

Anti-metastatic effect of rhodomyrtone from *Rhodomyrtus tomentosa* on human skin cancer cells

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Abstract. This study focused on the inhibitory effect of rhodomyrtone, a bioactive compound isolated from the leaves of *Rhodomyrtus tomentosa* (Aiton) Hassk., on cancer metastasis in epidermoid carcinoma A431 cells and on the verification of the underlying related molecular mechanisms of this event. We demonstrated that rhodomyrtone at the subcytotoxic concentration (0.5 and 1.5 $\mu\text{g/ml}$) exhibited pronounced inhibition of cancer metastasis by reducing cell migration, cell adhesive ability and cell invasion of A431 cells in a dose-dependent manner. Data demonstrated that rhodomyrtone could inhibit the focal adhesion kinase (FAK) and phosphorylation of protein kinase B (AKT), c-Raf, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK involved in the down-regulation the enzyme activities and protein expression of matrix metalloproteinase-2 (MMP-2) and MMP-9. Moreover, we found that rhodomyrtone increased the expression of TIMP-1 and TIMP-2, which are inhibitors of MMP-9 and MMP-2, respectively. Rhodomyrtone also inhibited the expression of NF- κ B and phosphorylation of NF- κ B in a dose-dependent manner. These results suggested that rhodomyrtone inhibited A431 cell metastasis by reducing MMP-2/9 activities and expression through inhibiting ERK1/2, p38 and FAK/Akt signaling pathways via NF- κ B activities. This finding suggested that rhodomyrtone may be a novel antimetastasis agent for treatment of skin cancer cells.

Introduction

Cancer metastasis is the major cause of death in most cancer patients. It is a complex cascade process, in which cancer cells detach from the primary tumor, migrate, adhere and invade through the basement membrane or extracellular

matrix (ECM), survive in the circulatory system, invade into distant secondary organs or tissues, and start to proliferate (1). During the metastatic process, proteolytic enzymes play critical roles in helping cancer cells to enter into the vascular and lymphatic systems and invading tissues at ectopic sites. Among all proteolytic enzymes, the members of matrix metalloproteinases (MMPs) are the main group of proteolytic enzyme that is involved in the tumor invasion, metastasis and angiogenesis. MMPs or matrixins are a group of zinc-dependent endopeptidase enzymes, that respond to ECM degradation and tumor cell invasion, metastasis and angiogenesis (2). MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are the key enzymes in degradation of the ECM components of basal membrane and type IV collagen, a major component of the basement membrane. Activities of MMPs are controlled by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) such as TIMP-1 and TIMP-2, in cancer cells (3,4). It was reported that when the balance of MMPs and TIMPs was disrupted, direct inhibition of MMPs and increase of TIMPs in cancer may be a particular attractive target for therapeutic intervention in tumor invasion and metastasis (5). Therefore, inhibition of MMP activity and expression is important for inhibiting cancer metastasis, which affects mortality in patients.

Mitogen-activated protein kinases (MAPKs) include extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK, play an important regulatory role in cell growth, differentiation, apoptosis, and metastasis (6). AKT (also known as PKB) is also involved in multiple cellular processes such as cell growth, cell proliferation, angiogenesis and metastasis in various cancers (7). They have a central role in regulating the expression of MMPs (8-11). In addition, the expression of MMPs is also regulated by nuclear-factor- κ B (NF- κ B) and the activator protein 1 (AP-1) as the MMP gene has an NF- κ B and AP-1 binding site in its promoter region (12,13). Inhibition of the MAPK and PI3K/Akt pathways as well as NF- κ B and AP-1 activities may lead to potential prevention of cancer cell proliferation, invasion, and metastasis.

Traditionally, *Rhodomyrtus tomentosa* (Aiton) Hassk., the family myrtaceae, has been used for anti-inflammation, to treat diarrhea, gastrointestinal, urinary tract infections and

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antiseptic wash for wounds (14,15). It is native to Southeast Asia and is a troublesome invader of native plant communities in Florida (16). Rhodomyrtone is a pure compound, isolated from *Rhodomyrtus tomentosa* leaves. Previous studies have shown that rhodomyrtone displays antibacterial activity against a wide range of gram-positive bacteria such as *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* spp., and methicillin-resistant *Staphylococcus aureus* (MRSA) (17-21). Moreover, rhodomyrtone stimulated pro- and anti-inflammatory cytokine responses (22) and reduced hyperproliferation and abnormal differentiation of HaCaT cells (23). However, the anti-metastatic activity of rhodomyrtone on cancer cells has not yet been reported.

Non-melanoma skin cancer (NMSC) is the most common cancer affecting white-skinned individuals and the incidence is increasing worldwide. There are two main types of NMSC including the basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) (24,25). SCC is the second most common skin cancer, accounting for ~20% of NMSC cases. It is more common in older people. The main risk factor for skin cancer is exposure to UV radiation, which causes cellular damage (26,27). Current treatments of SCCs consist of surgery, photodynamic therapy, radiation therapy, chemotherapy or combination therapy, but these treatments are unsatisfactory. Thus, it is necessary to search for a new effective therapeutic agent to treat SCCs.

In this study, we investigated the inhibitory effect of rhomylrtone on cancer metastasis in A431 cells. It was demonstrated that rhodomyrtone effectively inhibits cell migration, invasion and adhesion in human epidermoid carcinoma A431 cells.

Materials and methods

Chemical and antibodies. Rhodomyrtone was obtained from Dr Wilawan Mahabusarakum, Department of Chemistry, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. It was dissolved in dimethylsulfoxide (DMSO). MTT (3-(4,5-dimethyl-2,5-diphenyl tetrazolium bromide), DMSO and trypan blue were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco/BRL (Gaithersburg, MD, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). Immobilon Western Chemiluminescent HRP substrate was purchased from Merck Millipore Corp. (Merck KGa, Darmstadt, Germany). The protein assay kit and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Labs (Hercules, CA, USA). Antibodies (Abs) for immunoblotting analysis including rabbit monoclonal Abs against matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, RAS, growth factor receptor-bound protein-2 (GRB2), focal adhesion kinase (FAK), p-FAK (Try397), pPDK1 (Ser241), p-cRaf, extracellular signal regulation kinase 1/2 (ERK1/2), p-ERK1/2, p38, p-p38, c-Jun N-terminal kinase 1/2 (JNK1/2), pJNK1/2, AKT, pAKT (Ser473) pAKT (Thr308), c-Fos, c-Jun, p-cJun, nuclear factor κ B (NF- κ B), p-NF- κ B and anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies were obtained

from Cell Signaling Technology, Inc. (Danvers, MA, USA), and mouse monoclonal Abs against β -actin was obtained from Merck Millipore Corp. (Merck KGaA).

Cell line and cell culture. The human epidermoid carcinoma cell line (A431) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A431 cells were maintained as a monolayer in DMEM (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (GE Healthcare Life Science, Little Chalfont, UK), 100 U/ml penicillin and 100 μ g/ml streptomycin (GE Healthcare Life Science, Inc.) at 37°C in a humidified 5% CO₂.

Cell viability analysis. A431 cells (7x10³ cell/well) were seeded in a 96-well plate for 24 h. Then cells were treated with rhodomyrtone at various concentrations (0, 0.5, 1.5, 3, 5, 10 and 15 μ g/ml) for 24 h. After treatment 0.5 mg/ml of MTT solution was added to each well and incubated for 2 h at 37°C. The supernatant was removed and DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance was measured using a Epoch™ Microplate Spectrophotometer at 570 nm and survival percentage (%) was calculated relative to the control.

In vitro migration and invasion assay. Cells were pretreated with 0, 0.5, 1.5 and 3 μ g/ml rhodomyrtone for 24 h. The cells were harvested and seeded to the upper chamber of the Transwell insert [polyethylene terephthalate (PET) filters, Merck Millipore Corp.] at 10⁴ cell/well in serum-free medium. The lower chambers were filled with FBS medium as chemoattractant, and thereafter these Transwell inserts were incubated for 24 h at 37°C in 5% (v/v) CO₂. For invasion assay, the Transwell insert was coated with 30 μ g Matrigel (BD Biosciences, MA, USA) and lower chambers were filled with FBS medium. After incubation, the cells were removed with a cotton swab and those on the lower surface of the membrane were fixed with methanol and stained with 0.5% crystal violet. Cells that migrate through the membrane were viewed and photographed under an inverted microscope (Olympus). The percentage of the migratory cells for each treatment was calculated by NIH ImageJ software, version 1.46r.

Cell-matrix adhesion assay. Cells at 2x10⁵ cells/well were pretreated with various concentrations of rhodomyrtone (0, 0.5, 1.5 and 3 μ g/ml) for 24 h, then 1x10⁴ cells/well were seeded into the Matrigel-coated 96-well plate for 1 h. The non-adherent cells were then removed with PBS and the adherent cells were reacted with 0.5 mg/ml of MTT solution at 37°C for 2 h. After that 100 μ l of DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance was measured at 570 nm using a microplate reader. The percentage (%) of cell adhesion was calculated relative to the control.

Gelatin zymography. Cells were treated with various concentrations of rhodomyrtone (0, 0.5, 1.5 and 3 μ g/ml) for 24 h, the conditioned media were collected and mixed with non-reducing sample buffer. Samples were separated by SDS-PAGE containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min, 3 times

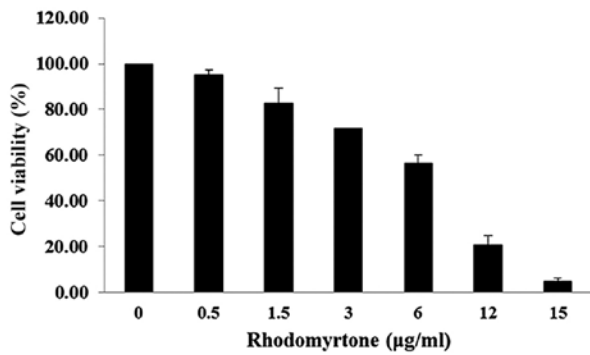


Figure 1. The effect of rhodomyrtone on cell proliferation at different concentrations for 24 h. The subtoxic concentration range of rhodomyrtone that resulted in >80% cell viability was selected for subsequent studies. Values represent mean \pm SD in three independent experiments.

and incubated in zymogram incubation buffer (50 mM Tris-HCl, pH 7.6, 10 mM CaCl_2 , 50 mM NaCl, 0.05% Brij35) for 48 h at 37°C. Then the gels were rinsed with distilled water and stained with Coomassie Brilliant Blue R-250. The bands of gelatinolytic activity were quantified using NIH ImageJ software, version 1.46r.

Western blot analysis. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100), after treatment with 0, 0.5, 1.5 and 3 $\mu\text{g/ml}$ of rhodomyrtone. Total proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore Corp., Billerica, MA, USA). After protein transferring, the

membranes were subsequently blocked with 5% non-fat milk for 1 h at room temperature to block non-specific binding. Then membranes were incubated with specific primary antibody against MMP-2, MMP-9, TIMP-1, TIMP-2, RAS, GRB2, FAK, p-FAK (Try397), pPDK1 (Ser241), p-cRaf, ERK1/2, p-ERK1/2, p38, p-p38, JNK1/2, pJNK1/2, AKT, pAKT (Ser473) pAKT (Thr308), c-Fos, c-Jun, p-cJun, NF- κB , p-NF- κB and β -actin at 4°C, overnight. Subsequently, membranes were incubated with anti-mouse or anti-rabbit antibody conjugated with horse-radish peroxidase (Cell Signaling Technology) for 1 h at room temperature. The protein bands were detected by chemiluminescence using enhanced chemiluminescence reagent (ECL) (Millipore) and exposed to CCD camera (Biotek Instruments, Winooski, VT, USA). The quantitative results for the protein of interest were expressed as relative to an internal housekeeping control such as β -actin.

Statistical analysis. To compare the data from different treatments, one-way ANOVA was used. All data presented were obtained from at least three independent experiments and were presented as mean \pm standard deviation (SD). A p-value of 0.05 was taken as minimum basis for assigning significance. The statistical analyses were performed using SPSS 17.0 software.

Results

Effect of rhodomyrtone on A431 cell proliferation. The effect of rhodomyrtone on A431 cell viability was analyzed by MTT assay. The viability of A431 cells treated with rhodomyrtone under different concentrations (0, 0.5, 1.5, 3, 5, 10 and 15 $\mu\text{g/ml}$) for 24 h as shown in Fig. 1. The result demonstrated that

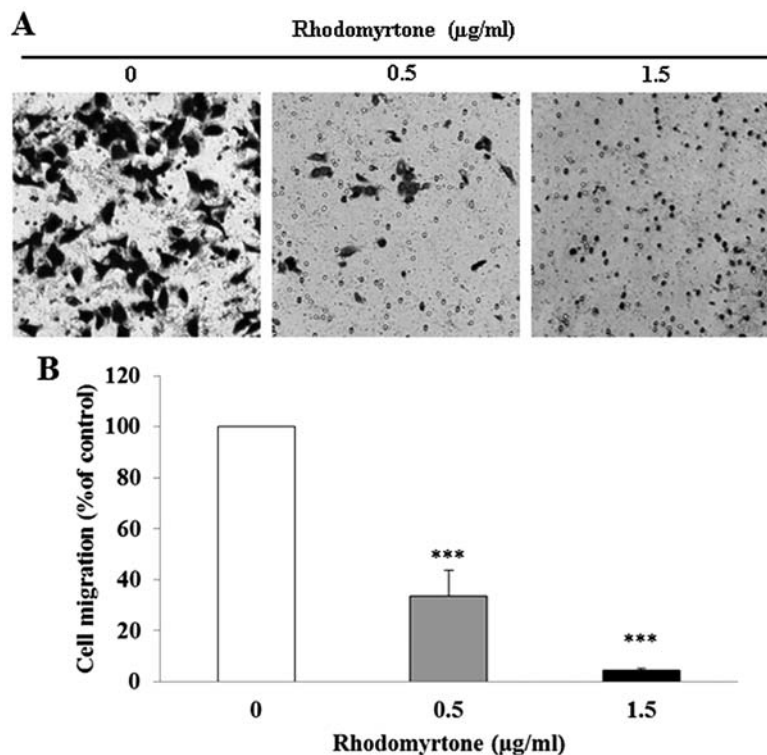


Figure 2. Effect of rhodomyrtone on A431 cell migration. Rhodomyrtone inhibited cell migration as tested by Transwell chamber assay. The migratory cells were stained with crystal violet and photographed (x40 magnification). Data are presented as mean \pm SD of three independent experiments. ***p<0.001 compared with the control group.

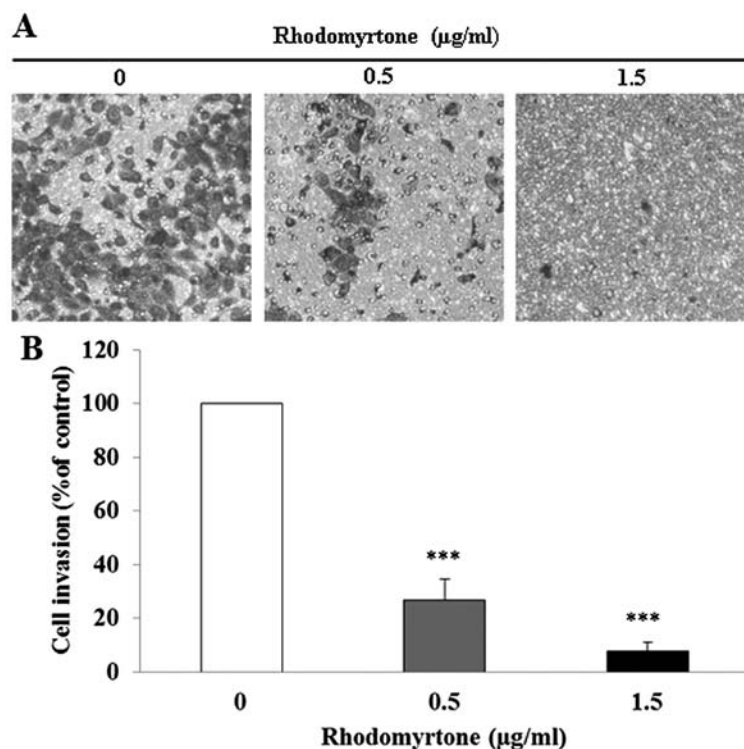


Figure 3. Effects of rhodomyrtone on A431 cell invasion. A431 cells treated with or without rhodomyrtone 24 h on Matrigel-coated filter (pore size, 8 µm) in Boyden chamber. (A) The invaded cells were stained with crystal violet and photographed (x40 magnification). (B) The invasion rate is presented as a percentage of the control. Data are presented as mean \pm SD of three independent experiments. *** $p < 0.001$ compared with the untreated control.

rhodomyrtone inhibited A431 cell viability in a time- and dose-dependent manner. At the high concentrations, rhodomyrtone significantly inhibited cell proliferation of A431 cells while at lower concentrations no significant effect was observed. The non-cytotoxic concentration and subcytotoxic concentration (< 1.5 µg/ml showing $> 80\%$ cell proliferation) was selected for the subsequent experiment.

Effect of rhodomyrtone on A431 cell migration and invasion. The effect of rhodomyrtone on A431 cell migration and invasion was determined by Transwell chamber assay. After A431 cells were treated with rhodomyrtone at 0.5 and 1.5 µg/ml for 24 h, rhodomyrtone significantly reduced cell migration in a dose-dependent manner (Fig. 2; $p < 0.001$). The percentage of cell migration was 33.6 ± 9.9 and $4.4 \pm 0.8\%$ after treatment with 0.5 and 1.5 µg/ml rhodomyrtone, respectively. Moreover, we found that rhodomyrtone significantly inhibited the invasion of A431 cell through Matrigel-coated filter in a dose-dependent manner ($p < 0.001$). Exposure of A431 cells to 0.5 and 1.5 µg/ml rhodomyrtone inhibited 73.2 and 92.3% of cell invasion, respectively (Fig. 3). These results revealed that rhodomyrtone markedly inhibits migration and invasion of A431 cells.

Effect of rhodomyrtone on adhesion ability of A431 cells. We examined the effect of rhodomyrtone on adhesion ability of A431 cells to Matrigel. The result demonstrated that the adhesive capacities of A431 cells to Matrigel were significantly decreased after treatment with 0.5 and 1.5 µg/ml rhodomyrtone when compared to the untreated control group as shown in Fig. 4.

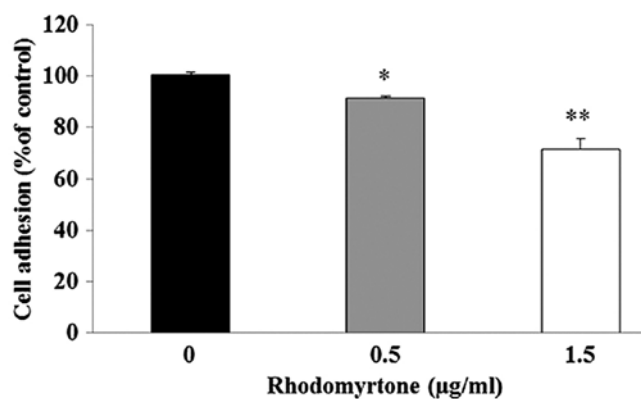


Figure 4. Effect of rhodomyrtone on adhesion ability of A431 cells. Adhesion of A431 cells to Matrigel were markedly inhibited by rhodomyrtone at concentration of 0.5 and 1.5 µg/ml as compared with control group. Data are presented as mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with the control group.

Effect of rhodomyrtone on MMP-9 and MMP-2 activities. To determine the possible mechanism of rhodomyrtone to inhibit cell migration and invasion, we investigated the activity of MMP-2 and MMP-9 in culture media of A431 cells using gelatin zymography. After treatment with 0.5 and 1.5 µg/ml of rhodomyrtone for 24 h, the conditioned medium was collected and MMP activity was estimated from densitometric analysis. The result showed rhodomyrtone reduced MMP-2 and MMP-9 activities in a dose-dependent manner (Fig. 5). MMP-2 activity was reduced by 53.5 and 37.8% and MMP-9 activity was reduced by 51.1 and 30.3% upon treatment with

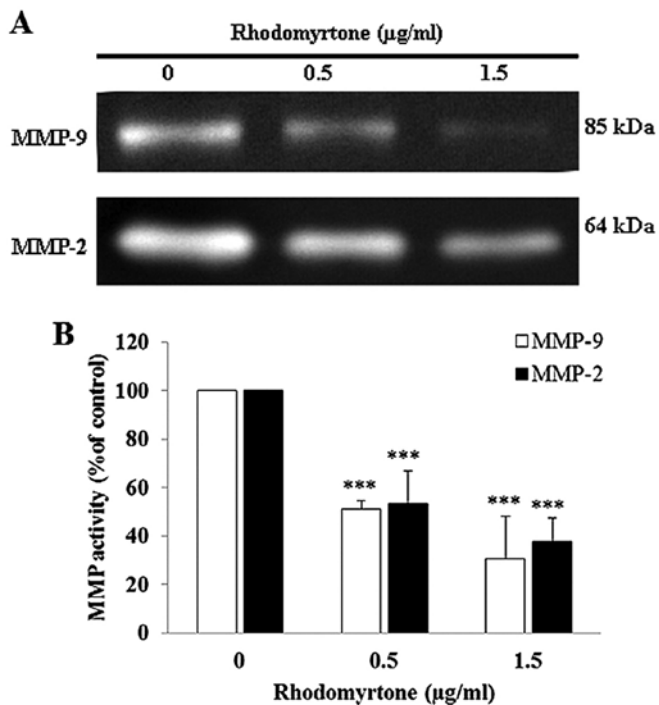


Figure 5. Effect of rhodomyrtone on MMP-9 and MMP-2 activities. (A) Cells were treated with the 0.5 and 1.5 $\mu\text{g/ml}$ of rhodomyrtone for 24 h. The conditioned media were collected and MMP-2 and MMP-9 activities were examined by gelatin zymography analysis. (B) The activities of MMP-2 and MMP-9 were quantified by using NIH ImageJ. Data are presented as mean \pm SD of three independent experiments. *** $p < 0.001$ compared with the untreated group.

0.5 and 1.5 $\mu\text{g/ml}$ of rhodomyrtone, respectively. The results indicated that rhodomyrtone inhibited MMP-2 and MMP-9 activities in A431 cells.

Effects of rhodomyrtone on MMP-2, MMP-9, TIMP-1 and TIMP-2 expression in A431 cells. MMP-2 and MMP-9 are involved in the degradation of ECM and are essential to the cell migration and invasion during metastasis. The effects of rhodomyrtone on MMP-2 and MMP-9 expression were detected by western blot analysis. As shown in Fig. 6A and B, rhodomyrtone suppressed the expression of MMP-2 and MMP-9 in a dose-dependent manner. Inhibition of MMP-2 was $\sim 15.3\%$ upon treatment with 1.5 $\mu\text{g/ml}$ rhodomyrtone and MMP-9 was ~ 33.1 and 54.2% with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone, respectively. Furthermore, we demonstrated that rhodomyrtone markedly increased TIMP-1 and TIMP-2 protein expression upon treatment with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h (Fig. 6C and D). TIMP-1 and TIMP-2 are known to be the specific endogenous inhibitors of MMPs.

Effect of rhodomyrtone on GRB2, RAS, MAPK signaling pathway and p-cRaf expression. We determined the mechanisms of rhodomyrtone for anti-metastatic effects on A431 cells. GRB2, RAS, p-cRaf and MAPK expression were investigated in A431 cells. The results showed that rhodomyrtone significantly reduced the phosphorylation of cRaf, ERK1/2 and p38 in a dose-dependent manner, but no significant alterations were observed in GRB2, RAS, ERK1/2 and p38 as shown in Fig. 7.

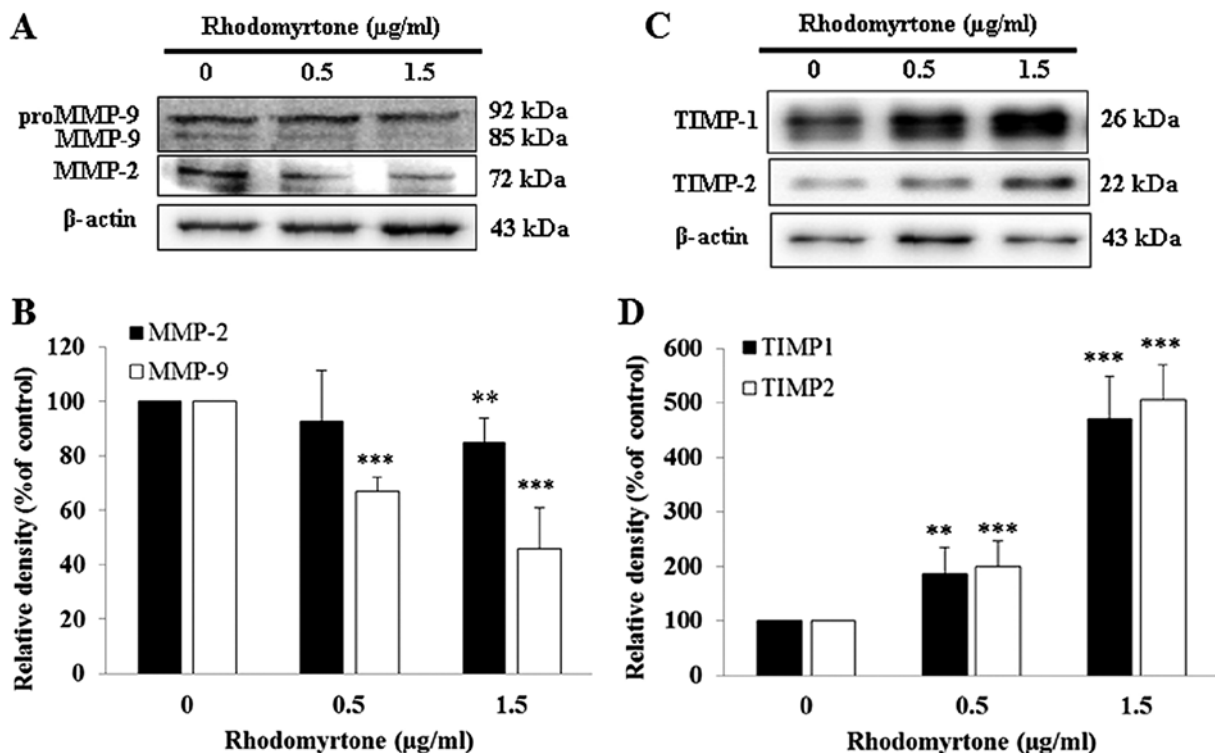


Figure 6. Rhodomyrtone inhibits MMPs expression and increases the protein level of TIMPs in A431 cells. (A and B) Cell were treated with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h, and the protein expression of MMP-2 and MMP-9 were detected using specific antibodies and β -actin was used as loading control. (C and D) The protein level of TIMP-1 and TIMP-2 were also detected when the cells were treated with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h, and β -actin was used as loading control. Data are presented as mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the untreated group.

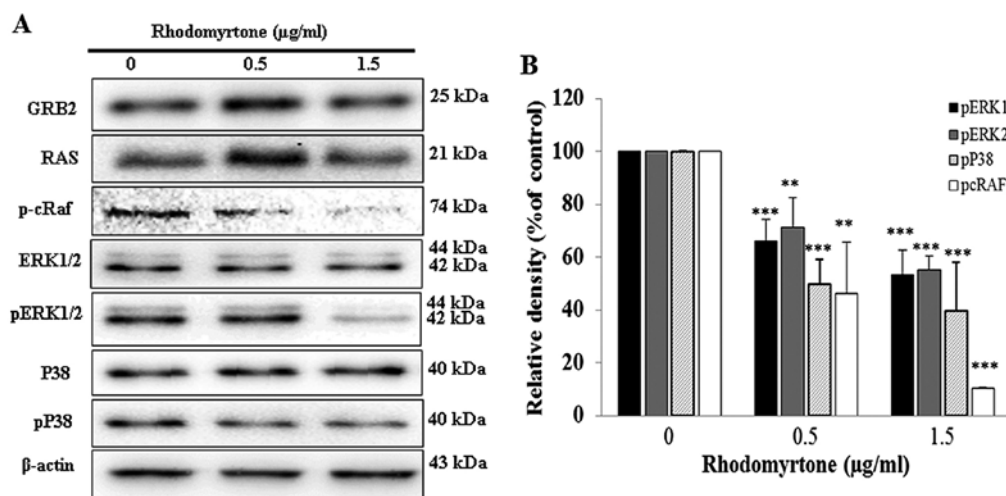


Figure 7. Effect of rhodomyrtone on GRB2, RAS, p-cRaf and MAPK signaling pathway proteins expression. Cell were treated with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h, and the protein expression of GRB2, RAS, p-cRaf, ERK1/2, pERK1/2, p38 and pp38 were detected with western blotting analysis using specific antibodies and β -actin was used as loading control. The values are presented as mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared with the untreated group.

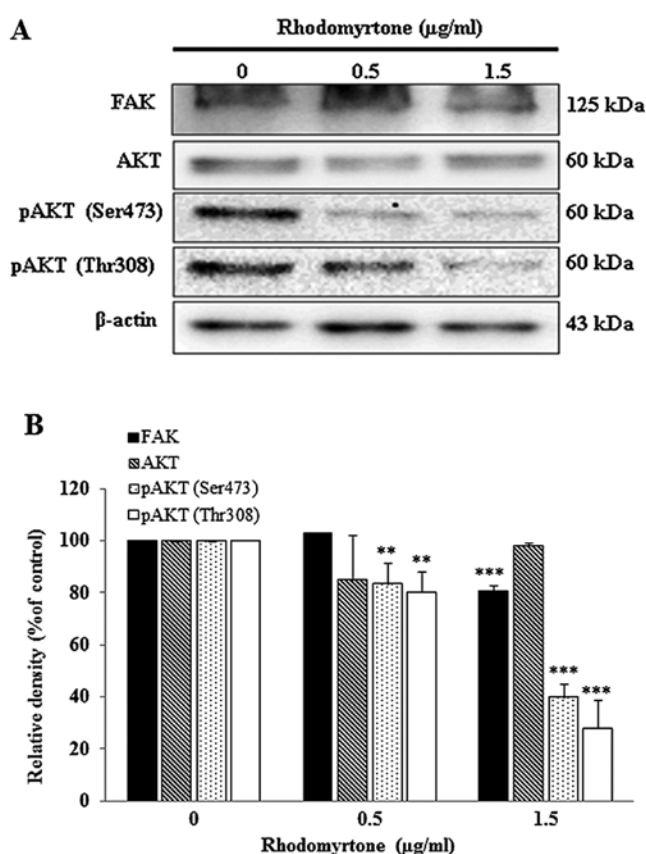


Figure 8. Effect of rhodomyrtone on FAK and AKT signaling pathway. Cells were treated with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h, and the protein expression of FAK, AKT, pAKT (Ser473) and pAKT (Thr308) were detected by western blot analysis using specific antibodies and β -actin was used as loading control. The values are presented as mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared with the untreated group.

Effect of rhodomyrtone on FAK and AKT signaling pathway. To determine whether rhodomyrtone inhibits A431 cells migration and invasion via FAK/PI3K/Akt, cells were treated

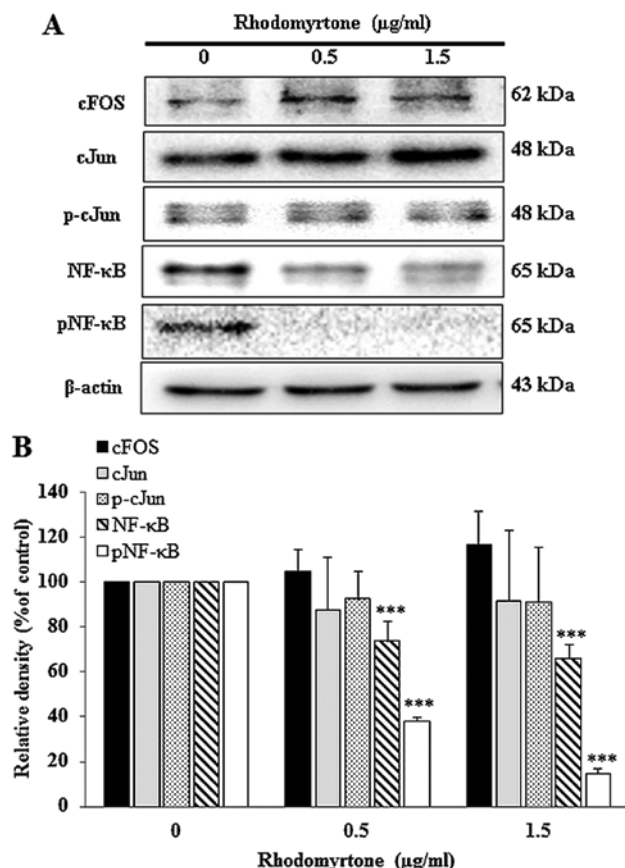


Figure 9. Effect of rhodomyrtone on AP-1 and NF- κ B protein expression. Cells were treated with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h, and the protein expression of cFos, cjun, p-cjun (components of transcription factor AP-1), NF- κ B and pNF- κ B were investigated by western blot analysis using specific antibodies and β -actin was used as loading control. The values are presented as mean \pm SD of three independent experiments. *** $p < 0.001$ compared with the untreated group.

with 0.5 and 1.5 $\mu\text{g/ml}$ rhodomyrtone for 24 h. FAK, AKT, pAKT (Ser473) and pAKT (Thr308) were detected by western blot analysis. Fig. 8A showed rhodomyrtone could suppress

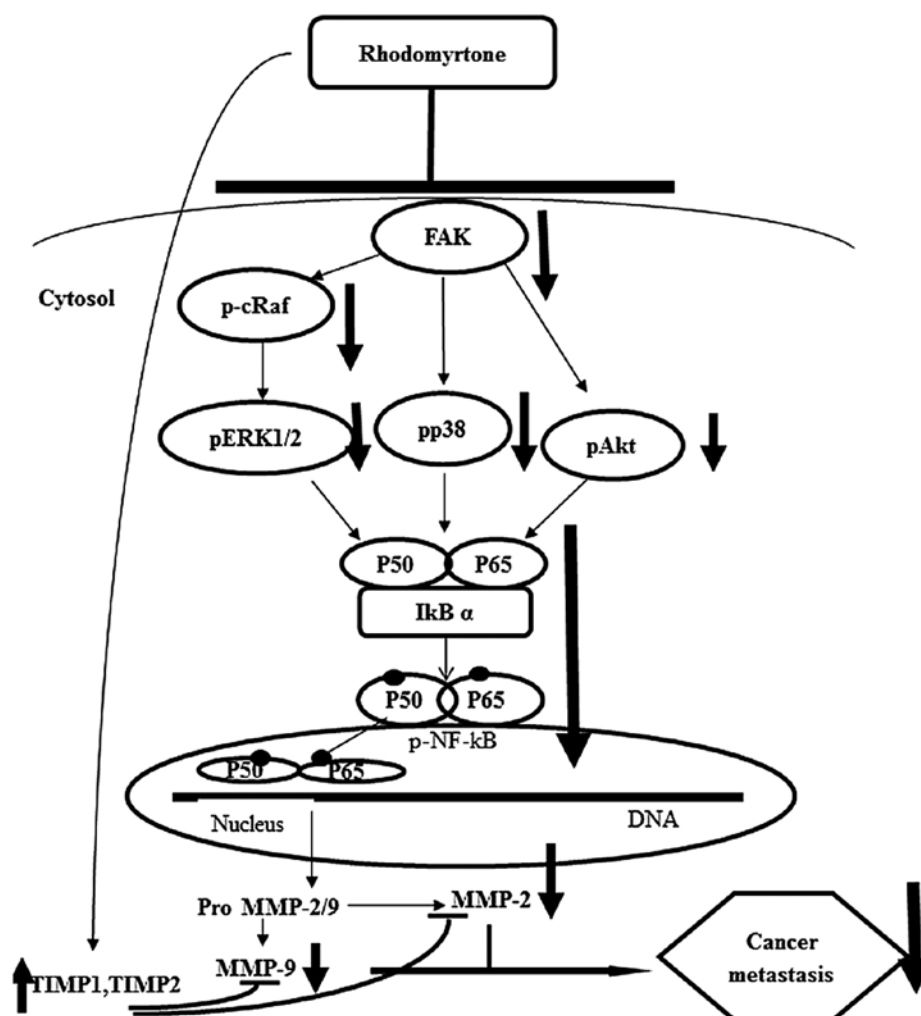


Figure 10. Rhodomyrtone inhibits cancer metastasis of A431 cells by suppressing MMP-2 and MMP-9 activities and protein expression and induces TIMP-1 and TIMP-2. Anti-metastatic effect of rhodomyrtone on A431 cells in human epidermoid carcinoma is through the inhibition of Raf/ERK, p38 MAPK and FAK/Akt signaling pathways via NF- κ B activities.

FAK and the phosphorylation of AKT. The quantitative results showed that rhodomyrtone significantly inhibited the FAK and the phosphorylation of AKT in a dose- and time-dependent manner (Fig. 8B).

Effect of rhodomyrtone on AP-1 and NF- κ B protein expression. The inhibitory effect of rhodomyrtone on transcription factor NF- κ B and AP-1 in A431 cells were investigated by western blotting. Data demonstrated that A431 cells treated with rhodomyrtone significantly decreased the level of NF- κ B and pNF- κ B expression in a dose-dependent manner (Fig. 9), but, there was no change in the expression of cFos, cJun and p-cJun (components of transcription factor AP-1) under the same conditions. The results indicated that rhodomyrtone significantly inhibited NF- κ B protein expression.

Discussion

Therapeutic agents to prevent development of metastases are an urgent therapeutic need. Present cancer chemotherapy is mainly targeted on primary tumors but the late stage patient survival has improved very little. We demonstrated that

rhodomyrtone could inhibit A431 cell proliferation in a dose-dependent manner (Fig. 1). Rhodomyrtone at non-cytotoxic concentration and subcytotoxic concentration (0-1.5 μ g/ml) significantly reduced A431 cell migration and cell invasion by Transwell chamber (Fig. 2) and the Matrigel-coated Boyden chamber assay in a dose-dependent manner (Fig. 3). Rhodomyrtone also exhibited the anti-adhesion in A431 cells on Matrigel as shown in Fig. 4. These results indicated that rhodomyrtone inhibits cell metastasis of A431 human skin cancer independent of cell cytotoxicity. Consistent with Lee *et al* who showed andrographolide at low-cytotoxic concentration inhibits the invasion and migration of human non-small cell lung cancer A549 cells (28). Thus, rhodomyrtone might be used as a chemotherapeutic agent for cancer treatment in skin cancer in the future.

This study showed that rhodomyrtone significantly inhibited MMP-2 and MMP-9 protein expression as well as MMP-2 and MMP-9 enzyme activity but increased TIMP-1 and TIMP-2 expression. Overexpression of MMP-2 and MMP-9 are involved in cancer angiogenesis, cancer invasion and metastasis. Thus inhibition of MMP-2 and MMP-9 expression or enzyme activity provide early targets of cancer metastasis

prevention (29-31). Reduction of MMP-2 and MMP-9 activities and protein expression have been shown to inhibit cell migration and invasion in various types of cancer cells (32-38). Moreover, Wang *et al* reported that upregulation of TIMP-1 could inhibit activity of MMP-2 and suppressed HepG2 and MHCC97L metastasis (34). In addition, invasion of hepatocellular carcinoma was inhibited by *Chrysanthemum indicum* ethanolic extract via the imbalance of MMPs and TIMPs (35). This finding supported possible anti-metastatic mechanism of rhodomyrtone in skin cancer.

Several studies have demonstrated the role of the MAPK and PI3K/AKT pathway in regulating MMPs expression (9,39). In this study, we found that rhodomyrtone significantly inhibited the cRaf, ERK1/2 and p38 phosphorylation in A431 cells in a dose-dependent manner (Fig. 7). Likewise, previous reports showed the inhibition of MMP-2 and MMP-9 expression in cancer cells via ERK1/2 pathway (9,32,39,40). Chen *et al* and Chien *et al* showed the expression of MMP-2 and MMP-9 were regulated by p38 α MAPK pathway (41,42). In addition, we showed that rhodomyrtone inhibited FAK and the phosphorylation of AKT (Ser473) and AKT (Thr308) in A431 cells. Previous studies showed the inhibition of cell invasion and migration of human non-small cell lung cancer through FAK/PI3K/AKT signaling pathway (43). Qin *et al* also showed the inhibition of breast cancer cell invasion by suppressing the expression of MMP-2 and MMP-9 through the integrin β 1/FAK/PI3K/AKT/ β -catenin signaling by excisatin A (44). The promoter regions of MMP genes show remarkable conservation of regulatory elements, including AP-1 and NF- κ B (12,13). NF- κ B is constitutively activated in various types of cancer, including breast cancer and has been shown to contribute to the development and progression of tumors including HCC cells (45). Herein, we found that rhodomyrtone significantly inhibited NF- κ B protein expression (Fig. 9). Similarly to the previous result demonstrated that tomatidine inhibited the invasion of A549 cells by reducing MMPs expression via ERK and AKT signaling pathways and NF- κ B activity (46). Consistent with Lu *et al* showed the inhibition of migration and invasion in melanoma cells by α -solanine via JNK, PI3K/AKT and NF- κ B pathway (47).

In conclusion, we demonstrated that rhodomyrtone inhibited cell migration, adhesion and invasion of A431 cells by suppressing MMP-2 and MMP-9 activities and MMP-2 and MMP-9 protein expression. Furthermore, we showed the mechanism of anti-metastatic effect of rhodomyrtone on A431 cells through the inhibition of Raf/ERK, p38 MAPK and FAK/Akt signaling pathways via NF- κ B activities (Fig. 10). These findings reveal that rhodomyrtone is a new therapeutic agent preventing cancer metastasis.

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