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

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Abstract

Hypothesis: Heptaocellular 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 immunobloting 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 .[1-2] Each year there are approximately 500,000 new cases of HCC worldwide, 80% of which happens in Asia and Africa.[3] 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.[4] 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,[5] and molecules targeted to these factors for the sake of cancer therapy have been reported.[6-8] 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,[9] anti-viral,[10] anti-bacterial[11] and anti-oxidative agent.[12] Our screening study demonstrated that CRAE exhibited the strongest inhibitory activity on the growth of tumor cells amongst sixteen anticancer traditional Chinese medicinal herbs.[13] 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.[14]. A lot of studies have been carried out on cytotoxic, apoptotic effects and mechanisms of CR and berberine

in vitro and *in vivo*,[15] 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 chinesis* 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[16]. 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℃ 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_{18} 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 Rat (abr):40; Auxiliary Gas Flow Rate (abr): 40; Spray voltage (kV): 4.5; Capillary Temperature (℃): 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.[17] 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_2 at 37 ℃.

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 μM) and incubated for 24 to 72h. All experiments were conducted parallel with controls (0.1% DMSO). Then cells were incubated with 15 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma, USA) at 37℃ for 4h. Then medium was removed and 200 μl 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 μM CRAE or berberine for 0, 24 or 48h. The migration of MHCC97-L cells were observed under fluorescent microscope with 40× magnification (Carl Ziess, USA).

Invasion chamber assay

Experiment was conducted under the manufacturer's instruction (QCM^{TM} 96-well cell invasion assay kit, Millipore, USA). Briefly, the invasion chamber was pre-activated by 100 μl of serum free medium for 1h. After rehydration of chamber, 150 μl growth medium was added to the feeder tray. 100 μl serum free media with 1×10^4 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 μM and incubated for 24h at 37℃, 5% CO_2. 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^2 flask in serum-free medium with 80% confluence and treated with 100 μM 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 μM) for 24h. Cells werer 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 μg/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 1μg/mL DAPI for 10 minutes and cells were visualized under fluorescent microscope with 400× magnification (Carl Ziess, USA).

RhoA-GTPase activity

Cells were seeded in 25 cm^2 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, 200μM) 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%apotinin and 0.5% leupeptine) and phosphatase inhibitor (1 mM Na_3VO_4 and 1mM NaF) on ice for 30 minutes and then centrifuged at 14,000 rpm at 4℃ 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℃. The membrane was then incubated with primary antibody uPA, ROCK1, β-actin (Abcam, UK) at 4℃ overnight followed by incubation with appropriate secondary antibody (Abcam, UK) at room temperature for 2 hours. The immunoreactivites were detected using ECL plus kit (GE Healthcare, UK) and visualized using a chemiluminenescence

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 ± 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, jatrorrhzine, 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,[18] peak 1 to 7 is identified as magnoflorine, columbamine, jatrorhizine, epiberberine, copsitine, 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 μM of CRAE, and then the IC_{50} of CRAE decreased to about 150 μM 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 200μM 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 μM 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, prostrate cancer and colon cancer,[19] 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_{50}, 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 μM 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.[20-21] 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.[22] 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.[23] CR extract can induce apoptosis by up-regulating of interferon-beta and TNF-alpha in human breast cancer cells.[24] 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.[25] 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[14], 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.[26] 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.[27-29] 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.[4] The activation of Rho small GTPases family, especially the RhoA-GTPase, is frequently found in the metastasis of different types of human cancers[30-32], and inhibition of Rho-GTPase and ROCK suppresses tumor invasion *in vitro* and *in vivo*.[33-36] 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[16,37]. 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.[38,39] 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.[40] 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.[41] 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 IC50 of berberine or CRAE was lower than 4 μg/ml, which is below the safety limit established by National Cancer Institute.[40, 42] The IC50 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.[16] 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 trail.[15] 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

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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 μM berberine (B), 100 μM 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.

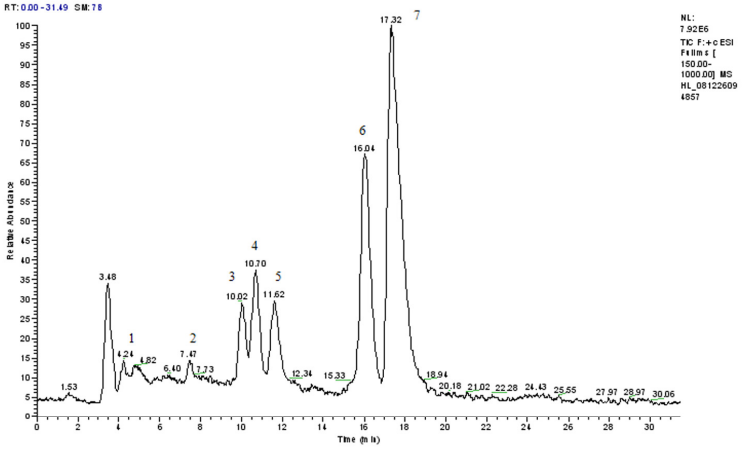
Fig.5 CRAE and berberine affect F-actin reorganization and therefore inhibit MHCC97-L cell motility.

Fig.6 Immunobloting 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)

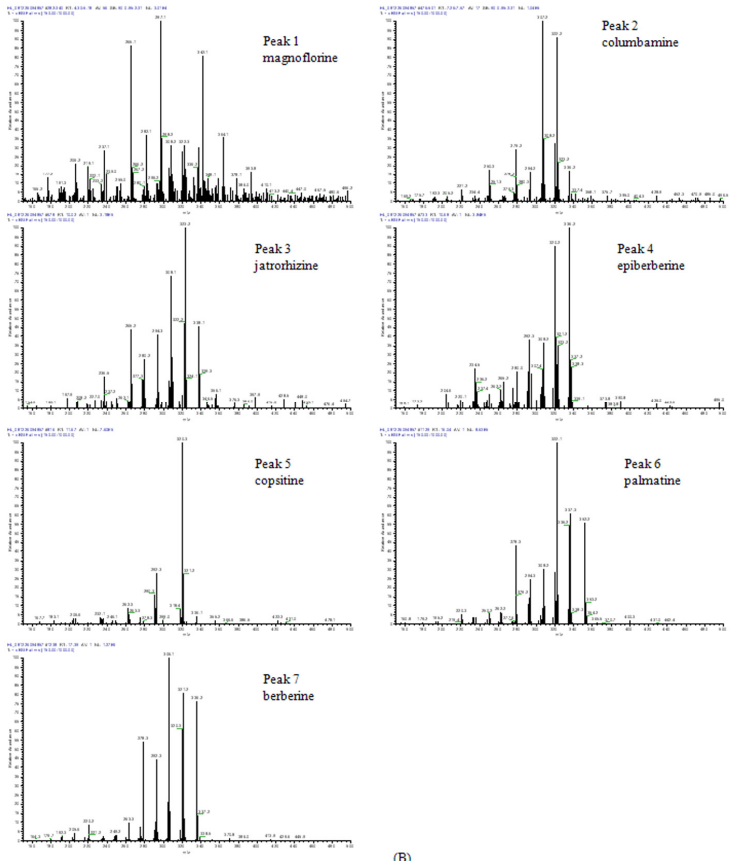
Table. 1 Chemical identification of Alkaloid in CRAE

Peak No.	Retention Time (min)	Characteristic Fragmentation (CF)	CR in literature[17]	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

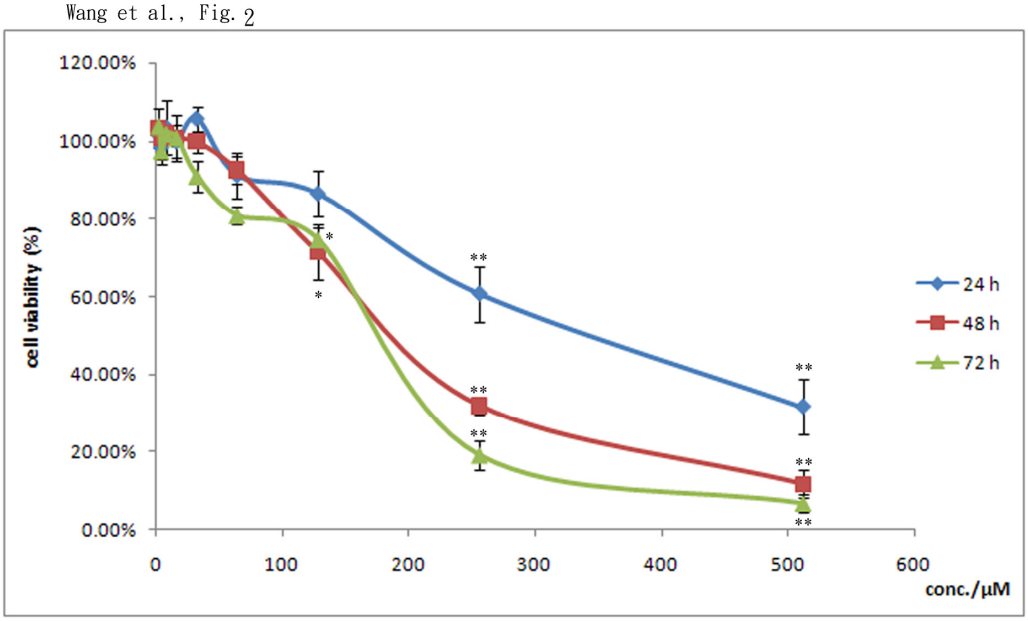


(A)

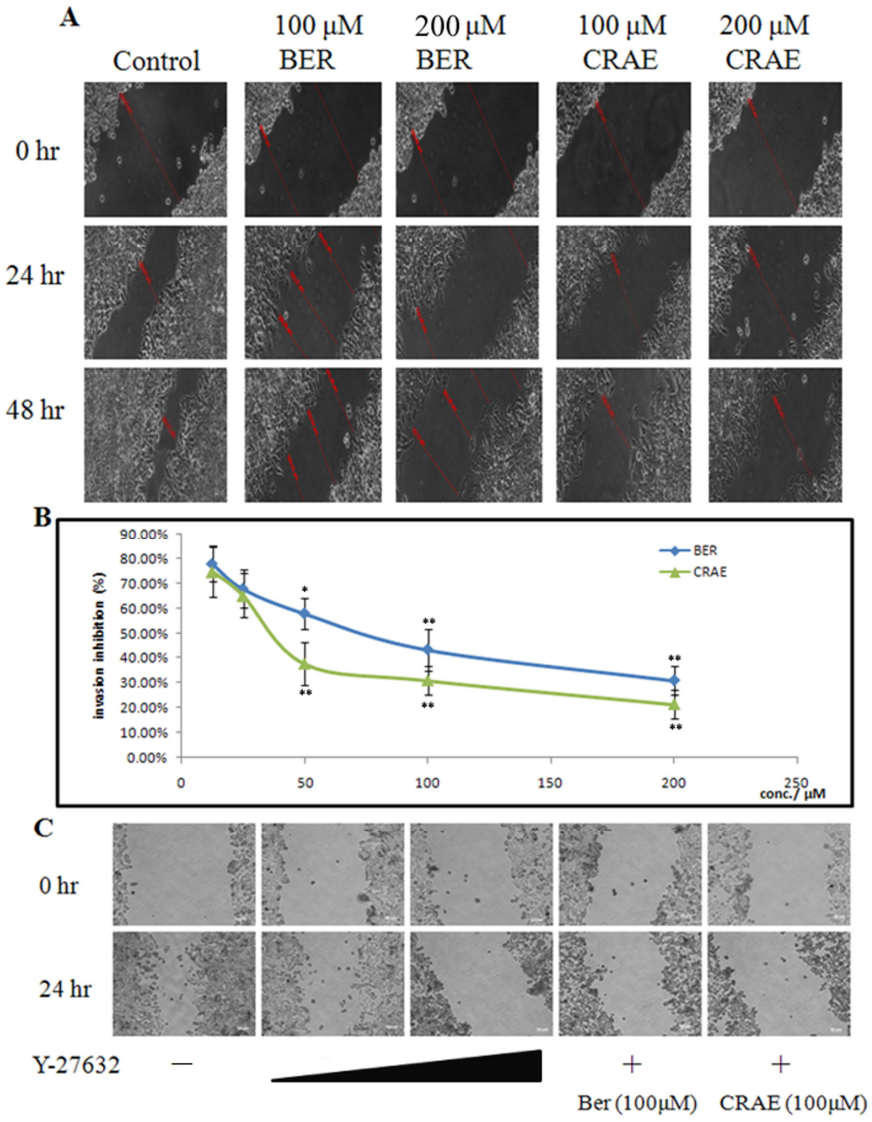


(B)

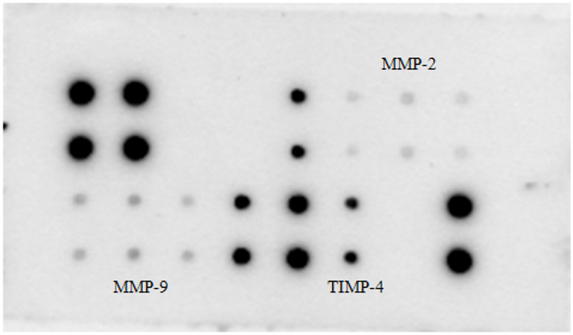
Wang et al., Fig. 2



Wang et al., Figure 3



Wang et al., Figure 4



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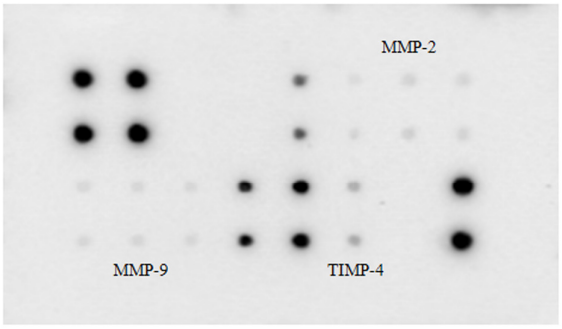

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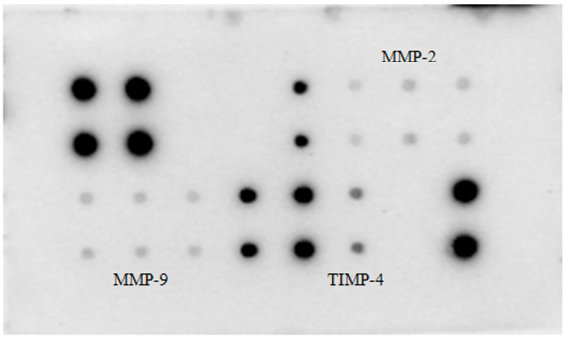
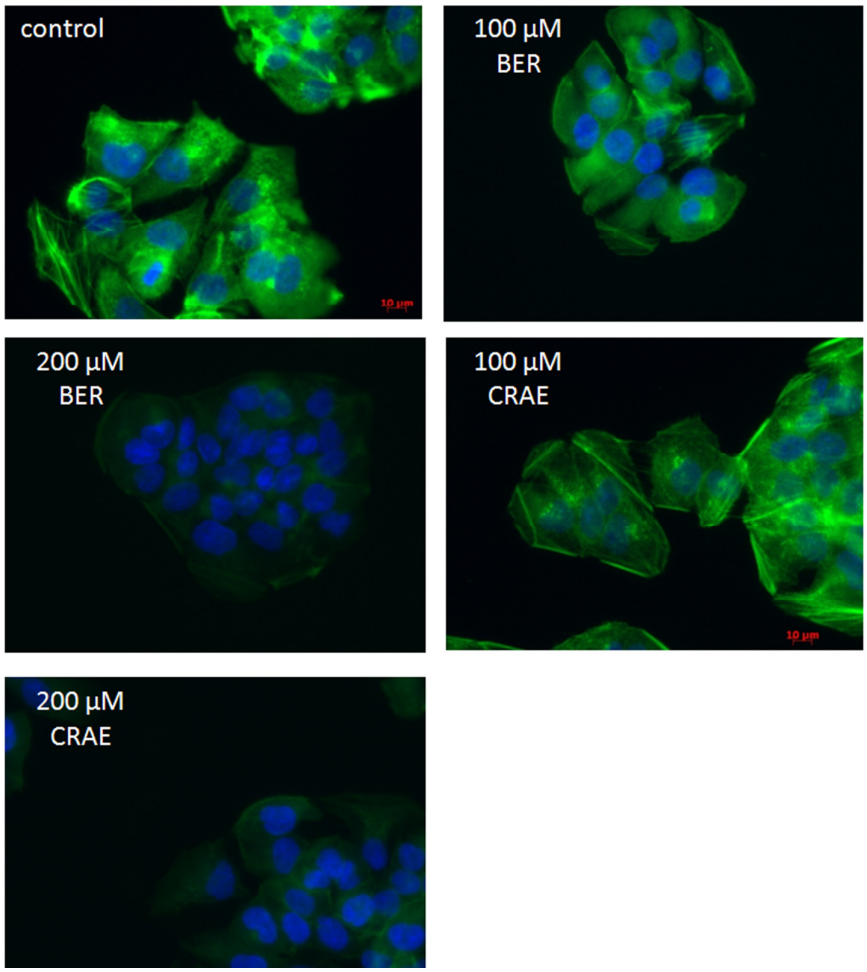
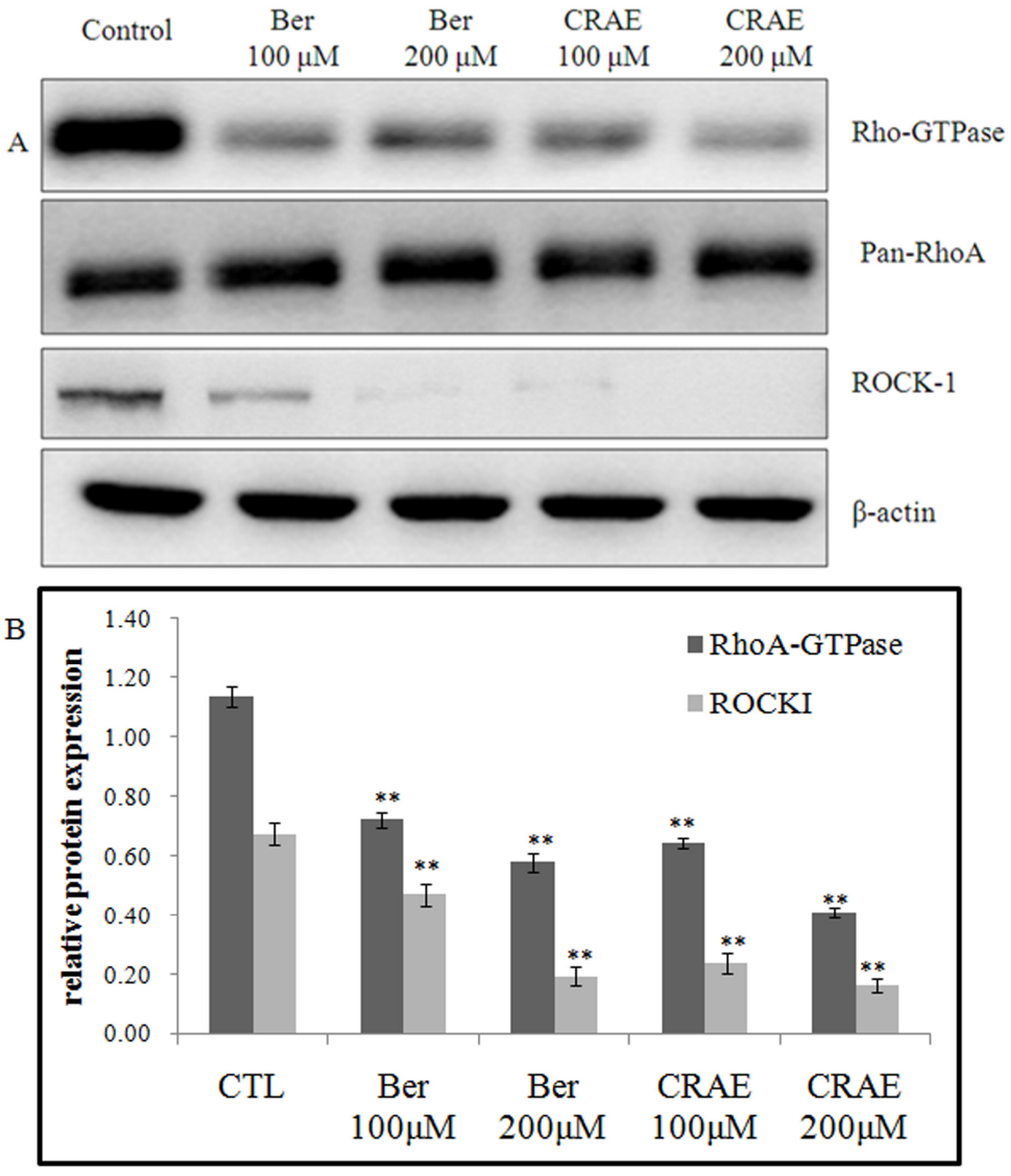


(C)

Wang et al., Figure 5



Wang et al., Figure 6