1   Anti-angiogenic potential of three triterpenic acids in human liver cancer cells

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14   running title: anti-angiogenic potential of triterpenic acids

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1   **ABSTRACT**

2   Three triterpenic acids, oleanolic acid, ursolic acid and maslinic acid, at 2 or 4 μmol/L

3   were used to study their anti-angiogenic potential in human liver cancer Hep3B, Huh7 and

4   HA22T cell lines.   The effects of these compounds upon the level and/or expression of

5   hypoxia-inducible factor (HIF)-1α, basic fibroblast growth factor (bFGF), vascular

6   endothelial growth factor (VEGF), interleukin (IL)-8, urokinase plasminogen activator

7   (uPA), reactive oxygen species (ROS), nitric oxide (NO) and cell invasion and migration

8   were examined.   Results showed that these triterpenic acids at 4 μmol/L significantly

9   suppressed HIF-1α expression in three cell lines ($P<0.05$); and these compounds at test

10 doses   failed   to   affect   bFGF   expression   ($P>0.05$).   Three   triterpenic   acids

11 dose-dependently decreased production and expression of VEGF and IL-8, retained

12 glutathione level, lowered ROS and NO levels, and declined cell invasion and migration in

13 test cell lines ($P<0.05$).   These compounds also dose-dependently reduced uPA production

14 and expression in Hep3B and Huh7 cell lines ($P<0.05$); but these agents only at 4 μmol/L

15 significantly suppressed uPA production and expression in HA22T cells ($P<0.05$).   These

16 findings suggest that these triterpenic acids are potent anti-angiogenic agents to retard

17 invasion and migration in liver cancer cells.

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19 KEYWORDS: angiogenesis; triterpenic acid; liver cancer cell; hypoxia-inducible factor;

20 vascular endothelial growth factor

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INTRODUCTION

Angiogenesis, also called as neovascularization, is an important process for tumor growth and metastasis. Liver cancer, also called as hepatocellular carcinoma (HCC), is a hypervascular tumor characterized by massive angiogenesis (*1*). The increased circulating levels and/or expression of several angiogenetic 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 angiogenetic 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*)

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1 also found that these two triterpenic acids at 8 μmol/L caused markedly apoptotic effects in

2 four human liver cancer HepG2, Hep3B, Huh7 and HA22T cell lines via increasing DNA

3 fragmentation, decreasing mitochondrial membrane potential and lowering

4 Na^+-K^+-ATPase activity. Juan et al. (*15*) reported that maslinic acid, another triterpenic

5 acid, could inhibit cell proliferation in human colon cancer cell, HT-29. These studies

6 suggest that these triterpenic acids are potent anti-cancer agents to cause apoptosis or

7 retard metastasis for liver cancer. Therefore, a further study was conducted to examine

8 the anti-angiogenic potential of these triterpenic acids. The major purpose of our present

9 study was to investigate the effects of triterpenic acids on the production and/or expression

10 of HIF-1α, bFGF, VEGF, IL-8 and uPA in liver cancer cell lines. The influence of these

11 compounds upon the production of ROS and NO in these cell lines was also evaluated.

12 On the other hand, the presence of ursolic acid and maslinic acid in apple peel has

13 been considered as major compounds responsible for the anti-cancer activity of apple (*16*).

14 So far, less information is available regarding the presence of these triterpenic acids in

15 fresh vegetables. In order to enrich the food component database, eleven locally available

16 fresh vegetables in Taiwan were used to analyze the content of oleanolic acid, ursolic acid

17 and maslinic acid.

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19 **MATERIALS AND METHODS**

20 **Chemicals.** Oleanolic acid (OA, 99%), ursolic acid (UA, 98%) and maslinic acid

21 (MA, 98.5%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

22 Medium, plates, antibiotics and chemicals used for cell culture were purchased from Difco

23 Laboratory (Detroit, MI, USA). All chemicals used in these measurements were of the

24 highest purity commercially available.

1   **Content of Triterpenic Acids.** Eleven locally available fresh vegetables, carrot

2   (*Daucus carota* L.), celery (*Apium graveolens* L.), okra (*Hibiscus esculentus*), eggplant

3   (*Solanum melongena* L.), spinach (*Spinacia oleracea* L.), leaf mustard (*Brassica juncea*),

4   gynura (*Gynura bicolor* DC.), Chinese chive (*Allium tuberosum* Rottler), fennel

5   (*Foeniculum vulgare*), basil (*Ocimum basilicum* L.) and daylily (*Hemerocallis fulva* L.),

6   were used to analyze the content of OA, UA and MA.   These vegetables, harvested in

7   summer of 2010, were purchased from farms in central area of Taiwan.   The content of

8   three triterpenic acids in these vegetables was analyzed by HPLC methods described in Liu

9   et al. (*17*).

10   **Cell Culture.** Human normal liver cell line (L-02 cell) and liver cancer Hep3B, Huh7

11   and HA22T cell lines were obtained from American Type Culture Collection (ATCC,

12   Rockville, MD, USA).   All cells were cultured in RPMI 1640 medium, containing 10%

13   fetal bovine serum (BSA), 100 units/mL of penicillin and 100 units/mL of streptomycin

14   (pH 7.4).   Hypoxia (1% O_2) was created using an anaerobic jar (AnaeroPack Series,

15   Mitsubishi Gas Chemical, Tokyo, Japan) with an AnaeroPack disposable O_2-absorbing and

16   CO_2-generating agent.   The culture medium was changed every three days, and cells were

17   subcultured once a week.   A phosphate buffer saline (PBS, pH 7.2) was added to adjust

18   the cell number to 10^5/mL for various experiments and analyses.

19   **Experimental Design.** Stock solution of OA, UA or MA was prepared in dimethyl

20   sulfoxide (DMSO) and diluted with medium.   An equal volume of DMSO (final

21   concentration <0.5%) was added to the controls.   Our previous study (*14*) found that OA

22   and UA at 2 and 4 μmol/L caused 55-70% cell viability in Hep3B, Huh7 and HA22T cells

23   (8 μmol/L caused 20-30% cell viability).   Thus, in order to observe the effects of these

24   triterpenic acids upon angiogenic factors in living cells, these compounds at these two

25   doses were used for present study.   The cells at 10^5/mL were treated with OA, UA or MA

1 at 2 or 4 µmol/L under hypoxia at 37 °C for 72 h.   Control group contained no OA, UA

2 or MA.

3 **Measurement of Glutathione (GSH), Reactive Oxygen Species (ROS) and Nitrite.**

4 After 72 h hypoxic treatment, cells were washed and suspended in RPMI 1640 medium.

5 GSH concentration (ng/mg protein) was determined by a commercial colorimetric GSH

6 assay kit (OxisResearch, Portland, OR, USA).   The dye DCFH2-DA was used to measure

7 level of intracellular cellular ROS according to the method of Fu et al. (*18*).   In briefly,

8 cells were incubated with 50 µmol/L dye for 30 min and washed with PBS.   After

9 centrifugation at 412 xg for 10 min, the medium was removed and cells were dissolved

10 with 1% Triton X-100.   Fluorescence changes were measured at an excitation wavelength

11 of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader.

12 The production of NO was determined by measuring the formation of nitrite.   Briefly,

13 100 µL of supernatant was mixed with 100 µL Griess reagent after centrifuged at 10,000

14 xg for 15 min at 4 ºC.   The optical absorbance at 540 nm was measured and compared

15 with a sodium nitrite standard curve.

16 **Determination of Angiogenic Factors.** VEGF and IL-8 levels in cell homogenates

17 were quantified by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

18 uPA level was assayed by commercial ELISA kit purchased from American Diagnostica

19 (Greenwich, CT, USA).   Each measurement was made in duplicate, and the level of each

20 angiogenic factor was determined from a standard curve.

21 **Real Time Polymerase Chain Reaction (RT-PCR) for mRNA Expression.** Total

22 RNA was isolated from cells using Trizol reagent (Invitrogen, Life Technologies, Carlsbad,

23 CA, USA).   Two µg of total RNA was used to generate cDNA, which was amplified

24 using Taq DNA polymerase.   PCR was carried out in 50 µL of reaction mixture

25 containing Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 mM

1 dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U Taq DNA polymerase. The

2 specific oligonucleotide primers for HIF-1α, bFGF, VEGF, IL-8, uPA and

3 glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) are as

4 follows. HIF-1α: forward, 5'-CTG CTG TCT TAC TGG TCC TT-3', reverse, 5'-GTC

5 GCT TCT CCA ATT CTT AC-3'; bFGF: forward, 5'-GTG TGC TAA CCG TAC CTG

6 GC-3', reverse, 5'-CTG GTG ATT TCC TTG ACC GG-3'; VEGF, forward, 5'-ATG AAC

7 TTT CTG CTC TCT GG-3', reverse, 5'-TCA TCT CTC CTA TGT GCT GGC-3'; IL-8,

8 forward, 5'-CAC TCT CAA TCA CTC TCA GTT CTT TGA T-3'; reverse, 5'-CTT CCT

9 GAT TTC TGC AGC TCT GT-3'; uPA, forward, 5′-TCA CAC CAA GGA AGA GAA

10 TGG CCT-3′, reverse 5′-AAT GAC AAC CAG CAA GAA AGC GGG-3′; GAPDH:

11 forward, 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3', reverse, 5'-CCT TGG

12 AGG CCA TGT AGG CCA T-3'. The cDNA was amplified under the following

13 reaction conditions: 95 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min. 28 cycles

14 were performed for GAPDH and 35 cycles were performed for HIF-1α, bFGF, VEGF,

15 IL-8 and uPA. Generated fluorescence from each cycle was quantitatively analyzed by

16 using the Taqman system based on real-time sequence detection system (ABI Prism 7700;

17 Perkin-Elmer Inc., Foster City, CA, USA). In this study, mRNA level was calculated as

18 percentage of the control group.

19   **Invasion Assay.** Invasion was measured using 8 μm pore BD BioCoat Matrigel

20 Invasion Chambers (BD Biosciences, San Jose, CA, USA), in which membrane was

21 coated with 500 ng/μL Matrigel. After 72 h hypoxic treatment, cells were resuspended in

22 RPMI 1640 medium containing 1% BSA, and at 10^5/100 μL were seeded into the upper

23 chamber. The chambers were incubated for 24 h at 37 ℃ in a humidified, 5% CO_2

24 atmosphere for cell invasion. Non-invasive cells were removed by Q-tip. Invasive cells

25 were stained with 0.2% crystal violet in 10% ethanol. Four independent fields of

1 invasive cells per well were photographed under the microscope to determine the number

2 of invasive cells.   Data were calculated as percentage of the control group.

3   **Migration assay.** Migration was measured using a transwell chamber (Corning, NY,

4 USA).   The underside of transwell membranes was precoated with fibronectin (10

5 μg/mL).   After 72 h hypoxic treatment, cells were resuspended in RPMI 1640 medium

6 containing 1% BSA, and at $10^5/100$ μL were seeded into the upper chamber of transwell

7 chambers separated by inserts with 6.5 mm polycarbonate filters of 8 μm pore size.   After

8 24 h incubation, non-migratory cells on the upper surface of membranes were removed

9 and migratory cells were stained with 0.1% crystal violet.   Four independent fields of

10 migratory cells per well were photographed under the microscope to determine the number

11 of migratory cells.   Data were calculated as percentage of the control group.

12   **Statistical Analysis.** The effect of each treatment was analyzed from nine different

13 preparations (n=9).   Data were reported as means ± standard deviation (SD), and

14 subjected to analysis of variance (ANOVA).   Differences among means were determined

15 by the Least Significance Difference Test with significance defined at $P<0.05$.

16

17 **RESULTS**

18   The content of three triterpenic acids in eleven fresh vegetables is shown in Table 1.

19 Among test vegetables, spinach had the highest content of OA and UA, and leaf mustard

20 had the highest MA content.   As shown in Figure 1, HIF-1α mRNA expression of three

21 liver cancer cells under hypoxic condition was greater than normoxic condition ($P<0.05$),

22 which indicated that hypoxia had been created.

23   OA, UA and MA at 4 μmol/L significantly suppressed HIF-1α mRNA expression in

24 three cell lines (Figure 2, $P<0.05$); and these compounds at test doses failed to affect bFGF

1 mRNA expression (Figure 3, $P>0.05$).   Three triterpenic acids dose-dependently

2 decreased production and mRNA expression of VEGF and IL-8 in test cell lines (Figures 4

3 and 5, $P<0.05$).   As shown in Table 2, three cancer cells had significantly lower GSH

4 level and higher production of ROS and NO than normal liver cells ($P<0.05$); however, test

5 compounds dose-dependently retained GSH level, and lowered production of ROS and NO

6 in test liver cancer cells ($P<0.05$).   These compounds also dose-dependently diminished

7 invasion and migration in three test cell lines (Figure 6, $P<0.05$).   As shown in Figure 7,

8 three test triterpenic acids dose-dependently reduced uPA production and mRNA

9 expression in Hep3B and Huh7 cell lines; but only at 4 μmol/L significantly

10 down-regulated uPA production and mRNA expression in HA22T cells ($P<0.05$).

11

12 **DISCUSSION**

13   Our previous study already indicated that OA and UA exhibited marked apoptotic

14 effects in liver cancer cell lines (*14*).   The results of our present study further found that

15 these two triterpenic acids and MA, another triterpenic acid, effectively down-regulated

16 mRNA expression of HIF-1α, VEGF, IL-8, uPA, retained GSH level, decreased ROS and

17 NO production, as well as declined invasion and migration in three liver cancer cell lines

18 under hypoxic condition.   These findings support that these triterpenic acids could

19 mitigate angiogenesis, which might attenuate liver cancer metastasis and deterioration.

20 Furthermore, we found that spinach, leaf mustard and daylily are rich in these triterpenic

21 acids.   It has been suggested that vegetable intake could reduce cancer risk (*19, 20*).   The

22 results of our present study further suggest that the vegetables rich in these triterpenic acids

23 may offer more protection against liver cancer.

24   Malignant tumor growth causes hypoxic condition within the tumor, which in turn

25 enhances the expression of HIF-1α in order to mediate an adaptive response through

1 increasing angiogenesis. Subsequently, HIF-1α, as an transcriptional activator,

2 up-regulates VEGF expression and leads to tumor neovascularization (*21, 22*). Thus,

3 regulation of HIF-1α and/or VEGF has been considered as promising target for cancer

4 therapy. In our present study, three test triterpenic acids down-regulated HIF-1α

5 expression at high dose, but decreased VEGF expression and production in dose-dependent

6 manner. Thus, the anti-angiogenic activity of these triterpenic acids could be partially

7 ascribed to these agents suppress HIF-1α and VEGF expression in test cell lines.

8 However, it is interesting to find that these agents at lower dose (2 μmol/L) failed to affect

9 HIF-1α expression, but they lowered VEGF expression. Obviously, other upstream

10 factor(s) was involved for VEGF transcription in these triterpene-treated cancer cells.

11 bFGF, another angiogenic activator, is more potent than VEGF for stimulating the vascular

12 endothelial mitogenesis because bFGF enhances the release and activity of collagenases,

13 proteases and integrins on the extracellular membrane to form nascent microvascular

14 networks (*23*). In our present study, three triterpenic acids at test doses failed to lower

15 bFGF expression. These results suggested that the anti-angiogenic activity of test

16 triterpenic acids did not involve bFGF associated pathway.

17 IL-8 from tumor cells is an autocrine growth factor and acts on endothelial cells

18 through their receptors to promote migration, invasion and angiogenesis, which

19 consequently elevated metastatic potential (*24*). Thus, decreasing IL-8 expression and

20 production might also alleviate cancer progression. In our present study, test triterpenic

21 acids reduced IL-8 expression and production in dose-dependent manner, which

22 subsequently contributed to diminish angiogenesis. IL-8 expression could be evoked by

23 HIF-1α and NO (*25, 26*). Since these triterpenic acids could suppress HIF-1α expression

24 and reduce NO production, it seems reasonable to observe the declined expression of

1 downstream target gene, IL-8. On the other hand, enhanced IL-8 expression could

2 increase the activity of some matrix metalloproteins and facilitate tumor cell invasion (*27*).

3 Thus, the lowered cell invasion as we observed in triterpene-treated liver cancer cells could

4 be partially ascribed to these agents already down-regulated IL-8.

5 It has been indicated that oxidative stress enhanced the expression of both HIF-1α and

6 VEGF in cancer cells (*28*). Intracellular ROS derived from NADPH oxidase or

7 mitochondria, acting a signaling messenger, is able to activate critical target molecules

8 such as intercellular adhesion molecule-1 and trigger cell adhesion,

9 epithelial-mesenchymal transition and migration in tumor cells (*29*). Nitrite is capable of

10 inducing VEGF expression and favors nitrite-driven formation of endothelial tubes in

11 hypoxic tumor microenvironment (*30*). Obviously, liver cancer cells released ROS and

12 NO to promote their migration and angiogenesis. In our present study, triterpenic acids

13 treatments effectively retained GSH level, and reduced ROS and NO release in test cell

14 lines, which in turn alleviated oxidative stress and diminished the expression of angiogenic

15 factors such as VEGF. Several studies have reported that OA, UA and MA could

16 attenuate oxidative stress via sparing GSH, inhibiting NO production and/or scavenging

17 ROS (*31-33*). Thus, the lower ROS and NO levels in triterpenic acid-treated groups as

18 we observed could be explained. These findings revealed that these triterpenic acids

19 could mitigate angiogenesis in liver cancer cells through their anti-oxidative actions.

20 uPA converts plasminogen to plasmin, which is responsible for the degradation of

21 fibrin and extracellular matrix components, and finally promotes cell migration and

22 invasion (*34*). In our present study, the production and expression of uPA in the liver

23 cancer cell lines could be down-regulated by triterpenic acid treatments, which

24 subsequently decreased plasminogen conversion and degradation of fibrin and matrix

25 proteins. In addition, ROS could stimulate uPA expression in tumor cells (*35*). Thus,

1   the reduced uPA expression from triterpenic acids could be partially due to these

2   compounds already decrease ROS production.   Since uPA expression was suppressed, the

3   observed lower cell invasion and migration in these triterpenic acid-treated groups could be

4   explained.   Furthermore, test triterpenic acids at 4 µmol/L inhibited 50-70% cell invasion

5   and migration in these three cell lines.   Thus, these agents might be able to retard HCC

6   progression via blocking metastatic process.

7   It is interesting to find that three test compounds at equal dose exhibited greater

8   suppressive effects upon VEGF and uPA expression in Hep3B and Huh7 cells than in

9   HA22T cells, but these agents were greater in declining IL-8 expression in HA22T cells

10   than in other two cell lines.   It has been reported that Hep3B expressed more

11   mesenchymal proteins for mesenchymal transition, Huh7 was highly associated with

12   hepatitis B virus- or C virus-related HCC, and HA22T was a poorly-differentiated cell line

13   (*36-38*).   Thus, these cells, based on different pathological features and biochemical

14   behavior, might exhibit different response to certain chemical(s).   These findings implied

15   that selecting any agent for anti-hepatoma therapy should consider the responsible cancer

16   cell line, and/or target molecules.   In addition, the results of our present study suggested

17   that any agent, like these triterpenic acids, with multiple anti-cancer actions toward

18   multiple HCC cell lines might be appropriate choice for anti-HCC therapy.

19   In summary, the content of oleanolic acid, ursolic acid and maslinic acid in daylily,

20   spinach and leaf mustard was in the range of 76-174 mg/100 g dry weight.   These three

21   triterpenic acids at 4 µmol/L markedly suppressed mRNA expression of angiogenic factors,

22   HIF-1α, VEGF, IL-8 and uPA.   These triterpenic acids also effectively alleviated

23   oxidative stress via retaining GSH level, lowering ROS and NO production, as well as

24   decreased cell invasion and migration in Hep3B, Huh7 and HA22T cell lines.   These

1 findings suggest that these triterpenic acids are potent anti-angiogenic agents to retard

2 invasion and migration in liver cancer cells.

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1 **Table 1**. Content (mg/100 g dry weight) of oleanolic acid (OA), ursolic acid (UA) and

2 maslinic acid (MA) in eleven vegetables, carrot, celery, okra, eggplant, spinach, leaf

3 mustard, gynura, Chinese chive, fennel, basil and daylily.   Data are mean ± SD, n=6.

	OA	UA	MA
carrot	25±4	31±6	-
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	-[a]	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

4 [a]means too low to be detected.

5

1   **Figure 1.** HIF-1α mRNA expression in human Hep3B, Huh7 and HA22T cell lines under

2   normoxia and hypoxia condition.   Data are mean±SD (n = 9). [a,b]Means among bars
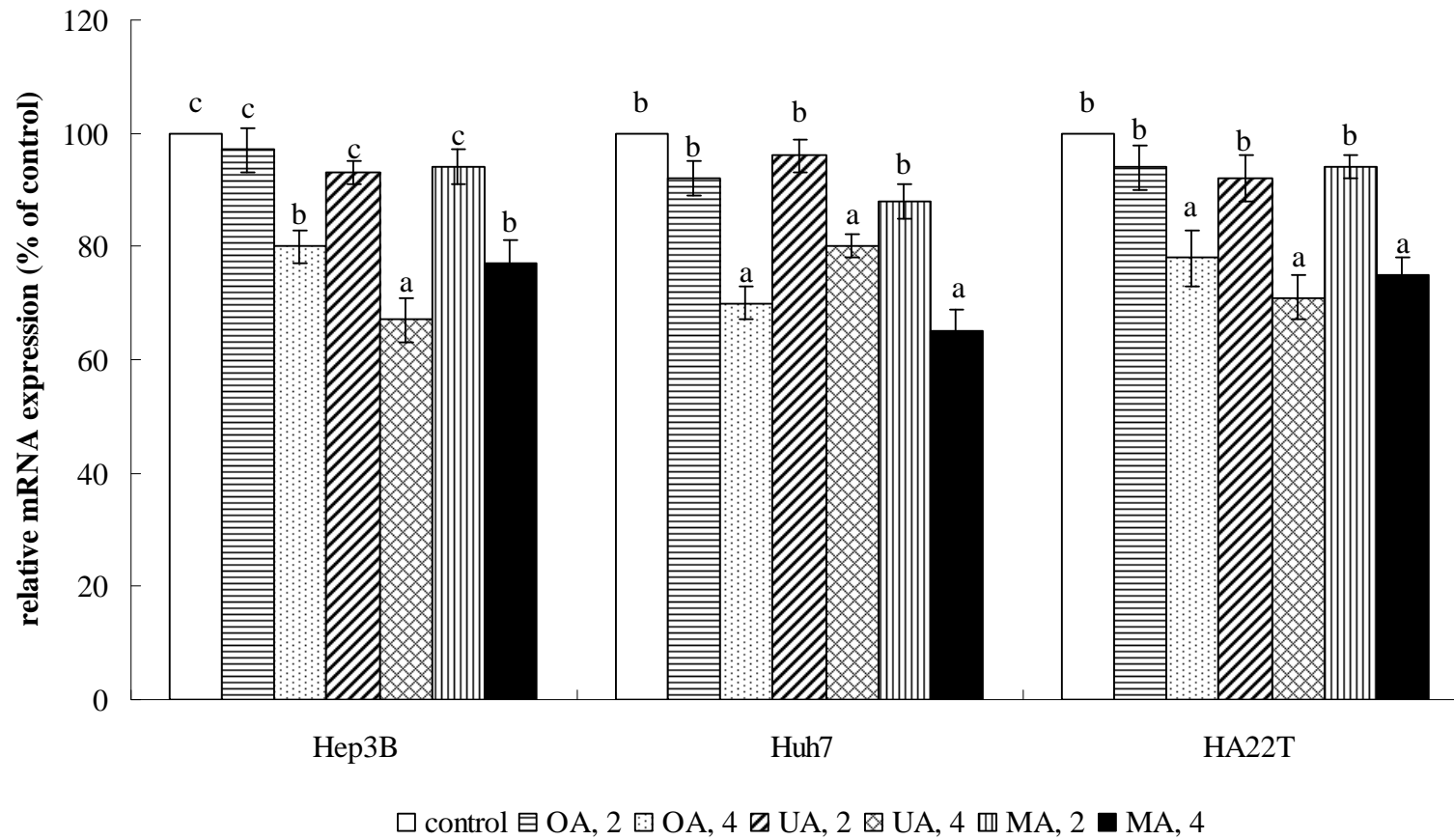
3   without a common letter differ, *P*<0.05.

1

relative mRNA expression

400

350

300

250

200

150

100

50

0

b

b

b

a   a   a

normoxia   hypoxia
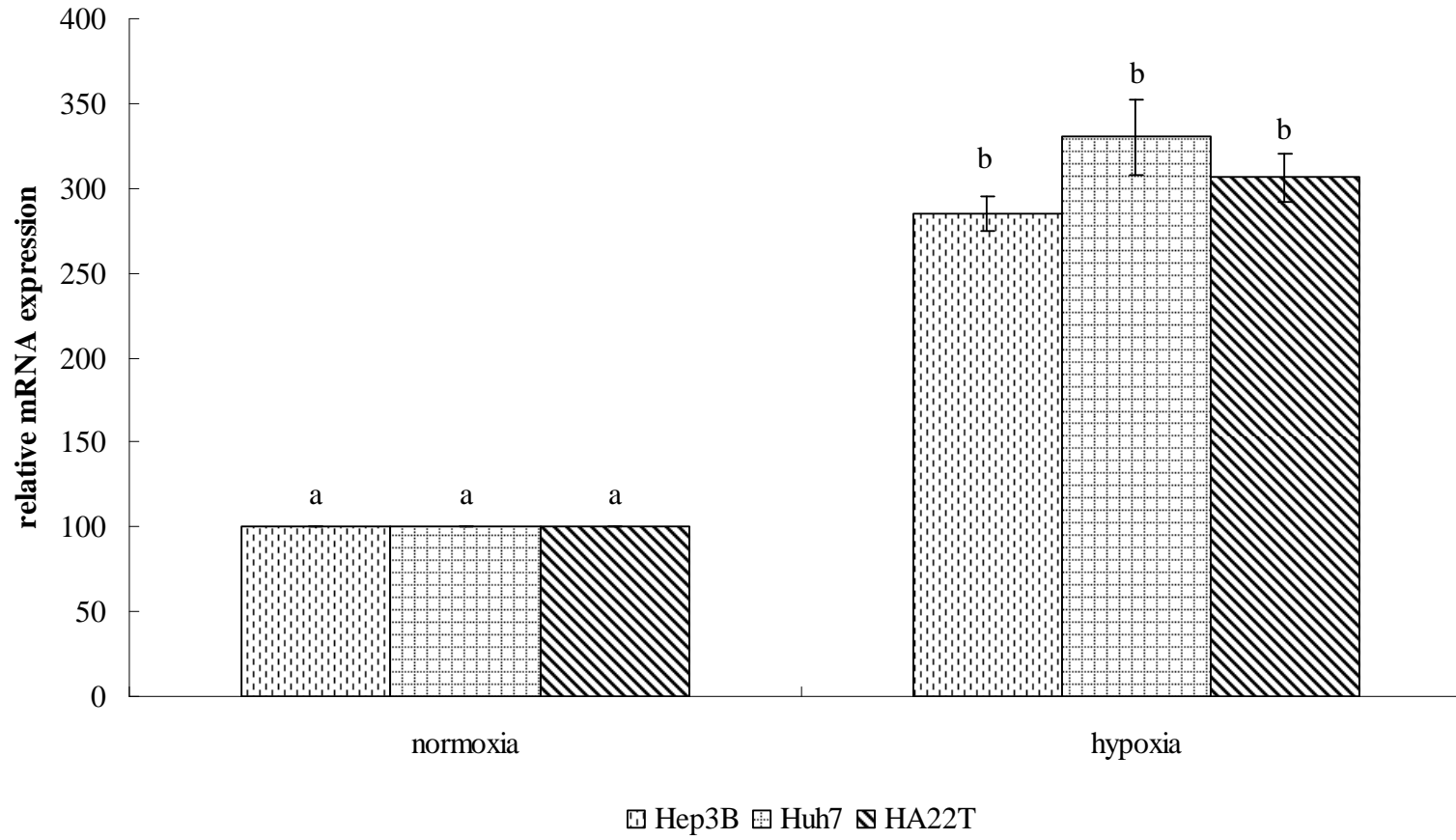
▥ Hep3B ▦ Huh7 ◩ HA22T

2

3

1   **Figure 2.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 μmol/L upon HIF-1α mRNA expression in human Hep3B, Huh7 and

3   HA22T cell lines.   Data are mean±SD (n = 9).   [a-c]Means among bars without a common

4   letter differ, *P*<0.05.
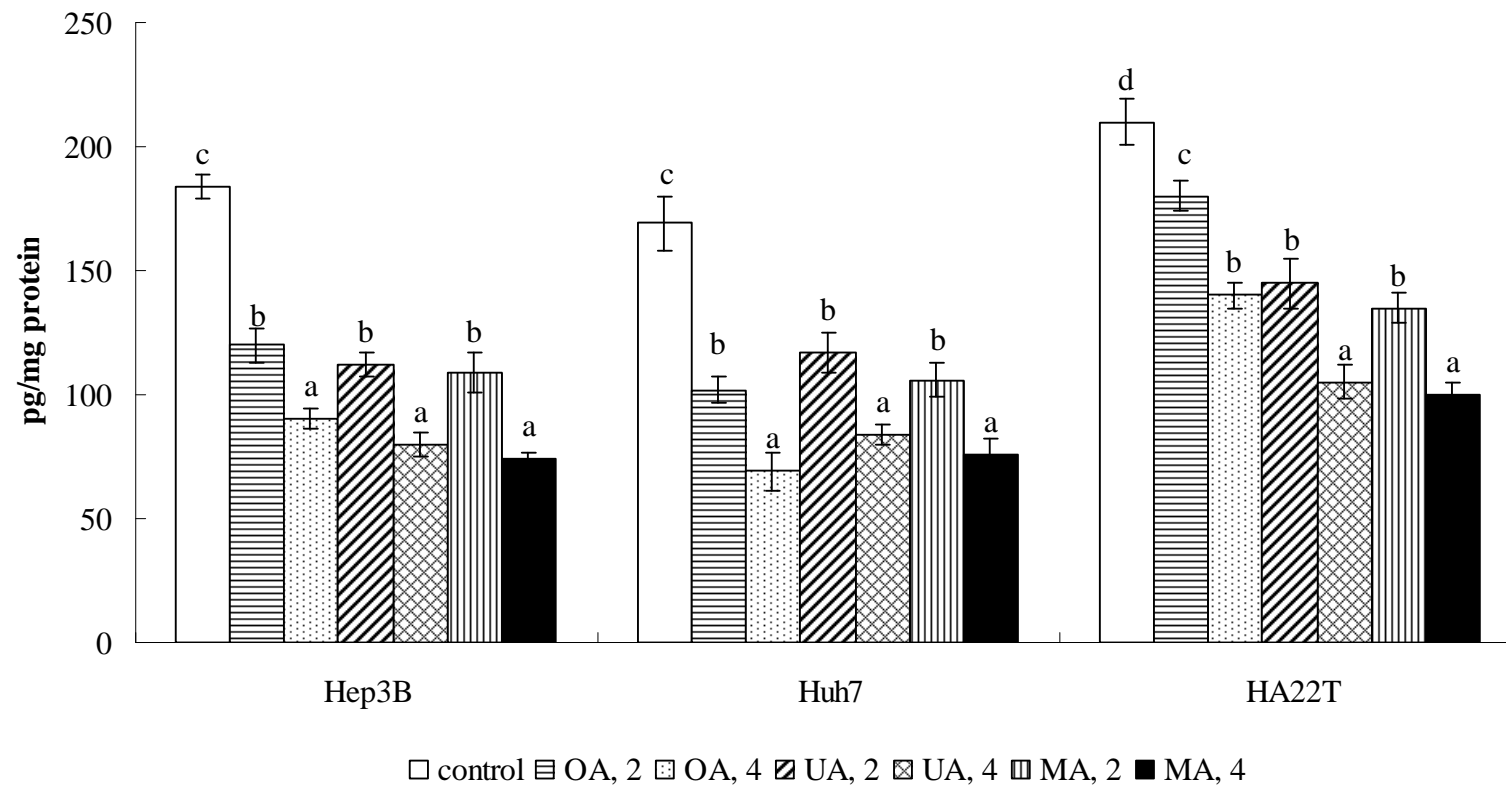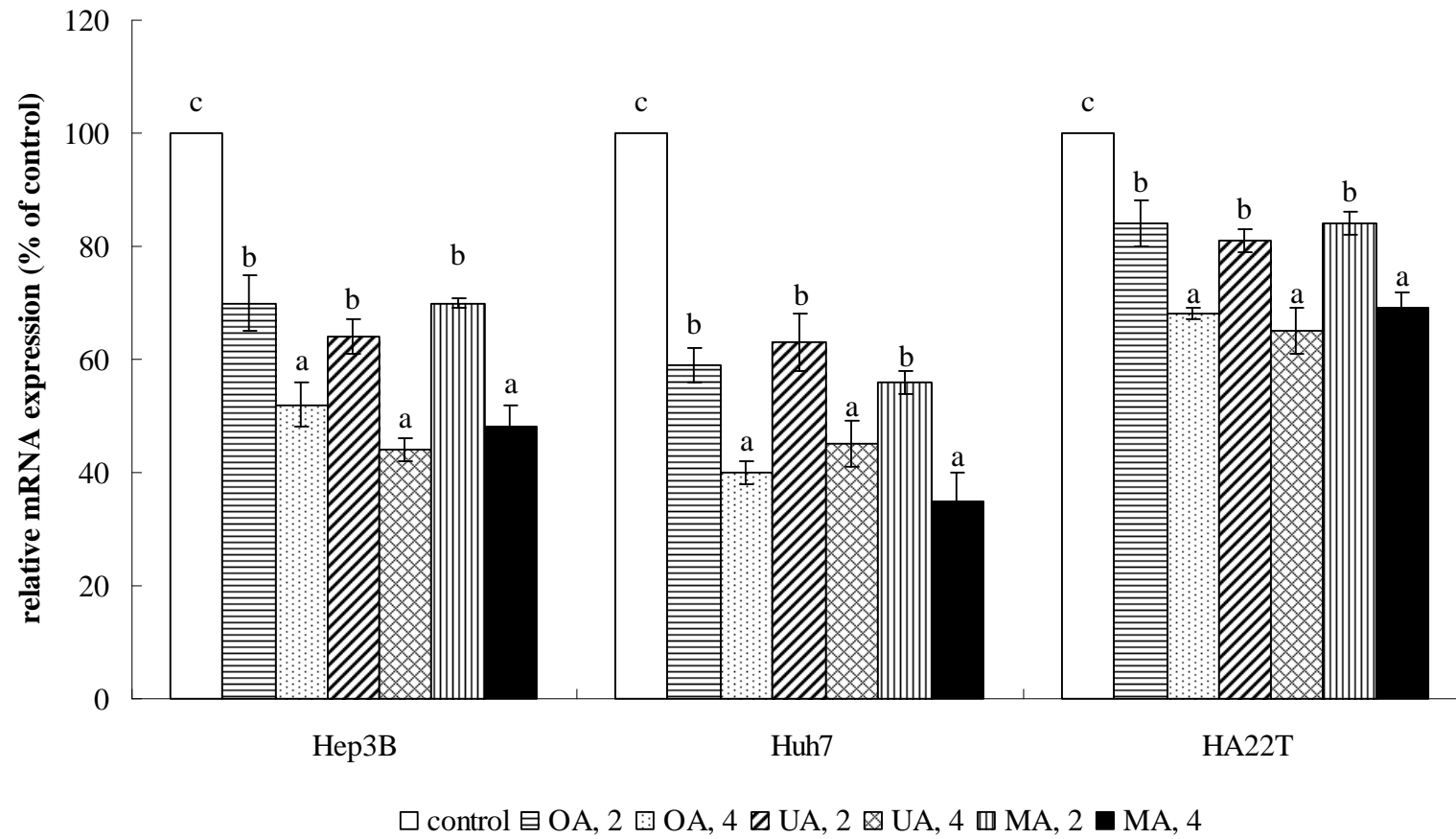
1

1   **Figure 3.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 μmol/L upon bFGF mRNA expression in human Hep3B, Huh7 and HA22T

3   cell lines.   Data are mean±SD (n = 9).   [a]Means among bars without a common letter differ,

4   *P*<0.05.
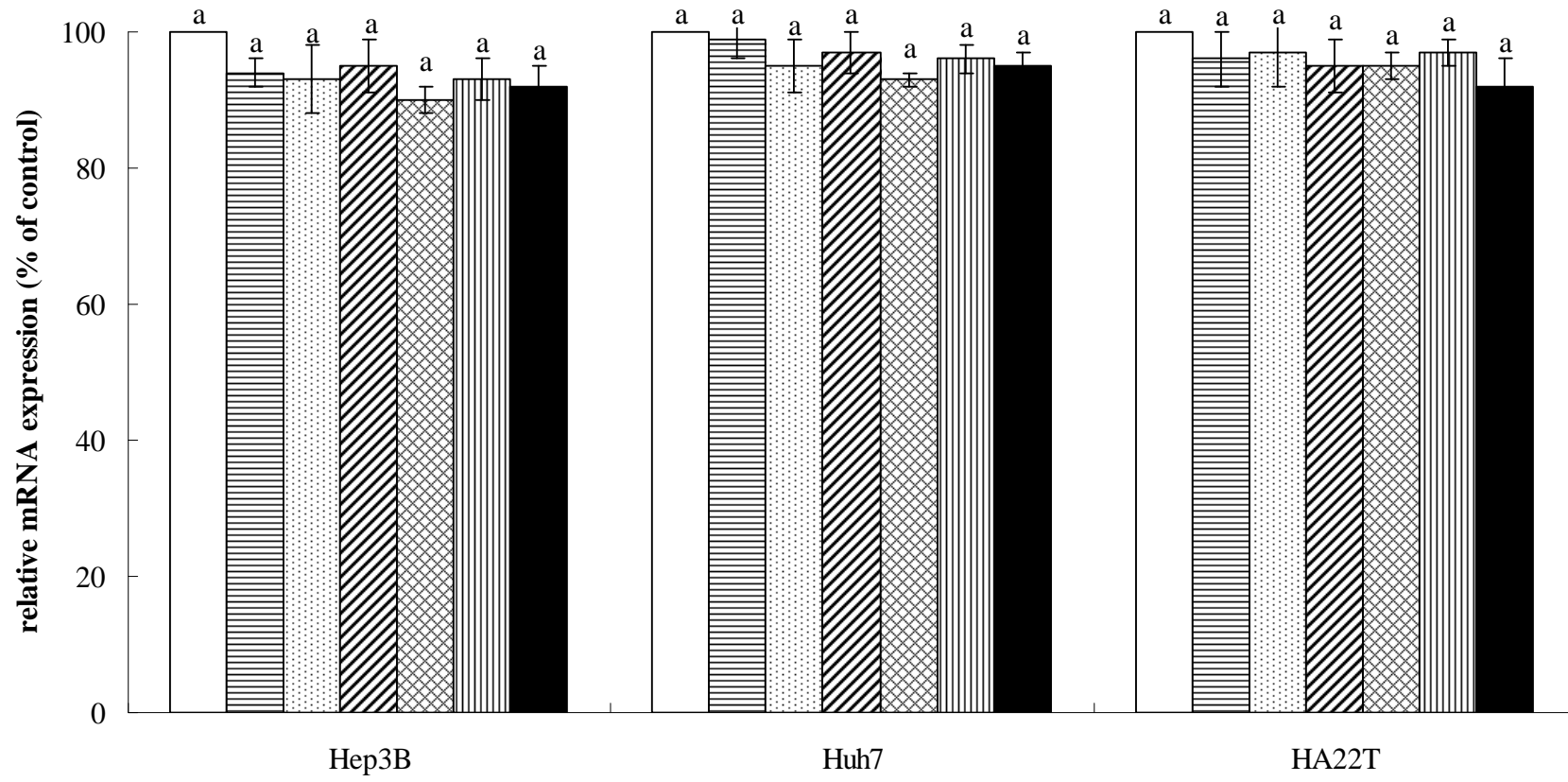
1   **Figure 4.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 μmol/L upon VEGF level (upper part) and mRNA expression (lower part) in

3   human Hep3B, Huh7 and HA22T cell lines.   Data are mean±SD (n = 9).   [a-d]Means among

4   bars without a common letter differ, *P*<0.05.

relative mRNA expression (% of control)

Hep3B   Huh7   HA22T

□ control ⊟ OA, 2 ⊞ OA, 4 ▨ UA, 2 ⊠ UA, 4 ▥ MA, 2 ■ MA, 4
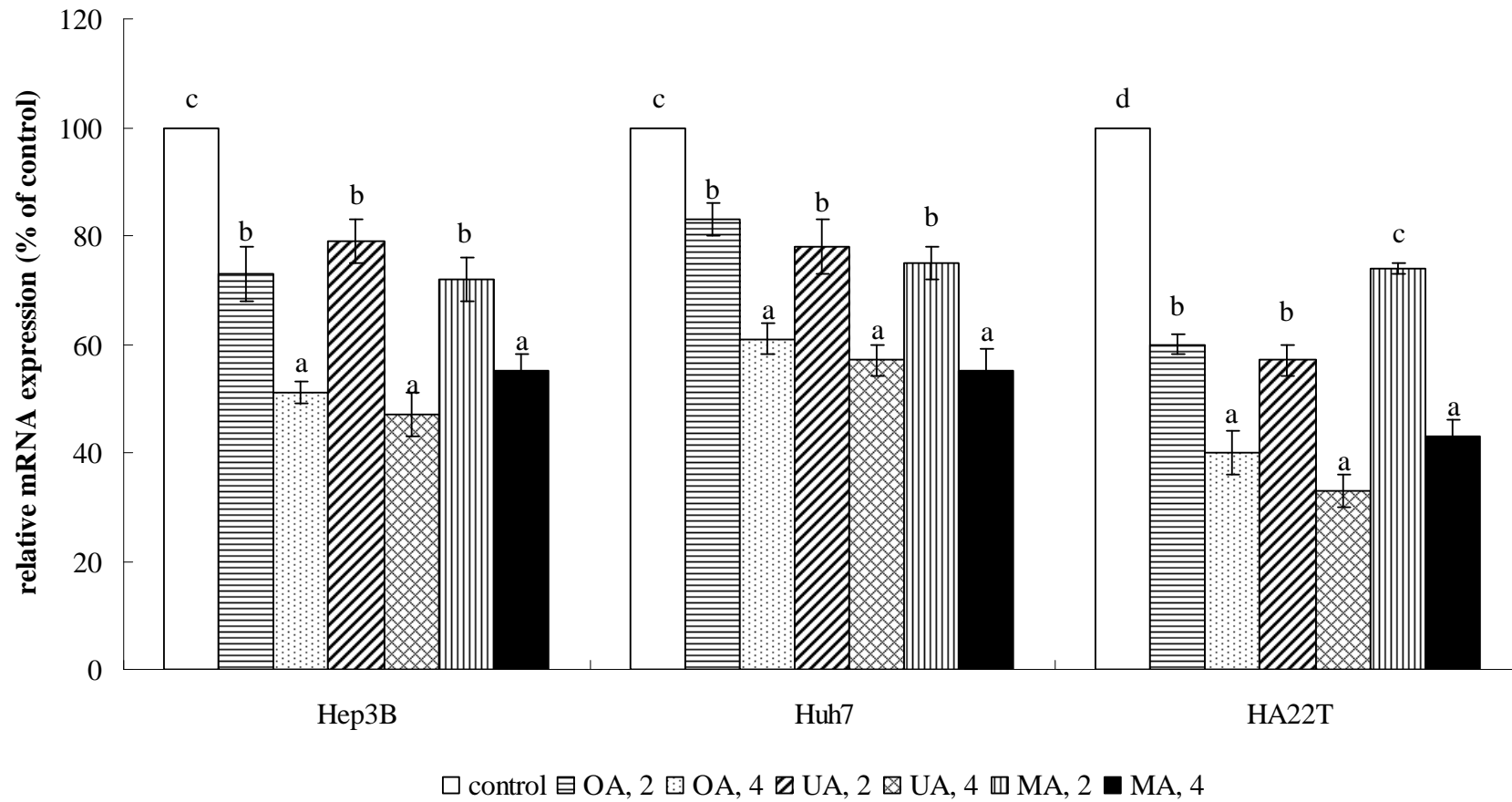
1

1   **Figure 5.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 µmol/L upon IL-8 level (upper part) and mRNA expression (lower part) in

3   human Hep3B, Huh7 and HA22T cell lines.   Data are mean±SD (n = 9).   [a-d]Means among

4   bars without a common letter differ, $P<0.05$.

relative mRNA expression (% of control)

control ☐ OA, 2 ☐ OA, 4 ☐ UA, 2 ☐ UA, 4 ☒ MA, 2 ☐ MA, 4 ■
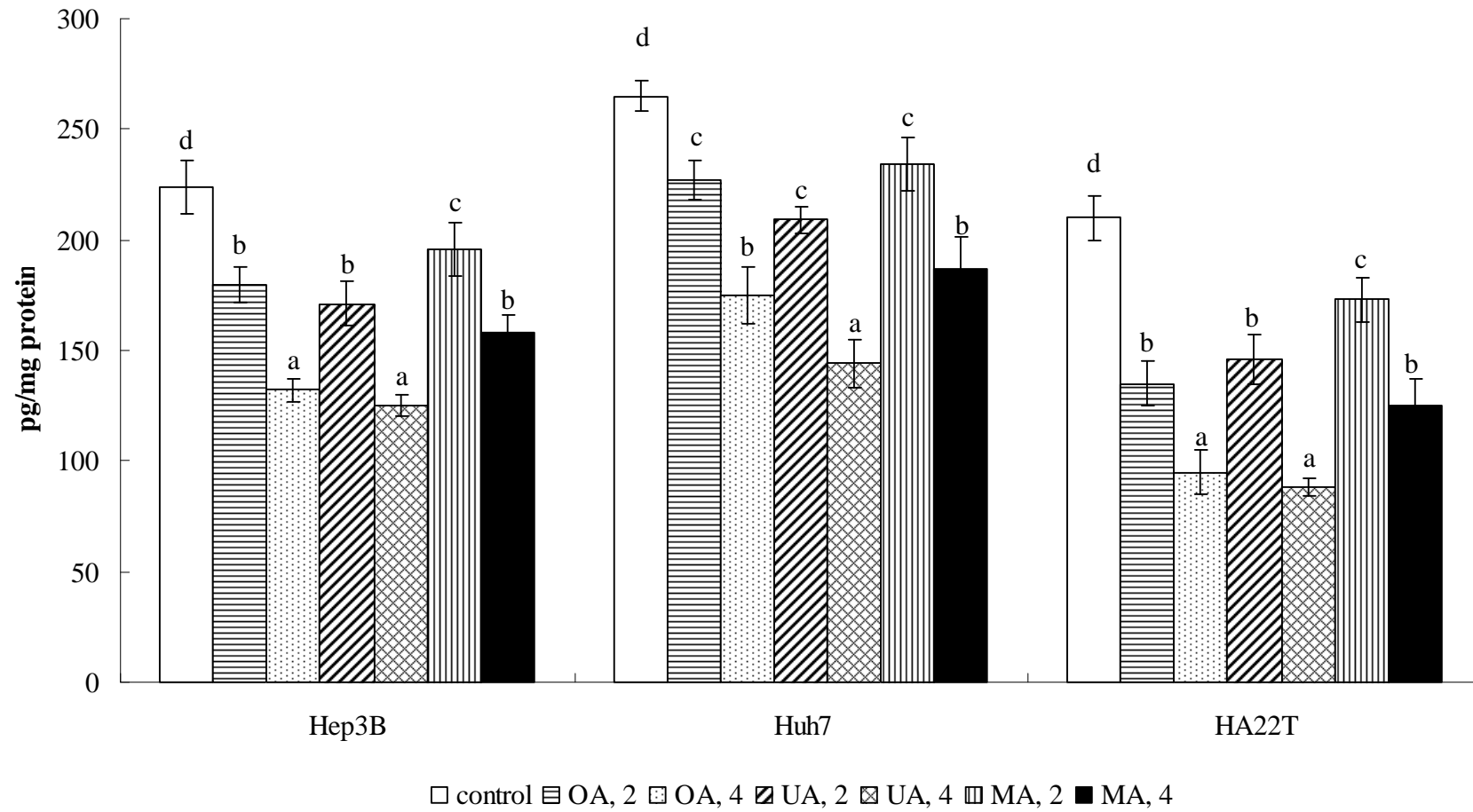
1

2

1 **Table 2**. Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2 (control), 2 or 4 μmol/L upon level of GSH, ROS and NO in human normal liver cell line

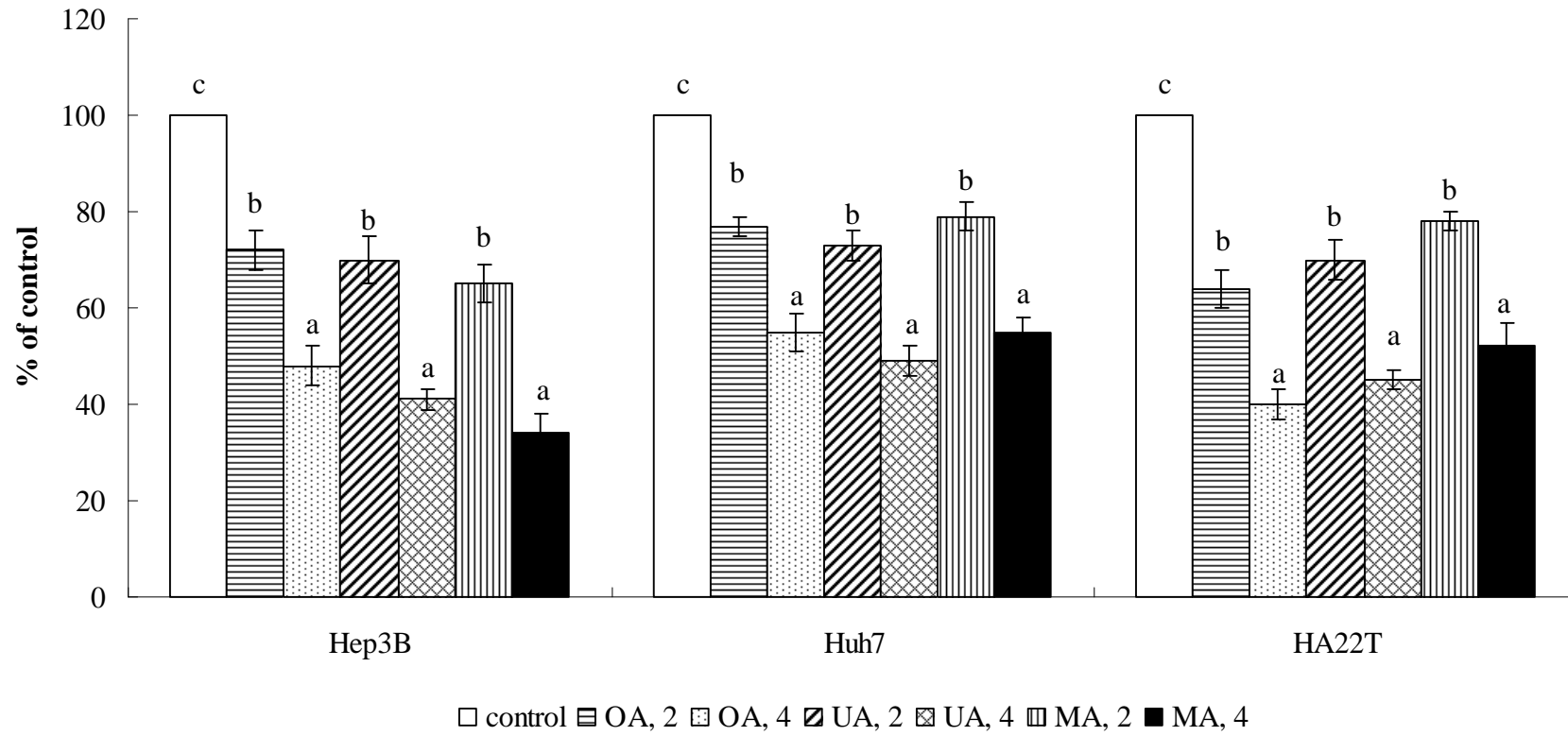3 (L-02 cells), Hep3B, Huh7 and HA22T cell lines.   Data are mean±SD (n = 9).

	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

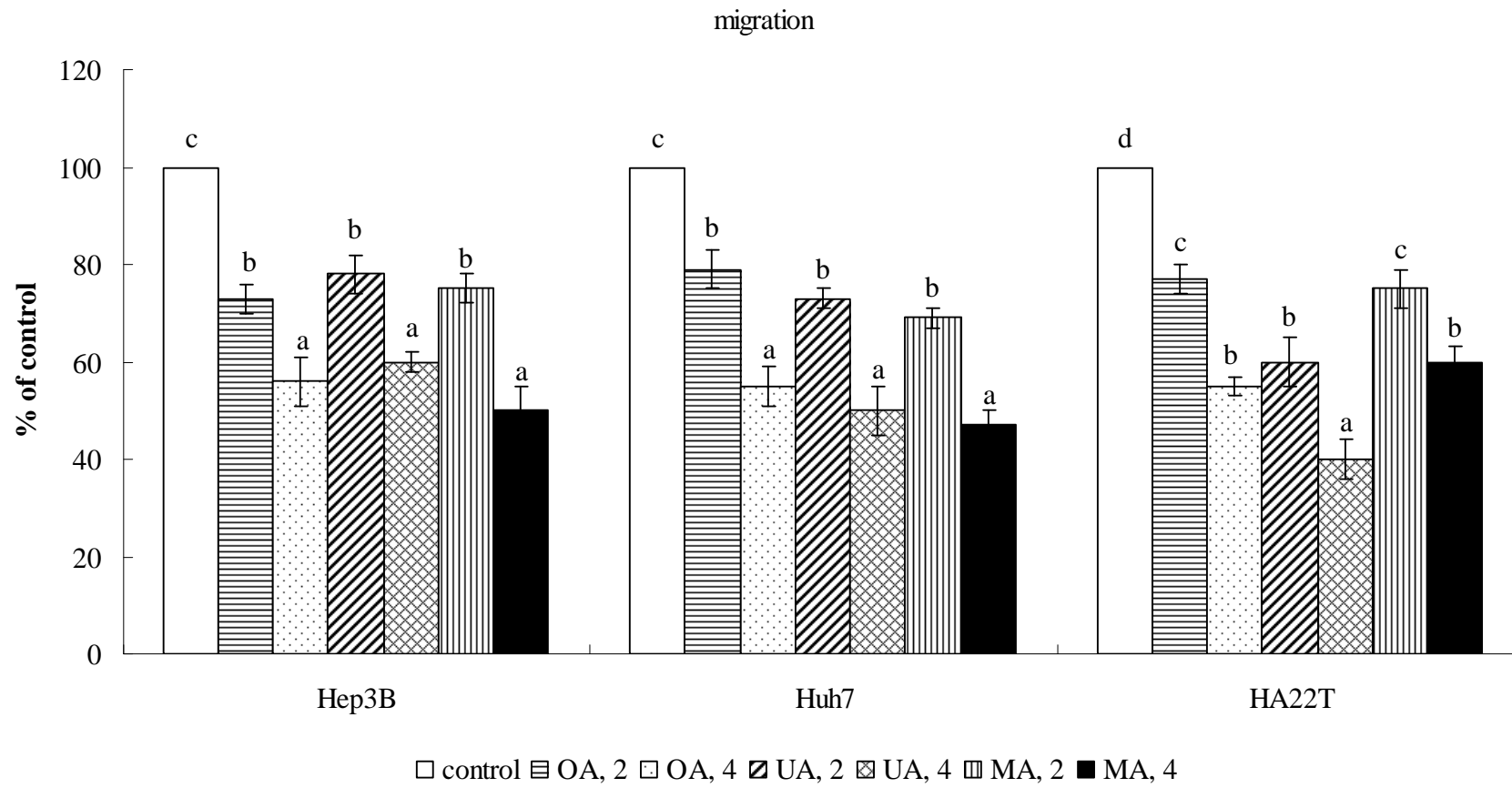|				
Control	1.3±0.3[a]	15.7±1.1[d]	14.6±1.0[c]	15.3±1.4[d]
OA, 2	1.6±0.5[a]	10.5±0.7[b]	12.1±0.6[b]	10.8±1.0[b]
OA, 4	1.4±0.2[a]	8.3±0.9[a]	8.5±1.1[a]	7.9±0.8[a]
UA, 2	1.2±0.4[a]	12.6±0.8[c]	11.8±0.9[b]	12.9±1.2[c]
UA, 4	1.5±0.2[a]	8.0±1.1[a]	8.3±0.7[a]	7.6±0.5[a]
MA, 2	1.4±0.5[a]	13.1±1.0[c]	12.2±1.2[b]	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]

1   [a-d]Means in a column without a common letter differ, $P<0.05$.

1   **Figure 6.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 μmol/L upon invasion and migration in human Hep3B, Huh7 and HA22T

3   cell lines.   Data are mean±SD (n = 9).   [a-d]Means among bars without a common letter
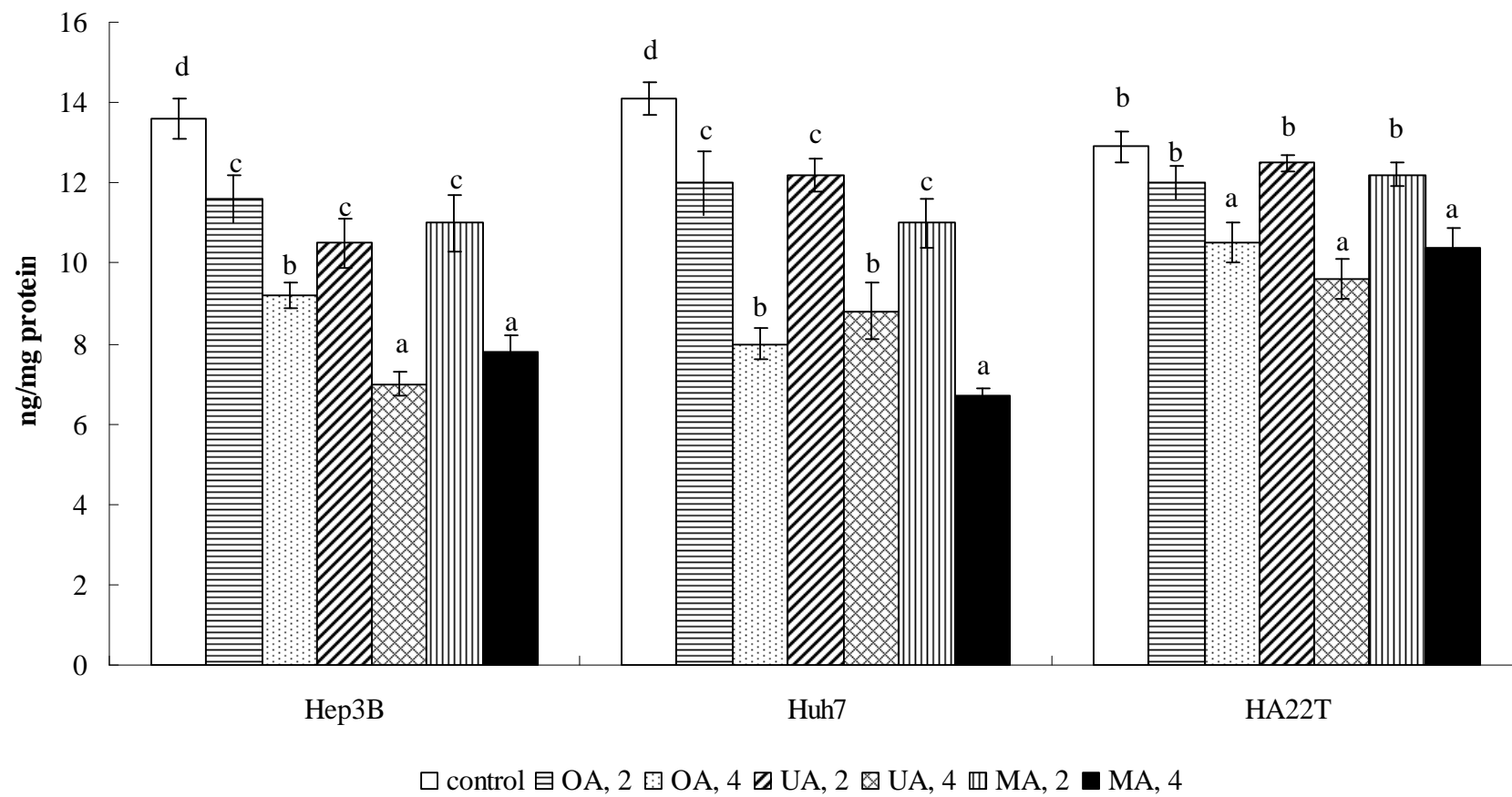
4   differ, $P<0.05$.

invasion

□ control ⊟ OA, 2 ⊞ OA, 4 ◪ UA, 2 ⊠ UA, 4 ⊞ MA, 2 ■ MA, 4

1

2

migration

% of control

Hep3B   Huh7   HA22T

□ control ☰ OA, 2 ▨ OA, 4 ▨ UA, 2 ▨ UA, 4 ▥ MA, 2 ■ MA, 4

1

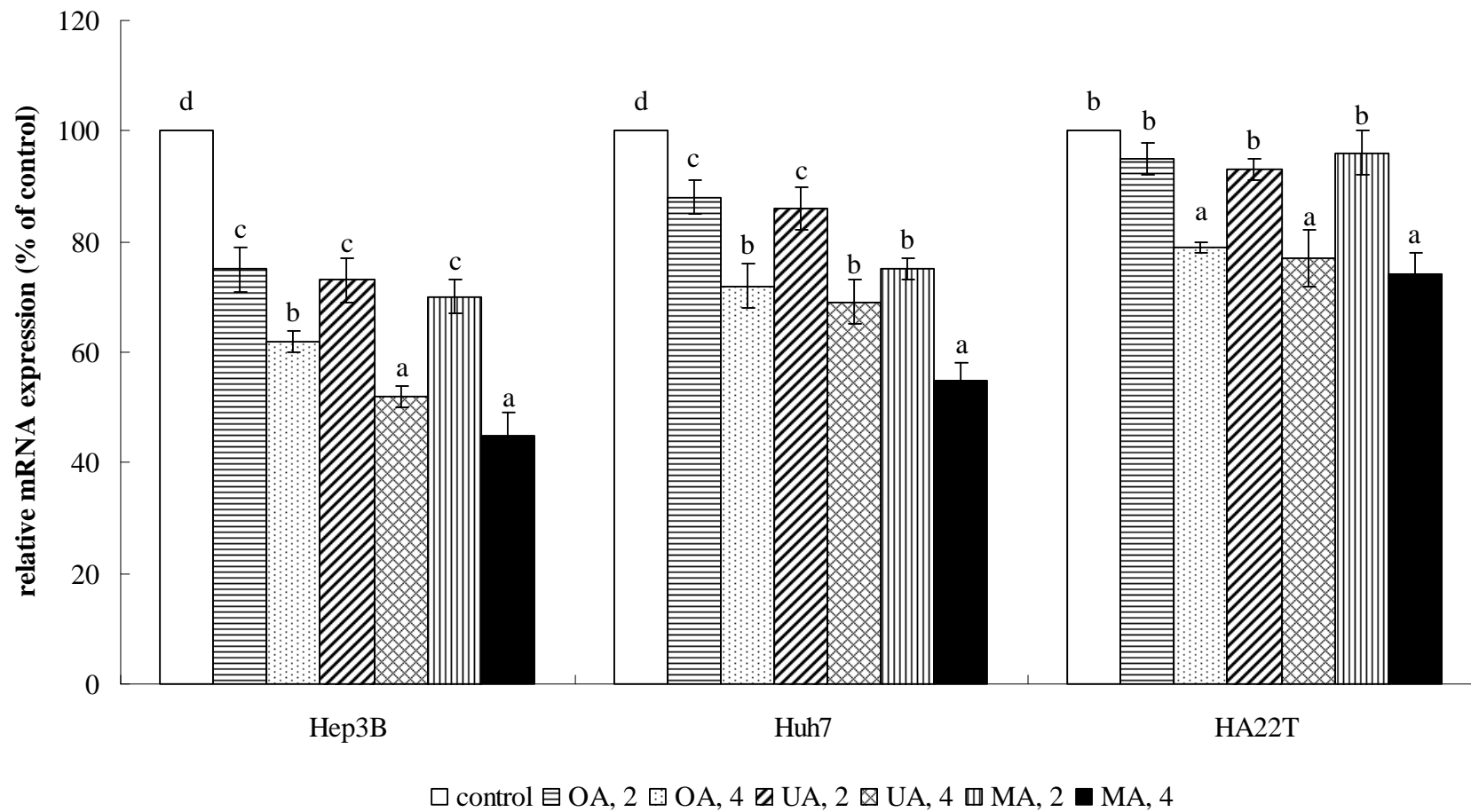1   **Figure 7.** Effect of oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) at 0

2   (control), 2 or 4 μmol/L upon uPA level (upper part) and mRNA expression (lower part) in

3   human Hep3B, Huh7 and HA22T cell lines.   Data are mean±SD (n = 9). [a-d]Means among

4   bars without a common letter differ, $P<0.05$.

1

2

1