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ABSTRACT: Diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are major organosulfur compounds exiting in garlic (*Allium sativum*). These compounds are reported to exhibit various pharmacological properties such as antibacteria, antiangiogenesis, anticancer, and anticoagulation, and they also induce cytotoxicity and induction of apoptosis in human cancer cells. Although these compounds show wide spec-

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trum of biological activities, there are no reports to show that DAS, DADS, and DATS affected migration and invasion of human colon cancer cells, and their exact molecular mechanisms are not well investigated. Therefore, the purpose of this study was to determine whether DAS, DADS, and DATS affected the invasion and migration abilities of colo 205 human colon cancer cells. The results indicate that DAS, DADS, and DATS at 10 and 25 μM inhibited the migration and invasion of colo 205 cells in the order of DATS $<$ DADS $<$ DAS. DATS is the highest for inhibition of migration and invasion of colo 205 cells. DAS, DADS, and DATS induce downregulation expression of PI3K, Ras, MEKK3, MKK7, ERK1/2, JNK1/2, and p38 and then lead to the inhibition of MMP-2, -7, and -9. DAS, DADS, and DATS inhibited NF-κB and COX-2 for leading to the inhibition of cell proliferation. Taken together, these results demonstrated that application of DAS, DADS, and DATS might serve as potential antimetastatic drugs. Environ Toxicol 00: 000–000, 2011.
Keywords: DAS; DADS; DATS; migration and invasion; colo 205 human colon cancer cells

INTRODUCTION

At distant sites, cancer metastasis and proliferation involves complex interactions between cancer cells and their microenvironment at the primary tumor site (Chiang and Massague, 2008), and cancer metastasis goes through many steps including vessel formation, cell attachment, invasion, and cell proliferation, and is regulated by many mechanisms (Fidler, 2002). The development and progression of distant metastases often determine patient survival. Therefore, to identify key regulators of metastasis is important for the development of treatments to patients with metastases. It is well-known that several key proteins involved in the mechanisms such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) are vital for devising new therapies.

Cancer invasion occurs after the cancer cells respond and migrate towards gradients of stimuli including growth factors and also requires proteolysis of basement membrane (BM) and extracellular matrix (ECM) proteins to create a path for migration. Proteolysis of ECMs and BM are considered a key event for tumor invasion and metastasis (Liotta et al., 1991; Duffy, 1992; Mignatti and Rifkin, 1993). MMPs are a group of enzymes (zinc-dependent endopeptidases) responsible for the proteolysis of BM and ECM proteins, and the expression level of MMPs appears to correlate with the invasiveness of cancer cells (Toda et al., 2006; Lai et al., 2010). Colon cancer metastasis also caused treatment to be more difficult, and in Taiwan about 18.5 persons per 100 thousand people die because of colon cancer from the reports of the ''Department of Health, R.O.C. (Taiwan).'' Surgery, chemotherapy, and radiotherapy are commonly used for clinical therapy in human colon cancer. Currently, the strategies for treatment of human colon cancer are not yet satisfactory.

Garlic, one of the most consumed plant medicine, possesses multiple biological activities including antimicrobial, hypolipidemic, antithrombotic, and antitumor activities (Augusti, 1996). Garlic contains oil-soluble compounds including diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), and these oil-soluble allyl sulfides (OASs) are thought to be more potent in inhibiting growth and induction of apoptosis of tumor cells *in vitro* studies (Sundaram and Milner, 1993, 1996). DAS, DADS, and DATS are volatile compounds produced after garlic bulb is cut and have been demonstrated to inhibit carcinogenesis (Wargovich, 1987; Wattenberg et al., 1989; Hosono et al., 2005). Garlic allyl sulfide suppresses oxidized LDL-induced vascular cell adhesion molecule and E-Selectin expression through protein kinase A- and B-dependent signaling pathways and they are in the order DATS $>$ DADS $>$ DAS (Lei et al., 2008). Currently there are no reports to address whether DAS, DADS, and DATS affected the migration and invasion of human colon cancer cells *in vitro*. In the present studies, we investigated the effects of DAS, DADS, and DATS on the migration and invasion of colo 205 human colon cancer cells *in vitro*. The results indicated that all examined DAS, DADS, and DATS inhibited the migration and invasion of colo 205 human colon cancer cells *in vitro*.

Abbreviations

AP-1	activation protein-1
BM	basement membrane
BSA	bovine serum albumin
DADS	diallyl disulfide
DAS	Diallyl sulfide
DATS	diallyl trisulfide
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
MMP	matrix metalloproteinases
OAS	oil-soluble allyl sulfides
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
uPA	urokinase-type plasminogen activator

MATERIALS AND METHODS

Chemicals and Reagents

DAS, DADS, dimethyl sulfoxide (DMSO), ribonuclease-A, Triton X-100, and propidium iodide (PI) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). DATS was from LKT Laboratories, Inc. (Paul, MN). RPMI 1640 medium, L-glutamine, fetal bovine serum, penicillin–streptomycin, and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA). Antibodies used for

Western analysis were obtained as follows: antibodies for MMP-2, MMP-7, MMP-9, Ras, NF-κB p65, NF-κB p50, COX-1 and COX-2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and antibodies for PI3K, GRB2, MEKK3, MKK7, ERK1/2, JNK1/2, p38-p, iNOS, and PKC were obtained from BD PharMingen (San Diego, CA).

Cell Cultures

Human colon cancer cell line (colo 205) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 75-cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere.

Cell Viability Assay

The effect of DAS, DADS, and DATS treatment on cell viability was determined by a PI exclusion assay. Briefly, the colo 205 cells (2×10^5 cells/well) were plated in 12-well plates and allowed for overnight. The RPMI 1640 medium was replaced with fresh complete medium containing different concentrations (0, 5, 10, 25, and 50 μM) of DAS, DADS, and DATS and incubation was continued for 24 h at 37°C. Stock solution of DAS, DADS, and DATS were prepared in DMSO, and an equal volume of DMSO (final concentration 1%) was added to controls. At the end of the incubation, both floating and adherent cells were collected and suspended in PBS by centrifugation. The cells were then added to PBS containing PI (4 μg/mL), and determined by using a FACSCalibur flow cytometer and BD CellQuest Acquisition software (San Jose, CA) as described previously (Lin et al., 2009; Lu et al., 2010).

In Vitro Migration Assay

The migration of colo 205 cells was measured by chemotactic directional migration, which was determined using a 24-well Transwell insert as described previously (Chen et al., 2010; Lin et al., 2010). The 8-μm pore filters (Millipore, Billerica, MA) were coated with 30 μg type (collagen (Millipore) for 1 h before colo 205 cells (10^4 cells/0.4 mL RPMI 1640 medium) were placed in the upper chamber with or without DAS, DADS, or DATS (0, 10, and 25 μM) and allowed to undergo migration for 48 and 72 h at humidified atmosphere with 95% air and 5% CO_2 at 37°C. At the end of experiments, the upper chamber containing nonmigrated cells were removed, and then filters were stained with 2% crystal violet (Sigma-Aldrich Corp.). Migrated cells adherent to the underside of the filter were counted and photographed under a light microscope at × 200.

In Vitro Invasion Assay

The invaded colo 205 cells were measured using Matrigel (BD Biosciences, San Jose, CA)-coated transwell cell culture chambers (8 μm pore size), as described previously (Chen et al., 2010; Lin et al., 2010). Cells were maintained in serum-free-RPMI 1640 medium for 24 h and then were trypsinized and resuspended in serum-free medium and placed in the upper chamber of the transwell insert (1×10^4 cells/well) and treated with 1% DMSO or DAS, DADS, or DATS (0, 10, and 25 μM). RPMI 1640 medium containing 10% FBS was placed in the lower chamber. The above treated cells were seeded to filters and then were incubated for 48 or 72 h at humidified atmosphere with 95% air and 5% CO_2 at 37°C. Invasive cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in ethanol. The noninvasive cells in the upper chamber were removed by wiping with a cotton swab. The cells in the lower surface of the filter which penetrated through the Matrigel were randomly selected fields and were counted under a light microscope at × 200.

Western Blotting Analysis

The colo 205 cells were cultured in six-well tissue culture plates and grown for 24 h. DAS, DADS, or DATS were individually added to cells at a final concentration of 0, 10, 25, 50, 75, and 100 μM, whereas DMSO (solvent) alone was added to control cells. Cells were incubated with DAS, DADS, or DATS in 90% RPMI-1640 medium with 0.5% FBS at 37°C for 24 h. Then cells were harvested, resuspended in the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea), and centrifuged at 13,000 × g for 10 min at 4°C to remove cell debris. The supernatant was collected and total protein concentration of each sample was determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin (BSA) as the standard. SDS gel electrophoresis and Western blotting were performed as described previously (Wu et al., 2010) to determine effects of DAS, DADS, or DATS on protein levels of MMP-2, MMP-7, MMP-9, PI3K, GRB2, Ras, PKC, MEKK3, MKK7, ERK1/2, JNK1/2, p38-p, iNOS, NF-κB p65, NF-κB p50, COX-1, and COX-2. The reduced and denaturized lysate was loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separated by electrophoresis and then transferred to nitrocellulose membranes. Relative quantization of proteins was determined by band densitometry using NIH ImageJ software as described previously (Chiang et al., 2011; Yu et al., 2011).

Statistical Analysis

All data are reported with means ± standard deviation of three experiments and evaluated by the Student's *t* test and considered significant at the $^*P < 0.05$

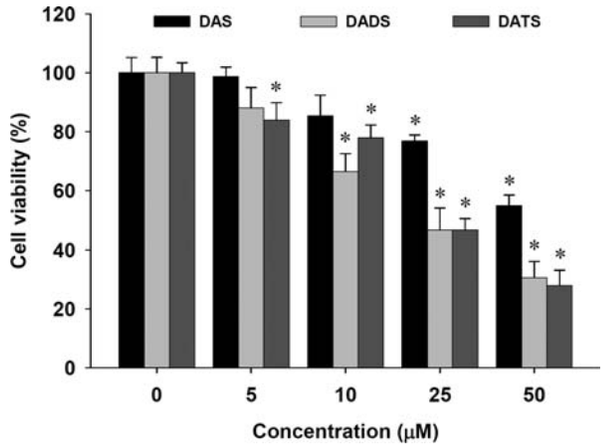

Fig. 1. DAS, DADS and DATS decreased the viability of colo 205 cells. Cells (2×10^5 cells/well) were placed in 12-well plates and incubated at 37°C for 24 h before each well was then cotreated with various doses of DAS, DADS and DATS for 24 h. DMSO (solvent) was used for the control regimen. Cells were stained with PI and were analyzed by flow cytometry as described in Materials and Methods. Each point is mean ± S.D. of three experiments. *$P < 0.05$ versus control.

RESULTS

The Effects of DAS, DADS, and DATS on the Percentage of Viable Colo 205 Cells

To examine whether DAS, DADS, and DATS exhibit cytotoxicity and cell growth inhibition in colon cancer cells, colo 205 cells were treated with various concentrations of DAS, DADS, and DATS, which markedly decreased cell viability dose dependently in colo 205 cells [Fig. 1(A–C)] ($P < 0.05$). The inhibition of cell viability are in the order DATS > DADS > DAS.

The Effects of DAS, DADS, and DATS on Colo 205 Cell Migration

To examine the effect of DAS, DADS, and DATS on the migration ability of colo 205 cells, Boyden chamber assay was used in a dose- and time-experiment. The colo 205 cells treated with 10 and 25 μM of DAS, DADS, and DATS for 48 and 72 h were plated in the upper chamber, and the number of cells moved to the underside of the coated membrane was counted under a light microscopy. The results shown in Figure 2(A–C) indicated that the number of migrated cells to the lower chamber was significantly decreased after the 48-h and 72-h treatment of DAS, DADS, and DATS. Also, the levels of colo 205 cell numbers decreased almost three-fold after treatment with 25 μM DAS, DADS, and DATS for 72 h when compared with the untreated cells. These results revealed that DAS, DADS, and DATS significantly inhibited the motility of colo 205 cells *in vitro*.

The Effects of DAS, DADS, and DATS on Colo 205 Cell Invasion

To further examine the effects of DAS, DADS, and DATS on the invasive ability of colo 205 cells, Boyden chamber coated with Matrigel was used in a dosage experiment. Colo 205 cells after exposure to 10 and 25 μM of DAS, DADS, and DATS for 48 and 72 h were plated in the upper chamber, and the number of cells moved to the underside of the coated membrane was counted under a light microscopy. The results are shown in Figure 3(A–C) and indicated that the number of invaded cells to the lower chamber was significantly decreased by the 48 h and 72 h treatment of DAS, DADS, and DATS. Such induction was concentration dependent with a three-fold increase ($P < 0.05$) when DAS, DADS, and DATS were at 25 μM [Fig. 3(A–C)].

The Effects of DAS, DADS, and DATS on the Levels of Associated Proteins with Invasion and Migration in Colo 205 Cells

We investigated the regulation of proteins that are associated with cell migration and invasion signaling pathways by Western blot analysis. After colo 205 cells were exposed to various concentrations of DAS, DADS, and DATS for 24 h, they were harvested for determining the proteins levels. Results are shown in Figure 4(A–D), which indicated that DAS, DADS, and DATS all decreased the GRB2, Ras, and PKC [Fig. 4(B)], affected MEKK3, MKK7, ERK1/2, JNK, and P38-p [Fig. 4(B,C)], and then decreased the levels of MMP-2, MMP-7, and MMP-9 [Fig. 4(A)]. All examined compounds also inhibited PI3K, iNOS, and COX-2 [Fig. 4(B,D)] and then led to the inhibition of cell proliferation. These results indicate that DAS, DADS, and DATS effectively inhibit MMP-2, MMP-7, and MMP-9 for causing inhibition of migration and invasion in colo 205 cells.

DISCUSSION

Cancer development involves multistep process as cancer eventually spreads from one area of the body to other organs or tissues during the late metastasic stage. It is well known that one important characteristic of metastasis is the invasive and migratory ability of tumor cells. In this study, we show for the first time that DAS, DADS, and DATS can inhibit the migration and invasion of colo 205 human colon cancer cells *in vitro* by using cell invasion and migration assays. In the present study, at 10 and 25 μM DAS, DADS, and DATS -treated group, the invasion and migration of colo 205 cells showed 60%, 76%, and 75%; 56%, 68%, and

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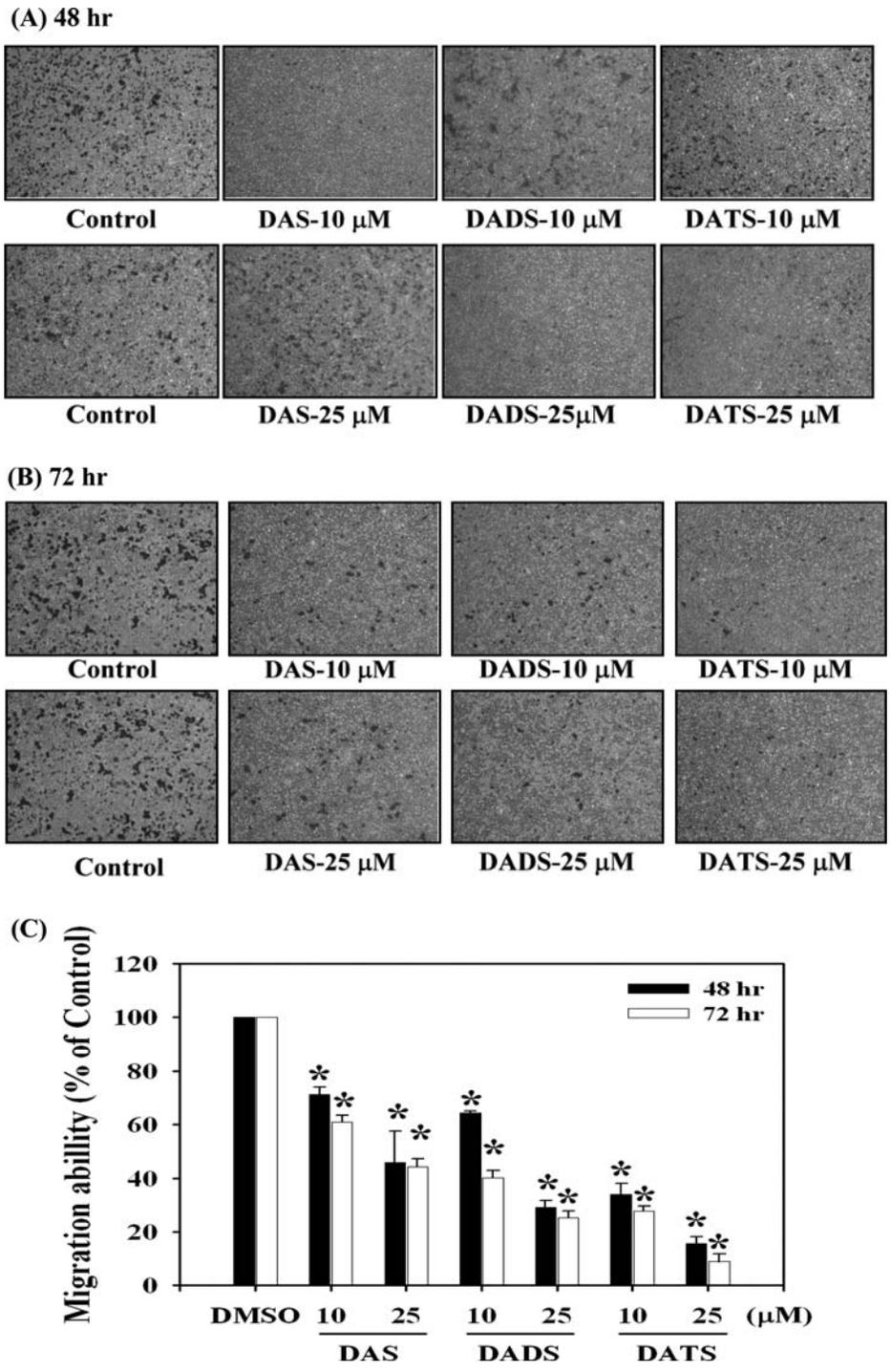

Fig. 2. Effects of DAS, DADS and DATS on the *in vitro* migration of colo 205 cells. Cells (5×10^4 cells/0.4 mL RPMI-1640 medium) were plated in the upper chamber in the absence or present of DAS, DADS and DATS (10 or 25 μM) and allowed to undergo migration for 48 and 72 h. The filters with a reconstituted basement and collagen type I was stained by 2% crystal violet, and migrated colo205 cell adherent to the underside of the filter were observed and counted under a Nikon phase-contrast microscope at $\times$ 200 (A: 48 h treatment; B: 72 h treatment). The percentage of inhibition was calculated (C). *$P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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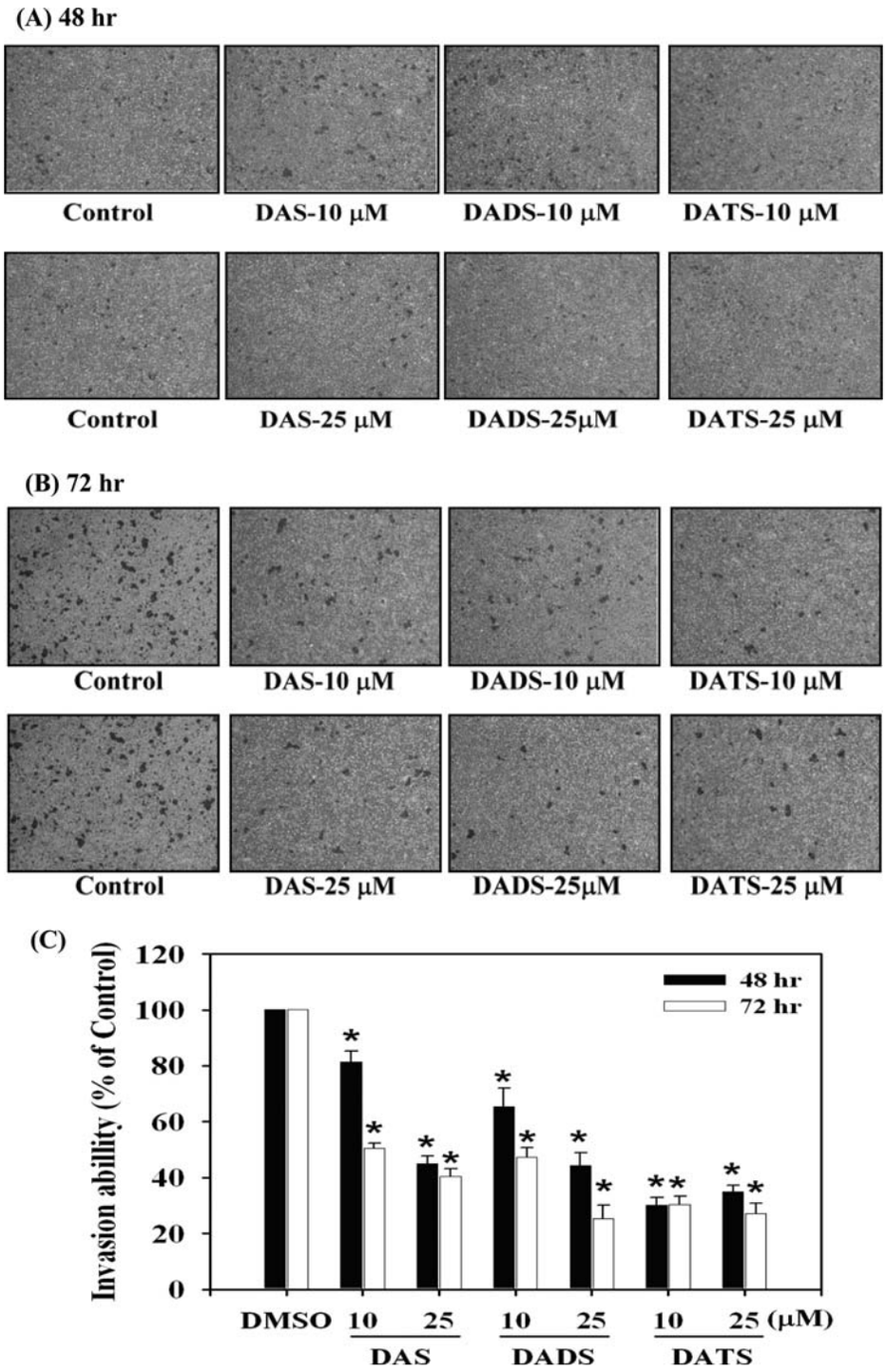

Fig. 3. Effects of DAS, DADS and DATS on the *in vitro* invasion of colo 205 cells. Cells (5×10^4 cells/0.4 mL RPMI-1640 medium) were placed and cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at 200× (A: 48 h treatment; B: 72 h treatment). Quantification of cells in the lower chamber was performed by counting cells under a Nikon phase-contrast microscope at 200× (C). *$P < 0.05$, significant difference between DAS, DADS and DATS-treated groups and the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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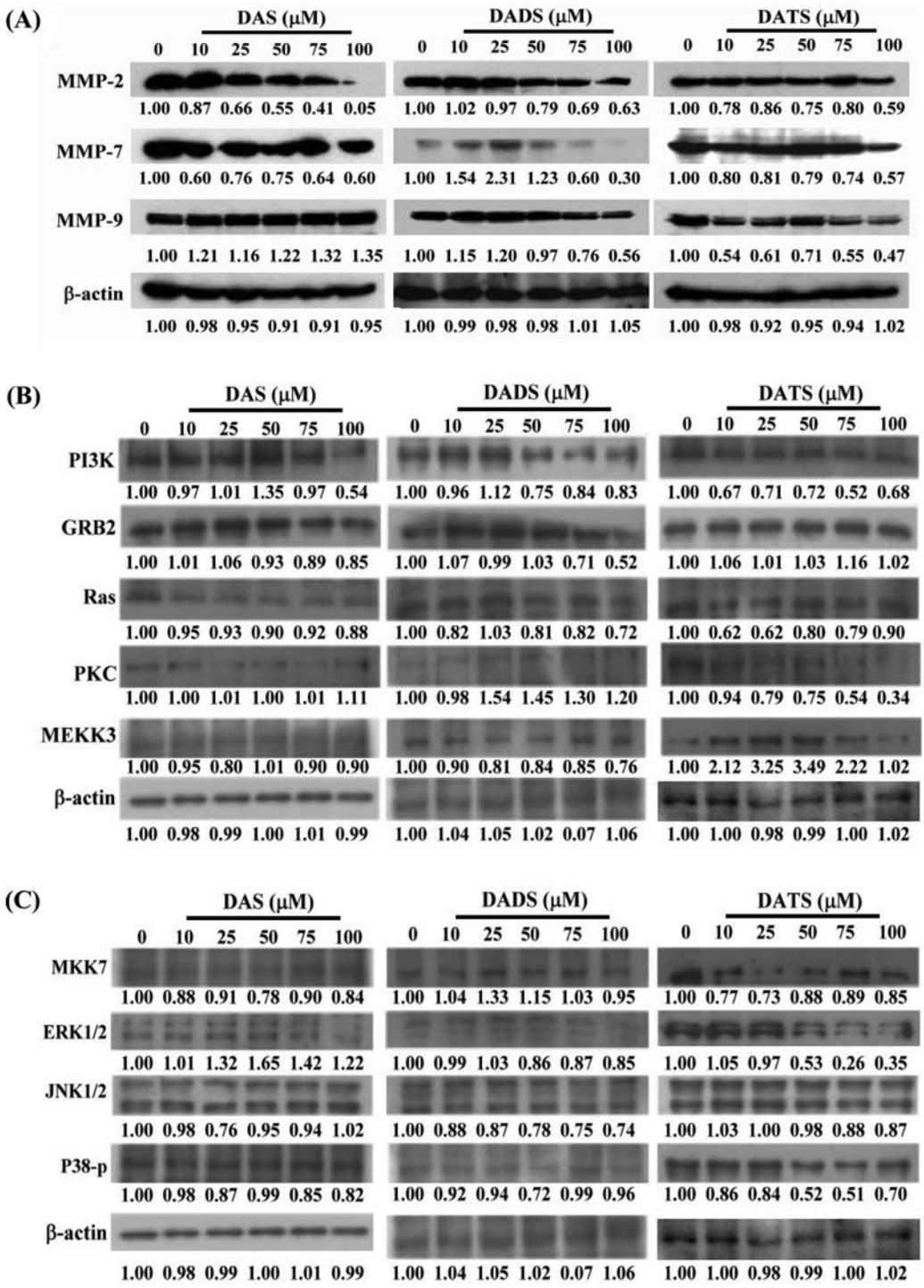

Fig. 4. Effects of DAS, DADS and DATS on the levels of associated proteins with invasion and migration in colo 205 cells. The total proteins were collected from colo 205 cells (1×10^6 cells/1 mL RPMI-1640 medium) treated with 0, 10, 25, 50, 75, and 100 μM of DAS, DADS and DATS for 24 h before and the proteins levels (A: MMP-2, MMP-7 and MMP-9; B: PI3K, GRB2, Ras, PKC, MEKK3; C: MKK7, ERK1/2, JNK, P38-p; D: iNOS, NF-κB p50, NF-κB p65, COX-1 and COX-2) were measured by SDS-PAGE and Western blotting as described in Materials and Methods.

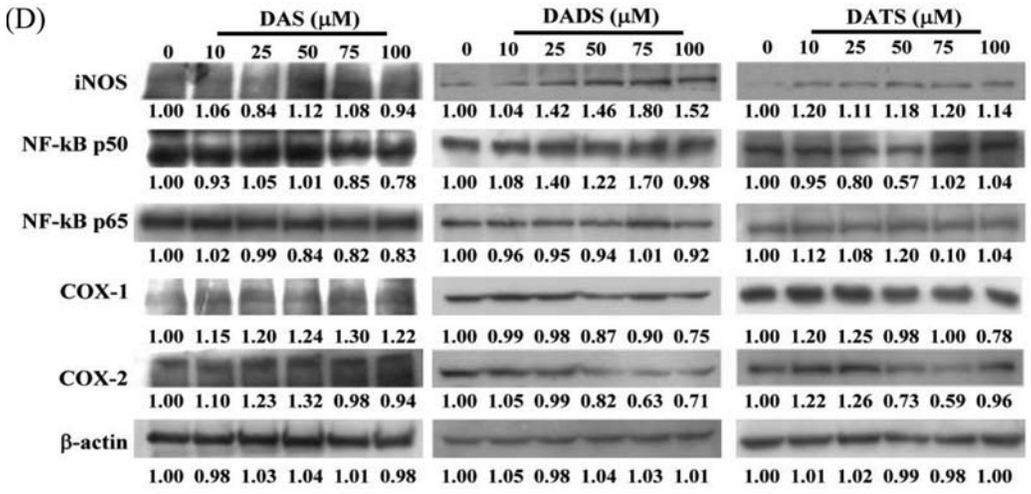

Fig. 4. (Continued)

91% inhibition, respectively, when compared with the non-DAS, DADS and DATS treated group [Figs. 2(C) and 3(C)]. The results also demonstrated that DAS, DADS, and DATS significantly inhibited the invasion and migration of highly metastatic colo 205 cells, and these effects are in the order of DAS < DADS < DATS.

The colo 205 cells treated with DAS, DADS, and DATS significantly inhibited migration and invasion. Protein production analysis showed that the inhibition of cell migration was because of the inhibition of MMP-2, -7, and -9. It could be argued that the observed inhibition of cell migration was because of the cytotoxicity of DAS, DADS, and DATS. However, that was ruled out by a cell proliferation assay and percentage of viable cells assay as shown in Figure 1. Note that at the doses and time frames employed in these experiments, more than 90% of the cells were viable. These results showed that DAS, DADS, and DATS might inhibit tumor cell migration *in vitro* [Figs. 2(A,B) and 3(A,B)]. Although the ability of DAS, DADS, and DATS to inhibit colo 205 cell migration and invasion has been shown in the present study, the mechanism by which DAS, DADS, and DATS inhibits cell migration and invasion was not clear. For further investigation, we used Western blotting method to examine the associated proteins levels of migration and invasion of colo 205 cells.

It was reported that the migration of tumor cells to distant organ sites plays a pivotal role in metastasis (Berens et al., 1994; Giese et al., 1999). Cell migration has been shown to be regulated by numerous molecules including PI3K, p38/MAPK, p44/42/MAPK, p-JNK, and FAK (Wang et al., 1999; Lakka et al., 2000; Goncharova et al., 2002; Neudauer and McCarthy, 2003). In this study, we found that DAS, DADS, and DATS inhibited cell migration by downregulating the production of the p38, PI3K, ERK, JNK, and NF-κB proteins (Fig. 4). It was reported that the inhibition of PI3K and MAPK activity by using specific inhibitors can impair cell migration of ovarian tumor cells (Meng et al., 2006). Except migration, the tumor cells also have to invade to establish metastasis successfully at a distant site. Tumor cell invasion involves the degradation of the extracellular matrix by proteolytic enzymes (Goldfarb and Liotta, 1986), which are named matrix metalloproteinases (MMPs) (Matrisian, 1992), which play an important role in tissue remodeling in normal cells and also in cancer cells. MMPs have been found in many invasive tumor cell lines such as the breast, lung, and colon cancer (Shapiro, 1998; Oberg et al., 2000; Pritchard et al., 2001; Nakopoulou et al., 2003). MMP-2 and -9 have been found in several cancer cell lines and surgical specimens (Ohbayashi, 2002).

In this study, we found that DAS, DADS, and DATS inhibited tumor cell invasion in colon cancer colo 205 cells. Furthermore, DAS, DADS, and DATS mediated inhibition of invasion occurring via the downregulation of MMP-2,

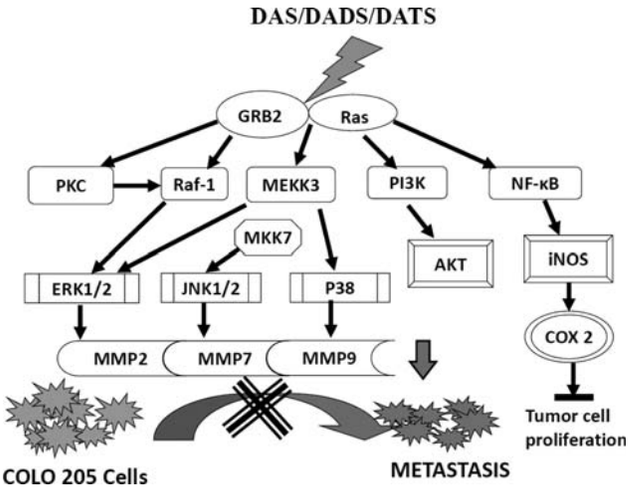

Fig. 5. The proposed mechanisms of DAS, DADS and DATS inhibited migration and invasion of colo 205 cells.

-7, and -9 through the inhibition of PKC, MKK3 which led to the inhibition of ERK1/2, JNK, and p-p38 as shown in Figure 4. DAS, DADS, and DATS inhibited the levels of NF-κB p50, which may also affect the MMP-2, -7, and -9 in colo 205 cells. Other investigators already showed that the expression of MMPs are primarily regulated at the transcriptional level through activation protein-1 (AP-1) or nuclear factor-kappaB (NF-κB), and at the protein level via their activators or inhibitors, and their cell surface localization (Aguirre Ghiso et al., 1999; Westermarck and Kahari, 1999). Based on the results, we observed from the Western blotting, the cause is not completely clear. However, one of the possibilities is that the concentration of DAS, DADS, and DATS required to inhibit the expression of the associated gene expression may vary among the cell types. Another possibility that the expression of MMP-2, -7, and -9 in colo 205 cells may be regulated by additional mechanisms heretofore unrecognized. However, further studies are warranted to explore these possibilities. Whatever the underlying mechanism, the ability of DAS, DADS, and DATS to inhibit tumor cell invasion has been clearly shown in this study.

REFERENCES

Aguirre Ghiso JA, Alonso DF, Farias EF, Gomez DE, de Kier Joffe EB. 1999. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. Eur J Biochem 263:295–304.

Augusti KT. 1996. Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.). Ind J Exp Biol 34:634–640.

Berens ME, Rief MD, Loo MA, Giese A. 1994. The role of extracellular matrix in human astrocytoma migration and proliferation studied in a microliter scale assay. Clin Exp Metastasis 12: 405–415.

Chen YY, Chiang SY, Lin JG, Ma YS, Liao CL, Weng SW, Lai TY, Chung JG. 2010. Emodin, aloe-emodin and rhein inhibit migration and invasion in human tongue cancer SCC-4 cells through the inhibition of gene expression of matrix metalloproteinase-9. Int J Oncol 36:1113–1120.

Chiang AC, Massague J. 2008. Molecular basis of metastasis. N Engl J Med 359:2814–2823.

Chiang JH, Yang JS, Ma CY, Yang MD, Huang HY, Hsia TC, Kuo HM, Wu PP, Lee TH, Chung JG. 2011. Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways. Chem Res Toxicol 24:20–29.

Duffy MJ. 1992. The role of proteolytic enzymes in cancer invasion and metastasis. Clin Exp Metastasis 10:145–155.

Fidler IJ. 2002. The organ microenvironment and cancer metastasis. Differentiation 70:498–505.

Giese A, Hagel C, Kim EL, Zapf S, Djawaheri J, Berens ME, Westphal M. 1999. Thromboxane synthase regulates the migratory phenotype of human glioma cells. Neuro Oncol 1: 3–13.

Goldfarb RH, Liotta LA. 1986. Proteolytic enzymes in cancer invasion and metastasis. Semin Thromb Hemost 12:294–307.

Goncharova EA, Ammit AJ, Irani C, Carroll RG, Eszterhas AJ, Panettieri RA, Krymskaya VP. 2002. PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 283:L354–L363.

Hosono T, Fukao T, Ogihara J, Ito Y, Shiba H, Seki T, Ariga T. 2005. Diallyl trisulfide suppresses the proliferation and induces apoptosis of human colon cancer cells through oxidative modification of beta-tubulin. J Biol Chem 280:41487–41493.

Lai KC, Huang AC, Hsu SC, Kuo CL, Yang JS, Wu SH, Chung JG. 2010. Benzyl isothiocyanate (BITC) inhibits migration and invasion of human colon cancer HT29 cells by inhibiting matrix metalloproteinase-2/-9 and urokinase plasminogen (uPA) through PKC and MAPK signaling pathway. J Agric Food Chem 58:2935–2942.

Lakka SS, Jasti SL, Kyritsis AP, Yung WK, Ali-Osman F, Nicolson GL, Rao JS. 2000. Regulation of MMP-9 (type IV collagenase) production and invasiveness in gliomas by the extracellular signal-regulated kinase and jun amino-terminal kinase signaling cascades. Clin Exp Metastasis 18:245–252.

Lei YP, Chen HW, Sheen LY, Lii CK. 2008. Diallyl disulfide and diallyl trisulfide suppress oxidized LDL-induced vascular cell adhesion molecule and E-selectin expression through protein kinase A- and B-dependent signaling pathways. J Nutr 138:996–1003.

Lin HJ, Su CC, Lu HF, Yang JS, Hsu SC, Ip SW, Wu JJ, Li YC, Ho CC, Wu CC, et al. 2010. Curcumin blocks migration and invasion of mouse-rat hybrid retina ganglion cells (N18) through the inhibition of MMP-2, -9, FAK, Rho A and Rock-1 gene expression. Oncol Rep 23:665–670. AQ3

Lin SS, Lai KC, Hsu SC, Yang JS, Kuo CL, Lin JP, Ma YS, Wu CC, Chung JG. 2009. Curcumin inhibits the migration and invasion of human A549 lung cancer cells through the inhibition of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor (VEGF). Cancer Lett 285:127–133.

Liotta LA, Steeg PS, Stetler-Stevenson WG. 1991. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327–336.

Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL, Lu HF, Lee TH, Wood WG, Chung JG. 2010. Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res 54:967–976.

Matrisian LM. 1992. The matrix-degrading metalloproteinases. Bioessays 14:455–463.

Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang BH. 2006. Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway. Cell Signal 18:2262–2271.

Mignatti P, Rifkin DB. 1993. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 73:161–195.

Nakopoulou L, Tsirmpa I, Alexandrou P, Louvrou A, Ampela C, Markaki S, Davaris PS. 2003. 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.

Neudauer CL, McCarthy JB. 2003. Insulin-like growth factor I-stimulated melanoma cell migration requires phosphoinositide 3-kinase but not extracellular-regulated kinase activation. Exp Cell Res 286:128–137.

Oberg A, Hoyhtya M, Tavelin B, Stenling R, Lindmark G. 2000. Limited value of preoperative serum analyses of matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2) in colorectal cancer. Anticancer Res 20:1085–1091.

Ohbayashi H. 2002. Matrix metalloproteinases in lung diseases. Curr Protein Pept Sci 3:409–421.

Pritchard SC, Nicolson MC, Lloret C, McKay JA, Ross VG, Kerr KM, Murray GI, McLeod HL. 2001. Expression of matrix metalloproteinases 1, 2, 9 and their tissue inhibitors in stage II non-small cell lung cancer: Implications for MMP inhibition therapy. Oncol Rep 8:421–424.

Shapiro SD. 1998. Matrix metalloproteinase degradation of extracellular matrix: Biological consequences. Curr Opin Cell Biol 10:602–608.

Sundaram SG, Milner JA. 1993. Impact of organosulfur compounds in garlic on canine mammary tumor cells in culture. Cancer Lett 74:85–90.

Sundaram SG, Milner JA. 1996. Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis 17: 669–673.

Toda D, Ota T, Tsukuda K, Watanabe K, Fujiyama T, Murakami M, Naito M, Shimizu N. 2006. Gefitinib decreases the synthesis of matrix metalloproteinase and the adhesion to extracellular matrix proteins of colon cancer cells. Anticancer Res 26:129–134.

Wang F, Nohara K, Olivera A, Thompson EW, Spiegel S. 1999. Involvement of focal adhesion kinase in inhibition of motility of human breast cancer cells by sphingosine 1-phosphate. Exp Cell Res 247:17–28.

Wargovich MJ. 1987. Diallyl sulfide, a flavor component of garlic (*Allium sativum*), inhibits dimethylhydrazine-induced colon cancer. Carcinogenesis 8:487–489.

Wattenberg LW, Sparnins VL, Barany G. 1989. Inhibition of *N*-nitrosodiethylamine carcinogenesis in mice by naturally occurring organosulfur compounds and monoterpenes. Cancer Res 49:2689–2692.

Westermarck J, Kahari VM. 1999. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 13:781–792.

Wu SH, Hang LW, Yang JS, Chen HY, Lin HY, Chiang JH, Lu CC, Yang JL, Lai TY, Ko YC, et al. 2010. Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. Anticancer Res 30:2125–2133.

Yu FS, Yang JS, Yu CS, Lu CC, Chiang JH, Lin CW, Chung JG. 2011. Safrole induces apoptosis in human oral cancer HSC-3 cells. J Dent Res 90:168–174.

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