



# Human urinary bladder cancer T24 cells are susceptible to the *Antrodia camphorata* extracts

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## Abstract

Bladder cancer has been cited to result from the neoplastic lesion with environmental and/or occupational factors identified as causatives. Transitional cell carcinoma (TCC) is the most common type of bladder cancer. Most of the bladder cancer patients die from the invasive, metastatic TCC that has turned out to be resistant to chemotherapy. T24 cells, a cell line established from a human urinary bladder cancer patient, are high-grade and invasive TCC. T24 cells were found very susceptible to ACCE at concentration of 50 µg/mL. MTT assay showed that the cell growth and proliferation were inhibited to 50% of the control when treated with ACCE for 72 h, at which the cell proliferation suppressing rate revealed  $-4.4 \times 10^3$  cells/µg per day. Comparing the expressions of the cell cycle biomarkers Cdc2 and Cyclin B1 by the western blot analysis, a phase G<sub>2</sub>M arrest was confirmed. Both the wound scratch assay and the transwell motility assay indicated that ACCE was very effective anti-metastatic against T24 cells. Furthermore, the active form of matrix metalloproteinase-9 (MMP-9) was also found totally suppressed as revealed by zymography at 72 h post-incubation with ACCE, while the light and electron microscopic images have apparently revealed cell membrane damages on T24 cells when treated with ACCE (50 µg/mL). Moreover, both the wound scratch and the transwell assays have demonstrated the migration capability of T24 cells has been significantly retarded to 1.5-fold at same dosage of ACCE used. In conclusion, ACCE is a good anti-cancer agent, being effective in inducing phase G<sub>2</sub>M arrest, acting as an anti-proliferative, and an anti-metastatic agent against bladder cancer cell T24 cells.

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**Keywords:** *Antrodia camphorata*; Transitional cell carcinoma (TCC); Migration; Matrix metalloproteinase (MMP)

## 1. Introduction

Bladder cancer, comprising a broad spectrum of tumors including transitional cell carcinoma (TCC),

has been cited to result from the neoplastic lesion with environmental and occupational factors identified as causatives [1]. Epidemiological studies have demonstrated with sufficient evidence that the risk factors associated with bladder cancer involve smoking, and exposures to aromatic amines, paints and solvents, leather dust, inks, heavy metals, polycyclic aromatic hydrocarbons, and diesel combustion and exhaust fumes, etc. [2]. Generally, bladder cancer develops by a multistage process, the symptoms of which are very

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similar to the common infections at the initial phase, hence the diagnosis at the initial stage always has been very confusing and misled. Although majority of the patients may present with superficial bladder tumors, yet 20–40% of the bladder cancer either present with or develop invasive diseases [3]. In Taiwan, TCC has been ranked as the top seven common type cancer in male, and still is rising in incidence and prevalence [4]. Most of the bladder cancer patients die from the invasive, metastatic TCC that has turned out to be resistant to chemotherapy. T24 cells, a cell line established from a high-grade and invasive human urinary bladder cancer patient, belong to one category of urinary bladder epithelial transitional carcinoma cells. T24 cells have several documented characteristics: (1) having extremely low-level of alkaline phosphatase (AKL) isozyme pattern comparing to other four cell lines derived from human bladder carcinomas including RT4, RT112, J82, and EJ [5]; (2) resembling the liver-type AKL in electrophoretic mobility and sensitivity to heat denaturation [5]; (3) containing high proportion of  $\beta$ 1–6 branched tri- and tetraantennary complex type glycans such as P-glycoprotein (P-gp) may facilitate the important function in adhesion and migration of T24 cells, a fact implying that oligosaccharides are involved in several steps of the metastatic process [6]. Several biomarkers for bladder cancer have been investigated in different bladder cancer cell lines including T24, RT4, TSGH 8301, and BFTC 905 [7]. Presence of wild type p53 was found only in cell lines derived from either a low-grade, papillary tumor, while the T24 cells were found to contain a novel p53 mutation in a panel of bladder carcinoma cell lines [8]. The subpopulations of human bladder cancer cell lines of T24, Hi-T24 and Lo-T24, were reported to exhibit similar gross morphology, cell growth rate, and adhesion activity to basement membrane extract (matrigel), but the Hi-T24 cells were 3.8-fold more haptotactic through matrigel than Lo-T24 cells. The amounts of MMP-2 protein secreted into the medium by Hi-T24 and Lo-T24 cells were  $7.8 \pm 0.2$  and  $3.8 \pm 0.3$  ng/mL, respectively, and correspondingly, the quantities of tissue inhibitor of metalloproteinase-2 (TIMP-2) protein secreted were  $133.2 \pm 4.3$  and  $168.7 \pm 5.6$  ng/mL, respectively. Furthermore, the expression of the matrix metalloproteinases (MMP) are imbalanced at the gene level in human urinary bladder cancer cells at the stage of tumor progression [9].

Recently, so-called ‘complementary alternative medicines’ have attracted attention from conventional

medical therapeutists [10]. Some treatment courses have been observed to be more effective and versatile and less toxic than routine clinical treatments. *Antrodia camphorata* is a well-known traditional Chinese medicine. It usually grows only on the inner heartwood wall of the Taiwanese endemic evergreen *Cinnamomum kanehirai* Hay (Lauraceae), seldom found in the wild and more importantly, unable to be cultivated easily in laboratories. *A. camphorata* has long been used as a remedy for chemical intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and hepatoma [11]. Recently, the culture broth of an AC strain (AC strain no. CCRC-35396) has been reported to possess an anti-HBV pyrrolidione, 3-isobutyl-4-{4-[(3-methyl-2-butenyl)oxy]phenyl}-1*H*-pyrrole-2,5-dione [12]. In addition, one of the constituent group, zhankunic acids (A–C) extracted by ethanol from the fruit bodies of AC, has shown to exhibit anti-inflammatory activities and immunomodulating effects in human leukocytes [13,14]. Another constituent group, polysaccharides extracted from the fruit bodies of *A. camphorata*, has shown anti-hepatitis B virus effects [15]. Recently, Nakamura et al. showed satisfactory cytotoxic effects of maleic and succinic acid derivatives from *A. camphorata* mycelia on the LLC tumor cell line, reaching ED50 (the 50% growth inhibition) at a level of 3.6  $\mu$ g/mL [16]. Moreover, in treatment of human premyelocytic leukemia HL-60 cells with fermented *A. camphorata* culture fluid, researchers observed marked apoptosis, which was dose- and time-dependent [17]. To our knowledge, the application of *A. camphorata* to the treatment of bladder cancer has never been documented, in this study, we try to investigate the effect of *A. camphorata* crude extract on bladder cancer T24 cells that have been considered to be a high grade transitional cell carcinoma.

## 2. Materials and methods

### 2.1. *Antrodia camphorata* crude extract (ACCE)

The pulverized crude extract of *Antrodia camphorata* (ACCE) was provided by Well shine Biotechnology Development Co., Ltd (Taipei, Taiwan) following the preparation method as previously described [18], which contained 15–20% of triterpenoids and 1–2% of polysaccharides, the remaining of which was fungal cell wall components (HPLC and GC/MS data not shown). A stock of ACCE solution was prepared by dissolving the ACCE powder in absolute EtOH to make a final concentration of 80 mg/mL and stored at  $-20$  °C. The concentrations of ACCE used in experimentation ranged between 10 and 150  $\mu$ g/mL.

## 2.2. Cell culture

The human urinary bladder cancer cell line T24 (HTB-4, ATCC) was purchased from the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan, ROC) and cultured in McCoY's 5A medium supplemented with 10% fetal bovine serum (FBS) (GIBCO). The mouse embryonic fibroblast (MEF) cell lines as normal cell control was kindly provided by Dr. Hung Li (Academia Sinica, Taipei, Taiwan) and cultured in DMEM medium (GIBCO) supplemented with 10% FBS. All the culture media, after further additions of 100 IU/mL penicillin and 100 µg/mL streptomycin (GIBCO), were used to incubate cells at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Cells were passaged for MTT assay, flow cytometric analysis, western blot analysis, cell migration assay and zymography after 1–3 days of ACCE treatment.

## 2.3. MTT assay

T24 cells ( $5 \times 10^3$ ) were plated onto each well (500 µL/well) of 24-well culture plates for 24 h and treated with ACCE at concentrations of 10, 20, 30, 50, 100, and 150 µg/mL, respectively, or vehicle alone for another 24–72 h. MTT solution (50 µL from 5.5 mg/mL) was then added to each well, and the plates were further incubated at 37 °C for 4 h in a humidified incubator with 5% CO<sub>2</sub>. The medium was then aspirated to ease the formation of the formazan product, which was then solubilized with the addition of 500 µL of DMSO. An aliquot of 200 µL was measured and transferred into 96-well plates. The optical density was measured at 570 nm with a Microplate Autoreader (Bio-Tek Instruments, Vermont, USA). Similar experiments were repeated in triplicates.

## 2.4. Cell cycle analysis

Cell cycle analysis was performed with a flow cytometer (FACS Calibur; BD Biosciences, CA, USA). Cells ( $2 \times 10^4$ ) were cultured in 6-cm culture plates and treated with 50, 100 and 150 µg/mL of ACCE or vehicle alone, respectively, for another 72 h. 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. Data acquisition and analysis were performed in the flow cytometer with the accompanying software (CellQuest; BD Biosciences). Appropriate gating was used to select the easily distinguished single population. Ten thousand events per sample were counted and at least triplicate determinations were performed to assure each cell cycle distribution. The final percentages of cells in each phase were calculated from the triplicate experimentations.

## 2.5. Scanning electron microscopy (SEM)

T24 cells were prepared by cultivating cells on 12-mm-diameter coverslips. Cells ( $2 \times 10^3$ ) were seeded and treated with 50 µg/mL ACCE or vehicle alone. Following 72 h-exposure to ACCE, the culture media were removed and the cells were rinsed three times with PBS. Cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 20 min at room temperature, and fixed with 1% osmium tetroxide in 0.1 M PBS for 30 min. After fixation, the samples were washed with PBS and dehydrated in an ethanol series of the concentrations of 70, 80, 90, 95 and 100% in a critical point dryer (HCP-2, Hitachi Ltd, Tokyo, Japan). A thin layer of palladium–gold was coated on to the samples in a sputtering apparatus (IB-2, Hitachi, Ltd, Tokyo, Japan). The morphology of the cells was then examined using Hitachi S-2400 electron microscope.

## 2.6. Western blot analysis

The cells in 10-cm culture plates were harvested at indicated time following 50 µg/mL of ACCE treatment or vehicle alone and washed with ice-cold PBS. The cells were incubated in ice-cold RIPA buffer [1 M Tris (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% SDS, 10% DOS, 10% NP40] with freshly added protease inhibitor cocktail tablets (Roche, USA) over ice for 30 min. The cells were scraped and the lysate was collected in an Eppendorff tube and cleared by centrifugation at 13,000 rpm for 30 min at 4 °C, and the supernatant was collected, aliquoted, and stored at –70 °C. The protein concentration in the lysates was measured by BCA protein assay kit (Pierce, USA) by following the manufacturer's protocol.

Proteins (30 µg) were loaded over 5–15% SDS-PAGE gels and transferred onto a nitrocellulose membrane (Amersham Biosciences, USA) at 30 V overnight. The blots were blocked by incubating 10% nonfat dry milk in PBS for 1 h at room temperature or overnight at 4 °C. The blots were washed with PBST (PBS with 0.1 Tween 20) thrice, each time for 5 min and incubated with appropriate primary antibody for 2 h at room temperature or overnight at 4 °C. The blots were washed with PBST thrice, each time for 5 min followed by incubating with the corresponding secondary antibody horseradish peroxidase conjugate for 1 h at room temperature. The blots were washed in PBST three times for 5 min each. Then the proteins were added with the ECL Western blotting detection reagents (Amersham Biosciences, USA) and analyzed using the Fuji LAS-3000 imaging system (Japan). In addition, the antibodies and dilutions used in this study included anti-Cyclin B1 mAb (Cat. 05-373; 1/3000; Upstate, USA), anti-Cdc2 Ab (Cat. PC25; 1/4000; Calbiochem, Germany), anti-Actin mAb (Cat. MAB1501; 1/4000; Chemicon, CA), anti-rabbit IgG, HRP-linked Ab (Cat. 7074; 1/5000; Cell signaling, USA) and anti-mouse IgG, HRP-linked Ab (Cat. 7076; 1/5000; Cell signaling, USA).

### 2.7. Wound scratch assay

T24 cells ( $2 \times 10^4$ ) were seeded into 6-well tissue culture dishes and cultured in medium containing 10% FBS to confluent cell monolayers, which were then carefully wounded using sterile 200  $\lambda$ -pipette tips and removed any cell debris with PBS [19]. The cells were then incubated in ACCE (50 and 100  $\mu\text{g}/\text{mL}$ ) or vehicle alone for 24 h and photographed under a phase contrast microscope. The cell migration ability can be expressed by the relative migration capability  $R_m$  derived and modified from Liu et al. [20].

$$R_m = S_{dt}/S_{ut} \quad (1)$$

where  $R_m$  is the relative migration capability (dimensionless),  $S_{dt}$  is the migration distance of drug-treated cells (mm), and  $S_{ut}$  is the migration distance of untreated cells (mm). Similar experiments were repeated in triplicates.

### 2.8. Transwell motility assay

T24 cells were plated at  $10^5$  cells/ $\text{cm}^2$  in the upper compartment of 8  $\mu\text{m}$  pore size Transwell migration chamber (Cat. 3422; Corning, Inc., USA) and cultured in medium containing ACCE (50 and 100  $\mu\text{g}/\text{mL}$ ) or vehicle alone for 72 h. The cells 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 ( $\times 400$ ) per filter. Similar experiments were repeated in triplicates.

### 2.9. Zymography

T24 MMP activity in conditioned medium was demonstrated by gelatin zymography (7.5% zymogram gelatin gels). Briefly, samples were diluted in buffer (0.12 M Tris-HCL, 20% glycerol, 0.1% bromophenol blue, 10% SDS), and 4  $\mu\text{g}$  of total proteins were electrophoresed through gelatin-impregnated zymogram gels at 125 V for 90 min, which were then incubated at room temperature in a Zymogram Renaturing Buffer (Invitrogen, CA) for 30 min and further washed in Zymogram Developing Buffer (Invitrogen, CA) for 30 min. Thereafter, fresh Zymogram Developing Buffer was added, and the gels were incubated overnight at 37  $^\circ\text{C}$ . Zymograms were developed by staining with 0.5% Coomassie blue (Bio-Rad, USA) for 90 min before destaining until clear bands of MMP activity appeared against a blue background.

### 2.10. Statistical analysis

Results were analyzed using a two-tailed Student's *t*-test to assess statistical significance. Values with  $p < 0.05$  were considered statistically significant.

## 3. Results and discussion

### 3.1. MTT assay indicates the optimum inhibitory concentration at 50 $\mu\text{g}/\text{mL}$

Growth inhibition of T24 cell could have been effected by ACCE at concentration as low as 10  $\mu\text{g}/\text{mL}$ . A dose- and time-dependent manner was observed up to 100  $\mu\text{g}/\text{mL}$  (Fig. 1A), showing a linearity between the percent inhibition and the ACCE dosages (from 56.25% at 50  $\mu\text{g}/\text{mL}$  to 61.25% at 100  $\mu\text{g}/\text{mL}$ ), from which the percent inhibitions were derived and tabulated in Table 1. With regard to the therapeutic conditions, the concentration of 50  $\mu\text{g}/\text{mL}$  can be considered to be optimum for a 72 h treatment period, which actually exhibited percent inhibitions of 23.70, 45.00, and 56.25

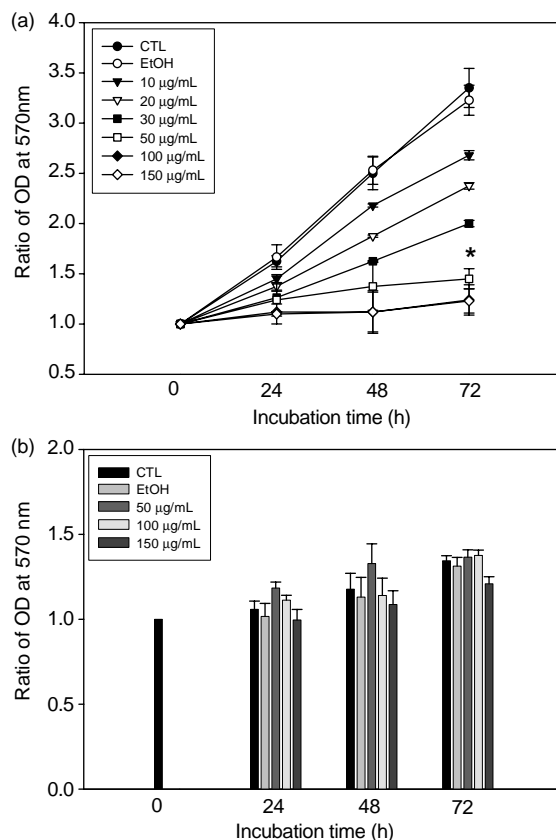


Fig. 1. MTT assay in the presence of ACCE at different concentrations, as indicated. A negative control, CTL (in the absence of ACCE), and a positive control with the addition of ethanol, EtOH are shown. All values of OD are relative to the OD at 0 h. The ratio of OD at 570 nm are shown for at 24 h intervals during an incubation time of 72 h. (A) T24 cells treated with ACCE at 10, 20, 30, 50, 100 and 150  $\mu\text{g}/\text{mL}$ , respectively (\* $p < 0.05$ ). (B) Mouse fibroblast cells were treated with ACCE at 50, 100 and 150  $\mu\text{g}/\text{mL}$ , respectively.

Table 1  
The dose- and time-dependent growth inhibition by ACCE on T24 cells

Dosage ( $\mu\text{g/mL}$ )	Inhibition % <sup>a</sup>		
	Period of treatment (day)		
	1	2	3
10	10.77	12.80	16.25
20	15.40	25.00	25.78
30	22.30	35.00	37.50
50	23.70	45.00	56.25
100	31.10	55.20	61.25
150	31.10	55.20	61.56 <sup>b</sup>

<sup>a</sup> Percent inhibitions were calculated based on the cell populations in the EtOH samples at the corresponding incubation times.

<sup>b</sup> A nonlinear relationship occurred above this concentration.

at 24, 48 and 72 h, respectively (Table 1). Above a concentration of 100  $\mu\text{g/mL}$ , the time–growth curve practically reached a plateau at which the therapeutic efficiency leveled off on 72 h of incubation, revealing a nonlinear relationship above a concentration of 100  $\mu\text{g/mL}$  (e.g. 61.25%, at 100  $\mu\text{g/mL}$ ; and 61.56% at 150  $\mu\text{g/mL}$ ) (Table 1). No similar inhibitory effects were observed in MEF cell lines (Fig. 1B). We suspect such a result can be ascribed to the alteration and aberration of cancer cells in signal transduction pathway comparing to the normal cell lines such as the MEF.

The cell proliferation data in Fig. 2 shows that originally inoculated populations ( $1.6 \times 10^5$  cells/mL) had been increased to  $4.4 \times 10^5$ ,  $1.03 \times 10^6$ , and  $1.6 \times 10^6$ ; and  $3.34 \times 10^5$ ,  $7.6 \times 10^5$ , and  $1.02 \times 10^6$ , respectively, for the control and that when cultivated with 50  $\mu\text{g/mL}$  of ACCE. At 72 h, the cell population in ACCE-treated well ( $1.02 \times 10^6$ ) was found to have been

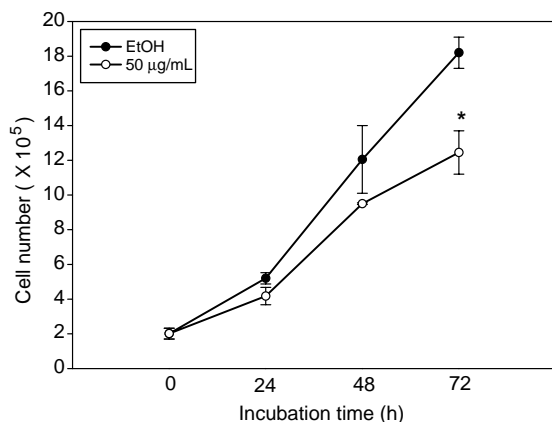


Fig. 2. Effects of ACCE (50  $\mu\text{g/mL}$ ) on cell proliferation of T24 cells. Cells were incubated for 72 h in the presence or absence of ACCE (50  $\mu\text{g/mL}$ ). Proliferation of ACCE-treated T24 cells was significantly suppressed  $n=3$ ,  $*p<0.05$

suppressed to almost half of the control ( $1.6 \times 10^6$ ). Linear regression of the two curves in Fig. 2 gave, respectively, for the control,

$$X_{\text{cntl}(t)} = X_{\text{cntl}(0)} + at \quad (2)$$

and for the ACCE-treated,

$$X_{\text{ACC}(t)} = X_{\text{ACC}(0)} + a't \quad (3)$$

where the terms  $X_{\text{cntl}(0)}$  and  $X_{\text{ACC}(0)}$  are the initial cell counts per milliliter at time  $t=0$  for the control and the ACCE-treated, respectively. On cultivation for a time period of  $t$ , the cell counts can be brought up to  $X_{\text{cntl}(t)}$  and  $X_{\text{ACC}(t)}$  cell counts per milliliter, and  $a$  and  $a'$  ( $\Delta$  cell counts)/( $\Delta$  mL) are the growth rates, respectively. Substitution of the parameters obtained from the experimentation into the Eqs. (2) and (3) yields Eqs. (4) and (5), respectively.

$$1.6 \times 10^6 = 1.6 \times 10^5 + at \quad (4)$$

$$1.02 \times 10^6 = 1.6 \times 10^5 + a't \quad (5)$$

Using the data at 72 h (Table 1) to solve for the corresponding growth rates  $a$  and  $a'$ , we have

$$a = 4.8 \times 10^5 \text{ cells/d mL} \quad (6)$$

and

$$a' = 2.87 \times 10^5 \text{ cells/d mL} \quad (7)$$

Dividing Eq. (6) by Eq. (7) leads to

$$a/a' = 1.672 \quad (8)$$

Surprisingly, over a 1.5-fold ( $>150\%$ ) inhibition on the T24 cell proliferation by ACCE (50  $\mu\text{g/mL}$ ) were observed (refer to Fig. 2).

In addition, the slopes  $a$  and  $a'$ , the dose-dependent relationships, could provide the information for the therapeutic efficiency per unit dosage of ACCE, thus the anti-proliferative rate was estimated to be  $-4.4 \times 10^3$  cells/ $\mu\text{g}$  per day, or synonymously, in general statement, ACCE at 50  $\mu\text{g/mL}$  of dosage, could effectively suppress T24 cell growth at a suppressing rate of  $-4.4 \times 10^3$  cells/ $\mu\text{g}$  per day.

### 3.2. Scanning electron microscopy (SEM) reveals membrane damaged by ACCE

In addition, the LM photos revealed rather distinct and apparent morphological changes, a distinct difference was observed between the control (Fig. 3A) and the ACCE-treated (50  $\mu\text{g/mL}$ ) at 72 h post-treatment (Fig. 3B), and more prominent in the

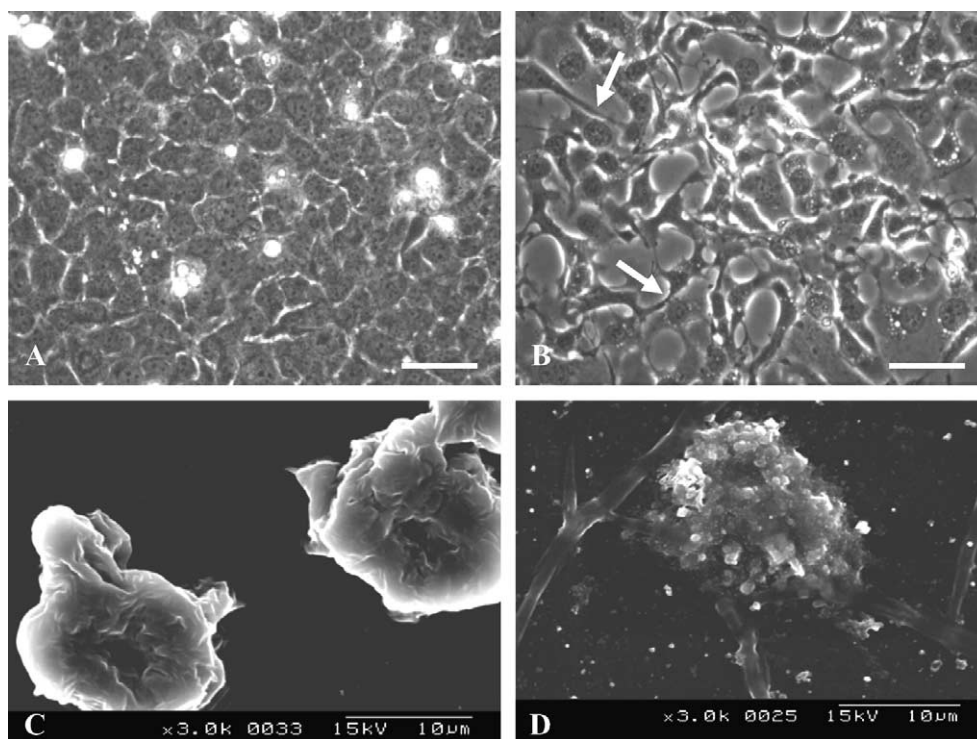


Fig. 3. The LM (A and B) and the SEM (C and D) images for the T24 cells incubated with EtOH alone (A and C) or ACCE at 50 µg/mL (B and D) for 72 h. The arrows point to the long cell extension after ACCE treatment (B). The blabbing occurs on the surface of cell membrane (D) (magnification 100×, A and B: scale bar=0.2 mm).

SEMs (Fig. 3C vs. D). The phenomenon that long cell extensions irradiating from the perikarya resulting in intercellular contacts through long, tenous secondary filopodial extension accompanied with the decrease in cell population was observed (Fig. 3B and D). The remainder of the cell cytoplasm was found to be blabbing and showing blisters on its surface as emitting long filopodia (Fig. 3D), indicating a slight degree characteristics of T24 cell morsellation by autoschizis. Previously, such a type of cell death has been cited in vitro with the human bladder (T24) [21,22], the prostate (DU-145) [23], the renal [24], as well as the ovarian carcinoma cell lines [25].

### 3.3. Cell cycle analysis shows phase G2M arrest

The flowcytometric analysis of T24 cell, after cultivated for 72 h, revealed apparently phase G2M arrests as shown in Fig. 4 in ACCE 50, 100, and 150 µg/mL treated groups. The percentages arrested in phase G2M by ACCE dosage of 50, 100 and 150 µg/mL were found to be 15.78, 15.50, and 13.26%, respectively, comparing to the vehicle control 7.11%. As a contrast, a

significant decrease in phase G1 between the control and the ACCE-treated was observed (Fig. 4).

### 3.4. Western blot analysis demonstrates down-regulations of Cdc2 and Cyclin B1

The regulations of Cyclin B1 and Cdc2 have been reported to be involved in the G2/M arrest [26]. Primarily, no apparent results were conceivable at the first 24 h post-addition of ACCE. However, the down-regulations of these two cell cycle related proteins and the up-regulation of phospho-Cdc2 (Tyr15) were simultaneously observed after 48 h of cultivation when treated with ACCE (50 µg/mL) (Fig. 5), a result being consistent with the flowcytometric analysis of T24 cells (Fig. 4).

### 3.5. Wound scratch assay reveals migration capability decreased 1.5-folds by ACCE

T24 cells, the cell lines derived from the grade III bladder carcinoma, are poorly differentiated and own higher potential of metastasis. The ability of tumor

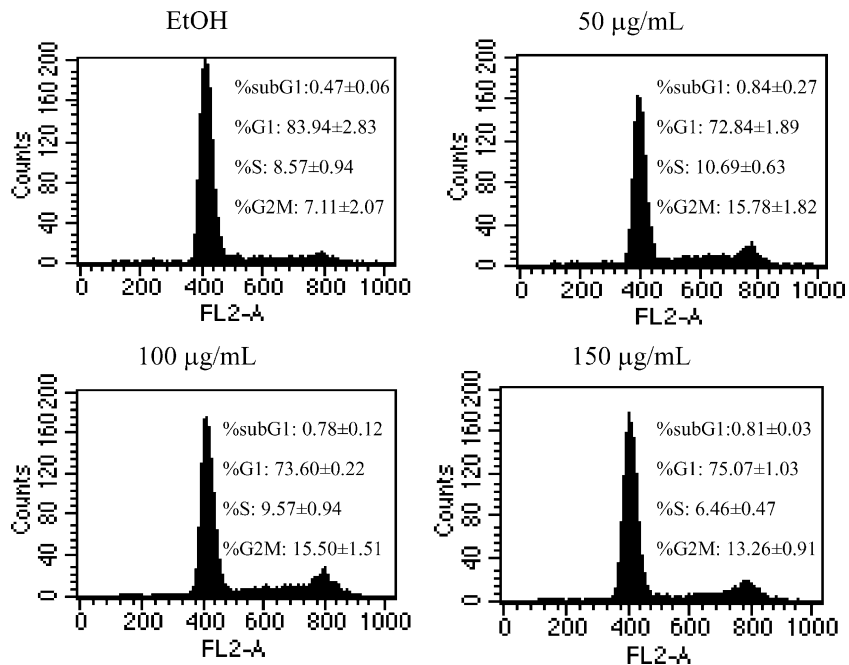


Fig. 4. The flow cytometric analysis of T24 cells in the presence or the absence of ACCE (50 µg/mL). Significant cell arrest occurs at phase G<sub>2</sub>M by ACCE, while cell numbers decrease significantly at phase G<sub>1</sub> (\**p*%<0.05).

cells to migrate is closely associated to their metastatic potentiality [27], hence the wound scratch assay was used for this purpose. Results revealed that the gap distances were ACCE dose-dependent (Fig. 6), which were distinctly observable even at

24 h post-addition of ACCE. Comparing to the absolute gap dimension of  $0.187 \pm 0.005$  mm for the control, the gap dimension with added ACCE (50 µg/mL) was  $0.289 \pm 0.026$  mm, while that by 100 µg/mL was  $0.354 \pm 0.048$  mm, almost doubled over the

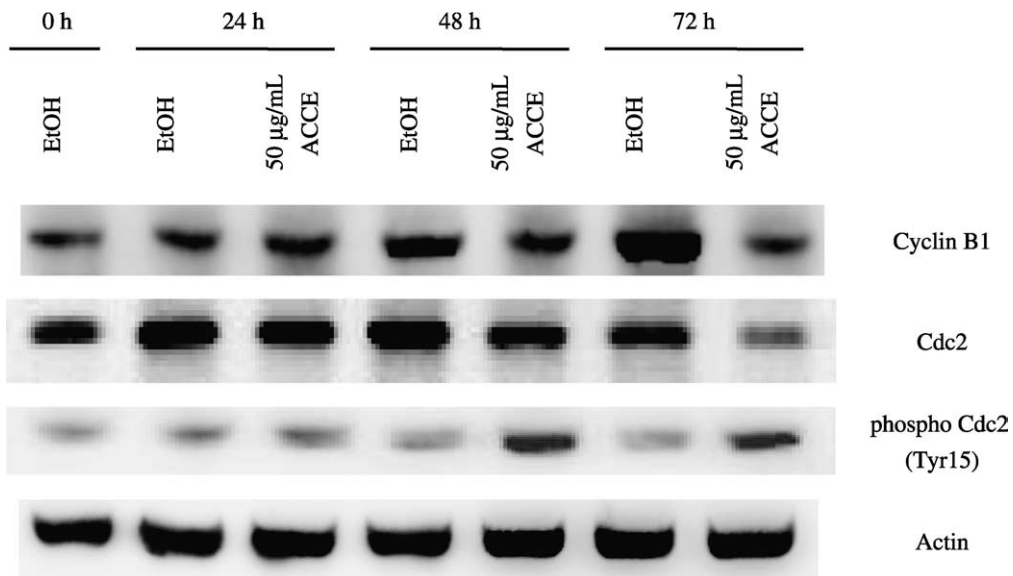


Fig. 5. Western blotting for cell cycle proteins Cdc2 and Cyclin B1. These proteins were down-regulated by ACCE at 50 µg/mL at 48 h post-incubation, and the more significant down-regulations persisted till 72 h post-incubation. Actin was used as the internal control.

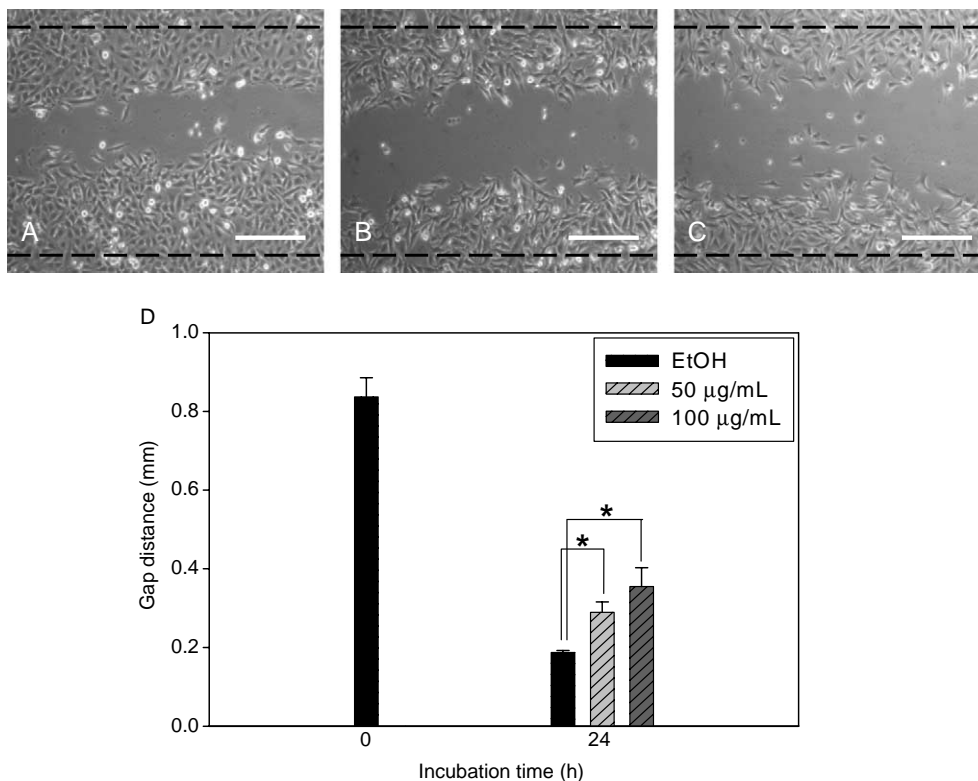


Fig. 6. The wound scratch assay for T24 cells. The confluent cells were scratched by 200 $\lambda$  pipette tips, then cells were incubated with EtOH alone (A) or ACCE at 50 (B) and 100 (C)  $\mu$ g/mL for 24 h (magnification 100 $\times$ , scale bar=0.2 mm; \* $p$ <0.05).

control. Substitution of these data into Eq. (1) gave the results of relative migration capability  $0.83 \pm 0.04$  and  $0.76 \pm 0.063$  for the dosages of 50 and 100  $\mu$ g/mL, respectively. This implicated that at higher dosages of ACCE, the T24 cell spreading along the wound edges would have become slower than that treated with vehicle alone. Thus, in the wound scratch tests, the larger gaps could mean the more effective as the anti-metastasis [20]. The anti-metastatic effect of ACCE on T24 cells was thus confirmed to be dose-dependent and accompanied by cell growth inhibition.

### 3.6. Transwell assay demonstrates migration capability being inversely dose-dependent to ACCE

Because of the scratch-space limitation in wound scratch assay, the transwell assay was used in parallel to investigate the metastatic potential of T24 cells for further 72 h post-ACCE. T24 cells are more motile than RT4 cells when applied to the transwell experiment (data not shown). The migration capability was found also to be inversely dose-dependent (Fig. 7) even without the addition of

any chemo-attractant as often cited to be required elsewhere [28,29]. The less cell counts were found along with the higher dosages of ACCE added (Fig. 7A–C). When expressed in the cell number of ACCE-treated/control, results from both dosages, 50 and 100  $\mu$ g/mL, were all found to be significantly different from that of control (Fig. 7D). In contrast, we failed to observe such a migration phenomenon in RT4 cells (data not shown), implying that the metastatic capability differs from each other for different bladder cancer cells.

### 3.7. Zymography exhibits active form of MMP-9 suppressed by ACCE

The matrix metalloproteinases (MMPs) form a family of at least 14 enzymes participating in matrix remodeling [30]. MMPs promote cancer progression by boosting cancer cell growth, migration with invasion, metastasis and angiogenesis [31]. The roles of MMP-2 and MMP-9 have been particularly highlighted in the last few years

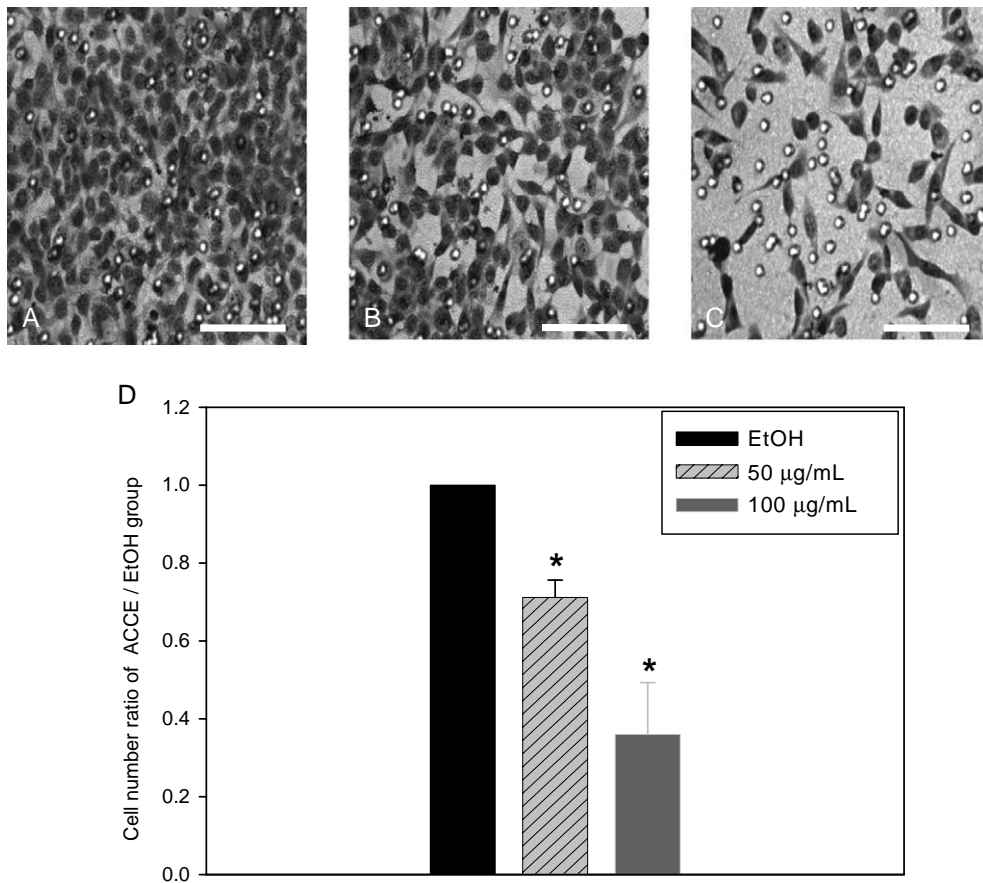


Fig. 7. The transwell motility assay for T24 cells. Cells ( $10^5$  cells/cm<sup>2</sup>) were incubated with ACCE at 50 (B) and 100 (C) µg/mL for 72 h, respectively, with EtOH alone used as for control (A). The motility ratios for cell numbers of ACCE-treated to that with EtOH alone are shown in (D) (magnification 400 $\times$ , scale bar=0.1 mm; \* $p$ <0.05).

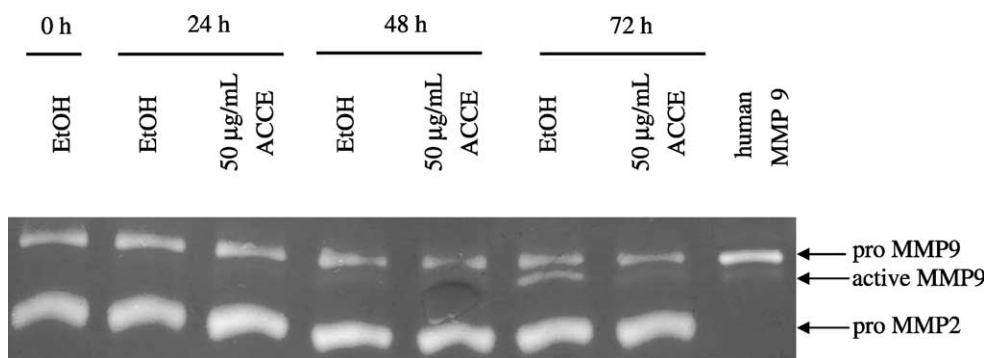


Fig. 8. The zymograms of MMP-2 and MMP-9. Cells were incubated in the presence of ACCE (50 µg/mL) or EtOH alone for 24, 48 and 72 h, respectively. Proteins (4 µg) in culture media were collected for zymography. The bands for pro MMP-9 (92 kDa) and pro MMP-2 (72 kDa) are all observed at each time period as indicated, while active MMP-9 (82 kDa) only apparently appeared in EtOH alone at 72 h post-incubation.

using different in vivo and in vitro experimental models [9,32]. MMP-2 mRNA was found expressed by human bladder cancer cells T24, and had been considered to be correlated with the

imbalance at the genetic level at stage of tumor progression [9]. Although the latent forms (pro-forms) of MMP-2 (72-kDa) and MMP-9 (92-kDa) all have been expressed during the time course

(0–72 h) of experimentation, yet the only active form of MMP-9 (82-kDa) that appeared in the vehicle-control at 72 h post incubation was found totally suppressed by ACCE at 50 µg/mL (Fig. 8), thus speculative of the appearance of active form (such as active MMP-9) can only be expressed and perceivable after a reasonable cultivation period, such as in the study, 48 h. Similar results were reported in liver metastasis from colorectal cancer [33], and in hepatocellular carcinoma [34]. In contrast, MMP-2 was totally unaffected in this respect (Fig. 8), implicitly indicating that ACCE may inhibit the cell migration of T24 cell by suppressing the expression of the active form of MMP-9. Remarkable high levels of both active and latent MMP-9 were often detected in urine samples of patients with bladder carcinoma, whereas hardly any activity has been observed in healthy urine samples [30]. While such an active form of MMP-9 can mean the invasive power of the bladder cancer, as exemplified and evidenced in the T24 cells, ACCE has indeed actually revealed a fantastic highlight with respect to its anti-metastatic potentiality.

#### 4. Conclusion

The *A. camphorata* crude extract (ACCE) is a good anti-cancer agent. At 50 µg/mL, it induces phase G2M arrest, acting as an effective anti-proliferative agent. The anti-proliferative rate was estimated to be  $-4.4 \times 10^3$  cells/µg per day. In addition, ACCE can suppress the expression of the active form of MMP-9, in such a way, exhibiting its anti-metastatic activity against bladder cancer T24 cells.

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