



Title	Suppression of Vascular Endothelial Growth Factor via Inactivation of Eukaryotic Elongation Factor 2 by Alkaloids in <i>Coptidis rhizoma</i> in Hepatocellular Carcinoma
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Suppression of Vascular Endothelial Growth Factor via Inactivation of Eukaryotic Elongation Factor-2 by Alkaloids in *Coptidis rhizoma* in Hepatocellular Carcinoma

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Keywords:	Hepatocellular carcinoma, <i>Coptidis rhizoma</i> aqueous extract, VEGF, eEF2, nascent protein synthesis

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Abstract

Aim of study: To investigate the inhibitory effect of *Coptidis rhizome* aqueous extract (CRAE) on vascular endothelial growth factor (VEGF) expression and tumour angiogenesis in hepatocellular carcinoma (HCC). **Methods:** Quality control of CRAE was determined. Secretion of VEGF protein and expression of its mRNA in MHCC97L and Hep G2 cells were measured with enzyme-linked immunosorbent assay and quantitative real time-polymerase chain reaction. Synthesis of nascent protein was determined by AHA-protein labelling technologies. The *in vivo* anti-angiogenic effect of CRAE was evaluated *with* xenograft model. **Results:** Absence of organochlorine pesticides in CRAE was found and phytochemical analysis showed that its components were in proportion of magnoflorine 2.2%, jatrorrhizine 1.68%, palmatine 4.4% and berberine 13.8%. CRAE exhibited significant inhibition on VEGF secretion from MHCC97L and HepG2 cells at non-toxic doses. The mRNA transcripts of VEGF could not be inhibited by CRAE, however, synthesis of VEGF nascent protein was potently blocked by CRAE. CARE intervention increased the phosphorylation of eukaryotic elongation factor 2 (eEF2) in HCC cells, which blocked eEF2 activity for proceeding nascent protein synthesis. The activity of eEF2 was restored in CRAE-treated HCC cells in the presence of A484594, leading to the recovery of VEGF expression. Berberine was found to be the major active component in CRAE, however, CRAE is more effective in inhibiting eEF2 activity compared to berberine treatment alone, suggesting the additive effect of other components present. Reduction of tumour size and neovascularization were observed in mice xenograft model. **Conclusion:** Our study postulates the anti-angiogenic effect of CRAE on hepatocellular carcinoma via eEF2 driven pathway.

Keywords

Hepatocellular carcinoma, *Coptidis rhizome* aqueous extract, VEGF, eEF2, nascent protein synthesis

Introduction

Liver cancer is the seventh most common cancer and third most cancer-related mortality for both genders in the world, causing about 700,000 deaths annually¹. Hepatocellular carcinoma (HCC) is the most prevalent type of death leading liver cancer, with only 15% 5-year survival rate of liver cancer patient². The major complications of HCC re ineffectiveness of existing drug against HCC, lack of biomarker to diagnose the disease in early and recurrence stages and other underlying liver diseases such as cirrhosis and liver fibrosis³, which all make the treatment become difficult. Even in HCC patients suitable for surgical reception, invasiveness and aggressive metastatic spread of HCC cells have rendered tumor recurrence after resection⁴. Evidences have showed detachment and spread of tumor cells from one location to another requires formation of new blood vessels⁵⁻⁶, the process was termed as angiogenesis. The loose and irregular structure of new blood vessels allows tumor cells to infiltrate in and migrate to distinct organs. Vascular endothelial growth factor (VEGF) is the most studied angiogenic factor which has a dominant role in responding the proliferation of endothelial cells and vascular permeability⁷.

Coptidis rhizome (Huang Lian in Chinese) has been well-reviewed thousands of years ago in *Shen Nong's Herbal Classic* for its effect in reducing *damp-heat, fire, and toxicity* in the human body. It is also used for treatment of diarrhea, eyes inflammation and abdomen pains of females⁸⁻⁹. Accumulating studies reported pharmacological benefits of *Coptidis rhizome* and its major component berberine on kidney injury improvement, inflammation prevention, cell growth arrest, autophagic cell death, chemoprevention, hepatoprotective and improvement in insulin resistance¹⁰⁻¹⁶. Studies have also demonstrated down-regulation of secretory protein and transcriptional level of VEGF by berberine. This was associated with the activation of ERK and suppression of MMP2 that affected VEGF-induced proliferation of endothelial cells¹⁷⁻¹⁸. Another study showed the inhibitory effect of berberine on endothelial cells tube formation through degradation of HIF-1 α protein, a transcriptional factor expressed during oxygen depletion¹⁹. In recent years, *Coptidis rhizome* has been reported to actively suppress cancer progression by inducing apoptosis in colorectal²⁰, oral²¹ and breast cancer²². Our previous study demonstrated anti-invasive effect of CRAE in HCC cells via deregulation of F-actin and Rho/ROCK signalling pathway²³. Up-regulation of miR23a and miR21 that associated with tumour suppression has also reported on CRAE intervened HCC cells²⁴. The hepatoprotective action of CRAE has been also addressed, which suggest its benefit to the liver organs²⁵⁻²⁶.

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3 However, whether *Coptidis rhizome* could suppress tumour angiogenesis of HCC remains
4 unclear. The underlying mechanism also request further elaboration..
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7 In this study, we targeted on VEGF to examine the effect of *Coptidis rhizome* on tumour
8 angiogenesis of HCC. We observed the toxicity of *Coptidis rhizome* aqueous extract on
9 MHCC97L and Hep G2 cells and reduction on subcutaneous growth of MHCC97L tumour size
10 in animal model. The effect of *Coptidis rhizome* aqueous extract in attenuating secretion of
11 VEGF was associated with inhibition of eEF2 and blockade of nascent protein synthesis. We
12 also proposed the additive effect of other compounds other than berberine in CRAE that
13 contribute to the activity. These findings have unravelled the new molecular mechanism of
14 regulating VEGF expression at post-transcriptional level by *Coptidis rhizome* and berberine on
15 hepatocellular carcinoma.
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24 **Methods**

25 **Chemicals and Reagents**

26 Organochlorine pesticides (OCPs), berberine, palmatine and 3-(4,5-Dimethylthiazol-2-yl)-2,5-
27 diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Jatrorrhizine
28 was purchased from NICPBP (China) and magnoflorine from Tauto Biotech (China). Rabbit
29 monoclonal antibodies against GADPH, eEF2 and peEF2 were purchased from Cell Signalling
30 Technologies (USA); and α -tubulin from AbCam (UK). Rabbit polyclonal antibody was
31 purchased from Santa Cruz (USA).
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40 **Sample preparation**

41 The dried rhizome of *Coptis chinensis Franch* species (Ranunculaceae) was collected from
42 Sichuan, China. The collected *Coptidis rhizome* crude herb was washed, dried and cut into small
43 pieces. 100g of herb was boiled in 1L of distilled water at 100⁰C for 1 hour. The crude extracts
44 were filtered and evaporated *in vacuo* using rotary evaporator. The dried concentrated crude
45 extract was stored in -20⁰C freezer.
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51 **Phytochemical Analysis**

52 To determine the presence of organochlorine pesticide in CRAE, the sample was subjected to
53 gas chromatography analysis. Determination was performed on Agilent 6890 N gas
54 chromatography system equipped with 5973 Network mass selective detectors and HP-5MS
55 30m x 0.25mm x 0.25 μ m column (Agilent Technologies). CRAE was developed in condition of
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3 50°C for 3 min; 50 °C- 100 °C at 20 °C/min; 100 °C- 210 °C at 10 °C/min; 210 °C- 250 °C at 8
4 °C/min; 250 °C for 4 min in helium carrier gas flow rate of 1 mL/min. The profile for
5 organochloride pesticides (OCPs) standards was previously optimized and developed.
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10 CRAE was subjected to high performance liquid chromatography for fingerprinting analysis.
11 The separation was performed using Waters model HPLC instrument, equipped with 626 pumps
12 controlled by 600s interface module and a photodiode array detector 996. 10µl of CRAE
13 (0.5mg/mL) was injected and eluted on ACE 5 aqueous column (250mm x 4.6mm i. d.) with
14 mobile phase of 0.05M potassium dihydrogen phosphate-HPLC graded acetonitrile (75:25). The
15 flow rate was 1mL/min. Berberine, jatrorrhizine, palmatine and magnoflorine (0.05mg/mL) were
16 dissolved in methanol and injected as standard markers.
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21 22 23 **Cell lines and Cell culture**

24 Two human hepatocellular carcinoma cell lines, Hep G2 and MHCC97L were chosen for the
25 following experiment. Hep G2 was purchased from ATCC while MHCC97L was a gift by Dr.
26 Man Kwan in Department of Surgery, The University of Hong Kong. Both cell lines were
27 maintained in DMEM medium with 10% FBS and 1% penicillin/streptomycin. The cells were
28 cultured in tissue culture flasks and maintained in humidified incubator at 37⁰C with 5% CO₂.
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33 34 35 **Cell Viability Assay**

36 Cell viability of Hep G2 and MHCC97L were determined by MTT assay based on the protocols
37 described by Mosmann²⁷. Both cells (7.0 x 10⁴cells/mL) were treated with CRAE in serial
38 concentrations (512, 256, 128, 64, 32, 16, and 8µg/mL) and incubated for 24 and 48 hours. 10µL
39 of 5mg/mL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-
40 Aldrich, USA) solution was added to each well. After 4 hours of incubation, the supernatant was
41 removed and 100µL of DMSO was added to dissolve the remaining formazan crystal. The
42 absorbance was measured at wavelength of 550nm by Multiskan MS microplate reader
43 (Labsystems, Finland).
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50 51 52 **VEGF Enzyme-linked Immunosorbent assay (ELISA)**

53 Secretion of VEGF was measured by Human VEGF ELISA kit (ExCell Biology, Shanghai)
54 according to the manufacturer's protocol. Briefly, the culture supernatant was incubated with
55 biotinylated antibody on anti-VEGF monoclonal antibody pre-coated 96 wells plate for 1 hour.
56 The wells were washed and HRP-conjugated streptavidin was added to all wells for 30 minutes
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3 of incubation. The plate was washed and TMB substrate solution was added to each well for
4 another 30 minutes. Lastly, stop solution was added to each well before absorbance
5 determination at 450 nm using Multiskan MS microplate reader (Labsystems, Finland). The
6 experiment was conducted in triplicate.
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10 11 **Quantitative Real-time PCR**

12 Firstly, the total RNA was extracted using RNA isoplus reagent (Takara, Japan) according to the
13 manufacturer's protocol. Each 0.5µg of RNA sample was then reverse transcribed into cDNA
14 (Reverse Transcription kit, Takara, Japan). The quantitative real-time PCR was performed by
15 SYBR Premix Ex Taq (Takara, Japan) with LightCycler 480 real-time PCR system (Roche,
16 USA). The primer used was VEGF 5'-CCTCCGAAACCATGAACTTT- 3' (forward) and 5'-
17 TTCTTTGGTCTGCATTACATT-3' (reverse). Relative quantification of mRNA levels was
18 normalised using β-actin as the endogenous control.
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26 **Immunoblotting**

27 The cell pellets were lysed with RIPA buffer supplemented with proteinase inhibitor (1% PMSF,
28 0.5% aprotinin and 0.5% leupitine) and phosphatase inhibitor (1mM Na₃VO₄ and 1mM NaF)
29 and protein concentration was determined using BSA as standard. 10µg of protein sample was
30 resolved by 10% sodium dodecyl-sulphate acrylamide gel and transferred to polyvinylidene
31 difluoride membrane (Biorad, USA). The membrane was blocked with 5% BSA and probed with
32 antibodies to α-tubulin, GADPH, eEF2 and pEF2 overnight. The membrane was then incubated
33 with HRP-conjugated rabbit antibody before visualised with ECL Advanced kit (GE Healthcare,
34 UK) by chemiluminescence imaging system (Biorad, USA).
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43 **Nascent protein synthesis**

44 To label CRAE-treated Hep G2 and MHCC97L cells with Click-iT L-azidohomoalanine (AHA),
45 the pre-treated cells were cultured in methionine free medium overnight. 30µM of AHA was
46 added to the cells followed by four hours of incubation. The nascent proteins were extracted with
47 Click-iT Protein reaction buffer kit (Invitrogen, UK).
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53 **Xenograft Model**

54 A xenograft tumour model was established in nude mice by subcutaneously injecting 1×10⁶
55 MHCC97L cells/ml into the right flank of the mice. The tumours were allowed to grow for
56 seven days. CRAE (50 mg/kg/two days, i.p for three weeks) treatment was initiated on one
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3 group of mice (n=5), and another group (n=5) was subjected with the same volume of PBS as
4 control. The mice were sacrificed after three weeks with intraperitoneal injection of overdose
5 pentobarbital (200mg/kg); tumours were then removed and measured. The animal experimental
6 protocol has obtained the approval from the Committee on the Use of Live Animal in Teaching
7 and Research (CULATR) in The University of Hong Kong. After that, the tumours were
8 subjected to immunohistochemistry staining to evaluate multi vessel density and cellular
9 proliferation of tumour cells with rat monoclonal antibody CD31 and Ki67 (AbCam).
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16 **Results**

17 **Quality assessment of CRAE via GC-MS and HPLC**

18 Comprehensive quality assessment was conducted to ensure the consistency and safety of the
19 components in this herbal extract. In order to determine any presence of organochlorine
20 pesticides in CRAE, it was subjected to GC-MS and analysed under optimised chromatographic
21 condition. The CRAE chemical profile was further compared to the previously obtained
22 fingerprint profile of OCP standards and there was no peak detected in CRAE (Supplementary
23 data). CRAE was subjected to HPLC analysis to confirm its quality before treatment. The HPLC
24 fingerprint profile of CRAE was compared to the four external standard profiles based on its
25 retention time and UV spectrum. The yield of four constituents was calculated and as follows:
26 magnoflorine 2.2%, jatrorrhizine 1.68%, palmatine 4.4% and berberine 13.8% (Figure 1).
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36 **Expression of VEGF was blocked by CRAE at non-toxic doses**

37 To evaluate the effect of CRAE on the expression of VEGF in HCC cells, we first of all
38 evaluated the toxicity of the extract to HCC cell lines, Hep G2 and MHCC97L. The cytotoxicity
39 of CRAE-treated MHCC97L and Hep G2 cells were determined after 24 hours and 48 hours of
40 incubation. CRAE exerted 50% of cytotoxicity against MHCC97L at the dose of 500µg/mL and
41 150µg/mL at 24 hours and 48 hours (Figure 2A). As to Hep G2 cells, 50% of cell death was
42 observed after treatment with 250µg/mL and 120µg/mL of CRAE at 24 hours and 48 hours,
43 respectively (Figure 2B). Based on the obtained results, the non-toxic dose of 75µg/mL and
44 150µg/mL of CRAE were employed for further experiments. MHCC97L and HepG2 cells were
45 then subjected to ELISA after exposure to CRAE for 24 hours. Results showed more than 2-fold
46 of secretory VEGF protein reduction in both cell lines compared to control (Figure 2C). These
47 results indicated that extract of *Coptidis rhizome* could inhibit the VEGF expression, which is
48 independent to its cytotoxicity.
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CRAE regulated VEGF expression through suppression of nascent protein synthesis in HCC cells

Previous studies¹⁹⁻²⁰ has reported that berberine, the component in CRAE, could suppress the expression of VEGF in transcriptional regulatory manner. Therefore, we further examined if CRAE could have the same effect on VEGF transcripts. The mRNA level of VEGF in CRAE-treated HCC cells was determined by quantitative real-time PCR. Unexpectedly, no significant reduction of VEGF mRNA transcripts was observed in HCC cells after CRAE treatment. Instead, the amounts of VEGF mRNA increased three-fold in CRAE-treated cells (Figure 2D). The result suggested that CRAE did not affect VEGF in transcriptional level while actively attenuated VEGF protein secretion. To further test the post-transcriptional effect of CRAE on VEGF, we carried out nascent protein synthesis on CRAE-treated HCC. The CRAE-treated cells were incubated with L-azidohomoalanine (AHA) in methionine-free medium for four hours. The AHA-incorporating nascent proteins were labelled with biotin-azide and further subjected to immunoblotting with streptavidin-HRP as secondary antibody. Pre-treatment of CRAE on both cell lines blocked global cellular protein synthesis (Figure 3A). The result indicated the involvement of CRAE in attenuating nascent protein translation in hepatocellular carcinoma.

Inhibition of VEGF nascent protein synthesis by CRAE was associated with eEF2 inactivation

We observed CRAE induced eEF2 phosphorylation in HCC cells, in which eEF2 was responsible for elongation process of cap-dependent translation. Western blot analysis showed phosphorylation of eEF2 in CRAE-treated MHCC97L and Hep G2 cells, suggesting the inactivation of eEF2 by CRAE (Figure 3B). This may indicate that blockade of VEGF nascent protein synthesis was associated with eEF2 inactivation in CRAE-treated HCC cells. To further confirm eEF2 phosphorylation by CRAE, HCC was incubated with or without eEF2k inhibitor, A-484954 (50 μ M) together with CRAE for 24 hours. Incubation of HCC with A-484954 neutralized the expression of eEF2 (Figure 4A) and normalised the secretion of VEGF (Figure 4B), suggesting CRAE attenuated eEF2-driven VEGF protein synthesis. Amounts of VEGF mRNA were increased in the presence of A-484954 and this correlated with the results obtained earlier, indicating CRAE has no inhibitory effect on VEGF mRNA (Figure 4C). Taken together, the data showed the inactivation of eEF2 activity by CRAE, led to disruption in translation process.

Berberine was the major active component of CRAE in inducing eEF2 phosphorylation

To further elucidate the responsible constituent in CRAE that contribute to eEF2 phosphorylation, we incubated HCC cells with individual compounds (i) Jatrorrhizine (2.52µg/mL) (ii) Berberine (20.7µg/mL) (iii) Palmatine (6.6µg/mL) for 24 hours. The compounds concentration was prepared based on its individual proportion present in CRAE. Cell lysates were then subjected for eEF2 phosphorylation. Western blot analysis showed berberine and mixture of four constituents were expressed at the same level in both cells, while berberine was the most active among them. However, higher expression level of phosphorylated-eEF2 was observed in CRAE, postulating that other constituents present in CRAE may contribute to the activity (Figure 5).

CRAE reduced tumour size and neovascularization in xenograft HCC model

To investigate the effect of CRAE on tumour growth *in vivo*, the MHCC97L xenograft mice were administrated with CRAE (50mg/kg/two days, i.p.) for three weeks. After 3 weeks of CRAE administration, the body weight of mice remain unchanged (results not shown), indicating CRAE has minimal toxicity to the animal at this dose. A significant reduction in tumour size was speculated in CRAE-treated mice in comparison to control (Figure 6A). The excised tumours were then measured. The tumour volumes of untreated and CRAE-treated mice were $1306.67 \pm 100.7 \text{ mm}^3$ and $656.67 \pm 485.2 \text{ mm}^3$. Approximately two-fold of size reduction was observed in CRAE-treated mice (Figure 6B). Multi vessel density and cellular proliferation was examined by haematoxylin and eosin staining on anti-CD31 and anti-Ki67 antibody. Histologic analysis against Ki-67 antibody showed a marked decrease in area of proliferating cells in CRAE-treated mice compared to control. Presence of CD31-positive cells was also apparently decreased in CRAE-treated mice (Figure 6C), indicating the reduced rate of new blood vessel formation. Compared to control group, CRAE-treated mice showed lower vascular density in tumour.

Discussion

The *in vitro* study has demonstrated that CRAE offsets VEGF secretion in HCC cells; however, it is not associated with down regulation of VEGF mRNA. Significant increase of VEGF mRNA after treatment may be the stabilization effect of hypoxia induced factor-1 alpha transcriptional factor (HIF-1α) in tumour cells²⁸. In our study, we surprisingly observed that the expression of VEGF could be inhibited by CRAE under normoxic condition, in which HIF-1α should not be

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3 present, indicating the CRAE-suppressed VEGF is mediated through HIF-1 α -independent
4 mechanism. This responsible effect was further justified where we found that CRAE has no
5 inhibition on mRNA transcripts of VEGF, which may rule out the presence of transcriptional
6 control in VEGF regulation by CRAE.
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10 We reported for the first time that CRAE could suppress the synthesis of nascent protein, which
11 was translated on decoded ribosomes; the unfolded and extended chain of nascent protein was
12 transported through translocon into endoplasmic reticulum for further folding. VEGF is a
13 glycoprotein that synthesized inside ER and ER stress could trigger VEGF up-regulation
14 transcriptionally and post-translationally²⁹. Our observation showed reduction of nascent protein
15 synthesis after treatment with CRAE in dose-dependent manner. Consistent with these findings,
16 CRAE triggered eEF2 phosphorylation was followed by blockade of nascent protein synthesis,
17 leading to the inhibitory effects of VEGF protein secretion. Eukaryotic elongation factor-2
18 (eEF2) is responsible on mediating ribosomal transfer in elongation process of mRNA
19 translation. eEF2 is inactivated by being phosphorylated at its S56 site, which leads to disruption
20 in protein synthesis³⁰. Overexpression and hyperactivity of eEF2 in cancer is associated with
21 cancer cell progression and early tumour recurrence³¹⁻³², and high level of protein synthesis is
22 vital to maintain cancer cell metabolism³³. Treatment with doxorubicin has reported to
23 phosphorylate eEF2 through interfering anti-apoptotic protein and promote cancer cell death³⁴.
24 Previous report has postulated a novel energy-conserved mechanism where translational arrest
25 caused by eEF2 phosphorylation in HIF-independent oxygen depletion condition³⁵.
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29 Interestingly, in our study, we discovered eEF2 phosphorylation by CRAE in dose dependent
30 manner and it has been further justified through inhibition by A-484954. This suggested that
31 CRAE may cause HCC to enter an energy saving mechanism that rendered post-transcriptional
32 arrest and halted the production of other growth and angiogenic factors, such as VEGF. Protein
33 synthesis is an ATP-exhaustive process and termination of mRNA translation turns out to be a
34 tumour cell survival strategy³⁶. Besides, elevation of VEGF protein in MHCC97L after A-
35 484954 addition was observed, suggesting post-transcriptional inhibitor may initiate cell
36 compensation pathway to produce more VEGF protein. Our assumption was further supported
37 by previous studies, which also reported that VEGF inhibition may trigger cell survival
38 adaptations to increase VEGF protein expression and receptor activity^{37, 38}. The *in vivo*
39 subcutaneous animal model showed a reduction of CD31 stained blood vessels and shrinkage of
40 tumour size after treatment with CRAE. Data obtained has signified inhibition of tumour
41 angiogenesis was involved in the anti-cancer effect of CRAE in HCC. The regulatory scheme
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3 underlying the inhibitory effect of CRAE on VEGF expression in HCC cells was showed in
4 Figure 7.
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7 Our previous study has addressed the greater potential of *Coptidis rhizome* compared to
8 treatment by berberine alone and the effect was contributed by other protoberberine-type
9 alkaloids present in *Coptidis rhizome*²³. Consistent with previous study, we observed higher
10 level of eEF2 phosphorylation in CRAE, suggesting other compounds may be responsible on the
11 activity but berberine was the most effective one. The current data indicated the additive effect
12 of other constituents present in *Coptidis rhizome*.
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17 18 19 **Conclusion**

20 In conclusion, we have elucidated the inhibition of VEGF protein synthesis by CRAE via eEF2
21 driven pathway. CRAE blocked nascent protein synthesis and thereby inhibited VEGF protein
22 formation. CRAE exhibits more potent inhibition on eEF2 activity than berberine alone;
23 suggesting the presence of other compounds in *Coptidis rhizome* may contributes additive effect
24 of eEF2 inactivation. This study postulated CRAE as a potential anti-angiogenic substance for
25 hepatocellular carcinoma.
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31 32 **Conflict of Interest**

33 The authors declare no conflict of interest for this study.
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37 38 **Acknowledgement**

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Figure legends:

Figure 1. Chemical Analysis of CRAE with other standard references by HPLC. (A) *Coptidis rhizome* aqueous extract. (B) Magnoflorine reference standard. (C) Jatrorrhizine reference standard. (D) Palmatine reference standard. (E) Berberine reference standard.

Figure 2. CRAE induced cytotoxicity and blocked VEGF expression. (A) MHCC97L cells. (B) Hep G2 cells were treated with CRAE at dose and time dependent manner. The cells were seeded to 96-wells plate and cell viability was determined by MTT assay. Cell viability were measured as average % of replicates (n=3) with standard deviation. (C) Amount of secretory VEGF of MHCC97L and Hep G2 cells. Approximately 2-fold of reduction in VEGF concentration was observed. The VEGF concentration was measured in average of ng/nL \pm SD. (D) Fold change of VEGF mRNA level in HCC cells. Threefold increase of VEGF mRNA was observed in CRAE-treated cells versus control. The VEGF mRNA was measured in fold change \pm SD.

Figure 3. CRAE induced blockade of nascent protein synthesis and phosphorylation of eEF2 in HCC cells. (A) Nascent protein synthesis in MHCC97L and Hep G2 cells was inhibited by CRAE. (B) MHCC97L and Hep G2 cells were treated with CRAE (0, 75, 150 μ g/mL). The lysates were immuno-blotted against anti-peEF2, anti-eEF2, and α -tubulin. Up-regulated expression of peEF2 was observed after treatment with CRAE.

Figure 4. A-484954 normalised the phosphorylation of eEF2 in HCC cells. (A) MHCC97L and Hep G2 cells were treated with CRAE (0, 75, 150 μ g/mL) in presence or absence of A-484954 (B) The secretion level of VEGF were normalised after treatment with A-484954 in HCC cells. The VEGF concentration was measured in average of ng/nL \pm SD. (B) Level of VEGF mRNA increased in A-484954 pre-treated HCC cells. The VEGF mRNA was measured in fold change \pm SD.

Figure 5. Berberine is the major active compound in eEF2 phosphorylation. HCC cells were treated with Jatrorrhizine (Jat), Berberine (Ber), Palmatine (Pal), CRAE and mixture of compounds (mix) and analysed for peEF2 and eEF2.

Figure 6. CRAE reduced tumour size and neovascularization in HCC xenograft model. (A) CRAE treated mice has decreased tumour volume compared with the untreated mice. Two groups of mice were treated with or without CRAE for three weeks (n=5). (B) The excised tumours were measured and established in average of mm³ \pm SD. Significant reduction of tumor volume was observed in CRAE treated mice. (C) Histological sections of HCC tumors in two groups of mice. Decreased expression of Ki67 (magnification of 200x) and CD31 (low magnification, bar = 20 μ m) was observed. Lesser blood micro vessels were detected in CRAE-treated section. Blood vessels of control section were indicated in arrows. The expression was measured in average of % \pm SD.

Figure 7. The schematic mechanism of eEF2 driven VEGF protein suppression. CRAE inactivates eEF2 through phosphorylation and elongation phase of mRNA translation is inhibited. The blockade of nascent protein synthesis leads to VEGF protein suppression.

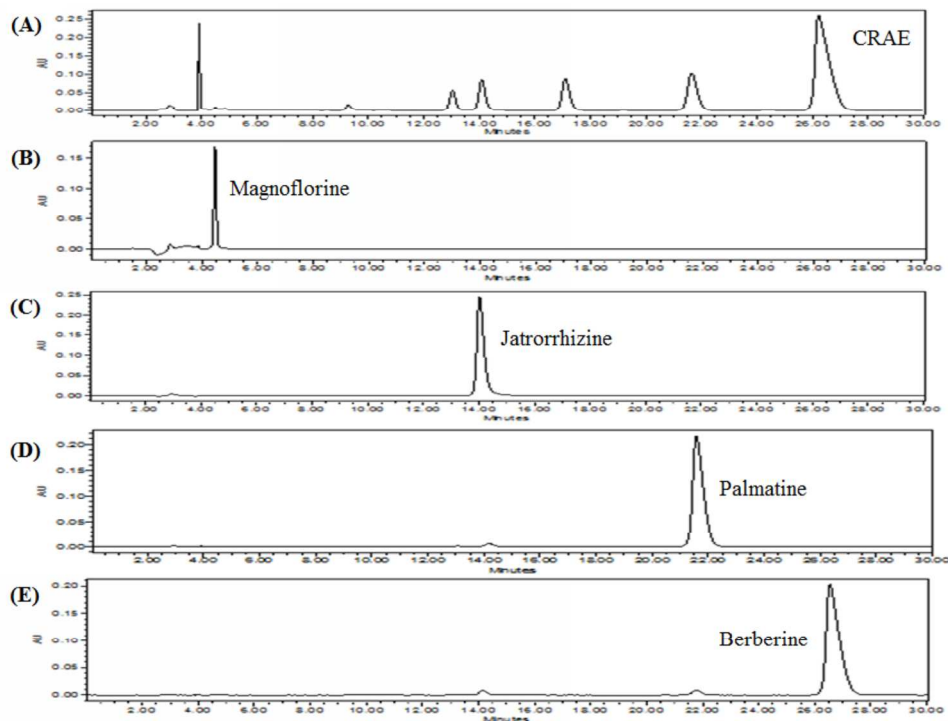


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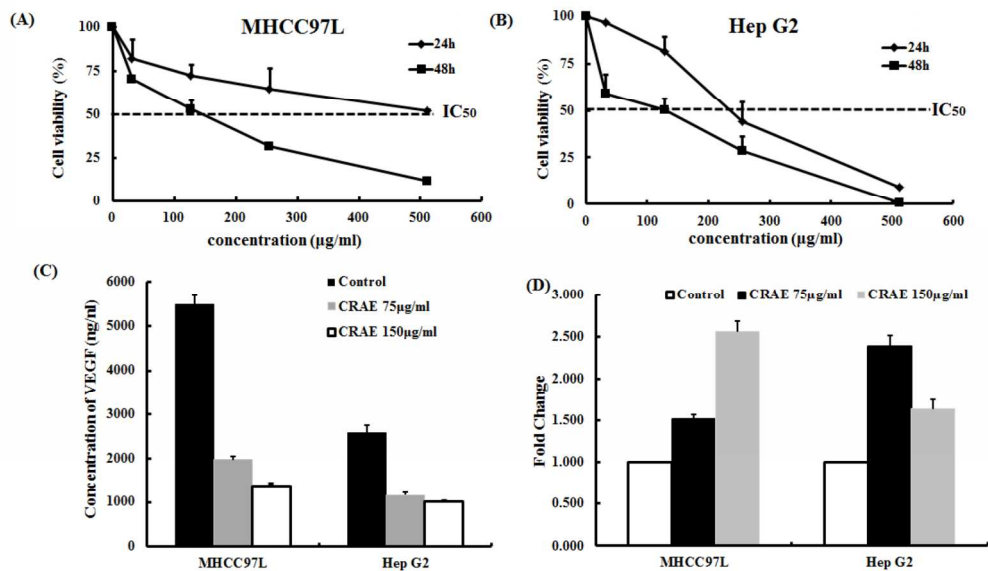
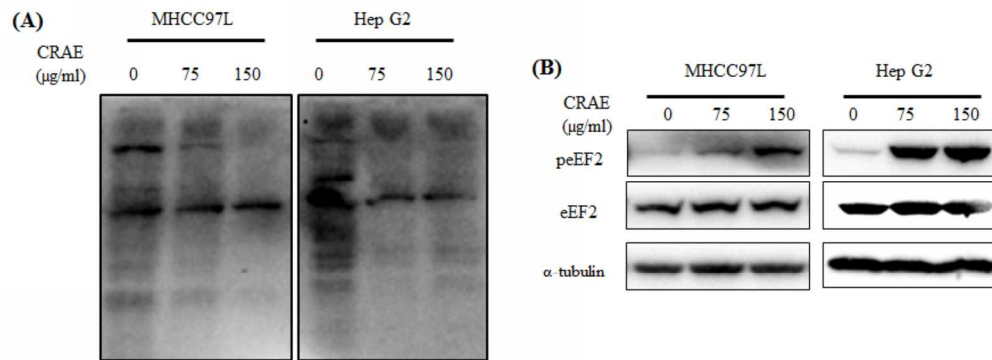


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160x93mm (300 x 300 DPI)



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24 160x58mm (300 x 300 DPI)

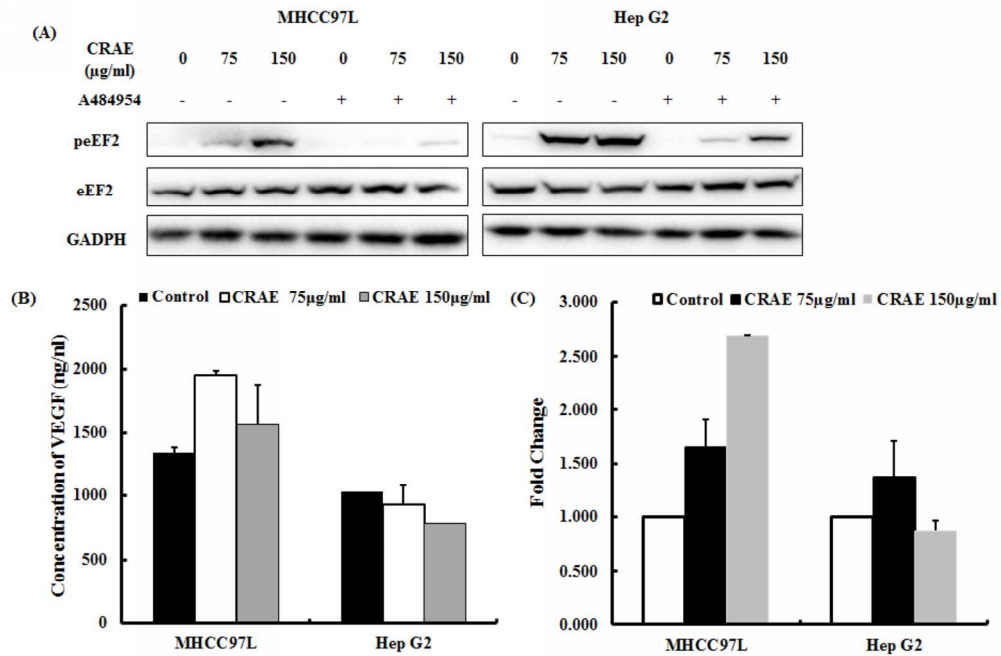


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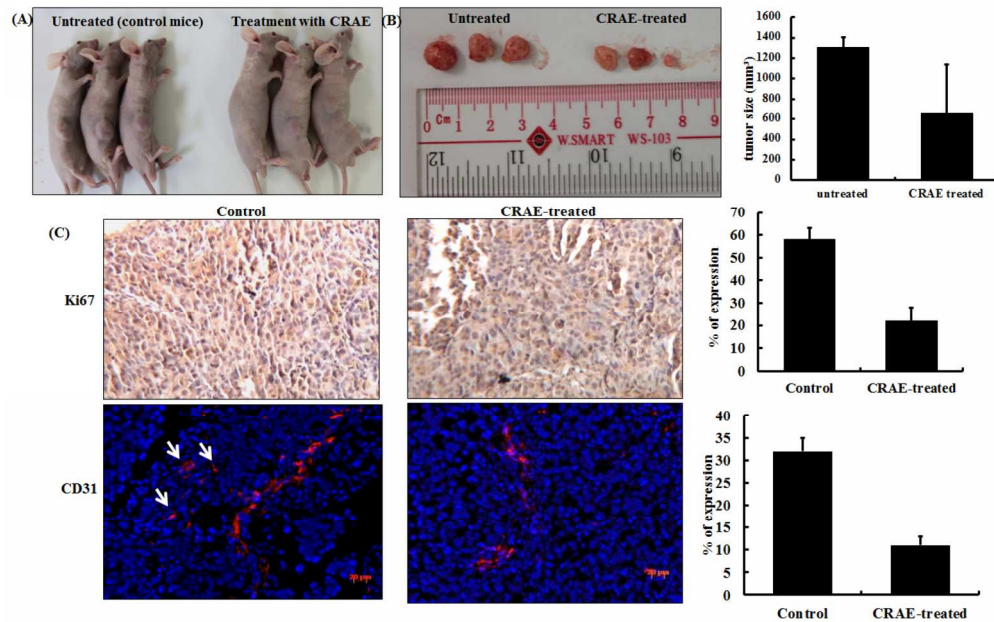


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