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Contributed Paper

Effect of The Thai Traditional Plant Formula, Hing-Hai on Colon Cancer Cell Growth and Metastasis

Rujirek Chaiwongsa and Preeyanat Vongchan*
Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand.
* Author for correspondence; e-mail: preeyanat.v@cmu.ac.th

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ABSTRACT

A Thai traditional formula named Hing-Hai is a water extracted mixtures of 7 particular medicinal plants. It is well-known and prescribed to treat 3 major cancers including colon, liver and lung. However, there was no scientific support for its effect and its detailed mechanism remained elusive. This work aimed to evaluate the anti-cancer effect of Hing-Hai and explore its mechanism of action. Hing-Hai was cytotoxic to Caco2 and SW620 in dose-dependent with IC_{50} of 234 and 438 µg/ml, respectively by MTT assay. Assay on apoptotic induction and cell cycle analysis using flow cytometry revealed that Hing-Hai did not induce apoptosis or arrest the cell cycle. Studies of cell adhesion in the presence of 3 major adhesion molecules including collagen I, fibronectin, and vitronectin, demonstrated that Hing-Hai inhibited adhesion of both cancer cells in dose-dependent manner. Scratch wound-healing motility assay and gelatin zymography also confirmed that Hing-Hai could delay cell migration and decrease gelatinolytic activity. Interestingly, at the same concentration, Hing-Hai effectively inhibited adhesion, migration, and decreased the gelatinolytic activity of SW620 more than that of Caco2 cell. These findings support anti-colon cancer activity of Thai traditional plant formula, Hing-Hai. The studies clearly confirmed anti-cancer activity based on the inhibition of cell adhesion and migration and decreasing the elatinolytic activity.

Keywords: traditional medicinal plant, anti-metastasis, anti-cancer activity, Caco2, SW620

1. INTRODUCTION

Cancer is a non-communicable disease responsible for 63% of deaths worldwide (1). WHO also estimated that almost 70% of cancer deaths occurred in low and middle-income countries (2a). In Thailand, the first four ranks which causes of death are cancer, followed by accidents and poisonings, diseases of heart and hypertension, and cerebrovascular diseases (2a). Total number of deaths of cancer is increasing every year. Among those, colon cancer is the third and fifth rank in male and female, respectively (2b) and metastasis is the most important cause of death for patients. Metastasis, a multi-step process including cellular adhesion, invasion, extravasation and proliferation at the distant site (3), requires the interaction between cancer cells and various host cells, extracellular matrix,

and basement membrane components. Therefore, preventing any of these steps would prevent metastasis and is considered to be an important therapeutic target of cancer treatment (4a-4c).

Thus, research and development in anti-cancer products has become a worldwide scientific effort with a major aim of target cell specific killing less toxic to normal cells. Moreover, mechanism of action should be revealed. Among those chemical and synthetic drugs used to treat and prevent cancer, natural compounds with anti-cancer benefits from medicinal plants have been widely studied in many countries all over the world (5a-5b). Use of medicinal plants is related to cultural and economic reasons. Besides those single and purified chemical compounds extracted from a particular plant, traditional medicine formula consisting of many plants has been used to treat a variety of cancers (6a-6d). Thai traditional plant formula named Hing-Hai consists of 7 plants including *Carissa cochinchinensis* Pierre (leaves), *Derris scandens* Benth (leaves), *Artocarpus heterophyllus* Lam. (core), *Cassia siamea* Lamk. (core), *Bridelia ovata* Decne (leaves), *Artocarpus lakoocha* Roxb. (core), and *Crotalaria quinquefolia* L. (leaves). Hing-Hai has been used for a number of years as a folk medicine for treatment of three major cancers including colon, liver and lung. It is formulated based on a specific ratio of seven plants through the process of boiling in water and concentrating under pressure. Hing-Hai was also used to modulate immune function and quality of life as well as to extend survival time of patients affected with cancers. Although it is recommended by oral tradition for patients with specific cancers, there is no scientific support for its clinical benefit. (respond to reviewer 1#1 and reviewer 2#2) Moreover, the mechanisms underlying its anti-cancer effect is poorly understood. In the present study, we studied the anti-cancer effect of Hing-Hai on Caco-2 and SW620, colon cancer cells derived from humans. In this study, we confirmed the anti-cancer activity of Hing-Hai. In addition, we demonstrated that the anti-cancer activity is based on the inhibition of cancer cell adhesion, migration, and metastasis.

2. MATERIALS AND METHODS

2.1 Materials and Cell Lines

Hing-Hai was prepared under boiling of all 7 plants with water. Water extract form is brown and clear in a ready-to-drink form. Before *in vitro* study, the product was frozen dried and kept frozen until assayed. The dried material was prepared in a stock concentration (5 mg/ml), and before any assay, the material was filtered through a 0.22 µM membrane (Sartorius, Goettingen, Germany) (1).

Two cell lines derived from human colon colorectal adenocarcinoma, Caco2 (colon) and SW620 (metastatic site, lymph node) were purchased from CLS Cell Lines Service (Germany). Cells were grown in complete DMEM, high glucose [DMEM, high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, UK)] at 37 °C, 5% CO_2, and 95% humidity. Before any assay, the culture medium was removed and cells were washed with Ca^{2+}/Mg^{2+} free phosphate buffered saline pH 7.2 (PBS, Sigma Aldrich, USA). Cells was then detached with 0.25% trypsin/0.02%EDTA/ Ca^{2+}/Mg^{2+} free PBS, washed twice and adjusted to a concentration as needed.

2.2 Cytotoxicity of Hing-Hai on Colon Cancer Cell Lines

Before assay, growth rate of Caco2 and SW620 cells was studied and their growth curves were analyzed. Caco2 and SW620 cells (1×10^4cells/ml) were cultured in 25-ml tissue culture flask with complete DMEM, high glucose. Cells were collected, counted,

and viable cells were determined every single day by trypan blue dye exclusion assay. Growth curve was plotted between cell concentration (cells/ml) and time of incubation (day).

Cytotoxicity assay was performed to determine the effect of Hing-Hai on Caco2 and SW620 cell viability. Cells were harvested with 0.25% trypsin/0.02%EDTA/ Ca^{2+}/ Mg^{2+} free PBS and washed with Ca^{2+}/Mg^{2+} free PBS before adjusting to 5×10^4 cells/ml with complete DMEM, high glucose. Cells were plated (100 mL/well) in 96-well tissue culture plate (Corning, USA) overnight at 37°C, 5% CO_2, and 95% humidity. Various concentrations of Hing-Hai in DMEM, high glucose (0-1000 µg/ml) were added 100 mL/well and cells were incubated for 4 days at 37 °C, 5% CO_2, and 95% humidity. After completion of cultivation, culture supernatant was discarded and methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Promega, USA) was added (20 mL/well). The reaction was incubated for 4 h (ordinarily, we use hours instead of h, but if these medical papers use h then keep it that way) at 37 °C, 5% CO_2, and 95% humidity. Sterile SDS (10% v/v in PBS) was added at 25 mL/well and plate was kept at room temperature for 18 h before measuring of the optical density at 540/630 nm. DMEM, high glucose was used as a blank control. The percentage of viable cells was calculated as [(O.D. of cell control-O.D. of treated cells)/(O.D. of cell control-O.D. of initiated cells)] ×100. IC_{50} was defined as the concentration of Hing-Hai resulting in 50% cell death. Dose response curve was plotted between concentration of Hing-Hai and viable cell count on X-axis and Y-axis, respectively and IC_{50} was determined. Three independent experiments were studied and each assay was performed in triplicate.

2.3 Apoptotic Induction of Caco2 and SW620 Cells in the Presence of Hing-Hai

Flow cytometric analysis of apoptosis was performed to explore whether Hing-Hai induced apoptosis of Caco2 and SW620 cells. Briefly, cells (5×10^4 cells/ml) in complete DMEM, high glucose were plated and incubated for 24 h. Cells were then washed with serum-free DMEM, high glucose before adding of Hing-Hai ($IC_{20)}$ and incubation was extended for another 3 days. After completion, cells were harvested with 0.25% trypsin in Ca^{2+}/Mg^{2+} free, 2% BSA-PBS and washed twice. Cells were adjusted to 5×10^5 cells/ml with binding buffer (ANNEXIN V:FITC Assay kit, Bio-Rad, Inc, USA). Five mL of Annexin V-FITC was added to 195 mL of cell suspension and mixed gently. The reaction was kept for 10 minutes at room temperature with light protection. Cells were then washed twice again and suspended to 190 mL with binding buffer before adding of propidium iodide solution (10 mL). Finally, apoptotic induction was analyzed by flow cytometry (Coulter, Danvers, MA, USA). Two independent experiments were studied and each assay was performed in triplicate.

2.4 Cell Cycle Analysis of Caco2 and SW620 in the Presence of Hing-Hai

To address whether Hing-Hai interferes with the cell cycle which results in arrested cell division 1, cell cycle was studied. Caco2 or SW620 cells (5×10^4 cells/ml) in complete DMEM, high glucose were plated and incubated overnight. Cells were then washed with serum-free DMEM, high glucose before adding of Hing-Hai ($IC_{20)}$ and incubation was extended for another 3 days. After completion, cells were harvested with 0.25% trypsin in Ca^{2+}/Mg^{2+} free, 2% BSA-PBS and washed twice. Cells were fixed with 1 ml of

-20 °C pre-cooled ethanol for 1 h and discarded. Five mL of 10% Triton X-100 was added following with 50 mL of RNase (2 mg/ml). The reaction was incubated for 5 minutes before adding 10 mL of propidium iodide (1 mg/ml) and kept at room temperature for another 30 minutes in the dark. Finally, PBS (440 mL) was added to stop reaction and cell cycle was analyzed by flow cytometry. Two independent experiments were studied and each assay was performed in triplicate.

2.5 Screening of the Major Adhesion Protein used in Caco2 and SW620

To identify the candidate molecules involved in the adhesion of Caco2 and SW620, various adhesion proteins were screened using CultreCoat® Adhesion Protein Array Kit (Trevigen, Inc, MD, USA) according to the instructions. Briefly, cells were grown in 10%FCS-RPMI-1640 until 80% confluent. Before assay, cells were harvested and washed twice with sterile PBS pH 7.2 and labeled with 2 uM Calcein AM for 1 h at 37 °C, 5% CO_2. Labeled cells were washed and seeded 100 µL of cells per well (1.5×10^5 cells/ml) with adhesion buffer before adding into microtiter plate precoating with various types adhesion protein. The reaction was performed for 1.15 h at 37 °C, 5% CO_2 incubator. At the end of incubation, total RFU was determined by fluorescent ELISA plate reader. Plate was then washed 3 times with washing buffer and determined again for remaining adherent cells (RFU after wash). Percentage of adhesion was calculated and presented compared to uncoated well.

2.6 Cell Adhesion Assay

Caco2 or SW620 cells were separately deprived 8 h in serum free DMEM high glucose before detaching with 0.5% trypsin/PBS. Cells were washed twice with the same medium and suspended to 2×10^5 cells/ml in 0.1% BSA DMEM, high glucose. Cell suspension (100 mL/well) was then separately plated onto 96-well culture plate pre-coated with 30 mL/well of collagen I (40 mg/ml in PBS pH 7.2), or fibronectin (30 mg/ml in PBS pH 7.2), or BSA (0.1% BSA in PBS). Various concentrations (0-1000 mg/ml) of Hing-Hai were then added 100 mL/well. Plate was incubated at 37 °C, 5% CO_2 and 95% humidity for 45 minutes before gently washing off the non-adherent cells with 100 mL/well of serum-free DMEM, high glucose for 4 times. Complete DMEM, high glucose was then replaced 200 mL/well and plate was cultured for 4 h at 37 °C. After completion of incubation, MTT assay was performed to determine the remaining adherent cells as previously described.

2.7 Scratch Wound-healing Motility Assay

Caco2 or SW620 were separately cultured 48 h until a monolayer formed. A wound was created by a sterile 200-mL pipette tip and floating cells were removed by washing with serum-free DMEM, high glucose. Complete DMEM, high glucose in the presence (50-1000 mg/ml) or absence of Hing-Hai was added. Treated Caco2 and SW620 cells were cultured for 24 and 72 h, respectively, at 37 °C, 5%CO_2 and 95% humidity. Wound healing was observed every 8 h under microscope (×10).

2.8 Gelatin Zymography

To explore whether Hing-Hai interfered with matrix metalloproteinase (MMP) activity, gelatinolytic activity of proteins from tissue culture media was assayed by electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a vertical gel apparatus according to the method previously described (7) with modification, so that gelatin was also included

in the resolving gel. Each sample was loaded to a well and the samples were electrophoresed for 200 min at 90 V. After electrophoresis, gel was washed twice with 2.5% Triton X-100 at room temperature (15 minutes each) to remove SDS following with incubating in activating buffer for 16 h at 37 °C. The gel was subsequently stained with 0.2% Coomassie Brilliant Blue R-250 for 5 h at room temperature, and destained with 50% methanol and 10% acetic acid to reveal zone of lysis within the gelatin matrix. Gelatinolytic activity was detected as clear band against a stained band. Inhibition of gelatinolytic activity (%) was calculated and compared to control.

2.9 Toxicity of Hing-Hai on Peripheral Blood Mononuclear Cells

Cytotoxic effect of Hing-Hai on normal peripheral blood mononuclear cells was investigated in this study. Whole blood was collected from healthy volunteers (N=3) using heparin (Leo Pharmaceutical Products Ballerup, Denmark) as anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation technique and washed with sterile RPMI-1640 (Gibco, USA). PBMCs (5×10^5 cells/ml) were cultured in 10% FBS DMEM, high glucose both with the absence and presence of various concentrations of Hing-Hai (0-1,000 mg/ml) for 3 days at 37 °C, 5% CO_2, and 95% humidity. After completion of cultivation, MTT assay was performed as mentioned previously. The cell concentration (cells/ml) was calculated and plotted between cell viability (%) and final concentration of Hing-Hai (mg/ml). Each assay was performed in triplicate.

2.10 Statistical Analysis

All data reported as the mean ±S.D. of three independent experiments. Analysis of differences was carried out by one-way ANOVA with a *p*-value of less than 0.05.

3. RESULTS

3.1 Hing-Hai was Cytotoxic to Colon Cancer Cells

Prior to exploring the effect of Hing-Hai on colon cancer cell viability, the growth rate and growth curve of Caco2 and SW620 were studied and were shown to be at day 5 and day 4.5, respectively (Figure 1). Anti-cancer activity of Hing-Hai on both cells was then studied in 4 day-incubation time after allowing cells to adhere for 24 hr. Figure 2 showed that Hing-Hai decreased viability of both cells tested in dose dependent manner with IC_{50} of 234 and 438 µg/ml for Caco2 and SW620, respectively.

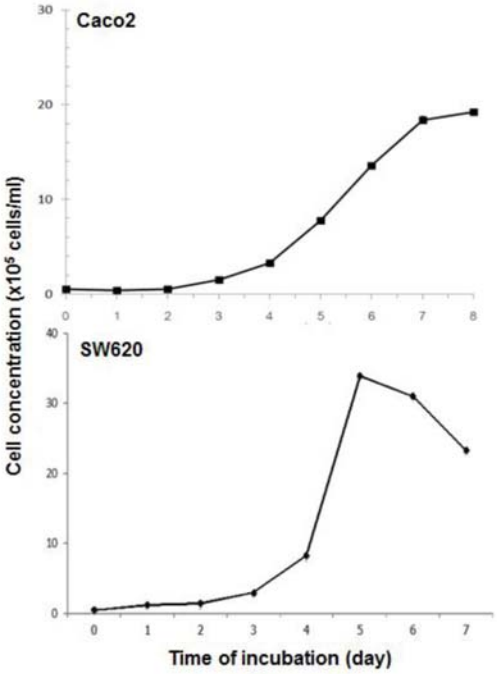

Figure 1. Growth curve analysis of Caco2 and SW620. Caco2 and SW620 (1×10^4 cells/ml) was cultured in complete DMEM, high glucose for 8 days. Aliquot of cells was collected and count every single day by trypan blue dye exclusion assay. Number of cells was plotted against day of incubation.

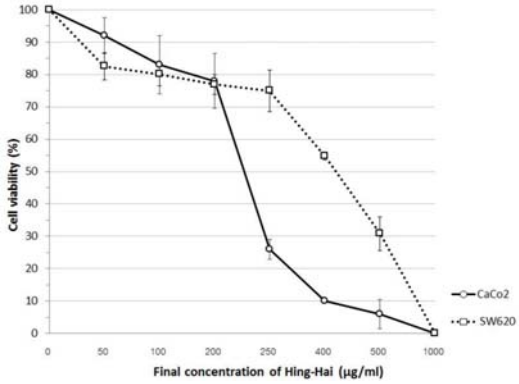

Figure 2. Anti-cancer activity of Hing-hai on Caco2 and SW620. Caco2 and SW620 (5×10^4 cells/ml) was cultured in complete DMEM, high for 4 days in a presence or absence of Hing-Hai. Viability of cell was determined by MTT. Cell viability (%) was plotted (Y-axis) against various final concentrations of Hing-Hai (µg/ml, x-axis). The results shown are representative of 3 independent experiments.

3.2 Hing-Hai did not Induce Apoptosis or Interfere with Cell Cycle of Caco2 and SW620

Programmed cell death or apoptosis is recognized by a pattern of morphological, biochemical, and molecular changes occurring in the affected cell (8). In order to evaluate the cause of growth inhibition of Caco2 and SW620 by Hing-Hai, apoptotic induction was studied and analyzed by flow cytometry. The result showed that there was no biochemical change in Hing-Hai treated cells compared to untreated group (Figure 3A-B). Moreover, analysis of cell cycle also clearly demonstrated that there was no change of DNA content in every phase of cell cycle compared between Hing-Hai treated cells and control group (Figure 3C-D).

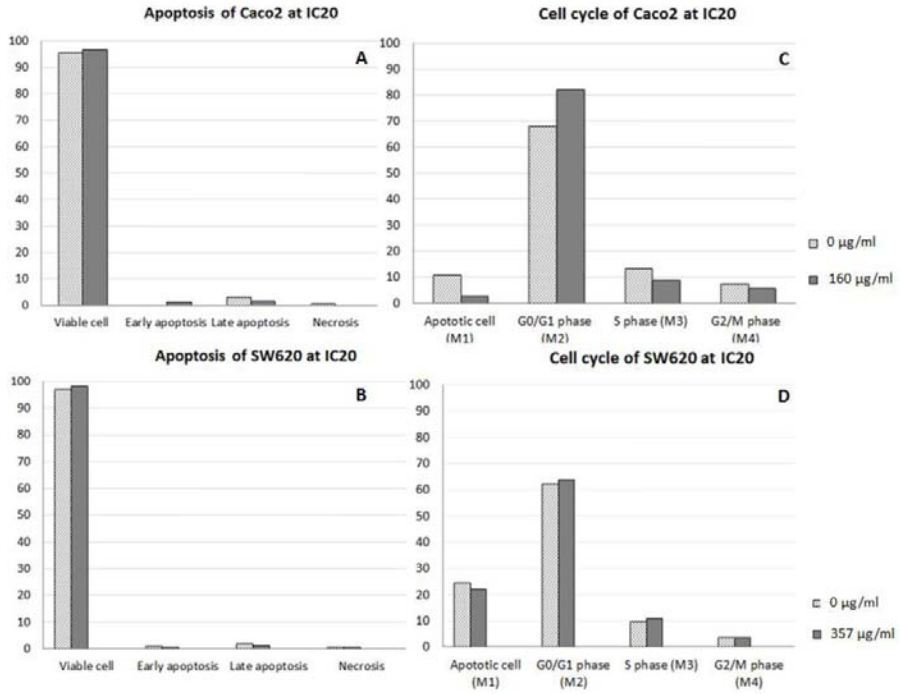

Figure 3. Hing-Hai did not induce apoptosis or arrest cell cycle of Caco2 and SW620. Caco2 cells or SW620 (5×10^4 cells/ml) was individually cultured in the presence of Hing-Hai (IC_{20}) for 3 days. Apoptosis and cell cycle were analyzed by flow cytometry. The results shown are mean of 2 independent experiments.

3.3 Adhesion of Caco2 and SW620 are Suppressed by Hing-Hai

To clarify that Hing-Hai may inhibit cell adhesion process, cell adhesion was assayed. First, expression of adhesion protein on Caco2 and SW620 was screened before an assay of adhesion inhibition. As shown in Figure 4, collagen I, fibronectin and vitronectin were 3 candidate adhesion proteins expressed on Caco2 and SW620 cells. Moreover, adhesion of both cells was increased in the presence of those 3 proteins compared to PBS control. Results showed that in the presence of Hing-Hai, adhesion of both cells was dose-dependently decreased. Interestingly, adhesion of Caco2 cells gradually decreased in the increasing concentration of Hing-Hai but less effective compared to that of SW620. In addition, it was found that adhesion inhibition of SW620 cells was dramatically observed at Hing-hai of 250 µg/ml and gradually increased upon higher concentration of Hing-Hai (Figure 5).

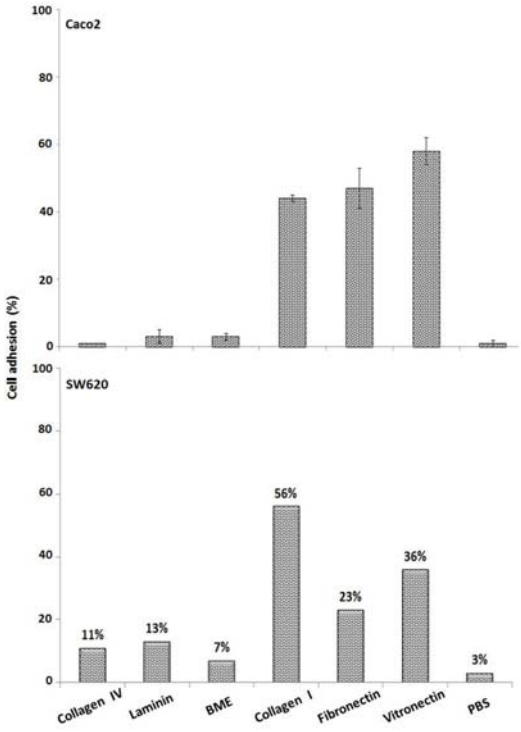

Figure 4. Screening of major adhesion molecules. Caco2 and SW620, were grown in 10% FCS-RPMI-1640 until 80% confluent. Cells were harvested and washed twice with sterile PBS pH 7.2 following with 2 µM Calcein AM labelling. Labeled cells were washed and seeded 100 µl of cells per well (1.5×10^5 cells/ml) with adhesion buffer before into microtiter plate precoating with various type of adhesion protein. The reaction was performed for another 1.15 h. Total RFU was determined by fluorescent ELISA plate reader. Plate was then washed 3 times with washing buffer and determined again for remaining adherent cells (RFU after wash). Percentage of adhesion was calculated and presented compared to uncoated well.

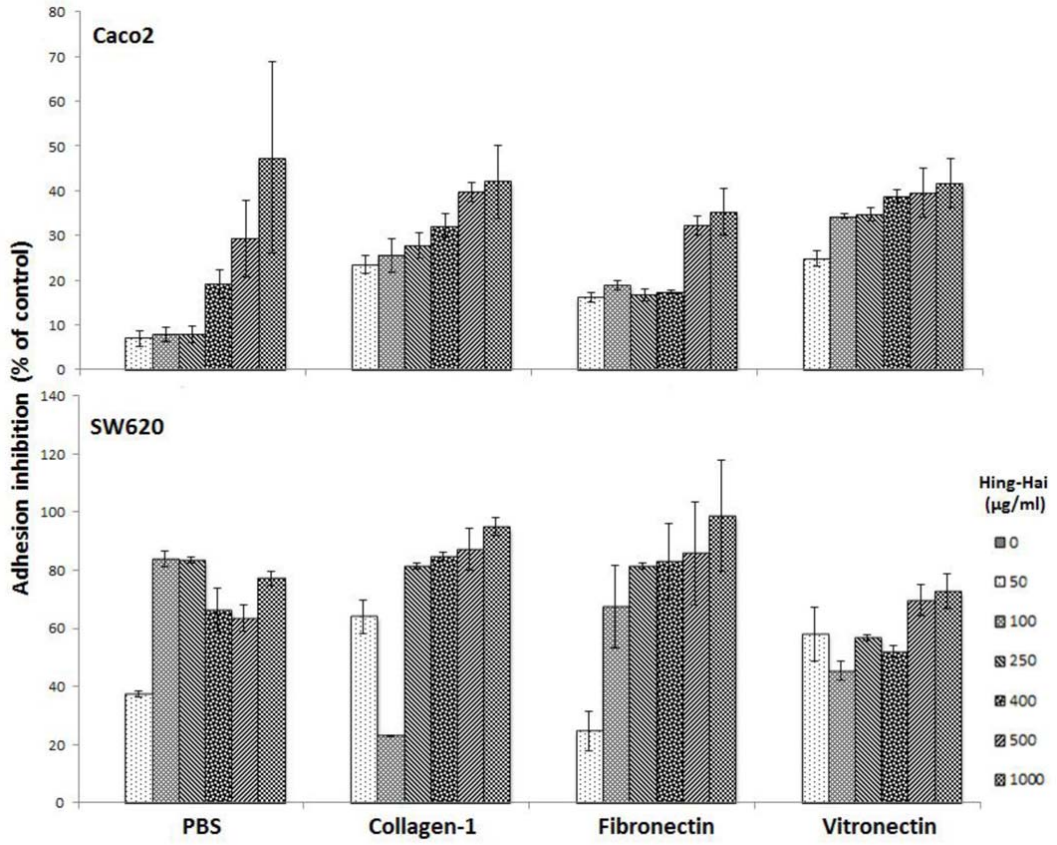

Figure 5. Hing-Hai inhibited adhesion of Caco2 and SW620 cells. Caco2 or SW620 cells were cultured in serum free DMEM, high glucose before detaching. Washed cells were suspended (2×10^5 cells/ml) in 0.1% BSA DMEM, high glucose and plated (100 mL/well) onto 96-well culture plate pre-coated with collagen I, fibronectin, vitronectin, or BSA (0.1% BSA in PBS). Various concentrations (0-1000 mg/ml) of Hing-Hai were then added 100 mL/well. Plate was for 45 minutes before gently washing off the non-adherent cells. Complete DMEM, high glucose was then replaced and plate was cultured for another 4 h. Remaining adherent cells were measured by MTT assay.

3.4 Hing-Hai Delayed Migration of Caco2 and SW620

To explore other mechanism in anti-cancer activity of Hing-Hai, scratch-wound healing assay was performed. It was found that Caco2 cells spent 24 h to complete the healing process while SW620 required more than 3 days to do so. As shown in Figure 6, in the presence of Hing-Hai, healing process in Caco2 cells was dose dependent and significantly observed at ≥200 µg/ml. The effect in migration inhibition of Hing-Hai was clearly addressed in SW620 cells at less concentration (50 µg/ml) than that of Caco2 cells.

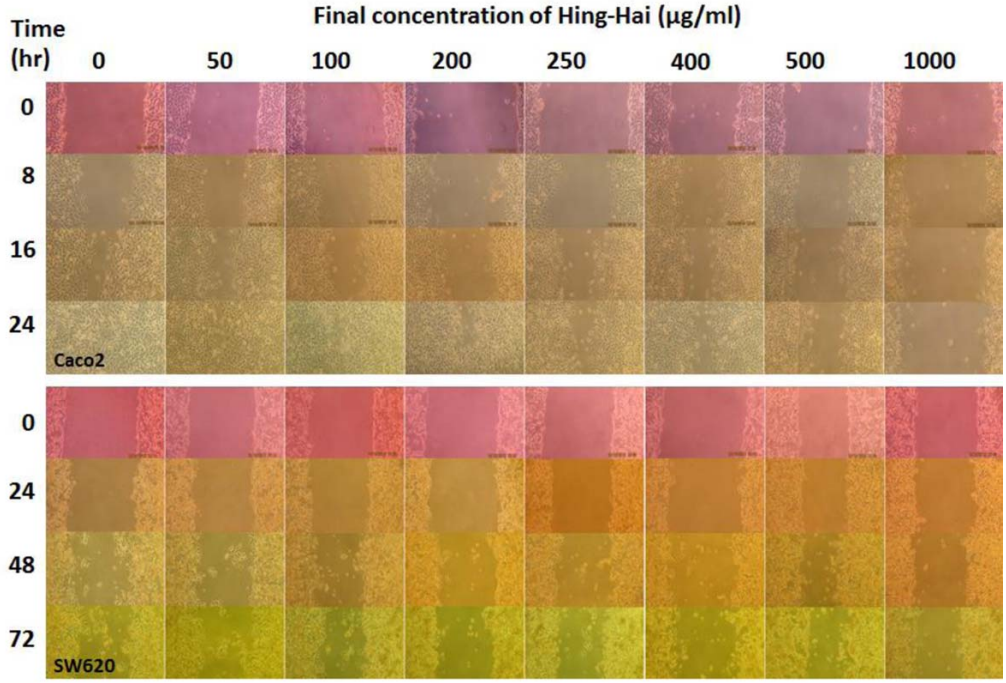

Figure 6. Hing-Hai inhibited migration of Caco2 and SW620 cells. Caco2 or SW620 were separately cultured 48 h until a monolayer formed. Wound was created by a sterile 200-mL pipette tip. Cells were cultured in complete DMEM, high glucose in the presence (0-1000 mg/ml) of Hing-Hai for 24 h and 72 h for Caco2 and SW620, respectively. Wound healing was observed every 8 h under microscope (×10).

3.5 Hing-Hai Suppressed Gelatinolytic Activity in Caco2 and SW620 Cells

As Hing-Hai delayed migration of both cells tested, we then clarified whether it may suppress the activity of matrix metalloproteinase enzymes using gelatin zymography technique. Figure 7 revealed that gelatinolytic activity was dose dependent and significantly decreased by change of dose. The effect was clearly observed and more effective in SW620 than in Caco2 cells.

3.6 Hing-Hai was Toxic to Normal Peripheral Blood Mononuclear Cells

The results mentioned confirmed cytotoxicity of Hing-Hai to colon cancer cell lines of both original and metastatic form. To study whether Hing-Hai may be cytotoxic to normal white blood cells, we then cultured peripheral blood mononuclear cells (PBMCs, n=3) in the presence of various concentrations of Hing-Hai for 3 days and tested viability. We found that Hing-Hai was also toxic to PBMCs, but with IC_{50} of >1000 µg/ml which was approximately <4-fold and <1.9-fold of toxic dose to Caco2 and SW620 cells, respectively (Figure 8)

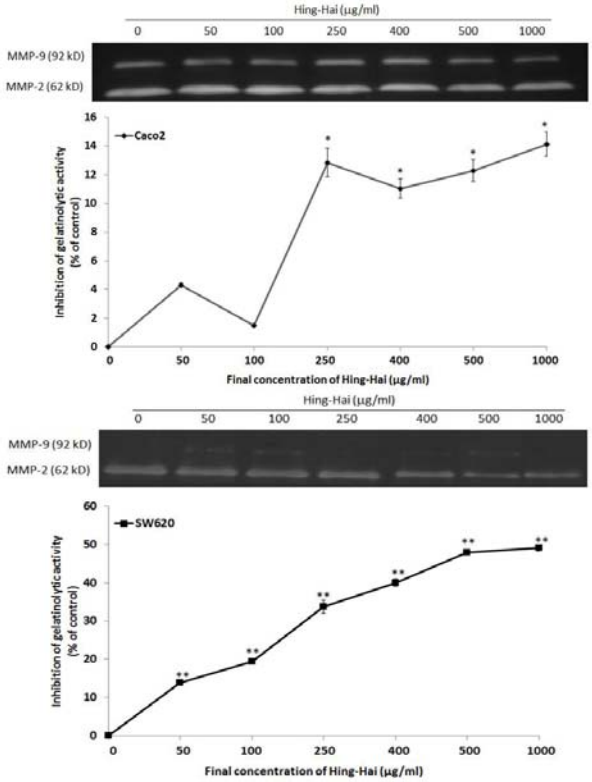

Figure 7. Hing-Hai decreased gelatinolytic activity in Caco2 and SW620 cells. Caco2 and SW620 cell were cultured in complete DMEM, high glucose. Cultured media was collected and assayed by electrophoresis. Electrophoresis was performed for 200 min at 90 V. Gel was washed twice with 2.5% Triton X-100 and incubated in activating buffer for 16 h at 37 °C. The gel was subsequently stained (0.2% Coomassie Brilliant Blue R-250) for 5 h at room temperature, and destained to reveal zone of lysis within the gelatin matrix. Gelatinolytic activity was detected as clear band against a stained. Inhibition of gelatinolytic activity (%) was calculated compared to control.

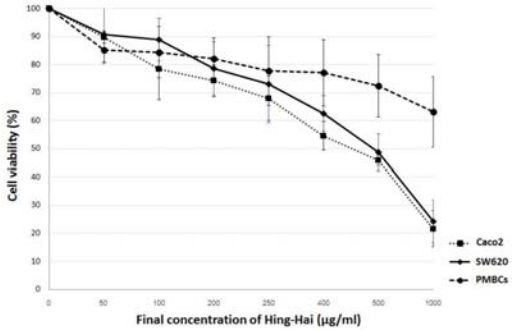

Figure 8. Determination of Toxicity of Hing-Hai to PBMCs. PBMCs (5×10^4 cells/ml) was cultured in the presence or absence of various concentrations of Hing-Hai for 3 days. Caco2 and SW620 (1×10^4 cells/ml) was cultured individually in the same condition. Viability of cell was determined by MTT. Cell viability (%) was plotted (y-axis) against various final concentrations of Hing-Hai (µg/ml, x-axis). The results shown are representative of 3 independent experiments.

4. DISCUSSION

Hing-Hai is a traditional formula consisting of 7 medicinal plants (leaves and cores) based on water extraction. It is well known and widely used in patients with cancers of colon, liver and lung but with no scientific support. This work is the first to explore anti-cancer effects of water-based extract, Hing-Hai in 2 types of colon cancer cell lines. Cells at exponential phase were cultured for 4 days in the presence of Hing-Hai and cell viability was tested. The result confirmed the anti-cancer effect, since viability of both cells was decreased in a dose dependent manner. Interestingly, Caco2, colon originate type, was more sensitive to Hing-Hai (IC_{50} 234 µg/ml) than that of SW620 which is lymph node derived type (IC_{50} 438 µg/ml). We then investigated the mechanism of Hing-Hai in inhibition of cancer cell growth. Many medicinal plants and/or medicinal formulas were reported to be toxic to various cancer cells through the cell cycle arrest and induction of apoptosis (9a-9b). To address whether Hing-Hai may arrest cell cycle and induce apoptosis and cause cell death, we individually cultured Caco2 and SW620 cells in the presence of Hing-Hai at a concentration of IC_{20} for 3 days and analyzed by flow cytometry after staining. The result showed that there was no change in both cell cycle and apoptosis compared to untreated control. We then considered whether the anti-cancer effects of Hing-Hai are based on other processes.

Primary tumor cells can digest surrounding extracellular matrix, migrate to interstitial spaces, and enter the blood and lymphatic system to other organs. Once entering the target organs, those cells migrate through interstitial space and continue growing and develop a secondary tumor (10a-10b). Thus, inhibition of these steps results in prevention of metastasis and tumor growth. Many reports demonstrated that water-based plant extracts are toxic to tumors by inhibition of cell adhesion, migration and invasion (11a-11c) through various mechanisms (12a-12d). Moreover, *Cudrania cochinchinensis* (Lour.) was reported to inhibit colon cancer cell proliferation, migration, and invasion (13) and one of 7 ingredients of Hing-Hai is leaves of *Carissa cochinchinensis* Pierre. In addition, *leaves from Derris scandens* Benth, the second ingredient in Hing-Hai, was reported to have anti-adhesion effect in squamous cell carcinoma (KKUM139) and adenosquamous carcinoma (KKU-M213) (unpublished data). Moreover, the previous studies revealed the potential anticancer activity of the composition of Hing-Hai, the lignans (-)-carinol, (-)-carissanol and (-)-nortrachelogenin derived from *Carissa cochinchinensis* Pierre exhibited cytotoxicity against breast (MCF7) and lung (A549) cancer cells (14). The ethanolic extract of *Derris scandens* Benth has been found to have antimigration activity in hepatoma cell line (HepG2), breast cancer cell line (MCF-7), and cholangiocarcinoma cell lines (KKU-M139 and KKU-M213). Hematulin A and her colleague showed that the stem extract of *D. scandens* could abolish G2/M checkpoint proficiency of colon cancer cell line (HT-29), resulting in strongly increased radiosensitivity of HT-29 cells (15). Veenavani M. reported the non-toxic to normal cells of methanolic extract of *Artocarpus heterophyllus* Lam, but showed excellent toxicity against MCF-7 and MDA-MB-231 cancer cell lines. The cytotoxicity of methanolic extract of *A. heterophyllus* may be due to the presence of flavonoids having mono to poly phenolic groups in the structure (16). *In vitro*, clinical trials demonstrated that *Cassia siamea* Lamk aqueous extracts inhibited human recombinant hepatic cytochrome P450 such as of 346.5 mg/ml and 50 mg/ml, respectively (17). This inhibition of GSTs

may be beneficial for cancer therapy. Also, petroleum ether, dichloromethane, ethanol and aqueous extracts of leave showed cytotoxicity against human epidermoid carcinoma (KB) cell lines value between 67 and 100 mg/ml (18). The anticancer properties of *C siamea* could be due to anthraquinones (emodin and its derivatives) and bianthraquinones (cassiamin B and its derivatives) (19). In 2015, Hifza B. showed a 80% survival (IC20) with 47 mg/ml as concentration of *Bridelia ovata* Decne ethanol extract inhibited invasion and migration of HepG2 cells (20). The heartwood of *Artocarpus lakoocha* Roxb has many pharmacological activities such as anti-inflammatory, antiviral, anticancer and anti-HIV (21). Moreover, *Crotalaria* species contain macrocyclic pyrrolizidine alkaloids which are considered as important secondary metabolites largely on account of their biological activities which include anticancer (22). (Respond to reviewer 1 #2,3 and reviewer 2#1)

We, therefore, explored the inhibition effect of Hing-Hai in cell adhesion, migration, and invasion based on MTT assay, scratch-wound healing assay, and gelatin zymography, respectively. The study showed that Hing-Hai could suppress all 3 steps including adhesion, migration and invasion. Concentration required for suppression of those steps was much less than that required for decreasing of cell viability. Moreover, SW620 was more sensitive to Hing-Hai in all processes compared to Caco2. We proposed that Hing-Hai provided an anti-metastasis of colon cancer cells. Hing-Hai is a water-extracted mixture of 7 medicinal plants but only *Derris scandens* Benth was scientific supported to have anti-adhesion. Hing-Hai is also recommended to promote immune response and for use as an anti-inflammatory remedy. The findings indicated an active molecule in water-extracted mixture. It is interesting to investigate the chemical profile and an active ingredient and its action involving cell adhesion, migration,and invasion. Clinically used anti-cancer drugs generally cause cellular cytotoxicity and related side effects. Therefore, drugs with no or less toxicity to normal cells are required. Peripheral blood mononuclear cells (PBMC) give selective responses to the immune system and are the major cells in the human body immunity. They contain several types of cells such as lymphocytes,monocytes or macrophages.Because peripheral blood is the place where exposure to chemicals occurs, these fundamentally important PBMCs are prone to be influenced by drugs and chemicals. This is why the availability of PBMCs from peripheral blood is very important for researchers studying toxicity of new drugs or chemical compounds (23). (Respond to reviewer 2 #3) Our study demonstrated that Hing-Hai was cytotoxic to normal PBMCs, but less than 4-fold and 1.9-fold to that of Caco2 and SW620, respectively. To apply as anti-cancer, thus, side effect to normal white blood cells may be considered. Moreover, purification of active ingredients and study anti-cancer of Hing-Hai in an animal model should be studied.

5. CONCLUSIONS

Taken together, our findings confirmed the anti-colon cancer activity of Thai medicinal formula named Hing-Hai. We demonstrated that Hing-Hai was dose-dependently toxic to both colon cancer cell types. It did not induce apoptosis or arrest cell cycle. Interestingly, the anti-cancer effect of Hing-Hai was verified to inhibit cell adhesion and migration and decrease the gelatinolytic activity. The effect was more potent in metastatic cell type, SW620, than for Caco2 which is colon originated. However, Hing-Hai was also toxic

to normal peripheral blood mononuclear cells, but less than 4-fold and 1.9-fold of toxicity to Caco2 and SW620 cells, respectively. Our study supports complementary and alternative medicines' utilization of Hing-Hai for the treatment of colon cancer. These scientific evidences will raise the value of Hing-Hai for therapeutic use in colon cancer.

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REFERENCE

[1] Alwan A., Maclean D.R., Riley L.M., d'Espaignet E.T., Mathers C.D., Stevens G.A. and Bettcher D., *Lancet*, 2010; **376(9755)**: 1861-1868.

[2] Health M.O.P., *Statistic of Death by Leading Cause Groups*, 2006-2010; 2011.

[3] WHO. Global status report on noncommunicable disease, 2010; 2011. WT 500.

[4] Fidler I.J. and Hart I.R., *Science*, 1982; **217(4564)**: 998-1003.

[5] Nicolson G.L., *Cancer Metastasis Rev.*, 1988; **7(2)**: 143-188.

[6] Price J.T. and Thompson E.W., *Expert Opin. Ther. Targets*, 2002; **6(2)**: 217-233.

[7] Iiizumi M., Liu W., Pai S.K., Furuta E., Watabe K., *Biochim. Biophys. Acta.*, 2008; **1786(2)**: 87-104.

[8] Han R., *Chin. Med. J. (Engl).*, 1995; **108(10)**: 729-731.

[9] Pezzuto J.M., *Biochem. Pharmacol.*, 1997; **53(2)**: 121-133.

[10] Farnsworth N.R., Akerele O., Bingel A.S., Soejarto D.D. and Guo A., *Bull. World Health Organ.*, 1985; **63(6)**: 965-981.

[11] Li J., Sun G.Z., Lin H.S., Pei Y.X., Qi X., An C., Yu J. and Hua B.J., *Int. Immunopharmacol.*, 2008; **8(9)**: 1196-1206.

[12] Ravi A., Mallika A., Sama V., Begum A.S., Khan R.S. and Reddy B.M., *J. Ethnopharmacol.*, 2011; **137(3)**: 1353-1359.

[13] Sangha J.S., Sun X., Wally O.S., Zhang K., Ji X., Wang Z., Wang Y., Zidichouski J., Prithiviraj B. and Zhang J., *PLoS One*, 2012; **7(8)**: e43990.

[14] Wilson M.J., *J. Urol.*, 1993; **149(3)**: 653-658.

[15] Elmore S., *Toxicol. Pathol.*, 2007; **35(4)**: 495-516.

[16] Tin M.M., Cho C.H., Chan K., James A.E. and Ko J.K.S., *Carcinogenesis*, 2007; **28(6)**: 1347-1355.

[17] Hsu H.F., *J. Ethnopharmacol*, 2001; **135(2)**: 492-500.

[18] Liotta L.A., *Cancer Res.*, 1986; **46(1)**: 1-7.

[19] Scherer R.L., Mcintyre J.O. and Matrisian L.M., *Cancer Metastasis Rev.*, 2008; **27(4)**: 679-690.

[20] Lirdprapamongkol K., Kramb J.P., Chokchaichamnankit D., Srisomsap C., Surarit R., Sila-asna M., Bunyaratvej A., Dannhardt G. and Svasti J., *In Vivo*, 2008; **22(3)**: 363-368.

[21] Lirdprapamongkol K., Mahidol C., Thongest S., Prawat H., Ruchirawat S., Srisomsap C., Surarit R., Punyarit P. and Svasti J., *J. Ethnopharmacol.*, 2003; **86(2-3)**: 253-256.

[22] Siripong P., *Asian Pac. J. Cancer Prev.*, 2012; **13(7)**: 3519-3528.

[23] Heo J.C., Park J.Y., Lee J.M., Kwon T.K., Kim S.U., Chung S.K. and Lee S.H., *J. Ethnopharmacol.*, 2005; **102(1)**: 10-14.

[24] Xia S.H., Wang J. and Kang J.X., *Carcinogenesis*, 2005; **26(4)**: 779-784.

[25] Wang L., *Int. J. Cancer*, 2010; **127(5)**: 1220-1229.

[26] Yang C.L., Liu Y.Y., Ma Y.G., Xue Y.X., Liu D.G., Ren Y., Liu X.B., Li Y. and Li Z., *PLoS One*, 2012; **7(5)**: e37960.

[27] Wang Y.H., Hou A.J., Zhu G.F., Chen D.F. and Sun H.D., *Planta Med.*, 2005; **71(3)**: 273-274.

[28] Wangteeraprasert R., *Phytother. Res.*, 2012; **26(10)**.

[29] Hematulin A., Ingkaninan K., Limpeanchob N. and Sagan D., *Asian Pac. J. Cancer Prev.*, 2012; **15(4)**: 1871-1877.

[30] Veenavani M., *The Am. J. Scu. Med. Res.*, 2016; **1(2)**: 223-228.

[31] Appiah-Opong R., Commandeur J.N., Axson C. and Vermeulen N.P., *Food Chem. Toxicol.*, 2008; **46**: 3598-3603.

[32] Nsonde-Ntandou G.F., Banzouzi J.T., Mbatchi B., Elion-Itou R.D., Etou-Ossibi A.W., Ramos S., Benoit-Vical F. and Abena A.A., *J. Ethnopharmacol.*, 2009; **127**: 108-111.

[33] Koyam J., Morita I., Tagahara K., Ogata M., Mukainaka T., Tokuda H. and Nishino H., *Cancer Lett.*, 2001; **170(1)**: 15-8.

[34] Hifza B., Penchatr D., Churat W.N., Monique P., Jisnuson S. and Chantragan S., *Naresuan Phayao J.*., 2015; **8(1)**: 6-10.

[35] Kirtikar K.R., *Indian Med. Plants*, 2007; **10**: 232.

[36] Kovach J.S., *Proc. Am. Assoc. Cancer Res.*, 1979; **20**: 357-362.

[37] Pourahmad J. and Salimi A., *Iran J. Pharm. Res.*, 2015; **14(4)**: 979.