

**F-actin reorganization and inactivation of Rho signaling pathway involved in the inhibitory effect of Coptidis Rhizoma on hepatoma cell migration**

**Ning Wang<sup>1</sup>, MS, Yibin Feng<sup>1\*</sup>, PhD, Echo Ping Woi Lau<sup>1</sup>, Mphil, Chiman Tsang<sup>2</sup>, PhD, Yickpang Ching<sup>2</sup>, PhD, Kwan Man<sup>3</sup>, PhD, Yao Tong<sup>1</sup>, MD, Tadashi Nagamatsu<sup>4</sup>, PhD, Weiwei Su<sup>5</sup>, MD, Saiwah Tsao<sup>2\*</sup>, PhD**

**1 School of Chinese Medicine, The University of Hong Kong, Pokfulam, 10 Sassoon Road, Hong Kong**

**2 Department of Anatomy, The University of Hong Kong, Pokfulam, 21 Sassoon Road, Hong Kong**

**3 Department of Surgery, The University of Hong Kong, Pokfulam, 21 Sassoon Road, Hong Kong**

**4 Department of Pharmacobiology and Therapeutics, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan**

**5 School of Life Science, Sun Yat-sen University, 135 Xingangxi Road, Guangzhou, P. R. China**

**\*Corresponding authors:**

**Dr. Feng Yibin: School of Chinese Medicine, The University of Hong Kong, Pokfulam, 10 Sassoon Road, Hong Kong; Tel: +852-25890482; Fax: +852-21684259 E-mail: yfeng@hku.hk,**

**Dr. Tsao Sai Wah, George: Department of Anatomy, The University of Hong Kong,**

**Pokfulam, 21 Sassoon Road, Hong Kong ; Tel: +852- 2819-9227; Fax: +852- 2817-0857;**

**E-mail: [gswtsao@hkucc.hku.hk](mailto:gswtsao@hkucc.hku.hk)**

## **Abstract**

**Hypothesis:** Hepatocellular Carcinoma is one of the most malignant human tumors and one of the risk factors is its high metastatic property. *Coptidis Rhizoma* Aqueous Extract (CRAE) is able to suppress the migration and invasion of hepatocellular carcinoma cells, MHCC97-L, and F-actin reorganization and Rho signaling inhibition is involved. **Main Methods:** CRAE was prepared and analyzed by High Performance Liquid Chromatography combined-Mass Spectrometry. The cytotoxicity and anti-migration action of CRAE on MHCC97-L cells were evaluated; Immunofluorescence and immunoblotting were used to investigate the proposed mechanism of CRAE's action. **Key findings:** Chemical analysis reveals that the active components in CRAE were berberine and berberine-like alkaloids. CRAE exhibits significant inhibitory effect on MHCC97-L cells migration as indicated by wound healing and invasion chamber assay. No significant alteration of matrix metalloproteinases and urokinase-type plasminogen activator (uPA) expression were observed in MHCC97-L cells exposed to CRAE. Reduction of F-actin polymerization and damage cytoskeleton network in MHCC97-L cells were observed after CRAE treatment. Furthermore, it was found that CRAE significantly down-regulated the Rho/ROCK signaling pathway. **Significance:** These results indicated that CRAE may act as Rho/ROCK signaling inhibitor to suppress MHCC97-L cells migration in vitro and suggested that total alkaloids in *Coptidis Rhizoma* may be a potential agent for suppressing liver cancer invasion.

**Keywords** *Coptidis Rhizoma*, berberine, hepatocellular carcinoma, anti-metastasis, F-actin, Rho/ROCK signaling

## Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human malignancies in the world.<sup>1-2</sup> Each year there are approximately 500,000 new cases of HCC worldwide, 80% of which happens in Asia and Africa.<sup>3</sup> HCC represents as the second common cancer in men and ninth in women in Hong Kong, approximately 11.8% of cancer death rate in 2002.<sup>4</sup> One of the obstacles in HCC therapy is the high metastatic property of liver cancer cells. Extensive studies have tried to elucidate the process and mechanism involved in cancer metastasis, during which cancer cells migrate from one site to another. Some molecules and signaling transduction pathways have been identified as critical factors, such as E-cadherin, catenin, matrix metalloproteinases and the actin cytoskeleton,<sup>5</sup> and molecules targeted to these factors for the sake of cancer therapy have been reported.<sup>6-8</sup> However, anti-metastasis drug with effective action and clear mechanism is far from development.

Coptidis Rhizoma (CR, *Huanglian* in Chinese) was a traditional Chinese medicinal herb with a long history of utilization in heat-clearing and toxic-scavenging. Extensive modern research on the pharmacological action of CR revealed its potential as an anti-inflammatory,<sup>9</sup> anti-viral,<sup>10</sup> anti-bacterial<sup>11</sup> and anti-oxidative agent.<sup>12</sup> Our screening study demonstrated that CRAE exhibited the strongest inhibitory activity on the growth of tumor cells amongst sixteen anticancer traditional Chinese medicinal herbs.<sup>13</sup> Moreover, our clinical study on the therapeutic effect of CR on liver diseases and cancer showed that CR may be used for liver cancer therapy.<sup>14</sup> A lot of studies have been carried out on cytotoxic, apoptotic effects and mechanisms of CR and berberine

*in vitro* and *in vivo*,<sup>15</sup> but there is no report on anti-invasive action of CR and its underlying mechanism. Thus, the following questions need to be clarified: Does CR have the anti-invasive effect? Are all signaling pathways of cancer cell migration involved in the anti-invasive effect of CR? Is Rho/ROCK signaling pathway specific for anti-invasive effect of CR?

In this study, we reported a significant inhibitory effect of CRAE on the migration of HCC cell line with high metastatic property MHCC97-L cells. Using High performance liquid chromatogram combined with mass spectrometry (HPLC/MS), seven components were identified as berberine-like alkaloids in CRAE. Studies on the proposed mechanism of CRAE's inhibition against MHCC97-L migration showed that the CRAE acts on actin cytoskeleton reorganization as Rho/ROCK inhibitor. These results shed lights on CRAE's potential for liver cancer therapy. At the same time, the results also implied that berberine is a main active compound and other berberine-like alkaloids have synergistic anti-migration effects in CRAE.

## **Materials and Methods**

### ***Sample preparation and phytochemical analysis***

CR was collected from Sichuan province of China and authenticated as the dried roots of *Coptis chinensis* Franch. by Dr. Feng Yibin under the guidance of the Pharmacopeia of China (2005). Authentication was described as previous study reported and toxic elements were tested<sup>16</sup>. To prepare the CRAE, raw material was cut into small pieces and 500 gram of crude huanglian was boiled in 10 times of distilled water (w/v) at 100°C for 1 hour (three times) and then filtered. The

filtrate was then evaporated to dryness and the dry extract powder was collected and stored at -20°C until used. Immediately before use, the extract powder was dissolved in DMSO and diluted to proper concentration in PBS and then sterilized by filtration through a 0.2 µm pore filter (Minisart®-plus, Sartorius).

High performance liquid chromatography combined with mass spectrometry (HPLC/MS) was introduced to identify the chemical profile of CRAE. Analysis was performed using a reverse-phase C<sub>18</sub> column (Alltech Alltima HP C18, 250 mm × 4.6 mm, 5 µm) as solid phase and methanol- Mill-Q water including 15 mM ammonium acetate (25-75) as mobile phase. The flow rate was 1.0 ml/min. Total ion chromatogram (TIC) of CRAE was obtained by LCQ Deca XP system (Thermo Finnigan, USA) using the condition as follows: Sheath Gas Flow Rate (abr):40; Auxiliary Gas Flow Rate (abr): 40; Spray voltage (kV): 4.5; Capillary Temperature (°C): 300; Capillary Voltage (V): 20; Tube Lens Offset Voltage (V):24. Immediately before analysis, berberine (Sigma, USA) and CRAE powder were dissolved in methanol and diluted to proper concentration. 1 µl of standard or sample solution was injected to HPLC-MS system and the content of berberine in CRAE was quantified.

#### ***Cell line and Cell culture***

Human liver cancer cell line with metastatic property MHCC97-L was used in our previous study.<sup>17</sup> In this study, MHCC97-L cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

### ***Cell viability assay***

Viable cell number after CRAE treatment was obtained by MTT assay. Briefly, cells were seeded in 96-well plate with supplemental medium and treated with series concentration of CRAE (2, 4, 8, 16, 32, 64, 128, 256 and 512  $\mu\text{M}$ ) and incubated for 24 to 72h. All experiments were conducted parallel with controls (0.1% DMSO). Then cells were incubated with 15  $\mu\text{l}$  of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma, USA) at 37°C for 4h. Then medium was removed and 200  $\mu\text{l}$  of DMSO was added to each well. The absorbance of formazan formed was measured at 595 nm by Multiskan MS microplate reader (Labsystems, Finland).

### ***Wound healing assay***

Cells were seeded in 6-well plate with 100% confluences. A gap was scrapped using a micro-pipette tip on the cell monolayer. Medium was refreshed and cells were exposed to 100  $\mu\text{M}$  CRAE or berberine for 0, 24 or 48h. The migration of MHCC97-L cells were observed under fluorescent microscope with 40 $\times$  magnification (Carl Zeiss, USA).

### ***Invasion chamber assay***

Experiment was conducted under the manufacturer's instruction (QCM<sup>TM</sup> 96-well cell invasion assay kit, Millipore, USA). Briefly, the invasion chamber was pre-activated by 100  $\mu\text{l}$  of serum free medium for 1h. After rehydration of chamber, 150  $\mu\text{l}$  growth medium was added to the feeder tray. 100  $\mu\text{l}$  serum free media with  $1 \times 10^4$  MHCC97-L cells were then placed into invasion

chamber. Then cells were exposed to CRAE or berberine with a series of concentrations, 12.5, 25, 50, 100, 200 and 400  $\mu\text{M}$  and incubated for 24h at 37°C, 5% CO<sub>2</sub>. The amount of cells that invaded from the upper chamber to the feeder tray was measured using 480/520 nm filters set using LS55 Fluorescence/Luminescence Spectrometer (PerkinElmer, USA).

### ***MMPs assay***

The expression of matrix metalloproteinases in MHCC97-L cells with or without CRAE and berberine treatment was evaluated using RayBio® Human Matrix Metalloproteinase Antibody Array (RayBiotech, USA) under the manufacturer's instruction. Briefly, cells were maintained in 75-cm<sup>2</sup> flask in serum-free medium with 80% confluence and treated with 100  $\mu\text{M}$  CRAE or berberine for 48h. Medium and cell supernatant were collected as sample solution for subsequent experiment. To conduct the experiment, membranes were first incubated in 2 mL blocking buffer for 30 minutes. Then membranes were incubated with sample solution overnight and then rinsed with washing buffer. Membranes were then subsequently incubated with biotin-conjugated anti-MMPs and HRP-conjugated streptavidin overnight. The expression of MMPs was detected using detection buffer and visualized under a chemiluminescence imaging system (Biorad, USA).

### ***Visualization on F-actin reorganization by Fluorescent Microscopy***

Cells were seeded in 35mm glass bottom microwell dishes (MatTek, USA) and then exposed to CRAE or berberine (100, 200  $\mu\text{M}$ ) for 24h. Cells were then fixed with 4% paraformaldehyde for 30 minutes and penetrated with 1% Triton X-100 in PBS for 5 minutes, followed by 30 minutes incubation of 5% BSA to block the unspecific binding. 50  $\mu\text{g}/\text{mL}$  FITC conjugated phalloidin



(Sigma, USA) was added to the chambers and cells were incubated in dark for 30 minutes. Nuclears were then stained with 1 $\mu$ g/mL DAPI for 10 minutes and cells were visualized under fluorescent microscope with 400 $\times$  magnification (Carl Ziess, USA).

### ***RhoA-GTPase activity***

Cells were seeded in 25 cm<sup>2</sup> flask with 80% confluence and then starved overnight in serum-free medium (Invitrogen, USA). After starvation, cells were treated with full growth medium with or without CRAE or berberine (100, 200 $\mu$ M) for 30 minutes. Cell lysate was collected and the RhoA-GTPase activity assay was conducted by Rho Activation Kit (Thermo-Pierce, USA) under the manufacturer's instruction. The RhoA-GTPase and total RhoA were detected by immunoblotting.

### ***Immunoblotting***

Cells were harvested using a micro-scrapper (Corning, USA) and then lysed with RIPA buffer supplemented with protease inhibitor (1% PMSF, 0.5%apoptinin and 0.5% leupeptine) and phosphatase inhibitor (1 mM Na<sub>3</sub>VO<sub>4</sub> and 1mM NaF) on ice for 30 minutes and then centrifuged at 14,000 rpm at 4 $^{\circ}$ C for 25 minutes. Equal amounts of protein were resolved by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF, Biorad). Then the membrane was blocked with 5% BSA overnight at 4 $^{\circ}$ C. The membrane was then incubated with primary antibody uPA, ROCK1,  $\beta$ -actin (Abcam, UK) at 4 $^{\circ}$ C overnight followed by incubation with appropriate secondary antibody (Abcam, UK) at room temperature for 2 hours. The immunoreactivities were detected using ECL plus kit (GE Healthcare, UK) and visualized using a chemiluminescence

imaging system (Biorad, USA).

### ***Statistic analysis***

All data were analyzed by Student-T tests for the significant interrelation between treatment and control groups. All data were presented in terms of mean  $\pm$  standard derivative (SD) of the mean. Differences between group means were considered to be statistically significant if values of  $P < 0.05$ .

## **Results**

### ***Phytochemical analysis on CRAE***

Coptidis Rhizoma contains different berberine-like alkaloids including berberine, palmatine, jatrorrhizine, columbamine, epiberberine, and coptisine. In this study, a rapid and efficient method was established to identify the chemical composition of the CRAE using HPLC-ESI-MS and the TIC profile (Fig. 1A) of the extract presents that the CRAE was mainly comprised of seven berberine-type compounds. Comparing the mass spectrums (Fig. 1B) with a literature report,<sup>18</sup> peak 1 to 7 is identified as magnoflorine, columbamine, jatrorrhizine, epiberberine, coptisine, palmatine and berberine, respectively (Table.1). In order to facilitate comparison on the bioactivity between berberine and equal content of berberine of CRAE, we determined the total alkaloids in CRAE at 348 nm by UV spectrometric approach, using berberine as standard. Total alkaloids content of CRAE was expressed by equal

amount of berberine in the following study.

#### ***The cytotoxicity of CRAE on MHCC97-L cells***

Fig. 2 shows decreased viability of MHCC97-L cells exposed to different concentrations of CRAE for 24, 48 or 72h, MHCC97-L cells survival was significantly inhibited after 24h incubation of approximately 300  $\mu$ M of CRAE, and then the IC<sub>50</sub> of CRAE decreased to about 150  $\mu$ M when cells were treated for 48h. This result indicates that CRAE could reduce the cell survival rate in dose- and time- dependent manner.

#### ***Effect of CRAE on the invasion and migration of MHCC97-L cells migration in vitro***

To study the anti-metastatic effect of CRAE on hepatocellular carcinoma cells MHCC97-L, wound healing assay and invasion chamber assay were introduced to qualitatively and quantitatively determine the migration of MHCC97-L cells with or without exposure of CRAE and its major component berberine. It was observed that MHCC97-L cells at the opposed edges of the wounds rapidly migrated towards each other after 48h incubation (Fig. 3A). With the increase of the doses, the speed of wound healing slowed down and the gap remained widely open and only minimum cell proliferation on the two edges of the wound was observed when cells were incubated with 200 $\mu$ M of CRAE or berberine. The cell motility was significantly inhibited in the presence of CRAE or its major component berberine. Similar result could be observed in invasion chamber assay, where both berberine and CRAE revealed inhibitory effect on the invasion of MHCC97-L through extracellular matrix (Fig. 3B). To further our knowledge on the CRAE's action on MHCC97-L cells' migration, cells were exposed to Y-27632, a Rho/ROCK signaling

inhibitor in the presence or absence of 100  $\mu$ M CRAE or berberine. The result showed that inhibition on Rho/ROCK signaling can significantly suppress the migration of liver cancer cells MHCC97-L in dose dependent manner, indicating that Rho/ROCK signaling activation may play an important role in MHCC97-L migration. The combination of Y-27632 with CRAE increased its inhibitory effect at its low dose, revealing that CRAE's action is similar to Rho/ROCK inhibitor in liver cancer cell migration (Fig.3C).

#### ***Effect of CRAE on Matrix Metalloproteinases and uPA Expression***

It was observed that expression of MMP-2 and MMP-9 in MHCC97-L cells remained low, indicating a loose connection between the MMPs expression and MHCC97-L cells migration ability. Furthermore, both CRAE and berberine did not reduce the expression of MMP-2 and MMP-9 in MHCC97-L cells, but it is interesting to observe that the tissue inhibitor of matrix metalloproteinase-4 (TIMP-4), which was reported as an indicator in breast cancer, prostate cancer and colon cancer,<sup>19</sup> was potently inhibited by CRAE and berberine treatment (Fig.4). No significant change in the expression of urokinase-type plasminogen activator (uPA) in MHCC97-L cells with or without exposure of CRAE and berberine was also observed in our study (Fig.6), indicating that uPA may not involve in CRAE's inhibitory effect on hepatocellular carcinoma cells' migration.

#### ***Effect of CRAE F-actin reorganization in MHCC97-L cell***

F-actin cytoskeleton in MHCC97-L was potently damaged by CRAE at the doses far lower than its IC<sub>50</sub>, indicating low dose of CRAE may prominently inhibit the F-actin polymerization and

induce damage of cytoskeleton network of MHCC97-L cells (Fig. 5).

#### *Effect of CRAE on Rho/ROCK signaling pathway*

The GTPase form of RhoA was potently suppressed in MHCC97-L with CRAE and berberine treatment while total RhoA expression remained constant (Fig.6), indicating that CRAE may suppress the activation of RhoA signaling by impeding the cycle between Rho-GDPase and Rho-GTPase. Moreover, CRAE exhibited prominent inhibition on the expression of ROCK-1 (Fig.6). This further proves CRAE as inhibitor of Rho/ROCK signaling to suppress MHCC97-L cells migration. To confirm the role of Rho/ROCK signaling pathway on effects of CRAE in MHCC97-L cell migration, Y-27632, a Rho/ROCK signaling inhibitor alone or in combination to 100  $\mu$ M CRAE was used in this study and the result showed that inhibition on Rho/ROCK signaling could significantly suppress the migration of MHCC97-L in dose dependent manner, indicating that Rho/ROCK signaling activation may play an important role in MHCC97-L migration (Fig.3C).

#### **Discussion**

HCC is one of the most malignant human cancers in the world.<sup>20-21</sup> Recurrence after surgical removal and metastasis are common in hepatocellular carcinoma and are associated with poor prognosis. Effective treatment is in urgent need for improvement of patients' survival. Traditional Chinese Medicine as a complementary and alternative treatment for cancer therapy has been widely used in daily clinical treatment.<sup>22</sup> As a commonly used medicinal herb, CR has been

extensively investigated for its potent anti-tumor action. It was reported that CR extract can inhibit cell proliferation by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells.<sup>23</sup> CR extract can induce apoptosis by up-regulating of interferon-beta and TNF-alpha in human breast cancer cells.<sup>24</sup> Recently, we reported that that anti-invasion of berberine, pure compound isolated from CR may inhibit RhoA signaling pathway at low dose, while apoptosis are induced by berberine via G2 arrest at high dose in NPC cell lines due to berberine distribution in cell nuclear and cytoplasm in dose dependent manner.<sup>25</sup> In this study, we reported the anti-invasive action of CRAE, and its major component berberine on hepatocellular carcinoma cells. Potent action on the F-actin reorganization and RhoA activity inhibition could be observed in cells with CRAE and berberine treatment. With screening on the major signal pathways that may involve in cancer cell migration and invasion, we found the inhibitory effect of CRAE and berberine on HCC cell migration is specific. Moreover, we found that CRAE, which is composed of seven berberine-like alkaloids, showed superior anti-invasive effect to its pure compound berberine. Considering that herbal extract rather than pure compound is more commonly used in Chinese Medicine practice<sup>14</sup>, our findings offer a potential complementary medication for the HCC invasion and metastasis.

F-actin is one type of stress fibers that regulates cell motility and polarization. With its constant state of flux with new monomers being added at the 'barbed' or 'plus' end, and depolymerization at the 'pointed' or 'minus' end, F-actin allows eukaryotic to migrate directionally.<sup>26</sup> In cancer cells with high metastatic property, active polymerization of F-actin is often reported and the reduction of F-actin cytoskeleton could inhibit the migration of cancer cells.<sup>27-29</sup> In this study, we showed for

the first time that CRAE could effectively suppress hepatocellular carcinoma tumor migration and invasion *in vitro* and as its mechanism, we observed that CRAE acts on F-actin, inducing filament reorganization and inhibits Rho/ROCK signaling pathway. In a Rho/ROCK signaling induced cancer cells migration, cell goes through the amoeboid mode of migration. In this mode, cell migration is independent of integrin function and cell-substrate adhesion, which exerts pivotal impacts in collective and mesenchymal modes of cell migration. Cells that go through amoeboid migration move within the extracellular matrix by squeezing the cell body, and a rounded morphology can be easily observed. Inhibition of Rho/ROCK signaling was reported to induce elongated morphology.<sup>4</sup> The activation of Rho small GTPases family, especially the RhoA-GTPase, is frequently found in the metastasis of different types of human cancers<sup>30-32</sup>, and inhibition of Rho-GTPase and ROCK suppresses tumor invasion *in vitro* and *in vivo*.<sup>33-36</sup> These studies indicated that the Rho/ROCK signaling pathway plays a pivotal role in cancer metastasis. Our result indicated CRAE's potential as an inhibitor of Rho/ROCK signaling to suppress liver cancer metastasis.

Standard CRAE was used in this study since the clinical use form of CR is its water extract and it is also a basic unit to make composite formulae<sup>16,37</sup>. To standardize the quality of CRAE, HPLC/MS/MS was introduced in this study and we have identified seven berberine-type alkaloids in CRAE, in which berberine amounts approximately 23% of the total extract (data not shown). These results suggested that the berberine and berberine-type alkaloids may be the active components in CRAE for the anti-invasive effect on MHCC97-L cells. Some studies have revealed that CR and berberine exhibit positive correlation in their anti-cancer action *in vitro* and

*in vivo*.<sup>38,39</sup> A report demonstrated that CRAE inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells and has better inhibitory effect than berberine.<sup>40</sup> Moreover, recent studies reported that berberine suppresses the metastasis and invasion lewis lung carcinoma and human lung cancer cells through the repression of expression of urokinase-type plasminogen activator (u-PA) or decreased production of u-PA and matrix metalloproteinase-2.<sup>41</sup> Our result indicated that anti-invasive effect of CRAE on MHCC97-L cell line only acts on F-actin via Rho/ROCK signaling pathway, but not other metastasis-related molecules such as integrin beta4, E-cadherine (data not shown), u-PA and MMPs, indicating that the Rho/ROCK inhibition may be one new mechanism involved in its action against cancer invasion. The inhibitory effect of CRAE on MHCC-97L cell migration was observed which is consistent with berberine, but the inhibitory effect of CRAE is more potent than that of berberine, indicating that berberine acts as the main active compound in CRAE and other berberine-like alkaloids have synergistic effect to berberine. Precise role of CRAE and berberine on anti-metastasis in animal models needs further investigation.

It was noted that berberine and CRAE used in this study were a relatively high doses. In some cases, the IC<sub>50</sub> of berberine or CRAE was lower than 4 µg/ml, which is below the safety limit established by National Cancer Institute.<sup>40, 42</sup> The IC<sub>50</sub> of berberine and CRAE in this study seem rather high and the reason need to be further explored in future. However, berberine and CRAE are natural products which have been widely used for many years. As an anti-microbial agent, berberine has been used to cure microbial-related gastric diseases. The dose can be very high, even up to 1 gram per day [China Pharmacopeia, 2005 edition]. Our



other studies also showed that berberine revealed very low cytotoxicity on rat hepatocyte (data not shown) and CRAE has liver protective effect in liver damage animal model.<sup>16</sup> These data suggested low toxicity of berberine and CRAE and indicated that both can be used at high dose. Due to the potential of berberine and Huanglian in vitro results and Chinese Medicine practice in the treatment of cancers, berberine and CRAE should be promising agents for clinical trial.<sup>15</sup> New molecular targets, dosages of berberine and CRAE used in this study will provide useful information for further study.

## **Conclusion**

In conclusion, this is the first time to show that CRAE is a potent anti-metastatic agent to inhibit MHCC97-L cells which is a high invasive cell model for liver cancer. CRAE has better antiinvasive effect than its main active compound, berberine, suggesting that other berberine-like alkaloids have synergistic effect to berberine. CRAE acts on F-actin, inducing filament reorganization and therefore inhibits MHCC97-L cell motility. The inactivation of Rho/ROCK signaling pathway involved in CRAE's inhibitory action on MHCC97-L migration indicated CRAE's role as Rho-small GTPase inhibitor. This study sheds light on CRAE as an alternative therapy for the treatment of metastatic hepatic carcinoma.

## **Conflict of interest**

The authors have declared no conflict of interest.

## **Acknowledgements**

The study was financially supported by grants from the research council of the University of Hong Kong (Project Codes: 200811159197 and 200907176140), The University Grant Committee (UGC) of Hong Kong SAR of China (Project Code: 764708M), Pong Ding Yueng Endowment Fund for Education & Research in Chinese-Western Medicine (Project Code: 20005274) and Government-Matching Grant Scheme (4th Phase, Project Code: 20740314). The cell line MHCC97-L was a kind gift from Liver Cancer Institute of Fudan University, Shanghai, China. The authors would like to express thanks to Dr. Ka-Yu Siu, Mr. Keith Wong, Ms Cindy Lee and Mr. Freddy Tsang for their technical support.

## **Reference**

1. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferrazzi E, Grazi GL, Croce CM, Bolondi L, Negrini M. Cyclin G1 Is a Target of miR-122a, a MicroRNA Frequently Down-regulated in Human Hepatocellular Carcinoma. *Cancer Res.* 2007; 67:8-15.
2. Budhu A, Jia, HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW. Identification of Metastasis-Related MicroRNAs in Hepatocellular Carcinoma. *Hepatology.* 2008;47:897-907.

3. Furuse J. Growth factors as therapeutic targets in HCC. *Crit Rev Oncol Hematol*. 2008;67:8-15.
4. Tamazaki D, Kurisu S, Takenawa T. Regulation of cancer cell motility through actin reorganization. *Cancer Sci*. 2005;96:379-386.
5. Cheng LF, Fung PY. Screening for hepatocellular carcinoma: the rationale behind. *The Hong Kong Practitioner*. 2005;27:469-474.
6. Chen HW, Lee JY, Huang JY, Wang CC, Chen WJ, Su SF, Huang CW, Ho CC, Chen JJ, Tsai MF, Yu SL, Yang PC. Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppressor HLJ1. *Cancer Res*. 2008;68:7428-7438.
7. Hu XW, Meng D, Fang J. Apigenin inhibited migration and invasion of human ovarian cancer A2780 cells through focal adhesion kinase. *Carcinogenesis*. 2008;29:2369-2376.
8. Singh RP, Raina K, Sharma G, Agarwal R. Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice. *Clin Cancer Res*. 2008;14:7773-7780.

9. Kim KS, Rhee HI, Park EK, Jung K, Jeon HJ, Kim JH, Yoo H, Han CK, Cho YB, Ryu CJ, Yang HI, Yoo MC. Anti-inflammatory effects of Radix Gentianae Macrophyllae (Qinjiao), Rhizoma Coptidis (Huanglian) and Citri Unshiu Pericarpium (Wenzhou migan) in animal models. *Chin Med.* 2008;3:10.
10. Kim HY, Shin HS, Park H, Kim YC, Yun YG, Park S, Shin HJ, Kim K. In vitro inhibition of coronavirus replications by the traditionally used medicinal herbal extracts, Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, and Phellodendron cortex. *J Clin Virol.* 2008;41: 122-128.
11. Kwon HA, Kwon YJ, Kwon DY, Lee JH. Evaluation of antibacterial effects of a combination of Coptidis Rhizoma, Mume Fructus, and Schizandrae Fructus against Salmonella. *Int J Food Microbiol.* 2008;127: 180-183.
12. Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Lin WL, Chu CY. Inhibitory effect of berberine on tert-butyl hydroperoxide-induced oxidative damage in rat liver. *Arch Toxicol.* 2002;76:664-670.
13. Luo WQ, Hui SC, Chan TY, Feng Y. Inhibitory effect of water extract from golden thread (Huanglian) on Leukemia L-1210 cells cultured in vitro. *Pharmacologist.* 2002;44: A126.
14. Feng Y, Luo WQ, Zhu SQ. Explore new clinical application of Huanglian and corresponding compound prescriptions from their traditional use. *Zhongguo Zhong Yao Za Zhi.*

2008;33:1221-1225. (Chinese)

15. Tang J, Feng Y, Tsao S, Wang N, Curtain R, Wang Y. Berberine and Coptidis Rhizoma as novel antineoplastic agents: a review of traditional use and biomedical investigations. *J Ethnopharmacol.* 1999;126:5-17.

16. Ye X, Feng Y, Tong Y, Ng KM, Tsao S, Lau GK, Sze C, Zhang Y, Tang J, Shen J, Kobayashi S. Hepatoprotective effects of Coptidis Rhizoma aqueous extract on carbon tetrachloride-induced acute liver hepatotoxicity in rats. *J Ethnopharmacol.* 2009;124:130-136.

17. Lee TK, Man K, Ho JW, Wang XH, Poon RT, Xu Y, Ng KT, Chu AC, Sun CK, Ng IO, Sun HC, Tang ZY, Xu R, Fan ST. FTY720: a promising agent for treatment of metastatic hepatocellular carcinoma. *Clin Cancer Res.* 2005;11:8458-8466.

18. Chen JH, Zhao HQ, Wang XR, Lee SC, Yang HH, Zheng L. Analysis of major alkaloids in Rhizoma coptidis by capillary electrophoresis-electrospray-time of flight mass spectrometry with different background electrolytes. *Electrophoresis* 2008;29:2135-2147.

19. Zhao YG, Xiao AZ, Park HI, Newcomer RG, Yan M, Man YG, Heffelfinger SC, Sang QX. Endometase/Matrilysin-2 in Human Breast Ductal Carcinoma in Situ and Its Inhibition by Tissue Inhibitors of Metalloproteinases-2 and -4: A Putative Role in the Initiation of Breast Cancer Invasion. *Cancer Res.* 2004;64:590-598.

20. Harris CC. Hepatocellular carcinogenesis: recent advances and speculations. *Cancer Cells*. 1999;5:146-148.
21. Kuper H, Ye W, Broomé U, Romelsjö A, Mucci LA, Ekblom A, Adami HO, Trichopoulos D, Nyrén O. The risk of liver and bile duct cancer in patients with chronic viral hepatitis, alcoholism, or cirrhosis. *Hepatology*. 2001;34:714-718.
22. Leung PC, Fong H. Alternative treatment for Cancer. World Scientific Publishing. Singapore, pp1-359. 2007.
23. Li Y, Tang ZY, Ye SL, Liu YK, Chen J, Xue Q, Chen J, Gao DM, Bao WH. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. *World J Gastroenterol*. 2001;7:630-636.
24. Kang JX, Liu J, Wang J, He C, Li FP. The extract of huanglian, a medicinal herb, induces cell growth arrest and apoptosis by upregulation of interferon-beta and TNF-alpha in human breast cancer cells. *Carcinogenesis*. 2005;26:1934-1939.
25. Tsang CM, Lau EP, Di K, Cheung PY, Hau PM, Ching YP, Wong YC, Cheung AL, Wan TS, Tong Y, Tsao SW, Feng Y. Berberine inhibits Rho GTPases and cell migration at low doses but induces G2 arrest and apoptosis at high doses in human cancer cells. *Int J Mol Med*.

2009;24:131-138.

26. Olson MF, Sahai E. The actin cytoskeleton cancer cell motility. *Clin Exp Metastasis*. 2009;26:273-287.

27. Chen PS, Wang MY, Wu SN, Su JL, Hong CC, Chuang SE, Chen MW, Hua KT, Wu YL, Cha ST, Babu MS, Chen CN, Lee PH, Chang KJ, Kuo ML. CTGF enhances the motility of breast cancer cells via an integrin- $\alpha$ v $\beta$ 3-ERK1/2-dependent S100A4-upregulated pathway. *J Cell Sci*. 2007;120:2053-2065.

28. Havaki S, Kouloukoussa M, Amawi K, Drosos Y, Arvanitis LD, Goutas N, Vlachodimitropoulos D, Vassilaros SD, Katsantoni EZ, Voloudakis-Baltatzis I, Aleporou-Marinou V, Kittas C, Marinos E. Altered expression pattern of integrin  $\alpha$ v $\beta$ 3 correlates with actin cytoskeleton in primary cultures of human breast cancer. *Cancer Cell Int*. 2007;7:16.

29. Smerling C, Tang K, Hofmann W, Danker K. Role of the  $\alpha$ (1) integrin cytoplasmic tail in the formation of focal complexes, actin organization, and in the control of cell migration. *Exp Cell Res*. 2007;313:3153-3165.

30. Banyard J, Nand-Apte B, Symons M, Zetter BR. Motility and invasion are differentially modulated by Rho family GTPases. *Oncogene*. 2001;19:580-591.

31. Fritz G, Just I, Kaina B. Rho GTPases are over-expressed in human tumors. *Int J Cancer*.

1999;81:682–687.

32. Yoshioka K, Nakamori S, Itoh K. Overexpression of small GTP-binding protein RhoA promotes invasion of tumor cells. *Cancer Res.* 2009;59:2004-2010.

33. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nat Med.* 1999;5:221-225.

34. Takamura M, Sakamoto M, Genda T, Ichida T, Asakura H, Hirohashi S. Inhibition of intrahepatic metastasis of human hepatocellular carcinoma by Rho-associated protein kinase inhibitor Y-27632. *Hepatology.* 2001;33:577-581.

35. Wong CC, Wong CM, Ko FC, Chan LK, Ching YP, Yam JW, Ng IO. Deleted in liver cancer 1 (DLC1) negatively regulates Rho/ROCK/MLC pathway in hepatocellular carcinoma. *PLoS ONE.* 2008;3:e2779.

36. Leung TH, Ching YP, Yam JW, Wong CM, Yau TO, Jin DY, Ng IO. Deleted in liver cancer 2 (DLC2) suppresses cell transformation by means of inhibition of RhoA activity. *Proc Natl Acad Sci U S A.* 2005;102: 15207-15212.

37. Feng Y, Siu K, Wang N, Ng KM, Tsao SW, Nagamatsu T, Tong Y. Bear bile: dilemma of traditional medicinal use and animal protection. *J Ethnobiol Ethnomed.* 2009;12:2-11.



38. Iizuka N, Miyamoto K, Okita K, Tangoku A, Hayashi H, Yosino S, Abe T, Morioka T, Hazama S, Oka M. Inhibitory effect of Coptidis Rhizoma and berberine on the proliferation of human esophageal cancer cell lines. *Cancer Lett.* 2000;148:19-25.
39. Iizuka N, Oka M, Yamamoto K, Tangoku A, Miyamoto K, Miyamoto T, Uchimura S, Hamamoto Y, Okita K.. Identification of common or distinct genes related to antitumor activities of a medicinal herb and its major component by oligonucleotide microarray. *Int J Cancer.* 2003;107:666-672.
40. Li XK, Motwani M, Tong W, Bornmann W, Schwartz GK. Huanglian, A chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. *Mol Pharmacol.* 2000;58:1287-1293.
41. Mitani N, Murakami K, Yamaura T, Ikeda T, Saiki I. Inhibitory effect of berberine on the mediastinal lymph node metastasis produced by orthotopic implantation of Lewis lung carcinoma. *Cancer Lett.* 2001;165:35-42.
42. Letasiová S, Jantová S, Cipák L, Múcková M. 2006. Berberine-antiproliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells. *Cancer Lett* 239:254-62.

## Figure legends

Fig.1 Chemical analysis of CRAE by HPLC/MS/MS. A shows the HPLC/MS/MS TIC chromatogram of CRAE; B shows the mass spectrums of particular peaks in TIC chromatogram of CRAE.

Fig.2 MHCC97-L cells viability after CRAE treatment in different dose and time (MTT assay)

(\* $p < 0.05$ , \*\* $p < 0.01$  when compared with control)

Fig.3 CRAE and Berberine significantly inhibits the migration of liver cancer cells MHCC97-L. A shows results of wound healing assay, presenting that low dose of CRAE and berberine can suppress MHCC97-L cells migrating from distinct edges to center. B shows results of invasion chamber assay, indicating that CRAE and berberine inhibit MHCC97-L cells migration in dose-dependent manner. (\* $p < 0.05$ , \*\* $p < 0.01$  when compared with control) C shows that addition of Y-27632, a Rho/ROCK inhibitor alone or in combination of CRAE, can dose-dependently suppress the migration of MHCC97-L. The left three panels showed cell migration in Fig. 3C, in which the first one is without any treatment, the second and third ones showed the effect of low and high dose of Y-27632 treatment alone. This figure indicates that high dose of Y-27632 could completely suppress MHCC97-L cell migration. The figure also reveals that Rho/ROCK inhibition, which showed berberine and CRAE have the similar action could suppress cancer cell migration. The right two panels in Fig. 3C showed that combination of low dose of berberine or CRAE and Y-27632 increased their inhibitory effect at their low dose compared with Fig.3A, revealing that

action of berberine and CRAE is similar to Rho/ROCK inhibitor in liver cancer cell migration.

Fig.4 The expression of MMPs in MHCC97-LL cell treated with 100  $\mu$ M berberine (B), 100  $\mu$ M CRAE (C) or vehicle (A). The graph shows the inhibition of CRAE on the expression of TIMP-4 but no suppression on MMP-2 and MMP-9.

Fig.5 CRAE and berberine affect F-actin reorganization and therefore inhibit MHCC97-L cell motility.

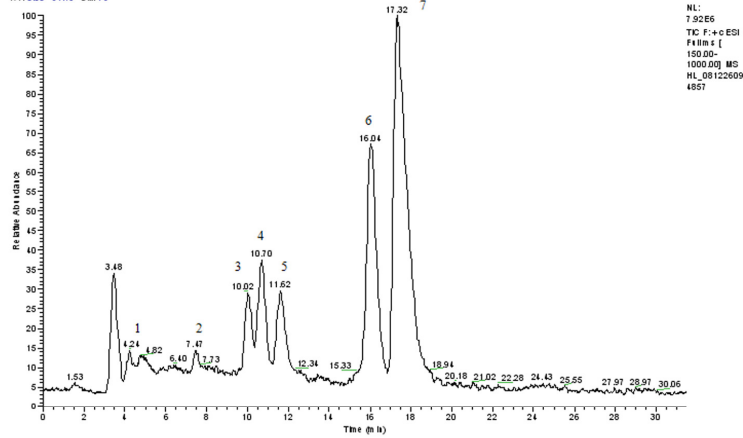
Fig.6 Immunoblotting of Rho/ROCK signaling pathway in MHCC97-L cell. A shows that CRAE and berberine exhibited a dose-dependent inhibitory effect on the activation of RhoA-GTPase and ROCK-1 expression, indicating its action on F-actin reorganization may be through its suppression on Rho/ROCK signal transduction. B shows a quantitative analysis on the western blot results. (\*\* $p < 0.01$  when compared with CTL group)

Table. 1 Chemical identification of Alkaloid in CRAE

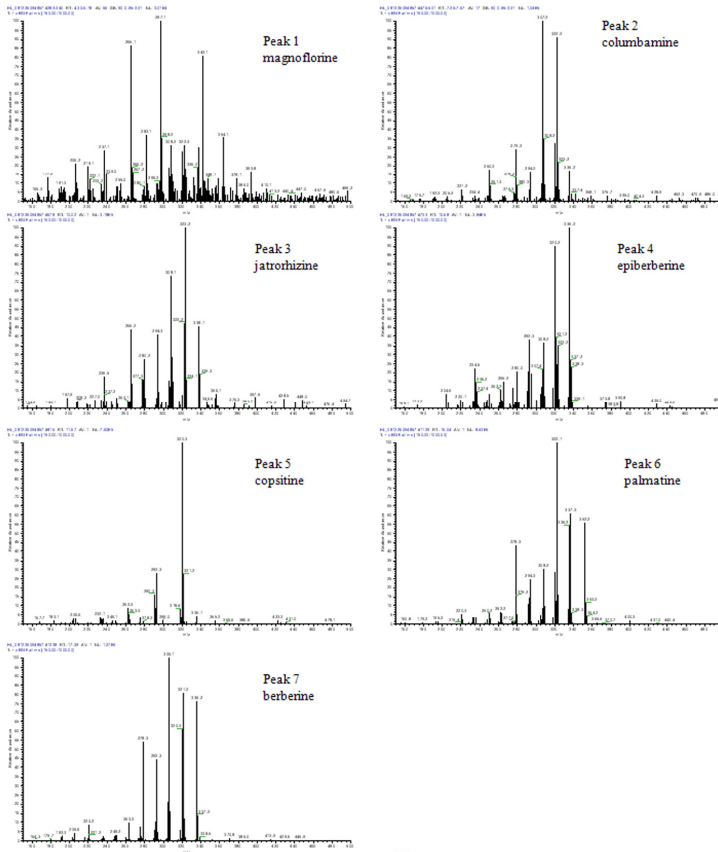
Peak No.	Retention Time (min)	Characteristic Fragmentation (CF)	CR in literature <sup>[17]</sup>	chemical identification	molecular weight
1	4.3	342.1,297.1,282.1,265.1,222.1	342.1705,297.1115,282.0886,265.0858,222.0672	magnoflorine	342
2	7.26	322.2,308.2,294.2,279.2	322.1063,308.0907,294.1107,280.0958	columbamine	338
3	10.02	338.1,323.2,308.1,294.3,280.2	338.1375,323.1135,308.0907,294.1107,280.0958	jatrorrhizine	338
4	10.68	336.2,320.2,292.3,	336.1219,320.0966,292.0950	epiberberine	336
5	11.67	320.3,292.3,290.3,262.3	320.0910,292.0959,290.0804,262.0856	copsitine	320
6	16.04	352.2,337.3,336.2,308.2,294.3	352.1536,337.1295,336.1224,308.1252,294.1113	palmatine	352
7	17.39	336.2,320.3,306.1,292.3,278.3	336.1217,320.0904,306.0755,292.0953,278.0801	berberine	336

Wang et al. Figure 1

RT: 0.00-31.49 SM: 78

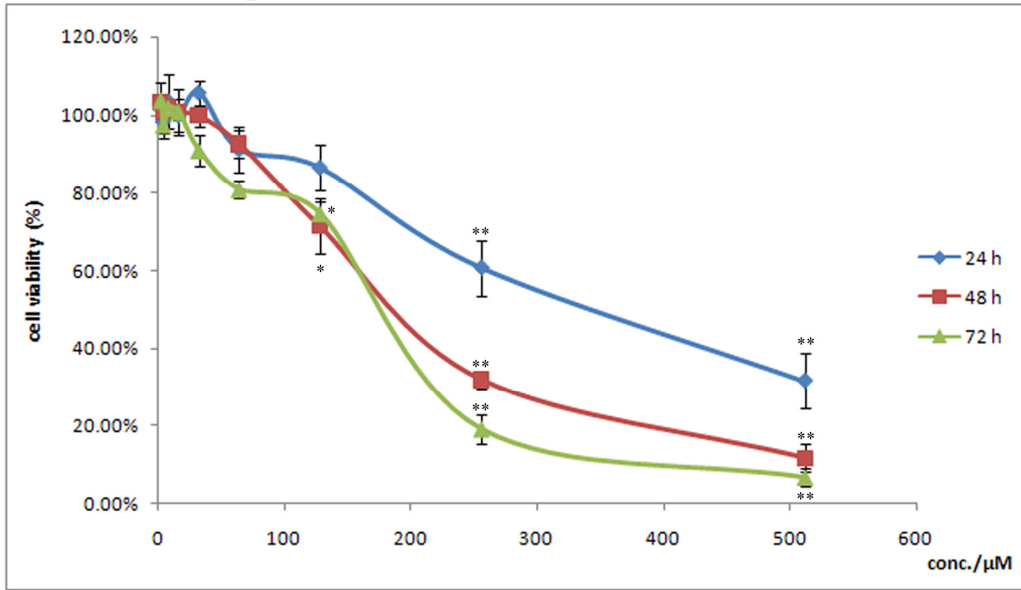


(A)

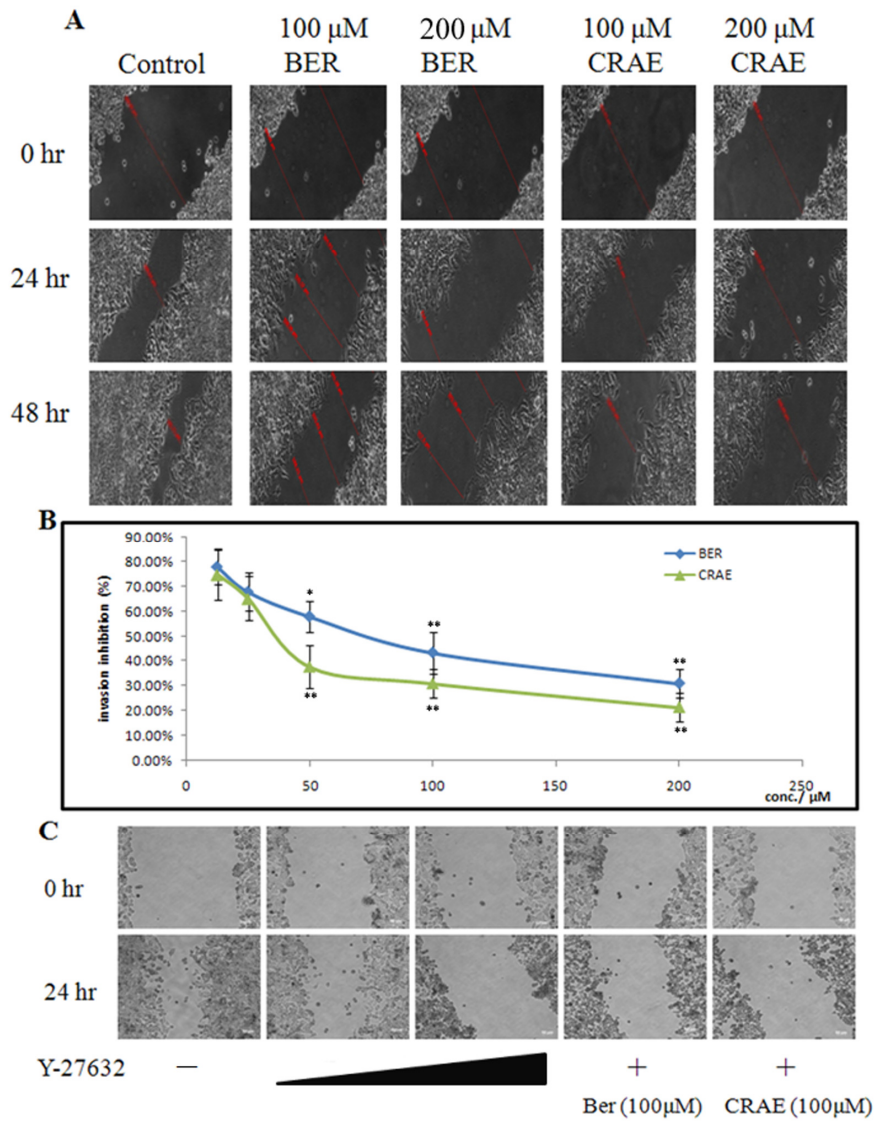


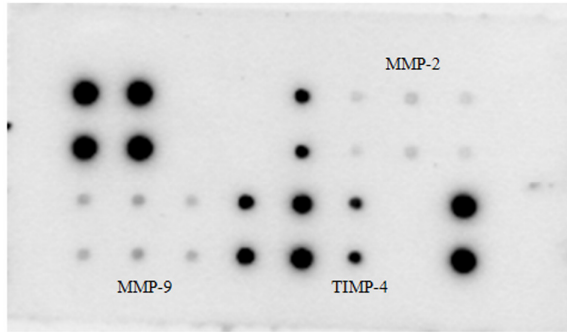
(B)

Wang et al., Fig. 2

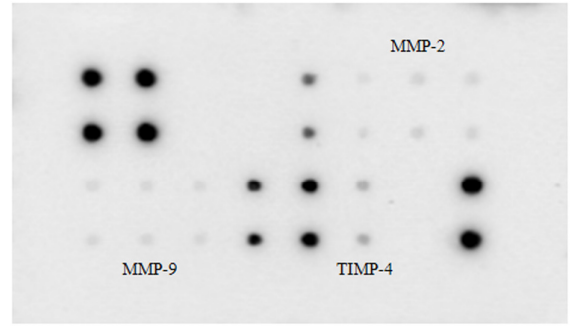


Wanget al., Figure 3

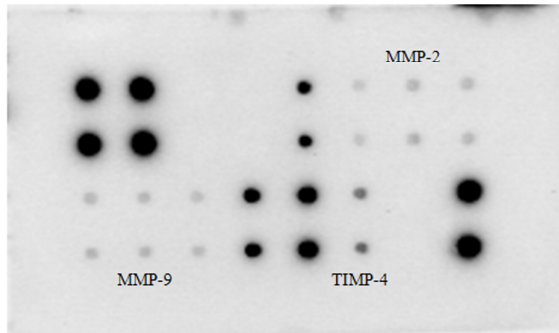




(A)



(B)



(C)



