Anti-invasive and anti-angiogenic activities of *Curcuma sp.* extracts

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Extracts of a herbal plant, *Curcuma sp.* (Zingiberaceae), were investigated for their anticancer activities. The rhizome of this plant is used in Thai folk medicine to treat cancers and to promote wound healing. In the present study, we performed preliminary bioassays to assess the anti-invasive and anti-angiogenic activities of the methanol (MeOH) and ethyl acetate (EtOAc) extracts. We found that both extracts produced moderate cytotoxic effects against murine hepatocellular carcinoma CBO140C12 cells. Interestingly, the EtOAc extract exhibited remarkable inhibitory effects on the invasion and migration of tumor cells *in vitro*, and on the adhesion of tumor cells to various extracellular matrix proteins. Moreover, the EtOAc extract also inhibited the formation of tube-like structures by hepatic sinusoidal endothelial (HSE) cells cultured on Matrigel-coated substrate, suggesting its anti-angiogenic activity. Altogether, our preliminary results indicate that the EtOAc extract contains active constituents that could potentially be developed into anticancer agents.

Key words adhesion, angiogenesis, cancer, *Curcuma*, curcuminoids, invasion, metastasis, migration, tube formation, *Zingiberaceae*.

Abbreviations EtOAc, ethyl acetate; MeOH, methanol; HSE, hepatic sinusoidal endothelial cells.

Introduction

The use of medicinal plants and their extracts for the prevention or treatment of human diseases is a feature of folk medicine in different parts of the world. In the present study, we investigated the anticancer activities of extracts of *Curcuma sp.* (Zingiberaceae) (Figure 1). This plant has no value as a spice but is used in folk medicine. The plant rhizome has been used by local people in Ubon Ratchathani province, Thailand to treat cancers and to promote wound healing (personal communication). The classification and chemical composition of this plant has not yet been investigated in detail, but preliminary analysis of the crude extract by thin-layer chromatography in our laboratory revealed several phytochemicals such as terpenes, curcuminoids, lavonoids, steroids, and coumarins. We speculate that curcuminoids are most likely the major active constituents in this plant as in other *Curcuma* plants.

Recently, curcuminoids have gained much interest in anticancer research. These compounds possess numerous biological activities that may contribute to their chemopreventive and anticancer effects. Important activities include free radical scavenging and anti-oxidation,[1-3] anti-inflammation,[4-6] and inhibition of tumorigenesis in animal models.[7-12] Curcuminoids have been shown to inhibit the proliferation of a wide variety of cancer cell lines,[13-16] to suppress angiogenesis *in vivo*[17,18] and to induce matrix metalloproteases-9 (MMP-9) expression.[18] In addition, curcuminoids inhibit the growth of normal cells such as thymocytes,[19] vascular smooth muscle cells,[20] osteoclasts[21] and endothelial cells.[22] Furthermore, our group has recently reported that curcumin prevented intrahepatic metastasis in an orthotopic implantation model, possibly mediated by a functional alteration of the cytoskeletal organization of tumor cells.[23]

Tumor invasion and metastasis are the major causes of treatment failure and death in cancer patients. Although there are several drugs available to control cancer growth in humans, no drug specifically inhibits tumor metastasis. Thus, development of new drugs that block metastatic events would significantly enhance the effectiveness of cancer therapy. The process of metastasis is a cascade of linked sequential events.[24-25] Any drug that can inhibit one of the steps in the cascade would be useful for the inhibition of tumor metastasis. This cascade includes the release of tumor cells from the primary site, invasion into the subendothelial basement membrane, dissemination through the blood or lymphatic circulation, adhesion to the basement membrane, and invasion and proliferation at distant sites. Tumor cell invasion involves the expression and/or activation of extracellular matrix protein (ECM)-degrading proteases, which can be categorized into four classes: serine proteases, aspartyl proteases, cysteine proteases and the largest class, MMPs.[26-28] Altered activity of each of these enzymes has been shown to be associated with tumor invasion, metastasis, and angiogenesis. In addition to proteolytic degradation of ECM, both cell-cell and cell-stroma interactions also play

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important roles in the invasive cascade.[29] Connections through cell adhesion molecules, integrins, and cadherins stabilize tissue integrity, whereas loss or alteration of these molecules has been shown to be associated with increased metastatic potential.

Another fundamental process required for tumor growth and metastasis is angiogenesis, which is the formation of new capillaries from preexisting vasculature. Angiogenesis proceeds in several steps that include degradation of the basement membrane, migration of endothelial cells towards an angiogenic stimulus, proliferation of endothelial cells, lumen formation and ECM remodeling.[30] Inhibition of angiogenesis has become a promising therapeutic method for cancers and other angiogenic diseases such as rheumatoid arthritis and diabetic retinopathy.[31-32]

In this study, we assessed the anticancer activities of *Curcuma* extracts. We showed here that the ethyl acetate (EtOAc) extract significantly inhibited the invasion and migration of tumor cells *in vitro*, and the adhesion of tumor cells to fibronectin, laminin and collagen type IV. Moreover, it also attenuated the formation of tube-like structures by hepatic sinusoidal endothelial cells on Matrigel *in vitro*, suggesting that it possesses anti-angiogenic activity. These findings indicate the potential of this plant as a natural source for anticancer drug development.

Materials and Methods

Plant materials. Rhizomes of *Curcuma* sp. (closer species to *Curcuma comosa* Roxb) (Figure 1) were collected in Ubon Ratchathani Province, Thailand in 2002. This plant is locally known as "Wan Mah Wao", and a voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand.

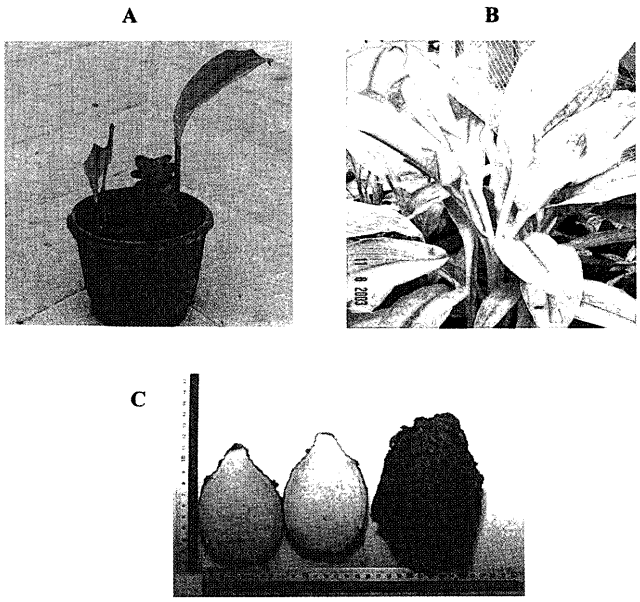

Figure 1 *a)* Flower and leaves of *Curcuma* sp.; *b)* Plant leaves; *c)* Plant rhizomes

Extraction and isolation. Dried rhizomes (690 g) were extracted successively in a Soxhlet apparatus with *n*-hexane, ethyl acetate, and methanol:H_2O (9:1). Concentration of the extracts using a rotary evaporator yielded *n*-hexane (22.43 g), ethyl acetate (EtOAC) (73.34 g), and methanol: H_2O (MeOH) (67.04 g) extracts, respectively.

Cells. Murine hepatocarcinoma (CBO140C12) cells were maintained in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Gibco BRL Products, Gaithersberg, MD). This medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-(+)-glutamine, 0.1% sodium hydrogen carbonate, and 2 mM glutamate (Wako Pure Chemicals Ind., Ltd., Kyoto, Japan). Hepatic sinusoidal endothelial cells (HSE) were maintained in T25 flasks precoated with Attachment factor™ (Cell Systems Corporation, Kirkland, WA, USA) in DMEM/F12 complete medium containing 5% FCS and 2 μg/ml of endothelial mitogen (Biomedical Technologies Inc. Stoughton, MA, USA). All cell types were cultured at 37℃ in a humidified 5% CO_2 incubator.

Cytotoxicity assay. Cellular viability in the presence or absence of the extracts was determined using a WST-1 Cell Counting Kit (Wako Pure Chemicals Ind., Ltd., Japan). In brief, murine CBO140C12 cells ($1x10^4$) were suspended in 100 μl of DMEM/F12 containing 1% FCS and seeded into the well of a 96-well culture plate (Coster, Cambridge, MA, U.S.A). After 12 h of incubation in a humidified 5% CO_2 incubator at 37℃, cells were treated with 100 μl of various concentrations of each extract and incubated for a further 24 h. At the end of the incubation, 10 μl of WST-1 solution were added to each well and incubated with the cells there for 2 h. The amount of soluble formazan formed was measured spectrophotometrically at 450 nm using an Immuno Mini NJ-2300 microplate reader. Extracts were dissolved in DMSO and then diluted with medium. Medium containing less than 0.1% of DMSO had no cytotoxic effect on the cells.

HSE cells ($5x10^3$ cells/well) were suspended in 100 μl of DMEM/F12 complete medium containing 5% FCS and 2 μg/ml of endothelial mitogen and seeded into a 96-well plate that has been precoated with Attachment factor™. Following 24 h of incubation, cells were treated with 100 μl of various concentrations of each extract in DMEM/F12 medium containing 0.2% FCS, and incubated for a further 24 h. At the end of incubation, cell viability was determined using the WST-1 Cell Counting kit as described above.

In vitro invasion and migration assays. Invasion of tumor cells through the reconstituted basement membrane, Matrigel, was assessed in Transwell cell culture chambers as described previously.[33] In brief, polyvinylpyrrolidone-free carbonate filters of 8.0-μm pore size (Nucleopore, Pleasanton, CA) were mounted in the Transwell chambers (Costar 3422, Cambridge, MA, U.S.A). The lower surface of the filters was coated with 2 μg of fibronectin (Iwaki Glass Co., Ltd., Japan) and the upper surface was coated with 5 μg of Matrigel (Collaborative Research Inc., Bedford, MA, U.S.A). These precoated filters were washed extensively in phosphate buffered saline (PBS) and air-dried at

room temperature before use. Exponentially growing CBO140C12 cells were harvested and resuspended in DMEM/F12 containing 1% FCS and various concentrations of the extracts. One hundred μl of cell suspension ($1x10^5$ cells/chamber) was then added to the upper chamber and incubated in a 24-well culture plate at 37°C for 12 h. At the end of incubation, the cells on the filters were fixed with 30% methanol and stained with 0.5% crystal violet for 5 min. After gentle rinsing with water, the cells remaining in the Matrigel were removed by wiping the upper chamber with a cotton swab. The filters containing the stained cells that had invaded or migrated through the lower surface were removed from the Transwell chambers and transferred to a separate well in a 96-well microplate. The crystal violet dye retained on the filters was extracted with 30% acetic acid and colorimetrically assessed by measuring its absorbance at 590 nm using an Immuno Mini NJ-2300 microplate reader.

The migration assay was performed using a procedure similar to that of the invasion assay except the upper surface of the filters was not coated with Matrigel.

Adhesion assay. For the cell adhesion assay, 96-well microculture plates were coated with matrix proteins at a concentration of 50 μg/ml overnight and then treated with 100 μl of 10% FCS-medium for 1 h to block the remaining protein binding sites. The matrix proteins included fibronectin, laminin, and collagen type IV. CBO140C12 cells were harvested and resuspended in FCS-free medium, and were pre-treated with extract at room temperature for 30 min prior to seeding into a 96-well culture plate at a density of $4x10^4$ cells/well. The cells were allowed to attach to the matrix proteins at 37°C for 1 h. The non-adherent cells were removed by vacuum aspiration. The adherent cells were washed with FCS-free medium and fixed with 20% formalin at 4°C for 1 h. After incubation, formalin was removed and the wells were washed with water 5 times, and dried at room temperature. The crystal violet dye in the stained cells was extracted with 30% acetic acid, and colorimetrically assessed by measuring its absorbance at 590 nm using an Immuno Mini NJ-2300 microplate reader.

Gelatin zymography. Conditioned media of CBO140C12 cells were electrophoresed under non-reducing conditions on gelatin zymogram containing 7.5% SDS-PAGE copolymerized with 0.1% type A gelatin (Sigma Chemical Co). After electrophoresis in 25 mM Tris-glycine, 0.1% SDS, gels were washed with rinsing buffer (50 mM Tris-HCl, pH 7.5, 2.5% Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05%NaN_3) at room temperature to remove SDS. The gels were cut into pieces and incubated with the desired concentrations of the extracts in incubation buffer, (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3). Following incubation at 37°C for 24 h, gels were stained with 0.1% Coomassie Blue R 250. The locations of the enzymes were visualized as clear bands on the blue background after destaining with 10% isopropranol in 10% acetic acid.

In vitro formation of tube-like structures. HSE cells (passage 2-3) were pre-treated with the desired concentrations of the extracts in conditioned medium (DMEM/F12 supplemented with 0.2% FCS) at 37°C for 24 h in a humidified 5% CO_2 incubator. After incubation, cells were harvested and resuspended with the desired concentrations of the extracts in conditioned medium. Cell suspensions ($4x10^4$ cells/well) were then applied to the wells of a 48-well culture plate precoated with 1 mg of Matrigel. The plate was incubated at 37°C for 4-6 h. The formation of tube-like structures was observed under the microscope and photographed at *x4* and *x10* magnifications. The degree of tube-like structure formation was measured in four different fields of each well from printed photographs. Data are expressed as a percentage of the length of tubes in untreated control wells.

Statistical analysis. Data from two or more independent experiments are expressed as mean values ± S.E.M. Data from single experiments (e.g., in the tube-formation assay) are expressed as mean values ± S.D. Statistical comparisons of the data were performed using the unpaired Student's *t* test (two-tailed). A p value < 0.05 was considered statistically significant.

Results

Cytotoxic activity of Curcuma extracts. The cytotoxic effect of *Curcuma* extracts against murine hepatocellular carcinoma CBO140C12 cells is shown in Figure 2. Both MeOH and EtOAc extracts at concentrations of more than 100 μg/ml showed clear cytotoxic effects against CBO140 C12 cells after 24 hours of incubation.

Curcuma extract inhibited the invasion and migration of tumor cells in vitro. The effects of *Curcuma* extracts on the invasion and migration of CBO140C12 cells *in vitro* are shown in Figure 3. The EtOAc extract inhibited the *in vitro* invasion (Figure 3a) and migration (Figure 3b) of CBO140C12 cells. At the concentration of 50 μg/ml, the levels of inhibition of tumor cell invasion and migration were approximately 62% and 50%, respectively, but no sig-

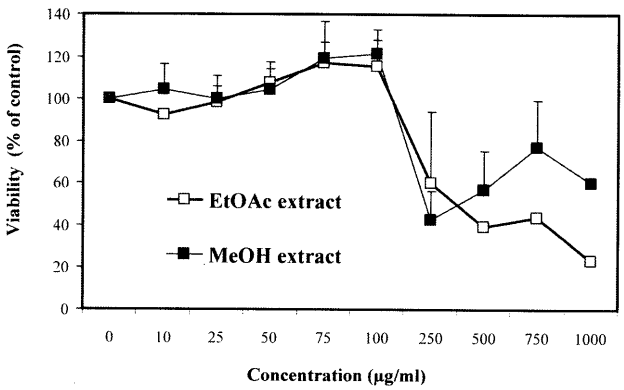

Figure 2 Cytotoxic effect of *Curcuma* extracts against CBO140C12 cells. Cells ($1x10^4$ cells/well) were grown in the absence or presence of various concentrations of ethyl acetate (EtOAc) or methanol (MeOH) extracts. Cell viability was assessed 24 h later by a WST-1 colorimetric assay. Experiments were done in triplicate wells and repeated at least two times. Data were expressed as mean±S.E.M.

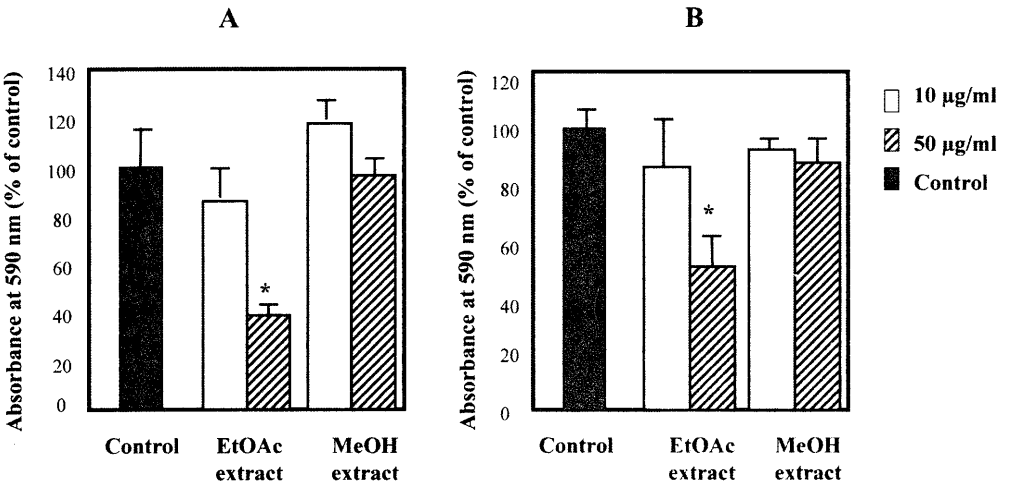

Figure 3 Inhibitory effects of *Curcuma* extracts on *in vitro* invasion (*a*) and migration (*b*) of tumor cells. The invasive and migrating properties of CBO140C12 cells were assessed in the absence or presence of the EtOAc or MeOH extract. Cells ($1x10^5$ cells/chamber) were added to the upper compartment of the chamber and incubated at 37°C for 12 h. For the migration assay, the upper surface of the filter was not coated with Matrigel. The number of invading or migrating cells was determined by crystal violet staining and colorimetrically quantitated by measuring the absorbance at 590 nm. Experiments were done in quadruplicate wells and repeated two times. Data were expressed as mean ± S.E.M. (*$p < 0.001$).

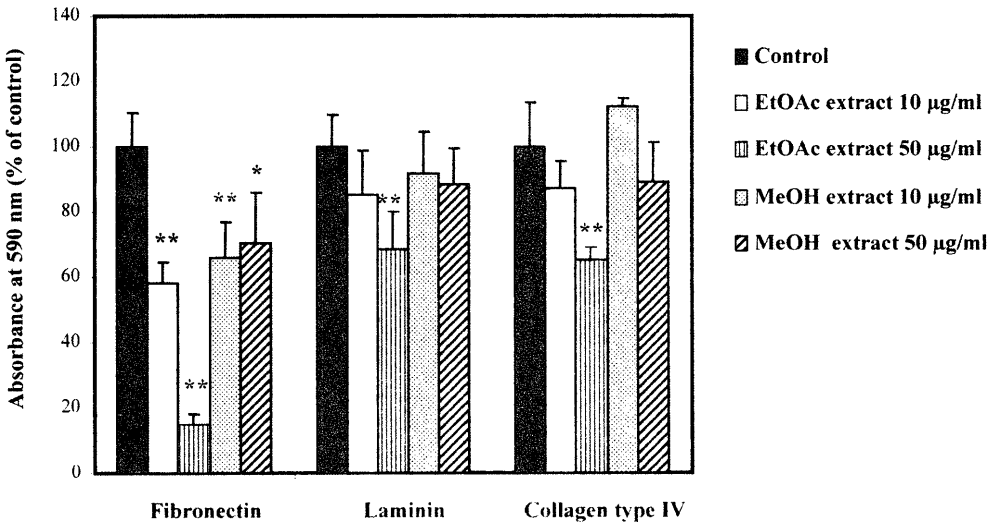

Figure 4 Inhibitory effects of *Curcuma* extracts on the adhesion of murine CBO140C12 cells to various matrix proteins. Cells were pre-treated with the extract for 30 min, and then $4x10^4$ cells/well were seeded into a 96-well plate that had been pre-coated with the indicated matrix protein. Following a 1-h incubation, non-adhered cells were removed and the adherent cells were fixed with formalin. The cell number was quantitated by the crystal violet staining method. Experiments were carried out in quintuplicate wells and repeated at least two times. Data were expressed as mean±S.E.M. (*$p < 0.01$, **$p < 0.001$)

nificant effects were observed at 10 μg/ml. In contrast, pre-treatment of CBO140C12 cells with the MeOH extract at concentrations of 10 and 50 μg/ml did not affect the invasion or migration of tumor cells.

Curcuma extracts inhibited the in vitro adhesion of tumor cells to matrix proteins. The ability of *Curcuma* extracts to interfere with tumor cell adhesion to fibronectin was observed when CBO140C12 cells were pre-treated with either the EtOAc or MeOH extract at non-cytotoxic concentrations of 10 and 50 μg/ml (Figure 4). Pretreatment of the cells with 50 μg/ml of the EtOAc extract showed the strongest activity (~ 85% inhibition). A less marked inhibition of the cell adhesion to laminin and collagen type IV was also observed at the concentration of 50 μg/ml. In contrast, the MeOH extract had only a slight effect on the adhesion to fibronectin (~30% inhibition) and no effect on the adhesion to laminin or collagen type IV.

Zymographic analysis of gelatinase activities in tumor cells. Zymographic analysis of the conditioned medium of CBO140C12 cells showed clear digested bands at 92 kDa and 72 kDa, which represented MMP-9 and MMP-2, respectively. These gelatinases activities were unaffected

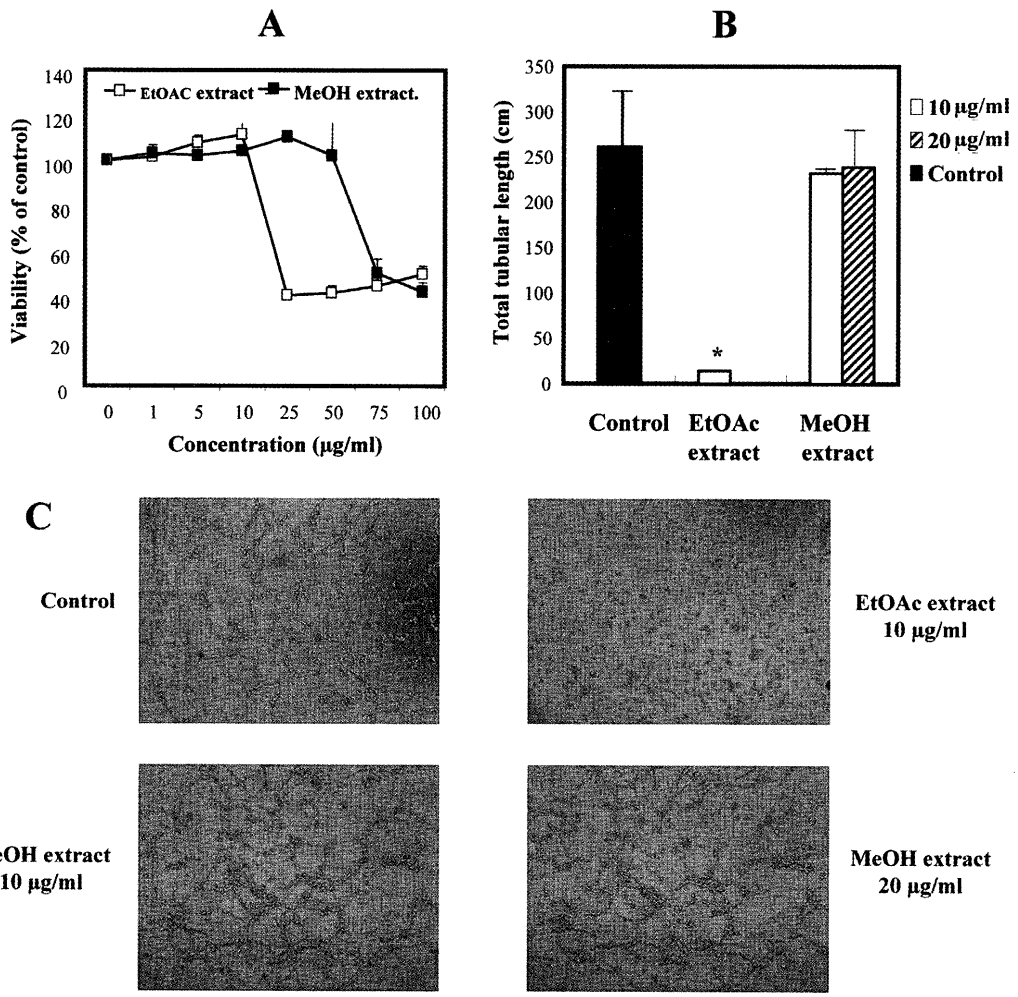

Figure 5 Cytotoxic effect of *Curcuma* extracts against HSE cells and inhibitory effect on the formation of tube-like structures *in vitro*. HSE cells ($4x10^4$ cells/well) were pre-treated for 24 h with the EtOAc or MeOH extract and seeded on Matrigel-coated wells. *a)* Cytotoxic activity of *Curcuma* extracts against HSE cells; *b)* Total tubular lengths measured from printed photographs; *c)* Photographs of tube-like structures were taken from four different corners at 6 h. Data were expressed as mean ± S.D. ($*p < 0.001$).

when *Curcuma* extracts were added to the culture at concentrations of 10 and 50 μg/ml (data not shown).

Curcuma extract inhibited the formation of tube-like structures by endothelial cells in vitro. To evaluate the anti-angiogenic activities of *Curcuma* extracts, their cytotoxic effects against endothelial cells were first determined. The EtOAc extract showed a stronger cytotoxicity against HSE cells (Figure 5a) than the MeOH extract. The cytotoxic effect of EtOAc extract against HSE cells was over 10-fold higher than that against CBO140C12 cells (Figure 2). The effect of *Curcuma* extracts on the formation of tube-like structures was then analyzed using non-cytotoxic concentrations of extracts. In cells pre-treated with 10 μg/ml of EtOAc extract for 24 h, the formation of tube-like structures in Matrigel-coated wells was almost completely inhibited (Figures 5b, 5c). In contrast, cell treatment with the MeOH extract at 10 or 20 μg/ml showed no effect.

Discussion

The present study demonstrated the *in vitro* anticancer activities of *Curcuma* extracts. The cytotoxic activities of the EtOAc and MeOH extracts against murine hepatocellular carcinoma CBO140C12 cells were dose-dependent. Interestingly, HSE cells were much more sensitive to the cytotoxic effects of the *Curcuma* extracts than CBO140C12 cells, suggesting that the *Curcuma* extracts may specifically inhibit the proliferation of endothelial cells. Although it is not yet known whether this effect is mediated by the plant curcuminoids, our finding is consistent with previous reports on the strong inhibi-tion of endothelial cell proliferation by curcuminoids.[22,34]

We further focused on the anti-invasive and anti-angiogenic properties of *Curcuma* extracts at non-cytotoxic concentrations. We demonstrated that the EtOAc extract at 50 μg/ml significantly inhibited the *in vitro* invasion of CBO140C12 cells (Figure 3a). This effect was not observed with the MeOH extract. Tumor invasion is a multistep process that consists mainly of tumor cell adhesion, migration, and enzymatic degradation of extracellular matrix proteins. To gain some insight into the mechanism of the tumor anti-invasive property of the EtOAc extract, we further assessed

the effect of *Curcuma* extracts on tumor cell migration toward a chemoattractant (fibronectin). The results revealed the migration of CBO140C12 cells was inhibited similarly to their invasiveness. We also tested the effect of *Curcuma* extracts on the adhesion of CBO140C12 cells to various matrix components, *i.e*, fibronectin, laminin, and collagen type IV. We successfully demonstrated, using an *in vitro* adhesion assay, a significant inhibition of CBO140C12 cell adhesion to fibronectin by the EtOAc extract and a substantial inhibition of adhesion to laminin and collagen type IV (Figure 4). These effects appeared to be concentration dependent. The MeOH extract also attenuated the adhesion of CBO140C12 cells to fibronectin, but not to laminin or collagen type IV.

The process of tumor invasion also requires proteolysis of the ECM by MMPs. In particular, MMP-2 and MMP-9 play important roles in the invasion of many aggressive types of cancer.[26,27] Therefore, we next investigated the effects of *Curcuma* extracts on gelatinase activities. We found that *Curcuma* extracts did not affect the activities of MMP-2 or MMP-9. Although we were unable to rule out the participation of other metalloproteinases, the changes in *in vitro* CBO140C12 cell invasion induced by *Curcuma* extracts are probably not due to reduced degradation of the ECM. Moreover, the strong inhibition of cell adhesion to matrix proteins, especially to fibronectin, and of cellular migration indicate that the EtOAc extract mainly affects tumor cell adhesion and migration properties.

Another important activity of the EtOAc extract revealed in our study was a significant inhibition of the formation of tube-like structures by HSE cells (Figures 5b, 5c). This effect occurred at a concentration as low as 10 μg/ml, which was not cytotoxic and did not affect cell viability (Figure 5a). The process of angiogenesis requires endothelial cell activation, migration, proliferation, and tube formation.[30] It appears that the effect of the EtOAc extract on tube formation was not due to reduced cellular proliferation because it occurred within 4-6 h after the cells were seeded on Matrigel. Although it has not yet been tested, we speculate that the inhibition of tube formation may be associated with the reduction of cellular migration. Similar to our finding, a number of studies have reported the inhibitory activity of curcuminoids on angiogenesis.[17,18,35] The molecular mechanism of this activity is not yet clearly understood but may involve inhibition of the expression of gelatinase B and some angiogenic factors such as vascular endothelial growth factor (VEGF), or angiopoietin 1 and 2. The inhibition by the EtOAc extract of the formation of tube-like structures by endothelial cells suggests its anti-angiogenic activity. The importance of this *in vitro* function to the clinical antitumor activity of this plant remains to be determined.

In conclusion, the results obtained in our study strongly indicate that the EtOAc extract of *Curcuma* possesses significant biological activities. The ability of this extract to inhibit *in vitro* tumor cell invasion, migration, and endothelial cell tube-like structure formation at relatively low concentrations suggests the potential for anticancer drug development. Further purification using bioassay-guided fractionation, structural elucidation of active compounds, and investigation of the molecular mechanisms of the anticancer activities will be carried out in our laboratory.

Acknowledgment

This work was supported in part by a Japanese-Thai Collaborative Scientific Research Fellowship (JSPS-NRCT), 2003, and also by a Grant-in-Aid for the 21th Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Japanese abstract

ショウガ科に属する *Curcuma* 種の根茎の抽出エキスを用いて抗腫瘍活性を検討した。この植物の根茎はタイの伝統医学において，癌の治療や創傷治癒を促進するために使われている。本研究では，メタノール及び酢酸エチルの抽出エキスを用いて，癌細胞の増殖，浸潤及び血管新生に対する抑制作用について予備的検討を行った。両エキスは，*in vitro* におけるマウスの肝細胞癌：CBO140C12 細胞の増殖に対して軽度の抑制効果を示した。興味あることに，酢酸エチル抽出エキスは，癌細胞の浸潤能，運動能及び種々の細胞外マトリックス成分に対する接着能に対して顕著な抑制効果を示した。さらに，マウス肝類洞内皮細胞（HSE）のマトリジェル基質上での管腔様構造の形成の抑制，すなわち，血管新生の阻害活性が認められた。このように，*Curcuma* 種の根茎の抽出エキスを用いた予備検討において，酢酸エチル抽出エキス中には抗腫瘍薬剤を開発するための有効な成分が含まれている可能性が示唆された。

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