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Full Paper

Thai perilla (*Perilla frutescens*) leaf extract inhibits human breast cancer invasion and migration.

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Abstract: Thai perilla (*Perilla frutescens*), also called Nga-mon, contains a substantial quantity of bioactive substances including phenolics and flavonoids. These phytochemicals have been linked to various bioactivities of *P. frutescens* such as *in vitro* and *in vivo* anti-inflammatory, anti-oxidative and anti-cancer capacities. In this study we evaluated anti-invasive and anti-migratory activities of Thai perilla leaf extract (PLE) on human breast cancer cells, MDA-MB-231. Our results demonstrate that rosmarinic acid is the main constituent of PLE. *In vitro* cytotoxicity analysis shows that PLE, at 24-hr exposure, is not toxic to MDA-MB-231 cells. A Boyden chamber-based transmembrane assay shows that PLE at a non-toxic dose (12.5-50 µg/mL) dramatically exhibits an inhibitory effect on cell invasion and migration. Gelatin zymography shows that PLE at a concentration of 100–400 µg/mL dose-dependently decreases matrix metalloproteinase-9 (MMP-9) secretion ($p < 0.05$–0.001) and activity ($p < 0.001$). Our data indicate that PLE can inhibit breast cancer cell invasion and migration through the reduction in activity and availability of MMP-9. Our observations also suggest that rosmarinic acid in PLE may account for the anti-invasion and anti-migration activities. In particular, rosmarinic acid as a food-derived chemotherapeutic agent can potentially be used in cancer chemotherapy.

Keywords: Nga-mon, *Perilla frutescens*, human breast cancer, matrix metalloproteinase, rosmarinic acid

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INTRODUCTION

Cancer cell metastasis generally arises from complex multistep processes that are initiated by invasion through the basement membrane and migration to distant sites, followed by adhesion to the endothelial cells of blood vessels, extravasation and colonisation. The critical step that promotes metastasis occurs via an action of proteolytic enzymes against an extracellular matrix (ECM) [1]. The matrix metalloproteinases (MMPs) are well known for their involvement in the degradation of the basement membrane components. In particular, the 72-kDa gelatinase A (MMP-2) and 92-kDa gelatinase B (MMP-9) play an important role in degrading type-IV collagen [1, 2].

Metastasis is seemingly a profound cause of cancer-related death. It has thus become one of the most important targets in cancer treatment. For this reason, inhibition of the ECM-degrading enzymes is an attempt to prevent or delay cancer metastasis. Most anti-cancer drugs are not-sufficiently specific to their targets and possibly involve serious side effects. Hence high efficacy drugs with low toxicity to normal tissues are required. The most likely candidates are among dietary phytochemicals that have anti-cancer properties [3, 4]. Available scientific evidence indicates that flavonoids and phenolics exert extensive *in vitro* anti-invasive and *in vivo* anti-metastatic efficacy [5, 6]. In dietary herbs many flavonoids including catechin, genistein, quercetin, kaempferol, luteolin and apigenin have anti-metastatic activities in tumours [5, 7]. Rosmarinic acid, a phenolic compound found in several plants, has also been reported to inhibit cancer cell metastasis as well as tumour cell growth *in vivo* [8, 9]. To effectively overcome the cancer metastatic cascade, natural phenolics and flavonoids can be considered as important anti-invasion and anti-migration agents against cancer cells.

Perilla (*Perilla frutescens*) is a native dietary and medicinal herb grown in parts of South-east Asian countries including northern Thailand, China, Korea and Japan. Besides being a food ingredient, perilla leaves have been used to treat several diseases including asthma, colds, cough and allergies [10, 11]. Recently, perilla has garnered significant attention due to its abundance of bioactive substances including phenolics and flavonoids such as rosmarinic acid, apigenin and luteolin [12, 13]. Several studies have reported that owing to its phytochemicals content perilla extracts display a range of biological functions including anti-inflammatory [14, 15], antioxidant [16, 17] and anti-allergic activities [18, 19] as well as growth inhibitory activity against cancer cells [20, 21]. Perilla leaf extract (PLE), which contains an abundance of rosmarinic acid, apigenin and luteolin [13], could possibly be involved in cancer metastatic cascade. However, to date the effects of PLE on human breast cancer cell migration and invasion have not been investigated.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

MDA-MB-231 human breast carcinoma cells and NIH3T3 fibroblast cells were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) fetal bovine serum and were maintained at 37°C in an atmosphere consisting of 5% CO_2.

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Preparation of *Perilla frutescens* Extract

P. frutescens leaves were collected from Wiang-Sa district, Nan province, Thailand. A voucher specimen number (QSBG-K2) was certified by the Queen Sirikit Botanic Garden Herbarium, Chiang Mai, Thailand. Fresh leaves were then dried, ground and soaked in 70% ethanol with continuous shaking at room temperature overnight. The extraction was performed twice. The solvent was subsequently removed using a vacuum rotary evaporator. The PLE was freeze-dried and kept at –20°C until use.

Determination of Total Phenolic Content

Total phenolic content was analysed using a Folin-Ciocalteu assay [22]. In brief, 50 mg/mL of PLE was prepared in dimethyl sulfoxide (Sigma, USA). Then 200 µL of PLE at different concentrations was mixed with 1,000 µL of 10% Folin reagent (Merck, Germany) and 800 µL of 7.5% sodium carbonate and incubated at room temperature for 30 min. The absorbance of the solution was measured at 765 nm using a spectrophotometer. A standard curve was prepared using gallic acid (Sigma, USA) as standard and the total phenolic content was expressed as milligram gallic acid equivalent per gram of PLE. The assay was run in triplicate for each sample.

Determination of Total Flavonoid Content

The total flavonoid content was analysed by an aluminium calorimetric method [23]. In brief, different dilution of PLE was mixed with 75 µL of 5% $NaNO_2$ and incubated in the dark at room temperature for 5 min. Then, 150 µL of 10% $AlCl_3$ and 500 µL of 1 M NaOH were added. Deionised water was added to adjust the volume to 2,500 µL. After incubation for 10 min. at room temperature, the absorbance of the supernatant was measured at 510 nm using a spectrophotometer. The procedure was performed in triplicate for each sample. Catechin (Sigma, USA) was used as standard and the total flavonoid content was expressed as milligram catechin equivalent per gram of PLE.

Determination of Apigenin, Luteolin and Rosmarinic Acid in PLE

The high performance liquid chromatography fingerprint of phenolic compounds in PLE was determined using the ultra-high performance liquid chromatography-H class (Waters, USA) analysis equipped with photo diode array detector as described by Theppakorn et al. [24] with modifications. Apigenin, luteolin and rosmarinic acid (Biopurify, China) were used as standards. The PLE and standards were loaded onto a C18-EPS Rocket column (53 mm × 7 mm, GRACE). The isocratic elution was carried out for 5 min. using 0.05% trifluoroacetic acid:acetonitrile at a ratio of 87:13. The flow rate was set at 1.0 mL/min. and the detection of PLE constituents was done at 210 nm.

Effects of PLE on Cell Viability

The MDA-MB-231 cells (1.5×10^3) were plated onto a 96-well plate. Cells were cultured for 24 hr in DMEM containing 10% fetal bovine serum. The PLE at different concentrations in DMEM (0–400 µg/mL) were then added to each well and incubation was continued for 24 and 48 hr. The cell viability was then tested using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA). After incubation, the MTT formazan crystals formed was dissolved

in dimethyl sulfoxide [25]. The absorbance was measured at 540 nm using a microplate reader with reference absorbance at 630 nm.

Effects of PLE, Apigenin, Luteolin and Rosmarinic Acid on MDA-MB-231 Cell Migration and Invasion

The effects of PLE, apigenin, luteolin and rosmarinic acid on the inhibition of MDA-MB-231 cell invasion and migration was determined as previously described by Pintha et al. [25]. Cell migration assay was performed using polyvinylpyrrolidone-free polycarbonate filters (Merck, Germany) coated with 0.01% (w/v) gelatin (Sigma, USA), while the filter coated with Matrigel (Corning, USA) (15 µg per filter) was used for cell invasion assay. The culture medium of NIH 3T3 fibroblast cells was added to the lower chamber to act as a chemoattractant. Then 1.5×10^5 MDA-MB-231 cells were seeded into the upper inserts containing different concentrations of PLE in DMEM (0–50 µg/mL) or 1 µg/mL of apigenin, luteolin and rosmarinic acid in DMEM. The chambers were then incubated at 37°C with 5% CO_2. After 18-hr incubation, cells that migrated or invaded through the lower surface of membrane were fixed with methanol and stained in toluidine blue. Cell images were photographed under phase-contrast microscopy. Indirect quantification was performed by re-dissolving the migrating or invading cells in 20% acetic acid and measuring the absorbance at 570 nm using a microplate reader.

Effects of PLE on MMP-9 Secretion from MDA-MB-231 Cells

Gelatin zymography was applied to analyse the effect of PLE on the secretion of MMP-9 from MDA-MB-231 cells [26]. The MDA-MB-231 cells were treated with different concentrations of PLE in DMEM (0–400 µg/mL). The incubation was carried out in a serum-free medium for 24 hr. The culture supernatant was harvested and subjected to electrophoresis on 10% polyacrylamide gels containing 0.1% (w/v) gelatin. The separating gels were washed with 2.5% (v/v) Triton X-100 and soaked in an activation buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.4) at 37°C for 18 hr. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R followed by de-staining until clear bands against a blue background were observed. The digested bands, representing the proteolytic activity of MMP-9, were measured and analysed using Bio 1D software (Viber Lourmat).

Effects of PLE on MMP-9 Activity from MDA-MB-231 Cells

The activities of MMP-9, which were secreted from MDA-MB-231 cells, were analysed using gelatin zymography as described by Pintha et al. [26]. The MDA-MB-231 cell culture supernatant, from PLE treatment (0 – 400 µg/mL), was subjected to gel electrophoresis on 10% polyacrylamide gels containing 0.1% (w/v) gelatin by loading in equal amounts of total protein. The electrophoretic gels were washed twice with Triton X-100 and cut into strips of single lane width. Each gel strip was re-incubated with PLE (0–400 µg/mL) in activation buffer at 37°C for 24 hr and then stained with 0.1% (w/v) Coomassie Brilliant Blue R. Clear bands, representing digested bands, against a blue background were considered as displaying the proteolytic activity of MMP-9. Bio 1D software (Viber Lourmat) was used to quantify the digested bands.

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Statistical Analysis

Data were shown as mean ± SD. The statistical analysis was determined using one-way ANOVA, and a p value of < 0.05 was defined as significant. Statistical analyses were performed using GraphPad Prism 5.0 software.

RESULTS AND DISCUSSION

Phenolics and Flavonoids Content of PLE

The PLE yield was 15.9±3.8%. The total phenolic content and total flavonoid content of PLE were 242.6±24.3 mg gallic acid equivalent per g of PLE and 296.7±34.3 mg catechin equivalent per g of PLE, respectively. Apigenin, luteolin and rosmarinic acid in PLE were quantitatively analysed by ultra-high performance liquid chromatography and the results are shown in Figure 1. Rosmarinic acid is predominant in PLE (8.8±4.2 %), whereas apigenin and luteolin were presented at 0.053±0.04 % and 0.62±0.40 % respectively.

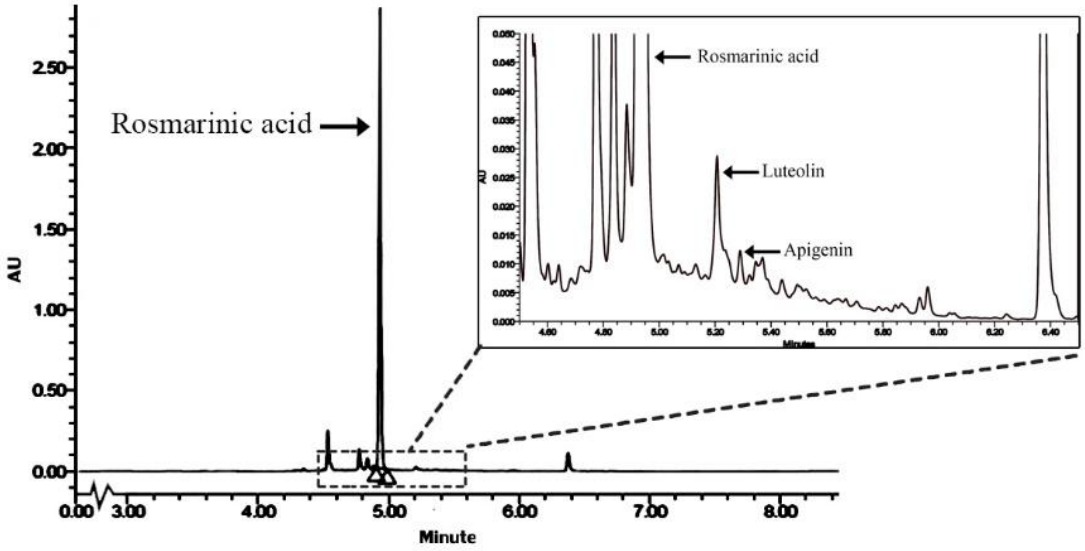

Figure 1. Ultra-high performance liquid chromatography profile of PLE

Cytotoxicity of PLE to MDA-MB-231 Cells

The effects of PLE on the viability of MDA-MB-231 cells were determined by MTT assay and the results are shown in Figure 2. There was no significant effect on the cell viability after PLE treatment for 24 hr. The 20% inhibitory concentration (IC_{20}) and 50% inhibitory concentration (IC_{50}) of PLE were greater than 400 µg/mL. In contrast, MDA-MB-231 cell viability was significantly decreased ($p < 0.001$) after treatment with 100–400 µg/mL of PLE for 48 hr with an IC_{20} value of 92±5.2 µg/mL and an IC_{50} value of higher than 400 µg/mL. These non-cytotoxic concentrations were applied for further study.

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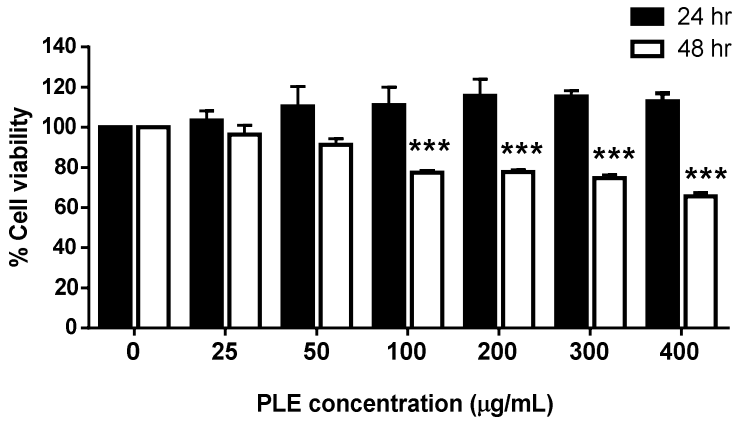

Figure 2. Cytotoxic effects of PLE on MDA-MB-231 cells. Data are presented as mean ± SD of three independent experiments. (*** $p < 0.001$ when compared to control.**)**

Inhibition of MDA-MB-231 Cells Migration and Invasion by PLE

Some studies have shown that PLE exhibits potent anti-oxidative and anti-inflammatory activities due to its phytochemical content including phenolics and flavonoids [27–29]. The inhibitory effects of PLE on the growth, migration and adhesion of human cancer cells have been studied previously, but the mechanism of inhibition remains unclear [10, 30]. Here, we aim to demonstrate the potential bioactive constituents in PLE and their inhibitory effects on human breast cancer cell metastasis. To elucidate the anti-metastatic properties of PLE, its effects on MDA-MB-231 cell invasion and migration were studied. On increasing PLE concentration, MDA-MB-231 cell invasion passing through the Matrigel was significantly reduced, as shown in Figure 3A. The IC_{50} value of PLE was 24.0±1.2 μg/mL. Likewise, the PLE also slightly decreased cell migration, assayed on the gelatin-coated filters, with an IC_{50} value of higher than 100 μg/mL (Figure 3B). The reduction in fixed cells on the membrane can be seen in the photographs shown (Figure 3) as PLE concentration increases. This represents the inhibitory efficiency of PLE against cell migration and invasion.

Effects of Apigenin, Luteolin and Rosmarinic Acid on MDA-MB-231 Cell Invasion

Cells were treated with apigenin, luteolin and rosmarinic acid at a non-toxic dose of 1 μg/mL for 18 hr. At this concentration only rosmarinic acid can effect a more than 30% reduction in the invasiveness of MDA-MB-231 cells (Figure 4). Our results confirm that rosmarinic acid at low concentration (1 μg/mL) can reduce MDA-MB-231 cell invasion, whereas at the same concentration apigenin and luteolin show no effect. These results are consistent with those of previous studies in which rosmarinic acid showed anti-migration activity [8, 31, 32] and inhibited cancer metastasis as well as tumour cell growth *in vivo* [8, 9]. It was also reported to slow down the progression of tumour cell invasion and migration via inhibition of MMP-2 and MMP-9 secretion *in vitro* [8]. Another study reported that a perillaketone-type compound, isoegomaketone, significantly inhibited hepatocellular carcinoma proliferation [33]. However, our study did not identify isoegomaketone in PLE. The potential inhibitory effect of isoegomaketone in PLE on breast cancer cell invasion and migration therefore still needs to be determined.

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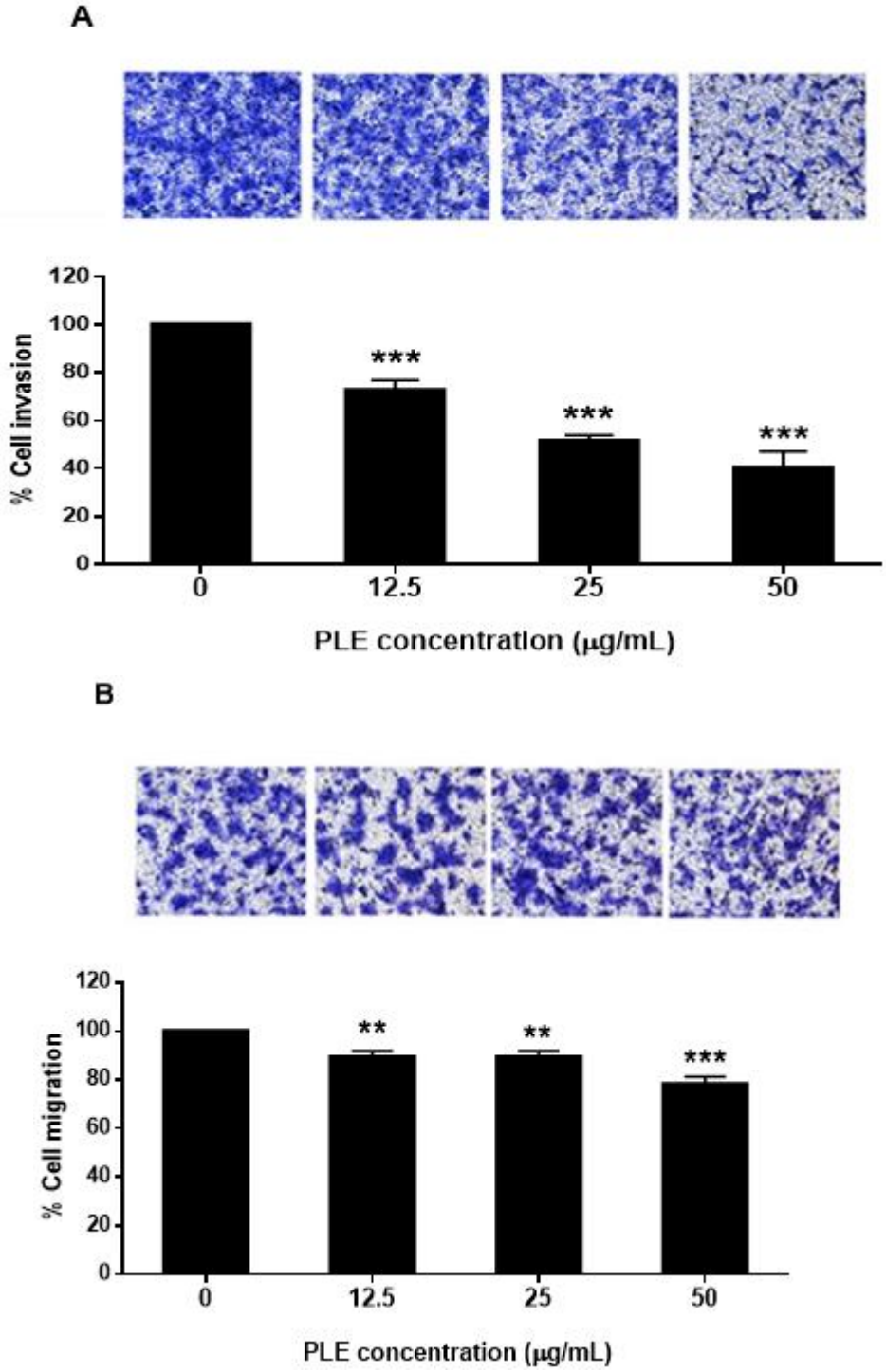

Figure 3. The anti-invasion (A) and anti-migration (B) effects of PLE on MDA-MB-231 cells. . Phase-contrast images of invading and migrating cells are shown in the upper panels. Invasion and migration were expressed as a percentage compared to the untreated control. Data are presented as mean ± SD of three independent experiments. (** $p < 0.01$, *** $p < 0.001$ when compared to control)

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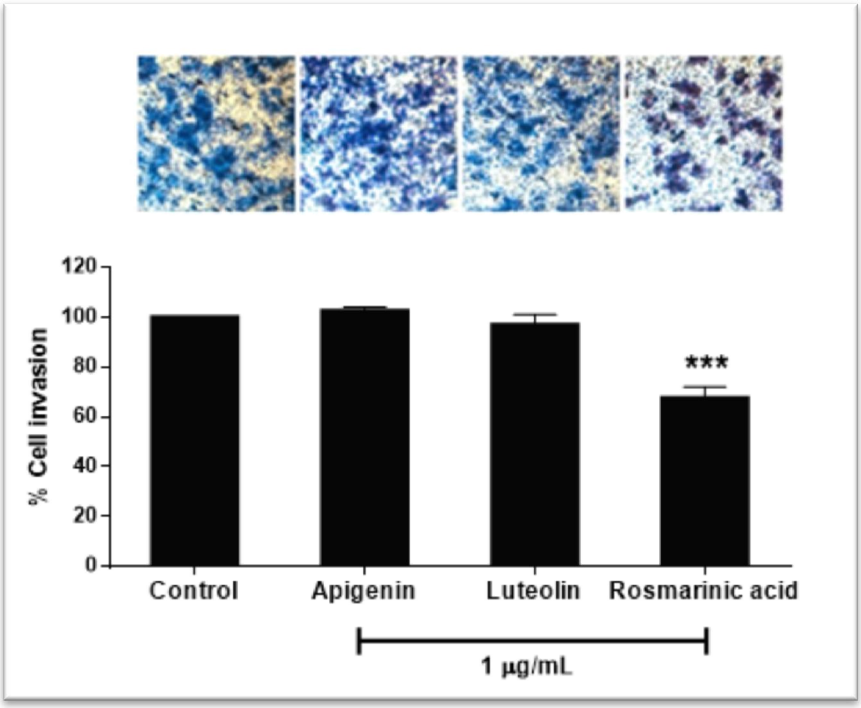

Figure 4. Effects of apigenin, luteolin and rosmarinic acid (at 1 µg/mL) on MDA-MB-231 cells invasion. Phase-contrast images of invading cells are shown in the upper panel. The invasion is expressed as a percentage compared to the untreated control. Data are presented as mean ± SD of three independent experiments. (*** $p < 0.001$ when compared to control)

Reduction of Secretion and Activity of MMP-9 by PLE

The metastatic cascade of cancer cells requires multi-cellular processes, namely cell adhesion, migration, invasion and proteolytic degradation. To prevent the progression of cancer cell metastasis, several studies have targeted at inhibiting MMPs' expression and blocking their activity. Investigating whether PLE can inhibit MMP-9 secretion, we analysed the MMP-9 levels in the culture supernatant from MDA-MB-231 cells treated with PLE by gelatin zymography. Our findings indicate that 21–55% of the MMP-9 secretions are significantly inhibited in MDA-MB-231 cells treated with 100–400 µg/mL of PLE. The IC_{50} value of PLE is 341.0±18.5 µg/mL, as shown in Figure 5.

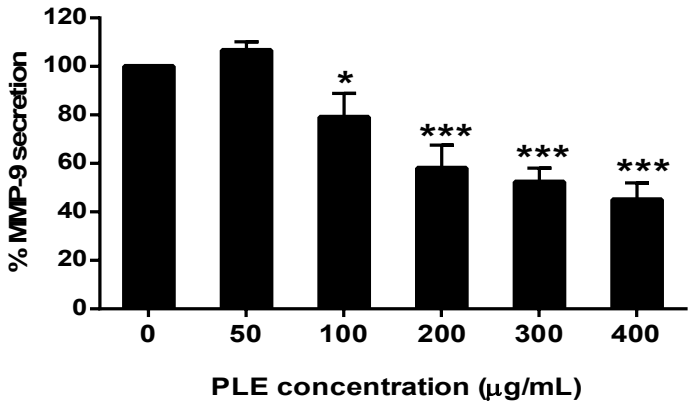

Figure 5. Effects of PLE on MMP-9 secretion from MDA-MB-231 cells. Data are presented as mean ± SD of three independent experiments. (* $p < 0.05$, *** $p < 0.001$ when compared to control)

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To determine the inhibitory effect of PLE on MMP-9 activity, MDA-MB-231 cells were incubated with different concentrations (0–400 μg/mL) of PLE. The result shows that the MMP-9 activity is significantly reduced in a dose-dependent manner (Figure 6). The IC_{50} value of PLE is 191.0±20.9 μg/mL. In another study Bae et al. [34] reported that PLE could reduce the expression of MMP-1 and MMP-3 in human dermal fibroblast cells. The treatment of the human dermal fibroblast cells with PLE markedly inhibited UV-induced MMP-1 and MMP-3 expression levels by suppressing activator protein-1 activation which occurred through a mitogen-activated protein kinase signalling pathway.

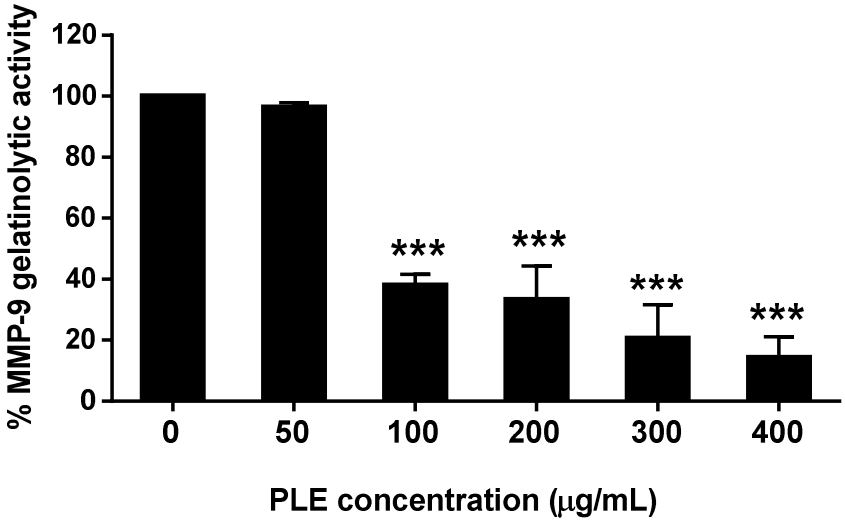

Figure 6. Effects of PLE on MMP-9 activity derived from MDA-MB-231 cells. Data are presented as mean ± SD of three independent experiments. (*** $p < 0.001$ when compared to control)

CONCLUSIONS

We have demonstrated that PLE can inhibit cancer cell migration and invasion. Indeed, it succeeds via two alternative pathways, firstly by decreasing the secretion and activity of ECM-degrading enzymes, and secondly by blocking the cell invasiveness to Matrigel. Rosmarinic acid, the main bioactive compound in PLE, is likely a major contributor in this regard, even though the exact mechanism remains elusive. These findings suggest that PLE at least plays a role in the inhibition of the proteolytic enzymes involved in ECM degradation and consequently reduces the process of migration and invasion of cancer cells.

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