

## Potentiating Effect of Oxymatrine and 5-fluorouracil on Cell Survival and Inhibition of Cancer Metastasis in SW-620 Colorectal Cancer Cells

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### Abstract

Colorectal cancer (CRC) is the most common cancer in the world, beginning in the cell lining of the colon and rectum. 5-fluorouracil (5-FU) is a first-line therapy for colorectal cancer patients. However, the response rate of 5-FU in advanced colorectal cancer patients is only 10 - 15 %, and can fail due to drug resistance. Therefore, the development of a new anticancer compound for improving the response rate or for reversing resistance to 5-FU is urgently needed. Oxymatrine is a major component of *Sophora flavescens*. Recently, many pharmacological effects have been exhibited. The objectives of the present study were to investigate the anticancer activity of oxymatrine, as well as to potentiate the effect of oxymatrine with 5-FU on cell viability, colony-forming, cell migration, cell invasion, and determined MMP-9 protein expression in SW-620 colorectal cancer cells. The results demonstrated that oxymatrine significantly inhibited the cell viability of SW-620 cells after 24, 48, and 72 h treatments. Oxymatrine and 5-FU interaction was synergistically greater in inhibiting the cell survival of SW-620 cells than 5-FU was alone. Oxymatrine also decreased colony formation, cell migration, and cell invasion through reducing the level of MMP-9 protein in SW-620 cells. These first findings elucidated an efficacious anticancer agent, which is derived from natural sources, which could be used to overcome 5-FU resistance in colorectal cancer, and may be beneficial for patients with colorectal cancer who are treated with 5-FU.

**Keywords:** Oxymatrine, cell viability, cell invasion, colorectal cancer, 5-fluorouracil

### Introduction

Colorectal cancer (CRC) is a type of malignant cancer that occurs in worldwide. Malignant cancer arises from the cell lining of the large intestine and rectum. In Thailand, the incidence rate of CRC has been rapidly increased, both in males and females. In 2012, the National Cancer Institute of Thailand (NCI) reported that CRC is the second most common type of cancer manifested, and the third leading cause of cancer-related death in patients [1]. 5-fluorouracil (5-FU) is the drug used first-line treatments of CRC, and is clinically useful for CRC therapy, as an adjuvant regimen administered after complete surgical resection; additionally, it has therapeutic effects on other types of cancer, such as breast cancer and head and neck cancer [2]. However, the overall response rate to 5-FU as a single drug in advanced CRC patients is only 10 - 15 % and CRC has been seen to be refractory to 5-FU, which may result from drug resistance, including alteration of drug influx and efflux, mutation of the drug target, and an increase in drug inactivation [3]. The combination of 5-FU with newer chemotherapies, such as irinotecan and oxaliplatin, can increase the response rate for metastatic colorectal cancer to 40 - 50 %, compared with 5-

5-FU treatment alone, leading to improvements in time to progression and for free-survival in patients [4]. Nowadays, combination therapy of 5-FU with oxaliplatin and leucovorin is a FOLFOX regimen that has widely been treated for CRC patients. However, only half of advanced CRC patients receive the benefit from this regimen because of acquired resistance to 5-FU, which is still the main cause of CRC therapy failure [5]. Therefore, the development of new strategies for the treatment or for the reversal of resistance to 5-FU is urgently required.

Cancer metastasis is in the main character and hallmark of cancer progression [6]. Metastasis is a multi-step process, beginning from detachments of cancer cells from the primary tumor and disruption of the basement membrane leading to invasion of the surrounding tissue. Subsequently, the cancer cells are able to enter the blood and lymphatic systems to spread into other parts of the body and to extravasation, leading to growth and proliferation in distant sites. Eventually, the progress of the disease is due to micrometastasis of the cancer, and the patient dies of their disease [7]. Matrix metalloproteinase (MMP) is a zinc-dependent endopeptidase, which is responsible for degradation of the component of extracellular matrix (ECM) proteins including collagen, elastin, and fibronectin. MMP can be secreted by inflammatory cells, osteoblast, fibroblast, and also cancer cells. MMP has a pivotal role in promoting cancer cell migration and invasion by degrading the surrounding tissue inside the ECM that is assisting cancer cells during metastasis [8,9]. MMP-9, also known as gelatinase B, is produced by many types of cells. Normally, the MMP-9 function is controlled by tissue inhibitors of MMPs (TIMPs). In pathological conditions, such as carcinogenesis, the balance between MMP and TIMPs is altered, resulting from the increase of MMP activity that promotes cancer metastasis [10,11]. MMP-9 protein is primarily expressed in the cytoplasm of both tumor and stromal cells [12]. In CRC, many studies reported a correlation between an increase in MMP-9 and the worst outcome in CRC patients. Recently, it has been demonstrated that MMP-9 is a marker of CRC invasion, because MMP-9 protein levels were expressed at significantly higher ratios in the sera of persons with CRC, compared to normal controls [13]. Since metastasis is the major cause of cancer-related death in CRC patients, the development of a new anticancer agent that reduces cancer migration and invasion through decreasing MMP-9 expression would be a promising target for CRC treatment.

Recently, an anticancer agent which is derived from traditional Chinese herb may potentially be an effective anticancer compound in combination therapy when used for standard treatments or for the prevention of tumors. Combination treatments using herbs not only reduce the dose of chemotherapy needed, but may also decrease the adverse effects of the main chemotherapy. Oxymatrine (molecular formula:  $C_{15}H_{24}N_2O_2$ , molecular weight: 264.36) is one of the kinds of alkaloids extracted from the dry roots of the traditional Chinese herb *Sophora flavescens*. Recently, it has been studied in terms of its pharmacological activities, including anti-inflammatory [14], anti-hepatitis [15], and anti-fibrosis [16,17]. Moreover, it has been reported to exhibit anticancer activity in human pancreatic cancer cells [18], human hepatoma cells [19], human colorectal cancer cells [20], human gastric cancer cells [21,22], and human breast cancer cells [23]. However, there has been no report that has examined the improvement of 5-FU efficacy or potentiating effects with 5-FU on SW-620 colorectal cancer cells. Therefore, the objectives of this study were to investigate the anticancer activity of oxymatrine, as well as to evaluate the potential and safety profile of combination therapy of 5-FU and oxymatrine on SW-620 colorectal cancer cells and MRC-5 normal fibroblast cells.

## Materials and methods

### Cell culture

An SW-620 human colorectal cancer cell line, derived from a metastatic site of the lymph node, was obtained from the American Type Culture Collection (ATCC, USA). SW-620 cells were cultured in RPMI-1640 medium (Invitrogen, U.S.A), supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco, U.S.A), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 250 µg/ml of amphotericin B, in a humidified 37 °C incubator containing 5 % CO<sub>2</sub>. The human fibroblast cell lines MRC-5 were purchased from ATCC. MRC-5 cells, derived from lung tissue, were maintained in DMEM medium (Invitrogen, U.S.A), supplemented with 10 % FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin,

and 250 µg/ml of amphotericin B, in a humidified 37 °C incubator containing 5 % CO<sub>2</sub>. 0.4 % trypan blue dye was stained for viable cell count using a hemocytometer. Cell viability at 95 % was used in the experiments.

### Test compounds

High purity (99 %) oxymatrine was purchased from Sigma-Aldrich (St. Louis, Mo, USA), as determined by high-performance liquid chromatography (HPLC), and dissolved in 100 % methanol to a final stock concentration of 1,000 µg/ml. 5-FU was obtained from Sigma-Aldrich (St. Louis, Mo, USA), and was solubilized by dimethyl sulfoxide (DMSO) to the final concentration of 1,000 µg/ml. The stock solution of oxymatrine and 5-FU was kept away from light and stored at -20 °C. The stock solution was freshly diluted by cell culture medium before application in the experiment. The final concentration of dissolving agents was not more than 0.1 % in cell culture medium during treated cells throughout the experiment. This concentration did not affect cell viability or cytotoxicity.

### MTT cell viability assay

Cells viability assays were performed based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique. Cells were seeded at a density of 5,000 cells/well into a 96-well plate and incubated at 37 °C in 5 % CO<sub>2</sub> atmosphere for 24 h. Cells were treated with 5, 10, 25, 50, and 100 µg/ml of oxymatrine in triplicate for 24, 48, and 72 h. Cells were treated with 1, 10, and 100 µg/ml of 5-FU were used as positive control in the experiment. Then, 10 µL of 5 mg/ml MTT (AMRESCO, U.S.A) in PBS solution was directly added into each well and incubated at 37 °C for 3 h. Mitochondrial dehydrogenase in viable cells can metabolize the yellow color of MTT to form purple formazan crystal, which is solubilized by DMSO and measured by spectrophotometer. Supernatant was aspirated and 100 µL aliquot of DMSO was then added; absorbance was measured by a microplate reader (Metertech Co., Taipei, Taiwan) at a wavelength of 570 nanometers. The percentage of cell viability was calculated by using the following equation;

$$(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100 \% \quad (1)$$

### Drug combination assay

Cells were seeded at a density of 5,000 cells/well into a 96-well plate in RPMI-1640 supplemented with 5 % FBS, 100 U/ml penicillin, and 100 µg/ml of streptomycin in an incubator at 37 °C in 5 % CO<sub>2</sub> atmosphere for 24 h. Cells were treated with a combination of 1, 2.5, 5, 7.5, 10, 25, and 50 µg/ml of 5-FU and 30, 40, and 50 µg/ml of oxymatrine for 72 h. Cell viability was determined by MTT cell viability assay.

### Drug combination index analysis

This method was utilized to determine the synergistic, additive, and antagonistic values of the drug-drug interaction between oxymatrine and 5-FU. Combination index values were determined by using the following equation from the Chou and Talalay method:  $CI = (C_{50}(5\text{-FU})/IC_{50}(5\text{-FU}) + C_{50}(\text{oxymatrine})/IC_{50}(\text{oxymatrine}))$ .  $C_{50}(5\text{-FU})$  and  $C_{50}(\text{oxymatrine})$  were the concentrations used in the combination treatment that generated percent of cell viability at 50 %.  $IC_{50}(5\text{-FU})$  and  $IC_{50}(\text{oxymatrine})$  were the concentrations that generated percent of cell viability at 50 % when the compound was used alone for treatment. CI value was interpreted as per the following: synergism (CI < 1) when the addition of oxymatrine apparently increases the effect of 5-FU more than 5-FU treatment alone, additive (CI = 1) when the combination of oxymatrine has an equally pharmacological effect to the sum of oxymatrine and 5-FU, antagonism (CI > 1) when the combination of oxymatrine with 5-FU is less effective than the sum of the oxymatrine and 5-FU, respectively.

### **Combination drug toxicity testing**

This assay was utilized to investigate the toxicity effect of oxymatrine combined with 5-FU on cell viability of MRC-5 fibroblast cells. Cells at a density of 5,000 cells/well were grown in 96-well plate for 24 h. Cells were cultured in a presence of 1, 2.5, 5, 7.5, 10, 25, and 50 µg/ml of 5-FU and 30, 40, and 50 µg/ml of oxymatrine for 72 h. Cell viability was evaluated by MTT cell viability assay.

### **Colony formation assay**

Cells were seeded at a density of 500 cells/well in 24-well plate and incubated at 37 °C in 5 % CO<sub>2</sub> atmosphere for 24 h. Cells were treated with 0.1 % methanol, 50 µg/ml of oxymatrine, and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU for 24 h. The medium was aspirated and the cells were cultured in RPMI-1640 supplemented with 10 % FBS for 7 days. Cells were fixed with acetic acid (1): methanol (3) for 15 min. Cells were stained with 0.5 % crystal violet for 30 min. Cells were photographed with an inverted microscope (objective 5×). The number of colonies were counted by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data was presented as % colonies being equal to a number of colonies in a treated group/number of colony in the control group × 100 %.

### **Scratch assay**

Cells were seeded at a density of 100,000 cells/well in a 24-well plate and were allowed to form a confluent monolayer until 100 % confluence. The 200 µl of yellow pipette tips generated a straight line wound crossing the monolayer of cancer cells in the 24-well plate. Afterward, the 24-well plate was washed with PBS solution to remove floating cells and photographed at time 0. Cells were incubated at 37 °C in RPMI-1640 medium containing 5 % FBS in the presence of the concentrations of 50 µg/ml oxymatrine and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU. Control cells were treated with 0.1 % methanol. Cells were then photographed at time 24 and 48 h with an inverted microscope (objective 5×, Olympus, Japan). The number of cells migrated to the wound were quantified by using Image J software (National Institutes of Health, Bethesda, MD, USA).

### **Matrigel invasion assay**

The invasiveness of SW-620 colorectal cancer cells was tested using BD Matrigel Invasion Chambers (pore size: 8 µm; Corning, Cambridge, MA). Briefly, cells were seeded at a density of 50,000 cells/well into the upper chambers of the system which contained 0.1 % methanol, 50 µg/ml of oxymatrine, and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU in serum-free RPMI-1640 medium. The bottom wells were fully filled with RPMI-1640 and supplemented with 10 % FBS as a chemo-attractant. The plate was placed in an incubator at 37 °C in 5 % CO<sub>2</sub> atmosphere for 72 h. Cells which migrated through the matrigel and invasion chamber were fixed with ice-cold methanol for 15 min. Non-penetrating cells were wiped off from the upper surface of the transwell with a cotton swab. Upper chambers were stained with crystal violet for 30 min and washed with 1× PBS until the bottom of the chamber was clear. Images were captured in 5 random fields per well under an inverted microscope (objective 20×, Olympus, Japan). Data was expressed as a percentage of proportional invasiveness by comparing treated cells with the control group. The percentage of proportional invasiveness is equal to the numbers of cells invaded in treated cells/numbers of cells invaded in the control group ×100 %.

### **Human MMP-9 enzyme-linked immunosorbent assay (ELISA)**

Cells were seeded at a density of 50,000 cells/well for 24 h in 24-well plate. Cells were treated with 0.1 % methanol, 50 µg/ml of oxymatrine, and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU for 48 h. The supernatant was collected and kept at -80°C until use. The MMP-9 protein was secreted into the cell culture medium and measured using MMP-9 ELISA assay kits (Invitrogen, U.S.A.) following the manufacturer's instructions. The absorbance of each well was read at 450 nm by using a microplate reader (Metertech Co., Taipei, Taiwan), and MMP-9 protein levels were expressed as pg/ml.

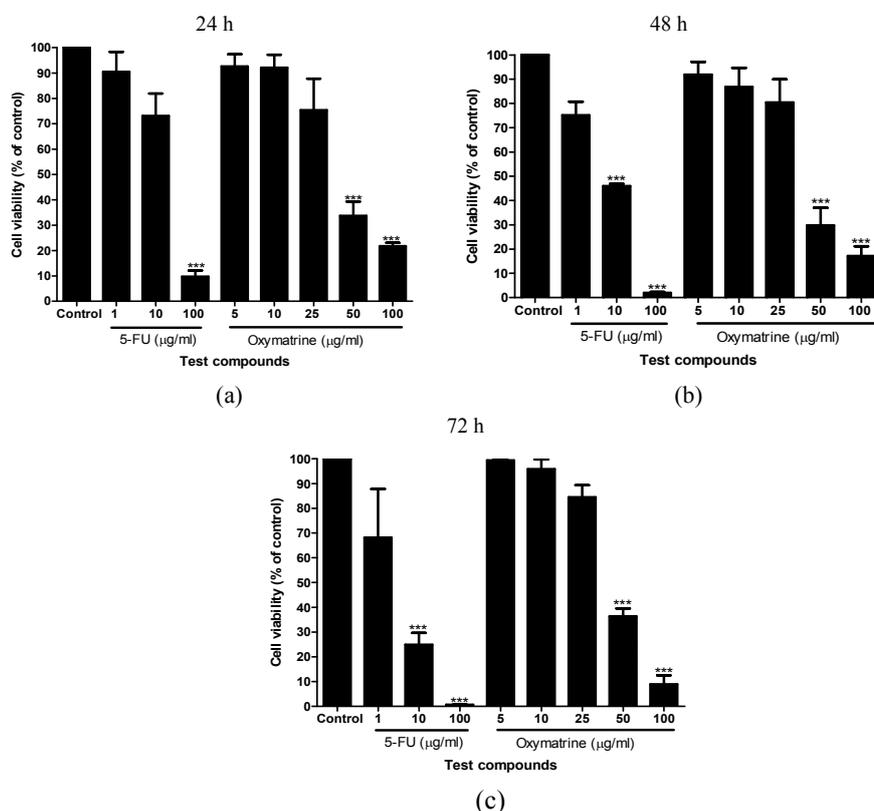
### Statistical analysis

The data was performed at least 3 times ( $n = 3$ ) and presented as means  $\pm$  SEM from 3 independent experiments determined by one-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test. A  $p$ -value of less than 0.05 was considered to be statistically significant. All graphs were created using GraphPad prism software Ver.5.0 (GraphPad Software, San Diego, CA).

### Results and discussion

#### Effect of oxymatrine on cell viability in SW-620 colorectal cancer cells

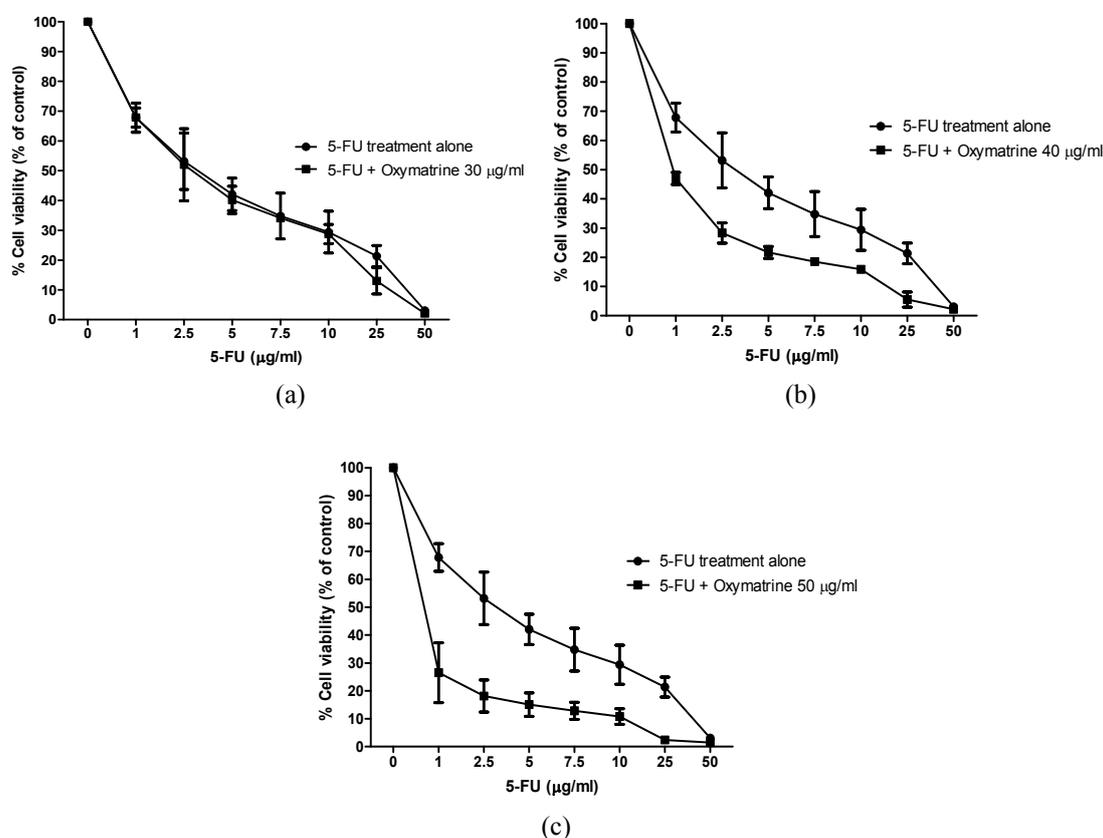
The anticancer effects of oxymatrine on SW-620 colorectal cancer cells were evaluated by MTT assay. As shown in **Figure 1**, oxymatrine decreased cell viability of SW-620 colorectal cancer cells in a concentration- and time- dependent manner at 24 h (**Figure 1(a)**), 48 h (**Figure 1(b)**), and 72 h (**Figure 1(c)**) exposures. Especially, 50 and 100  $\mu\text{g/ml}$  of the oxymatrine significantly decreased cell viability in SW-620 colorectal cancer cells ( $p < 0.001$ ). The half maximum inhibitory concentrations ( $IC_{50}$ ) value of oxymatrine in SW-620 colorectal cancer cells at 24, 48, and 72 h were 54.29, 51.27, and 46.41  $\mu\text{g/ml}$ . In this study, it was demonstrated that the cell viability of SW-620 human colorectal cancer cells was largely inhibited by the extract of traditional Chinese herb oxymatrine in a time- and dose-dependent manner after 24, 48, and 72 h treatment. It is possible that the cell viability of another type of colorectal cancer is also decreased by oxymatrine.



**Figure 1** shows the effect of oxymatrine on the inhibition of cell viability in SW-620 colorectal cancer cells. Cells were treated with 5, 10, 25, 50, and 100  $\mu\text{g/ml}$  of oxymatrine for 24, 48, and 72 h. 5-FU at 1, 10, and 100  $\mu\text{g/ml}$  were used as a positive control in the experiment. The experiment was performed at least 3 times ( $n = 3$ ). \*\*\*Significantly different at  $p < 0.001$ .

### Oxymatrine increases the cytotoxic effect of 5-FU in SW-620 colorectal cancer cells

The results show the percentage of cell viability curve when compared between the range of 5-FU concentrations from 1, 2.5, 5, 7.5, 10, 25, and 50  $\mu\text{g/ml}$ , and 5-FU combined with 30  $\mu\text{g/ml}$  (**Figure 2(a)**), 40  $\mu\text{g/ml}$  (**Figure 2(b)**), and 50  $\mu\text{g/ml}$  (**Figure 2(c)**) of oxymatrine. As shown in **Figure 2**, the results show that oxymatrine was able to increase the cytotoxic effect of 5-FU in SW-620 colorectal cancer cells, when compared to 5-FU treatment alone. The  $\text{IC}_{50}$  value of 5-FU was decreased from 1.25  $\mu\text{g/ml}$  to 0.02  $\mu\text{g/ml}$  for the combination of 30  $\mu\text{g/ml}$  of oxymatrine with 5-FU in SW-620 colorectal cancer cells. In addition, the CI value of 5-FU combined with 30  $\mu\text{g/ml}$  oxymatrine is 0.665, which demonstrates that the interaction between 5-FU and oxymatrine was synergistic (**Table 1**). Interestingly, the results show that oxymatrine at lower concentrations combined with 5-FU was superior to 5-FU treatment alone. The results indicated that oxymatrine could increase the cytotoxic effect of 5-FU on SW-620 colorectal cancer cells. Consistently, many studies have explored the concept that the extraction of traditional Chinese herbs, including resveratrol and ginsenoside, could sensitize human CRC cells to 5-FU and enhance the anti-proliferative effect of 5-FU to CRC cells [24,25]. Therefore, the combination of oxymatrine with 5-FU may be an alternative strategy to increase the anticancer effect of 5-FU, as well as to reduce the dose of these drugs in colorectal cancer patients.

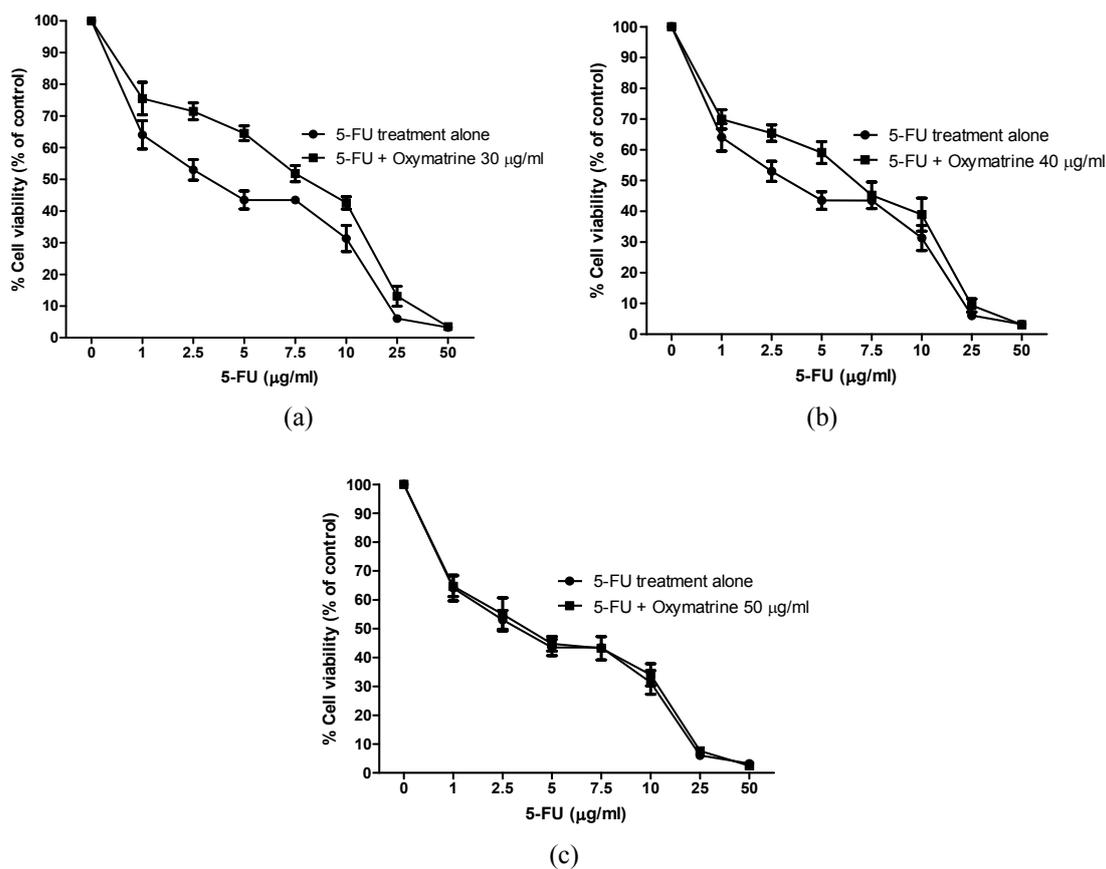


**Figure 2** Combined effects of oxymatrine and 5-FU on cell viability in SW-620 colorectal cancer cells. Cells were treated with a combination of 1, 2.5, 5, 7.5, 10, 25, and 50  $\mu\text{g/ml}$  of 5-FU and 30, 40, and 50  $\mu\text{g/ml}$  of oxymatrine for 72 h. Cell viability was determined by MTT cell viability assay. The experiments were performed at least 3 times ( $n = 3$ ).

**Table 1** represents the IC<sub>50</sub> values and combination index (CI) values of either 5-FU treatment alone or combined with 30 µg/ml of oxymatrine in SW-620 human colorectal cancer cells after treatment for 72 h.

Test compound	IC <sub>50</sub> (µg/ml)	CI value	Description
Oxymatrine	46.41	-	-
5-FU alone	1.25	-	-
Oxymatrine 30 µg/ml +5-FU	0.023	0.665	synergism

\*CI < 1, CI = 1, and CI > 1 were synergism (CI < 1), additive (CI = 1), antagonism (CI > 1), respectively.



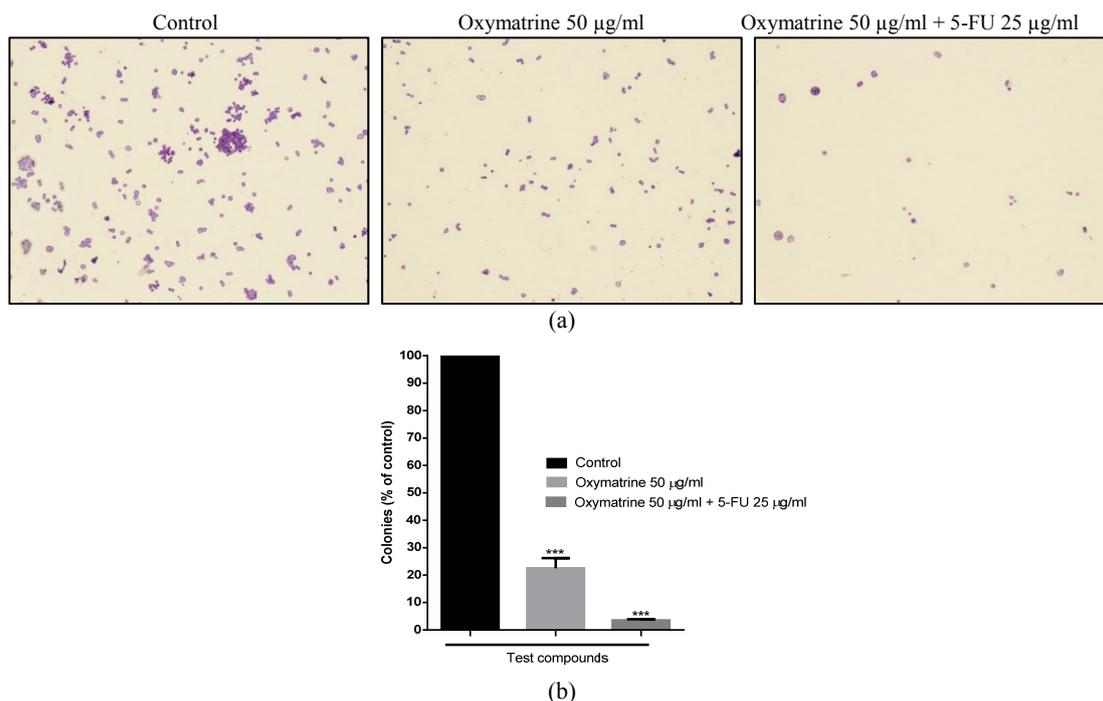
**Figure 3** Effect of oxymatrine combination with 5-FU on cell viability of MRC-5 normal fibroblast cells. Normal cells were treated with a combination of 1, 2.5, 5, 7.5, 10, 25, and 50 µg/ml of 5-FU and 30, 40, and 50 µg/ml of oxymatrine for 72 h. Cell viability was determined by MTT cell viability assay. The experiments were performed at least 3 times (n = 3).

### Oxymatrine did not have synergistic effects with 5-FU on cell viability of normal fibroblast cells

The results from a combination of oxymatrine with 5-FU had less cytotoxic effect on the cell viability of MRC-5 fibroblast cells. As shown in **Figure 3**, the IC<sub>50</sub> values of 5-FU combined with 30, 40, and 50 µg/ml of oxymatrine were 11.78 µg/ml (**Figure 3(a)**), 8.29 µg/ml (**Figure 3(b)**), and 4.67 µg/ml (**Figure 3(c)**), compared with the IC<sub>50</sub> value of wide ranging concentrations of 5-FU treatment alone, which were 4.12 µg/ml after treatment for 72 h. These results indicated that oxymatrine has a high selectively cytotoxic effect on cancer cells more than normal fibroblast cells. Additionally, this result found that the toxicity of oxymatrine at a low dose, when the oxymatrine was used as combination treatment, was less harmful to normal fibroblast cells. These results will be beneficial for further study in using this regimen in colorectal cancer treatment and developing it as an adjuvant therapy in the future.

### Effect of oxymatrine on colony formation in SW-620 colorectal cancer cells

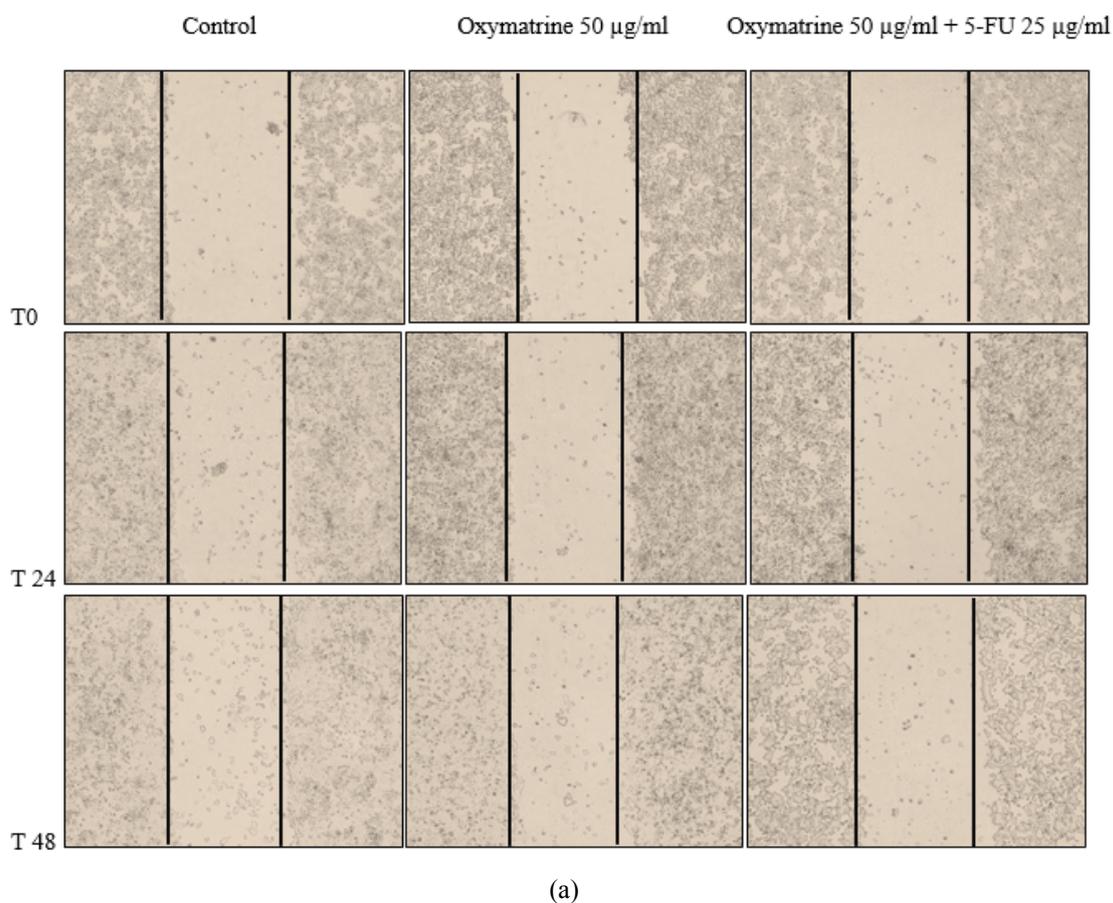
The colony formation assay was utilized to evaluate the inhibitory effects of oxymatrine and oxymatrine combined with 5-FU on cell proliferation to form a large colony. As shown in **Figure 4**, the results of 50 µg/ml of oxymatrine significantly decreased the percent of colony formation ( $22.45 \pm 3.73$  %), compared with the control group ( $100 \pm 0.00$  %,  $p < 0.001$ ), in SW-620 colorectal cancer cells after being treated for 24 h and cultured in RPMI-1640 medium for 7 days. In addition, 50 µg/ml of oxymatrine combined with 25 µg/ml of 5-FU could extremely reduce the percent of colony formation ( $3.47 \pm 0.44$  %) in SW-620 colorectal cancer cells ( $p < 0.001$ ) (**Figures 4(a)** and **4(b)**). These results indicated that oxymatrine combined with 5-FU inhibited the ability of a single cell to grow into a large colony, as defined by the loss of reproductive integrity.

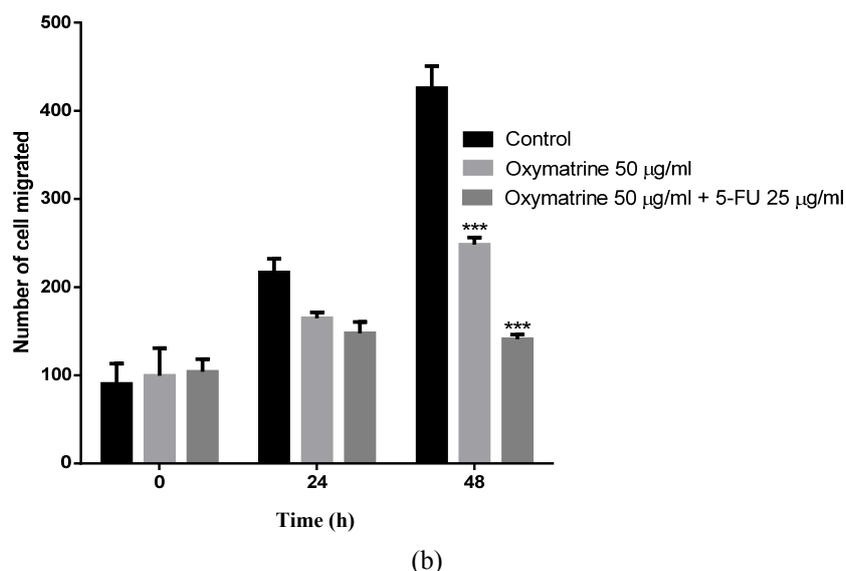


**Figure 4** Effect of oxymatrine and combination of oxymatrine with 5-FU on colony formation in SW-620 colorectal cancer cells. Cells were treated with 50 µg/ml of oxymatrine and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU in 24-well plates for 24 h. Cells were cultured in RPMI-1640 medium with 10 % FBS for 7 days. Cells were fixed with acetic acid: methanol and stained with 0.5 % crystal violet. The number of colonies were photographed with an inverted microscope (5×). The experiments were performed at least 3 times (n = 3). \*\*\*Significantly different at  $p < 0.001$ .

**Effect of oxymatrine on cell migration in SW-620 colorectal cancer cells**

Metastasis is an ability of cancer cells, starting with cell migration in the primary tumor, leading to invasion, extravasation to the blood and lymphatic systems, and colonization in distant organs. The ability of oxymatrine to decrease cell migration was investigated by scratch assay. As displayed in **Figure 5**, the results demonstrated that 50  $\mu\text{g/ml}$  of oxymatrine reduced numbers of cell migration in SW-620 colorectal cancer cells in a time-dependent manner after 24 h ( $164.7 \pm 6.69$  versus  $216.70 \pm 15.6$  in the control group) and 48 h ( $248.0 \pm 8.33$  versus  $425.7 \pm 25.22$  in the control group,  $p < 0.001$ ) treatments. Moreover, 50  $\mu\text{g/ml}$  of oxymatrine combined with 25  $\mu\text{g/ml}$  of 5-FU dramatically decreased numbers of cell migration in SW-620 colorectal cancer cells after 24 h ( $147.70 \pm 12.77$  versus  $216.70 \pm 15.6$  in the control group), 48 h ( $141.0 \pm 5.20$  versus  $425.7 \pm 25.22$  in control group,  $p < 0.001$ ) of wound scratch (**Figures 5(a)** and **5(b)**).

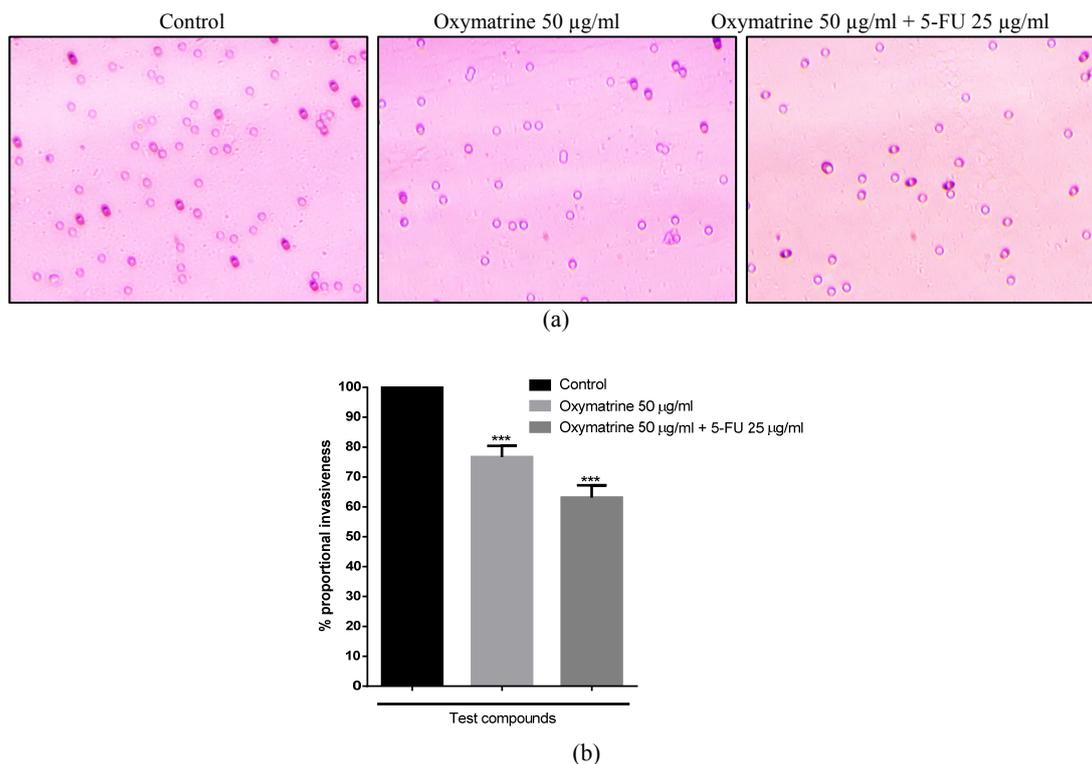




**Figure 5** Effect of oxymatrine and combination of oxymatrine with 5-FU on cell migration in SW-620 colorectal cancer cells. The confluent monolayer of SW-620 cells was scratched with a 200 µl pipette tip. Cells were cultured in 50 µg/ml of oxymatrine and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU for 24 and 48 h. The width of the wound was photographed with an inverted microscope. The experiments were performed at least 3 times (n = 3). \*\*\*Significantly different at  $p < 0.001$ .

#### Effect of oxymatrine on cell invasion in SW-620 colorectal cancer cells

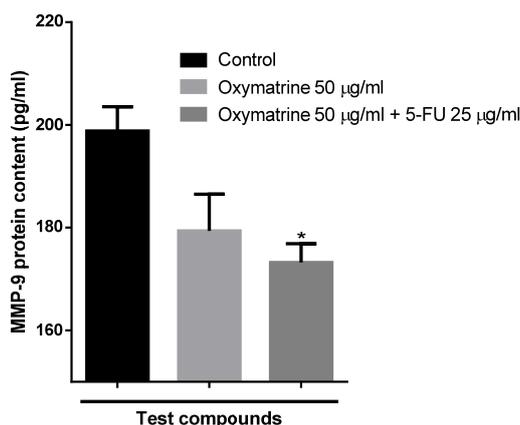
Cell invasion plays a crucial role in cancer metastasis; however, it is also the main problem after the surgical removal of tumors. The invasion of cancer cells begins by cancer cells secreting proteolytic enzymes for the degradation of the extracellular matrix, leading to the cause of cancer cells spreading to distant organs. The ability of oxymatrine to decrease cell invasion through the matrigel-coated invasion chamber was determined by using the matrigel invasion assay to confirm the anti-invasive effect of oxymatrine. As shown in **Figure 6**, the results demonstrated that oxymatrine at 50 µg/ml could inhibit numbers of cell invasion and percent proportional invasiveness in SW-620 colorectal cancer cells after 72 h treatment, when compared with the control group ( $76.70 \pm 2.16\%$  versus  $100.0 \pm 0.00\%$  in the control group,  $p < 0.001$ ) (**Figures 6(a)** and **6(b)**). These effects can be exerted by combination treatment with 5-FU at 25 µg/ml, compared to oxymatrine treatment alone ( $63.10 \pm 2.36\%$  versus  $100.0 \pm 0.00\%$  in the control group,  $p < 0.001$ ). These finding significantly indicate the anti-metastatic effect of oxymatrine on colorectal cancer cells.



**Figure 6** Effect of oxymatrine and combination of oxymatrine with 5-FU on cell invasion in SW-620 colorectal cancer cells. Cells were treated on the matrigel-coated invasion chambers in the presence of 0.1 % methanol, 50 µg/ml of oxymatrine, and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU for 72 h. Migrated cells were fixed with cold methanol and stained with crystal violet. Migrated cells were photographed with inverted microscope and counted in 5 random fields. The experiments were performed at least 3 times ( $n = 3$ ). \*\*\*Significantly different at  $p < 0.001$ .

#### Effect of oxymatrine on the expression of MMP-9 protein level in SW-620 colorectal cancer cells

MMP-9 is closely linked to the degradation of the extracellular matrix, supporting a process of cancer metastasis [8]. To better understand the mechanism of action of oxymatrine on cell migration and invasion in SW-620 colorectal cancer cells, as shown in **Figure 7**, this is the first study which focuses on MMP-9 protein expression in SW-620 colorectal cancer cells. These results clearly demonstrate for the first time that oxymatrine significantly decreased MMP-9 protein expression in SW-620 colorectal cancer cells ( $179.4 \pm 7.18$  pg/ml versus  $198.8 \pm 4.70$  pg/ml in the control group). These effects can be increased by combination with 5-FU, compared to oxymatrine treatment alone and control group ( $173.2 \pm 3.70$  pg/ml versus  $198.8 \pm 4.70$  pg/ml in the control group,  $p < 0.05$ ) (**Figure 7(a)**). This data indicates that oxymatrine inhibited cell migration and invasion through reducing the MMP-9 protein level in SW-620 colorectal cancer cells. These results are extremely consistent with other studies. Zhou *et al.* showed that epigallocatechin-3-gallate inhibited proliferation and migration by suppressing MMP-9 secretion in SW-620 colorectal cancer cells [26]. Taken together with the previously reported glaucine, it is an alkaloid extracted from the plant, inhibiting PMA-induced MMP-9 secretion and proteolytic activity, leading to a decrease in tumor metastasis and invasion in breast cancer cells [27]. It is possible that oxymatrine can decrease other types of MMP or other proteins which assist cancer cells during the metastasis process.



**Figure 7** Effect of oxymatrine and combination of oxymatrine with 5-FU on the expression of MMP-9 protein levels in SW-620 colorectal cancer cells. Cells were treated with 50 µg/ml of oxymatrine and 50 µg/ml of oxymatrine + 25 µg/ml of 5-FU for 48 h. The cell supernatant was measured for MMP-9 protein by the MMP-9 ELISA kit. The data was presented as pg/ml. The experiments were performed at 3 times (n = 3). \*Significantly different at  $p < 0.05$ .

## Conclusions

In conclusion, the results of the present study revealed that oxymatrine has anticancer activity against SW-620 colorectal cancer cells, with oxymatrine strongly decreasing the cell viability in a dose- and time- dependent manner. Moreover, the compound appears to exert the cytotoxic activity of 5-FU on human colorectal cancer cells, and has no cytotoxic effect on normal fibroblast cells. Oxymatrine also potently represses colony-formation, cell migration, and cell invasion, and has a synergistic effect with 5-FU in human colorectal cancer cells through decreasing the MMP-9 protein expression in cell culture medium. These findings suggest that the anti-metastatic activity of oxymatrine in colorectal cancer cells is involved with the alteration of the MMP-9 enzymatic activity. These results indicate that oxymatrine, which is an alkaloid extraction derived from *Sophora flavescens*, can be a novel therapeutic strategy to develop as an anticancer agent for improving the efficacy of 5-FU treatment or for resistance reversal to 5-FU in colorectal cancer patients. Furthermore, the data from this study will be used as a foundation for clarifying the molecular mechanism of action and the safety data of oxymatrine in animal tumor models and colorectal cancer patients in the future.

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