Crude extract of *Euphorbia formosana* inhibits the migration and invasion of DU145 human prostate cancer cells: The role of matrix metalloproteinase-2/9 inhibition via the MAPK signaling pathway

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Abstract. Prostate cancer is a common worldwide health problem in males with a poor prognosis due in part to tumor invasion and migration. The crude extract of *Euphorbia formosana* (CEEF) has been used for the treatment of numerous diseases, however, its effects on the migration and invasion of prostate cancer cells have yet to be examined. In the present study, we investigated the effects of CEEF on the migration and invasion of DU145 human prostate cancer cells *in vitro*. The wound healing assay and the Matrigel-uncoated migration assay were used to examine the migration of cancer cells. Western blotting was used to examine the levels of proteins associated with migration and invasion, and gelatin zymography was used to examine the secretion levels of matrix metalloproteinases-2 and -9 (MMP-2/9) from DU145 cells following exposure to CEEF. The results indicated that CEEF suppressed the migration and invasion of DU145 prostate cancer cells and that these effects are exerted in a concentration- and time-dependent manner. CEEF inhibited the ERK1/2, p38, JNK, SOS1, PKC, PI3K and MMP-2/9 protein expression in DU145 cells. The results demonstrated that CEEF suppressed the migration and invasion of DU145 cells through inhibition of the mitogen-activated protein kinase (MAPK) signaling pathway resulting in the inhibition of MMP-2/9 in DU145 human prostate cancer cells.

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Introduction

Prostate cancer is the second leading cause of cancer-related mortality in males worldwide (1-3). In the Western world, prostate cancer is the second leading cause of cancer-related mortality in males due to its high prevalence and metastatic rate (4-5). In Taiwan, prostate cancer is the seventh leading cause of cancer-related mortality (6). Prostate cancer has a poor prognosis due in part to tumor invasion and migration. The propensity of prostate cancer cells to metastasize to the bone greatly reduces the effectiveness of available treatment options (7-9).

It is well documented that cancer patients succumb to the disease primarily as a result of metastasis of the cancer to distant organs (10,11). Metastasis is a multistep process, including cancer cell adhesion, migration, intravasation, extravasation and colonization to a secondary site (12-15). Thus, disruption of the metastatic process is key to reducing cancer mortality in patients. Mitogen-activated protein kinase (MAPK) signaling contributes to human cancer cell migration, invasion and metastasis (16-18). The molecular mechanics of cancer cell migration

has been reported to involve cell cytoskeletal remodeling and focal adhesion dynamics (19,20). Matrix metalloproteinases (MMPs) are important in cell growth, angiogenesis, invasion and metastasis of cancer cells (16,21-23). Therapeutic agents that are capable of inhibiting MMPs may be efficacious. However, studies of MMP inhibitors, including batimastat (BB-94) for rat mammary carcinoma (24,25) and FYK-1388 for human fibrosarcoma have thus far been disappointing due to serious adverse effects (26). Therefore, much attention has been focused on developing new MMP inhibitors.

Crude extract of *Euphorbia formosana* (CEEF) has been used for the treatment of several diseases in the Chinese population (27). However, the effects of CEEF on the migration and invasion of prostate cancer cells has not been examined. In the present study, we investigated the effects of CEEF on the inhibition of cell migration and invasion in DU145 human prostate cancer cells. We demonstrated that CEEF inhibited cell migration and invasion in DU145 cells by suppressing the MAPK signaling pathway resulting in the inhibition of MMP-2/9.

Materials and methods

Cell culture and reagents. The DU145 human prostate cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Minimum Essential Medium (MEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), propidium iodide (PI), trypan blue and Tris-HCl were purchased from Sigma (St. Louis, MO, USA). The crude extract of *Euphorbia formosana* was kindly provided by Dr Kuo (Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan) (16).

Cell viability. DU145 cells ($2x10^5$ cells/well) were seeded in a 12-well plate at 37˚C with 5% CO_2 in MEM (Gibco BRL), supplemented with 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin for 24 h. The cells were incubated with 100 μg/ml CEEF or vehicle control (0.5% DMSO) for 0, 6, 12, 24, 48 and 72 h. The cells were then detached with trypsin, harvested, washed with PBS and stained with trypan blue. The cell number for each treatment was manually counted using a hemocytometer and presented as a percentage of viable cells per ml (28).

Wound healing assay. DU145 cells at a density of $1x10^6$ cells/well were cultured in 10 cm petri dishes until cells reached 90-95% confluency. The surface of the plate was then scratched with a pipette tip and washed three times. Cells were incubated in the absence and presence of CEEF for 0, 6, 12 and 24 h and then imaged using a Nikon Eclipse TS100 microscope (29).

Cell migration and invasion assays. The migration of DU145 cells was determined using Matrigel-uncoated transwell cell culture chambers (8 μm pore size) as described previously (30). DU145 cells were incubated with 0, 25, 50 and 75 μg/ml of CEEF for 24 and 48 h. The upper surface of the membrane containing the non-invaded cells were removed and the invaded cells on the lower surface of the membrane were fixed and stained with hematoxylin and eosin (H&E). Migrating cells in the chamber were counted. For the determination of cell invasion, the same migration assay was used although the membrane was coated with Matrigel (29).

Western blotting assay for the detection of migration and invasion associated proteins in DU145 cells. DU145 cells at a density of $5x10^6$ cells were maintained in a 6-well plate overnight and then treated with 100 μg/ml CEEF for 0, 6, 12, 24, 48 and 72 h. Cells were harvested and lysed with lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol and 0.1% Nonide P-40. The protein concentration of the lysate was determined using the Bio Red kit. Proteins from each sample were separated using sodium dodecyl sulfate-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by electroblotting. The membranes were probed with primary antibodies for 24 h and then washed and stained with secondary antibody for enhanced chemiluminescence (NEN Life Science Products, Inc., Boston, MA, USA) as previously described (29).

Real-time PCR of mRNA expression levels of MMP-2/9 in DU145 cells. Cells ($1x10^6$ cells/well) were placed in 6-well plates and incubated with CEEF (50 μg/ml) for 24 h. The cells were then collected and total RNA was extracted, as previously described (31). Collected RNA samples were reverse transcribed using the High Capacity cDNA Reverse Transcription kit at 42˚C for 30 min according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The primers used were: MMP-2 forward: CCCCAGACAGGTGATCTTGAC and reverse: GCTTGCGAGGGAAGAAGTTG; MMP-9 forward: CGCTGGGCTTAGATCATTCC and reverse: AGGTTGGATACATCACTGCATTAGG; GAPDH forward: ACACCCACTCCTCCACCTTT and reverse: TAGCCAAATTCGTTGTCATACC. An Applied Biosystems 7300 Real-Time PCR system was used for each assay in triplicate and expression fold changes were derived using the comparative CT (threshold cycle) method (31).

Statistical analysis. Experiments were repeated at least three times. Results are shown as the mean ± SD. The Student's t-test was performed to determine the statistical difference between the control- and CEEF-treated groups. $P<0.05$ was considered to indicate a statistically significant result.

Results

CEEF affects the percentage of viable DU145 cells. DU145 cells were treated with 100 μg/ml of CEEF for 0, 6, 12, 24, 48 and 72 h, and the percentage of viable cells was determined by trypan blue exclusion assay. The results are shown in Fig. 1. Fewer viable cells were present with increasing time in CEEF-treated cells when compared with controls.

CEEF inhibits migration of DU145 cells. Monolayers of DU145 cells were scratched and treated with 50 and 75 μg/ml of CEEF for 0, 6, 12 and 24 h, and allowed to recover to determine the rate of migration using the wound healing assay (Fig. 2). CEEF suppressed the migration of DU145 cells. The

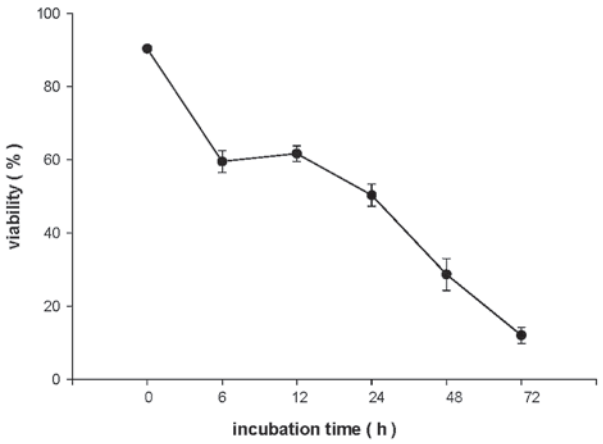

Figure 1. CEEF affects the percentage of viable DU145 cells. Cells in a 12-well plate were treated with 0 and 100 μg/ml of CEEF for 0, 6, 12, 24, 48 and 72 h. Cells were harvested and the percentage of viability was measured using the trypan inclusion assay as described in Materials and methods. CEEF, crude extract of *Euphorbia formosana*.

time required for wound closure of DU145 cells treated with CEEF for 24 h was significantly longer than that for the control cells.

CEEF inhibits the migration and invasion of DU145 cells. The effects of CEEF on the migration and invasion of DU145 cells are shown in Fig. 3. CEEF at concentrations between 25, 50 and 75 μg/ml significantly suppressed the migration of DU145 cells. The percentage of migration inhibition was 40-84% and 60-92% for cells incubated with CEEF for 24 and 48 h, respectively, when compared with the control cells. CEEF inhibited the invasion of DU145 cells (Fig. 3B). Percent inhibition at CEEF concentrations of 25, 50 and 75 μg/ml were 60-90% and 66-94% for 24 and 48 h, respectively, when compared with the controls.

CEEF alters the levels of proteins associated with migration and invasion in DU145 cells. Data shown in Fig. 4A demonstrate that CEEF decreased the levels of ERK1/2, p38, JNK, SOS1, PKC and PI3K protein expression. Protein levels of MMP-2/9 were also reduced by CEEF treatment in DU145 cells (Fig. 4B). Based on these results, CEEF inhibits the ERK and PI3K/AKT signaling pathways which leads to the suppression of MMP-2/9 expression in DU145 cells.

CEEF alters mRNA expression levels of MMP-2/9 in DU145 cells. Cells incubated with 100 μg/ml CEEF for 24 h and mRNA expression levels of MMP-2/9 were determined using real-time PCR. Fig. 5 demonstrates that CEEF significantly inhibits gene expression levels of MMP-2/9.

Discussion

Metastasis is one of the major causes of cancer-related mortality (31-35). At present, there is an intense effort to identify potential therapeutic agents that inhibit metastasis (35-38). We previously demonstrated that CEEF treatment significantly inhibits cell proliferation and induces cell cycle arrest

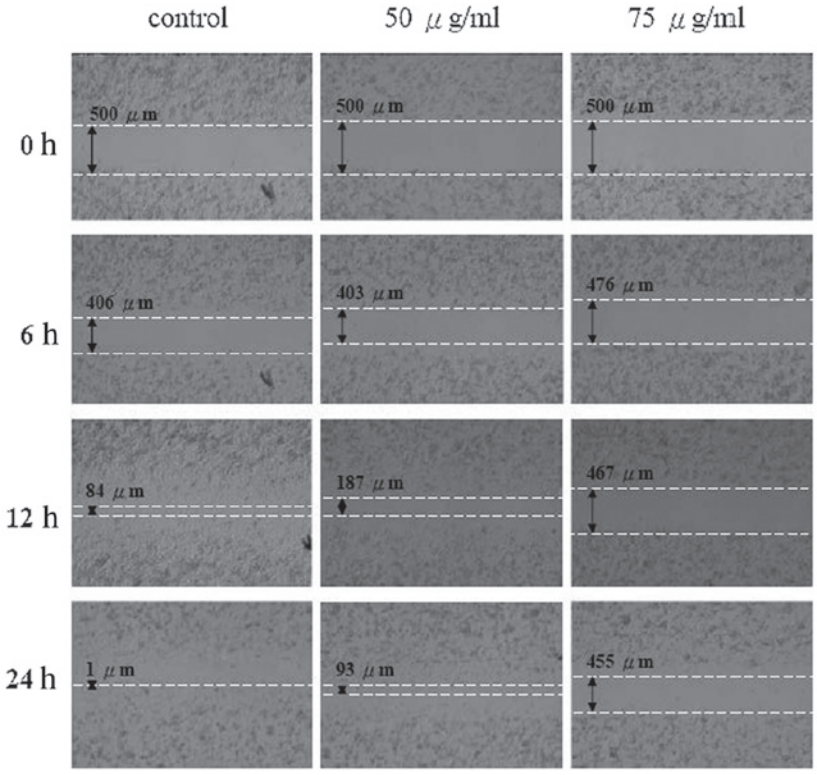

Figure 2. CEEF inhibits the migration of DU145 cells. The migration of DU145 cells was measured using the wound healing assay. Cells in a 6-well plate were treated with 0, 50 and 75 μg/ml of CEEF for 0, 6, 12 and 24 h. Cells in the plate were examined and imaged using a contrast-phase microscope as described in Materials and methods. CEEF, crude extract of *Euphorbia formosana*.

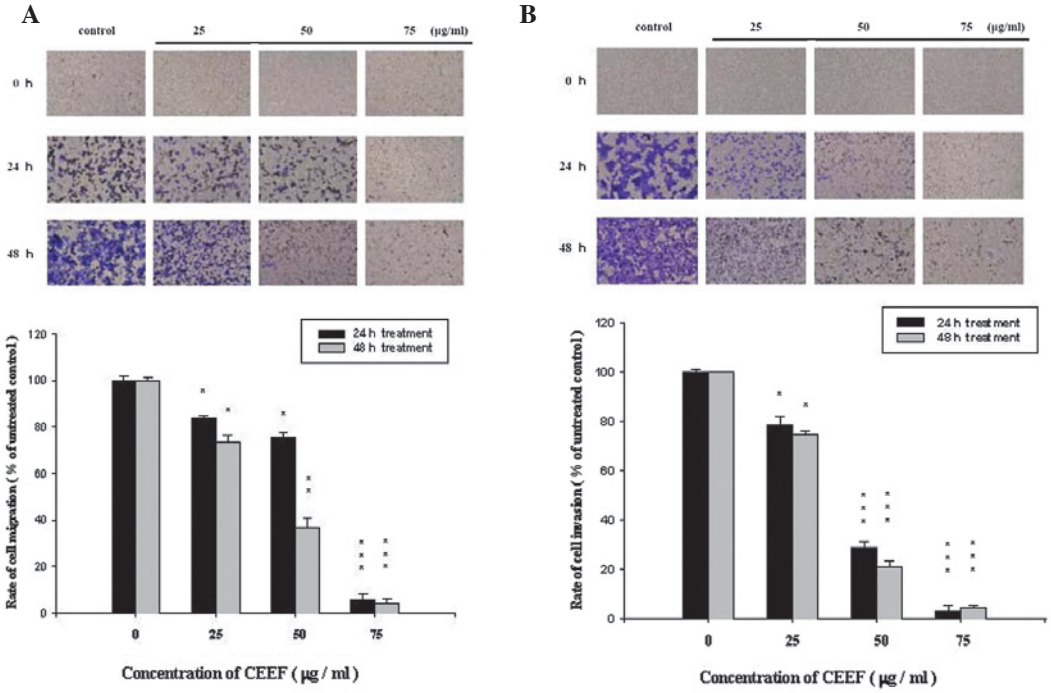

Figure 3. CEEF suppresses the migration and invasion of DU145 cells *in vitro*. Cells that penetrated through (A) without the Matrigel (migration) or (B) with the Matrigel (invasion) to the lower surface of the filter were stained with crystal violet and were imaged under a light microscope at x200. Quantification of cells in the lower chambers was performed by counting cells at x200. Columns repeat the mean from three independent experiments. CEEF, crude extract of *Euphorbia formosana*.

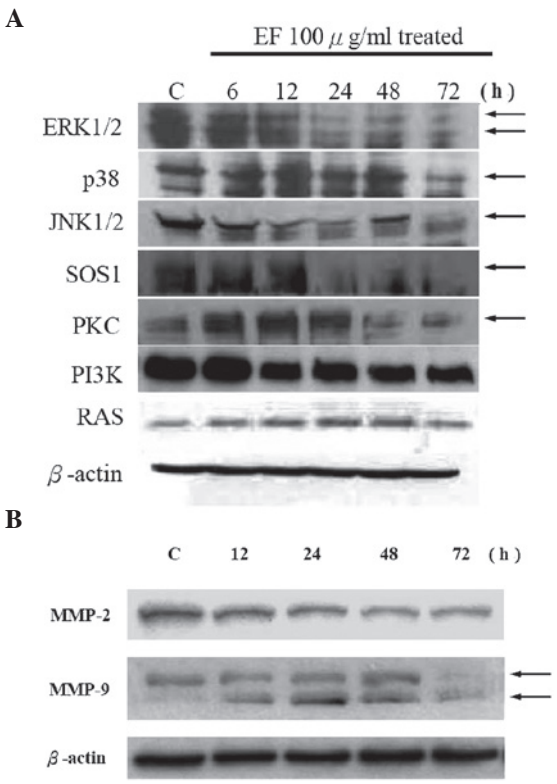

Figure 4. CEEF affects the levels of proteins associated with the migration and invasion of DU145 cells. Cells were treated with CEEF at 0 and 100 μg/ml for 0, 6, 12, 24, 48 and 72 h and then collected. The total protein was obtained as described in Materials and methods. The levels of (A) ERK, JNK, p38, SOS1, PKC and PI3K, and (B) MMP-2/9 expression were estimated by Western blotting as described in Materials and methods. CEEF, crude extract of *Euphorbia formosana*; MMP, matrix metalloproteinases.

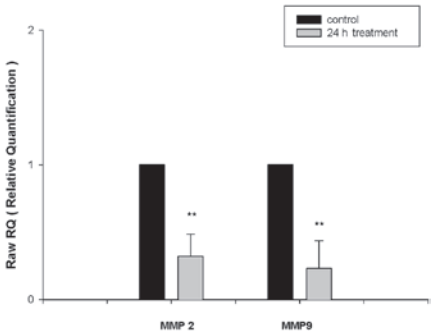

Figure 5. CEEF inhibits the mRNA expression levels of MMP-2/9 in DU145 cells. Cells ($1x10^6$ cells/ml) in a 12-well plate were treated with 100 μg/ml CEEF for 24 h. Cells were washed with PBS and collected. Total RNA was obtained for real-time PCR as described in Materials and methods. Significantly different from the control- and CEEF-treated groups at $^{**}P<0.01$. CEEF, crude extract of *Euphorbia formosana*; MMP, matrix metalloproteinases.

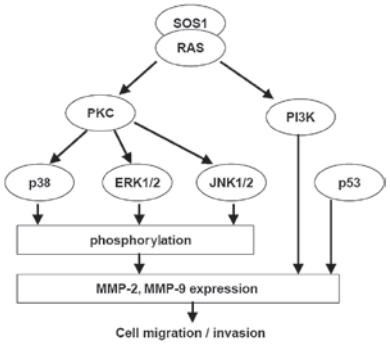

Figure 6. Potential signaling pathways involved in the inhibition of cell invasion and migration of DU145 human prostate cancer cells by CEEF. CEEF, crude extract of *Euphorbia formosana*; MMP, matrix metalloproteinases.

and apoptosis in DU145 prostate cancer cells (data not shown). In the present study, we investigated the mechanisms of the anti-migratory and anti-invasive effects of CEEF in DU145 prostate cancer cells. At the CEEF concentrations used, no effects on the growth rate of DU145 cells were observed (Fig. 1A). However, anti-migratory and anti-invasive effects were observed at CEEF concentrations which did not significantly inhibit cell growth (between 12.5 and 200 μg/ml). We conclude that the effects of CEEF on migration and invasion were not due to cytotoxicity.

It is well known that the uncontrolled degradation of the extracellular matrix and basement is associated with tumor cell invasion and migration, and that MMPs are important in cancer cell migration and invasion (23,27). In the present study, the secretion levels of MMP-2/9 (Figs. 4B and 5) were downregulated by CEEF treatment and these effects were concentration- and time-dependent. Western blotting also demonstrated that MMP-2/9 protein levels were reduced by CEEF (Fig. 5B). Overexpression of MMP-2/9 has been observed in different human cancer types and those proteins are associated with a high potential for metastasis (31,39,40). We hypothesize that a significant downregulation of MMP-2/9 secretion levels in CEEF-treated DU145 cells may be involved in reducing protein levels of MMP-2/9. In addition, CEEF reduced the protein levels of ERK1/2, JNK1/2, p38, SOS1 and PI3K (Fig. 4A). MAPK pathways involving ERK, JNK, p38 and ERK signaling have been reported to upregulate the expression of MMPs (31,41).

A model of the potential action of CEEF on the migration and invasion of DU145 cells is shown in Fig. 6. CEEF may inhibit the migration and invasion of DU145 cells via the MAPK (ERK1/2, JNK1/2 and p38) signaling pathway resulting in the subsequent downregulation of MMP-2/9 expression levels. Future studies are needed to address whether CEEF inhibits tumor migration and invasion in animal models.

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