Abstract. Background: The invasion of cancer cells is critical for metastasis. The effects of Eclipta prostrata, a Thai medicinal plant, on invasion, migration and adhesion of cancer cells were investigated and the anti-angiogenic activity in vivo was evaluated. Materials and Methods: In vitro invasion and migration assays were performed in modified Boyden chambers. In vivo anti-angiogenic activity was determined using the chick chorioallantoic membrane (CAM) assay. Results: E. prostrata juice inhibited cancer invasion and migration, without affecting cell adhesion. Cell migration was inhibited in a variety of cancer cell types and in endothelial cells, with IC_{50} values of 31-70 μg/ml, much lower than the IC_{50} values for cytotoxicity of 203-1,217 μg/ml for cancer cells and >4,000 μg/ml for endothelial cells. Fifty percent inhibition of angiogenesis by E. prostrata juice was observed at 200 μg/egg. Conclusion: E. prostrata juice inhibited cancer and endothelial cell migration in vitro and also showed in vivo anti-angiogenic activity.

Metastasis or the spread of cancer in the body is a multi-step phenomenon, by which cancer cells invade surrounding tissues and blood or lymphatic vessels, enabling them to reach distant organs to form metastatic colonies. Metastasis, rather than primary tumor growth, is a major cause of death in cancer patients. Thus the invasion of cancer cells is an important step in metastasis and is considered to be an important therapeutic target in cancer treatment (1). Antimetastasis therapy is a new approach to cancer treatment, requiring agents to retard the invasion of cancer cells, which have little or no cytotoxic activity, so that they can be used for long-term treatment, in combination with conventional short-term treatment with cytotoxic anticancer drugs (2).

The growth of a solid tumor beyond the size of 1-2 mm³ requires the formation of new blood vessels from pre-existing capillaries at the tumor site (tumor angiogenesis) to provide oxygen and nutrients, as well as an exit path, so inhibition of angiogenesis should reduce tumor growth and metastasis (3). Angiogenesis requires a series of sequential events, so that after activation, endothelial cells proliferate, degrade the basement membrane (BM) and extracellular matrix (ECM), and migrate into new areas, differentiating to form new vascular tube structures. Such events in angiogenesis parallel those in cancer invasion.

Eclipta prostrata Linn (syn: E. alba (Linn.) Hassk., E. erecta Hassk.) is a common weed widely distributed throughout tropical and subtropical countries in Asia. The plant has been used in traditional medicines in India, China and Thailand. In Thai traditional medicine, it has been used as a tonic for liver and blood, and in the treatment of jaundice, liver and spleen diseases, bronchitis and chronically infected skin diseases (4, 5). An anti-inflammatory compound, isolated from the plant, was identified to be wedelolactone, an inhibitor of a crucial kinase enzyme in the activation of inflammation response, IκB kinase (IKK) (6). The plant is also used in self-medication by AIDS patients in Thailand, and a recent report has shown the inhibition of HIV-1 protease and HIV-1 integrase by compounds isolated from the plant (7).

In this study, the effects of the plant on the in vitro invasion, migration and adhesion of cancer cells, as well as on in vivo angiogenesis were investigated using the chick chorioallantoic membrane (CAM) assay in fertilized hen eggs.
Materials and Methods

**Chemicals.** Matrigel was purchased from BD Biosciences (Bedford, MA, USA). Calcein-AM was purchased from Molecular Probes (Eugene, OR, USA). Cell culture media were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Utah, USA). Agarose type I was purchased from Sigma-Aldrich (Steinheim, Germany). Injectable Taxol™ (Paclitaxel) was purchased from Bristol-Myers Squibb (Princeton, NJ, USA), and was diluted with DMSO to a concentration of 5 μg/ml and kept as stock at 4°C. Before testing in bioassays, the stock was diluted with culture media and sterilized by filtration through a 0.2 μm membrane filter. The final concentration of DMSO was kept below 0.2% throughout the study.

**Plant material.** *E. prostrata* plants were collected from Nonthaburi Province in June 2003, and kindly identified by Assoc. Prof. Rungravi Temsiririrkkul of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

The aerial parts of the fresh plants were chopped into small pieces and divided into 3 parts for preparation of juice, aqueous extract and methanol extract. To prepare the juice, 3 kg of chopped plants were ground in a mortar and squeezed through cheesecloth and the juice thus obtained was then lyophilized to yield 58.6 g of powder. To prepare the aqueous extract, the chopped plants (1 kg) were added to hot water (60°C) and maintained at that temperature for 1 hour before cooling overnight, followed by lyophilization to yield 13.6 g of dried extract. To prepare the methanol extract, the chopped plants (1 kg) were macerated with methanol overnight twice at room temperature, and the extract was dried by a rotary evaporator, yielding 16.5 g of dried extract. The extracts were dissolved in water (for the lyophilized juice powder and aqueous extract) or DMSO (for the methanol extract) at 50 mg/ml and kept as stock aliquots at –80°C. Before testing in bioassays, each was diluted with culture media and sterilized by filtration through a 0.2 μm membrane filter. The final concentration of DMSO was kept below 0.2% throughout the *in vitro* studies.

**Cell culture.** HCC-S102 human hepatocellular carcinoma was established from a Thai patient and kindly provided by Dr. Sumalee Tungpradabkul (the Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand). SK-Hep-1 human hepatocellular carcinoma, A549 human lung adenocarcinoma, MDA-MB-231 human breast carcinoma and MRC-5 human embryonic lung fibroblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI 1640 (HCC-S102, SK-Hep-1 and A549) or DMEM (MDA-MB-231 and MRC-5) supplemented with 10% FBS and antibiotics. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA), and cultured in M199 containing 50 μM heparin, 10 ng/ml insulin, 100 ng/ml cortisol, 5 ng/ml vascular endothelial growth factor, 10 ng/ml basic fibroblast growth factor, 0.25 ng/ml epidermal growth factor, 50 ng/ml insulin-like growth factor, 4% human serum albumin, 10% FBS and antibiotics. All the cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Conditioned medium from MRC-5 was collected after 3 days incubation and sterilized by filtration through a 0.2 μm membrane filter. The conditioned media were used as the chemoattractant for invasion and migration assays.

**Cell survival assay.** The number of surviving cells after treatment was determined by crystal violet staining as previously described (8). Briefly, cell suspensions in culture medium were seeded in 96-well plates (100 μl/well), and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 24 hours, additional medium (100 μl) containing the test sample was added to each well, followed by further incubation for 3 days. After that, the cells were fixed with 95% EtOH, stained with crystal violet solution, and lysed with a solution of 0.1 N HCl in MeOH, after which absorbance was measured at 550 nm in a microplate reader. The number of surviving cells was determined from the absorbance. The assays were performed in quadruplicate wells. The data were expressed as percent survival compared with the control.

**Invasion and migration assay.** The invasion and migration of cells were assayed in modified Boyden chambers as previously described with some modifications (8). Briefly, for the invasion assay, fluorescence blocking cell culture inserts with membrane filters of 8 μm pore size (Fluoroblok™; BD Biosciences) were coated with 30 μg of Matrigel on the upper-surface. The cell suspensions containing the test samples (50 μg/ml) were incubated for 30 min, at 37°C, and then seeded into the inserts at 1x10⁵ cells (200 μl)/insert. The MRC-5 conditioned medium (500 μl) containing the same concentration of the test sample was added into the chamber as the chemoattractant. The plate was incubated for 20 hours, at 37°C in a humidified atmosphere of 5% CO₂. The migration assay was performed in the same way except that the insert was used directly without Matrigel coating. The HUVEC migration was assayed in the same manner as the cancer cells, except that culture medium was added into the chamber instead of MRC-5 conditioned medium.

The number of invaded or migrated cells on the under-surface of the insert was determined by the fluorescence technique as previously described (9) with some modification. Briefly, after incubation, the insert was transferred to a new chamber filled with fresh media containing 4 μg/ml calcine-AM, a vital staining calcium-binding fluorescent dye, and further incubated for 1 hour, at 37°C. Then, the insert was washed twice in phosphate-buffer saline (PBS) in the new chambers to remove excess dye, and the fluorescence of calcine-AM labeled cells on the lower-surface of the insert was measured from the bottom of the plate in a microplate reader (PerkinElmer HTS 7000 BioAssay Reader, Perkin Elmer, Wellesley, MA, USA) using 485-nm excitation and 535-nm emission filters. The assays were performed in duplicate wells. The data were expressed as percent invasion or migration compared with the control.

**Adhesion assay.** Adhesion of cancer cells to Matrigel was performed in 96-well plates coated with 30 μg of Matrigel/well. HCC-S102 cells, suspended in culture medium containing the test sample (50 μg/ml), were seeded into the plate at 5x10⁴ cells (100 μl)/well and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 1 hour. Then, the cells were washed with cold PBS and fixed with 95% EtOH, stained with crystal violet solution, and lysed with a solution of 0.1 N HCl in MeOH, after which absorbance was measured at 550 nm in a microplate reader. The number of attached cells was determined from the absorbance. The assays were performed in quadruplicate wells. The data were expressed as percent adhesion compared with the control.
Agarose pellet preparation. The lyophilized powder of the juice was dissolved in water and sterilized by filtration through a 0.2 μm membrane filter, and then mixed with warm agarose solution to a final concentration of 1.8% agarose solution. The sample gel solution (10 μl) was applied onto 5 mm diameter Teflon rods and allowed to solidify.

Chick chorioallantoic membrane assay. In vivo anti-angiogenic activity was assessed by the CAM assay as previously described (10). The anti-angiogenic activity is determined from inhibition of minor blood vessel formation on CAM between day 3 and day 4 of chick embryo development. Briefly, fertilized chicken eggs (White leghorn, Freddy’s Hühnerhof GmbH & Co. KG, Mainz, Germany) were incubated for 3 days in an automatically turning egg incubator (Ehret, Germany) at 37.8˚C and 75% relative humidity. On day 3, 8-10 ml albumen was removed from the eggs via a hole at the base of the egg, and a window was created in the upper surface of the egg to uncover the underlying CAM. Then the window was sealed with transparent tape, the eggs were further incubated at 100% humidity without turning. After 2 hours, agarose pellets containing the sample were placed on the area vasculosa of the CAM, and the eggs were returned to the incubator. After 24 hours, the pellets were removed and the CAMs were photographed using a digital camera attached to a stereomicroscope (Sony XC ST 50 monochrome CCD/Stereomicroscope Leica MZ 7-5, Germany). The images were analyzed by a customized image analysis software Leica QWin™ (Leica Microsystems, Bensheim, Germany) and the areas of blood capillaries (angiogenesis) were calculated. At least 10 eggs for each sample were used for this calculation. The data were expressed as percent angiogenesis compared with the control.

Statistical analysis. The data were analyzed for statistically significant differences between groups by using the Student’s two-tailed t-test. A p-value of lower than 0.05 was considered to be significant.

Results

Effects of E. prostrata juice on invasion, migration and adhesion of cancer cells. At a non-cytotoxic concentration (50 μg/ml), the juice significantly inhibited the invasion of HCC-S102 cells (6% invasion remaining compared to control), while the aqueous and methanol extracts showed moderate inhibition (69% and 64% invasion remaining, respectively) (Figure 1A).

A significant reduction of cell migration was obtained by treatment with the juice (15% migration remaining compared to control), while lesser inhibitory effect was observed by treatment with the aqueous and methanol extracts, where 76% and 72% migration remained, respectively (Figure 1B). Treatment with the juice and the extracts did not inhibit adhesion of the cancer cells to Matrigel (Figure 1C).

Collectively, our results indicated that the plant, either juice or extracts, has inhibitory effects on invasion and migration of cancer cells, with the most effective inhibition being obtained with the juice. Since the juice was more potent than the other extracts in inhibiting cell migration, the juice was therefore used for further studies.

Anti-migration activity of E. prostrata juice against cancer and endothelial cells. Long-term treatment with anti-metastasis therapy requires compounds with little or no cytotoxic activity. E. prostrata juice showed a dose-dependent inhibition of HCC-S102 cell migration in the non-cytotoxic range of concentrations (Figure 2A). In contrast, the inhibitory effect on cancer cell migration by Taxol, a standard cancer chemotherapeutic drug, was obtained in the cytotoxic range of concentrations (Figure 2B).

We further determined anti-migration activity of the juice on various human cancer cell lines of different tissue origins (liver, lung and breast), as well as human endothelial cells. The juice inhibited cell migration in all the tested cancer cell lines (of liver, lung and breast origins) and in the endothelial cells, with IC₅₀ values of 31-70 μg/ml, whereas IC₅₀ values for cytotoxicity were generally much higher but differed between different cell types, as follows: liver cancer (617-1,217 μg/ml);
lung cancer (1.183 μg/ml) and breast cancer (203 μg/ml) (Table I). The ratio of IC₅₀ values for cytotoxicity to IC₅₀ values for anti-migration activity is a useful indicator for predicting anti-metastatic potential of the juice against different cancer types. This ratio ranged between 5-32 folds for the various cancer cell types, as follows: liver cancer (20-32 fold); lung cancer (17 fold) and breast cancer (5 fold) (Table I).

The juice also showed an inhibitory effect on the migration of the HUVEC human endothelial cells (IC₅₀=66 μg/ml), but had very little cytotoxic effect (IC₅₀>4,000 μg/ml) (Table I).

**Effect of E. prostrata juice on angiogenesis in vivo.** The examination of the CAMs of day 4 eggs indicated that the juice showed a dose-dependent inhibition of minor blood vessel formation in the areas under the agarose pellets (Figure 3A), with 32-50% inhibition being observed at dosage ranges of 10-200 μg/pellet/egg (Figure 3B).

**Discussion**

Traditional anti-cancer screening of medicinal plants is focused on cytotoxic agents. Because of their low cytotoxic activity (IC₅₀>100 μg/ml), both the juice and the extracts of *E. prostrata* were ignored in our cytotoxic screening program. However, anti-invasion screening, using a single concentration (50 μg/ml) to screen non-cytotoxic screening samples indicated that *E. prostrata* juice had interesting inhibitory effects on cancer invasion.

The pharmacological activities of *E. prostrata* have been investigated using different extraction methods. Juice prepared from fresh green leaves of the plant have shown a protective effect on carbon tetrachloride-induced liver damage in guinea pigs (11). Methanol extracts of the plant have shown immunomodulatory activity in mice (12). Ethanol extracts of the plant have shown antibacterial and antioxidant activities (13), moreover, they also exhibited analgesic activity in mice (14), and lowered the lipid levels in rats (15). Aqueous and 50% ethanol extracts of the plant have shown neuropharmacological activities in rats (16).

The anti-invasion screening using hepatocellular carcinoma HCC-S102 cells, in the present study, showed strong inhibition by treatment with the juice, while the aqueous and methanol extracts showed moderate effects, suggesting that the juice had higher concentrations of the active ingredients than the other extracts.

The active ingredients in the juice inhibited cancer invasion via suppression of cell migration without an inhibitory effect on cell adhesion to Matrigel. The *in vivo* anti-angiogenic activity of the juice is likely to be, at least in part, due to its anti-migration activity towards endothelial cells as revealed by inhibition of HUVEC migration *in vitro*.

Cell migration is a complex process, by which activation of cell surface receptors after ligand binding stimulates downstream signaling pathways, resulting in cytoskeletal reorganization and modulation of the motility machinery of the cell (17). Medicinal plant extracts appear to have different mechanisms for inhibiting cell migration, for example, the extract of *Wisteria foetida* galls appears to inhibit cancer cell migration by, at least two mechanisms, down-regulating CD44 expression and activating RhoA GTPase (18). This suggests that the process of cell migration provides a variety of molecular targets for the development of anti-metastatic and anti-angiogenic agents.

In conclusion, *E. prostrata* juice has anti-migration activity against a variety of cancer cell types and endothelial cells *in vitro*, as well as also showing anti-angiogenic activity *in vivo*. The juice has potential for use in anti-metastatic and anti-angiogenic treatment. Isolation and characterization of the active ingredients in the juice are being performed. Since the plant has been shown to stimulate the white blood cell count, phagocytic index and antibody titer in mice (12), the
immunostimulating effect combined with anti-metastatic and anti-angiogenic effects make this plant a promising agent for inhibiting tumor progression and enhancement of immune function in cancer patients.

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**References**


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