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ARTICLE

Anti-invasive effect of β-myrcene, a component of the essential oil from *Pinus koraiensis* cones, in metastatic MDA-MB-231 human breast cancer cells

Jeong-Ho Lee · Kicheol Lee · Da Hyun Lee · Soon Young Shin · Yeonjoong Yong · Young Han Lee

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Abstract The invasive potential of malignant tumor cells is critical for their metastasis. This study was undertaken to evaluate the anti-invasive activity of β-myrcene, a natural compound found in the essential oil from *Pinus koraiensis* cones (EOPC), in metastatic MDA-MB-231 human breast cancer cells. Among four major constituents that included α-pinene, β-myrcene, 3-carene, and *d*-limonene, β-myrcene showed the most potent inhibition of tumor necrosis factor-α (TNFα)-induced nuclear factor κB (NF-κB) activity. Pretreatment with β-myrcene suppressed TNFα-induced phosphorylation of inhibitor of κB kinase and NF-κB as well as matrix metalloproteinase-9 (MMP-9) gene expression in a dose-dependent fashion. Furthermore, β-myrcene inhibited TNFα-induced invasion of MDA-MB-231 cells as determined by three-dimensional spheroid invasion assays. These findings suggest that EOPC may promote anti-metastatic activity in breast cancer cells through its downregulation of NF-κB-mediated MMP-9 expression.

J.-H. Lee · K. Lee
Useful Plant Resources Center, Korea National Arboretum, Yongmun-myeon, Gyeonggi-do 476-845, Republic of Korea

D. H. Lee · S. Y. Shin · Y. H. Lee (✉)
Department of Biological Sciences, Cancer and Metabolism Institute, College of Biological Science and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea
e-mail: yhlee58@konkuk.ac.kr

Y. Yong (✉)
Division of Bioscience and Biotechnology, BMIC, Konkuk University, Seoul 143-701, Republic of Korea
e-mail: dragonnmr@naver.com

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Introduction

The basement membrane is a fibrous sheet-like extracellular matrix on which various epithelial cells are anchored. Matrix metalloproteinase-9 (MMP-9) is a zinc-dependent 92-kDa type IV collagenase (also known as gelatinase-B) that plays a critical role in migration and invasion of tumor cells through its breakdown of basement membranes (Deryugina and Quigley 2006). Tumor necrosis factor alpha (TNFα) is a major inflammatory cytokine that is produced primarily by activated immune cells to control systemic inflammation. In the tumor microenvironment, TNFα is produced chiefly by tumor and tumor-associated stromal cells, and it promotes tumor progression through activation of several transcription factors, including nuclear factor-κB (NF-κB) (Balkwill 2009). NF-κB mediates the expression of a number of genes involved in cell survival, proliferation, tumor invasion, and metastasis (Van den Steen et al. 2002).

Pinus koraiensis, commonly known as the Korean nut pine, is a type of conifer native to far-eastern Asia, eastern Canada, and the northeastern USA. Its seed has been used in food as well as in medicines (Lee et al. 2008). It has been demonstrated that essential oil extracted from *P. koraiensis* leaves displays antiproliferation and antidiabetic properties(Hong et al. 2004). However, the biological effects of the essential oil from *P. koraiensis* cones (EOPC) have not been fully characterized. Our previous study demonstrated that EOPC exerts antimicrobial effects (Lee et al. 2008). The aim of this study was to investigate the

effect of EOPC on NF-κB activity. Our results demonstrate that EOPC inhibits TNFα-induced NF-κB-dependent transcription, leading to the suppression of TNFα-induced tumor invasion.

Materials and methods

Materials

Authentic standards were purchased from INDOFINE chemical company (Hillsborough, USA). MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (USA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (Thermo Scientific Hyclone, USA).

Preparation of essential oil from *P. koraiensis* cones (EOPC)

Pinus koraiensis cones after removing the seeds were collected in November 2013 from Korea and its specimen was authenticated by Dr. Jeong-Ho Lee, Head of Useful Plant Resources Center, Korea National Arboretum, Korea. The geometrical coordinates of the plant are given as latitude 37.5622N and longitude 127.5518E. A voucher specimen (UPRC-2013-10) was deposited in the herbarium of Useful Plant Resources Center for future reference. After removing the seeds, the cone (1000 g) was dried. The essential oil from the *P. koraiensis* cone was obtained by using the hydrodistillation method as reported previously. Its yield was 1.07 % (v/v).

Identification of four major constituents from EOPC

High performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1100 Series (USA) with Symmetry C18 column (Waters, USA, 4.6 × 250 mm, 5 μM). The mobile phase was a gradient system of aqueous acetonitrile: 25 % (0–5 min), 50 % (5–25 min), 60 % (25–60 min). The flow rate was 1.0 mL/min and the injection volume ranged from 5 to 20 μL. The wave length of UV/VIS detector was varied at 210, 230, and 254 nm. The peaks of 3-carene (at 210 nm; retention time: 41.6 min), β-myrcene (at 230 nm; retention time: 35.6 min), *d*-limonene (at 230 nm; retention time: 38.6 min), and α-pinene (at 254 nm; retention time: 8.6 min) were identified, based on a comparison of their authentic samples. Each component was collected using an Agilent 1100 Series prep-HPLC system with Luna C18 100 Å column (Phenomenex, USA, 10 × 250 mm, 5 μM). The condition of the mobile phase was same as the analytical HPLC system. The flow rate and injection volume were 3.0 mL and 80 μL, respectively. The fractions were dried using speed vacuum and their dried amounts were at least 1 mg. The samples for the biological test were prepared at 20 mM dissolved in dimethylsulfoxide.

MMP-9 promoter reporter assay

Construction of MMP-9 promoter constructs, wild-type pMMP(−925/+13)_Luc and NF-κB site-mutated pMMP9 (−925/+13)mtNFκB_Luc, was described elsewhere (Shin et al. 2010). MDA-MB-231 cells were seeded onto 12-well plates and transfected with 0.5 μg of the pMMP9(−925/+13)_Luc or pMMP9(−925/+13)mtNFκB_Luc using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. To monitor transfection efficiency, 50 ng of the pRL-null plasmid encoding *Renilla* luciferase was included in all of the samples. At 48 h post-transfection, cells were treated with 10 ng/mL TNFα in the absence or presence of β-myrene. After 8 h, cells were collected and the firefly luciferase activities were measured and normalized to *Renilla* activities using the Dual-Glo Luciferase Assay System (Promega, USA). Luminescence was measured using a Centro LB960 luminometer.

Cytotoxicity assay

MDA-MB-231 cells were seeded onto 96-well plates (2×10^3 cells/well) and treated with either vehicle (dimethyl sulfoxide) or various constituents of EOPC for 24 h. Cellular cytotoxicity assay was performed using a Cell Counting Kit-8 Assay (Dojindo Molecular Technologies, JAPAN) according to the manufacturer's instructions.

NF-κB-dependent transcriptional activity assay

MDA-MB-231 cells cultured in 12-well plates were transfected with 0.1 μg of the 5 × NF-κB-Luc plasmid. At 24 h post-transfection, cells were treated with 10 ng/mL TNFα in the absence or presence of β-myrene along with 50 ng of the pRL-null plasmid encoding *Renilla* luciferase. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) and normalized to *Renilla* activity. The luminescence was measured with a Centro LB960 luminometer (Berthold Technologies, Germany).

Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

MDA-MB-231 cells were treated with 100 μM β-myrcene for 24 h, and total RNA was extracted using Isol-RNA Lysis Reagent (5 PRIME). The first-strand cDNA was

synthesized from 500-ng total RNA using an iScript cDNA synthesis kit (Bio-Rad, USA). PCR conditions were as follows: hold for 5 min at 94 °C, followed by 30 cycles consisting of denaturation at 94 °C (30 s), annealing at 55 °C (30 s), and elongation at 72 °C (1 min). The amplified products were subjected to electrophoresis in a 1 % agarose gel. Relative expression levels of mRNAs were measured by quantitative real-time PCR with a TaqMan-iQ^{TM} supermix Kit (Bio-Rad) using the Bio-Rad iCycler iQ^{TM} according to the manufacturer's instruction. The TaqMan^{TM} fluorogenic probes and gene specific PCR primers for MMP-9 and GAPDH were described elsewhere (Shin et al. 2013a). The relative fold changes were normalized for GAPDH mRNA in the same samples.

Immunoblot analysis

Immunoblotting was performed as described previously (Shin et al. 2013b). Briefly, MDA-MB-231 cells were either untreated or treated with 50 or 100 μM β-myrcene for 30 min before the addition of 10 ng/mL TNFα for a further 30 min. Cell lysates containing 10–20 μg protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The blots were probed with phospho-specific antibody against IKKα/β (Ser176/180) or p65/RelA (Ser536), then incubated with secondary antibodies conjugated to horseradish peroxidase. The anti-GAPDH antibody was used as an internal control. Results were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc., USA).

Immunofluorescence staining

MDA-MB-231 cells seeded on coverslips were pretreated with 100 μM β-myrcene for 30 min before stimulation with 10 ng/mL TNFα. After 30 min, the cells were fixed and permeabilized as described previously (Shin et al. 2011). Antibody against β-tubulin or phospho-p65/RelA (Ser536) was added for 2 h, and then an Alexa Fluor 488-conjugated (Invitrogen; green signal for β-tubulin) or Alexa Fluor 555-conjugated (Invitrogen; red signal for phospho-RelA) secondary antibody, respectively, was added for 30 min. Nuclear DNA (blue signal) was stained with 1 μg/mL Hoechst 33,258 (Sigma-Aldrich, USA) for 10 min. Stained cells were examined under an EVOSf1 fluorescence microscope (Advanced Microscopy Group, USA).

Three-dimensional spheroid cell invasion assay

Three-dimensional invasion assay was performed using Cultrex 3-D Spheroid Cell Invasion Assay kit (Trevigen, Inc., USA). Briefly, MDA-MB-231 cells were cultured for 3 days in Spheroid Formation Extracellular matrix to drive aggregation and spheroid formation of cells, followed by addition of Invasion Matrix composed of basement membrane proteins and medium without or with 10 ng/mL TNFα in the absence or presence of 100 μM β-myrcene. Cell invasion was visualized with a Eclipse TS100 microscope equipped with a digital sight camera.

Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.0 software (GraphPad Software, USA; www.graphpad.com). A p value of <0.05 was considered statistically significant.

Results and discussion

Previously, we analyzed the chemical components of EOPC and revealed that 96.8 % of the oil contains 87 different compounds; however, five compounds make up the bulk (over 70 %) of the total composition: *d*-limonene (27.90 %), α-pinene (23.89 %), β-pinene (12.02 %), 3-carene (4.95 %), β-myrcene (4.53 %), and camphene (1.54 %) (Hong et al. 2004). In the present study, we analyzed the principal components of EOPC and identified the presence of four major constituents, including α-pinene at 254 nm, β-myrcene at 230 nm, 3-carene at 210 nm, and *d*-limonene at 230 nm, using analytical HPLC system (Fig. 1).

MMP-9 plays an important role in the locomotion and invasion of tumor cells (Deryugina and Quigley 2006). To investigate whether the major constituents of EOPC affect TNFα-induced tumor invasion, highly metastatic MDA-MB-231 human breast cancer cells were pretreated with these constituents prior to the addition of TNFα, after which MMP-9 gene promoter activity was measured. We found that all tested major constituents of EOPC (camphene, 3-carene, *d*-limonene, β-myrcene, and α-pinene) significantly inhibited TNFα-induced MMP-9 promoter reporter activity (Fig. 2A), but had no effect on cell viability assessed using a Cell Counting Kit-8^{TM} (Fig. 2B), suggesting that inhibitory effect of EOPC on MMP-9 promoter activity is not associated with cytotoxicity. Of these compounds, β-myrcene exerted the most potent inhibitory effect and has not been well studied on the effect of tumor invasion. We thus focused on characterizing the molecular mechanism underlying β-myrcene-mediated inhibition of TNFα-induced tumor invasion using highly metastatic human breast adenocarcinoma MDA-MB-231 cells.

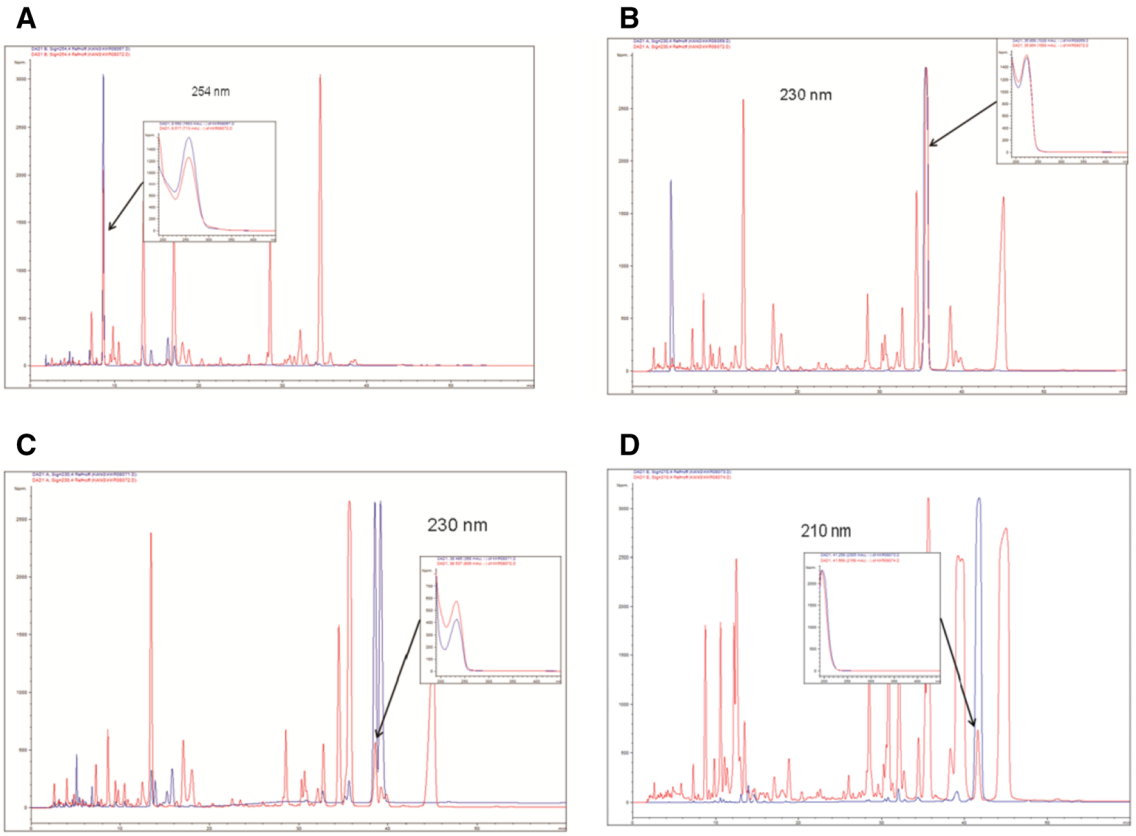


Fig. 1 A comparison of the chromatogram of the essential oil (*red*) obtained at 254 nm (**A**), 230 nm (**B**, **C**), and 210 nm (**D**) with an authentic sample (*blue*); α-pinene (**A**), β-myrcene (**B**), *d*-limonene (**C**), and 3-carene (**D**). The *inset* figures show the ultraviolet spectra of the peak observed in the chromatogram of the EOPC (*red*) and the authentic sample (*blue*). *x*-axis, retention time (min); *y*-axis, UV absorbance

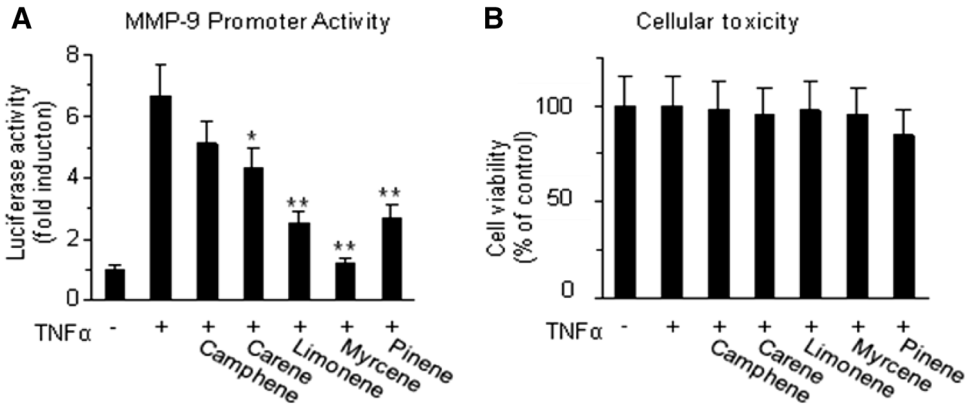


Fig. 2 Effect of major EOPC constituents on MMP-9 promoter activity. **A** MMP-9 promoter activity assay. MDA-MB-231 cells were transiently transfected with pMMP9(−925/+13)_Luc. At 48 h post-transfection, the cells were either left untreated or treated with 10 ng/mL TNFα in the absence or presence of EOPC components (100 μM). **B** Cytotoxicity assay. MDA-MB-231 cells were treated with TNFα or EOPC components as in (**A**) for 24 h. The data shown represent the mean ± SD of three independent experiments performed in triplicate. $^{**}p < 0.01$

NF-κB plays an important role in the regulation of the MMP-9 gene transcription (Han et al. 2001). TNFα stimulates the inhibitor of κB kinase (IKK), which subsequently phosphorylates IκB on serine-32, leading to the degradation of IκB and eventual activation of p65/RelA NF-κB. Pretreatment with β-myrcene prevented TNFα-

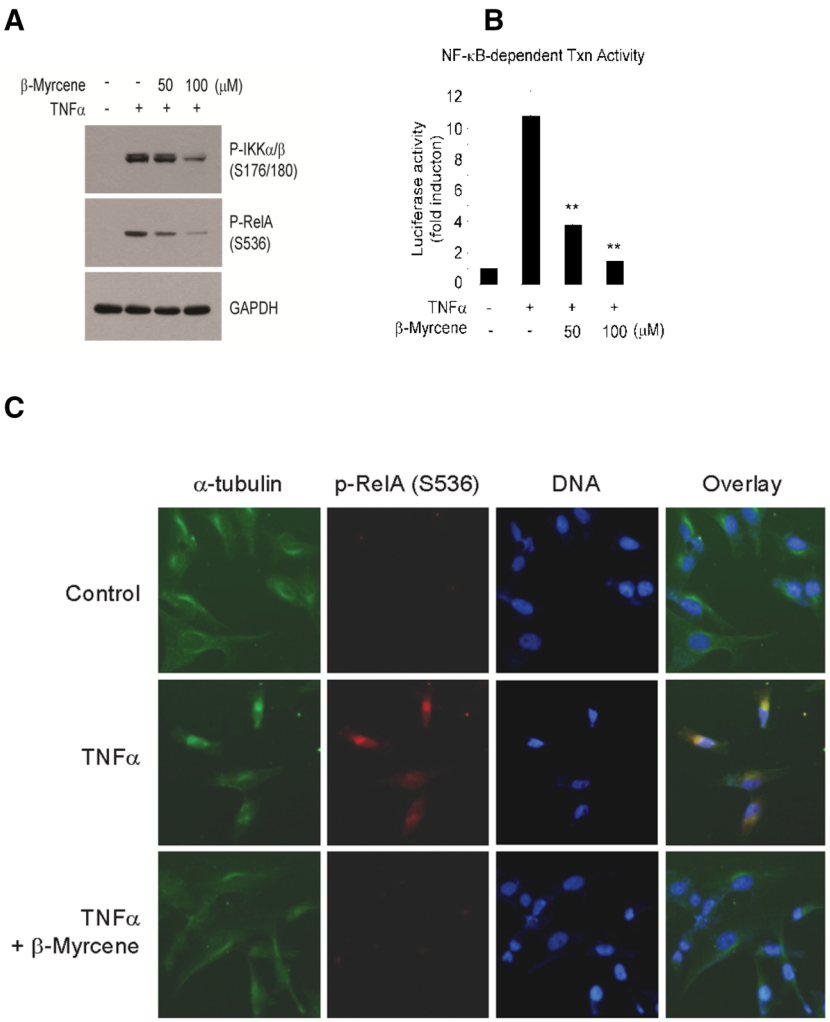


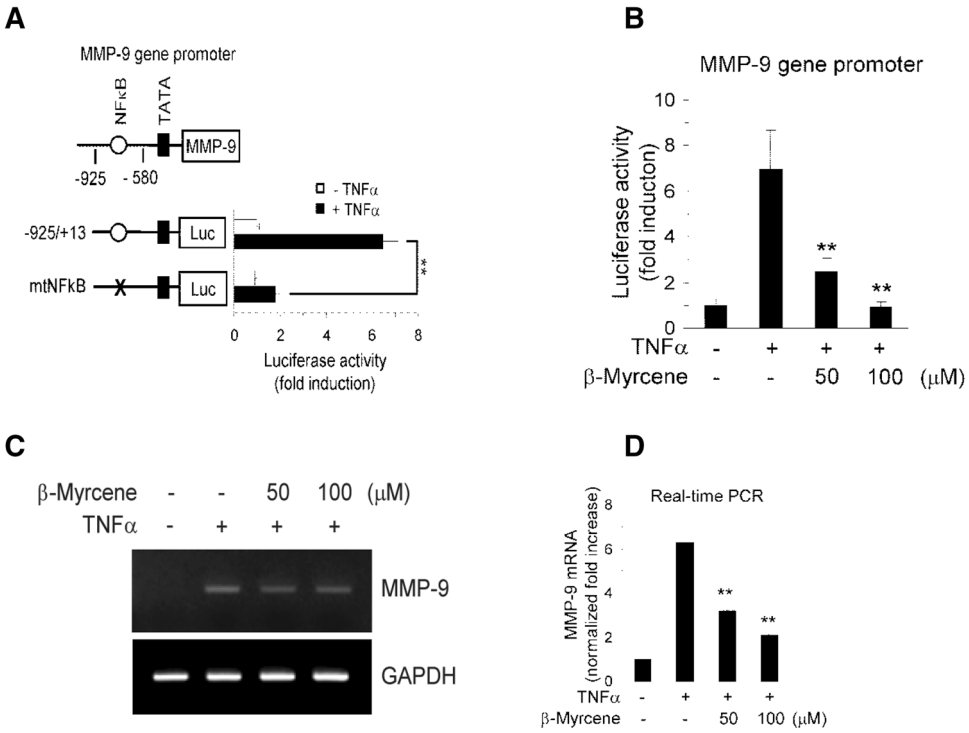
Fig. 3 Effect of β-myrcene on the inhibition of TNFα-induced NF-κB activity. **A** Immunoblot analysis. **B** NF-κB-dependent transcriptional activity assay. The data shown represent the mean ± SD of three independent experiments performed in triplicate. ** $p < 0.01$ versus TNFα-only treatment ($n = 9$). **C** Immunofluorescence microscopic analysis using Alexa Fluor 488-conjugated (*green signal*) or Alexa Fluor 555-conjugated (*red signal*) secondary antibody. Nuclear DNA was stained with 1 μg/mL Hoechst 33,258 (*blue signal*)

induced phosphorylation of IKK (serine-176/180) and p65/RelA NF-κB (serine-536) (Fig. 3A), and inhibited NF-κB-dependent transcriptional activity (Fig. 3B) in a dose-dependent fashion. Immunofluorescent microscopic analysis showed that phosphorylation of p65/RelA (serine-536) in the nucleus was evident upon TNFα stimulation, whereas phosphorylation was barely detectable in the presence of β-myrcene (Fig. 3C). These results indicate that β-myrcene inhibits TNFα-induced NF-κB activation through the inactivation of IKK.

Disruption of NF-κB binding sequences within the MMP-9 gene promoter caused substantial attenuation of MMP-9 promoter activity (Fig. 4A), indicating that NF-κB participates in TNFα-induced transcriptional activation of the MMP-9 gene. We further demonstrated that β-myrcene dose-dependently attenuated TNFα-induced MMP-9 promoter activity (Fig. 4B). RT-PCR analysis shows that TNFα increased MMP-9 mRNA level; this increase was suppressed by the addition of β-myrcene (Fig. 4C). Relative expression levels of mRNAs were measured by quantitative real-time PCR. Treatment with TNFα alone resulted in a 6.3-fold increase of MMP-9 mRNA level; however, this was reduced to 3.2- and 2.1-fold by pretreatment with 50 and 100 μM β-myrcene, respectively (Fig. 4D). These results demonstrate that β-myrcene prevents TNFα-induced MMP-9 expression by suppressing NF-κB activity.

We next investigated whether β-myrcene affects the invasive capability of MDA-MB-231 cells using a three-dimensional spheroid culture system. Under control conditions, the cells maintained a noninvasive spheroidal phenotype (Fig. 5, top panels). Upon TNFα stimulation, cells in the spheroid invaded into the surrounding basement membrane-like gel matrix (Fig. 5, middle panels). However, TNFα-induced invasion was markedly reduced in the presence of β-myrcene (Fig. 5, bottom panels). These data demonstrate that β-myrcene blocks TNFα-induced tumor cell invasion.

In summary, we found that β-myrcene, a component of the EOPC, inhibits TNFα-mediated NF-κB activity through



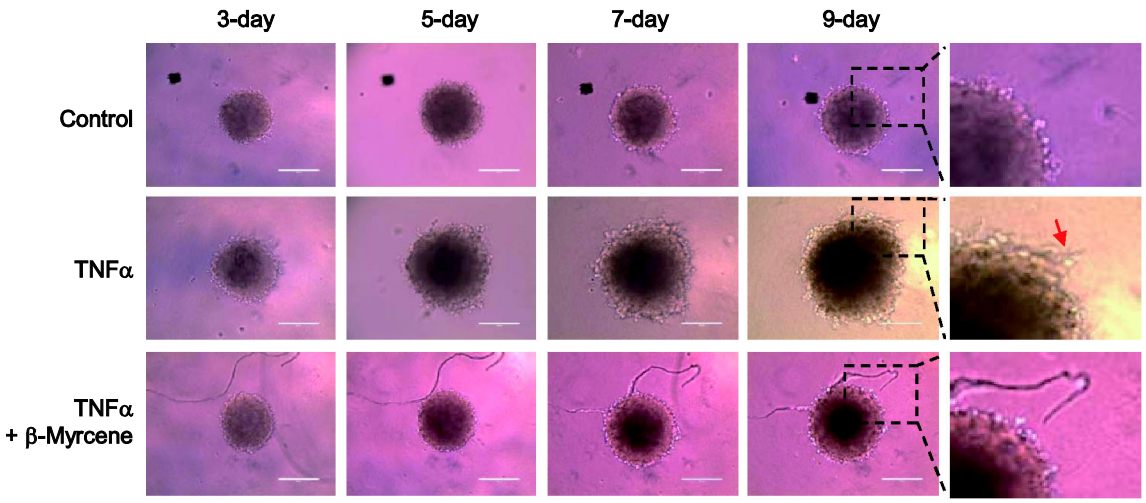


Fig. 4 Effect of β-myrcene on the inhibition of TNFα-induced MMP-9 gene expression. **A** Role of NF-κB in TNFα-induced activation of the MMP-9 promoter activity. The data shown represent the mean ± SD of three independent experiments performed in triplicate. ** $p < 0.01$. **B–D** Effect of β-myrcene on the inhibition of TNFα-induced MMP-9 promoter activity (**B**), MMP-9 mRNA expression by RT-PCR (**C**), and quantitative real-time PCR (**D**). GAPDH mRNA was used as an internal control. The data shown represent the mean ± SD of three independent experiments performed in triplicate. ** $p < 0.01$ versus TNFα-only treatment ($n = 9$)

Fig. 5 Effect of β-myrcene on the inhibition of invasive capability of MDA-MB-231 cells. MDA-MB-231 cells growing in three-dimensional spheroids in extracellular matrix were either untreated (control) or treated with 10 ng/mL TNFα in the absence or presence of 100 μM β-myrcene. *Arrow* indicates cells invading into the surrounding matrix

suppression of IKK and subsequent downregulation of MMP-9 expression, resulting in the inhibition of invasion of metastatic MDA-MB-231 human breast cancer cells. These findings suggest that EOPC may promote anti-metastatic activity in breast cancer cells, possibly through the down-regulation of NF-κB-mediated MMP-9 expression.

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References

Balkwill F (2009) Tumour necrosis factor and cancer. Nat Rev Cancer 9:361–371

Deryugina EI, Quigley JP (2006) Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25:9–34

Han YP, Tuan TL, Hughes M, Wu H, Garner WL (2001) Transforming growth factor-beta-and tumor necrosis factor-alpha-mediated induction and proteolytic activation of MMP-9 in human skin. J Biol Chem 276:22341–22350

Hong EJ, Na KJ, Choi IG, Choi KC, Jeung EB (2004) Antibacterial and antifungal effects of essential oils from coniferous trees. Biol Pharm Bull 27:863–866

Lee JH, Yang HY, Lee HS, Hong SK (2008) Chemical composition and antimicrobial activity of essential oil from cones of Pinus koraiensis. J Microbiol Biotechnol 18:497–502

Shin SY, Kim JH, Baker A, Lim Y, Lee YH (2010) Transcription factor Egr-1 is essential for maximal matrix metalloproteinase-9 transcription by tumor necrosis factor alpha. Mol Cancer Res 8:507–519

Shin SY, Hyun J, Lim Y, Lee YH (2011) 3′-Chloro-5,7-dimethoxyisoflavone inhibits TNF alpha-induced CXCL10 gene transcription by suppressing the NF-kappaB pathway in HCT116 human colon cancer cells. Int Immunopharmacol 11:2104–2111

Shin SY, Lee JM, Lim Y, Lee YH (2013a) Transcriptional regulation of the growth-regulated oncogene alpha gene by early growth response protein-1 in response to tumor necrosis factor alpha stimulation. Biochim Biophys Acta 1829:1066–1074

Shin SY, Lee KS, Choi YK, Lim HJ, Lee HG, Lim Y, Lee YH (2013b) The antipsychotic agent chlorpromazine induces autophagic cell death by inhibiting the Akt/mTOR pathway in human U-87MG glioma cells. Carcinogenesis 34:2080–2089

Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G (2002) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 37:375–536