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Brain Derived Metastatic Prostate Cancer DU-145 Cells Are Effectively Inhibited In Vitro by Guava (*Psidium gujava* L.) Leaf Extracts

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Abstract: *The aqueous extract of Psidium guajava* L. *(PE) inhibited the cancer cell DU-145 in a dose- and time-dependent manner. At 1.0 mg/mL, PE reduced the viability of PCa DU-145 (the androgen independent PCa cells) to 36.1 and 3.59%, respectively after 48 h and 72 h of incubations. The absolute cell viability suppressing capability (VSC)*$_{AC}$ *could reach 262.5 cells-mL-h/mg on exposure to PE for 72 h, corresponding to the safe ranges, i.e. the percent viability suppressing rates (PVSR) of 2.72 and 2.41 folds for DU-145 comparing to PZ-HPV-7 cells when treated with PE at 0.5 and 1.0 mg/mL respectively for 72 h. In addition, the colony forming capability of DU-145 cells was apparently lowered. The suppressing rates of which reached 8.09 and 5.96 colony/mg/day for D-145 and PZ-HPV-7 cells, respectively within the concentration range of PE at 0.1∼0.25 mg/mL. Cell cycle arrests at* G_0/G_1 *phase in both cells were observed by TUNEL assay and flow cytometric analysis, yet more prominently evident in DU-145. In addition, suppression of the matrix metalloproteinases MMP-2 and MMP-9, and the upregulation of active caspase-3 at 0.10 to 1.0 mg/mL in DU-145 were also effected in a dose-dependent manner by PE at 0.25 to 1.0 mg/mL, implicating a potent anti-metastasis power of PE. Conclusively, we ascribe the anticancer activity of PE to its extraordinarily high polyphenolic (165.61 ± 10.39 mg/g) and flavonoid (82.85 ± 0.22 mg/g) contents. Furthermore, PE might be useful for treatment of brain derived metastatic cancers such as DU-145, acting simultaneously as both a chemopreventive and a chemotherapeutic.*

Introduction

Psidium guajava L. commonly known as guava, belongs to the Order of Myrtales; Family of Myrtaceae-Myrtle; Genus of *Psidium guajava* L.-guava (1–3). *P. guajava* L. is an important tropical fruit widely spreading in Taiwan, Hawaii, Thailand, Philippines, and Malaysia. It, including the fruits, leaves, and bark, has been traditionally used as folkloric herbal medicines for treatment of a diversity of diseases (1,4). The active components in guava fruits involve ursolic acid, oleanolic acid, arjunolic acid, and glucuronic acid (5), saponin combined with oleandolic acid: morin-3-O-α-L-lyxopyranoside and morin-3-O-α-L-arabinopyranoside; pentane-2-thiol (6,7); and flavonoids: guaijavarin and quercetin (7). Among 16 flavonoids tested, luteolin, possessing the highest antiglycative activity, together with epigallocathechin gallate, kaempferol, quercitrin (quercetin 3-rhamnoside), and peltatoside (quercetin 3-arabinoglucoside), exhibited extraordinary high degree of antiglycative capability. In comparison, huge amounts of β-sitosterol glucoside, brahmic acid, and polyphenolics (165.61 ± 10.39 mg/g dried crude extract) including gallic acid, ferulic acid, and quercetin (2,4); and flavonoids (82.85 ± 0.22 mg/g dried crude extract) (4), and triterpenoids (8) are existing in guava leaves (2,4,8).

Currently, the unique antiglycative effects exhibited by the aqueous extract of guava leaves (PE) have been ascribed to its unusual free radical scavenging and antioxidative capabilities (4,8). As well cited, glycative reactions are relevantly associated with many progressive complications involved in cardiovascular (9,10) and neurodegenerative diseases (11). Hyperglycemic plasma glucose reacts with serum proteins (a reaction named literally as "glycation"), resulting in the formation of an irreversible advanced glycation end products (AGEs) (8,12,13). Experiments have shown that PE at 0.5 mg/mL can reduce over 40% of AGEs formation in bovine serum albumin and plasma LDL induced by glucose, glyoxal,

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and methyl glyoxal, respectively (4). Ishiguro et al. (14) also have shown that the interaction between AGE and AGE receptor (RAGE) is important in prostate cancer development, to inhibit such an interaction can be a new molecular target for cancer therapy or prevention. Even today, guava leaves till remain cited in the Dutch Pharmacopeia, its leaves are still used as an antidiarrheal therapy in Latin America, Central and West Africa, and Southeast Asia (15); and as antihyperglycemic medicines in Taiwan and Japan (5,16). Recently, we have confirmed a nontoxic behavior of PE by the acute toxicity test (2000 mg/kg, single dosage per os, 28 days) (4).

Prostate cancer (PCa) has caused the top second mortality of cancer frequently encountered by males in the United States (17), a comparable incidence of PCa also has risen rapidly in the past two decades in most of the Asian countries. Clinically, androgen-ablation therapy (ABT) is usually a routine methodology for treating PCa, however, many patients relapse thereafter and develop androgen-independent tumors such as DU-145 cells, the brain-metastasis PCa cells. Brain metastasis from PCa is often considered to be a terminal event which represents a poor prognosis, and death usually occurs within few months from diagnosis (18). Some case reports even indicated PCa with a solitary brain metastasis as a sole site of recurrence after a total prostatectomy (19). Moreover, the blood brain barrier (BBB) and the metastatic invasiveness may severely interfere with or lower the drug transport and activity. Eventually, the androgen independent PCa cells may become completely autonomous to androgenic effects on growth by acquiring a distinct set of mutated growth factors (over 80% metastasis has been reported) (20). Consequently, in order to avoid the possible drawbacks that may result from the anti-hormonal therapy, to develop a novel therapeutic or an adjuvant complementary therapeutic approach for such an androgen-independent PCa is currently imperative.

Thus, we evaluated the anti-proliferative effects of PE on the cell line DU-145, using the PZ-HPV-7 cells to serve as the control. Concomitantly, the matrix metalloproteinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), being important regulators of tumor growth at the primary site and in distant metastases, and related with cancer invasion and metastasis (21), were also examined to elucidate the anti-metastatic capability of PE.

Materials and Methods

2.1. Preparation of *Psidium guajava* L. Budding Leaf Extract (PE)

Desiccated budding leaves of *Psidium guajava* L. (200 g) were extracted thrice with boiling water, each time with 200 mL for 30 min, the extracts were combined and filtered through Whatman No. 2 filter papers. The filtrate was lyophilized and pulverized to obtain the solid dried extract (PE) with an yield of about 9.10 g.

2.2. Reagent and Antibodies

Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), trypsin–EDTA, and phosphate buffer saline (PBS) were purchased from Gibco (Langley, OK). Acrylamide and the protein assay kit were obtained from Bio-Rad (Hercules, CA). Polyclonal anti-MMP-2 and anti-MMP-9 were purchased from Sigma Chemicals (St. Louis, MO). Antibodies to caspase-3 were obtained from BD Bioscience (Santa Cruz, CA). An enhanced chemiluminescence (ECL) reagent kit, PhastGel Blue R and protein molecular weight markers were purchased from Amersham Pharmacia Biotech (Piscataway, IL). All other chemicals used in this study were purchased from authentic sources and of highest grade and purity.

2.3. Cell Lines and Cell Cultures

Virally transformed normal human prostate epithelial PZ-HPV-7 cells and human prostate carcinoma DU-145 cells were purchased from the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan, ROC). DU-145 cells were cultured in RPMI 1640 media with 5% FBS and 1% penicillin–streptomycin cocktail (Cellgro, Mediatech, Inc., Herndon, VA) at 37°C in a humidified atmosphere of 5% CO_2. The PZ-HPV-7 cells were cultured in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant EGF and 0.05 mg/mL bovine pituitary extract (Gibco).

2.4. Cell Viability Assay

MTT assay was performed mainly by following the method described by Mosmann (22) yet modified by the manufacturer (Bio-Tek Instruments, VT).

To quantify the results expected from the MTT assay, two calculation methods can be adopted: the percent viability suppressing rate (PVSR) and the absolute cell viability suppressing capability $(VSC)_{AC}$.

2.4.1. The percent viability suppressing rates (PVSR)

$$PVSR = [V_{(t)} - V_{(0)}]/\Delta t \quad (1)$$

where

$V_{(0)}$ is the control percent viability suppressing rates (without PE added)
$V_{(t)}$ is the percent viability suppressing rates (with PE added)
Δt is the time interval for incubation of the cells with a specified concentration of PE

2.4.2. The absolute cell viability suppressing capability $(VSC)_{AC}$

Assume that the proliferation rate per each vital cell is constant during the incubations regardless in the control or

in the PE tested samples, we have

$$(VSC)_{AC} = N_{(0)}[p(t)_c - p(t)_s)]/100[C \bullet \Delta t] \quad (2)$$

where

$(VSC)_{AC}$ = the absolute cell viability suppressing capability (cells-mL-h/mg)
$N_{(0)}$ = the initial cell number inoculated (cell count/well)
$p(t)_s$ = the percent viability of cells per well of the tested sample at time t = t, (cell count/well)
$p(t)_c$ = the percent of viability of cells per well of the control at time t = t, (cell count/well)
C = the concentration of PE in the tested system, (mg/mL), and
Δt = the total length of incubation time, (h)

2.5. Colony-Forming Assay

The anti-proliferative effect of PE on target cells were measured in parallel by a colony formation assay (23). Approximately 500 cells were seeded each onto a Petri dish (6 cm) and allowed to adhere for 24 h. For each incubations, the culture media were changed fresh and the cells were treated with or without PE, and then incubated at 37°C while keeping undisturbed for another 2 days. Thereafter, cells were further cultured with PE-free media and the media were changed every three days. During incubation, individual surviving cells would proliferate and form isolated distinct colonies. On completion of incubation on day 15, the colonies were washed with ice-cold PBS, fixed with ethanol (70%) and then stained with aqueous trypan blue solution (0.02%). The colonies that had 50 or more cells per colony with and without the treatment of PE were counted. The experimentation was performed in triplicates.

2.6. Cell Cycle Analysis

Cells (2×10^4) were cultured in 6-cm culture plates and treated with PE. The floating and adherent cells post trypsinization were collected and washed with ice-cold PBS, fixed and permeabilized with 70% ethanol at –20°C overnight, then incubated with 30 μg/mL propidium iodide (PI) and 100 μg/mL RNase for 30 min at room temperature in the dark after washing with ice-cold PBS on the next day. Ten thousand events per sample were counted and at least triplicate determinations were performed to assure each cell cycle distribution. Data acquisition and analysis were performed in the flow cytometer (FACS Calibur; BD Biosciences, CA) with the accompanying software (CellQuest; BD Biosciences). Appropriate gating was used to select the easily distinguished single population. The final percentages of cells in each phase were calculated from the triplicate experimentations.

2.7. Detection of Apoptosis

For detection of apoptosis, cytocentrifuge preparations were obtained and fixed with 1% formaldehyde, and the TUNEL staining was performed using the ApoDirect *In Situ* DNA Fragmentation Assay Kit (BioVision, CA). Fluorescence microscopy was used to confirm the existence of positively stained cells.

2.8. Western Blotting Analysis

After having been incubated with or without PE, the cells were washed and lyzed following the standard procedure of cell preparation for Western Blotting. The total protein extraction and quantification were performed as directed (BCA protein assay, Pierce, USA). The blotted membranes were incubated with a rabbit polyclonal antibody against the 17-kDa subunit of active caspase-3 as described by Kwong et al. (24). The immunoblotting signals were detected by the chemiluminescence method as instructed (The ECL Western Blotting System, Amersham Pharmacia Biotech). The β-actin was treated as the internal control and the caspsase-3 and β-actin protein amounts in the gel slabs were quantified using a densitometer (ImagePro Plus 5.0 Medica Cybernetics).

2.9. Gelatinolytic Zymography

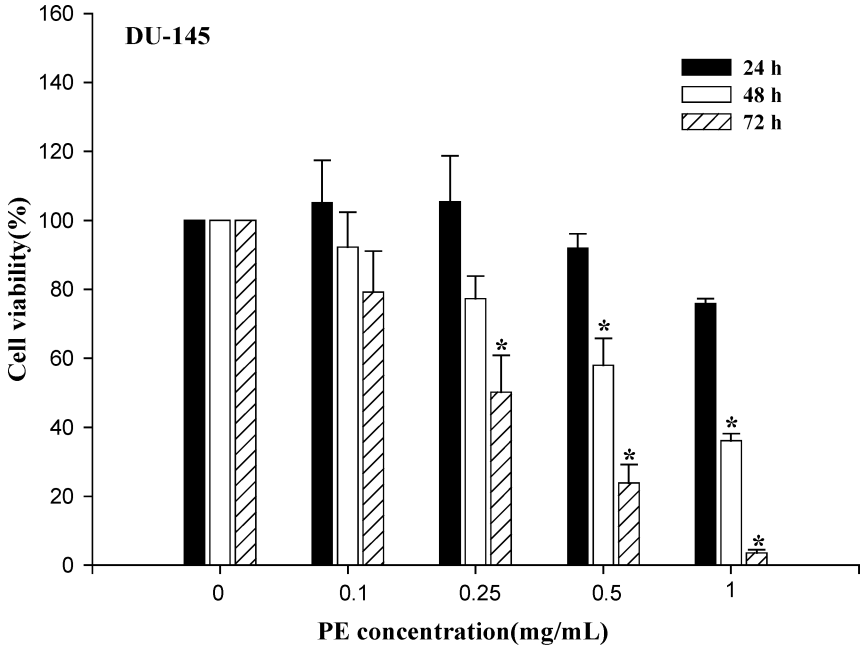
Gelatinolytic zymography was used to detect the expression of MMP in the supernatant obtained from the media with or without the treatment of PE as described by Leber and Balkwill (25). Briefly, the collected media (10 μL) after treatment were loaded on to 10% sodium dodecyl sulfate (SDS)- polyacrylamide gel copolymerized with 0.1% gelatin and subjected to electrophoresis at 100 V for 1.5 h. In order to remove SDS, the gel was washed twice with 2.5% Triton X-100 solution for 30 min each, rinsed with incubation buffer (0.05 M Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$ plus 5 mM $ZnCl_2$), and incubated at 37°C overnight. The gel was stained with PhastGel Blue R at room temperature for 2 h as described by Leber and Balkwill (25). Gelatinases in the media were detected as unstained gelatin degraded zones on the gel and were quantified using a densitometer (ImagePro Plus 5.0 Medica Cybernetics).

2.10. Cell Migration Assay

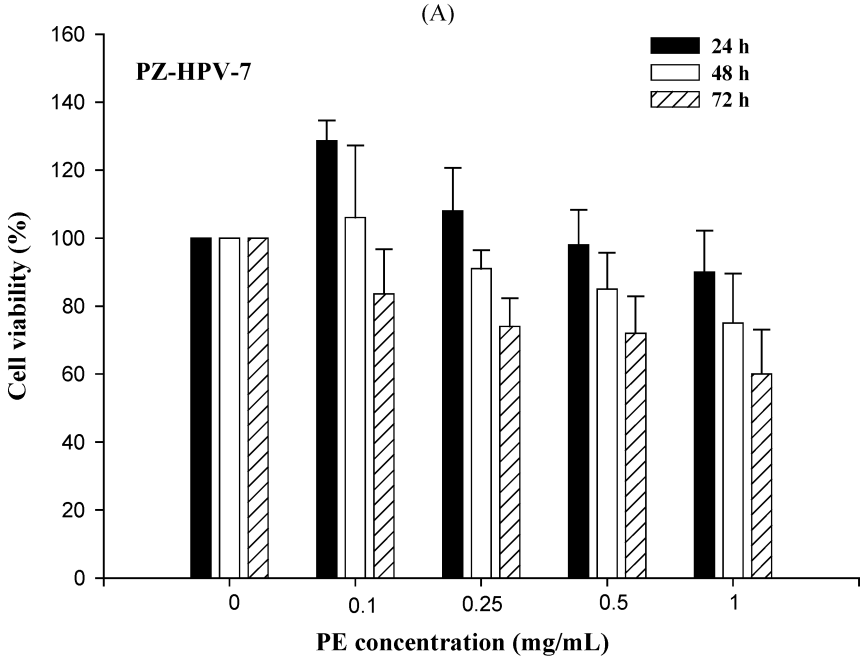
DU-145 Cells were plated at 5×10^4 cells/cm^2 in the upper compartment of 8 μm-pore size Transwell Migration Chamber (Cat. 3422; Corning Inc., USA) and cultured in medium containing PE (0.5 and 1.0 mg/mL) or vehicle alone for 48 h. The cells retained on the upper surface were then removed by wiping with a cotton swab and the filter was gently removed from the chamber and mounted on glass slides. The cells that invaded the filter and attached to the lower surface of the filter were fixed, stained with hematoxylin, and counted in 10 randomly selected microscopic fields (×400) per filter. Similar experiments were repeated in triplicates.

2.11. Statistical Analysis

The values were expressed as means ± SE. The significance between the control and treated groups was determined



(A)



(B)

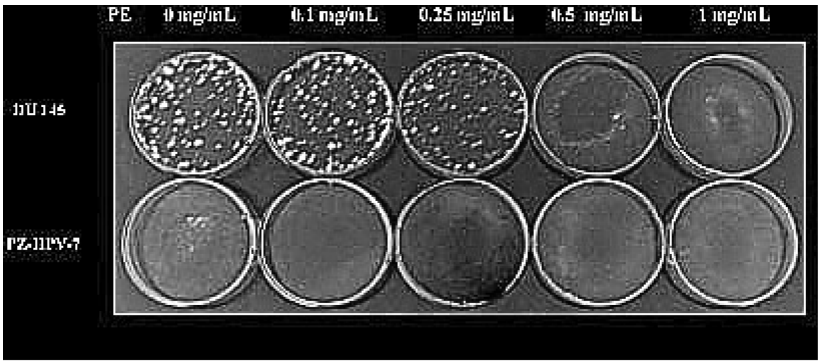
Figure 1. Effect of PE on cell viability in DU-145(A) and PZ-HPV-7 cells (B). The cells were exposed to PE at 0.1, 0.25, 0.50, and 1.0 mg/mL, respectively, for 24 h, 48 h, and 72 h. The viability (%) was determined by MTT assay. Vehicle-treated cells were regarded as 100% control viability. Error bars and all data were expressed in mean ± SD of the triplicates. *: $P < 0.05$, comparison for cell viability between non-PE and PE groups.

by Student's t test and P values less than 0.05 were taken as significant in all the experiments.

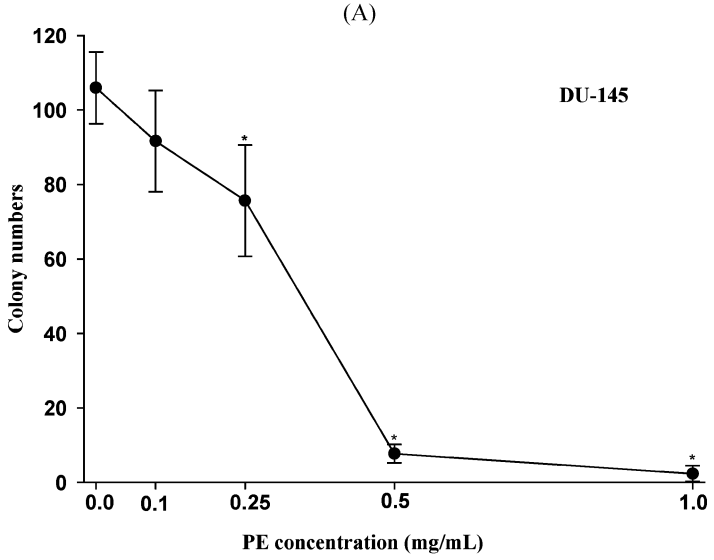
Results

3.1. MTT Assay Discriminated the Normal and the Cancer Cells

As can be seen in Fig. 1A, the viability of DU-145 cells was suppressed in a both dose-and time-dependent manner by PE with 0.0 to 1.0 mg/mL at 24, 48, and 72 h, respectively, post addition of PE. At the initial 24 h with lower concentrations such as 0.1 and 0.25 mg/mL (Figs. 1A and 1B), the two cells proliferated slightly, with averaged increases of about 5.28% and 29.22% respectively for DU-145 (Fig. 1A), and PZ-HPV-7 cells (Fig. 1B) at 0.1 mg/mL (Figs. 1A and 1B). Interestingly, the normal PZ-HPV-7 cells were seen still viable to some extent even at higher concentrations of PE (0.5 and 1.0 mg/mL) up to 72 h (Fig. 1B), whereas significant cell



(A)



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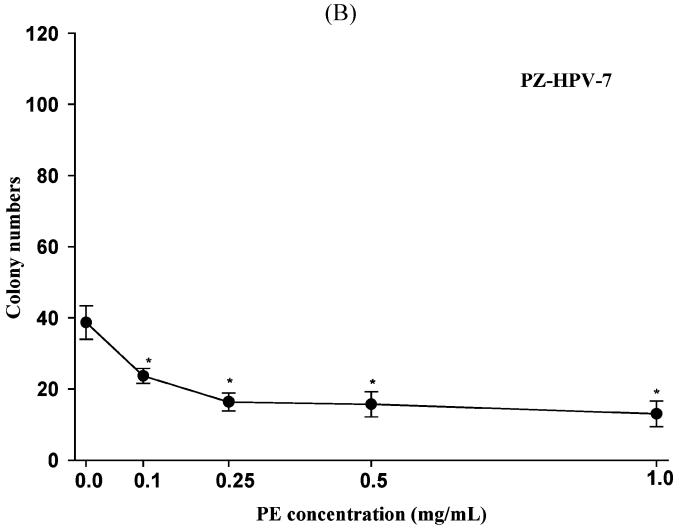
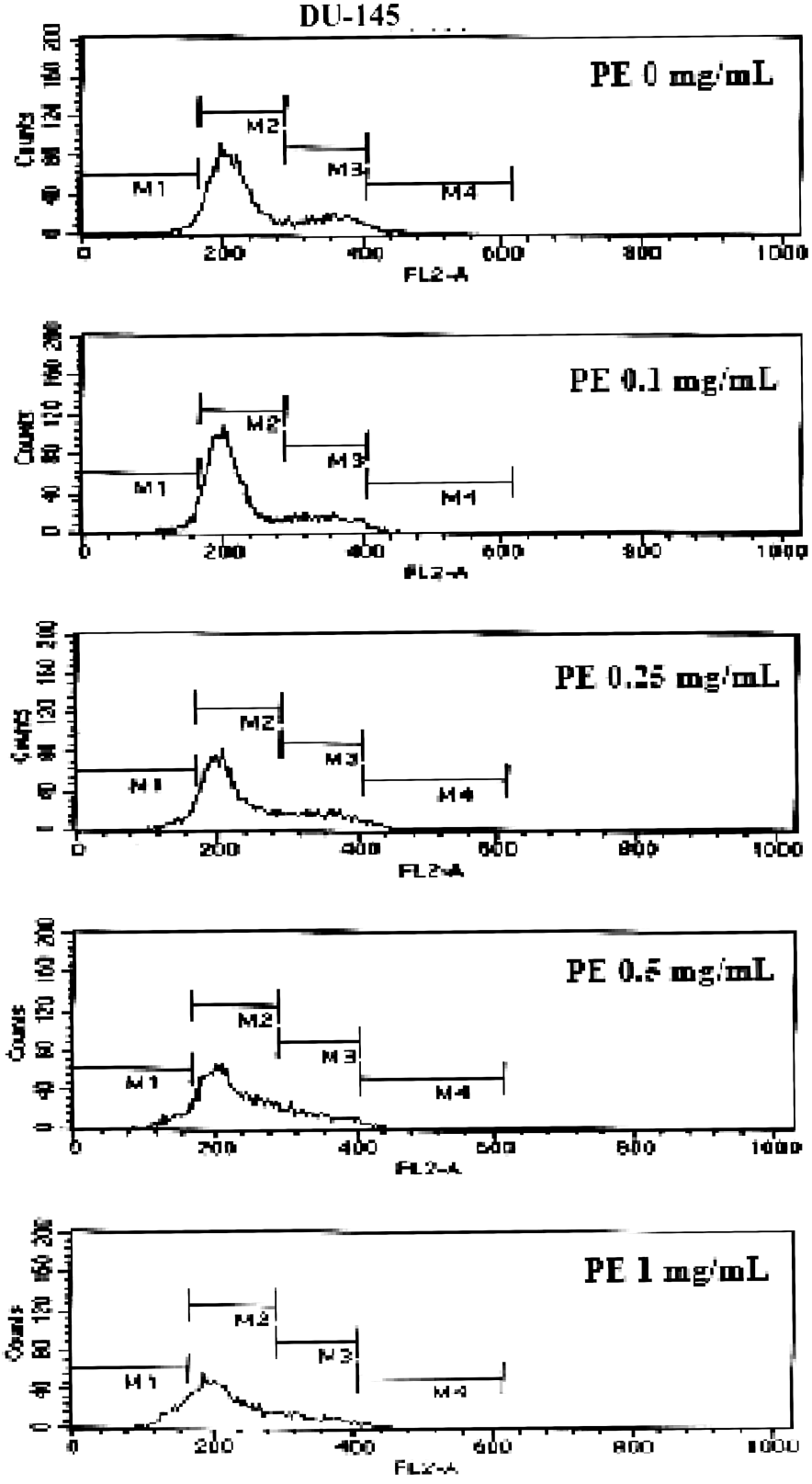


(C)

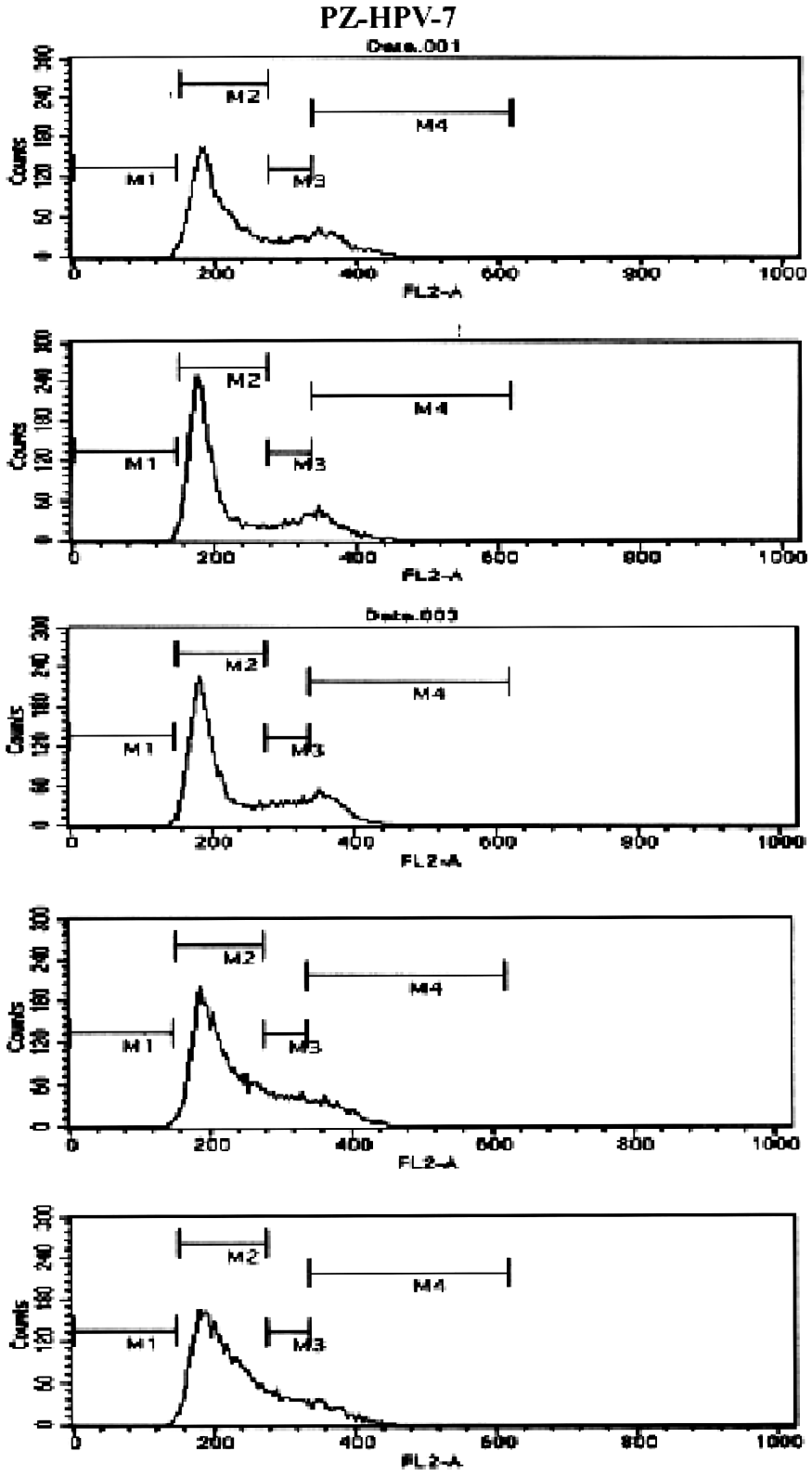
Figure 2. Effect of PE on anchorage-independent growth assay estimated by soft agar colony formation (A); in DU-145 (B); and PZ-HPV-7 cells (C). Cells were grown over agar medium with PE at 0.1, 0.25, 0.50, and 1.0 mg/mL or with vehicle only. The number of colonies was recorded after 14 days of treatment. Each point represents mean number of colonies on soft agar ± SE of triplicates. Asterisks indicate a significance $P < 0.05$ in each cell line.

viability suppressing effect, more prominently at 72 h, was observed in DU-145 by the same concentrations (Fig. 1A). For better quantification of DU-145 effected by PE, the values of PVSR at 72 h were found to be 0.288, 0.6925, 1.057, and 1.389 for PE at concentrations of 0.1, 0.25, 0.50, and 1.0 mg/mL respectively (Fig. 1A). While the corresponding values of PVSR for PZ-HPV-7 cells were 0.361, 0.388, and 0.556 for PE at 0.25, 0.50, and 1.0 mg/mL, respectively. Yet



(A)

Figure 3. Effect of PE on cell cycle in asynchronously growing DU-145 (A); and PZ-HPV-7 cells (B). Growing cells in log phase were incubated in a complete medium with PE of different concentrations for 48 h, stained with PI (30 μg/mL) and analyzed by flow cytometry. Percentages of cells in sub G_1, G_0/G_1, S, and G_2/M phase were calculated as described in Materials and Methods. Data were obtained from triplicates.



(B)

Figure 3. Continued

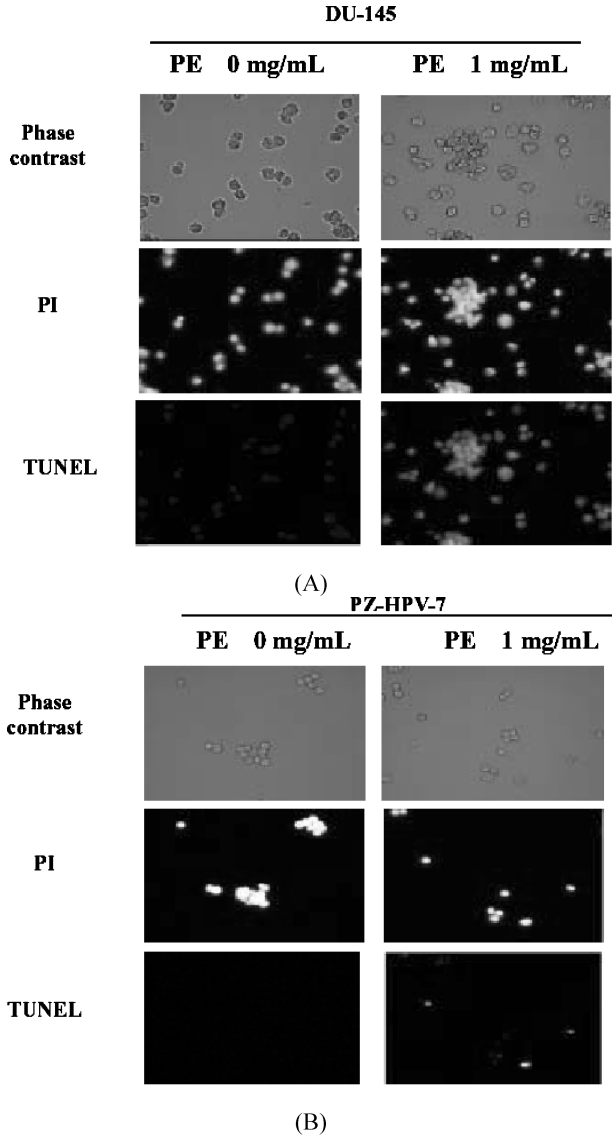


Figure 4. DU-145 (A) and PZ-HPV-7 (B) were treated with 1 mg/mL PE for 48 h and compared with the vehicle control groups. TUNEL assay was performed to detect apoptotic cells. Fluorescence microscopy was used to confirm the existence of positively stained cells, magnification ×100.

the proliferative effect was apparently observed at 48 h for PZ-HPV-7 cells treated by PE 0.1 mg/mL (Fig. 1B). Taking these data at 72 h post treatment with PE 0.5 and 1.0 mg/mL respectively and fitting into Eq. 1, the safe ranges were 2.72 and 2.41 folds respectively comparing the results obtained from the control PZ-HPV-7 (Fig. 1B) with those in DU-145 cells (Fig. 1A).

Alternatively, the absolute cell viability suppressing capability $(VSC)_{AC}$ can be evaluated from Eq. 2. Thus for instance, substitution of the relevant experimental data (not shown here) for DU-145 into Eq. 2 led to

$$(VSC)_{AC} = [(20000 \times 94)\ \text{cells}]/100(1\ \text{mg/mL}) \bullet 72\ \text{h})$$
$$= 262.5\ \text{cells-mL-h/mg}$$

Data indicate that PE at a concentration of 1 mg/mL was capable to kill a minimum of 262.5 DU-145 cells per hour per mg of PE. In summary, DU-145 cells were more susceptible to PE than PZ-HPV-7 cells.

3.2. PE Suppressed Colony Formation More Significantly in DU145

In comparing the effect exerted by PE on the anchorage-independent growth of the PZ-HPV-7 and DU-145 cells, the colony formation assay was performed (Fig. 2A). Correspondingly, the colony formation in the vehicle treated controls reached 106 colonies/field, comparing to those of 91, 75, 7, and 2 colonies/field in DU-145 cells when treated with PE at 0.1, 0.25, 0.5, and 1.0 mg/mL, respectively (Fig. 2B). As a contrast, PZ-HPV-7 cells also demonstrated 23, 16, 16, and 15 colonies/field at PE 0.1, 0.25, 0.5, and 1.0 mg/mL respectively comparing to 38 colonies/field for the vehicle alone treated controls (Fig. 2C). On regression of the two curves within the dosages 0.1∼0.25 mg/mL in Figs. 2B and 2C, the slopes (which indicates the suppressing rate for colony

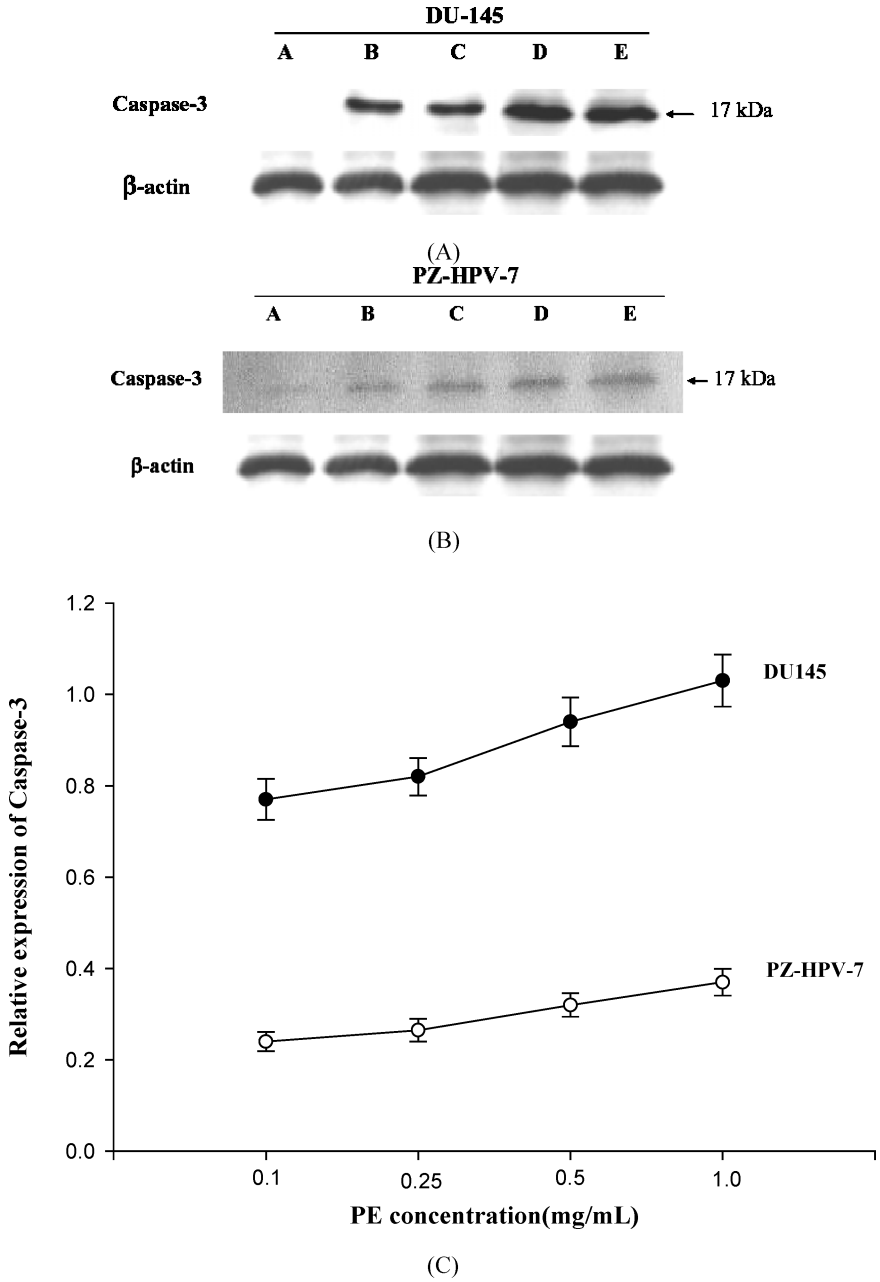


Figure 5. Expression of active caspase-3 in PE-treated DU-145 (A), PZ-HPV-7 cells (B), and the quantified data (relative expression of caspase-3 affected by PE, data expressed by ratio of caspase-3 vs. β-actin (C) when treated with PE at 0∼1.0 mg/mL(A∼E) for 48 h. Total cell protein was collected, 40 μg of which were loaded per lane and subjected to Western blot for active caspase-3.

formation) obtained were 8.09 and 5.96 colony/mg/day, respectively. Apparently an inhibition of 35.7% has been exerted by PE at 0.1∼0.25 mg/mL.

3.3. PE Induces Apoptosis More Significantly in DU145

The cell cycle flow cytometric analysis demonstrated that treatment of PE for 48 h has resulted in an appreciable arrest of these two cell lines at the G_0/G_1 phase. The exposure to PE for 48 h caused an arrest of 6.98% cells in the sub G_1 phase by PE at 0.1 mg/mL, and further increased to 11.5%, 21.9%, and 36.7% at 0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/mL respectively in DU-145 cells (Fig. 3A). Similar yet less affected dose-dependent results were observed in PZ-HPV-7 cells, resulting in 2.3%, 3.8%, 4.7%, and 5.4% arrest in the sub G_1 phase on exposure of the cells to PE at 0.1, 0.25, 0.5, and 1.0-mg/mL (Fig. 3B). The TUNEL assay was used to further confirm that the PE-mediated loss of cell viability in both the PZ-HPV-7 and DU-145 cells could have resulted from apoptosis. As can be seen in Fig. 4A, more nuclear apoptotic cells were found in DU-145 cells treated with PE at 1 mg/mL comparing to those non-PE treated cells. As a contrast, less extent of apoptosis was with PV-HPV-7 cells at the comparable dosages (Fig. 4B). Compared to vehicle treated controls, exposure of PZ-HPV-7 and DU-145 to PE at 1.0 mg/mL for 48 h resulted in a consequence of apoptosis while the

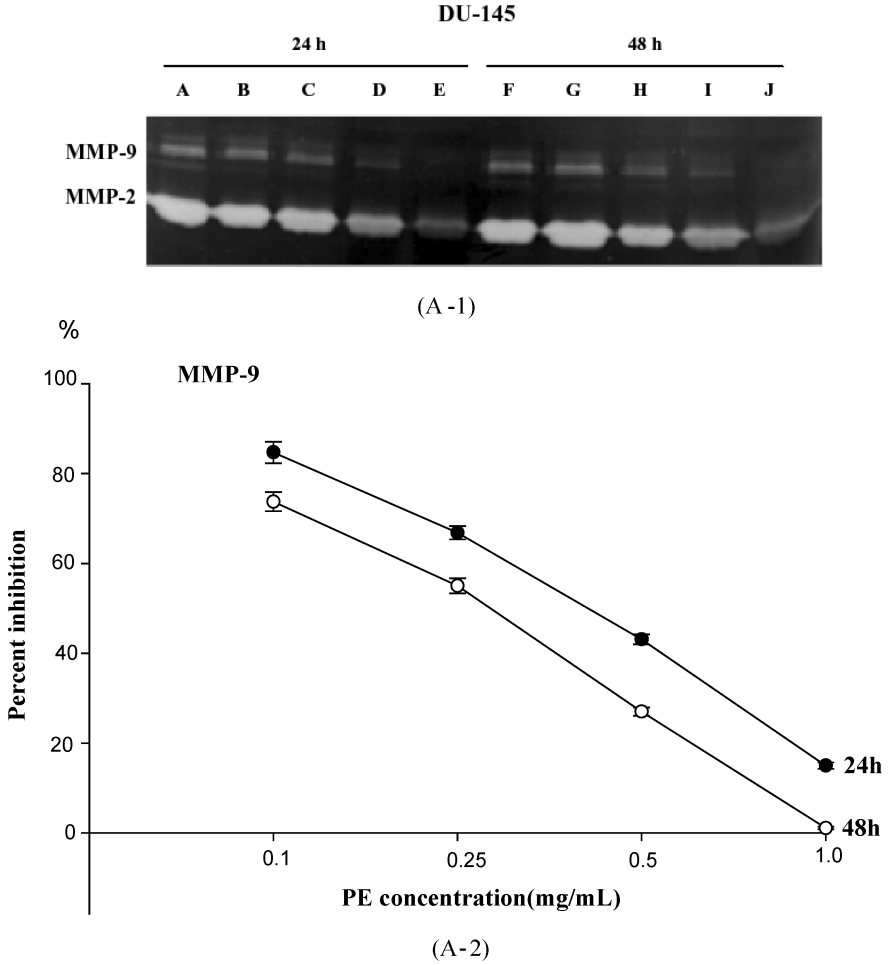


Figure 6. MMP-2 and MMP-9 assay determined by gelatinolytic zymography for DU-145 cells (A-1), their quantified data for MMP-9 (Data was expressed by percent inhibition of MMP-9 under 0.1∼1.0 mg/mL PE treatment) (A-2), and MMP-2 (Data was expressed by percent inhibition of MMP-2 under 0.1 ∼ 1.0 mg/mL PE treatment) (A-3); and for PZ-HPV-7 cells (B). The MMPs in the supernatant (10 μL) obtained from the media with PE treatment at 0 ∼ 1.0 mg/mL(A∼E in 24 h, F∼J in 48 h) were loaded onto a 10% sodium dodecyl sulfate (SDS)- polyacrylamide gel copolymerized with 0.1% gelatin and subjected to electrophoresis at 100 V for 1.5 h. The gel was washed to remove SDS with 2.5% Triton X-100 solution for 30 min, rinsed with incubation buffer (0.05 M Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$plus 5 mM $ZnCl_2$), and incubated at 37°C overnight. The gel was stained with PhastGel Blue R at room temperature for 2 h. Gelatinases in the media were detected as unstained gelatin degraded zones on the gel.

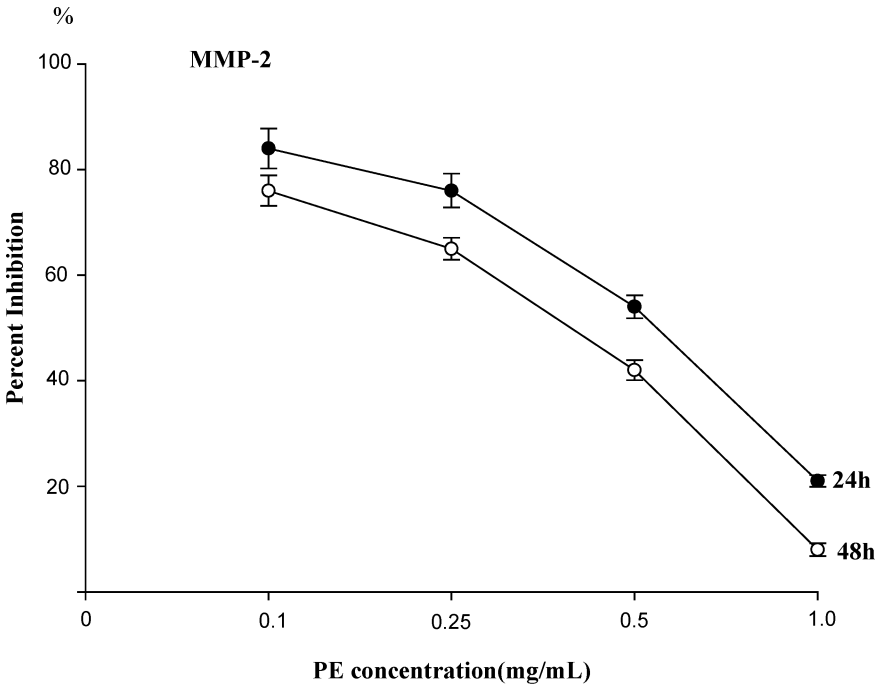
apoptosis was always more significant in DU-145 than PZ-HPV-7 (Figs. 4A and 4B).

3.4. PE Induced The Dose-Dependent Expression of Active Caspase-3 More Significantly in DU-145

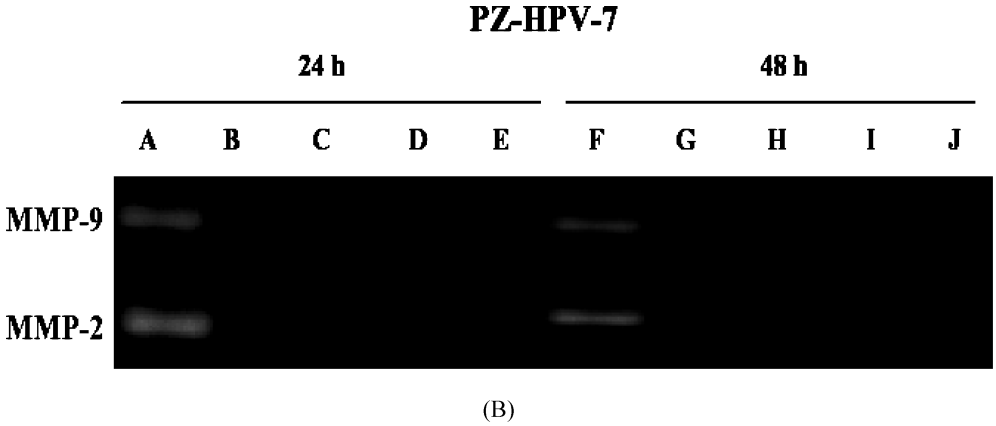
PE at concentrations of 0.1 to 1.0 mg/mL showed a dose dependent inducing capability on active caspase-3 (17 kDa protein band) in both DU-145 (Fig. 5A) and PZ-HPV-7 cells (Fig. 5B), more prominently revealed in DU-145 at dosages of 0.5 and 1.0 mg/mL. Quantitatively, the ratio of caspase protein to that of β-actin were 0.77, 0.82, 0.94, and 1.03; and 0.24, 0.27, 0.32, and 0.37, when treated with PE 0.10, 0.25, 0.50, and 1.0 mg/mL, respectively (Fig. 5C).

3.5. PE Suppressed the Expressions of Both MMP-2 and MMP-9 in DU-145

These two enzymes are normally overexpressed in DU-145 cells (Fig. 6A) compared with PZ-HPV-7 cells that usually exhibit only very low level of MMPs (Fig. 6B). On treatment of DU-145 cells with PE for 24 and 48 h, respectively, the appearance of MMP-9 band first disappeared at 0.25 mg/mL, then steadily downregulated in a dose-dependent fashion until totally abolished at 1.0 mg/mL (shown in column E's and J's incubated for 24 and 48 h, respectively, in Fig. 6A-1). While in the PZ-HPV-7 cells the original non-PE treated cells showed only slight extent of expressions of these two enzymes, which were completely suppressed on addition of PE at 0.1 mg/mL, a dosage far lower than that needed for the suppression of these two MMPs in the DU-145 cells (columns B, C, D and E's and G, H, I, and J's of Fig. 6B). On quantification, the ratio (treated to untreated) were 0.847, 0.668, 0.431, and 0.1560 for MMP-9 after 24 h; and 0.737, 0.550, 0.270, and 0.011 after 48 h on treatment with PE at 0.1, 0.25, 0.50, and 1.0 mg/mL, respectively (Fig. 6A-2). Similar results for MMP-2 were: 0.840, 0.765, 0.543, and 0.217 after 24 h; and 0.761, 0.653, 0.420, and 0.081 after 48 h, respectively effected by PE at 0.1, 0.25, 0.50, and 1.0 mg/mL, respectively (Fig. 6A-3).



(A-3)



(B)

Figure 6. Continued

3.6. Transwell Assay Demonstrates Migration Capability Being Inversely Dose-Dependent to PE in DU-145

The transwell assay is an indication of the metastatic potential of cells. As can be found, on exposure to PE at dosages of 0.5∼1.0 mg/mL for 48 h, DU-145 cells (Fig. 7) were shown to be more motile than PZ-HPV-7 cells (data not shown) when expressed in ratios of the cell number of PE-treated to the cell number of control (Fig. 7). The migration capability of DU-145 was found also to be inversely dose-dependent (Fig. 7). In contrast, we failed to observe such a migration phenomenon in PZ-HPV-7 cells (data not shown).

Discussion

The *Psidium guajava* extract (PE) was confirmed to be very effective in suppressing the viability of cancer cell DU-145, comparing to the normal PZ-HPV-7cells (Figs. 1A and 1B). Such a differential response implicates the potential of PE for treatment of PCa.

The anticancer activity of PE may be ascribed to its extraordinarily high polyphenolic (165.61 ± 10.39 mg/g) and flavonoid (82.85 ± 0.22 mg/g) contents (4) and gallic acid, one of the major active constituents of guava leaf that also has been identified in PE by our group (4), which had shown a very strong dose- and time-dependent growth inhibition and apoptotic death in DU145 cells (26).

The colony formation capability which belongs to a trait of the malignant or transformed phenotype (27) can be considered as the loss of contact inhibition of the cells, resulting in the anchorage-independent cell growth. As shown, PE inhibited the colony formation more significantly in DU-145 than in PZ-HPV-7 cells (Figs. 2A–2C). Attractively, the susceptibility of DU-145 was apparently revealed by the dras-

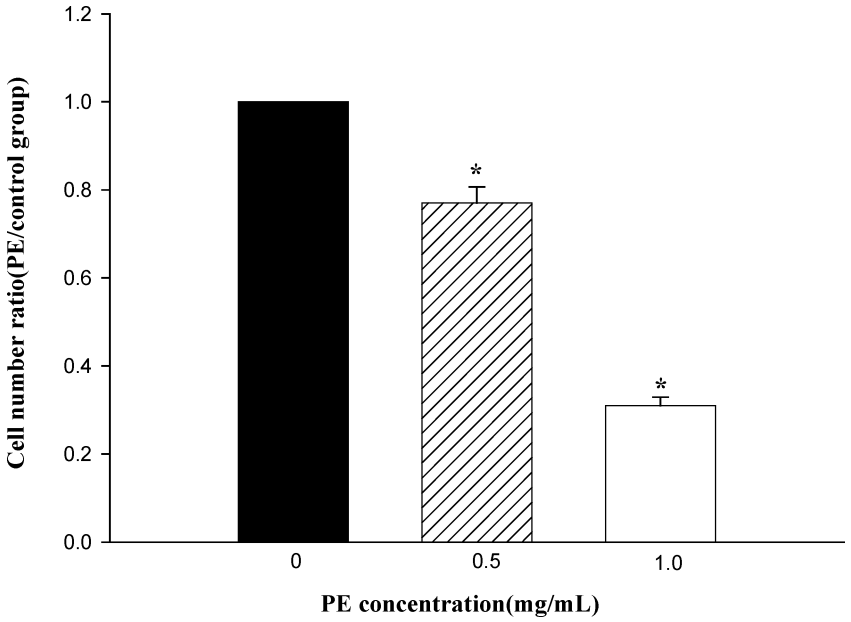


Figure 7. The transwell motility assay for DU-145 cells. Cells (5×10^4 cells/cm^2) were incubated with PE at 0.5 and 1.0 mg/mL for 48 h, respectively. Data were obtained from triplicates, and the motility ratios for cell numbers of PE-treated to that of control group are shown.

tically declining slopes in Fig. 2B than the PZ-HPV-7 cells (Fig. 2C), implicating the more specific targeting of PE on the DU-145 cells than normal cells.

The data obtained from the flowcytometric analysis, further confirmed by TUNEL assay, revealed that the decreases in viability of both the DU-145 and PZ-HPV-7 cells were a consequence of G_0/G_1 arrest and apoptosis, while the apoptosis is more significant in DU-145 than in PZ-HPV-7 cells (Figs. 3A and 3B), indicating that PE may issue simultaneously a powerful chemopreventive and chemotherapeutic effect in treating the refractory androgen independent PCa cells such as DU-145. Moreover, the significant cell population increase in sub-G_1 increase exerted by PE at 0.5 and 1 mg/mL respectively was consistent with the results obtained for colony forming assay shown in Figs. 2B and 2C.

Caspase-3 is the key executioners of apoptosis, as it is either partially or totally responsible for the downstream proteolytic cleavage and induce apoptosis. Agarwal et al. (28) considered caspase-3 to be the central caspase in the GSE-activated caspase cascades, whose activity when blocked may reveal a similar effect on GSE induced apoptosis, a result comparable to the effect induced by general caspase inhibitor. PE has evidently induced apoptotic death of DU-145 cells through caspase-3 activation (Fig. 5A) in a dose-dependent manner, more seriously affected in DU-145 than PZ-HPV-7 cells (judging by the slopes in Fig. 5C), implicating the potential effect of PE to act as an anti-prostate cancer agent. However, the direct evidence of the downstream pathway involved is still obscured, yet may possibly involve the dissipation of mitochondrial membrane potential and cytochrome C release from mitochondria into the cytosol.

The expression of MMPs in the prostate is related to normal and pathological tissue organization changes (29). Normal development of the prostate in the rat is marked by expression of activated MMP-2 during morphogenesis of the gland (30). In human prostate, MMP-2 has been localized by immunohistochemistry to the basal cells, and to a lesser extent to the secretory epithelial cells, but not to stromal cells (31). Primary human prostatic carcinomas express greater levels of MMP-2 and MMP-7 proteins and RNAs than normal prostate (32). While levels of MMP-2 and MMP-9 are increased in higher Gleason grade tumors and in tumors that are no longer organ confined (33,34). Both levels had been found elevated to a greater extent in human prostate cancer cell lines with higher metastatic potential (35). As seen from Figure 6A-1–6A-3, the overexpression of MMPs in DU-145 were steadily inhibited by PE at dosages of $0.1 \sim 1.0$ mg/mL in a dose- and time-dependent manner (Fig. 6A-1–6A-3). In contrast, the MMPs that were only slightly expressed in the normal prostate PZ-HPV-7 cells were readily suppressed on addition of PE at 0.1 mg/mL (Fig. 6B). Such a differential suppression of PE on the two MMPs implies the different action mechanisms and sources of these two enzymes. Overall, PE at 1 mg/mL was the most favorable inhibitory condition for suppressing the DU-145 cells in this regard (Fig. 6A-1–6A-3). Experimentally, down regulation of MMP-9 reduces hematogenous metastasis of murine prostatic carcinoma cells (36), and up-regulation of MMP-9 enhances in vitro invasion of prostate cancer cells (37). As mentioned, matrix metalloproteinases (MMPs) are important regulators of tumor growth, both at the primary site and in distant metastases. Given the clear implications of MMPs in many human cancers, MMPs and their regulatory pathways have been considered promising targets for anticancer drugs and chemopreventive agents (38). Inhibition of metastasis-specific MMP-2 and MMP-9 in DU-145 cells by grape seed

proanthocyanidins (GSP) has been reported to be associated with the inhibition of activation of the MAPK and NFκB pathways (39). Whether a similar mechanism could have been exerted by PE remains to further study.

Finally, the absolute cell viability suppressive capability $(VSC)_{AC}$ was found to be 262.5 cells-mL-h/mg for PE at concentration of 1 mg/mL (from Eq. 2). As well known pharmcokinetically, the differentiated responses of the time- and the dose-dependency in cell viability as found in the control PZ-HPV-7 and the PCa DU-145 cells indeed may strongly support the use of PE as the anti-prostate cancer agent.

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