

## Amentoflavone Induces Anti-angiogenic and Anti-metastatic Effects Through Suppression of NF- $\kappa$ B Activation in MCF-7 cells

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**Abstract.** *The aim of the present study was to investigate whether amentoflavone induces anti-angiogenic and anti-metastatic effects through suppression of NF- $\kappa$ B activation in breast cancer in vitro. Effects of NF- $\kappa$ B inhibitor 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine (QNZ) and amentoflavone on the expression and secretion of angiogenesis- and metastasis-related proteins and cell invasion were investigated by western blotting, enzyme-linked immunosorbent assay (ELISA), and invasion assays. We also verified the effects of QNZ and amentoflavone on lipopolysaccharides (LPS)-activated cell invasion. Obtained results indicated that both QNZ and amentoflavone reduce NF- $\kappa$ B activation, expression and secretion of angiogenesis- and metastasis-related proteins, and cell invasion. QNZ and amentoflavone also reverse LPS-activated cell invasion. In conclusion, inhibition of NF- $\kappa$ B activation decreases expression and secretion of angiogenesis- and metastasis-related proteins. Amentoflavone may induce anti-angiogenic and anti-metastatic effects through suppression of NF- $\kappa$ B activation.*

Angiogenesis, the process of new blood vessels formation, is an essential component of tumor growth and metastasis in breast cancer (1). Vascular endothelial growth factor (VEGF) and many inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ) and

interleukin-6 (IL-6) are constitutively expressed in breast cancer and have been shown to contribute to angiogenesis. Furthermore, inappropriate expression of angiogenic proteins is associated with lower survival rate in breast cancer patients (2-6). Metastasis is the major cause of death in breast cancer patients (7). Matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) are gelatinases capable of degrading Type IV collagen, that is a major component of the basement membrane acting as a barrier for cancer invasion and metastasis. Certain clinical studies indicated that MMP-2 and MMP-9 overexpression, which implicate more aggressive cell invasion and metastasis behavior, result in poor prognosis in breast cancer patients (8-9).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a family of transcription factors which act as important modulators of inflammation, immune response, and tumor progression (10). NF- $\kappa$ B p50/p65 heterodimer is generally restricted to the cytoplasm by inhibitor of kappaB (I $\kappa$ B). In order to induce NF- $\kappa$ B activity and up-regulate the transcription of downstream genes, I $\kappa$ B should first be phosphorylated and degraded by I $\kappa$ B kinase (IKK) and 26S proteasome, respectively, and finally unleashed NF- $\kappa$ B translocates into the nucleus (11). Many angiogenesis- and metastasis-related proteins are encoded by NF- $\kappa$ B-regulated genes (12). Expression of NF- $\kappa$ B-regulated gene products can be enhanced by chemotherapeutic agents and radiation therapy, both of which are standard breast cancer treatment modalities. In addition, constitutive activation of NF- $\kappa$ B is observed in breast cancer and related to aggressive tumor progression (13-15). Inhibition of NF- $\kappa$ B activation not only suppresses tumor angiogenesis and metastasis but also sensitizes breast cancer to chemo-radiotherapy both *in vitro* and *in vivo* (16-19). Therefore, the development of NF- $\kappa$ B inhibitors may be important to increase the treatment efficacy for breast cancer patients.

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**Key Words:** Amentoflavone, anti-angiogenesis, anti-metastasis, NF- $\kappa$ B.

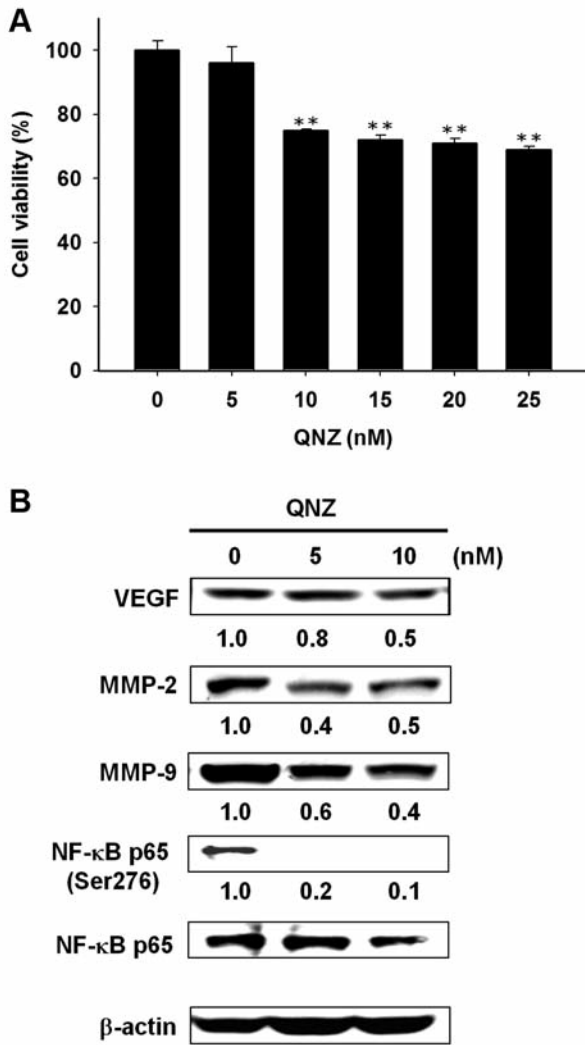


Figure 1. Effects of QNZ on cell viability, NF-κB activation, and expression of angiogenesis- and metastasis-related proteins in MCF-7 cells. Cells were treated with different concentrations (0, 5, 10, 15, 20, and 25 nM in 0.1% DMSO) of QNZ for 24 h. (A) Cell viability was evaluated by the MTT assay. (B) Change of protein levels (VEGF, MMP-2, MMP-9, pNF-κB p65 Ser 276, and NF-κB p65) were demonstrated with western blotting. \*\* $p < 0.01$  compared to control.

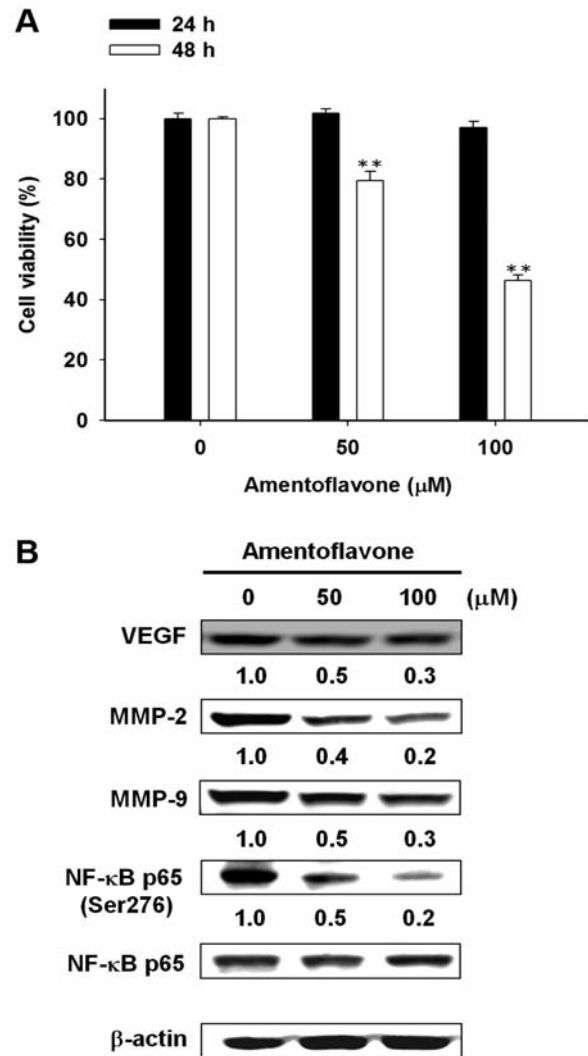


Figure 2. The effects of amentoflavone on cell viability, NF-κB activation, and expression of angiogenesis- and metastasis-related proteins in MCF-7 cells. Cells were treated with different concentrations (0, 50, and 100 μM in 0.1% DMSO) of amentoflavone for 24 or 48 h. (A) Cell viability was evaluated by the MTT assay. (B) Change of protein levels (VEGF, MMP-2, MMP-9, pNF-κB p65 Ser 276, and NF-κB p65) were demonstrated with western blotting. \*\* $p < 0.01$  compared to control.

Amentoflavone, a polyphenolic compound extracted from *Selaginella tamariscina*, has been shown to possess many biological properties, including anti-oxidation, anti-inflammation, neuroprotection and anti-tumor effects (20-21). Experiments using B16F-10 melanoma cells *in vivo* showed that amentoflavone modulates expression of anti-metastatic proteins and inhibits NF-κB activation (22). Pei *et al.* suggested that cell proliferation can be restrained by amentoflavone-induced cell-cycle arrest and apoptosis, that were dependent on mitochondrial pathway in breast cancer

cells *in vitro* (20). However, the effects of amentoflavone on angiogenesis and metastasis in breast cancer are ambiguous. The present study goals were to investigate whether amentoflavone can inhibit expression of angiogenesis- and metastasis-related proteins and cell invasion *via* suppression of NF-κB activation in breast cancer MCF-7 cells by using western blotting, ELISA, gelatin zymography and matrigel invasion assay. The role of NF-κB inactivation on anti-angiogenic and anti-metastatic effects in MCF-7 was also herein verified.

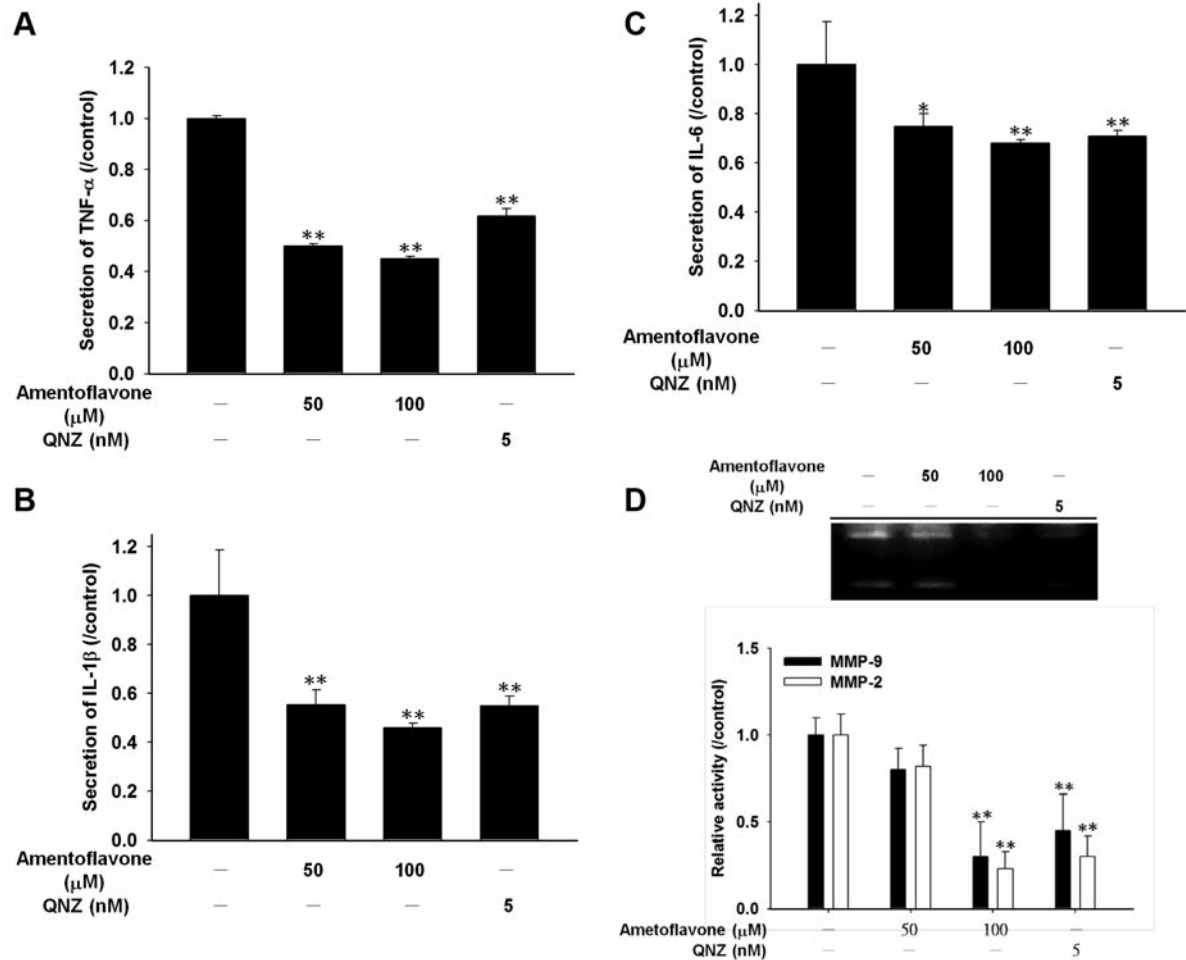


Figure 3. The effects of amentoflavone and QNZ on secretion of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and active MMP-9 and MMP-2 in MCF-7 cells. Cells were treated with different concentrations (50 and 100  $\mu$ M) of amentoflavone or 5 nM QNZ for 24 h. The secretion of cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) was evaluated with ELISA and activity of MMP-9 and MMP-2 was demonstrated with gelatin zymography assay. (A) Change of TNF- $\alpha$  secretion. (B) Change of IL-1 $\beta$  secretion. (C) Change of IL-6 secretion. (D) Change of active MMP-9 and MMP-2. \*\* $p < 0.01$  compared to control.

## Materials and Methods

**Chemicals.** Amentoflavone, crystal violet gelatin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, and penicillin-streptomycin (PS) were bought from Gibco/Life Technologies (Carlsbad, CA, USA). Matrigel and NF- $\kappa$ B inhibitor 4-N-[2-(4-phenoxyphenyl)ethyl] quinazoline-4,6-diamine (QNZ) were purchased from Selleckchem (Houston, TX, USA) and Apexbio (Houston, TX, USA), respectively. Primary antibody of  $\beta$ -actin, MMP-2, and NF- $\kappa$ B p65 Ser536 were obtained from Thermo Scientific (Hudson, NH, USA), OriGene Technologies (Rockville, MD, USA), and Cell Signaling Technology (Danvers, MA, USA), respectively. Primary antibody of NF- $\kappa$ B p65, MMP-9, and VEGF were purchased from Merck Millipore (Billerica, MA, USA). Secondary antibodies were obtained from Jackson immunoresearch (West Grove, PA, USA).

**Cell culture.** Human breast cancer cell line MCF-7 cells were used in the study. MCF-7 cells were generously provided by professor Jing-Gung Chung of Department of Biological Science and Technology, China Medical University, Taichung, Taiwan. Cells were cultured in DMEM with supplemental 10% FBS, 2 mM L-glutamine, and PS (100 U/ml and 100  $\mu$ g/ml) and maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub> atmosphere.

**MTT assay.** MTT powder was dissolved in phosphate-buffered saline (PBS) (1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 145 mM NaCl, 2.7 mM KCl, and 4.3 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.3). MCF-7 cells at a density of  $2 \times 10^4$  cells/well were seeded into 96-well plates and cultured overnight then treated with different concentration of amentoflavone or QNZ for different times. The cytotoxicity of amentoflavone and QNZ in MCF-7 cells was evaluated using MTT assay with the same protocol, as described previously (23).

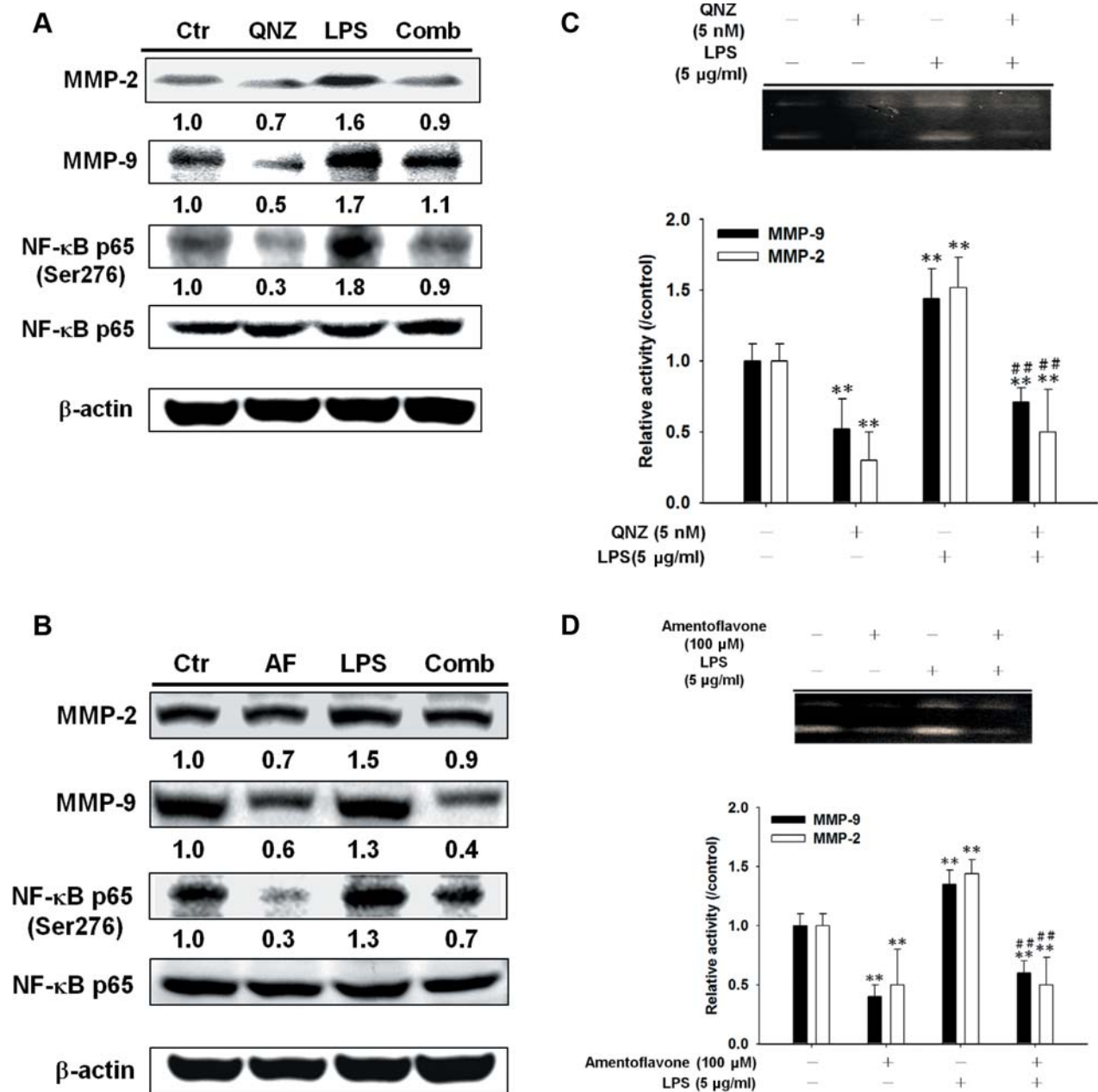


Figure 4. Continued

**Western blotting.**  $3 \times 10^6$  MCF-7 cells were cultured in a 10-cm diameter dish for 24 h. Detailed treatment conditions for MCF-7 cells in different groups were described in figure legends. The total protein from cells in different groups was extracted with lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM phenylmethanesulfonyl fluoride). The protein levels of VEGF, MMP-2, MMP-9, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , pNF- $\kappa$ B, and NF- $\kappa$ B were demonstrated by western blotting as described previously (24). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for the quantification of the protein levels.

**Enzyme-linked immunosorbent assay (ELISA).**  $1 \times 10^5$  MCF-7 cells/well were cultured in a 12-well plate for 24 h then treated with different concentration of amentoflavone or 5 nM QNZ for 24 h. Concentration of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 released by cells in medium was determined using ELISA kits (eBioscience, San Diego, CA, USA). The procedure followed the protocols provided by manufacturer.

**Gelatin zymography assay.**  $1 \times 10^5$  MCF-7 cells/well were cultured in a 12-well plate for 24 h. Detailed treatment conditions for MCF-



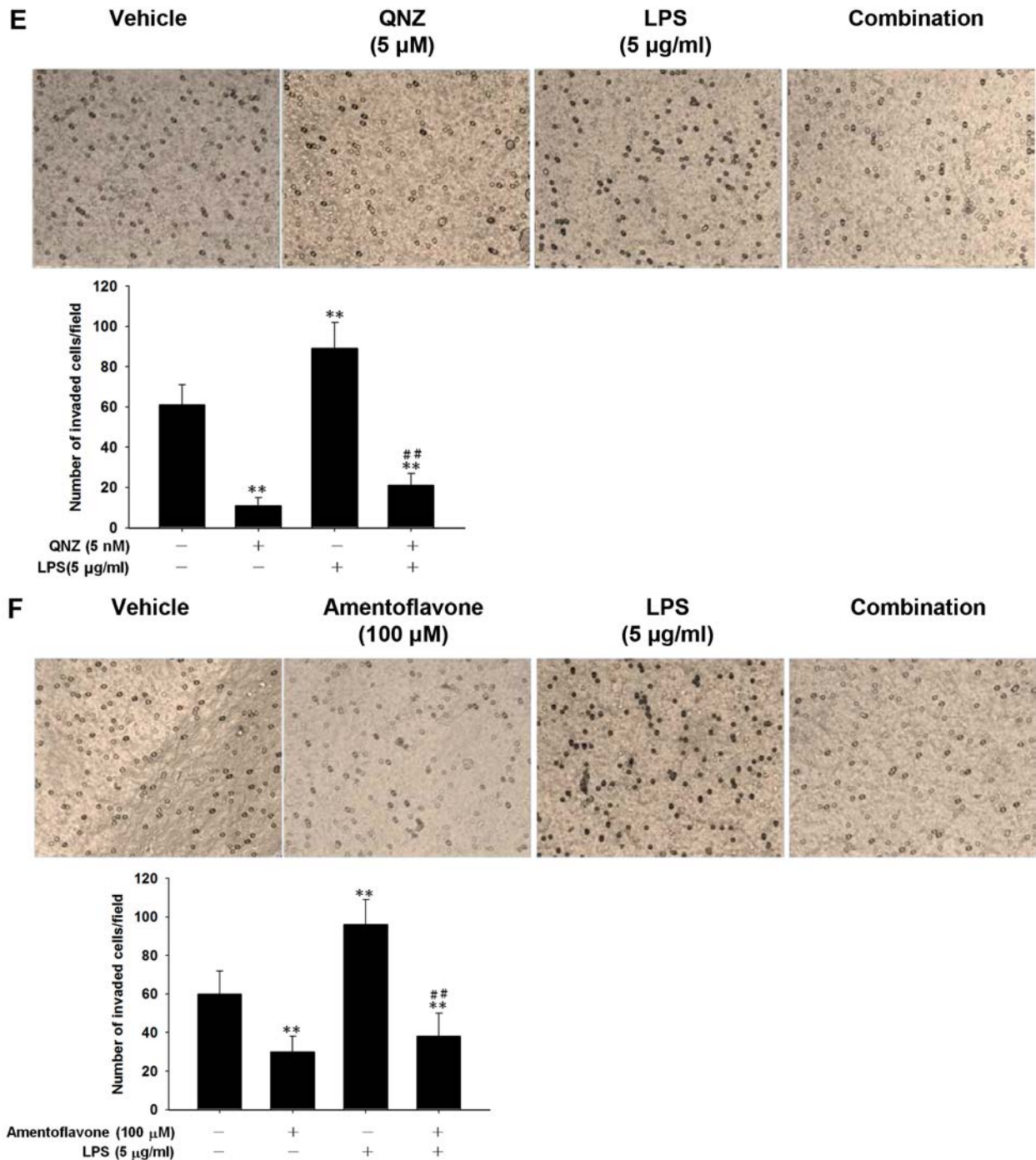


Figure 4. The effects of QNZ and amentoflavone on LPS-induced NF- $\kappa$ B activity, expression and activity of MMP-9 and MMP-2, and cell invasion in MCF-7 cells. Cells were treated with vehicle, 5 nM QNZ, 100  $\mu$ M amentoflavone, LPS, QNZ plus LPS (pretreated with 5 nM QNZ prior to the addition of 5  $\mu$ g/ml LPS), and amentoflavone plus LPS (pre-treated with 100  $\mu$ M amentoflavone prior to addition of 5  $\mu$ g/ml LPS), respectively, and then incubated for 24 h. (A) The effects of QNZ, LPS, and combination of both (comb) on proteins expression of MMP-2, MMP-9, pNF- $\kappa$ B p65 Ser 276, and NF $\kappa$ B p65 were evaluated with Western blotting. (B) The effects of amentoflavone (AF), LPS, and combination of both (comb) on proteins expression of MMP-2, MMP-9, pNF- $\kappa$ B p65 Ser276, and NF $\kappa$ B p65 were evaluated with western blotting. (C) The effects of QNZ, LPS, and combination of both on activity of MMP-2 and MMP-9 were investigated by gelatin zymography assay. (D) The effects of amentoflavone, LPS, and combination of both on activity of MMP-2 and MMP-9 were investigated by gelatin zymography assay. (E) The effects of QNZ, LPS, and combination of both on cell invasion were evaluated with matrigel invasion assay (Magnification: 100x). (F) The effects of amentoflavone, LPS, and combination of both on cell invasion were evaluated with matrigel invasion assay (Magnification: 100x). \*\* $p < 0.01$  compared to control, ## $p < 0.01$  compared to LPS alone. Ctr: control.

7 cells in different groups are described in Figure legends. Activation of MMP-2 and MMP-9 released by cells in medium was determined using gelatin zymography assay as described previously (23). ImageJ software was used to quantify bands of MMP-2 and MMP-9 activity.

**Invasion assay.** The cell-permeable membrane of 8  $\mu$ m transwell (Corning, Tewksbury, MA, USA) is coated with 50 ml matrigel. MCF-7 cells were resuspended with serum-free medium first and cells suspension was treated for 24 h with 5  $\mu$ g/ml LPS, 100  $\mu$ M amentoflavone, 5 nM QNZ, and amentoflavone or QNZ for 30 min prior to the addition of LPS.  $2 \times 10^5$  cells/well was placed in the apical chamber of the transwell insert while 10% serum medium was added into the basolateral chamber and then incubated for 24 h. The matrigel was removed with sterile cotton swabs on permeable membrane of transwell. The invasive cells in the permeable membrane were fixed with mixture of methanol and acetic acid for 15 min and then stained with 0.5% crystal violet for 30 min. The cell number per light microscopy field in the permeable membrane was counted and photographed at a magnification  $\times 100$ .

**Statistical analysis.** Student's *t*-test was used to verify significance of difference between two groups of data. Differences between means were considered significant when *p*-value was equal to or less than 0.05. Data shown represented the mean  $\pm$  standard error.

## Results

**NF- $\kappa$ B inhibitor induces cytotoxicity and inhibits expression of angiogenesis- and metastasis-related proteins in MCF-7 cells.** In order to verify whether NF- $\kappa$ B inactivation can suppress expression of angiogenesis- and metastasis-related proteins, MCF-7 cells were treated with different concentration of QNZ for 24 h. The effects of QNZ on cell viability, expression of angiogenesis- and metastasis-related proteins, and NF- $\kappa$ B activation in MCF-7 cells were investigated with the MTT assay and western blotting. Figure 1A shows that cell viability was significantly decreased gradually, while escalation of QNZ concentration (10-25 nM) in comparison to that of the control (vehicle treatment). Figure 1B indicates that the protein expression of VEGF, MMP-2, MMP-9, and NF- $\kappa$ B p65 Ser276 phosphorylation (pNF- $\kappa$ B p65 Ser 276) were inhibited by QNZ treatment in MCF-7 cells.

**Amentoflavone reduces cell viability, NF- $\kappa$ B activation and expression of angiogenesis- and metastasis-related proteins in MCF-7 cells.** After MCF-7 cells were treated with different concentration of amentoflavone for 24 h or 48 h. The effects of amentoflavone on cell viability, angiogenesis- and metastasis-related proteins, and NF- $\kappa$ B activation in MCF-7 cells were investigated with MTT assay and western blotting. Figure 2A shows that cell viability was significantly decreased by amentoflavone at 48 h (50-100  $\mu$ M) while there was no observed cytotoxicity when cells were treated with amentoflavone for 24 h (0-100  $\mu$ M). Figure 2B indicates that

amentoflavone inhibits protein expression of VEGF, MMP-2, MMP-9, and pNF- $\kappa$ B p65 Ser 276 in a dose-dependent manner.

**Amentoflavone and QNZ decrease secretion of angiogenesis and metastasis related proteins in MCF-7.** We confirmed whether amentoflavone and NF- $\kappa$ B inactivation can influence secretion and activation of angiogenesis- and metastasis-related proteins. MCF-7 cells were treated with different concentration of amentoflavone or 5 nM QNZ for 24 h and then secretion of angiogenesis- and metastasis-related proteins was evaluated by using ELISA and gelatin zymography assay. We found that 50 or 100  $\mu$ M amentoflavone (Figure 2A) and 5 nM QNZ (Figure 1A) do not reduce cell viability, but still induce a significant decrease in secretion of angiogenic proteins TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Figure 3A-C). Gelatin zymography assay can be used for detection of active forms of MMP-2 and MMP-9 (25). In the present study, the effects of amentoflavone or QNZ on activities of MMP-2 and MMP-9 were also evaluated by gelatin zymography assay. Activities of MMP-9 and MMP-2 were significantly decreased by 100  $\mu$ M amentoflavone and 5 nM QNZ treatments for 24 h, respectively (Figure 3 D).

**Amentoflavone and QNZ inhibit LPS-induced NF- $\kappa$ B activity, protein expression and activation of MMP-2/MMP-9, and cell invasion in MCF-7 cells.** Huang *et al.* suggested that LPS can induce cell invasion via increasing NF- $\kappa$ B activity (26). We also investigated the effects of amentoflavone and QNZ on LPS-induced NF- $\kappa$ B activity, protein expression and activation of MMP-2/MMP-9, and cell invasion in MCF-7 cells. MCF-7 cells were treated for 24 h with 5  $\mu$ g/ml LPS, 100  $\mu$ M amentoflavone, 5 nM QNZ, and amentoflavone or QNZ for 30 min prior to addition of LPS. NF- $\kappa$ B activation, protein expression and activation of MMP-2/MMP-9, and cell invasion in MCF-7 cells were evaluated by western blotting, gelatin zymography, and matrigel invasion assay, respectively. Figure 4A and 4B indicate QNZ and amentoflavone reversed LPS-induced protein expression of MMP-2/MMP-9 and pNF- $\kappa$ B p65 Ser 276. Figure 4C and D indicate that LPS significantly induced activities of MMP-9 and MMP-2 compared to the control. QNZ and amentoflavone significantly inhibited LPS-induced activities of MMP-9 and MMP-2. We also found that LPS significantly increased the number of invasion cells compared to the control. QNZ and amentoflavone significantly inhibited LPS-induced cell invasion (Figure 4E and F).

## Discussion

Amentoflavone has been indicated to induce cell-cycle arrest and apoptosis resulting in suppression of cell proliferation in breast cancer cells *in vitro* (20). However, the effects of amentoflavone on angiogenesis and metastasis in breast

cancer are unclear. Therefore, in the present study we tested whether or not amentoflavone can inhibit expression of angiogenesis- and metastasis-related proteins and cell invasion *via* suppression of NF- $\kappa$ B activation in MCF-7 cells. We first found that NF- $\kappa$ B inhibits cell viability, expression of angiogenesis- and metastasis-related proteins (*i.e.*, VEGF, MMP-2, and MMP-9), and NF- $\kappa$ B p65 activation (Figure 1A-B). Secondly, amentoflavone also reduces cell viability and protein expression of VEGF, MMP-2, MMP-9, and NF- $\kappa$ B p65 activation (Figure 2A-B). Thirdly, both QNZ and amentoflavone significantly inhibit secretion of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, while reducing MMP-9 and MMP-2 activity (Figure 3A-D). Finally, both QNZ and amentoflavone can suppress LPS-induced NF- $\kappa$ B p65 activity, proteins expression and activity of MMP-9 and MMP-2, and cell invasion (Figure 4A-F).

Breast cancer can be divided into two main groups according to the expression status of estrogen and progesterone receptors; hormone-positive and hormone-negative breast cancers (27). Many clinical studies have shown that over-expression of NF- $\kappa$ B found in both hormone-positive and negative-breast cancer is correlated with poor prognosis (15, 28, 29). Overactive NF- $\kappa$ B signaling pathway leads to transcription of NF- $\kappa$ B target genes, that can induce anti-apoptosis, cell proliferation, and angiogenesis, and cell invasion, and results in aggressive tumor progression (30). Therefore, Wu *et al.* suggested inhibition of NF- $\kappa$ B pathway as a novel approach in breast cancer therapy (30). In the present study, we used QNZ, an inhibitor of NF- $\kappa$ B activation in many cancer cell lines, to verify the role of NF- $\kappa$ B inactivation on anti-angiogenic and anti-metastatic effects in MCF-7 cells (31). The data indicated that QNZ not only inhibited cell viability but also reduced expression and secretion of angiogenesis- and metastasis-related proteins (VEGF, MMP-2, MMP-9, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) (Figures 1 and 3). LPS, a strong activator of NF- $\kappa$ B, has been indicated to induce cell invasion and mRNA expression of MMP-9 *via* increasing NF- $\kappa$ B activity in breast cancer cells (32). In Figure 4B, D and F, LPS-induced NF- $\kappa$ B activity, protein expression and activity of MMP-9 and MMP-2, and cell invasion in MCF-7 cells were reversed by QNZ co-treatment. The above findings indicate that expression and secretion of angiogenesis- and metastasis-related proteins and cell invasion can be reduced by inhibition of NF- $\kappa$ B activation in MCF-7 cells.

Amentoflavone has been found to reduce expression and secretion of inflammatory mediators and cytokines while suppressing NF- $\kappa$ B activation in different cell types (22, 33, 34). Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) in active inflammatory cells is a significant mediator in inflammation (33). Woo *et al.* found that amentoflavone inhibits LPS-induced expression of iNOS by suppressing NF- $\kappa$ B activation in macrophages (33). Sakthivel

*et al.* also suggested that amentoflavone has protective effects in acetic acid-induced ulcerative colitis in rats *via* inhibition of expression of inflammatory mediators (iNOS and cyclooxygenase-2, COX-2) and cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) together with NF- $\kappa$ B signal transduction pathway (34). The study of amentoflavone on B16F-10 melanoma demonstrated that amentoflavone triggers expression of tissue inhibitor of metalloprotease (TIMP)-1 and TIMP-2, reduces expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and VEGF, and suppresses activation of many transcriptional factors including NF- $\kappa$ B, c-Fos, activated transcription factor-2 (ATF-2), and cyclic adenosine monophosphate (AMP) response element-binding protein (CREB). All aforementioned molecules together result in inhibition of pulmonary metastasis of melanoma cells in C57BL/6 mice (22).

The goal of the present study was to investigate whether amentoflavone can inhibit expression of angiogenesis- and metastasis-related proteins and cell invasion *via* suppression of NF- $\kappa$ B activation in breast cancer MCF-7 cells. We first confirmed that NF- $\kappa$ B inactivation by QNZ can quell expression and secretion of angiogenesis and metastasis related proteins and cell invasion in MCF-7 cells. Next we used amentoflavone to reproduce the same findings as QNZ did. Figure 2A presents that cell viability was significantly repressed by 50 and 100  $\mu$ M amentoflavone treatment for 48 h, while no significant cytotoxicity was observed in cells treated with 50 and 100  $\mu$ M amentoflavone for 24 h. In Figure 2B and 3A-D, it is shown that amentoflavone reduced NF- $\kappa$ B activation and expression and secretion of angiogenesis- and metastasis-related proteins (VEGF, MMP-2, MMP-9, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6). We also evaluated the effects of amentoflavone on LPS-induced NF- $\kappa$ B activity, protein expression and activity of MMP-9 and MMP-2, and cell invasion in MCF-7 cells. The results showed that amentoflavone inhibits LPS-induced NF- $\kappa$ B activity, protein expression and activity of MMP-9 and MMP-2, and cell invasion (Figure 4B, D and F). Tarallo *et al.* used human umbilical vein endothelial cell (HUVEC) to evaluate anti-angiogenic effects of amentoflavone. Their data demonstrated that VEGF-A or placental growth factor 1 (PlGF-1)-induced endothelial cell migration and capillary-like tube formation were reduced by amentoflavone selectively binding to VEGF family members, blocking interaction between VEGF and VEGF receptor 1 and 2 (VEGFR-1, VEGFR-2), and finally resulting in dephosphorylation of VEGFR-1 and VEGFR-2 (35). We, therefore, suggest that amentoflavone reduces expression and secretion of angiogenesis and metastasis related proteins and cell invasion *via* suppression of NF- $\kappa$ B activation in MCF-7 cells *in vitro*.

In conclusion, it was herein proven that amentoflavone inhibits expression and secretion of VEGF, MMP-2, MMP-9, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 *via* suppression of NF- $\kappa$ B activation. Therefore, we propose the potential application for



amentoflavone as an NF- $\kappa$ B inhibitor to repress angiogenesis and metastasis in human breast cancer.

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## References

- Schneider BP and Miller KD: Angiogenesis of breast cancer. *J Clin Oncol* 23: 1782-1790, 2005.
- Zhu L, Loo WT and Louis WC: PTEN and VEGF: possible predictors for sentinel lymph node micro-metastasis in breast cancer. *Biomed Pharmacother* 61: 558-561, 2007.
- Tripsianis G, Papadopoulou E, Romanidis K, Katotomichelakis M, Anagnostopoulos K, Kontomanolis E, Botaitis S, Tentes I and Kortsaris A: Overall survival and clinicopathological characteristics of patients with breast cancer in relation to the expression pattern of HER-2, IL-6, TNF- $\alpha$  and TGF- $\beta$ 1. *Asian Pac J Cancer Prev* 14: 6813-6820, 2013.
- Burton ER and Libutti SK: Targeting TNF-alpha for cancer therapy. *J Biol* 8: 85.1-85.5, 2009.
- Snoussi K, Strosberg AD, Bouaouina N, Ben Ahmed S and Chouchane L: Genetic variation in pro-inflammatory cytokines (interleukin-1beta, interleukin-1alpha and interleukin-6) associated with the aggressive forms, survival, and relapse prediction of breast carcinoma. *Eur Cytokine Netw* 16: 253-260, 2005.
- Salgado R, Junius S, Benoy I, Van Dam P, Vermeulen P, Van Marck E, Huget P and Dirix LY: Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* 103: 642-6, 2003.
- Scully OJ, Bay BH, Yip G and Yu Y: Breast cancer metastasis. *Cancer Genomics Proteomics* 9: 311-320, 2012.
- Ranuncolo SM, Armanasco E, Cresta C, Bal De Kier Joffe E and Puricelli L: Plasma MMP-9 (92 kDa-MMP) activity is useful in the follow-up and in the assessment of prognosis in breast cancer patients. *Int J Cancer* 106: 745-751, 2003.
- Nakopoulou L, Tsimpa I, Alexandrou P, Louvrou A, Ampela C, Markaki S and Davaris PS: MMP-2 protein in invasive breast cancer and the impact of MMP-2/TIMP-2 phenotype on overall survival. *Breast Cancer Res Treat* 77: 145-155, 2003.
- Liu YC, Chiang IT, Hsu FT and Hwang JJ: Using NF- $\kappa$ B as a molecular target for theranostics in radiation oncology research. *Expert Rev Mol Diagn* 12: 139-146, 2012.
- Chen YJ, Yeh MH, Yu MC, Wei YL, Chen WS, Chen JY, Shih CY, Tu CY, Chen CH, Hsia TC, Chien PH, Liu SH, Yu YL and Huang WC: Lapatinib-induced NF- $\kappa$ B activation sensitizes triple-negative breast cancer cells to proteasome inhibitors. *Breast Cancer Res* 15: R108, 2013.
- Bassères DS and Baldwin AS: Nuclear factor- $\kappa$ B and inhibitor of  $\kappa$ B kinase pathways in oncogenic initiation and progression. *Oncogene* 25: 6817-6830, 2006.
- Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelfanov V, Boswell SH, Goulet RJ Jr, Sledge GW Jr and Nakshatri H: Paclitaxel sensitivity of breast cancer cells with constitutively active NF- $\kappa$ B is enhanced by IkappaBalpha super-repressor and parthenolide. *Oncogene* 19: 4159-4169, 2000.
- Nam JM, Ahmed KM, Costes S, Zhang H, Onodera Y, Olshen AB, Hatanaka KC, Kinoshita R, Ishikawa M, Sabe H, Shirato H and Park CC:  $\beta$ 1-Integrin via NF- $\kappa$ B signaling is essential for acquisition of invasiveness in a model of radiation treated in situ breast cancer. *Breast Cancer Res* 15: R60, 2013.
- Jana D, Das S, Sarkar DK, Mandal S, Maji A and Mukhopadhyay M: Role of nuclear factor- $\kappa$ B in female breast cancer: a study in Indian patients. *Asian Pac J Cancer Prev* 13: 5511-5515, 2012.
- Shibata A, Nagaya T, Imai T, Funahashi H, Nakao A and Seo H: Inhibition of NF- $\kappa$ B activity decreases the VEGF mRNA expression in MDA-MB-231 breast cancer cells. *Breast Cancer Res Treat* 73: 237-243, 2002.
- Rahman KM, Sarkar FH, Banerjee S, Wang Z, Liao DJ, Hong X and Sarkar NH: Therapeutic intervention of experimental breast cancer bone metastasis by indole-3-carbinol in SCID-human mouse model. *Mol Cancer Ther* 5: 2747-2756, 2006.
- Kang HJ, Lee SH, Price JE and Kim LS: Curcumin suppresses the paclitaxel-induced nuclear factor- $\kappa$ B in breast cancer cells and potentiates the growth inhibitory effect of paclitaxel in a breast cancer nude mice model. *Breast J* 15: 223-229, 2009.
- Ahmed KM, Cao N and Li JJ: HER-2 and NF- $\kappa$ B as the targets for therapy-resistant breast cancer. *Anticancer Res* 26: 4235-4243, 2006.
- Pei JS, Liu CC, Hsu YN, Lin LL, Wang SC, Chung JG, Bau DT and Lin SS: Amentoflavone induces cell-cycle arrest and apoptosis in MCF-7 human breast cancer cells via mitochondria-dependent pathway. *in vivo* 26: 963-970, 2012.
- Shin DH, Bae YC, Kim-Han JS, Lee JH, Choi IY, Son KH, Kang SS, Kim WK and Han BH: Polyphenol amentoflavone affords neuroprotection against neonatal hypoxic-ischemic brain damage via multiple mechanisms. *J Neurochem* 96: 561-572, 2006.
- Guruvayoorappan C and Kuttan G: Effect of amentoflavone on the inhibition of pulmonary metastasis induced by B16F-10 melanoma cells in C57BL/6 mice. *Integr Cancer Ther* 6: 185-197, 2007.
- Chiang IT, Liu YC, Wang WH, Hsu FT, Chen HW, Lin WJ, Chang WY and Hwang JJ: Sorafenib inhibits TPA-induced MMP-9 and VEGF expression via suppression of ERK/NF- $\kappa$ B pathway in hepatocellular carcinoma cells. *in vivo* 26: 671-681, 2012.
- Wang WH, Chiang IT, Ding K, Chung JG, Lin WJ, Lin SS and Hwang JJ: Curcumin-induced apoptosis in human hepatocellular carcinoma j5 cells: critical role of ca(+2)-dependent pathway. *Evid Based Complement Alternat Med* 2012: 512907, 2012.
- Snoek-van Beurden PA and Von den Hoff JW: Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques* 38: 73-83, 2005.
- Huang T, Chen Z and Fang L: Curcumin inhibits LPS-induced EMT through downregulation of NF- $\kappa$ B-Snail signaling in breast cancer cells. *Oncol Rep* 29: 117-124, 2013.
- Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B and Senn HJ: Panel members. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* 22: 1736-1747, 2011.
- Zhou XL, Fan W, Yang G and Yu MX: The clinical significance of PR, ER, NF- $\kappa$ B, and TNF- $\alpha$  in breast cancer. *Dis Markers* 2014: 494581, 2014.



- 29 Frasca J, Weaver A, Pradhan M, Dai Y, Miller LD, Lin CY and Stanculescu A: Positive cross-talk between estrogen receptor and NF-kappaB in breast cancer. *Cancer Res* 69: 8918-8925, 2009.
- 30 Wu JT, and Kral JG: The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. *J Surg Res* 123: 158-169, 2005.
- 31 Choi S, Kim JH, Roh EJ, Ko MJ, Jung JE and Kim HJ: Nuclear factor-kappaB activated by capacitative Ca<sup>2+</sup> entry enhances muscarinic receptor-mediated soluble amyloid precursor protein (sAPPalpha) release in SH-SY5Y cells. *J Biol Chem* 281: 12722-12728, 2006.
- 32 Zhao Y, Kong X, Li X, Yan S, Yuan C, Hu W and Yang Q: Metadherin mediates lipopolysaccharide-induced migration and invasion of breast cancer cells. *PLoS One* 6: e29363, 2011.
- 33 Woo ER, Lee JY, Cho IJ, Kim SG and Kang KW: Amentoflavone inhibits the induction of nitric oxide synthase by inhibiting NF-kappaB activation in macrophages. *Pharmacol Res* 51: 539-546, 2005.
- 34 Sakthivel KM and Guruvayoorappan C: Amentoflavone inhibits iNOS, COX-2 expression and modulates cytokine profile, NF-kappaB signal transduction pathways in rats with ulcerative colitis. *Int Immunopharmacol* 17: 907-916, 2013.
- 35 Tarallo V, Lepore L, Marcellini M, Dal Piaz F, Tudisco L, Ponticelli S, Lund FW, Roepstorff P, Orlandi A, Pisano C, De Tommasi N and De Falco S: The biflavonoid amentoflavone inhibits neovascularization preventing the activity of proangiogenic vascular endothelial growth factors. *J Biol Chem* 286: 19641-19651, 2011.

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