human melanoma cells. A recent study reported that melanoma grade, indicated by tumor thickness, mitotic index, lymph node metastasis, and distance metastasis, was positively correlated with higher HMGB1 levels (21). To our knowledge, we have shown for the first time that HCT extract suppresses HMGB1 release from human melanoma cells via p38 activation.

Quercetin is a major flavonoid found in many foods (14). Our HCT extract contained 1.042 mg quercetin/g, so the maximum concentration of HCT used in this study (100 μg/ml)
contained 0.1 μg/ml quercitin. The concentration-dependency in our results is dissimilar from those reported by others in B16-BL6 cells (21), melanoma cells (31), and primary colorectal cancer cells (30). The responses to HCT extracts in our study differed from those of the previous studies most likely due to differences among HCT species and cultivars, as well as differences in the extraction, separation, and purification protocols, and cell types.

The MAPK pathway regulates key processes such as cell proliferation, invasion, metastasis, survival, and angiogenesis, which are involved in melanoma development (32). A previous study showed that a p38-specific inhibitor could block hepatocyte growth factor-induced melanoma cell proliferation (33) and increase the levels of Fas expression, ultraviolet-induced apoptosis in late stage melanoma cells (34). The p38-dependence of HCT-induced apoptosis in our study is consistent with these earlier reports. In apoptotic processes, caspase-3 has been shown to play a pivotal role in the terminal and execution phase of apoptosis induced by diverse stimuli (35). Caspase-3, a downstream caspase, is activated by caspase-8 (36). Our time course of caspase-3 and caspase-8 expression is consistent with this order of activation because caspase-8 mRNA up-regulated earlier than caspase-3 mRNA did. In this circumstance, both caspase-3 and caspase-8 protein cleavage is maximized at 12 h after HCT exposure, but, unlike caspase 8, caspase 3 activation persists until 24 h. Our study suggests that caspase-dependent transcription plays a role in HCT-induced apoptosis in A375 cells.

Increased expression of HMGB1 is correlated with progression of human cutaneous melanoma (21). HMGB1 overexpression is observed in a number of malignancies, but its role in melanoma has not been fully elucidated. In this study, we found, for the first time, that HCT extract can suppress H2O2-induced HMGB1 levels in A375 cells, and this was reversed by treatment with p38 or JNK inhibitors. Our observations are consistent with the earlier study showing that high levels of HMGB1 are associated with cancers. However, the role of HMGB1 in the development of melanoma proliferation, metastasis, and potential targets for therapy should be further investigated. In conclusion, HCT is a potential compound that can be used for melanoma treatment; however, in depth in vivo and clinical trial studies are needed to elucidate its physiological mechanisms.

Acknowledgements

This research was supported in part by grants-in-aid from Mahidol University and School of Anti-Aging and Regenerative Medicine, Mae Fah Luang University, Thailand. The Authors would like to thank Ms. Pompen Dararat and Ms. Janine Kaewbai-ngam for their assistance with the statistical analysis and literature references.

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