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Antiangiogenic Potential of Three Triterpenic Acids in Human Liver Cancer Cells

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Three triterpenic acids, oleanolic acid, ursolic acid and maslinic acid, at 2 or 4 μmol/L were used to study their antiangiogenic potential in human liver cancer Hep3B, Huh7 and HA22T cell lines. The effects of these compounds upon the level and/or expression of hypoxia-inducible factor (HIF)-1α, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin (IL)-8, urokinase plasminogen activator (uPA), reactive oxygen species (ROS), nitric oxide (NO) and cell invasion and migration were examined. Results showed that these triterpenic acids at 4 μmol/L significantly suppressed HIF-1α expression in three cell lines ($P < 0.05$); and these compounds at test doses failed to affect bFGF expression ($P > 0.05$). Three triterpenic acids dose-dependently decreased production and expression of VEGF and IL-8, retained glutathione level, lowered ROS and NO levels, and declined cell invasion and migration in test cell lines ($P < 0.05$). These compounds also dose-dependently reduced uPA production and expression in Hep3B and Huh7 cell lines ($P < 0.05$); but these agents only at 4 μmol/L significantly suppressed uPA production and expression in HA22T cells ($P < 0.05$). These findings suggest that these triterpenic acids are potent antiangiogenic agents to retard invasion and migration in liver cancer cells.

KEYWORDS: Angiogenesis; triterpenic acid; liver cancer cell; hypoxia-inducible factor; vascular endothelial growth factor

INTRODUCTION

Angiogenesis, also called as neovascularization, is an important process for tumor growth and metastasis. Liver cancer, also called hepatocellular carcinoma (HCC), is a hypervascular tumor characterized by massive angiogenesis (*1*). Increased circulating levels and/or expression of several angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin (IL)-8 in patients with HCC have been reported (*2*, *3*). Hypoxia-inducible factor (HIF)-1α and basic fibroblast growth factor (bFGF) are two crucial activators responsible for cancer angiogenesis, in which HIF-1α regulates the essential adaptive responses of cancer cells to hypoxia, the major pathophysiological condition promoting angiogenesis; and bFGF mediates extracellular matrix degradation (*4*, *5*). In addition, VEGF through increasing vascular permeability could enhance endothelial cell migration and proliferation, which are required for vascular remodeling and sprouting development of new blood vessels (*6*). Overexpression of urokinase plasminogen activator (uPA), a gene related to invasion, also contributed to HCC angiogenesis (*7*). On the other hand, reactive oxygen species (ROS) and nitric oxide (NO) play important roles in cancer angiogenesis because these free radicals enhance oxidative stress and stimulate VGEF production, which in turn favors cancer progression (*8*, *9*). Obviously, these factors provide vascular support for the growth of already established malignant tumor. Thus, any agents with suppressive effects on the production or expression of these angiogenic factors may be beneficial in retarding tumor progression.

Oleanolic acid, ursolic acid and maslinic acid are pentacyclic triterpenic acids naturally occurring in many herbs and plant foods such as glossy privet fruit (*Ligustrum lucidum* Ait.), hawthorn fruit (*Crataegi Pinnatifidae* Fructus) and olive (*10*, *11*). The inhibitory effects of oleanolic acid and ursolic acid against the growth of human bladder, prostate and colorectal cancer cells have been observed (*12*, *13*). Our previous study (*14*) also found that these two triterpenic acids at 8 μmol/L caused markedly apoptotic effects in four human liver cancer HepG2, Hep3B, Huh7 and HA22T cell lines via increasing DNA fragmentation, decreasing mitochondrial membrane potential and lowering Na^+-K^+-ATPase activity. Juan et al. (*15*) reported that maslinic acid, another triterpenic acid, could inhibit cell proliferation in human colon cancer cell, HT-29. These studies suggest that these triterpenic acids are potent anticancer agents to cause apoptosis or retard metastasis for liver cancer. Therefore, a further study was conducted to examine the antiangiogenic potential of these

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triterpenic acids. The major purpose of our present study was to investigate the effects of triterpenic acids on the production and/or expression of HIF-1α, bFGF, VEGF, IL-8 and uPA in liver cancer cell lines. The influence of these compounds upon the production of ROS and NO in these cell lines was also evaluated.

On the other hand, ursolic acid and maslinic acid present in apple peel have been considered as major compounds responsible for the anticancer activity of apple (*16*). So far, less information is available regarding the presence of these triterpenic acids in fresh vegetables. In order to enrich the food component database, eleven locally available fresh vegetables in Taiwan were used to analyze the content of oleanolic acid, ursolic acid and maslinic acid.

Table 1. Content (mg/100 g dry weight) of Oleanolic Acid (OA), Ursolic Acid (UA) and Maslinic Acid (MA) in Eleven Vegetables, Carrot, Celery, Okra, Eggplant, Spinach, Leaf Mustard, Gynura, Chinese Chive, Fennel, Basil and Daylily[a]

	OA	UA	MA
carrot	25 ± 4	31 ± 6	–[b]
celery	17 ± 2	–	–
okra	61 ± 6	23 ± 3	40 ± 4
eggplant	53 ± 4	32 ± 3	84 ± 7
spinach	167 ± 13	133 ± 8	126 ± 11
leaf mustard	135 ± 11	92 ± 6	174 ± 12
gynura	–	45 ± 6	37 ± 5
Chinese chive	–	–	38 ± 4
fennel	54 ± 8	17 ± 5	–
basil	96 ± 7	83 ± 12	35 ± 4
daylily	118 ± 10	76 ± 5	105 ± 9

[a] Data are mean ± SD, $n = 6$. [b] Too low to be detected.

MATERIALS AND METHODS

Chemicals. Oleanolic acid (OA, 99%), ursolic acid (UA, 98%) and maslinic acid (MA, 98.5%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Medium, plates, antibiotics and chemicals used for cell culture were purchased from Difco Laboratory (Detroit, MI, USA). All chemicals used in these measurements were of the highest purity commercially available.

Content of Triterpenic Acids. Eleven locally available fresh vegetables, carrot (*Daucus carota* L.), celery (*Apium graveolens* L.), okra (*Hibiscus esculentus*), eggplant (*Solanum melongena* L.), spinach (*Spinacia oleracea* L.), leaf mustard (*Brassica juncea*), gynura (*Gynura bicolor* DC.), Chinese

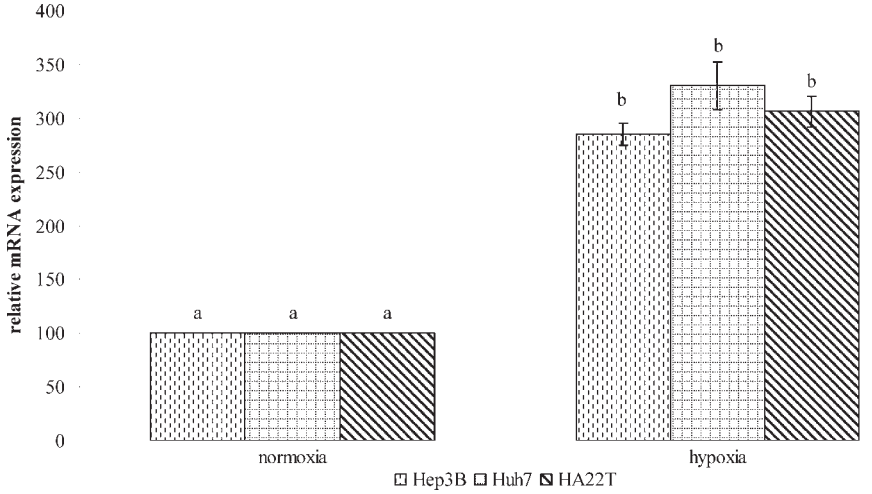

Figure 1. HIF-1α mRNA expression in human Hep3B, Huh7 and HA22T cell lines under normoxia and hypoxia condition. Data are mean ± SD ($n = 9$). Means among bars without a common letter (a, b) differ, $P < 0.05$.

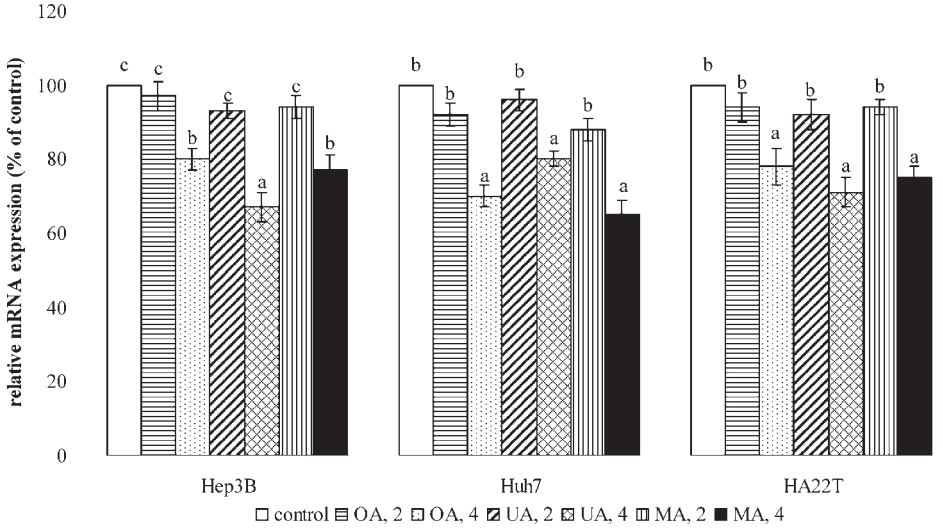

Figure 2. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon HIF-1α mRNA expression in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD ($n = 9$). Means among bars without a common letter (a–c) differ, $P < 0.05$.

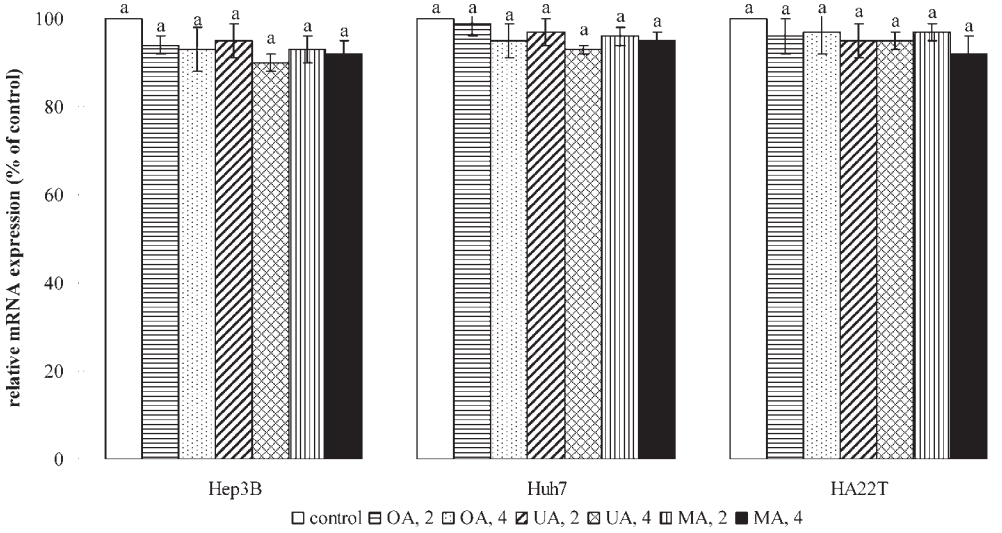

Figure 3. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon bFGF mRNA expression in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD (n = 9). Means among bars without a common letter (a) differ, $P < 0.05$.

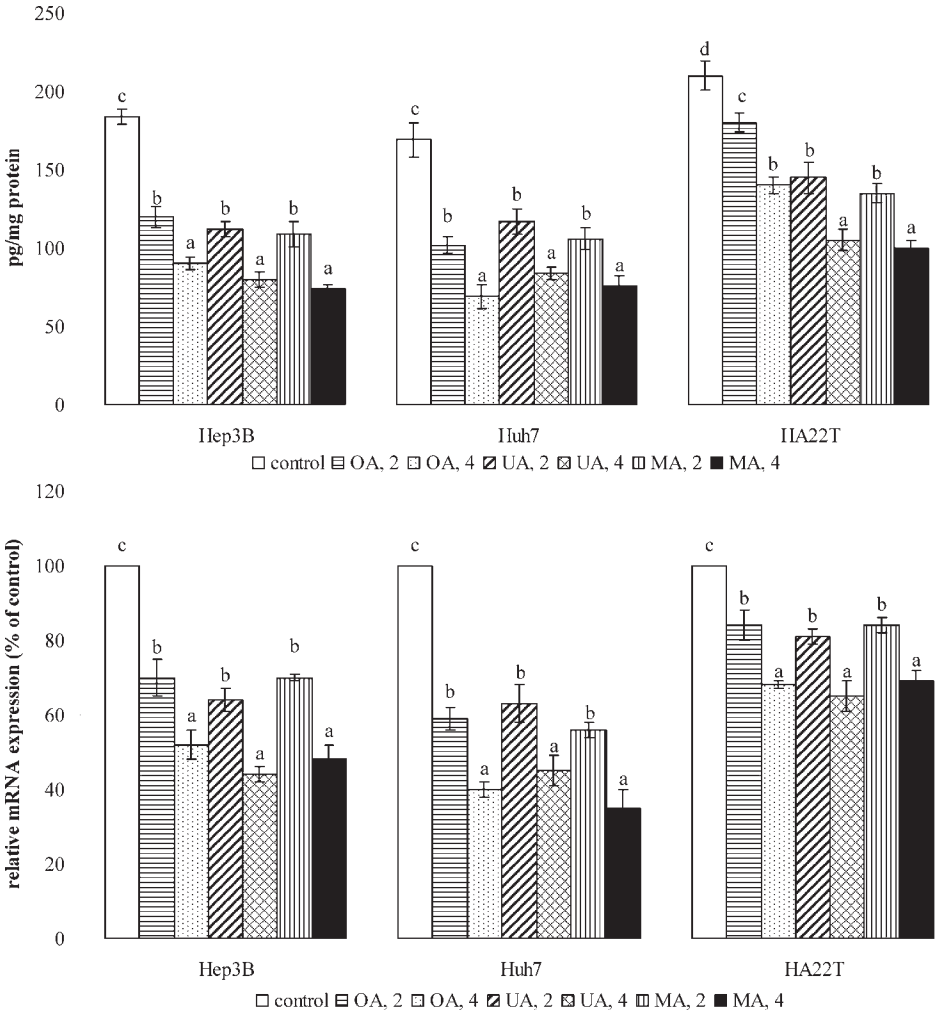

Figure 4. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon VEGF level (upper part) and mRNA expression (lower part) in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD (n = 9). Means among bars without a common letter (a−d) differ, $P < 0.05$.

chive (*Allium tuberosum* Rottler), fennel (*Foeniculum vulgare*), basil (*Ocimum basilicum* L.) and daylily (*Hemerocallis fulva* L.), were used to analyze the content of OA, UA and MA. These vegetables, harvested in summer of 2010, were purchased from farms in the central area of Taiwan. The content of three triterpenic acids in these vegetables was analyzed by HPLC methods described in Liu et al. (*17*).

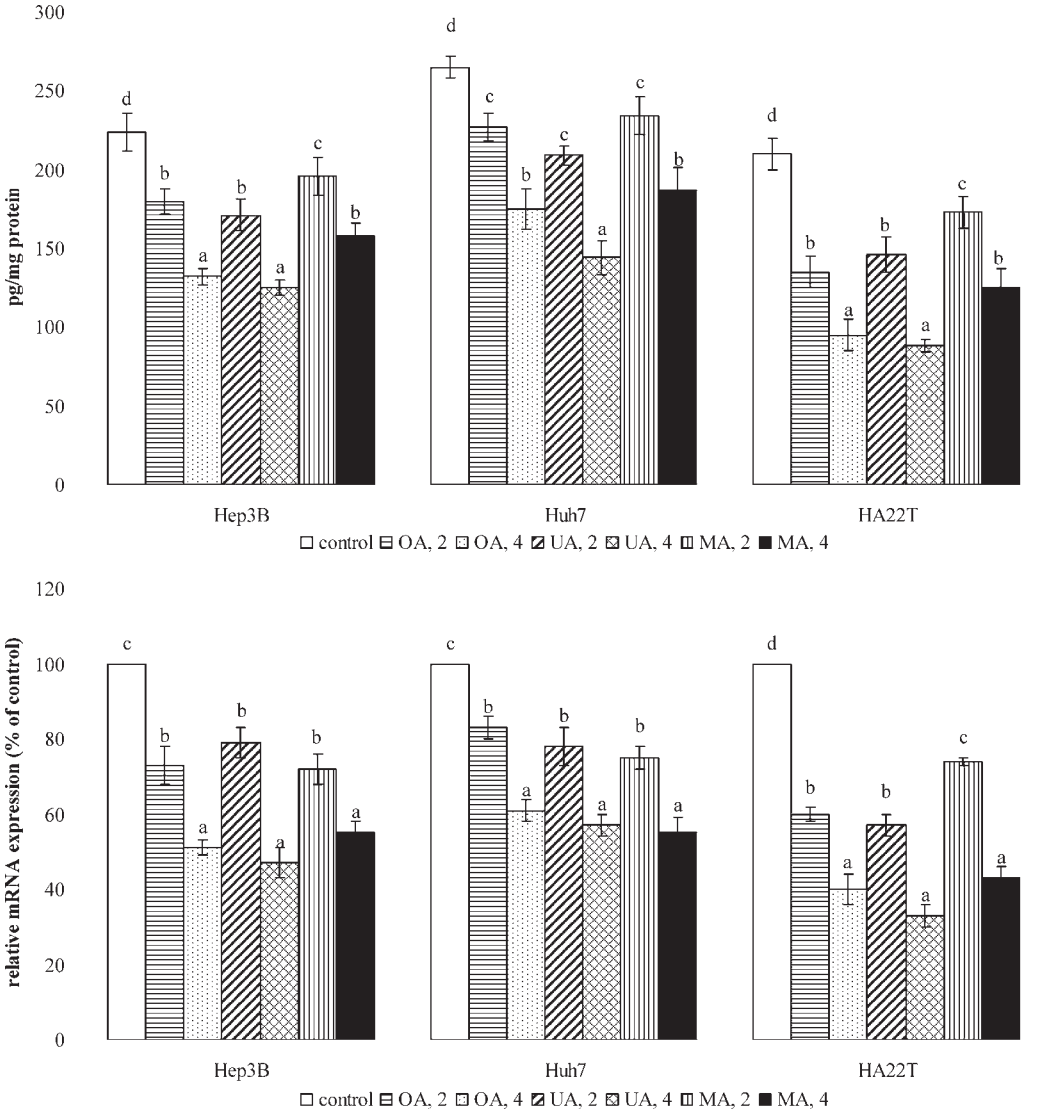

Figure 5. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon IL-8 level (upper part) and mRNA expression (lower part) in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD ($n = 9$). Means among bars without a common letter (a−d) differ, $P < 0.05$.

Cell Culture. Human normal liver cell line (L-02 cell) and liver cancer Hep3B, Huh7 and HA22T cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (BSA), 100 units/mL of penicillin and 100 units/mL of streptomycin (pH 7.4). Hypoxia (1% O_2) was created using an anaerobic jar (AnaeroPack Series, Mitsubishi Gas Chemical, Tokyo, Japan) with an AnaeroPack disposable O_2-absorbing and CO_2-generating agent. The culture medium was changed every three days, and cells were subcultured once a week. A phosphate buffer saline (PBS, pH 7.2) was added to adjust the cell number to 10^5/mL for various experiments and analyses.

Experimental Design. A stock solution of OA, UA or MA was prepared in dimethyl sulfoxide (DMSO) and diluted with medium. An equal volume of DMSO (final concentration < 0.5%) was added to the controls. Our previous study (*14*) found that OA and UA at 2 and 4 μmol/L caused 55−70% cell viability in Hep3B, Huh7 and HA22T cells (8 μmol/L caused 20−30% cell viability). Thus, in order to observe the effects of these triterpenic acids upon angiogenic factors in living cells, these compounds at these two doses were used the for present study. The cells at 10^5/mL were treated with OA, UA or MA at 2 or 4 μmol/L under hypoxia at 37 °C for 72 h. The control group contained no OA, UA or MA.

Measurement of Glutathione (GSH), Reactive Oxygen Species (ROS) and Nitrite. After 72 h hypoxic treatment, cells were washed and suspended in RPMI 1640 medium. GSH concentration (ng/mg protein) was determined by a commercial colorimetric GSH assay kit (Oxis-Research, Portland, OR, USA). The dye DCFH2-DA was used to measure level of intracellular cellular ROS according to the method of Fu et al. (*18*). In brief, cells were incubated with 50 μmol/L dye for 30 min and washed with PBS. After centrifugation at 412*g* for 10 min, the medium was removed and cells were dissolved with 1% Triton X-100. Fluorescence changes were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader. The production of NO was determined by measuring the formation of nitrite. Briefly, 100 μL of supernatant was mixed with 100 μL of Griess reagent after centrifuging at 10000*g* for 15 min at 4 °C. The optical absorbance at 540 nm was measured and compared with a sodium nitrite standard curve.

Determination of Angiogenic Factors. VEGF and IL-8 levels in cell homogenates were quantified by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). uPA level was assayed by commercial ELISA kit purchased from American Diagnostica (Greenwich, CT, USA). Each measurement was made in duplicate, and the level of each angiogenic factor was determined from a standard curve.

Real Time Polymerase Chain Reaction (RT-PCR) for mRNA Expression. Total RNA was isolated from cells using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). Two micrograms of total RNA was used to generate cDNA, which was amplified using Taq DNA polymerase. PCR was carried out in 50 μL of reaction mixture

Table 2. Effect of Oleanolic Acid (OA), Ursolic Acid (UA) and Maslinic Acid (MA) at 0 (control), 2 or 4 μmol/L upon level of GSH, ROS and NO in Human Normal Liver Cell Line (L-02 Cells) and Hep3B, Huh7 and HA22T Cell Lines[a]

	GSH (ng/mg protein)			
	L-02	Hep3B	Huh7	HA22T
control	80 ± 4 a	31 ± 2 a	23 ± 3 a	38 ± 4 a
OA, 2	84 ± 5 a	42 ± 3 b	36 ± 4 b	47 ± 5 b
OA, 4	88 ± 3 a	57 ± 5 c	50 ± 5 c	60 ± 5 c
UA, 2	82 ± 6 a	40 ± 4 b	40 ± 3 b	45 ± 4 b
UA, 4	85 ± 3 a	56 ± 4 c	60 ± 4 d	58 ± 2 c
MA, 2	79 ± 5 a	47 ± 5 b	33 ± 4 b	50 ± 3 b
MA, 4	87 ± 4 a	69 ± 3 d	48 ± 2 c	63 ± 5 c
	ROS (nmol/mg protein)			
	L-02	Hep3B	Huh7	HA22T
control	0.35 ± 0.04 a	2.06 ± 0.26 d	2.14 ± 0.13 c	1.90 ± 0.18 c
OA, 2	0.33 ± 0.06 a	1.67 ± 0.20 c	1.75 ± 0.17 b	1.58 ± 0.10 b
OA, 4	0.31 ± 0.02 a	1.03 ± 0.14 a	1.39 ± 0.15 a	1.27 ± 0.12 a
UA, 2	0.29 ± 0.05 a	1.72 ± 0.21 c	1.65 ± 0.09 b	1.65 ± 0.15 b
UA, 4	0.32 ± 0.03 a	1.35 ± 0.16 b	1.23 ± 0.10 a	1.19 ± 0.07 a
MA, 2	0.30 ± 0.06 a	1.70 ± 0.18 c	1.72 ± 0.15 b	1.52 ± 0.14 b
MA, 4	0.33 ± 0.05 a	1.38 ± 0.13 b	1.40 ± 0.11 a	1.23 ± 0.09 a
	NO (μM/mg protein)			
	L-02	Hep3B	Huh7	HA22T
control	1.3 ± 0.3 a	15.7 ± 1.1 d	14.6 ± 1.0 c	15.3 ± 1.4 d
OA, 2	1.6 ± 0.5 a	10.5 ± 0.7 b	12.1 ± 0.6 b	10.8 ± 1.0 b
OA, 4	1.4 ± 0.2 a	8.3 ± 0.9 a	8.5 ± 1.1 a	7.9 ± 0.8 a
UA, 2	1.2 ± 0.4 a	12.6 ± 0.8 c	11.8 ± 0.9 b	12.9 ± 1.2 c
UA, 4	1.5 ± 0.2 a	8.0 ± 1.1 a	8.3 ± 0.7 a	7.6 ± 0.5 a
MA, 2	1.4 ± 0.5 a	13.1 ± 1.0 c	12.2 ± 1.2 b	13.0 ± 0.9 c
MA, 4	1.3 ± 0.6 a	8.2 ± 0.6 a	9.0 ± 0.8 a	7.4 ± 0.6 a

[a] Data are mean ± SD (n = 9). Means in a column without a common letter (a−d) differ, $P < 0.05$.

containing Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U Taq DNA polymerase. The specific oligonucleotide primers for HIF-1α, bFGF, VEGF, IL-8, uPA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) are as follows. HIF-1α: forward, 5′-CTG CTG TCT TAC TGG TCC TT-3′, reverse, 5′-GTC GCT TCT CCA ATT CTT AC-3′. bFGF: forward, 5′-GTG TGC TAA CCG TAC CTG GC-3′, reverse, 5′-CTG GTG ATT TCC TTG ACC GG-3′. VEGF: forward, 5′-ATG AAC TTT CTG CTC TCT GG-3′, reverse, 5′-TCA TCT CTC CTA TGT GCT GGC-3′. IL-8: forward, 5′-CAC TCT CAA TCA CTC TCA GTT CTT TGA T-3′; reverse, 5′-CTT CCT GAT TTC TGC AGC TCT GT-3′. uPA: forward, 5′-TCA CAC CAA GGA AGA GAA TGG CCT-3′, reverse 5′-AAT GAC AAC CAG CAA GAA AGC GGG-3′. GAPDH: forward, 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′, reverse, 5′-CCT TGG AGG CCA TGT AGG CCA T-3′. The cDNA was amplified under the following reaction conditions: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Twenty-eight cycles were performed for GAPDH, and 35 cycles were performed for HIF-1α, bFGF, VEGF, IL-8 and uPA. Generated fluorescence from each cycle was quantitatively analyzed by using the Taqman system based on real-time sequence detection system (ABI Prism 7700; Perkin-Elmer Inc., Foster City, CA, USA). In this study, mRNA level was calculated as percentage of the control group.

Invasion Assay. Invasion was measured using 8 μm pore BD BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA), in which membrane was coated with 500 ng/μL Matrigel. After 72 h hypoxic treatment, cells were resuspended in RPMI 1640 medium containing 1% BSA, and at 10^5/100 μL were seeded into the upper chamber. The chambers were incubated for 24 h at 37 °C in a humidified, 5% CO_2 atmosphere for cell invasion. Noninvasive cells were removed by cotton swab. Invasive cells were stained with 0.2% crystal violet in 10% ethanol. Four independent fields of invasive cells per well were photographed under the microscope to determine the number of invasive cells. Data were calculated as percentage of the control group.

Migration Assay. Migration was measured using a Transwell chamber (Corning, NY, USA). The underside of Transwell membranes was precoated with fibronectin (10 μg/mL). After 72 h hypoxic treatment, cells were resuspended in RPMI 1640 medium containing 1% BSA, and at 10^5/100 μL were seeded into the upper chamber of Transwell chambers separated by inserts with 6.5 mm polycarbonate filters of 8 μm pore size. After 24 h incubation, nonmigratory cells on the upper surface of membranes were removed and migratory cells were stained with 0.1% crystal violet. Four independent fields of migratory cells per well were photographed under the microscope to determine the number of migratory cells. Data were calculated as percentage of the control group.

Statistical Analysis. The effect of each treatment was analyzed from nine different preparations (n = 9). Data were reported as means ± standard deviation (SD), and subjected to analysis of variance (ANOVA). Differences among means were determined by the least significance difference test with significance defined at $P < 0.05$.

RESULTS

The content of three triterpenic acids in eleven fresh vegetables is shown in **Table 1**. Among test vegetables, spinach had the highest content of OA and UA, and leaf mustard had the highest MA content. As shown in **Figure 1**, HIF-1α mRNA expression of three liver cancer cells under a hypoxic condition was greater than that under a normoxic condition ($P < 0.05$), which indicated that hypoxia had been created.

OA, UA and MA at 4 μmol/L significantly suppressed HIF-1α mRNA expression in three cell lines (**Figure 2**, $P < 0.05$); and these compounds at test doses failed to affect bFGF mRNA expression (**Figure 3**, $P > 0.05$). Three triterpenic acids dose-dependently decreased production and mRNA expression of VEGF and IL-8 in test cell lines (**Figures 4** and **5**, $P < 0.05$). As shown in **Table 2**, three cancer cells had significantly lower GSH level and higher production of ROS and NO than normal liver cells ($P < 0.05$); however, test compounds dose-dependently retained GSH level, and lowered production of ROS and NO in test liver cancer cells ($P < 0.05$). These compounds also dose-dependently diminished invasion and migration in three test cell lines (**Figure 6**, $P < 0.05$). As shown in **Figure 7**, three test triterpenic acids dose-dependently reduced uPA production and mRNA expression in Hep3B and Huh7 cell lines; but only at 4 μmol/L significantly downregulated uPA production and mRNA expression in HA22T cells ($P < 0.05$).

DISCUSSION

Our previous study already indicated that OA and UA exhibited marked apoptotic effects in liver cancer cell lines (*14*). The results of our present study further found that these two triterpenic acids and MA, another triterpenic acid, effectively downregulated mRNA expression of HIF-1α, VEGF, IL-8, uPA, retained GSH level, decreased ROS and NO production, and declined invasion and migration in three liver cancer cell lines under a hypoxic condition. These findings support that these triterpenic acids could mitigate angiogenesis, which might attenuate liver cancer metastasis and deterioration. Furthermore, we found that spinach, leaf mustard and daylily are rich in these triterpenic acids. It has been suggested that vegetable intake could reduce cancer risk (*19*, *20*). The results of our present study further suggest that the vegetables rich in these triterpenic acids may offer more protection against liver cancer.

Malignant tumor growth causes a hypoxic condition within the tumor, which in turn enhances the expression of HIF-1α in order to mediate an adaptive response through increasing angiogenesis.

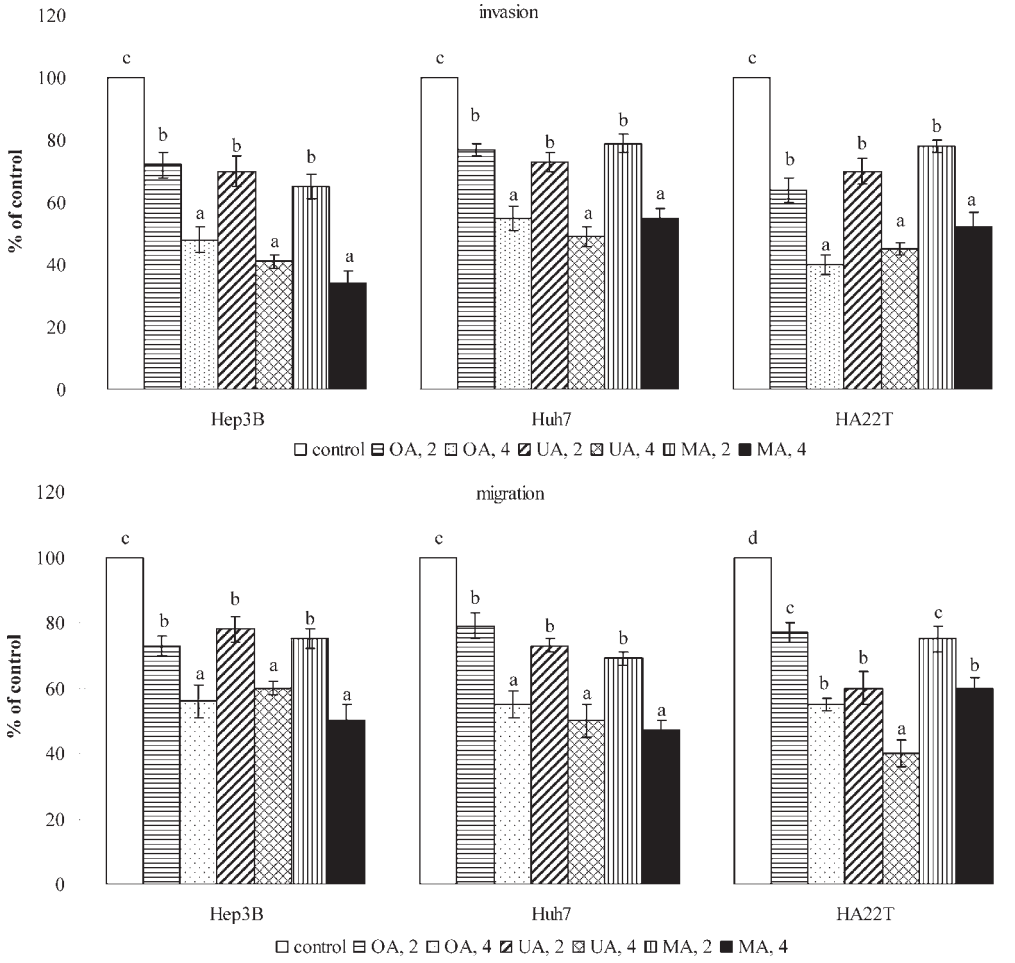

Figure 6. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon invasion and migration in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD (n = 9). Means among bars without a common letter (a−d) differ, $P < 0.05$.

Subsequently, HIF-1α, as a transcriptional activator, upregulates VEGF expression and leads to tumor neovascularization (*21*, *22*). Thus, regulation of HIF-1α and/or VEGF has been considered as a promising target for cancer therapy. In our present study, three test triterpenic acids downregulated HIF-1α expression at high dose, but decreased VEGF expression and production in a dose-dependent manner. Thus, the antiangiogenic activity of these triterpenic acids could be partially ascribed to suppression by these agents of HIF-1α and VEGF expression in test cell lines. However, it is interesting to find that these agents at lower dose (2 μmol/L) failed to affect HIF-1α expression, but they lowered VEGF expression. Obviously, one or more other upstream factors were involved for VEGF transcription in these triterpene-treated cancer cells. bFGF, another angiogenic activator, is more potent than VEGF for stimulating the vascular endothelial mitogenesis because bFGF enhances the release and activity of collagenases, proteases and integrins on the extracellular membrane to form nascent microvascular networks (*23*). In our present study, three triterpenic acids at test doses failed to lower bFGF expression. These results suggested that the antiangiogenic activity of test triterpenic acids did not involve bFGF associated pathway.

IL-8 from tumor cells is an autocrine growth factor and acts on endothelial cells through their receptors to promote migration, invasion and angiogenesis, which consequently elevated metastatic potential (*24*). Thus, decreasing IL-8 expression and production might also alleviate cancer progression. In our present study, test triterpenic acids reduced IL-8 expression and production in a dose-dependent manner, which subsequently contributed to diminish angiogenesis. IL-8 expression could be evoked by HIF-1α and NO (*25*, *26*). Since these triterpenic acids could suppress HIF-1α expression and reduce NO production, it seems reasonable to observe the declined expression of the downstream target gene, IL-8. On the other hand, enhanced IL-8 expression could increase the activity of some matrix metalloproteins and facilitate tumor cell invasion (*27*). Thus, the lowered cell invasion as we observed in triterpene-treated liver cancer cells could be partially ascribed to these agents having already downregulated IL-8.

It has been indicated that oxidative stress enhanced the expression of both HIF-1α and VEGF in cancer cells (*28*). Intracellular ROS derived from NADPH oxidase or mitochondria, acting as a signaling messenger, is able to activate critical target molecules such as intercellular adhesion molecule-1 and trigger cell adhesion, epithelial−mesenchymal transition and migration in tumor cells (*29*). Nitrite is capable of inducing VEGF expression and favors nitrite-driven formation of endothelial tubes in hypoxic tumor microenvironment (*30*). Obviously, these liver cancer cells depleted GSH, and released ROS and NO to promote their migration and angiogenesis, which favors cancer progression. In our present study, triterpenic acid treatments effectively retained GSH level, and reduced ROS and NO release in test cell lines, which in turn alleviated oxidative stress and diminished the expression of angiogenic factors such as VEGF. Several studies have reported that OA, UA and MA could attenuate oxidative stress via sparing GSH, inhibiting NO production and/or scavenging ROS (*31*−*33*). Thus, the lower ROS

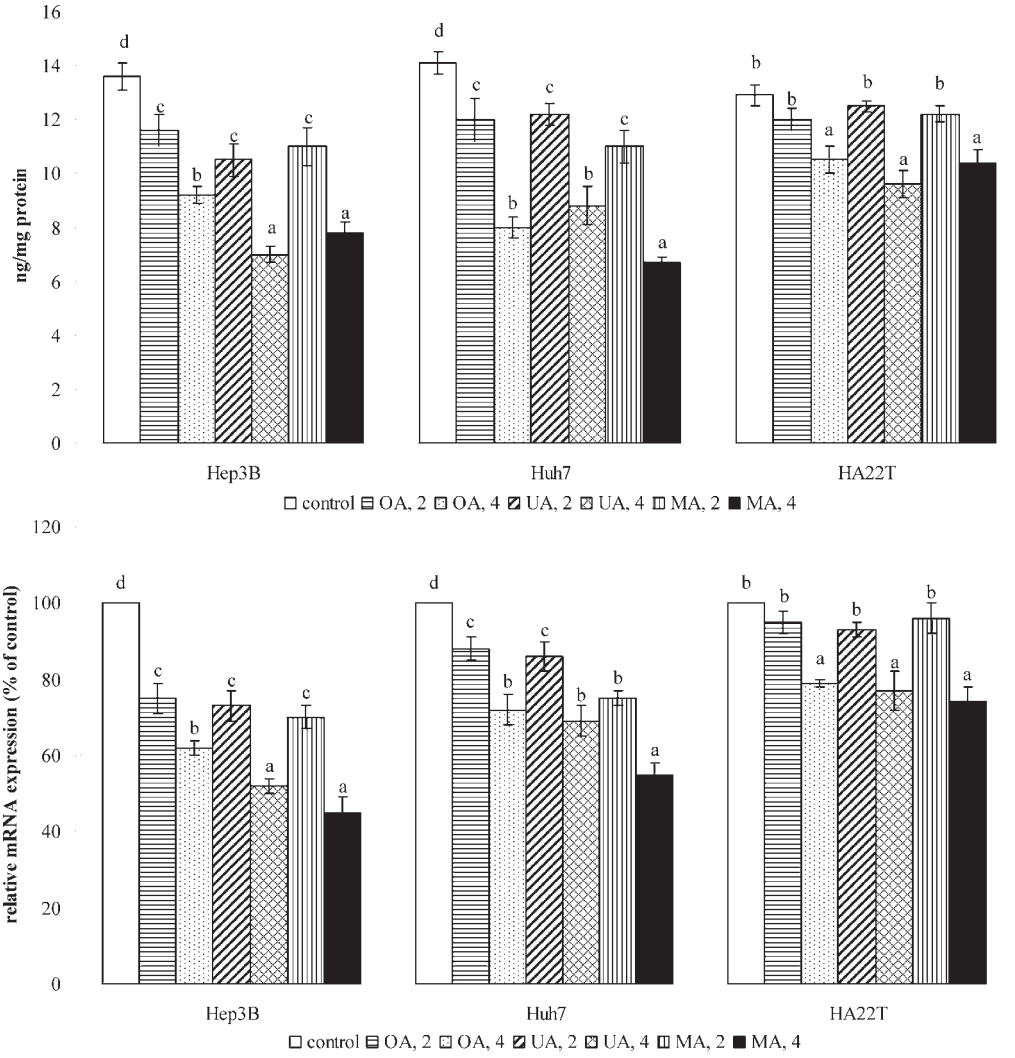

Figure 7. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0 (control), 2 or 4 μmol/L upon uPA level (upper part) and mRNA expression (lower part) in human Hep3B, Huh7 and HA22T cell lines. Data are mean ± SD ($n = 9$). Means among bars without a common letter (a−d) differ, $P < 0.05$.

and NO levels in triterpenic acid-treated groups as we observed could be explained. These findings revealed that these triterpenic acids could mitigate angiogenesis in liver cancer cells through their antioxidative actions.

uPA converts plasminogen to plasmin, which is responsible for the degradation of fibrin and extracellular matrix components, and finally promotes cell migration and invasion (*34*). In our present study, the production and expression of uPA in the liver cancer cell lines could be downregulated by triterpenic acid treatments, which subsequently decreased plasminogen conversion and degradation of fibrin and matrix proteins. In addition, ROS could stimulate uPA expression in tumor cells (*35*). Thus, the reduced uPA expression from triterpenic acids could be partially due to these compounds having already decreased ROS production. Since uPA expression was suppressed, the observed lower cell invasion and migration in these triterpenic acid-treated groups could be explained. Furthermore, test triterpenic acids at 4 μmol/L inhibited 50−70% cell invasion and migration in these three cell lines. Thus, these agents might be able to retard HCC progression via blocking metastatic process.

It is interesting to find that three test compounds at equal dose exhibited greater suppressive effects upon VEGF and uPA expression in Hep3B and Huh7 cells than in HA22T cells, but these agents were greater in declining IL-8 expression in HA22T cells than in other two cell lines. It has been reported that Hep3B expressed more mesenchymal proteins for mesenchymal transition, Huh7 was highly associated with hepatitis B virus- or C virus-related HCC, and HA22T was a poorly differentiated cell line (*36*−*38*). Thus, these cells, based on different pathological features and biochemical behavior, might exhibit different response to certain chemical(s). These findings implied that selecting any agent for antihepatoma therapy should consider the responsible cancer cell line, and/or target molecules. In addition, the results of our present study suggested that any agent, like these triterpenic acids, with multiple anticancer actions toward multiple HCC cell lines might be an appropriate choice for anti-HCC therapy.

In summary, the content of oleanolic acid, ursolic acid and maslinic acid in daylily, spinach and leaf mustard was in the range of 76−174 mg/100 g dry weight. These three triterpenic acids at 4 μmol/L markedly suppressed mRNA expression of angiogenic factors, HIF-1α, VEGF, IL-8 and uPA. These triterpenic acids also effectively alleviated oxidative stress via retaining GSH level, lowering ROS and NO production, as well as decreased cell invasion and migration in Hep3B, Huh7 and HA22T cell lines. These findings suggest that these triterpenic acids are potent antiangiogenic agents to retard invasion and migration in liver cancer cells.

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