RESEARCH ARTICLE

Suppression of Human Fibrosarcoma Cell Metastasis by *Phyllanthus emblica* Extract *in Vitro*

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Abstract

***Phyllanthus emblica* (PE) is known to exhibit various pharmacological properties. This study aimed to evaluate the antimetastatic potential of a PE aqueous extract. Cytotoxicity to human fibrosarcoma cells, HT1080, was determined by viability assay using the 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. Cell migration and invasion were investigated using chemotaxis chambers containing membranes pre-coated with collagen IV and Matrigel, respectively. Cell attachment onto normal surfaces of cell culture plates was tested to determine the cell-adhesion capability. The molecular mechanism of antimetastatic activity was assessed by measuring the gene expression of matrix metalloproteinases, MMP2, and MMP9, using reverse transcription-polymerase chain reaction (RT-PCR) assay. The mRNA levels of both genes were significantly down-regulated after pretreatment with PE extract for 5 days. Our findings show the antimetastatic function of PE extract in reducing cell proliferation, migration, invasion, and adhesion in both dose- and time-dependent manners, especially growth arrest with low IC_{50} value. A decrease in the expression of both MMP2 and MMP9 seems to be the cellular mechanism for antimetastasis in this case. There is a high potential to use PE extracts clinically as an optional adjuvant therapeutic drug for therapeutic intervention strategies in cancer therapy or chemoprevention.**

Keywords: *Phyllanthus emblica* - migration - invasion - adhesion - metastasis

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Introduction

Phyllanthus emblica Linn. (PE) or *Emblica officinalis* Gaertn., Euphorbiaceae, is an euphorbiaceous plant grown worldwide in the tropical and subtropical areas. It is commonly known as Indian gooseberry or amla. The plant contains several useful compounds, including phenolic compounds, tannins, phyllembelic acid, phyllembelin, rutin, curcuminoids, and emblicol. Because of really high nutrition, its fruit can be used for dietary source of vitamin C, amino acids, and minerals. All parts of PE have been used for Chinese, Tibetan, and Ayurvedic traditional medicine to treat a range of diseases (Krishnaveni and Mirunalini, 2011).

The improvement of endogenous antioxidant systems in HepG2 cells leads to the hypothesis that it may enhance antioxidant defenses *in vivo* (Shivananjappa and Joshi, 2012). The hepatoprotective mechanism might be due to the function of gallic acid or progallin A isolated from leaves of PE to up-regulate the expression of Bax and down-regulate the expression of Bcl-2 which may block G2/M period in cell life cycle and induce apoptosis of human hepatoma BEL-7404 cells (Huang and Zhong, 2011; Zhong et al., 2011). PE supplementation attenuates N-nitrosodiethylamine-induced liver injury via its antioxidant, anti-inflammatory, antiapoptic and anti-autophagic properties (Chen et al., 2011).

Many other reports mentioned PE in the view of radiomodulatory, chemomodulatory, chemopreventive, free radical scavenging, antioxidant, anti-inflammatory, antimutagenic and immunomodulatory activities. These properties are very useful for the treatment and prevention of cancer (Baliga and Dsouza, 2011; Huabprasert et al., 2012). The antitumor potential of PE extract was proved to inhibit some cancer cell growth, induce apoptosis, decrease mouse skin tumourigenesis and invasiveness (Ngamkitidechakul et al., 2010). Polyphenol compounds in the *Phyllanthus* plant play a major role in chemoprevention, inhibition of the invasion, migration and adhesion of cancer cells, along with the involvement in apoptosis induction (Lee et al., 2011). PE can be used either alone or in combination with other plants (Baliga, 2010; Jain et al., 2011), as it shows synergistic growth inhibitory effects with conventional cytotoxic agents, doxorubicin and cisplatin (Pinmai et al., 2008). This is another interesting point where even the mechanism remains unclear.

In this study, fibrosarcoma cell line, HT1080, was chosen because of its highly invasive activity with the expression of MMP2 and MMP9 (Said et al., 2012). The aim of this research is to study the chemopreventive capabilities of PE extract on fibrosarcoma cells by

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elucidating its effects to cell proliferation, migration, invasion and adhesion including the cellular and molecular mechanism through gene expression of matrix metalloproteinases (MMPs). It could be useful to mention the versatility of PE in cancer chemoprevention via the antimetastatic potential.

Materials and Methods

Crude extract

PE extract was a generous gift from the Research and Development Institute, The Government Pharmaceutical Organization, 75/1, Rama 6 Rd., Ratchathevi, Bangkok, Thailand. Briefly, the fresh fruits were crushed and then extracted with purified water. The extract was filtered and dried by spraying process. The dried extract was stored at 2-10°C, under low humidity and protected from light until used.

Cell culture

The human fibrosarcoma cells, HT1080, were a generous gift from Professor Thompson EW, Department of Surgery, St. Vincent's Hospital, the University of Melbourne, Australia. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 100 µg/ml streptomycin (Gibco BRL). The cells were grown maintained in a tissue culture incubator at 37°C in humidified air containing 5%CO_2 until 80% confluence and then subcultured twice a week.

Cytotoxic assay

To determine the cytotoxic effects of PE extract on the proliferative capability of the human fibrosarcoma cells, we performed this assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously with some modifications (Supabphol et al., 2009). Briefly, the cells were seeded in 24-well tissue culture plates at a density of 4×10^4 cells/well with serum-containing medium and grown to 80% confluence. Then, they were treated with increasing concentrations of PE extract in a humidified atmosphere of 5%CO_2 at 37°C for 24-72 h. After the incubation, the cells were washed twice with phosphate-buffered saline (PBS) and 150 µl of culture medium containing 1 mg/ml of MTT (Sigma) was added to each well and incubated further for 4 h. The medium containing MTT was then replaced with 150 µl of DMSO. The blue crystals of the oxidized MTT (formazan) were quantified by spectrophotometry at 570 nm using ELISA microplate reader (Biotex-synergy-HT). Percentage of survival was plotted against control (untreated) group. Each sample was assayed in triplicate.

Cell migration assay

Migration of fibrosarcoma cells was determined by using 48-well chemotaxis chamber (Neuroprobe Inc. Gaithersburg, MD) and a polycarbonate membrane of 8 µm pore size coated with collagen IV as described previously (Supabphol et al., 2009). Briefly, the cancer cells adjusted to the concentration of 2×10^5 cells/ml in cultured medium without FBS containing PE extract at various concentrations were seeded on the upper wells and allowed to migrate toward the chemoattractant, 0.1% FBS, in the lower wells. After 4-h incubation at 37°C in a humidified atmosphere of 5%CO_2, the remaining cancer cells on the upper surface of the membrane were wiped off with a cotton swab. Cells that did migrate and adhere onto the lower surface were fixed with methanol and stained by using a Diff-Quick Stain Kit (Baxter). The migrated cells attached to the lower side of the membrane were quantitatively assessed and expressed as the mean number of cells in five random fields (40×) under a light microscope. Migration of the untreated fibrosarcoma cells, being suspended on the upper well with DMEM without PE extract, served as a reference value (control) and is referred to as 100% migration.

Cell invasion assay

The direct invasion of fibrosarcoma cells was performed by using 48-well chemotaxis chamber, a membrane invasion culture system (Neuroprobe Inc. Gaithersburg, MD) as migration assay with slightly modifications. The membrane filters were coated with basement membrane extract, Matrigel (Becton Dickinson), and the assays were subsequently performed similar to those of the migration assay.

Cell adhesion assay

The adhesiveness tests were performed as described previously (Supabphol et al., 2009) with some modifications. The cultured cells were trypsinized, washed with serum-free medium, incubated with various concentrations of PE extract for 0, 3, and 5 h. Cells were then reseeded at a density of 4×10^4 cells/well on the ninety-six-well flat bottomed culture plates (Corning) and allowed to adhere for 50 min at 37°C in a humidified atmosphere of 5%CO_2. After washing three times with PBS to remove the non-adherent cells, the adherent cells were fixed with methanol, stained with 0.1% crystal violet (Merck) for 30 min, washed with tap water and air-dried. The stained cells were lysed with 10% acetic acid (Merck) to release the dye which was directly proportional to the number of adherent cells. Dye intensity was quantified by measuring the absorbance at 595 nm using an ELISA microplate reader.

RNA extraction and RT-PCR

Cells were treated with PE extract and total cellular RNA from treated and non-treated fibrosarcoma cells were isolated using Trizol reagent (Invitrogen, USA). The purity and concentration of the isolated RNA were estimated by measuring the absorption at 260 and 280 nm. Same amounts of RNA from each sample were reverse transcribed into cDNA using SuperScript RT kit (Invitrogen, USA) according to the manufacturer's instructions.

The following primers were designed based on the sequences in The GenBank and used to amplify the target genes using i-Taq kit (iNtRON Biotechnology): MMP2: 5'-ACATCAAGGGCATTCAGGAG-3', 5'-AGTTAAAGGCGGCATCCAC-3', 411 bp;

MMP9: 5'-TCTTCCCTGGAGACCTGAGA-3', 5'-CACCAAACTGGATGACGATG-3', 426 bp; β-actin: 5'-AGAGCTACGAGCTGCCTGAC-3', 5'-ACATTGTGAACTTTGGGGGA-3', 622 bp. Amplification products obtained by PCR in thermocycler (C1000 Thermal Cycler, Bio-Rad, USA) were electrophoretically separated on a 2% agarose gel, stained by 2% ethidium bromide and photographed.

Statistical analysis

Statistical analyses were performed with SPSS (IBM Singapore Pte Ltd; Registration No.1975-01566-C). Data are expressed as the mean of at least three individual experiments±standard deviation of control. Statistical significance was considered when $p<0.05$. Statistical comparisons between groups were performed using the Student's t-test.

Results

Effect of PE extract on HT1080 viability

The units used in our studies were μg in viability and RT-PCR assays, and mg in migration, invasion, and adhesion assays, due to the convenience in expressing the mean and standard deviation. Initially, to estimate the toxic doses of PE extract to fibrosarcoma cell growth and viability, MTT was used to differentiate the viable and dead cells. Cell survival decreased significantly ($p<0.05$) with increasing concentration (0, 1, 5, 10, 20, 30, 40, and 50 μg/ml) and exposure time (24, 48, and 72 h) as shown in Figure 1. Half of the cells were dead at PE extract concentration (IC_{50}) of 48.53±3.21, 3.47±0.35, and 3.05±0.15 μg/ml for 24, 48, and 72 h, respectively.

Effects of PE extract on the migration of HT1080

We subsequently investigated the effect of PE extract to HT1080 migration. Figure 2 indicated a dose-dependent manner with obvious reduction of cell migrated through collagen IV-precoated membrane at IC_{50} 0.64±0.07 mg/ml. It seemed to be higher concentration compared to proliferation assay. However, this could be attributed to the short treatment time, only 4 h-incubation, instead of 24-72 h in viability assay.

Effects of PE extract on the invasion of HT1080

As shown in Figure 3, a dose-dependent decrease in a number of cell invading through Matrigel-precoated membrane can be found. Dose-dependent manner was also observed and the obtained IC_{50} was in the same range with migration assay, 0.75±0.06 mg/ml.

Effects of PE extract on the adhesion of HT1080

Figure 4 shows both dose- and time-dependent fashion of the adhesiveness capability of HT1080 to adhere on the new surface after exposed to PE extract with increasing

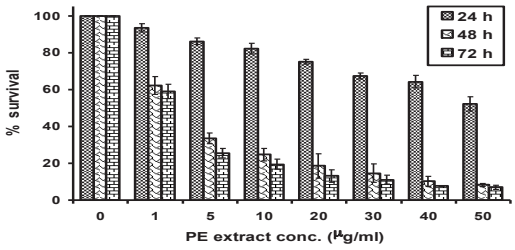

Figure 1. Effect of PE Extract on Proliferation of HT1080. Fibrosarcoma cells were treated with PE extract at the concentration of 0, 1, 5, 10, 20, 30, 40, and 50 μg/ml for 24, 48, and 72 h. PE extract significantly reduced the viability of HT1080 at all concentration with both dose- and time-dependent fashion, *$p<0.05$. Percentage of survival was calculated compared to control (untreated cells). Data are expressed as mean±standard deviation. The percentage of survival at all concentrations of PE were significantly different when compared to that of the control

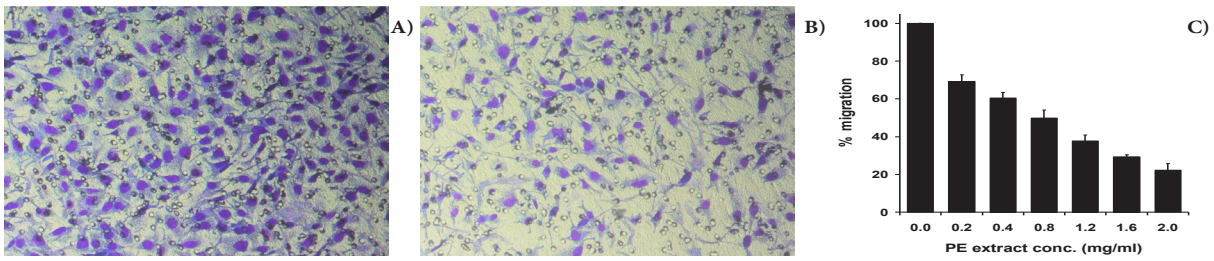

Figure 2. Effects of PE Extract on the Migratory Ability of Human Fibrosarcoma Cells, HT1080. A) Untreated fibrosarcoma cells, 0.0 mg/ml PE extract; **B)** Fibrosarcoma cells were treated with PE extract 1.0 mg/ml; and **C)** Each data point represents mean±SD from three independent experiments. Each experiment was done in triplicate. All values were significantly different from the control

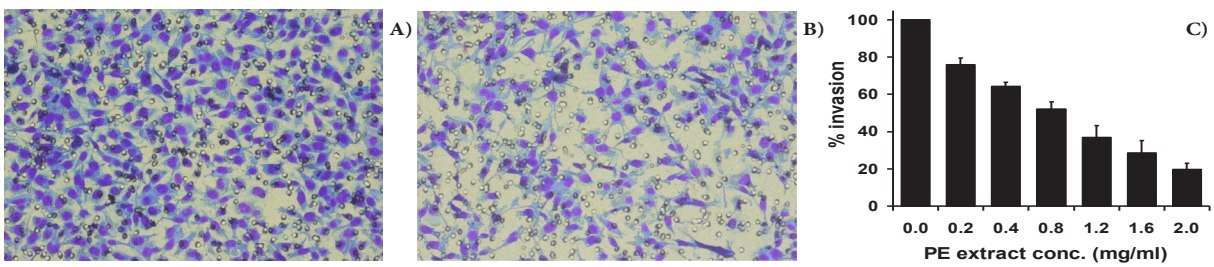

Figure 3. Effects of PE Extract on the Invasiveness Ability of Human Fibrosarcoma Cells, HT1080. A) Untreated fibrosarcoma cells, 0.0 mg/ml PE extract; **B)** Fibrosarcoma cells were treated with PE extract 1.0 mg/ml; and **C)** Each data point represents mean±SD from three independent experiments. Each experiment was done in triplicate. All values were significantly different from the control

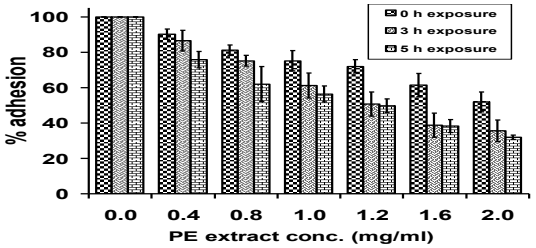

Figure 4. Effects of PE Extract on the Adhesiveness Ability of Human Fibrosarcoma Cells, HT1080. Each data point represents mean±SD from three independent experiments. Each experiment was done in triplicate. All values were significantly different from the control

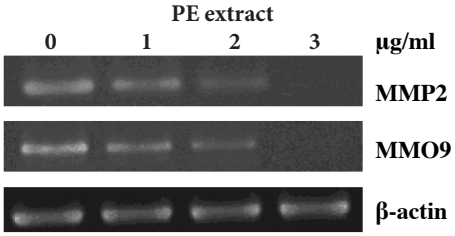

Figure 5. The Effect of PE Extract on the Expression of MMP2 and MMP9 Genes in Human Fibrosarcoma Cells. HT1080 were treated with PE extract for 5 days. Six independent experiments were performed

dose and pretreatment time. IC_{50} values of PE extract able to reduce the attachment of cancer cells are 1.95±0.34, 1.35±0.25, and 1.16±0.28 mg/ml for the pretreatment time 0, 3, and 5 h, respectively.

Effect of PE extract on the expression of MMP genes

The antimetastatic potential at cellular level of PE extract was expected to occur through a change in the MMP activity, MMP2 and MMP9. mRNA of HT1080 exposed to PE extract at 1, 3, and 5 days were extracted for RT-PCR to examine the expression of the two genes normalized to the mRNA level of the housekeeping gene, β-actin. The exposure to PE extract (1, 2, and 3 µg/ml) at 1 and 3 days showed no significant difference of both MMP gene expression compared to control, untreated cells (data not shown). The down regulation of MMP2 and MMP9 expression in HT1080 exposed to PE extract 2 µg/ml was observed when the exposure time was extended to 5 days. MMP2 and MMP9 expression were abolished when the concentration of extract increased to 3 µg/ml as shown in Figure 5.

Discussion

Some cancers can be cured with good prognosis in the early state of the disease. Chemotherapy often results in a high failure rate if the tumor mass is metastasized. Cancer metastasis is the spread of cancer cells to grow in the other organs or tissues and produce the secondary tumor. Detachment and migration from primary cancer mass, invasion through extracellular matrix or basement membrane, and attachment at the remote tissue are the important events that occur before the existence of new secondary tumor mass. The new drug or phytochemical compound possessing capability to inhibit any step or more than one step of metastasis has been interested, especially substance producing non-serious side effect (Supabphol et al., 2009).

Our data obviously showed cytotoxic effect of PE extract to fibrosarcoma HT1080, occurring in a dose- and time-dependent manner. Increasing the exposure time from 24-48 or 72 h (Figure 1), a considerably decrease in cell viability was observed with obvious reduction of IC_{50}. The low concentration of IC_{50} implies to the sensitivity of cancer cells to the extract and cytotoxicity might be a major effect to fibrosarcoma cells. Some other cancer cell lines were previously reported to be sensitive to aqueous PE extract at the range of 50-100 µg/ml with apoptotic marker in extrinsic pathway, caspase 8 and caspase 3/7. PE extract offers an advantage over other conventional chemotherapy as it is non-toxic to normal cells such as lung fibroblast, MRC5 (Ngamkitidechakul et al., 2010; Krishnaveni and Mirunalini, 2011).

Results from migration (Figure 2) and invasion (Figure 3) assays showed the antimetastatic potentials of the extract. Both IC_{50} values were higher than that of cytotoxic assay. It might be due to the shorter exposure time only 4 h-incubation while that of cytotoxic assay was extended to 24-72 h. The lower IC_{50}, not more than 50 µg/ml, was found in 48 h-incubation for invasion assay in the previous report. It is possible that if cancer cells were persistently exposed to the extract as in our body, the IC_{50} value might be down to microgram scale. Moreover, in case that we can do advanced extraction and isolate pure compound, IC_{50} could be down to nanogram scale as found in anticancer drug, taxol (Ngamkitidechakul et al., 2010).

The adhesion of escaped cancer cells from primary cancer mass to remote tissues and organs constitutes another interesting event in cancer research field because it is another crucial step to determine the existence of a secondary cancer mass. Observations in this study showed a significant reduction of adhesiveness with the longer pretreatment HT1080 with PE extract at any concentration (Figure 4). This experiment was done *in vitro* with a static condition of culture media. In the body with a consistent blood flow, we believe that the IC_{50} should be markedly lower.

Tumor microenvironment is considered to be critical for successful outgrowth of a metastatic mass. Several studies had explained that the relevant microenvironment factors include those related to extracellular matrix, reactive oxygen species, and angiogenesis (Catalano et al., 2013). The high antioxidant activities of PE extract in the *in vitro* radical scavenging assays have previously shown potential sources of natural antioxidants (Liu et al., 2008; Charoenteeraboon et al., 2010). Furthermore, the inhibitory effect of this extract on UV-induced ROS and collagen damage in normal human dermal fibroblasts suggest promising cosmeceutical benefits against photoaging (Majeed et al., 2011). The extract was proposed to suit as a natural anti-aging ingredient as it contains type I pro-collagen promoting and anti-collagenase activities in mouse fibroblasts (Fujii et al., 2008; Chanvorachote et al., 2009). Pretreatment with the extract before irradiation showed a significant depletion in lipid peroxidation and elevation in glutathione and catalase levels in mice (Jindal et al., 2009). PE extract can

also inhibit ovarian cell growth both *in vitro* and *in vivo* possibly via inhibition of angiogenesis and activation of autophagy in ovarian cancer (De et al., 2013).

This study focused on the extracellular matrix metabolism and found that the most likely PE extract cellular mechanism seems to occur via MMPs. The reduction of MMP2 and MMP9 expression found provides good clues for future research to test the antimetastatic activity. Previous studies have reported that the PE aqueous extract from fruit contains 30-35% gallic acid equivalents (Naik et al., 2005; Charoenteeraboon et al., 2010). Polyphenols in PE extract may be the key molecules to mediate the MMP expression level as found previously in polyphenols from olive oil (Hassan et al., 2012). Purification of the active ingredients is interesting and valuable for further development of an antimetastatic therapy.

In conclusion, our results show the antimetastatic potentials of PE extract on fibrosarcoma cells via the modification of MMP2 and MMP9 expression. A potent antiproliferative effect is expected to inhibit cancer growth of both primary and secondary mass. The inhibition of migration and invasion through extracellular matrix including the adhesion onto the new surface makes a promising metastatic event. Future studies to delineate the cellular mechanism should be pursued.

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