JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Inhibitory Effects of Dried Longan (*Euphoria longana* Lam.) Seed Extract on Invasion and Matrix Metalloproteinases of Colon Cancer Cells

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ABSTRACT: The critical step in colorectal cancer progression and associated mortality is cancer invasion, which depends on two key gelatinase enzymes, matrix metalloproteinases-2 and -9. Dried longan (*Euphoria longana* Lam.) seed is a rich natural source of antioxidant polyphenols.This study evaluated the effect of dried longan seeds on colon cancer cell invasion via gelatinase function and expression. Three dried longan seed fractions were collected by Sephadex LH-20 column chromatography. They showed a potent inhibitor on colorectal cancer cell invasion and gelatinase activity. The antigelatinase activities of fractions 1 and 2 were a direct effect via Zn^{2+} chelation, whereas fraction 3 modulated indirectly through suppression of zymogen activators. Among the fractions, only fraction 3 reduced the gelatinase expression, which was correlated with the levels of tissue inhibitor of metalloproteinase-1 and may as well involve the p38 mitogen-activated protein kinases and the c-Jun N-terminal kinase signaling pathways. This primary research has manifested and encouraged the anticancer properties of dried longan seed extracts with potential inhibitory effects on cancer cell invasion as well as antigelatinase activity and expression in colon cancer cells.

KEYWORDS: *dried longan seed fraction, cell invasion, MMP-2, MMP-9, MAPKs pathway*

INTRODUCTION

The incidence rates of colorectal cancers (CRC) are increasing throughout Asia, including Thailand. Most cases of colorectal cancer are diagnosed at an advanced stage, when the cancer has metastasized to distant sites[1] and is usually not curable.[2] To produce metastases, cancer cells require degradation of extracellular matrix (ECM) and/or basement membrane by protease enzymes. Among the different proteinases, matrix metalloproteinases (MMPs) seem to be primarily responsible for the degradation of the ECM, along with other substrates such as cytokines, growth factor receptors, and cell−cell and cell−matrix adhesion molecules.[3,4]

MMPs are a family of secreted and transmembrane Zn^{2+} endopeptidases that are produced by host connective tissue or tumor cells. They can degrade the surrounding basement membrane and ECM barriers, resulting in tumor cell migration. This family currently includes over 25 proteins that are produced as inactive forms (proMMP) or as zymogen via the binding of Zn^{2+} atom in the catalytic domain, with cysteine in the propeptide domain. Among all MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are considered to be the key enzymes for tumor invasion and metastasis, due to their ability to degrade type IV collagen, which is a major component of the basement membrane.[5,6] Generally, the proteolytic activity of MMPs is able to be modulated at three levels: transcription, proteolytic activation of proMMP, and inhibition of the active enzyme.[7] Three common mitogen-activated protein kinase (MAPKs) signal transduction pathways, including p38 mitogen-activated protein kinases (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK), have been associated with MMP-2 and MMP-9 transcription.[8] Meanwhile, active MMPs act as activators for other proMMPs, mostly through MT-MMP and MMP-3.[9] In the case of MMP-2, activation is accomplished by the formation of a multiprotein cell-surface cluster between MT1-MMP (MMP-14), TIMP-2, and proMMP-2. Specific endogenous inhibitors of MMPs or tissue inhibitor of metalloproteinases (TIMPs) have been identified. There are four TIMPs that bind MMPs via noncovalent bonds in a 1:1 stoichiometric complex.[10] Among the TIMPs, TIMP-1 is a specific inhibitor to both MMP-2 and MMP-9 and is highly expressed in colon adenocarcenoma tissue.[11] Additionally, the plasminogen system has been shown to proteolytically activate proMMPs. Activation is through pro-urokinase plasminogen activator (pro-uPA) interacting with the urokinase plasminogen receptor (uPAR) on the cell surface. Upon binding, active uPA stimulates the conversion of plasminogen to plasmin, which has been shown to play a role in MMP-9 and MMP-2 activation.[12−16] Moreover, the proteolytic capacity relies on a balance between protease and inhibitor activities; therefore, the

Received: December 11, 2012
Revised: March 18, 2013
Accepted: March 25, 2013
Published: March 25, 2013

dx.doi.org/10.1021/jf3052863 | J. Agric. Food Chem. 2013, 61, 3631−3641

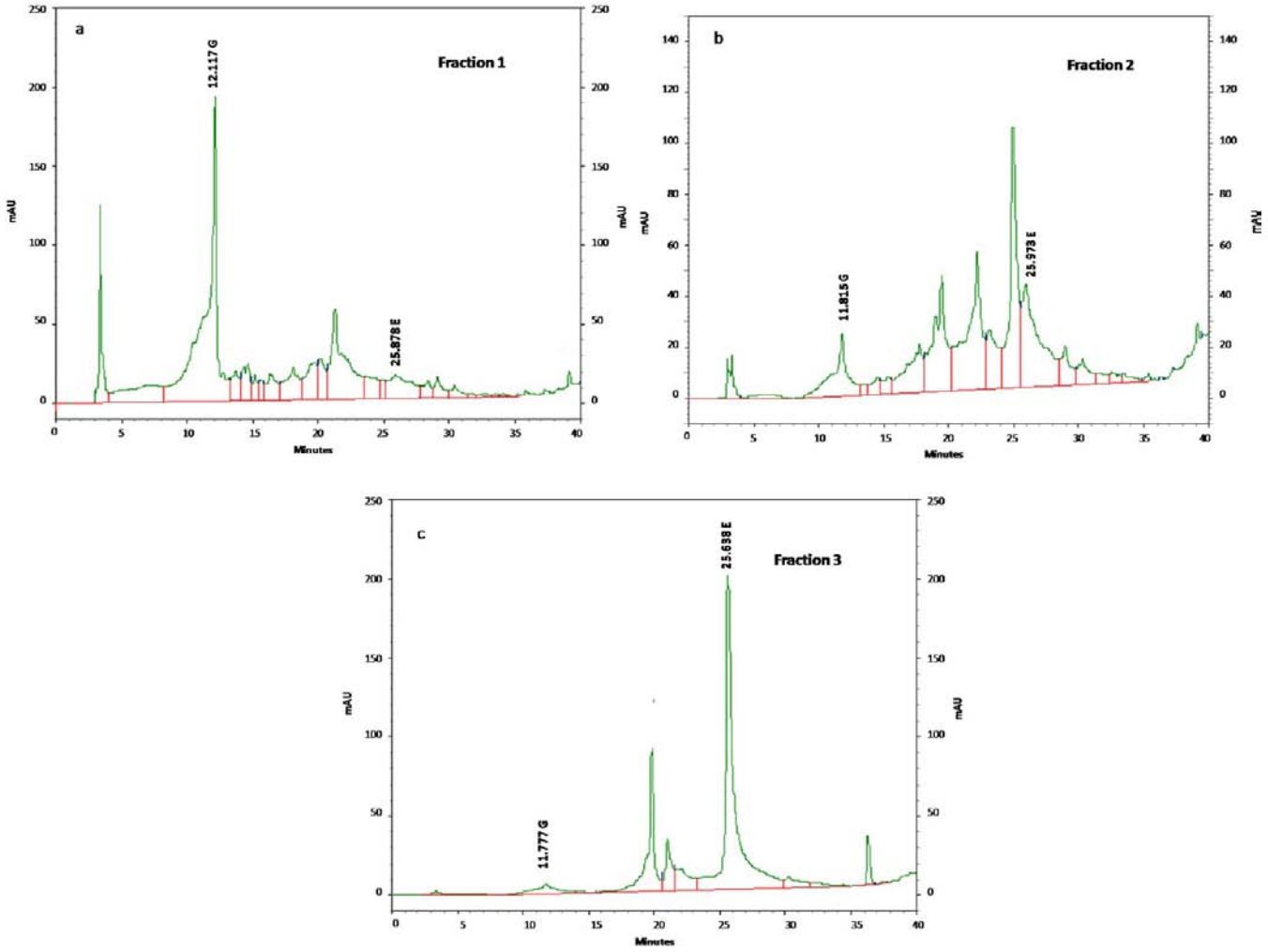

Figure 1. HPLC chromatogram of dried longan seed fractions: (a) fraction 1; (b) fraction 2; (c) fraction 3. Three dried longan seed fractions were quantified for content of gallic acid and ellagic acid (μg/mL) by comparison with the retention time (RT) of standard gallic acid (RT = 11.3 min) and ellagic acid (RT = 25.6 min) using HPLC at 270 nm.[21] E = Ellagic acid, G = Gallic acid.

TIMP level is generally correlated with decreasing tumorigenesis.[17]

Longan, *Euphoria longana* Lam. (syn. *Dimocarpus longan* Lour.), is a major Thai fruit export and is widely grown in southern China, India, and Southeast Asia.[18] A previous study demonstrated that a dried longan seed extract contained high levels of antioxidant polyphenolic compounds such as corilagin, gallic acid, and ellagic acid.[19] Longan seed extracts are capable of inducing apoptosis in various colorectal cancer cells.[20] Our previous research has shown that longan seed fractions obtained via Sephadex-LH-20 column chromatography possessed MMP inhibitory activity.[21] The current study explores the role of dried longan seed extracts on anti-invasion of colon cancer cells, with the goal to determine possible mechanisms of action.

MATERIALS AND METHODS

Plant Material and Extraction. Dried longan (*E. longana* Lam. cv. Biew Kiew) seeds were ground to a powder and then extracted with 80% acetone in a ratio of 1:10 (w/v) at room temperature for 24 h. The resulting slurries were filtered through a Whatman no. 1 filter paper. The filtrate was evaporated and lyophilized to yield dried longan seed extracts or crude extracts. The crude extracts were dissolved to 20 mg/mL in methanol and subjected to Sephadex LH-20 column chromatography. Three fractions were collected according to the absorbance at 360 nm, and these were labeled fractions 1, 2, and 3.[21]

Cell Lines. SW480 and SW620 cells (moderately invasive and highly invasive human colon adenocarcinoma cell lines, respectively) as well as human normal colon epithelial cells (CCD841 CoN cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). SW480 and SW620 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen Corp., NY, USA) with 10% fetal bovine serum (Invitrogen Corp.) at 37 °C in humidified atmospheric air and 5% CO_2 addition. CCD841 CoN cells were used as normal cell control and were cultured in minimum essential medium (MEM) (Invitrogen Corp.) with 10% fetal bovine serum under the same conditions as for the colon cancer cells.

Cell Cytotoxicity Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay was used to investigate the cytotoxicity of each fraction on human colon cancer and normal colon cells. Briefly, SW480, SW620, and CCD841 CoN cells were seeded into 96-well plates at a cell density of 5×10^3cells/well and incubated for 24 h. Then, cells were treated with each fraction at various concentrations (0−400 μg/mL). After incubation at 37 °C for 24 and 48 h, 15 μL of MTT solution was added to each well (5 mg/mL in PBS). The reaction was continued at 37 °C for 3−4 h and then formazan (purple) crystals were dissolved with dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm, and cell viability was calculated by comparison with controls.

dx.doi.org/10.1021/jf3052863 | J. Agric. Food Chem. 2013, 61, 3631−3641

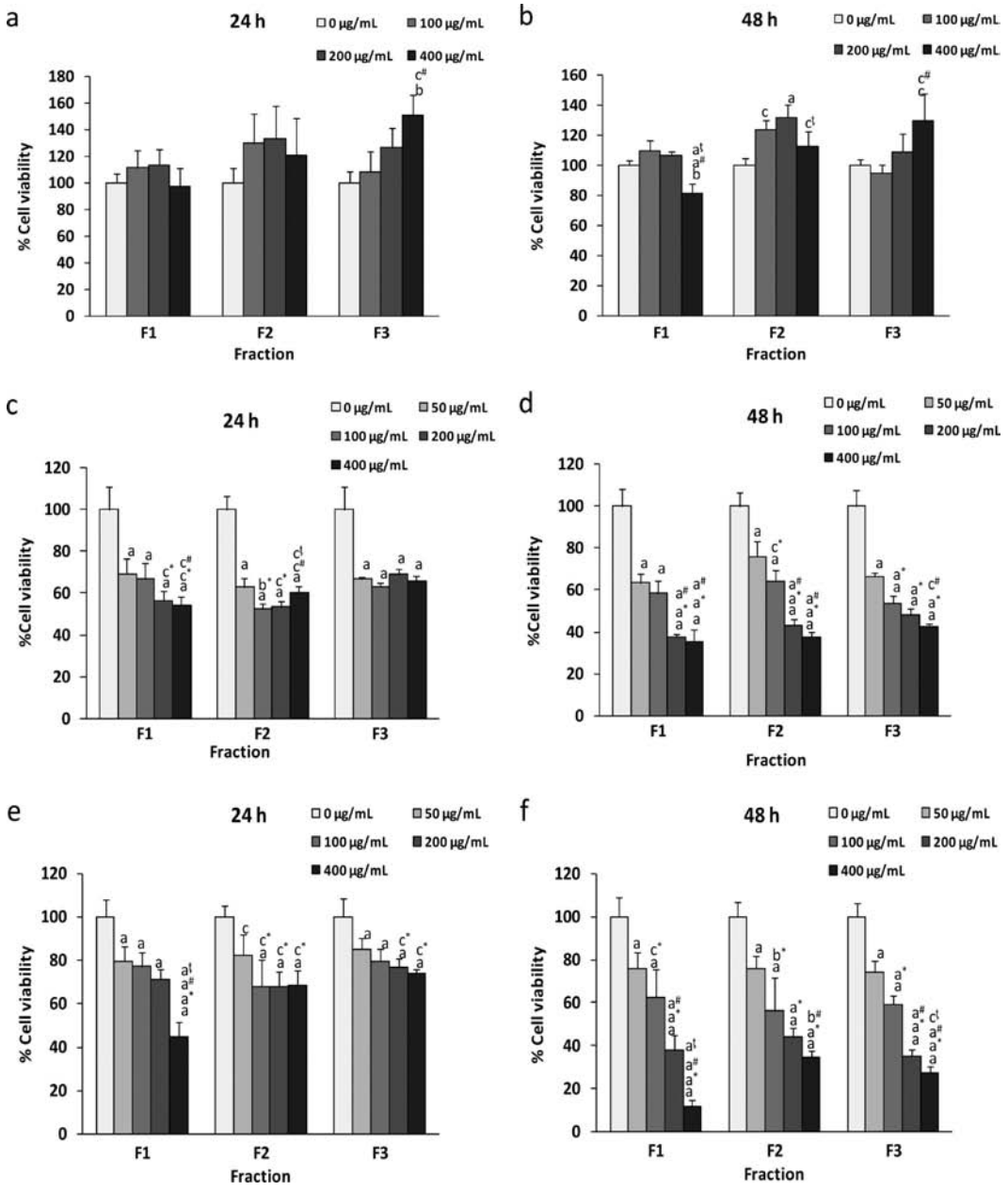

Figure 2. Cytotoxic effect of dried longan seed fractions on cell viability of SW480, SW620, and CCD841 CoN cells. CCD841 CoN cells (a, b), SW480 cells (c, d), and SW620 cells (e, f) were treated with each fraction at various concentrations (0−400 μg/mL) at different times (24 and 48 h). The data are shown as the average percent cell viability ± SD and compared with untreated controls. The results are from three independent experiments. Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, above the bars indicate significance as compared with controls, whereas symbols ∗, #, and ţ were compared with treated conditions at 50, 100, and 200 μg/mL of each fraction, respectively.

Cell Invasion Assay. In vitro cell invasion assay was a modified Boyden chamber assay using Matrigel as a matrix substrate for cell invasion. In brief, a polycarbonate membrane of a 24-transwell plate (Costar Transwell, NY, USA) was precoated with diluted Matrigel (BD Bioscience, CA, USA) in serum-free DMEM, and then colon cancer cells at 1×10^5 cells/well in serum-free DMEM with the fractions at various concentrations (0−100 μg/mL) were added into the upper compartment. Meanwhile, the lower chamber contained DMEM in the presence of 10% FBS. The plate was incubated at 37 °C for 36 h. Next, the transwell membrane was stained with 1% crystal violet in 50% methanol. Noninvaded cells were scraped off with a cotton swab so that only invasive cells remained under the membrane. The membrane was dissolved with 20% acetic acid in a 96-well plate, and then the absorbance was read at 570 nm.

Gelatin Zymography. The activities of MMP-2 and MMP-9 or gelatinases were examined by gelatin zymography as described previously.[22] In the case of gelatinase secretion analysis, SW480 and SW620 cells (6×10^5 cells/60 mm dish) were treated with each fraction at various concentrations (0−100 μg/mL) in serum-free media for 24 h, and then each conditioned medium was collected. Equal amounts of concentrated protein samples were mixed with sample buffer under nonreducing conditions and then loaded onto 10% sodium dodecyl sulfate (SDS)−polyacrylamide gels containing 0.1% gelatin. After gel electrophoresis, gels were soaked in 2.5% Triton X-100 (Thermo Scientific, Rockford, IL, USA) at room temperature for 1 h to remove SDS, and then gelatinases in the gel were activated in reaction buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4) at 37 °C for 24 h. For determination of the inhibitory effect to gelatinase activity, the culture supernatants were collected and

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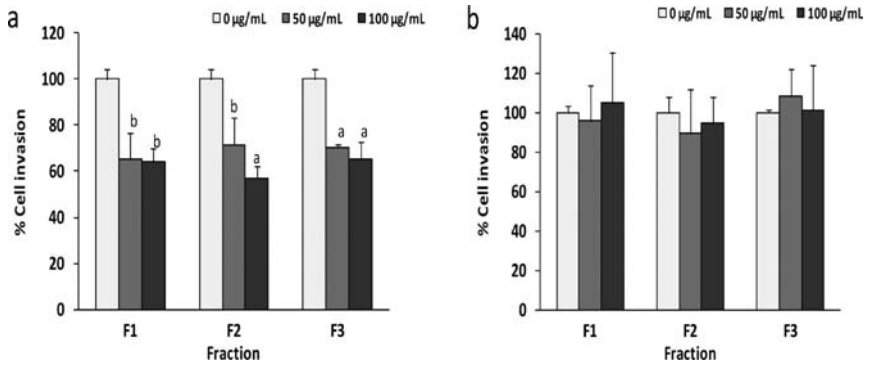

Figure 3. Dried longan seed fractions abrogate the invasion of SW480 cells. Treated SW480 (a) and SW620 (b) cells (at 0−100 μg/mL for 36 h) were examined for invasive ability by the Matrigel invasion assay. Three independent results are expressed as the average percent cell invasion ± SD versus untreated controls. Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, above the bars indicate significance as compared with controls, whereas symbols ‡ and ∗ indicate comparison with 25 and 50 μg/mL of each fraction, respectively.

analyzed following the same protocols as in the secretion analysis. During incubation at 37 °C for 24 h, gels were activated in reaction buffer, and each fraction was also added at different doses (0−100 μg/mL). After incubation, each gel was stained with 0.1% Coomassie brilliant blue R-250 (Sigma-Aldrich) and destained with 10% acetic acid in 30% methanol until a clear band against the blue background was visualized. The clear bands represented active gelatinases (MMP-2 and MMP-9) and were quantified using the Gel-Pro Analyzer 32 program (Media Cybernetics, MD, USA).

Reverse Gelatin Zymography $ZnCl_2$. The inhibitory effect of each fraction on MMP-2 and MMP-9 activity through Zn^{2+} chelation capacity was performed by reverse gelatin zymography by $ZnCl_2$.[23] This method is similar to the gelatinase activity assay described above, but during the step of MMP activation each gel was co-incubated with each fraction at 50 μg/mL with $ZnCl_2$ at different concentrations (0, 1, 10 μM) in the reaction buffer at 37 °C for 24 h. The clear bands represented gelatinase (MMP-2 and MMP-9) activity, and the Gel-Pro Analyzer 32 program was used to quantify their intensity.

Western Blot Analysis. SW480 cells (6×10^5 cells/60 mm dishes) were treated with each fraction at various concentrations (0−100 μg/mL) for 24 h under starved condition, and then whole cell lysates were collected with lysis buffer. Equal amounts of proteins (35−40 μg/lane) were mixed with sample buffer and then subjected to 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was soaked in blocking solution (5% nonfat dry milk) at room temperature for 1 h followed by incubation with the corresponding primary antibodies against MMP-2, MMP-9, uPA, uPAR, MT1-MMP, TIMP-1, p38, pp38, ERK, pERK, JNK, pJNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin. Next, the membranes were incubated with the appropriate horseradish peroxidase-conjugated specific secondary antibodies and visualized using an enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Amersham, UK).

Statistical Analysis. The results were expressed as the mean ± SD of three independent experiments. Statistical significance was calculated by a one-way analysis of variance (ANOVA), with (a) $p \leq 0.001$, (b) $p \leq 0.005$, and (c) $p \leq 0.05$ considered to be significant differences.

RESULTS

Cytotoxic Effect of Dried Longan Seed Fractions on Cell Viability of SW480, SW620, and CCD841 CoN Cells. A previous study concerned three dried longan seed fractions that were obtained by Sephadex LH-20 column chromatography. These fractions were quantified for gallic acid and ellagic content by high-performance liquid chromatography (HPLC). Gallic acid was dominant in fraction 1 (GA = 206 μg/mL, EA = 22 μg/mL) (Figure 1a), whereas fraction 3 was an ellagic acid-rich fraction (GA = 7.7 μg/mL, EA = 217 μg/mL) (Figure 1c). In addition, both compounds were found in fraction 2 in a low concentration (GA = 32 μg/mL, EA = 30 μg/mL) (Figure 1b).[21] In our preliminary data there was no cytotoxicity of crude acetone extract on human colon cancer HCT-15 cells and RKO cells at 100 μg/mL for 24 and 48 h (data not shown). In this study, each dried longan seed fraction was also used in a cytotoxicity analysis to human normal colon cells (CCD841 CoN cells) and human colon cancer cells (SW480 and SW620 cells) using the MTT assay. None of the fractions (at 0−200 μg/mL for 24 h) affected CCD841 CoN cell survival. Interestingly, it seemed that high concentration (400 μg/mL) of fractions 3 and 2 (at 0−200 μg/mL for 48 h) could significantly increase the proliferation rate of normal cells (Figure 2a,b). On the other hand, in SW480 and SW620 cell survival was clearly reduced (Figure 2c−f). The cytotoxicity of each treatment appeared to be dependent on dosage and time of exposure. The maximal percentages of growth inhibition for fractions 1, 2, and 3 at 100 μg/mL in SW480 cells were 33.1, 47.6, and 36.7% of untreated controls for 24 h treatment (a, $p \leq 0.001$) (Figure 2c) and 41.5, 36.1, and 46.2% of untreated controls for 48 h (a, $p \leq 0.001$) (Figure 2d). In SW620 cells, percentages of survival inhibition of each fraction at 100 μg/mL were in order 22.8, 31.9, and 20.1% of untreated controls for 24 h (a, $p \leq 0.001$) (Figure 2e) and 37.5, 43.8, and 40.7% for 48 h, respectively (a, $p \leq 0.001$) (Figure 2f).

Dried Longan Seed Fractions Abrogate the Invasion of SW480 Cells. The Matrigel invasion assay was used to determine the potential effect of dried longan seed fractions on colon cancer cell invasion using various nontoxic concentrations (0−100 μg/mL). Each fraction significantly reduced the invasion of SW480 cells from 50 μg/mL, and percentages of invasive inhibitory effect were approximately 30−40% of controls (Figure 3); there was no effect on SW620 cells. SW480 and SW620 cells differ in the degree of invasive ability; SW620 cells are more aggressive than SW480 cells. The results suggest that dried longan seed fractions have greater inhibitory effect on moderately invasive colon cancer cells (SW480).

Dried Longan Seed Fractions Inhibit the Secretion of MMP-2 and MMP-9 in SW480 Cells. Gelatin zymography was utilized to determine the effect of each fraction on gelatinase secretion in SW480 and SW620 cells. Conditioned media of treated SW480 and SW620 cells at different concentrations (0−100 μg/mL) for 24 h were collected for identifying gelatinase secretion. Each fraction inhibited

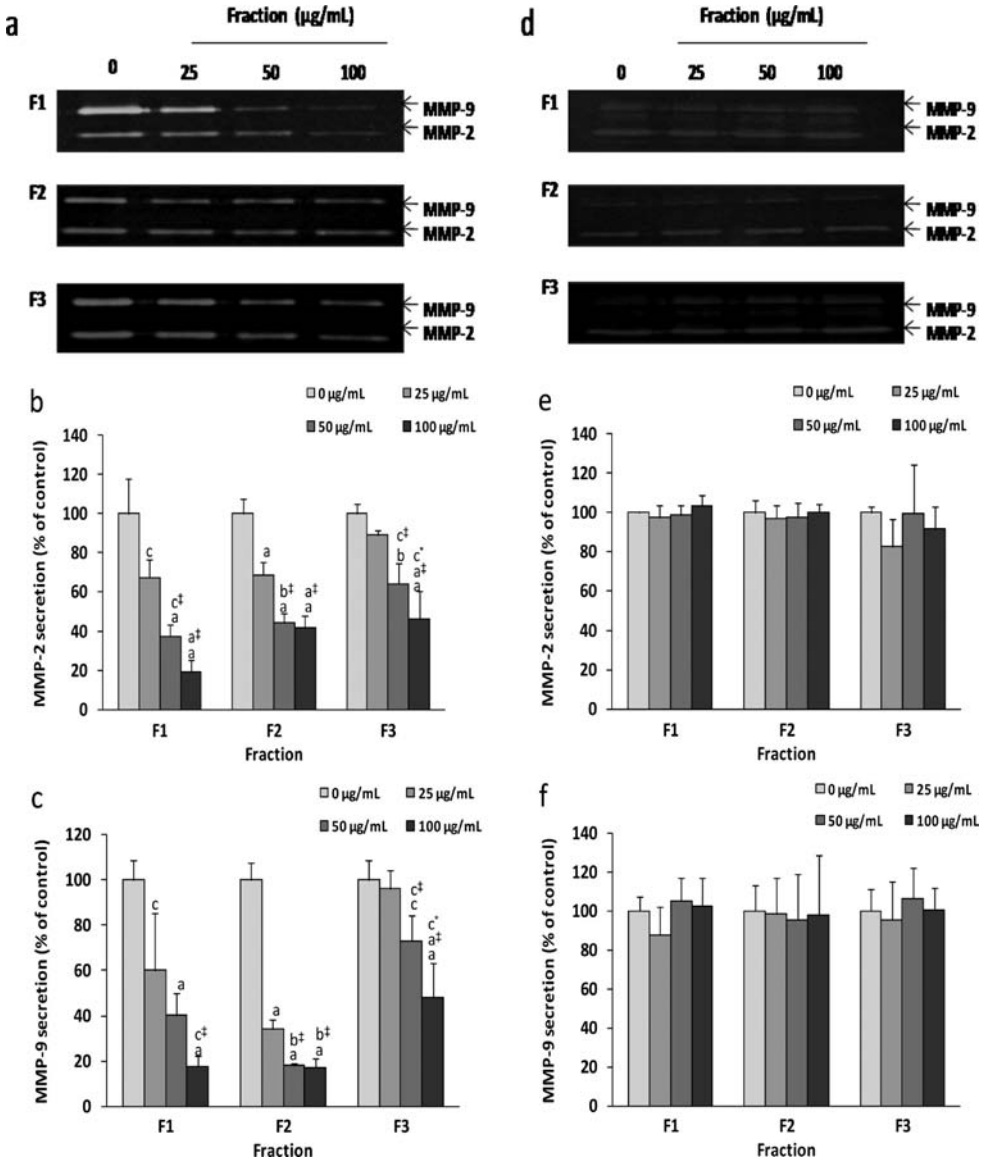

Figure 4. Dried longan seed fractions inhibit gelatinase secretion in SW480 cells. Conditioned media of treated SW480 (a) and SW620 (b) cells at various concentrations (0−100 μg/mL) for 24 h were collected and assessed for gelatinase secretion by gelatin zymography. The band intensity of MMP-2 and MMP-9 in SW480 (b, c) and SW620 (e, f) cells was analyzed and is shown as the mean percent intensity ± SD from triplicate independent experimental values (vs untreated controls). Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, above the bars indicate significance as compared with controls, whereas symbols ‡ and ∗ indicate comparison with treated conditions at 25 and 50 μg/mL of each fraction, respectively.

gelatinase secretion, clearly in a dose-dependent manner in SW480 cells (Figure 4a−c) but not in SW620 cells (Figure 4d−f). This antigelatinase secretion effect of the fractions was correlated with anti-invasion in SW480 cells, suggesting that the effect of dried longan seed fractions was greater in SW480 cells than the more aggressive SW620 cells.

Dried Longan Seed Fractions Inhibit MMP-2 and MMP-9 Activity in SW480 Cells: Relationship to Zn^{2+} Chelation Activity. Gelatin zymography was used to analyze the ability of each fraction on gelatinase activity of SW480 cells. The gelatinase activity significantly declined by dose−response with 70−80% inhibition observed for each fraction at 100 μg/mL (a, $p \leq 0.001$) (Figure 5a−c). The Zn^{2+} atom of matrix metalloproteinase plays a crucial role in the proteolytic activity of MMP. Reverse gelatin zymography by $ZnCl_2$ was used to verify the inhibitory effect of each fraction on gelatinase activity via Zn^{2+} chelation. After the addition of $ZnCl_2$, the inhibitory effect of each fraction was abolished, particularly in MMP-2, with almost full recovery at 10 μM $ZnCl_2$ (Figure 5d−f). Remarkably, for most fraction 3 treatments there was no significant recovery of gelatinase activity even after the addition of $ZnCl_2$ up to10 μM in MMP-9 activity but not in MMP-2 activity. These results indicate that dried longan seed fractions 1 and 2 suppressed gelatinase activity by directly attacking the Zn^{2+} atom of the MMP structure. Surprisingly, the anti-gelatinase property of the EA-rich fraction 3 did not occur through Zn^{2+} chelation activity, whereas the antigelatinase activity of EA treatment alone was clearly due to its Zn^{2+} chelation.

Dried Longan Seed Fraction 3 Regulates the Proteolytic Activity of MMP-2 and MMP-9 via Abolishing the Expression of Their Zymogen Activators, Including MT1-MMP, uPA, and uPAR in SW480 Cells. Fractions 1 and 2 could reduce gelatinase activity by Zn^{2+}

dx.doi.org/10.1021/jf3052863 | J. Agric. Food Chem. 2013, 61, 3631−3641

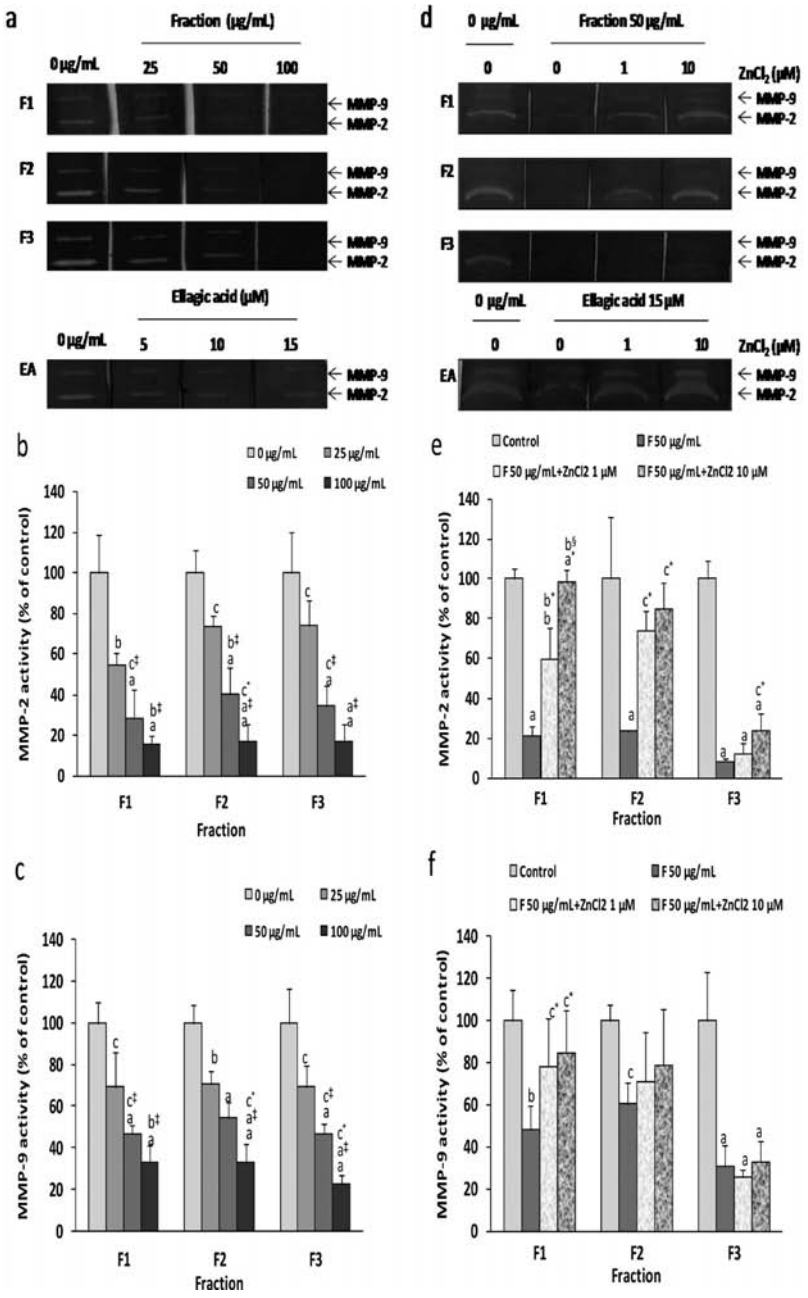

Figure 5. Dried longan seed fractions restrain gelatinase activity related with Zn^{2+} chelation ability. To determine gelatinase activity, culture supernatants of SW480 cells were collected and analyzed by gelatin zymography (a). Each fraction at various concentrations (0−100 μg/mL) or EA (0−15 μM) was added in reaction buffer and incubated with MMP-2 and MMP-9 for 24 h. The band intensities of MMP-2 (b) and MMP-9 (c) in SW480 cells were analyzed. In the case of reverse inhibitory effect, $ZnCl_2$ was added to the reaction buffer at 1 and 10 μM for each fraction at constant concentration (50 μg/mL) or EA (15 μM) (d). The appearance of clear bands was quantified for MMP-2 (e) and MMP-9 (f) activity with GelPro32 software. All values are shown as the mean percent intensity ± SD from three independent experiments (vs untreated controls). Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, above the bars indicate significance as compared with controls, whereas symbols ‡ and * indicate comparison with treated conditions at 25 and 50 μg/mL of each fraction and §, comparison with 1 μM $ZnCl_2$.

chelation directly, and it thus seemed unnecessary to determine other possible means of regulation. Because the action of fraction 3 was not via Zn^{2+} chelation, there might be other possible inhibitory mechanisms, for example, through the activation of proMMP. Some active MMPs and the plasminogen system commonly mediated this process, which is composed of MT1-MMP, uPA, and uPAR. In this study fraction 3 demonstrated indirect inhibitory action through significant attenuation of these mediators expression in SW480 cells, especially at 100 μg/mL (Figure 6a−c). Moreover, inhibition of active MMPs by specific endogenous inhibitors is another regulation level to proteolysis activity. Tissue inhibitors of metalloproteinases (TIMPs) are a group of inhibitors to MMP activity, especially TIMP-1, which is specific for both MMP-2 and MMP-9. These data showed that fraction 3 at 100 μg/mL dramatically reduced TIMP-1 expression in SW480 cells nearly 80% inhibition (a, $p \leq 0.001$) (Figure 6d). These results implied that the indirect inhibitory effect of fraction 3 on gelatinase activity was through blocking the expression of

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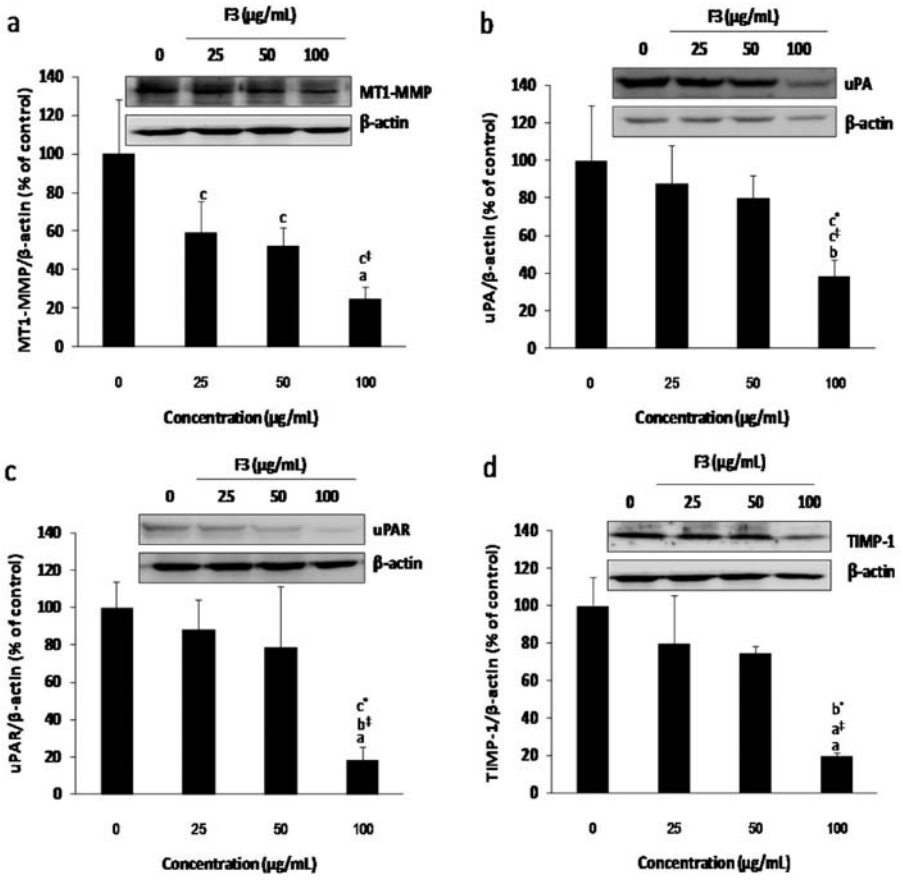

Figure 6. Dried longan seed fraction 3 regulates the proteolytic activity of MMP-2 and MMP-9 via abolishment of their zymogen activators, including MT1-MMP, uPA, and uPAR in SW480 cells. Lysates from cells treated with fraction 3 at 0, 25, 50, and 100 μg/mL for 24 h were collected for analyzing the expression of MT1-MMP (a), uPA (b), uPAR (c), and TIMP-1 (d) by Western blot analysis. β-Actin was used for normalized and internal control to ensure the equality of protein sample loading. The results are given as the mean percent relative intensity ± SD of three independent experiments (vs untreated controls). Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, indicate significance as compared with controls; symbols ‡ and ∗ indicate comparison with treated conditions at 25 and 50 μg/mL of each fraction, respectively.

progelatinase activators but not via enhancing their inhibitor (TIMP-1) expression.

Dried Longan Seed Fraction 3 Diminishes the Expression of MMP-2 and MMP-9 in SW480 Cells. MMP-2 and MMP-9 are overexpressed in many cancer cell types. Only EA-rich fraction 3 significantly attenuated MMP-2 and MMP-9 expression (Figure 7a) at 100 μg/mL, with nearly 40% (c, $p \leq 0.05$) (Figure 7b) and 80% (b, $p \leq 0.005$) (Figure 7c) inhibition compared with controls, respectively. Interestingly, a single EA treatment did not affect MMP-2 and MMP-9 expression in SW480 cells. This result not only supported the beneficial effect of a combination of compounds in fraction 3 but also distinguished the inhibitory mechanisms among the dried longan seed fractions to SW480 cell invasion. Among the TIMPs, TIMP-1 seems to be up-regulated in colon cancer tissue and is correlated with levels of MMPs.[11] In our study the ratios of MMP-2/TIMP-1 were 1.0, 1.3, 1.3, and 3.1, whereas MMP-9/TIMP-1 ratios were 1.0, 1.1, 0.9, and 1.0 at 0, 25, 50, and 100 μg/mL of fraction 3, respectively (Figure 7d). These close ratios of MMPs and TIMP-1 levels were also observed in our results, particularly MMP-9/TIMP-1 in SW480 cells.

Dried Longan Seed Fraction 3 Down-regulates the p38 MAPK and JNK Pathway in SW480 Cells. The MAPK pathways p38 MAPK, JNK, and ERK are major signaling pathways controlling gelatinase expression in many cell types[8] as well as tumor invasion and metastasis in colorectal cancer.[24] In the present study the phosphorylated forms of p38 MAPK and JNK of untreated controls in SW480 cells were slightly increased at 3 and 24 h as compared with at 0 h under starved condition, whereas there was no effect on ERK or pERK (Figure 8a–c). However, in fraction 3 treated cells (at 100 μg/mL) the phosphorylated forms of p38 MAPK were reduced by 42.1, 47.1, and 60.5% inhibition at 3, 6, and 24 h, compared with untreated controls at 0 h (Figure 8b). Meanwhile, the phosphorylated form of JNK was also inhibited by 30.4, 45.5, and 69.1% inhibition at 3, 6, and 24 h versus untreated controls at 0 h (Figure 8d). These data suggest that the possible regulatory pathway on anti-invasion and antigelatinase expression of fraction 3 might act through attenuation of phosphorylation of p38 MAPK and JNK pathways in SW480 cells.

■ DISCUSSION

Previous studies demonstrated the ability of gallic acid and ellagic acid to induce apoptosis as well as inhibit cell proliferation in human colon adenocarcinoma COLO-205[25] and Caco-2 cell lines.[26−28] Dried longan seeds are rich in these compounds and possess antiproliferation activity in both human colon cancer invasive SW480 and SW620 cells, but not normal colon cells (Figure 2). Our study has not only demonstrated the inhibitory effect of dried longan seeds on colon cancer cell invasion (Figure 3) but also suggested

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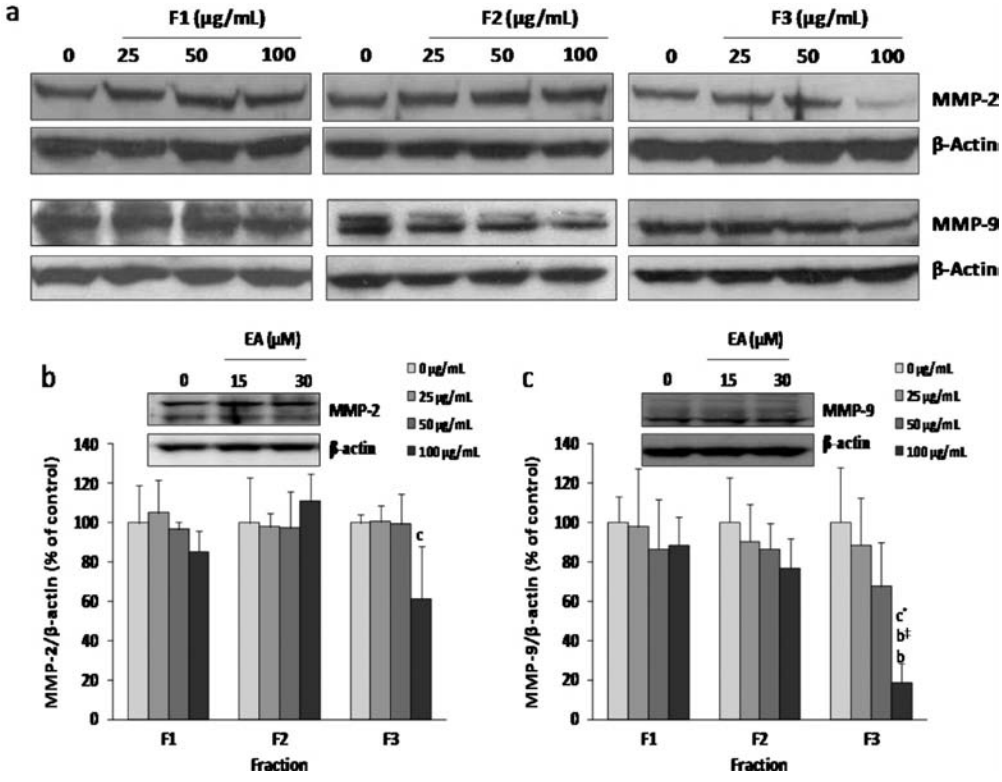

Figure 7. Dried longan seed fraction 3 diminishes the expression of MMP-2 and MMP-9 in SW480 cells. SW480 cells treated with dried longan seed fractions at various concentrations (0−100 μg/mL) and EA (0−30 μM) for 24 h were analyzed for MMP-2 and MMP-9 expression using Western blot analysis (a). Results are shown as the average percent relative intensity of MMP-2/β-actin (b) or MMP-9/β-actin (c) of three independent experiments (vs untreated controls). Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, indicate significance as compared with controls, whereas symbols ‡ and ∗ indicate comparison with treated conditions at 25 and 50 μg/mL of each fraction, respectively.

possible mechanisms of action, because the action of dried longan seed extract appears to depend on the type of colon cancer cell.

To understand such regulation, MMPs were chosen due to their crucial enhancing role in tumor invasion.[29] Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are key enzymes in the proteolytic process of ECM degradation during metastasis in colon carcinoma.[30] The effect of each fraction on gelatinase secretion was related to its effect on the invasive ability of SW480 and SW620 cells (Figure 4). These results also supported a cell type dependent action, with greatest effect on moderately invasive colon cancer cells (SW480) or nonmetastatic cells as compared to metastatic cells (SW620).

Many phenolic compounds have been found in longan seed[31] and have been reported to inhibit invasive ability, which in turn is responsible for their antigelatinase activity and expression in various cancer cell types. These include compounds such as gallic acid,[32−34] ellagic acid,[23,28,35] the aglycoside form of quercitrin (quercitin),[36] and procyanidin B2.[37] Our study showed the antigelatinase activity of dried longan seed fractions and single EA on SW480 cells (Figure 5a−c). The fact that Zn^{2+} is necessary for proteolytic activity of MMPs was the rationale for first-generation Zn^{2+} chelators being used for inhibiting MMPs activity.[38] There are abundant flavonoids possessing metal chelation activity.[39,40] The phenol group of EA has been reported to directly bind to the Zn^{2+} atom of MMP-2, leading to direct inhibition of MMP-2 activity.[23,41] Our results demonstrated that the inhibitory effects on gelatinase activity of fraction 1, fraction 2, and EA could be reversed by $ZnCl_2$, but not by the EA-enriched fraction 3 (Figure 5d−f). These data suggest that the Zn^{2+} chelating property of fraction 1, fraction 2, and EA seemed to be via direct attenuation of gelatinase activity. Although the addition of Zn^{2+} could not reverse the gelatinase activity of fraction 3, this could be explained by noting that MMPs are Zn^{2+}-dependent Ca^{2+}-containing endopeptidases for which Ca^{2+} is also essential for activity and conformation.[42] Fraction 3 possibly contained not only Zn^{2+} chelating EA but also many other flavonoids, which might possess Ca^{2+} chelating ability and thus inhibit MMP activity. In other words, the inhibitory regulation of fraction 3 on gelatinase activity probably does not depend on metal chelation. Generally, MMP-2 and MMP-9 proteolytic activity is regulated through proMMP activation and their inhibitors.[43] The present study addressed the possibility of indirect inhibitory regulation of fraction 3 on gelatinase activity by seemingly abolishing expression of their zymogen activators, including MT1-MMP, uPA, and uPAR in SW480 cells (Figure 6a−c). In the case of their inhibitors, our data showed that TIMP-1 was also suppressed by fraction 3 and that this was not associated with the inhibitory effect on MMP-2 and MMP-9 activity in SW480 cells (Figure 6d). Recently, TIMP-1 has been suggested as a novel target for anticancer therapy[44] because it facilitates induction of cell proliferation[45] and inhibition of apoptosis,[44,46−48] and is associated with poor patient prognosis.[49−51] Thus, down-regulation of TIMP-1 might be related to the antitumorigenesis of fraction 3 but not its antigelatinase activity. Additionally, TIMP-1 is overexpressed in colon adenocarcinoma tissue and is associated with the levels of MMPs.[11] Our result showed that MMP-2 and MMP-9 protein

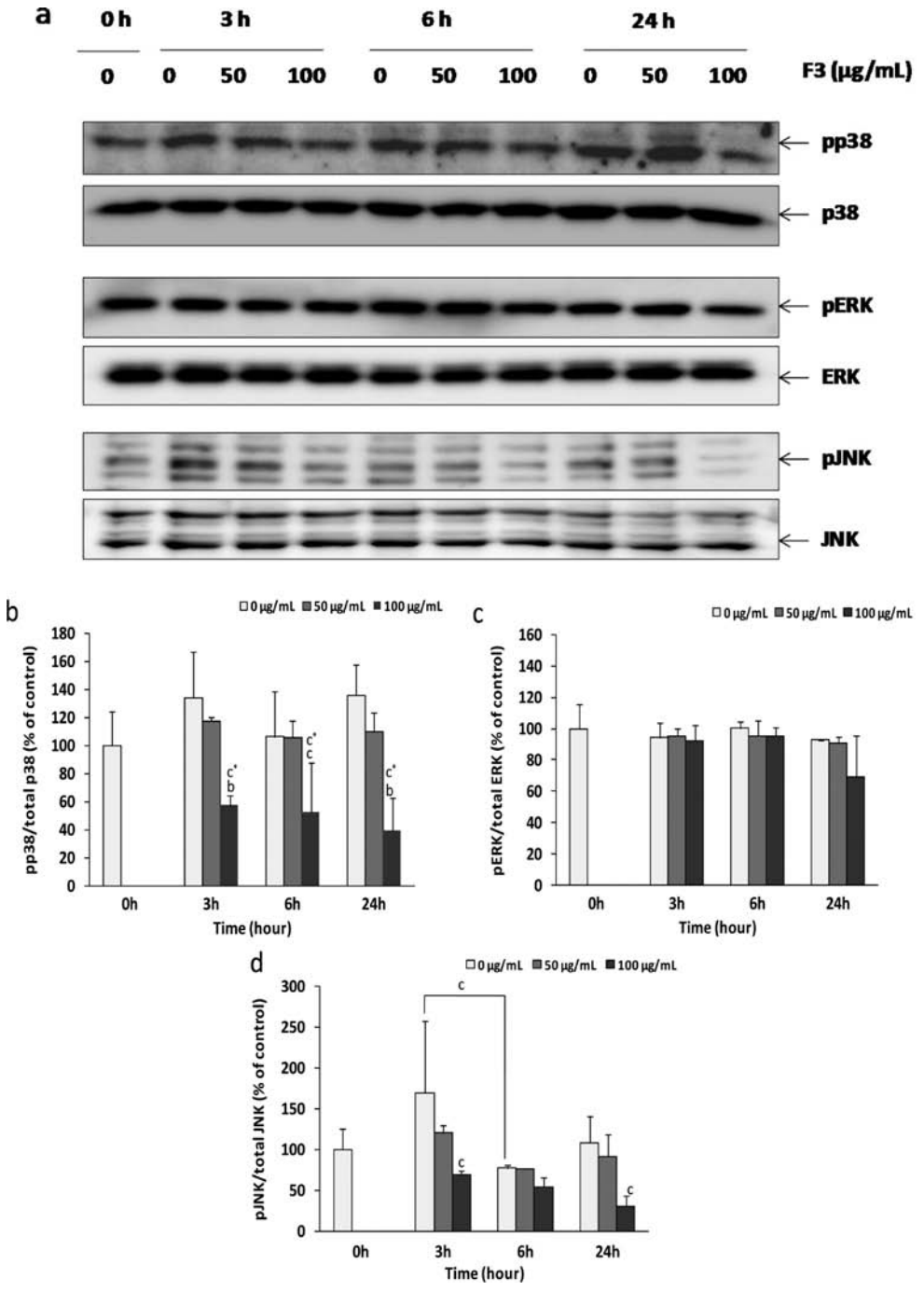

Figure 8. Dried longan seed fraction 3 attenuates phosphorylation of p38 MAPK and JNK pathway in SW480 cells. SW480 cells treated with fraction 3 at various concentrations (0, 50, and 100 μg/mL) and different times (0, 3, 6, and 24 h) were analyzed to determine the expression of p38, pp38, JNK, pJNK, ERK, and pERK using Western blot analysis (a). The results are the mean percent relative intensity of pp38/total p38 (b), pERK/total ERK (c), and pJNK/total JNK (d) ± SD of two independent experiments (vs untreated controls at 0 h) that showed the same results. Letters a, $p \leq 0.001$, b, $p \leq 0.005$, and c, $p \leq 0.05$, above the bars indicate significance as compared with untreated controls of each time treatment, whereas $*$ indicates comparison with treated conditions at 50 μg/mL for each time.

expressions were also down-regulated by the EA-enriched fraction 3 (Figure 7a–c). Consequently, the ratios of MMP-2/TIMP-1 and MMP-9/TIMP-1 in SW480 cells seemed undisturbed by fraction 3 treatment. Actually, the dried longan seed fractions contain a cocktail of bioactive compounds that were unidentified and that might interact with multiple targets in signal transduction of MMP expression. In other words, a combination of polyphenol compounds that contribute to increase biological activity more than a single compound is probably quite important. Three common subfamilies of MAPK signaling pathways consist of p38 MAPK, JNK, and ERK, which mainly regulate MMP-2 and MMP-9 expression. Some papers in the literature have addressed the inhibition of MAPK pathways with specific inhibitors as being closely related with negative regulation of MMP expression, but differing in specific cell types.[8,52−54] Our present research showed that phosphorylation of p38 MAPK and JNK pathways was attenuated by fraction 3 in SW480 cells (Figure 8a–d). This result suggested

dx.doi.org/10.1021/jf3052863 | J. Agric. Food Chem. 2013, 61, 3631−3641

that the possible regulation of fraction 3 on antigelatinase expression as well as the anti-invasive ability on SW480 cells seemed likely to be via negative regulation of the phospholylated form of p38 MAPK and JNK pathways.

Our results comprise the first evidence of the potent inhibition of dried longan seed fractions on colon cancer cell invasive capacity and that the mechanism is related to the attenuation of MMP-2 and MMP-9 activity and expression. However, their antigelatinase activities were modulated through their Zn^{2+} chelation activity and zymogen activation. The EA-enriched fraction 3 displayed down-regulation of gelatinase expression, which indicated a possible regulatory mechanism via interruption of the phosphorylated forms of p38 MAPK and JNK pathways. Distinguishing the mechanism of each fraction can increase the selective and specific effects on MMP-2 and MMP-9 activity and expression. Therefore, the active ingredients and their possible interaction (synergistic or antagonistic action) of dried longan seed extracts should be further identified and developed for their anti-invasion potential of colorectal carcinoma.

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Funding

This study was supported by a grant of the Royal Golden Jubilee (RGJ) Ph.D. program and the Graduate School, Chiang Mai University.

Notes

The authors declare no competing financial interest.

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