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trans-3-Methoxy-5-hydroxystilbene (MHS) from the rhizome of *Alpinia nantonensis* inhibits metastasis in human lung cancer cells

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ABSTRACT

Background: Alpinia nantoensis (Zingiberaceae) is an aromatic plant endemic to Taiwan, which is used as food flavoring and traditional herbal medicine. The biological activities of compounds isolated from this plant are rarely investigated.

Purpose: The present study was aimed to investigate the anti-metastatic potential of *trans*-3-methoxy-5-hydroxystilbene (MHS) a major stilbene isolated from the rhizomes of *A. nantonensis.*

Methods: We investigated the anti-metastatic potential of MHS on human non-small cell lung carcinoma (A549) cell line using wound healing, trans-well, western blot, zymography and immunofluorescence assays.

Results: Initial cytotoxicity assay showed that treatment with MHS did not exhibit cytotoxicity to A549 cells up to the concentration of 40 μM. Further *in vitro* wound healing and transwell chamber assays revealed that MHS significantly inhibited tumor cell migration in a dose-dependent manner, which is associated with inhibition of matrix mettalloprotinase-2 (MMP-2) and matrix mettalloprotinase-9 (MMP-9) at both enzyme and protein levels. The inhibition of MMPs activity by MHS was reasoned by suppression of their corresponding transcription factor, β-catenin as indicated by reduced levels of β-catenin in the nucleus. MHS also regulates epithelial-to-mesenchymal transition (EMT) by increasing E-cadherin and occludin as well as decreasing N-cadherin levels in A549 cells. Furthermore, pre-treatment with MHS significantly inhibited A549 cells migration and EMT in TGF-β induced A549 cells.

Conclusion: To the best of our knowledge, this is the first report demonstrating that MHS, a plant-derived stilbene has a promising ability to inhibit lung cancer cell metastasis *in vitro*.

Keywords: Alpinia nantoensis, *trans*-3-methoxy-5-hydroxystilbene, Metastasis, Lung cancer, Epithelial-to-mesenchymal transition

Abbreviations

MMP-2: mettalloprotinase-2 (MMP-2), MMP-9: mettalloprotinase-9 (MMP-9), EMT: epithelial-to-mesenchymal transition (EMT), MHS: *cis*-3-methoxy-5-hydroxystilbene (MHS), MTT: 3-(4,5-imethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide

Introduction

Rhizomatous plants of genus *Alpinia* were widely distributed in tropical and sub-tropical regions of Asia and South America. Many *Alpinia* plants were mostly used as food, food adjunct, spices, aromatic oils, folk medicine, and as cultivated ornamentals (Padalia et al., 2010). Traditionally, *Alpinia* plants were used to treat various illness, including fever, influenza, bronchitis, rheumatism, arthritis, colic, dysentery, stomach ache, diabetes, dyspepsia, urinary infection, inflammation, hypertension, and diuresis (Victório, 2011). In Taiwan, *A. pricei* Hyata is used to make traditional zongzi (a glutinous rice dumpling) (Lin et al., 2009); and in Japan, one of the staples of the local diet is made from rice and *A. zerumbet* leaves (Victório, 2011). Number of scientific reports also indicate that *Alpinia* plants possessed various biological activities including anti-inflammatory, antioxidant, antimicrobial, antidermatophytic, antinociceptive, hepatoprotective, immunostimulatory, and anticancer (Ghosh and Rangan, 2013; Hsu et al., 2010; Lin et al., 2009; Victório, 2011)

Alpinia nantonensis F. Y. Lu & Y. W. Kuo is a newly identified species of genus *Alpinia*, which is native to the mountain area of central Taiwan. *A. nantonensis* can be distinguished from *A. pricei* and *A. shimada var.* Shimada by the presence of bracteole and the rhombic labellum (Yu-Wen Kuo, 2008). *A. nanotensis* has various medicinal and commercial uses, including the leaves of *A. nantonensis* is used to make traditional Zhonzi (a glutinous rice dumpling); rhizomes were used to treat analgesia, rheumatoid arthritis, bruises, toothache and stomach pain; flowers are used to treat poor digestion, gastric problems and food or drug intoxication; fruits are used for the treatment of dampness, vomiting diarrhea, nausea and loss of appetite. However, to the best of our knowledge the phytochemistry and biological activities of *A. nantonensis* have not yet been investigated until now.

Lung cancer, especially non-small cell lung carcinoma (NSCLC) remains the leading cause of cancer-related death worldwide. Despite there being multiple kinds of therapies were currently practiced, such as surgery, chemotherapy, radiotherapy, immunotherapy, percutaneous ablation and palliative care, alone or with a combination. However, most lung cancers are diagnosed at an advanced stage when treatment is relatively ineffective; thus, the mortality for lung cancer patients are still high (Molina et al., 2008). Tumor cell metastasis is the primary cause of treatment failure and increase of mortality in cancer patients. To achieve metastasis, tumor cells require an ability to migrate from initial to the secondary site. This progress is characterized by a series of complicated process, including phenotypic changes (epithelial to mesenchymal transition, EMT) or mesenchymal to epithelial transition, MET), extracellular matrix (ECM) degradation, invasion, migration, and adhesion (Hunter et

al., 2008). Therefore, prevention of tumor cell metastasis is a promising strategy for lung cancer treatment.

Chemopreventive properties have long been attributed to phytocompounds present in the human diet and the use of agents to prevent, suppress, delaying the onset or reverse the carcinogenesis (Surh, 2003). Meanwhile, epidemiological studies have reported that regular consumption of fruits, vegetables, red wine and green tea is associated with lower incidence of many chronic diseases, such as cardiovascular diseases and cancer (Block et al., 1992). Recent studies have suggested that bioactive components found in food or herbal medicine can activate molecular signaling cascades by targeting small molecules in cancer cells. For example, phenolic compounds, such as lignans, flavonoids, benzenoid, and stilbenes have been identified to be chemopreventive, and their practical health benefits are an active field of research (Lee et al., 2011; Surh, 2003). Stilbenes are a group of natural phenolic compounds found in very few edible plants such as grapes and blue berries (Bavaresco et al., 1999). Stilbenes are not common as other polyphenols, and there are only two stilbenes have been well studied, they are resveratrol (3,5,4'-trihydroxystilbene) and pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene) (McCormack and McFadden, 2013). Many studies have demonstrated that stilbenes may possess potentially beneficial effects, including reducing the risk of cardiovascular diseases and cancers with their potent anti-oxidative, anti-inflammatory, and chemopreventive properties (De Filippis et al., 2017; Reinisalo et al., 2015; Rimando and Suh, 2008).

In the present study, a stilbene, *cis*-3-methoxy-5-hydroxystilbene (MHS) was isolated from the rhizome of *A. nantonensis* (Fig. 1). The objective of this study was to investigate the anti-cancer activity of MHS. Notably, the anti-metastasis property of MHS was verified in human non-small cell lung carcinoma (A549) cell line *in vitro*. The molecular mechanisms involved in the protective effects were also elucidated.

Materials and methods

Chemicals and reagents

Ham's F12 medium, fetal bovine serum (FBS), sodium pyruvate and penicillin-streptomycin-neomycin were obtained from GIBCO BRL., (Carlsbad, CA). Antibodies against MMP-2 and MMP-9 were obtained from Santa Cruz Biotechnology Inc., (Dallas, TX). Antibodies against β-catenin, E-cadherin, N-cadherin, occludin and GAPDH were purchased from Cell Signaling Technology, (Danvers, MA). HRP-conjugated goat anti-rabbit IgG was purchased from Millipore

(Temecula, CA) and HRP-conjugated goat anti-mouse IgG was obtained from Cell Signaling Technology. Plumbagin and 4′,6-diamidino-2-phenylindole (DAPI) were bought from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma.

Extraction and purification

A. nantonensis was collected in October 2014 from Nantou County, Taiwan, and was identified by Professor Yen-Hsueh Tseng (NCHU). The voucher specimen (TCF Tseng4568) was deposited in the herbarium of the same university. Air-dried rhizome of *A. nantonensis* (2.74 kg) was extracted with EtOH (50 L) at ambient temperature and concentrated under vacuum to yield the EtOH extract (50 g). The EtOH extract was partitioned between EtOAc-H_2O to give EtOAc-soluble (35 g) and H_2O-soluble fractions. The EtOAc-soluble fraction was further chromatographed over silica gel eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc. The eluent was collected in constant volumes, and combined into 10 fractions based on TLC properties. Fraction 7 was separated by HPLC with a mixture of acetonitrile: H_2O = 30:70 at a flow rate of 0.1 ml/ min to obtain *trans*-3-methoxy-5-Hydroxystilbene (MHS) (retention time 5 min). The purity of compound isolated from *A. nantonensis* was above 99% as confirmed by HPLC and 1H-NMR analysis. MHS was isolated earlier from *Alnus sieboldiana* (Yoshinori, 1971) and *Alpinia katsumadai* (Ngo and Brown, 1998), but this is the first record of this compound from *A. nantonensis*.

Cell culture and sample treatment

Human non-small cell lung carcinoma (A549) cell line was obtained from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in Ham's F-12 medium, 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin in a 37 °C humidified incubator supplemented with 5% CO_2. Cells were maintained in serum-free Ham's F-12 medium for 24 h, followed by treatment with or without samples for 24 h.

Cell viability assay

The effect of compounds isolated from the rhizome of *A. nantonensis* on A549 cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide) colorimetric assay.

A549 cells at a density of 1 × 10^4 cells/well were seeded in a 96-well cell culture plate. Then, cells were incubated with increasing concentrations of test samples (compounds **1-3**) for 24 and 48 h. After treatment, the cells were incubated with 100 µL of 0.5 mg/ml MTT in Ham's F-12 medium for 3 h. The Culture supernatant was then removed and resuspended in 100 µL of dimethyl sulfoxide (DMSO) to dissolve the MTT formazan, and the absorbance was measured at 570 nm using ELISA microplate reader (Bio-Tek Instruments, Winooski, VT). The effect of compounds **1-3** on cell viability was assessed as the percent of viable cells compared with the vehicle (DMSO)-treated cells, which were arbitrarily assigned viability of 100%.

In vitro wound healing assay

A549 cells at a density of 1 × 10^4 cells/well were seeded into a 24-well culture plate with silicon cell-free gap insert (ibidi GmbH, Martinsried, Germany). After monolayer formation, the insert was removed, washed with PBS, and then the cells were incubated with MHS (5, 10, 20 and 40 µM) for 3-36 h. The migrated cells were photographed (100 × magnification) at 0, 12 and 24 to monitor the migration of cells into the wounded area, and the closure of the wounded area was calculated.

Trans-well migration assay

The migration ability of the A549 cells was quantified using a Trans-well assay. The A549 cells were seeded in a 6-well culture plate at a density of 2 × 10^5 cells/well and then treated with various concentrations of MHS (5, 10, 20 and 40 µM) for 24 h. On the other hand, A549 cells were pre-treated with MHS (40 µM) for 6 h and then incubated with TGF-β (5 ng/ml) for 24 h. After treatment with MSH in the presence or absence of TGF-β, cells were collected by trypsin. A total of 2 × 10^5 cells in Ham's F-12 medium without serum was added to each upper chamber, and Ham's F-12 supplemented with 20% FBS was added to the lower chamber as a chemoattractant. After 12 h of treatment, the cells remained on the upper surface of the membrane were removed with cotton swabs, and the cells that had migrated to the lower-side of the membrane were fixed using methanol and stained with Giemsa solution for 15 min at room temperature. The migrated cells on the bottom of the membrane were washed with PBS and photographed under a confocal microscope (magnification, ×100).

Gelatin zymography

Gelatin zymography determined intercellular matrix metalloproteinases (MMPs) activity. Briefly, A549 cells were cultured in a 10-cm dish at a density of 1 × 10^6 cells/dish. Then, the cells were incubated with or without MHS (5–40 μM) for 24 h. The culture supernatant was collected, and the protein content in culture media was determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. An Equal amount of protein samples in culture supernatants were separated by10% SDS-PAGE, which contains 1 mg/ml gelatin. After electrophoresis, the gels were washed and incubated with re-nature buffer (2.5% Triton-X) for 1 h, and then incubated with developing buffer for overnight at 37 °C. After overnight incubation, the bands were incubated with 0.5% Coomassie Brilliant Blue for 1 h. After appeared as white bands against a blue background with intensity in proportion to the MMPs activity. The zymography bands were visualized by VL Chemi-Smart (Viogene Biotek, Sunnyvale, CA).

Protein isolation and western blot analysis

A549 cells at a density of 2 × 10^6 cells/dish were cultured in 10-cm dishes and treated with or without MHS (5-40 μM) for 2-48 h. On other hands, A549 cells were pre-treated with MHS (40 μM) for 6 h and then incubated with TGF-β (5 ng/ml) for 24 h. Cells were lysed in RIPA lysis buffer (Thermo Fisher Scientific). Protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories). Equal amounts of protein samples were separated by 7-12% SDS-PAGE, and the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane overnight. Then the membranes were blocked with 5% non-fat dried milk for 30 min, followed by incubation with specific primary antibodies for 2 h or overnight, and either horseradish peroxidase conjugated goat anti-rabbit or anti-mouse antibodies for 1 h. The immune blots were visualized using VL Chemi-Smart 3000 (Viogene Biotek) with the enhanced chemiluminescence (ECL) immunoblotting reagent (Millipore, Billerica, MA).

Immunofluorescence

A549 cells at a density of 1 × 10^4 cells/well were cultured in a 24-well cell culture plate and incubated with MHS (5–40 μM) for 90 min. After treatment, the culture media was removed, and the cells were fixed in methanol for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, washed and blocked with 10% FBS in PBS, and then incubated for 2 h with anti-β-catenin antibody in 1.5% FBS. The cells were then incubated with the fluorescein isothiocyanate (FITC) -conjugated secondary antibody

(Thermo Fisher Scientific) for another 1 hour in 6% BSA. Next, the nucleus was stained with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, washed with phosphate buffer saline (PBS), and visualized using fluorescence microscopy (Motic Electric Group, Xiamen, P.R. China) at 40 × magnification.

Statistical analysis

Data are expressed as means ± SD. All data were analyzed using the statistical software GraphPad Prism version 6.0 for windows (GraphPad Software, La Jolla, CA). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test with a *P* values of < 0.05*, < 0.01**, and < 0.001*** were considered statistically significant for MHS treatment group *vs* control group; <0.05$^{\Delta}$ control *vs* TGF-β and <0.05$^{\Phi}$ TGF-β *vs* MHS pre-treatment groups.

Results

Effects of MHS on cell viability and anti-metastatic effects on human lung cancer cells

In the present study, a major compound, MHS (Fig. 1), in EtOH extract was isolated from the rhizome of *A. nantonensis*. Therefore, we continued our bioactivity screening with MHS. Initially, the cytotoxic effect of MHS was determined by MTT assay. A549 cells were incubated with increasing concentrations of MHS (5-40 µM) for 24 and 48 h and the cell viability were analyzed. As shown in Fig. 2, MHS revealed that up to a concentration of 40 µM, there was no significant reduction in cell viability, when compared with vehicle control (0.1% DMSO). Whereas, the positive drug control, pulmbagin (5 µM) showed significant cytotoxicity to the A549 cells. Therefore, in subsequent experiments, MHS was used within the dosage range of 5-40 µM.

MHS inhibits migration in A549 cells

To further examine whether MHS could affect A549 cell migration, A549 cells were incubated with increasing concentrations of MHS for 12 and 24 h, and wound healing assay determined the cell migration. The dotted yellow lines indicating the margin of wounded area and the wound closure was photographed at 0, 12 and 24 h after MHS treatment. As shown in Fig. 3A, untreated control cells exhibited a time-dependent increase of wound closure. However, MHS significantly decreased the

wound-healing ability of A549 cells in a dose-dependent manner. Indeed, treatment with 40 μM of MHS almost halted wound closure process in A549 cells. The percentage of wound healing after 12 and 24 h were compared with 0 h, and the values were plotted in histogram form. The rate of wound closure in control cells after 12 and 24 h were 82.6 ± 1.6% and 92.5 ± 1.2%, respectively. However, treatment with MHS dose-dependently reduced wound closure ability to 62.6 ± 10.8%, 51.8 ± 5.7%, 46.2 ± 2.0% and 32.8 ± 1.3% by 5, 10, 20 and 40 μM, respectively for 12 h and 82.3 ± 1.2%, 65.5 ± 4.2%, 48.1 ± 6.1% and 38.9 ± 3.9% by 5, 10, 20 and 40 μM for 24 h, respectively (Fig. 3A). To further confirm this effect, trans-well migration assay was performed. As shown in Fig. 3B, compared with control group, treated A549 cells with MHS significantly as well as dose-dependently reduced the migratory potential of A549 cells. Briefly, the number of migrated cells in control group was 255 ± 16, whereas treatment with MHS dose-dependently decreased to 238 ± 9, 129 ± 16, 83 ± 2 and 77 ± 5 by 5, 10, 20 and 40 μM of MHS, respectively (Fig. 3B).

MHS down-regulates MMPS activity in A549 cells

Next, we hypothesize that inhibition of migration and invasion by MHS might associate with the suppression of MMPs activity in A549 cells. Initially, the enzyme activity of MMP2 and MMP9 were examined by gelatin zymography. As shown in Fig. 4A, the enzyme activity of MMP2 was significantly decreased by MHS. Notably, at a dose of 40 μM remarkable reduced the MMP2 activity. Indeed, the histogram analysis shows that treatment with MHS dose-dependently decreased the MMP2 activity to 0.72-fold, 0.50-fold, 0.39-fold and 0.044-fold by 5, 10, 20 and 40 μM, respectively. A similar result was also observed in the MMP9 activity that treatment with MHS dose-dependently decreased MMP9 activity in A549 cells. Almost a complete loss of MMP9 activity was observed after treatment with 20 and 40 μM of MHS in a same course of time (Fig. 4B). Also, histogram analysis exhibit that treatment with MHS decrease the MMP9 activity to 0.93-fold, 0.84-fold, 0.004-fold and 0.002-fold by 5, 10, 20 and 40 μM, respectively (Fig. 4B). To further confirm this effect, protein expression levels of MMP2 and MMP9 were examined by western blot analysis. Consistency with the result of zymography, treatment with MHS dose-dependently decreased MMP2 expression at both pro-and active forms. MHS reduced the MMP2 protein levels to 0.72-fold, 0.33-fold, 0.33-fold and 0.28-fold by 5, 10, 20 and 40 μM, respectively (Fig. 4C). Besides, MHS also significantly decreased MMP9 protein expression to 0.88-fold, 0.36-fold, and 0.39-fold by 10, 20 and 40 μM, respectively (Fig. 4D). In contrast, at a low concentration of MHS (5 μM) significantly increased MMP-9 expression (1.42-fold) in A549 cells.

MHS suppress β-catenin activities in A549 cells

Previous studies have reported that β-catenin a transcription factor regulates MMP2 and MMP9 expression in a variety of cell lines (Wu et al., 2007). Therefore, we further determined whether MHS modulates β-catenin activity in A549 cells. The result of western blot analysis showed that a dose-dependent decrease in β-catenin level was observed after treatment with MHS. Indeed, MHS decreased β-catenin protein levels to 0.98-fold, 0.86-fold, 0.68-fold, and 0.36-fold by 5, 10, 20 and 40 μM, respectively (Fig. 4E). It has been implicated that nuclear translocation and association with transcription factors such as TCF/LEF facilitate transcription of their target genes, including MMPs. Thus, we sought to determine the effect of MHS on β-catenin nuclear translocation by immunofluorescence analysis. In control cells showing nuclear accumulation of β-catenin as supported by high level of immunostaining in the nucleus, whereas treatment with MHS reduced the nuclear translocation of β-catenin as evidenced by reduced levels of β-catenin in the nucleus as well as increased level of β-catenin expression in the cytoplasm (Fig. 4F). Our data suggested that treatment with MHS down-regulates β-catenin expression as well as nuclear translocation in A549 cells.

MHS modulates epithelial-to-mesenchymal (EMT) processes in A549 cells

To further clarify whether inhibition of migration and invasion by MHS involved in dysregulation of EMT regulatory proteins, we examined the expression levels of both epithelial and mesenchymal marker proteins such as E-cadherin and N-cadherin, respectively in A549 cells. As shown in Fig. 5A, E-cadherin expression was barely observed in control cells, whereas treatment with MHS significantly increased to 1.06-fold, 1.34-fold, 2.15-fold and 3.3-fold by 5, 10, 20 and 40 μM, respectively. On the other hand, treatment with MHS dose-dependently decreased N-cadherin expression levels to 0.90-fold, 0.88-fold, 0.55-fold and 0.40-fold by 5, 10, 20 and 40 μM, respectively (Fig. 5B). These data support that MHS could inhibit morphological changes in A549 cells.

MHS inhibits TGF-β-induced migration in A549 cells

Tumor growth factor-β (TGF-β) is known to stimulate malignant tumor cell migration and invasion in a variety of cancer cells through the activation of MMPs. Therefore, we further examined whether MHS also affects TGF-β-induced A549 cell migration. As shown in Fig. 6A, TGF-β-induced cell migration was determined by trans-well migration assay. Compared with untreated control cells (331 ± 5), a remarkable increase of cell migration was observed in TGF-β-treated cells (402 ± 7), whereas pre-

treatment with MHS significantly abrogated TGF-β-induced cell migration as evidenced by decrease the number of migrated cells (271 ± 6). Further, we examined the effect of MHS on β-catenin expression under TGF-β-stimulated condition. Compared with untreated control cells, cell exposed to TGF-β significantly increased β-catenin expression (1.2-fold) in A549 cells. However, co-treatment with MHS significantly reduced TGF-β-induced β-catenin expression to 0.5-fold (Fig. 6B).

MHS inhibits TGF-β-induced EMT in A549 cells

Next, we examined whether MHS could modulate TGF-β-induced EMT. The protein expression levels of E-cadherin, occludin and N-cadherin were determined. Western blot analysis showed that cells exposed to TGF-β significantly down-regulated the protein levels of E-cadherin (0.72-fold), whereas pre-treatment with MHS significantly prevented the TGF-β-induced reduction in E-cadherin and raised to (1.47-fold) (Fig. 6C). Also, a similar result was also observed in occuldin levels that treatment with TGF-β remarkably reduced occluding level to 0.57-fold, whereas pre-treatment with MHS significantly prevented this reduction and increased to 1.44-fold (Fig. 6D). Furthermore, a dramatic increase of N-cadherin level was observed after exposure to TGF-β (2.49-fold). However, pre-treatment with MHS significantly inhibited the TGF-β-mediated up-regulation of N-cadherin in A549 cells (Fig. 6E). These data were providing another positive feedback that MHS could inhibit lung cancer metastasis even though in stimulated condition.

Discussion

Alpinia nantonenis is a newly identified species of genus Alpinia, which is endemic to Taiwan. Traditionally, the leaves and rhizomes of *A. nantonenis* were used as food and herbal medicine. In this study, we isolated a known stilbene, *trans*-3-methoxy-5-hydroxystilbene (MHS), from the rhizome of *A. nantonensis*. MHS was identified as an active compound, and it was previously isolated from other species such as *A. katsumadai* (Ngo and Brown, 1998), *Pephiopedilum exul* (Napphatsawan, 2016) and *Didymochlaena truncatula* (Cao et al., 2006). However, the biological effects of MHS were poorly elucidated. In this study, we have examined the anti-metastatic potential of MHS in human lung cancer cell line. An initial cytotoxic analysis with MHS against A549 cells shows that there was no noticeable reduction in cell viability up to a concentration of 40 μM, which supports that MHS is not cytotoxic to A549 cells.

Cancer cells migrate away from the primary tumor site and to invade the blood or lymphatic circulation is a considerable hallmark of cancer cell metastasis. Particularly, migration is a complex and organized event essential to normal physiological and morphogenetic process such as wound healing and embryogenesis. However, the abnormal activation of this critical process leads to multiple pathogenic events, including cancer cell metastasis (Friedl and Gilmour, 2009). To examine the migratory potential of cancer cells *in vitro*, a simple wound healing assay was widely practiced. In this study, the wound healing activity of A549 cells was observed 0, 24 and 48 h after treatment with MHS. Results from the wound healing assay, we found that treatment with MHS significantly inhibits A549 cells migration. In other hands, determination of the inhibition of A549 cell migration by trans-well chamber assay is widely accepted *in vitro* assay for screening agent that can inhibit migration (Justus et al., 2014). Trans well migration assay also strongly supports that MHS exerted A549 cell migration *in vitro*.

To further explore the underlying molecular mechanisms involved in this protective effect, the expression levels of metastatic regulatory molecules were examined. It is well described that matrix metalloproteinases (MMPs) belong to a family of highly homologous, zing- or calcium-dependent endopeptidases are the principal activators of gelatinase A followed by the degradation of gelatin and collagen in the extracellular matrix, which is the crucial events trigger tumor cell migration (Deryugina and Quigley, 2006). Therefore, inhibition of MMPs has been considered an important step in preventing cancer metastasis. To investigate the whether MHS modulates MMPs expression thereby, it could inhibit A549 cell metastasis, a gelatin zymography assay was performed. It is noteworthy that MHS significantly inhibited the enzyme activity of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in A549 cells. Also, western blot analysis also supports that MHS down-regulated the protein levels of MMP-2 and MMP-9 in A549 cells.

Recent studies have reported that β-catenin, a dual functional transcription factor coordinates cell-cell adhesion and gene transcription, which regulates proliferation, survival and metastasis in various normal and cancer cell types, including lung cancer cells (Jin et al., 2017; MacDonald et al., 2009; Pongracz and Stockley, 2006). Pongracz and Stockley (2006) have demonstrated that matrix metalloproteinases, including MMP2, MMP3, MMP7, and MMP9 are the down-stream target genes of the canonical β-catenin pathway. Therefore, next we determined whether MHS inhibits transcriptional activation of β-catenin in A549 cells. The result from western blot analysis showed that treatment with MHS significantly inhibited β-catenin expression in A549 cells. It has been implicated that during metastasis, β-catenin translocate into the nucleus, where it can associate with transcription factors such as TCF/LEF and promotes transcription of target genes (Cadigan and Waterman, 2012; MacDonald et

al., 2009). Meanwhile, increase of β-catenin in the cytoplasm act as a key component of E-cadherin cell adhesion complex and the microtubule network (MacDonald et al., 2009). Thereby, β-catenin could inhibit ECM degradation and cancer cell migration. Thus, we determined the effect of MHS on the cellular location of β-catenin using immunofluorescence analysis. Treatment with MHS significantly reduced nuclear translocation of β-catenin as indicated by decreased staining of nuclear β-catenin. Concurrently, the cytosolic β-catenin, especially membrane bound β-catenin level was significantly increased in the MHS treated cells. This increase of membrane bound β-catenin giving a positive feedback that MHS could promote cell-cell adhesion and inhibits ECM degradation.

Epithelial-to-mesenchymal transition (EMT) has been identified as one of the crucial events involved in metastasis, as it is the first step in the migration of tumor cells (Heerboth et al., 2015). During the EMT process, most of the tumor suppressor proteins or epithelial markers such as E-cadherin, occluding, claudin, desmoplakin, cytokeratin-8, -9, -18 and mucin-1 were down-regulated, and the oncoproteins or mesenchymal marker proteins, including N-cadherin, vimentin, vitronectin, fibronectin, FSP1are up-regulated (Lamouille et al., 2014). Particularly, E-cadherin promotes homotypic tumor cell adhesion function to maintain intercellular contacts that confine cells to the primary tumor site and prevent metastatic potential (Zetter, 1993). To further explain the inhibitory mechanism of MHS in A549 cell metastasis, the effect of MHS on epithelial marker protein E-cadherin and mesenchymal marker protein N-cadherin were examined. Interestingly, treatment with MHS significantly up-regulated E-cadherin and down-regulated N-cadherin expression in A549 cells. These data strongly suggest that MHS can modulate EMT process in A549 cells.

There are number of factors regulate EMT and tumor cell metastasis, such as hypoxia, tumor-stromal cell interactions, and growth factors, such as transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF) (Lamouille et al., 2014). Among them, TGF-β, a multifunctional cytokine plays the critical role in the metastatic spread of various cancer types (Heldin et al., 2012) including lung cancer (Toonkel et al., 2010). To further examine the efficacy of MHS on TGF-β-induced tumor cell metastasis, A549 cells were pre-treatment with MHS and then stimulated with TGF-β, cell migration and regulatory protein levels were determined. Here, we found that A549 cells exposed to TGF-β alone significantly increased tumor cell migration, which is consistent with a previous study (Zhao et al., 2015). Whereas, pre-treatment with MHS significantly inhibited TGF-β-induced migration in A549 cells. In line with others observation (Sato, 2006) cells exposed to TGF-β significantly increased β-catenin levels in A549 cells. However, pre-treatment with MHS significantly attenuated the TGF-β-mediated increase of β-catenin. Next, we found that a significant loss of E-cadherin expression when exposed to TGF-β, which is well

correlated with a previous report (Kim et al., 2007), whereas pre-treatment with MHS significantly blocked TGF-β-mediated loss of E-cadherin in A549 cells. These data further support the efficacy of MHS against TGF-β-induced metastasis in lung cancer cells.

In conclusion, the present results indicate that MHS inhibits migration in human lung cancer A549 cells through down-regulating the expression levels of MMPs and their transcription factor β-catenin in normal and TGF-β-stimulated conditions. Also, MHS regulates EMT process by modulating EMT regulatory proteins, including E-cadherin and N-cadherin under normal and TGF-β-induced conditions. Taken together, these results strongly suggest that MHS could be a potential candidate for the development of anti-metastatic agents against human lung cancer. However, these findings warranted further analysis with MHS in clinically relevant models to explore its potential role in the treatment of metastatic lung cancer.

Conflict of interest

The authors declare that there is no conflict of interest

Acknowledgment

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Figure legends

Fig. 1. Chemical structure of *trans*-3-methoxy-5-hydroxystilbene (MHS).

Fig. 2. Cytotoxic effect of MHS. A549 cells were seeded at density of 1×10^4 cell/well in a 96-well plate. After overnight incubation, cells were treated with various concentrations of MHS (5, 10, 20 and 40 μM) for 24 h. Cell viability was measured by MTT colorimetric assay as described in materials and methods. Plumbagin (5 μM) was used as a positive drug control. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{**}P < 0.01$ compared to control *vs.* sample treatment group.

Fig. 3. MHS inhibits A549 cells migration *in vitro*. (A) A549 cell migration was determined by wound-healing assay. A549 cells were seeded into a 24-well culture plate with silicon cell-free gap insert. After monolayer formation, the insert was removed, washed with PBS, and then the cells were treated with MHS (5, 10, 20 and 40 μM) for 0, 12 and 24 h. The migrated cells were photographed (100 × magnification) and the closure of the wounded area was calculated. (B) A549 cells were seeded in the upper chamber of the trans-well filters after treatment with various concentrations of MHS (5, 10, 20 and 40 μM) for 24 h. After cells had migrated to the bottom chamber, cells were fixed in methanol and stained with coomassive brilliant blue. Migrated cells were photographed, and the number of migrated cells/field was counted. Values represent the mean ± SD of three independent experiments. Statistical significance was set at *P* values of $< 0.05^*$, $< 0.01^{**}$, and $< 0.001^{***}$ compared to control *vs* sample treatment group.

Fig. 4. MHS down-regulates MMPs expression via suppression of β-catenin activity. A549 cells were treated with MHS (5, 10, 20 and 40 μM) for 24 h. (A, B) Gelatin zymography was subjected to measure the MMP-2 and MMP-9 enzyme activity. The histogram shows the relative enzyme activity of MMPs. (C-E) Western blot analysis was performed to determine the protein expression levels of MMP-2, MMP-9, and β-catenin. Relative protein levels of MMP-2, MMP-9, and β-catenin were normalized with GAPDH. (F) The Immunofluroscence analysis was utilized to examine the localization of β-catenin in A549 cells. DAPI was used to stain the nucleus. Values represent the mean ± SD of three independent experiments. Statistical significance was set at *P* values of $< 0.05^*$, $< 0.01^{**}$, and $< 0.001^{***}$ compared to control *vs* sample treatment group.

Fig. 5. MHS modulates EMT regulatory proteins in A549 cells. Cells were treated with MHS (5, 10, 20 and 40 μM) for 24 h. The protein expression levels of E-cadherin (A) and N-cadherin (B) were determined by Western blot analysis. Relative protein levels of E-cadherin and N-cadherin were normalized with GAPDH. Values represent the mean ± SD of three independent experiments. Statistical significance was set at *P* values of < 0.05*, < 0.01**, and < 0.001*** compared to control *vs* sample treatment group.

Fig. 6. MHS inhibits TGF-β-induced migration and EMT in A549 cells. Cells were pre-treated with MHS (40 μM) for 6 h and then exposure to TGF-β (5ng/ml) for 24 h. (A) Cell migration was measured by trans-well chamber migration assay. (B-E) Protein expression levels of β-catenin, E-cadherin, occluding and N-cadherin were determined by western blot analysis. Relative protein levels of β-catenin, E-cadherin, occluding and N-cadherin were normalized with GAPDH. Values represent the mean ± SD of three independent experiments. Statistical significance was set at *P* values of < 0.05* control *vs* MHS treatment group; < 0.01^{Δ} control *vs* TGF-β treatment group and < 0.001^{Φ} TGF-β treatment group *vs* MHS pre-treatment group.

Fig. 1.

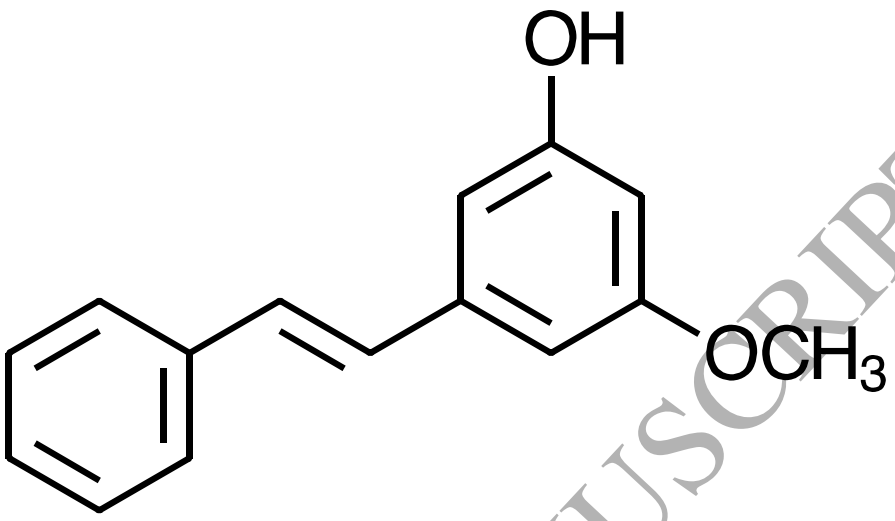


Fig. 2.

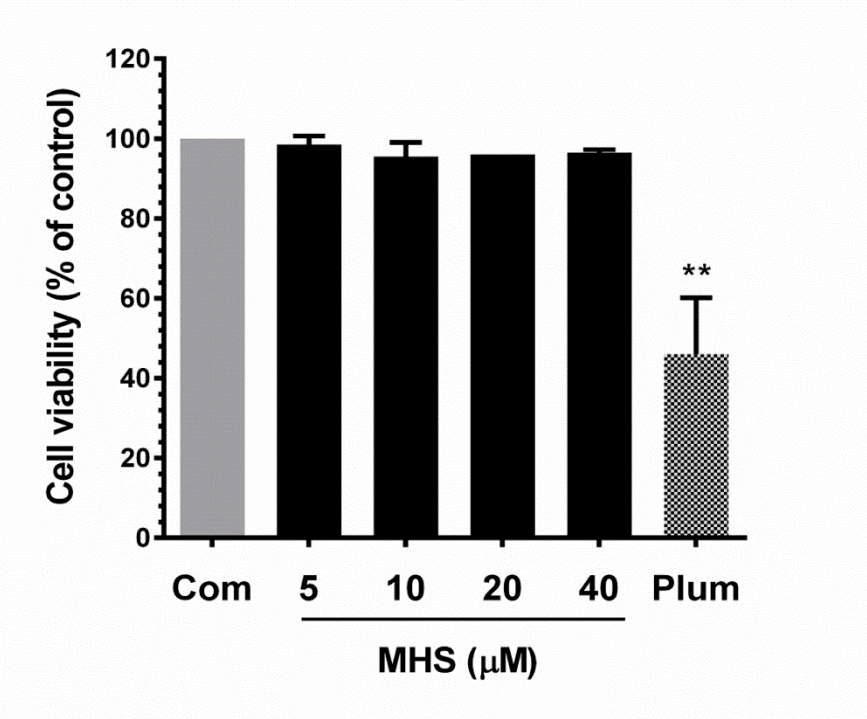


Fig. 3.

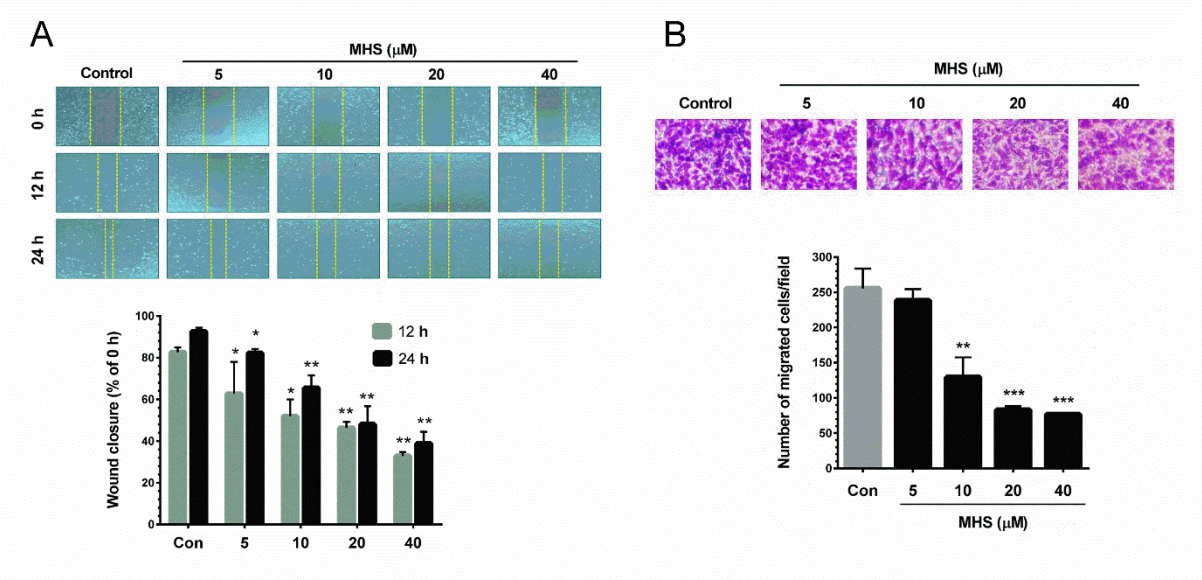
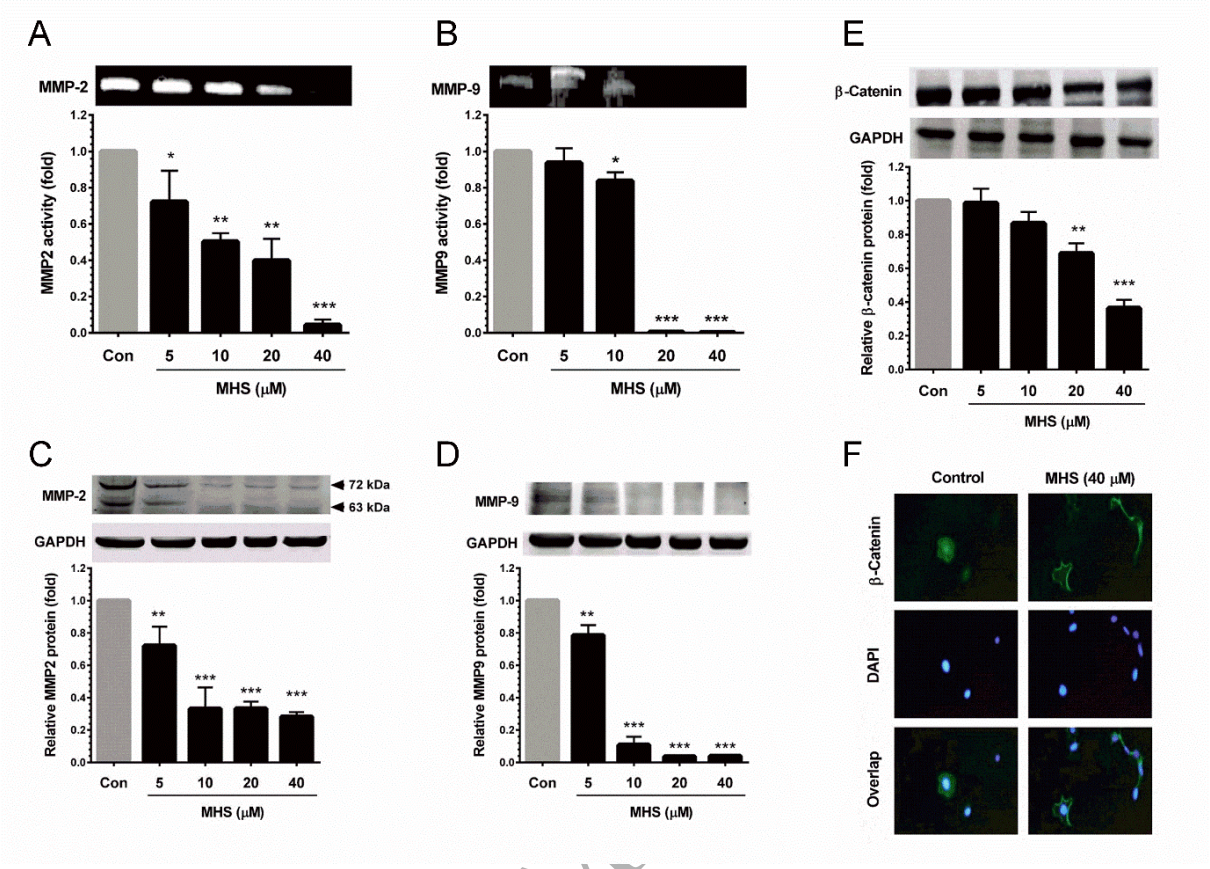


Fig. 4.



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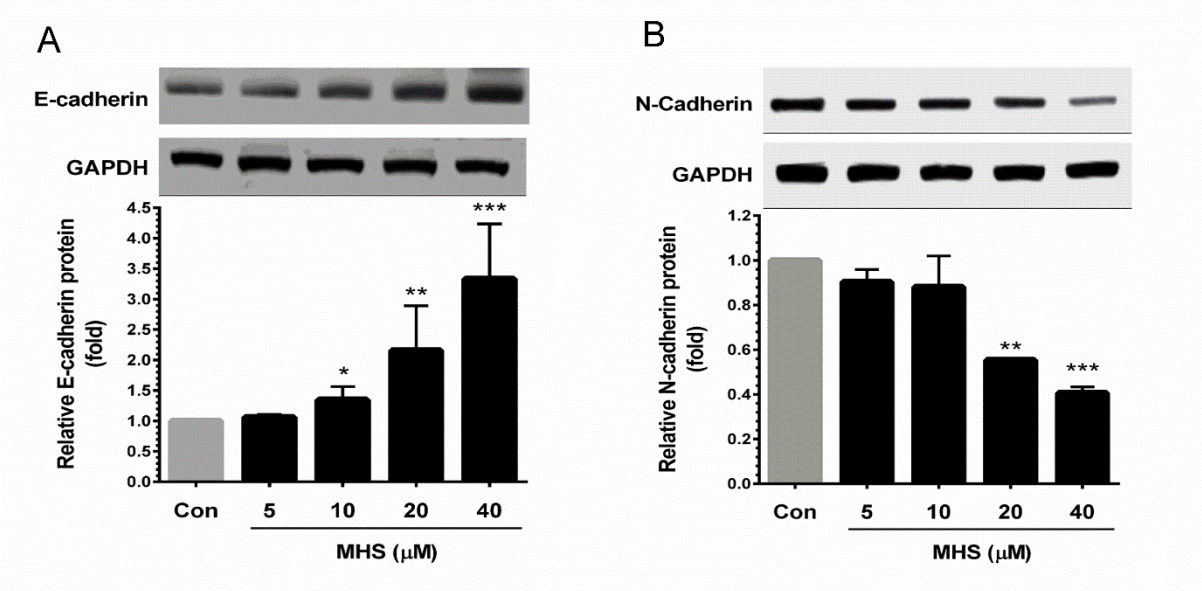

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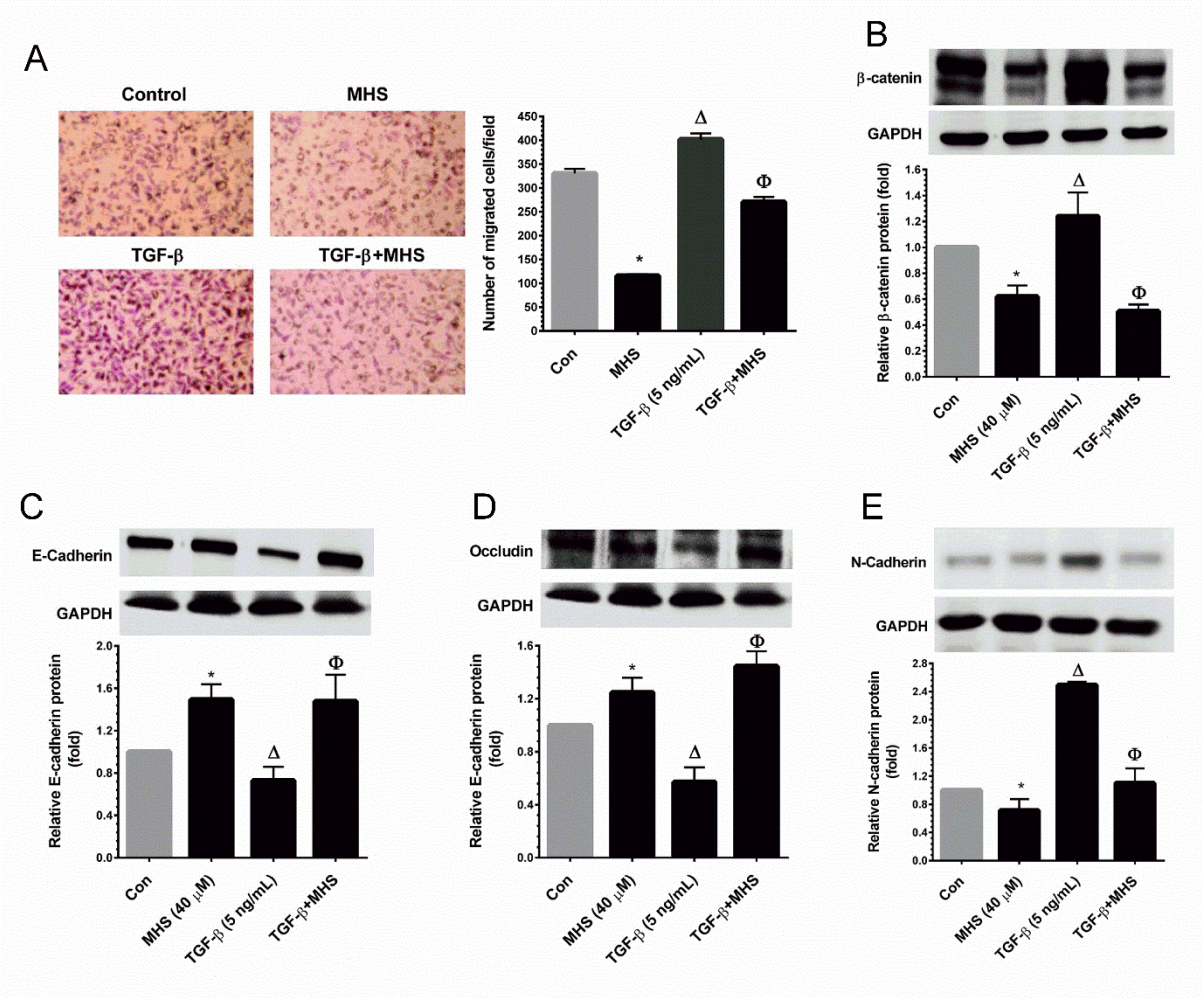

Graphical abstract