

Comparison of Components and Anti-Liver Cancer Activity *In vitro* between *Huanglian* and *Yunlian*

Chingshan To¹, Caylan Liew^{1,2}, Xuanbin Wang^{1,3}, Ning Wang¹ and Yibin Feng^{1*}

¹School of Chinese Medicine, The University of Hong Kong, China

²Chemical and Biological Sciences, Polytechnic Institute of New York University, USA

³Laboratory of Chinese Herbal Pharmacology, Renmin Hospital, Hubei University of Medicine, Shiyan, P. R. China, 442000

Abstract

Objective: Coptidis Rhizoma is a famous Chinese medicinal herb for its potential to combat cancer, inflammation, fibrosis, viral and bacterial infections, and oxidation. *Coptis chinensis* Franch (*Huanglian* in Chinese HL) and *Coptis teeta* Wall (*Yun Huanglian* or *Yunlian* YHL) are two species of Coptidis Rhizoma commonly used in Traditional Chinese Medicine. *Huanglian* is produced from Sichuan Province, while *Yunlian* comes from the Yunnan Province. Both provinces are located in China. There are indications that both herbs possess similar characteristics and functions in clinical use. However, there are no signs of prior research to compare the effects of HL and YHL on hepatocellular carcinoma. In this study, we compared the components and anti-liver cancer effects *in vitro* between *Huanglian* and *Yunlian*.

Methods: HPLC was introduced to analyze the active components in *Huanglian* and *Yunlian* aqueous extracts. Liquid chromatography with mass spectrometry and High performance liquid chromatography with UV detector were used to identify and compare the main ingredients ratio between *Huanglian* and *Yunlian* extracts. Cytotoxicity and apoptosis affected by the two extracts on MHCC97L cell line was observed by using MTT assay and flow cytometry, respectively. The inhibition of metastasis and invasion in hepatocellular carcinoma cell line MHCC97L by *Huanglian* and *Yunlian* were compared as well.

Results: Results of phytochemical analysis showed there were no significant differences between the two herbs' ingredients and ratios. Also, there were no significant differences between *Huanglian* and *Yunlian* on cytotoxicity, apoptosis and metastasis in MHCC97L.

Conclusion: *Yunlian* and *Huanglian* share great similarities in both chemical composition and bioactivity *in vitro*.

Keywords: Coptidis rhizoma; *Coptis chinensis* Franch (*Huanglian*); *Coptis teeta* Wall (*Yunlian*); Quality control; Quantity control; Cytotoxicity; Metastasis

Introduction

Since antiquity, Coptidis Rhizoma (*Coptis*) has been administered as an ancient remedy. In Traditional Chinese Medicine (TCM), *coptis* is used to treat conditions associated with *Qingre Zaoshi* (clearing heat and removing dampness), *Xiehuo Jiedu* (purging fire to eliminate toxin) such as diarrhea, eye inflammation and women's abdomen ailments [1,2]. Recent studies have disclosed its potential to combat cancer, inflammation, fibrosis, viral and bacterial infections, and oxidation [3-10]. *Coptis* and its main ingredient, berberine, show significant effects on cancer with a multiple targets including mitochondrial [11], NAG-1 [12,13], topoisomerases [14], estrogen receptors [15], p53 [16,17], NF- κ B [18,19] and ROS [20]. Our previous studies indicated that *Coptis* extracts activated miR-21 and miR-23a in human liver cancer [21], we also found that *Coptis* and berberine initiated autophagy in liver cancer by activating Beclin-1 and blocking mTOR-signaling way by suppressing the activity of Akt and inducing p38 MAPK activation [22].

According to China Pharmacopoeia (2010), three species could be the resources of *Coptis*, including *Coptis chinensis* Franch, *Coptis deltoidea* C.Y. Cheng et Hsiao and *Coptis teeta* Wall [23], among which *Coptis chinensis* Franch (*Huanglian* in Chinese, HL) and *Coptis teeta* Wall (*Yun Huanglian* or *Yunlian*, YHL) are two main medical products from Sichuan Province and Yunnan Province, respectively. Chemical analysis have determine Berberine to be *Huanglian*'s active components, and other trace alkaloids include palmatine, columbamine, jatrorrhizine, epiberberine, copsitine, and an

aporphinoid alkaloid-magnoflorine [24]. However, it shall be necessary to provide scientific evidence to show the bioequivalence of HL and YHL in certain pharmacological aspects.

In this study, we compared the chemical compositions and tumor suppressive effects *in vitro* between *Huanglian* and *Yunlian*. High performance liquid chromatography (HPLC) and HPLC combined with mass spectrometry (LC-MS) were facilitated to determine berberine and other alkaloids and their ratio. As we previously found that berberine effectively inhibited cell migration and invasion at low doses by induction of Rho GTPases including RhoA, Cdc42 and Rac1 [25]. Inhibition of *huanglian* and *yunlian* on migration and invasion in human hepatocellular carcinoma cell line MHCC97L was investigated in this study as well.

Materials and Methods

Preparation of HL and YHL Aqueous Extracts

Dried HL and YHL herbs were collected from Sichuan and Yunnan

*Corresponding author: Yibin Feng, School of Chinese Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong, China, Tel: 852-25890482; Fax: 852-28725476; E-mail: yfeng@hku.hk

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Province of China respectively and authenticated under the guidance of Chinese Pharmacopoeia (2010). They were identified by School of Chinese medicine, the University of Hong Kong. 500 g of each was boiled in 10 times of distilled water (w/v) at 100°C for 1 hour and percolated through filter paper (Whatman, pleated filter grade 597 1/2, 4–7 µm) and then sterilized by filtration through a 0.2 µm pore filter (Minisart®-plus, Sartorius). The filtrate was further concentrated and the dry extract powder was collected and stored at -20°C until used.

Cell line

Human hepatocellular carcinoma cell line MHCC97L was kindly gifted by Dr. Man Kwan, Department of Surgery, and The University of Hong Kong. Cells were cultured in DMEM medium (Invitrogen, USA), and incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Identification of chemical composition of HL and YHL extracts by LC-MS

LC-MS (Thermo Finnigan, USA) was used to identify the chemical composition of HL and YHL extracts. Both HL and YHL extract powders were dissolved in methanol and diluted to proper concentration. 10 µl of sample solution was injected to the LC-MS [26-28].

Quantification of active components of HL and YHL extracts by HPLC

HPLC (Water 996 Photodiode Array Detector, Waters 717plus Autosampler, Water 600S Controller, Empower Pro Analysis system, USA) was introduced to analyze the active components in herb extracts as described previously [29-31].

Cell viability assay

In vitro cytotoxicity of HL and YHL extracts on MHCC97L cells was observed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay system. 1×10⁴ cells with 100 µl of medium per well were seeded in 96-well plates, incubated, and treated with different concentrations of HL and YHL for 24, 48, and 72 hrs, respectively. At the end of each treatment, 10 µl of MTT (5 mg/mL) was added to the well and incubated for 4 hrs. The absorbance was read at 575 nm with Multiskan MS microplate reader (Labsystems, Finland).

Annexin v/PI staining

Cells were seeded in 35-mm petri-dish with 80% confluence and treated with different concentrations of HL and YHL for 24 hrs. After treatment, cells were collected using micro-scraper (Corning, USA) and then centrifuged. To detect apoptosis in MHCC97L cells, Annexin V/PI double staining kit (Sigma-aldrich, USA) was introduced to quantify cell amount in different stages of cell death. Briefly, cells were resuspended in 1 mL of binding buffer containing 100 mM HEPES/NaOH, 1.4 M NaCl, 25 mM CaCl₂, pH 7.5. Then 5 µl of FITC-conjugated Annexin V (50 µg/mL) and 10 µl of propidium iodide (100 µg/mL) were added to the cell suspension. Incubation of cells was performed in dark for exactly 10 minutes at room temperature and detected by flow cytometer (Epics XL, Beckman Coulter, USA).

Wound healing assay

MHCC97L cells were seeded in 6-well plates and waited for full confluence attained. To introduce uniform wound on the confluent monolayer cells, a gap was scraped by using a micro-pipette tip on each well. After wound introduction, culture medium was replaced with

fresh medium containing different concentrations of HL and YH. To compare the speed of wound closure between berberine treated and untreated cells, photographs were taken under 100x magnifications using phase-contrast microscopy immediately after wound incision (0 h) and after 24 hrs.

Invasion chamber assay

The experiment was conducted under the manufacturer's instruction (QCMT 96-well cell invasion assay kit, Millipore, USA). Briefly, 100 µl of pre-warmed serum free media was placed into the invasion chamber and the plate was incubated for 1 hour at room temperature. After rehydration of chamber, media were removed and 150 µl serum free media in the presence of 10% fetal bovine serum were added to the feeder tray. 100 µl serum free media with 1×10⁴ MHCC97L cells were then placed into invasion chamber. A series of concentrations of agents were added to cell suspension and incubated for 24 hours at 37°C, 5% CO₂. Then media with cell suspension in each chamber were discarded and the chamber plate was plated onto a new feeder tray containing 150 µl PBS in each well and incubated for 1 minute at room temperature. After rinsing with PBS as described, PBS was removed and 150 µl of pre-warmed cell detachment solution were added to the feeder tray and the chamber plate was incubated for 30 minutes at 37°C. This step was followed by addition of 50 µl of lysis buffer/dye and 15 minutes incubation at room temperature. 150 µl of the mixture was transferred to a new 96-well black plate and read it using 480/520 nm filters set using a fluorescence plate reader.

Statistical analysis

Data was computed using student T-test and expressed as mean ± SD. Experiments were repeated three times. P<0.05 was considered significantly difference.

Results

Identification of chemical composition in HL and YHL extracts by LC-MS

7 peaks, magnoflorine, columbamine, jatrorrhizine, epiberberine, coptisine, palmatine, and berberine were identified as major alkaloids in both HL and YHL extracts as reported previously [26,27]. No significant difference of chemical composition between HL and YHL extracts was observed (Table 1).

Quantification of active components in HL and YHL extracts by HPLC

Area of 5 main peaks were determined and calculated as a percentage of total area. Data indicated there was no difference between the two herbal extracts (Figure 1 and Table 1). It was shown that berberine is the major active compound in both HL and YHL. The yields of berberine in HL and YHL were 32.14% and 30.96% respectively.

	Magnoflorine	Jatrorrhizine	Coptisine	Palmatine	Berberine
% in HL	4.49	7.84	10.22	12.89	32.14
% in YHL	2.33	4.81	8.21	9.76	30.96

Table 1: Summary of active components in HL and YHL. The phytochemical analysis was performed under the following condition: Reserve C18 column (Symmetry®, 250 mm x 4.0 mm, 5 µm) was used as solid phase; elution was performed using acetonitrile-25 mM potassium dihydrogen phosphate (23:77) as mobile phase. Detecting wavelength and flow rate was 254 nm and 1.0 ml/min respectively. The analysis was performed under room temperature. Area of 5 main peaks were calculated as a percentage of total area. There is no significant difference between two herbs.

Cytotoxicity effects of HL and YHL extracts on MHCC97L cells

MHCC97L cells treated with HL and YHL extracts for 24, 48 and 72 hrs were conducted and calculated. To better examine the bioequivalence between HL and YHL, the IC₅₀ value of HL and YHL was determined and the data was presented by showing the yield of berberine contained by the extracts. IC₅₀ value for HL was 340, 170 and 170 μM for 24, 48 and 72 hours, respectively, while for YHL was 380, 150 and 180 μM. Both of HL and YHL extracts exhibited potent cytotoxicity on MHCC97L with a dose-dependent manner in 48 hours (Figure 2).

HL- and YHL- induced apoptosis in MHCC97L Cells

The results showed that HL and YHL significantly induced early and median apoptosis in MHCC97L cells after 24 hours treatment. The induction of apoptosis by HL and YHL was slightly enhanced in line with increased doses. There was no large difference in potency between HL and YHL for inducing apoptosis in the MHCC97L cell line (Figure 3).

HL and YHL inhibit metastasis of MHCC97L cells by wound healing assay

It was observed that MHCC97L cells at the opposite edges of the wounds rapidly migrated toward each other after 24 hrs of incubation. With increasing doses, the speed of wound healing slowed down, the gap remained open, and only minimal cell proliferation on the 2 edges of the wound was observed when cells were incubated with 200 μM of HL and YHL. Cell motility was remarkably inhibited with HL and YHL exposure. Photographs of cells with different treatment were taken and displayed in Figure 4.

HL and YHL inhibit invasion of MHCC97L cells

The results of chamber assay combined with MTT assay showed promising inhibitory effects of HL and YHL on the invasion of MHCC97L cell line. The results also showed that HL and YHL suppressed the metastasis of MHCC97L at the doses far lower than their IC₅₀ values. HL and YHL showed no difference in their capability to suppress cell invasion (Figure 5).

Discussion

In Chinese Pharmacopoeia, HL and YHL belong to the same genus,

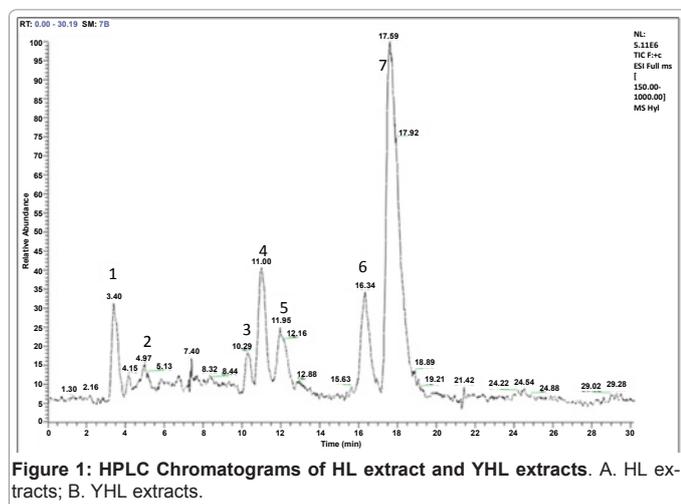


Figure 1: HPLC Chromatograms of HL extract and YHL extracts. A. HL extracts; B. YHL extracts.

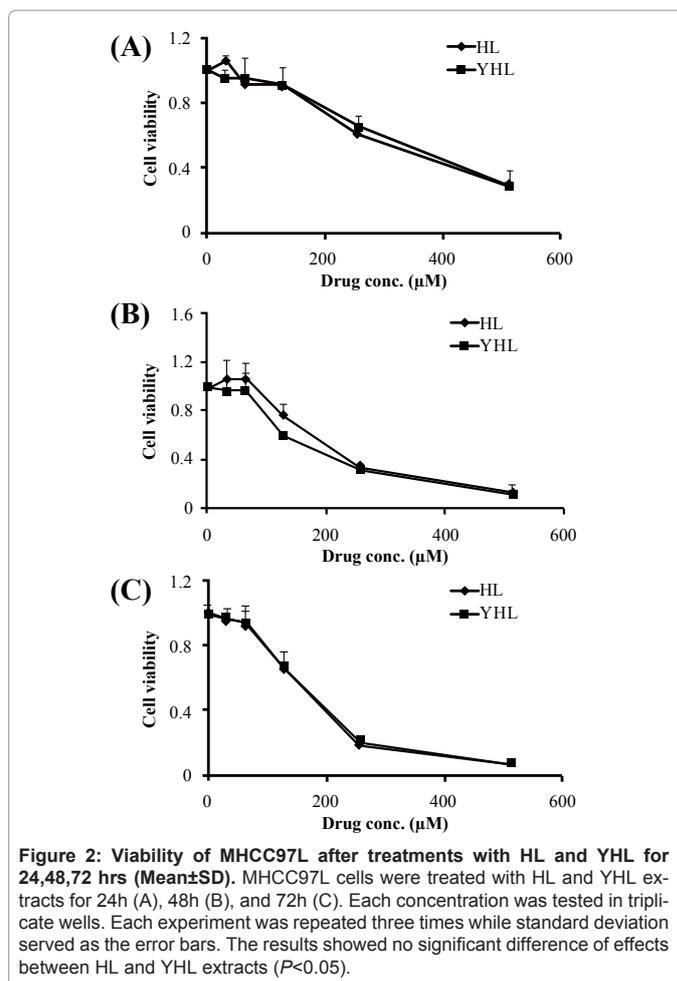


Figure 2: Viability of MHCC97L after treatments with HL and YHL for 24, 48, 72 hrs (Mean±SD). MHCC97L cells were treated with HL and YHL extracts for 24h (A), 48h (B), and 72h (C). Each concentration was tested in triplicate wells. Each experiment was repeated three times while standard deviation served as the error bars. The results showed no significant difference of effects between HL and YHL extracts ($P < 0.05$).

Coptis, and both herbs could be administered in Traditional Chinese Medicine clinics, and are important components in Chinese medicine herbal formulae. There are indications that both herbs possess similar characteristics and functions in clinical use. However, there are no signs of prior research to provide any scientific observation on the pharmacological similarities of HL and YHL. In this study, we have been able to identify the active ingredients in HL and YHL by LC-MS. The HPLC-UV chromatograms of HL and YHL extracts showed that there was no significant difference in the chemical composition between the two extracts. The quantification of active components in HL and YHL was obtained by HPLC where there were no remarkable variation of the yields of major active alkaloids observed between HL and YHL, indicating that HL and YHL share great similarities in chemical composition.

To compare the bioactivity of HL and YHL, MTT Assay and flow cytometry were employed. As we previously found that coptis and berberine effectively inhibit cell metastasis and invasion at low doses by induction of Rho GTPases including RhoA, Cdc42 and Rac1 [25]. Wound healing and invasion chamber assay were conducted to compare the inhibition of metastasis and invasion between HL and YHL. Both of HL and YHL showed similar cytotoxicity in serial concentrations. We used Annexin V/PI double-staining to monitor apoptosis induced by HL and YHL. Both HL and YHL can significantly

induce apoptosis in MHCC97L cells after 24 hours treatment. There was no large difference in potency between HL and YHL for inducing apoptosis in the MHCC97L cell line. Wound-healing assay was introduced to qualitatively determine the migration of MHCC97L cells with exposure to HL and YHL. Cell motility was remarkably inhibited under exposure with either HL or YHL. Moreover, the results of

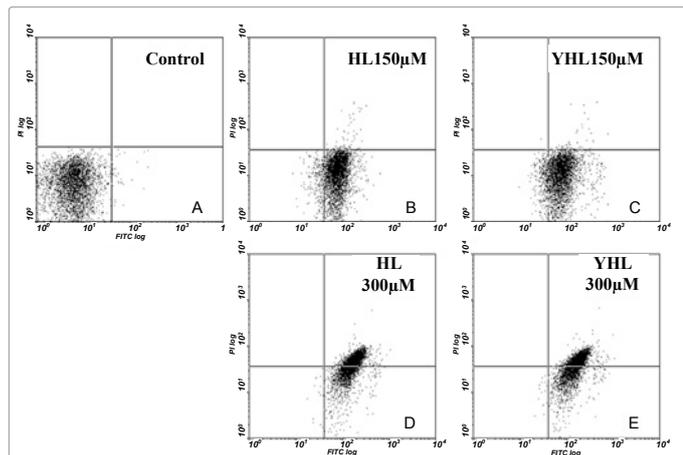


Figure 3: HL- and YHL- induce apoptosis in MHCC97L cells. MHCC97L cells were treated with HL and YHL for 24hrs. Annexin V/PI double-staining was used for flow cytometry (FCM). A. control; B. HL extracts 150μM; C. YHL extracts 150μM; D. HL extracts 300μM; E. YHL extracts 300μM.

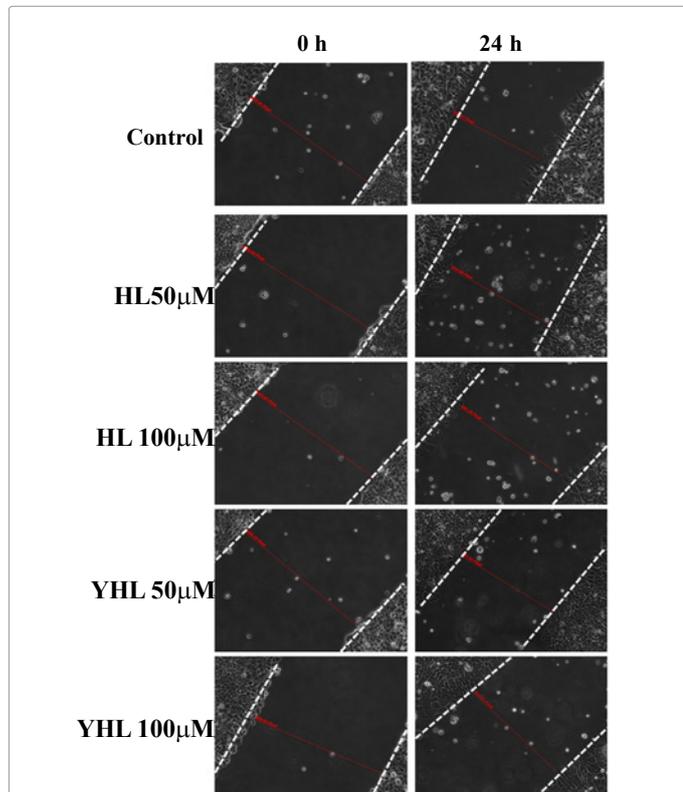


Figure 4: Anti-metastasis of agents in different time points. MHCC97L cells were treated with HL and YHL at different concentrations for 0 and 24 hrs. Distance between the two opposite edges wound was captured and recorded at the relative time points. There is no significant difference between two herbal extracts.

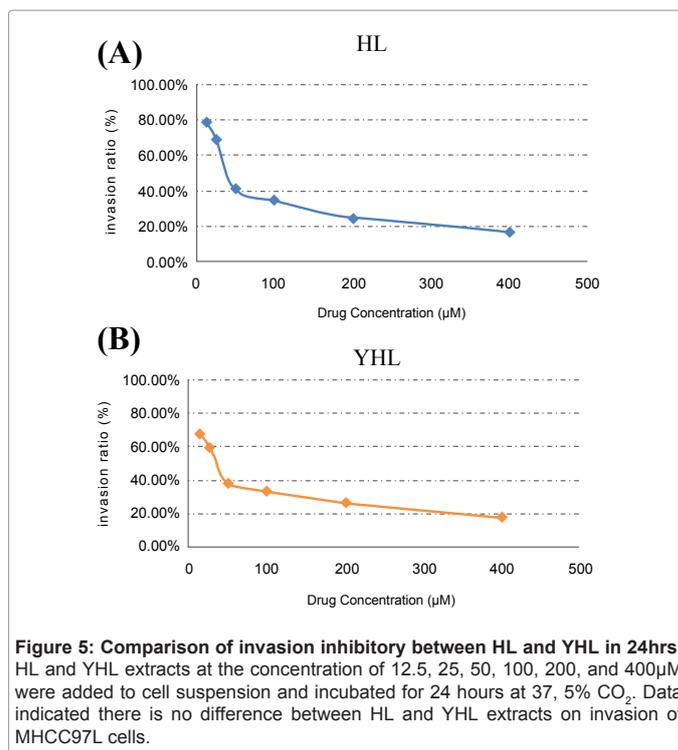


Figure 5: Comparison of invasion inhibitory between HL and YHL in 24hrs. HL and YHL extracts at the concentration of 12.5, 25, 50, 100, 200, and 400μM were added to cell suspension and incubated for 24 hours at 37, 5% CO₂. Data indicated there is no difference between HL and YHL extracts on invasion of MHCC97L cells.

invasion chamber assay indicated HL and YHL showed no difference in their capability to suppress cell invasion.

In conclusion, HL and YHL share great similarities in both chemical composition and inhibitory effect on HCC growth and metastasis. In addition, we also found that other ingredients in HL and YHL presented a synergetic interaction with berberine which increased the late effects on cancer (data not shown). This sheds light on the application of HL and YHL as HCC therapeutic agents and deserves further studies.

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