Migration Inhibition Induced by Gypenosides and Its Combination Effect with 5-fluorouracil on Human Colon Cancer SW-620 Cells

Xiaoxia Cheng*, **Shao'an Xue, Zijian Wang, Fengqin He, Bo Wang**
Key Laboratory of Natural Product Development and Anticancer Innovative Drug Research in Qinling, Xi'an 710065, Shaanxi, China
Genetic Engineering Laboratory, College of Biological and Environmental Engineering, Xi′an University, Xi′an 710065, Shaanxi, China
DOI: 10.32629/jcmr.v2i4.547

Abstract: In this study we investigate the migration inhibition of Gypenosides (Gyp) and its combined effects with 5-fluorouracil (5-FU) on human colon cancer SW-620 cells, hoping to explore more potential clinical use of Gyp. Our data implied Gyp could significantly inhibit the migration potential of SW-620 cells including down-regulating matrix Metalloproteinases expression and decreasing cells adhesion ability. What's more, evidence showed cells treated with Gyp exerted serious microfilament network collapse as well as a significant decline in the number of microvilli. A significant migration inhibitory effect was seen in Gyp groups along with the decline of cell adhesion. Further, the combination studies suggested Gyp could synergistically enhance the antitumor effect of 5-FU in SW-620 cells through the apoptosis way. The present study indicated Gyp could prevent cell migration and further enhance the cell killing effect of 5-FU on human colon cancer SW-620 cells.
Keywords: Gypenosides, SW-620 cells, anti-migration, 5-FU, synergisticeffect

1. Introduction

Recent years, colorectal cancer (CRC) has been considered as the third most common malignant tumor and the third cause of death by cancer in the USA [1]. Metastasis has been regarded as the major factor to induce the death with CRC [2], which remains one of the most enigmatic aspects of cancers [3, 4]. Migration of cancer cells plays an important role in the occurrence and development of the tumor [5]. Many patients retain residual disease after surgery, and this can eventually lead to metastasis. Therefore, suppressing the tumor cell metastasis is a crucial target for improving patients' prognosis.

Gypenosides (Gyp), a popular folk medicine in China, is the major components in extracts from Gynostemmapentaphyllum Makino. It exists mainly as dammarane type-triterpene glycosides. Gyp has been widely known for its beneficial effects for treating hepatitis, hyperlipoproteinemia, myocardial ischemia and cardiovascular disease [6]. Studies have demonstrated that Gyp has the activity of anti-inflammatory, anti-oxidative and anti-cancer actions [7]. To our best knowledge, the migration inhibitory effect of Gyp on tumor cells hasn't been sufficiently investigated, thus, in this study, the primary aim was to evaluate the changes in migration of SW-620 cells after treated by Gyp.

5-Fluorouracil (5-FU) is an antimetabolite-based chemotherapeutic drug widely used in treating solid tumors, especially colorectal cancer [8]. But it has been reported only 10-15% of patients with advanced CRC would respond to the administration of 5-FU alone [9]. Therefore, developing innovative strategies to overcome drug resistance in conventional chemotherapy is urgently needed. Botanical medicines are generally plentiful, low cost, and relatively non-toxic in clinical practice, and in many cases, plant extracts are thought to be therapeutically superior to their single isolated constituents [10, 11]. Therefore, plant medicine is increasingly combined with chemical medicines in anticancer drugs, especially in countries where botanical medicines are well-accepted [12, 13]. Some studies have suggested by combination with some botanical and chemical medicines. Chemotherapeutic drugs would exhibit much enhanced anti-cancer efficacy with diminished side effects and complications [14]. The aim of this study was to evaluate the synergistic efficacy of Gyp and 5-FU, hoping to show the utility for identifying herb-chemotherapeutic drug combinations.

2. Materials and methods

2.1 Cell culture

Human colon cancer SW-620 cells were obtained from the cell bank of Chinese Academy of Science, Shanghai, China. The cell line was cultured under standard conditions in L-15 medium containing 10% fetal bovine serum (FBS, HYCLONE, USA), 1% penicillin-streptomycin (100U/mL penicillin and 100μg/mL streptomycin), and 1 mM/L-glutamine solution (Sigma-Aldrich). The cells were sub-cultured every day and incubated at 37°C in humidified atmosphere in an incubator containing 5% CO_2.

2.2 Reagents

Gyp was kindly provided by Ankang Pharmaceutical Institute of the Beijing University. The powder was dissolved in 80% ethanol (EtOH) to get a stock solution of 100mg/mL and finally sterilized by a 0.22 μm membrane filtration. MTT, Paraformaldehyde, Triton X-100, crystal violet and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC-phalloidine was supplied by Molecular Probes Inc. (Carlsbad, CA, USA). Guava Nexin Reagent and Guava ViaCount kits were obtained from Guava Technologies (Hayward, CA, USA). Anti-MMP-9 and -MMP-2 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). All the other reagents were commercial products of analytical grade.

2.3 Cytotoxicity evaluation

Cells (1×105 cells/mL) in 96-well plates were treated with Gyp alone (0, 40, 70, 100 and 130μg/mL; 80% ethanol was used as the solvent control or with Gyp/5-FU, and then the treated cells were incubated for various periods of time (Gyp alone for 24 h; 5-FU alone and Gyp/5-FU for 24, 48 and 72 h), respectively. The cell cytotoxicity was determined through conventional MTT reduction assay [15]. The absorbance at 570 nm was recorded in a microplate reader (Bio-Tek ELX800, USA). The results were expressed as the percentage of MTT reduction, assuming that the absorbance of the control group was 100%.

Another way to test Gyp-induced cytotoxicity is ViaCount assay, which is a rapid, convenient and reliable method to determine the cell viability. Viable cells are shown in left of the bias, but dead cells are shown in right of the bias. Briefly, after Gyp incubation for 24 h, SW-620 cells were harvested and stained with ViaCount reagent kit (4000-0040, Millipore, USA) according to the manufacturer's instructions. The ViaCount reagent differentially stains viable and nonviable cells based on the differential permeability of DNA-binding dyes in the ViaCount reagent. Finally, the cell viability was determined by flow cytometry (Guava easyCyte 8HT, Millipore, Billerica, MA).

2.4 Analysis of cell apoptosis

Cell apoptosis induced by Gyp (70, 100 and 130 μg/mL) and Gyp/5-FU (10 μg/mL 5-FU, 70μg/mL Gyp and 10μg/mL 5-FU/ 70μg/mL Gyp) was quantified by Guava Nexin assay, which utilizes Annexin V-PE to detect the phosphatidylserine on the external membrane of apoptotic cells. The membrane-impermeant dye, 7-amino-actinomycin D, is used as an indicator of cell membrane integrity. Briefly, 100 μL cells in each sample were suspended in a mixture of 100 μL Annexin V-PE and 7-ADD binding buffer. After incubation at room temperature for 20 minutes, samples were analyzed by flow cytometry (Millipore, Boston, MA, USA). The population was separated into four groups: living cells with low-level fluorescence (lower left), the apoptotic cells in earlier stages (lower right), the late apoptotic cells (upper right) and the mechanic injury cells (upper left).

2.5 Immunofluorescence

Cells were treated with different concentrations of Gyp for 24 h. Then, the treated cells were harvested and fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 and blocked with 5% BSA. After cells were blocked, they were incubated with anti-MMP-9and -MMP-2antibodies overnight at 4 °C. After washing with PBS, the samples were incubated with the corresponding secondary antibody performed by immunoglobulin FITC (Zhong Shan Golden Bridge Biotechnology Co., Beijing, China) at 37 ℃ in the dark for 1 h. Finally, the cells were observed by fluorescence microscope (Nikon E600, Japan).

2.6 Cell motility

For trans-well assay, after Gyp incubation (70, 100 and 130 μg/mL) for 24 h, 5 × 105 viable cells were seeded in top chamber with serum-free medium containing 0.3% BSA and medium containing 10% serum was added to the lower chamber of the Corning chamber (polycarbonate filter with 8 mm pore size inserts, Corning Pharmingen, San Diego, CA). After being incubated for 72 h, cells moved to the underside of the membrane were detected by 0.1% crystal violet solution. Cells move to the underside of the membrane were observed by microscope, and the crystal violet adhered in the underside cells were dissolved in 33 % acetic acid, the OD ratio of the solution was measured at 570 nm by microplate reader.

2.7 Adhesion ability detection

After incubation with different doses of Gyp (0, 70, 100 and 130μg/mL) for 24 h, cells were harvested and suspended in fresh L-15 medium containing 10 % FBS, then cells were seeded into the same type of culture dish. Cell adhesion condition was observed with Nikon phase contrast microscope (Nikon E-600) at 0, 6 and 12 h after re-seeded.

Gyp treated cells were firstly fixed with 4 % paraformaldehyde in PBS for 15 minutes at 37 ℃, then permeabilized

with 0.1% Triton X-100 in PBS for 7 minutes and blocked with 1% BSA in PBS for 1 h at 37 ℃. Finally, blocking cells were stained with 5μg/mL FITC-phalloidine for 1h in the dark at 37 ℃. Images were obtained by a laser scanning confocal microscope (TCS SP5, Leica, Germany)

2.8 Scanning electron microscope (SEM) observation

After various treatment, cells in each group were fixed with 2.5% glutaraldehyde, washed with PBS, dehydrated by graded alcohol, displaced, dried at the critical point, gold evaporated, and finally observed under a scanning electron microscope (S-3400N, Hitachi, Tokyo, Japan).

2.9 DAPI staining

In order to observe changes of nuclei morphology of tumor cells after treatment, DAPI staining was used to detect with SW-620 cells after Gyp/5-FU co-treatment for 24 and 48 h. The treated cells were stained by 10 μM DAPI for 15min at 37℃, then observed using a fluorescence microscopy with standard excitation filters (Nikon, Japan). Excitation wavelength was 346nm and emission wavelength was 460 nm.

2.10 Statistical analysis

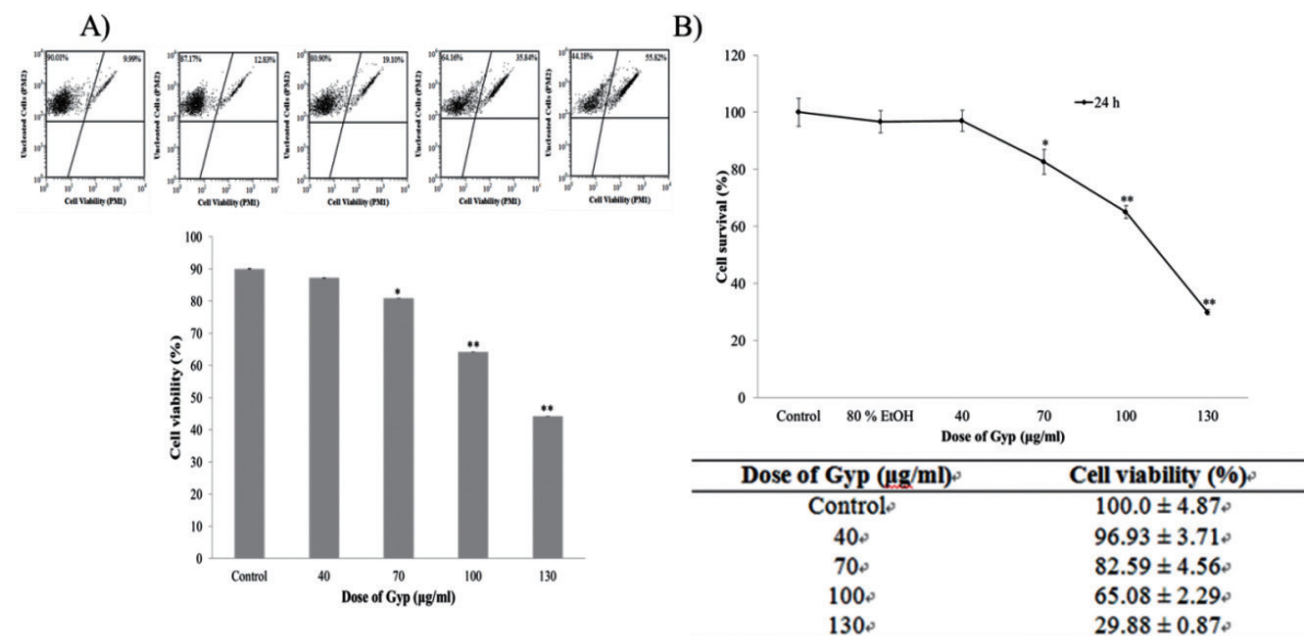
Data are expressed as mean ± standard deviation of at least three independent experiments. Statistical analysis was performed by one-way analysis of variance. Statistical significance was established at* $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1 Effects of Gyp on cell viability

In order to investigate cell viability, after Gyp incubation, MTT assay was employed in the present study. As it showed in Figure 1A, after 24 h Gyp incubation, the proliferation inhibition rate of SW-620 cells increased from 12.83% to 55.82% following the dose of Gyp increased from 40 to 130μg/ml.

To further confirm the proliferation inhibition induced by Gyp, ViaCount assay was accessed. As it plotted in Figure 1B, Gyp inhibited SW-620 cells proliferation in a visible dose manner. At 24 h post treatment, the cell survival ability loss was about 11.61% ($p>0.05$), 17.85% ($p<0.05$), 33.43 % ($p<0.01$) and 51.40% ($p<0.01$) when the concentration of Gyp was 40, 70, 100, and 130μg/ml, respectively. After calculation, at 24 h, the IC50 was 128.59μg/ml. Considering the above results, Gyp could obviously reduce SW-620 cell survival in a dose-dependent manner.



Dose of Gyp (μg/ml)	Cell viability (%)
Control	100.0 ± 4.87
40	96.93 ± 3.71
70	82.59 ± 4.56
100	65.08 ± 2.29
130	29.88 ± 0.87

Figure 1. Effects of Gyp on cell growth inhibition. Cytotoxicity induced by Gyp

Note: SW-620 was measured by MTT assay (1A). Cell viability detection through Viacount assay (1B). * $p < 0.05$, ** $p < 0.01$.

3.2 Influence of Gyp on apoptotic cell death in SW-620

To determine cell death induced by Gyp, apoptotic ratio was measured by Annexin V-PE and 7-ADD staining. The fractions of cells in each quadrant were analyzed by quadrant statistics [16]. As showed in Figure 2, the percentages of cells with Annexin V-positive staining increased gradually in a concentration-dependent manner following 24 h Gyp incubation, suggesting Gyp could induce apoptotic response in SW-620 cells. The apoptotic cells (lower right + upper right) in the untreated control group were only 3.65%. While, the apoptotic cell population (lower right + upper right) increased from 28.25% to 43.55% as Gyp dose increased from 70μg/ml to 130μg/ml.

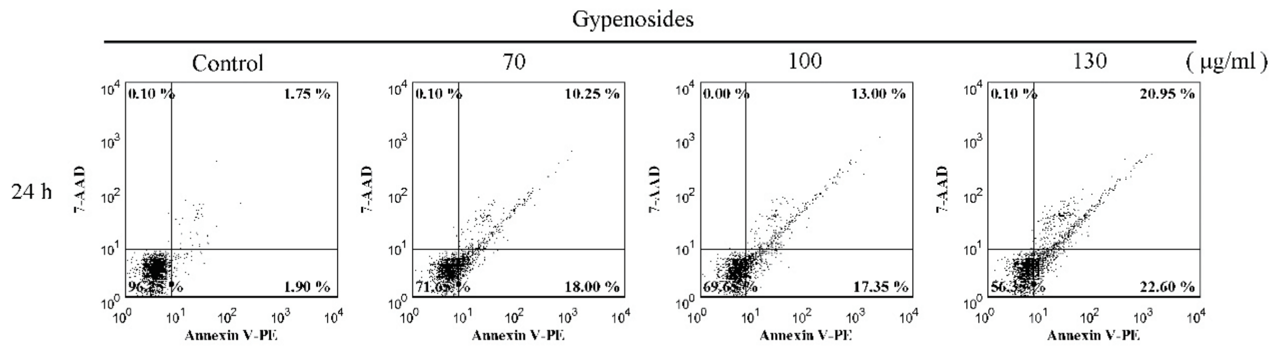


Figure 2. Gyp induced apoptosis on SW-620 cells by Annexin V and 7-AAD staining

3.3 Effect of Gyp on cells motility

Trans-well assay was utilized to explore the migration ability of SW-620 cells after treated by Gyp. As plotted in Figure 3, after incubation with Gyp for 24 h, the migration ability of SW-620 cells was significantly declined in a dose-dependent manner. Cells in 40 and 70 μg/ml Gyp-treated groups (both < IC50 at 24 h) showed an obvious decrease in the ability to move to the underside of the well comparing with the control. When the dose of Gyp increased to 100 and 130μg/ml, nearly all the treated cells lose their ability to migrate the underside of the well.

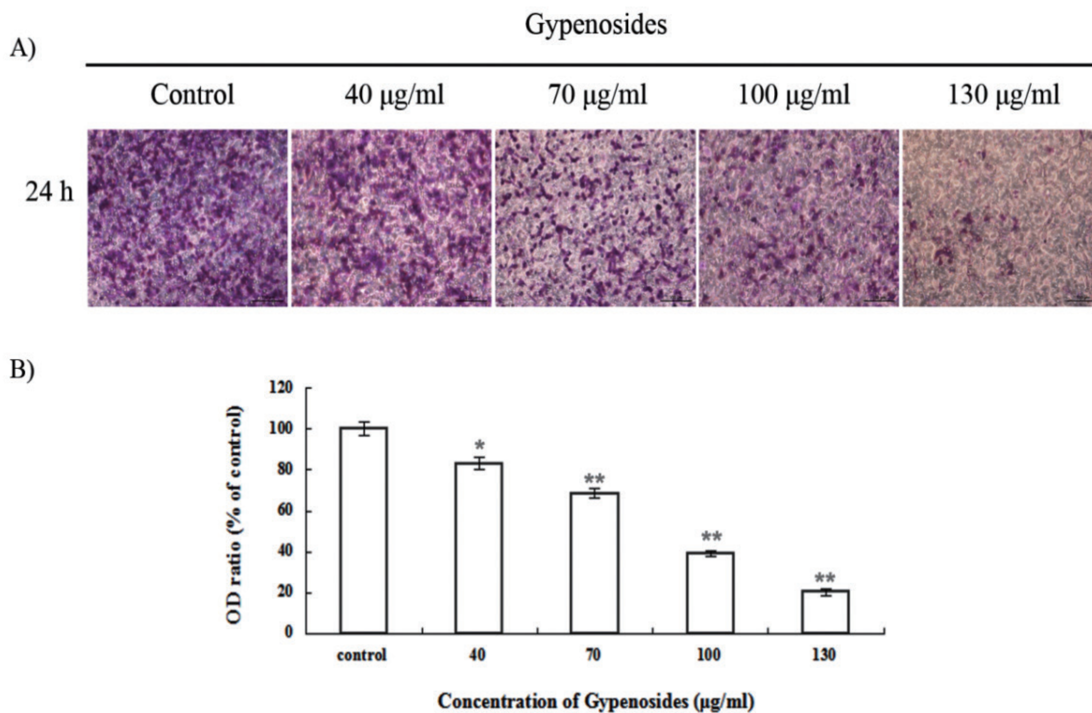


Figure 3. Analysis of changes in migration on SW-620 cells by Transwell assay

Note: * $p < 0.05$, ** $p < 0.01$.

3.4 Effect of Gyp on MMP-2 and MMP-9 expression

MMPs are vertical in cell migration and movement. The level of MMP-2 and MMP-9 expression was detected by immunofluorescence staining in the present study. Figure 4 revealed MMP-2 and MMP-9 were high expressed with bright green fluorescence in control group. For Gyp-treated groups, both enzymes decreased sharply compared to the control, which suggested Gyp could obviously inhibit SW-620 cells migration through down regulating the expression of MMPs.

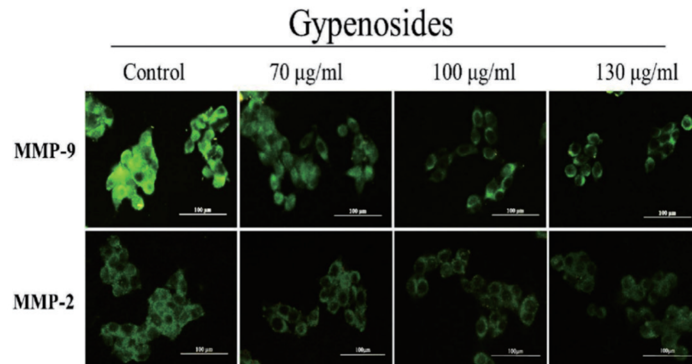


Figure 4. Measurement of MMP-2/9 expression level in SW-620 cells after Gyp treatment

3.5 Effect of Gyp on cell adhesion

After treated with Gyp for 24 h, the adhesion ability of SW-620 cells was observed at 0, 6 and 12 h. Data presented in Figure 5 implied different treated cells performed different adhesion ability. Cell adherent ability increased with the extension of incubation time. In control group, most adhered cells were observed and they started to become the spindle shaped at 6 h. But a few cells in 70μg/ml Gyp treated group could keep their adhesion ability until 12 h. More seriously, after treated by 100 and 130μg/ml Gyp, most cells changed into irregular shape and lose their adhesion capability totally at any time point post Gyp treatment.

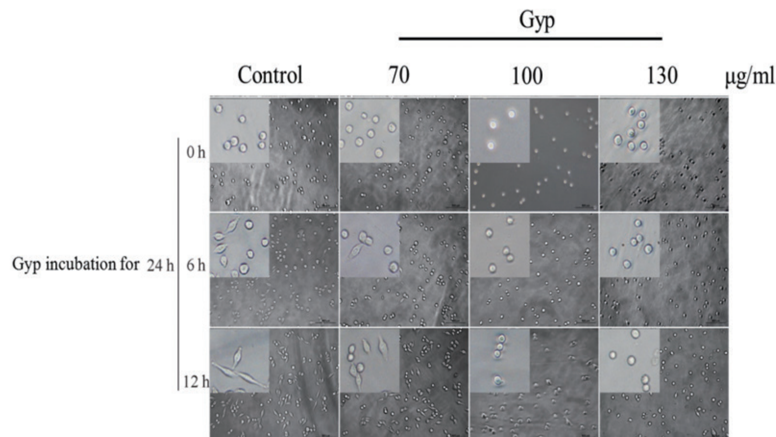


Figure 5. Cell adhesion condition after Gyp at different times

3.6 Effect of Gyp on actin network

Actin cytoskeleton, as one of structural proteins, is essential in cell contraction and cell motility because the functional/structural change of cellular compartments may affect the dynamics of cell adhesion [17, 18]. The change of F-actin microfilaments organization in SW-620 cells after Gyp treatment was investigated in this study. As showed in Figure 6A, control cells showed a regular array of defined actin filaments along the cells, evenly distributed in the cytoplasm; while cells in 70 and 100μg/ml Gyp showed a disorganization of actin filaments. An increase of actin stree fibers and green fluorescence spots were observed. Cells treated with 130μg/ml Gyp displayed an absolute damage of actins network and complete disappearance of actin filaments.

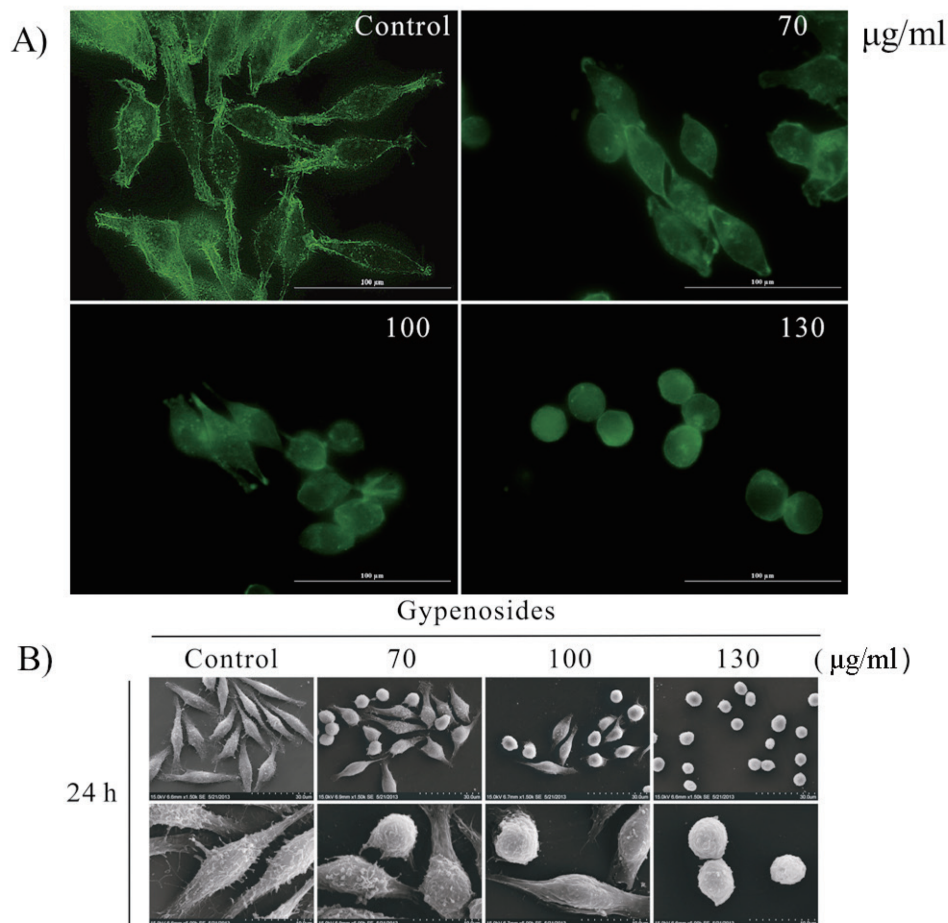


Figure 6. Changes in the actin cytoskeleton and morphology of SW-620 cells

Note: Changes of actin cytoskeleton with phalloidin (6A). Scanning electron microscopic images of SW-620 cells at 24 h after Gyp (6B).

3.7 Effect of Gyp on cell morphological changes

The morphological changes were observed under SEM (Figure 6B). In control group, cells appeared normal spindle cell morphology in shape with numerous microvilli over the surface. While in 70μg/ml, cells showed a significant decrease in the number of microvilli, and the surface of many cells became relatively smooth with no obvious microvilli. When Gyp dose was 100μg/ml, SW-620 cells were seriously damaged with apparent deformation and shrunken to abnormal round type. At the same time, cell number was significantly decreased. While in 130μg/ml Gyp-treated group, some papillary protuberances were observed on the surface of cells where the cytoplasm seemed to have extruded through the membrane boundary.

3.8 Combination of 5-FU and Gyp produced synergistic effects on SW-620 cells

In order to investigate the co-anticancer activity of Gyp and 5-FU, cytotoxic studies were performed in SW-620 cells. As shown in Figure 7, the viability levels of SW-620 cells decreased in a dose-dependent manner. The survival rate of SW-620 cells decreased from 52.2% to 34.7% with increasing doses (5-300μg/ml) of 5-FU at 72 h (Figure 7A). Such proportions decreased from 53.3% to 11.98% when SW-620 cells were co-administrated with Gyp and 5-FU for 24 h (Figure 7B). We also found the cell viability loss in combination cultures (5 μg/ml and 70 μg/ml Gyp) at 24 h was closed to that in 300 μg/ml 5-FU group at 72 h, which inferred Gyp could significantly decreased the concentration and the functional time of 5-FU.

The combination index (CI) for determining synergism and antagonism between Gyp and 5-FU was calculated with CompuSynR 3.01 software (Paramus, NJ) according to the manufacturer's instructions. CI<1, CI=1, and CI>1 indicated synergism, additivity and antagonism, respectively. After calculating, we found 5-FU and Gyp combination yielded synergistic effects with CI values ranging from 0.812 to 0.414 at different effect levels from IC50 to IC70 in SW-620 cells. These results showed the cytotoxic effect of the combination of Gyp and 5-FU was strongly synergistic in human colon cancer SW-620 cells.

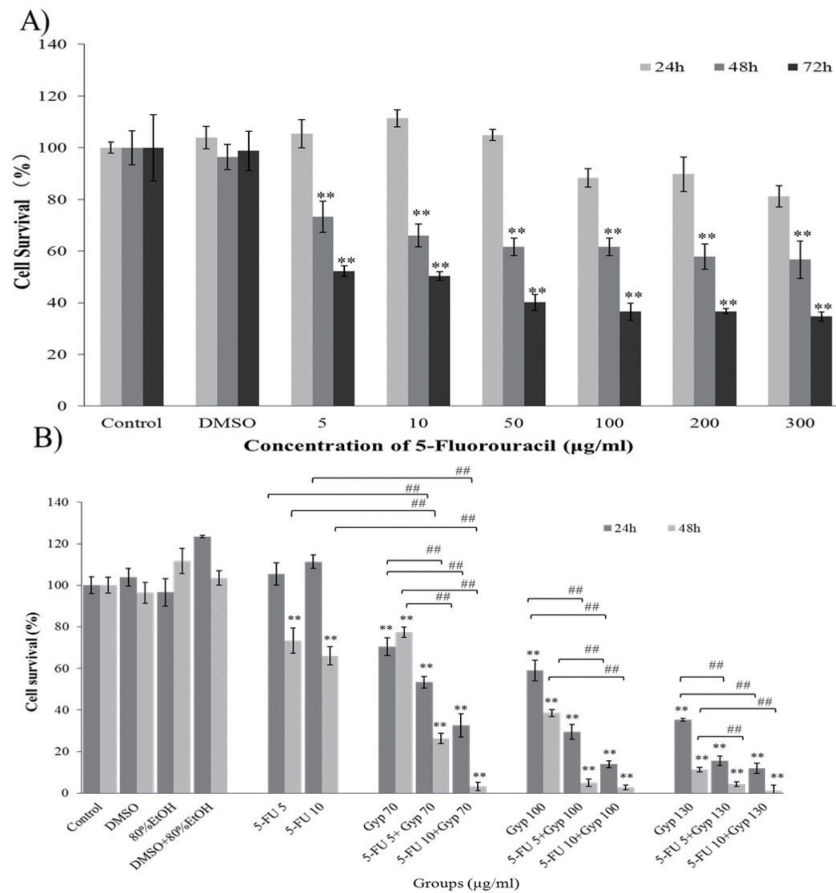


Figure 7. Cell cytotoxicity induced by 5-FU (A) and Gyp/5-FU (B).

Note: * $p < 0.05$, ** $p < 0.01$.

3.9 Combined treatment of SW-620 with 5-FU and Gyp promoted strong apoptosis

It has been well documented that 5-FU could induce cancer cells apoptosis through causing DNA damage [19]. Gyp has also been well demonstrated to trigger cancer cells apoptosis through mitochondria-dependent way [20]. Considering that, Guava Nexin assay was used to qualify the apoptosis ratio after co-incubation. As shown in Figure 8A, 97.15% cells were viable in control group. While, after 24 h incubated with 10μg/ml 5-FU alone and 70μg/ml Gyp alone, the apoptotic cell population (lower right + upper right) increased to 5.1% and 20.55%, respectively. Interestingly, such proportions increased to 40.5% in the co-treatment cultures.

Nuclear condense has been regarded as the hall marker of cell apoptosis, thus, cell morphology was observed under microscope in our hands. As shown in Figure 8B, nuclei of control cells were uniformly stained, and the contrast phase indicated normal SW-620 cell morphology with small islands of epithelial cells. However, cells after co-treatment for 24 and 48h showed significant morphological changes: condensed chromatin and fragmented punctuate blue nuclear fluorescence were seen in a dose- and time-dependent manner. Interestingly, there were no obvious changes in 5-FU and Gyp alone groups, which confirmed the above data.

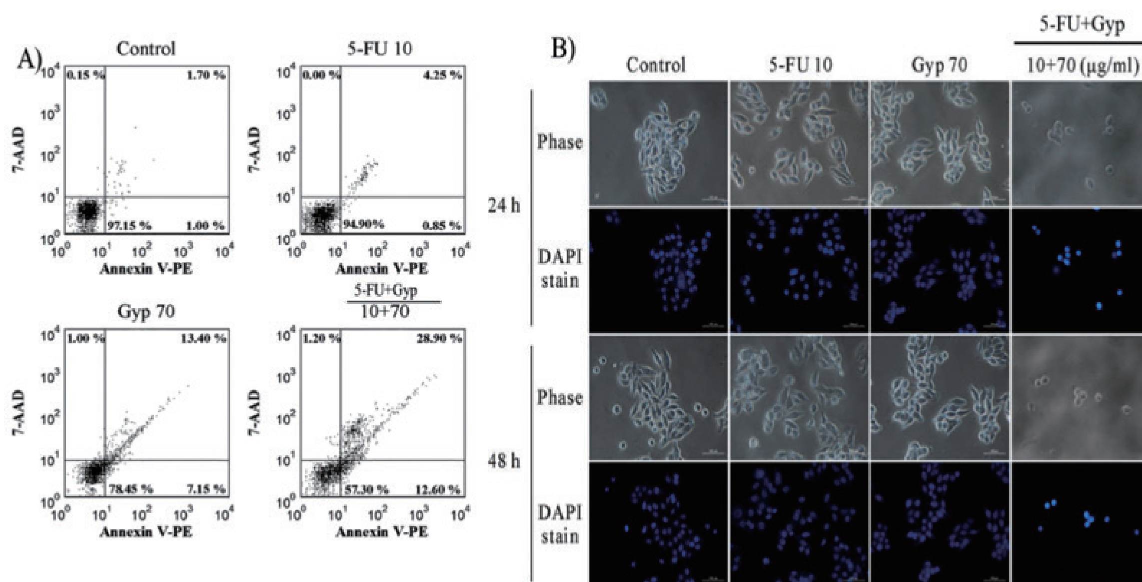


Figure 8. Apoptosis induced by Gyp and 5-FU on SW-620 cells

Note: Annexin V and 7-AAD flow cytometry assay after Gyp/5-FU treatment (8A). The co-incubation of 5-FU and Gyp on cell morphological changes and nuclear DAPI staining. SW-620 cells treated for 24 and 48 h were stained with DAPI (8B).

4. Discussion

Gyp has served as a traditional Chinese herbal medicine for hundreds of years because of its variety benefits on human health [21]. As far as we know, Gyp has been documented to exert migration inhibitory potential on some cancer cells such as human oral cancer SAS and human tongue cancer SCC4 cells [22]. However, due to the fact that different cells may response distinctively even subjected to the same stimulus, in our study, we mainly focused on the responses of colon cancer SW-620 cells post Gyp treatment. Previously, we primarily found Gyp could inhibit cell migration by wound healing assay [23].In the present study, we further carefully explored how Gyp performed the inhibition effect through various biochemical analyses and morphological observations. More importantly, in the present paper, enhanced antitumor potency of Gyp on SW-620 cells have been demonstrated when it was co-administered with 5-FU. We believe the results will provide more evidence to apply Gyp in clinical.

Initially, our data obtained from MTT and ViaCount assay showed Gyp could produce proliferation inhibition on SW-620 cells. Meanwhile, the previous studies in our lab have demonstrated Gyp showed little damage on normal PBMC cells [23]. All the results suggest that Gyp is capable of exerting different alternative cytotoxicity in cancer cells and normal cells, which might well be useful as a cancer preventive or treatment agent.

SW-620 has been always regarded as the cell model to investigate the migration of CRC because it belongs to Duck's stage C (Dukes' C means the cancer has spread to at least one lymph node in the area) [24]. Therefore, in this study, SW-620 cells were selected as the cell model to investigate the anti-metastasis function of Gyp. Our data indicated Gyp could inhibit cells migration and down-regulate the expression of MMP-2 and MMP-9 in a significant dose-dependent manner. And there also has some defects, in the follow-up research, we will continue to carry out the experiment, use the QRT-PCR and Western-blotting assay to dig in the mechanism.

Further, in order to confirm the anti-metastatic activity of Gyp, other important parameters which are related to cell migration, such as the alterations of cell adhesion, cytoskeleton and morphology, were tested in this study. Cell adhesion, together with cell migration and invasion, is an essential process involved in cancer metastasis [25]. Adhesion ability played an important role in cancer metastasis. Our study found there was a significant decline of cell adhesion after Gyp incubation. What's more, cell adhesion is always affected by the functional and structural changes of intermediate filaments. It has been well documented that actin cytoskeleton is a structural network of proteins, which are essential for multiple biological functions including cell contraction, cell motility and vesicle trafficking, et al [26, 27]. Thus, the alterations of actin cytoskeleton after Gyp treatment were assessed to evaluate the cell migration capability. Our results revealed Gyp could cause a complete disappearance of microfilament network. As we know, the collapse of microfilament network firstly injures the cell shape and migration ability, on the other hand, the actin cytoskeleton has been reported to have the dramatic changes in actin

filament organization accompanying different stages of apoptosis [28]. Interestingly, the obtained data revealed Gyp could significantly increase the apoptotic percentage of SW-620 cells, which might be induced by the disorder of F-actin. Besides, the present study also found the number of microvilli was significantly decreased when Gyp dose was 70μg/ml or above, implying Gyp could induce microfilament network collapse, and eventually, injure the cell shape and migration ability.

Nowadays, the traditional treatment for CRC involves surgical bowel resection and chemotherapy, most often by 5-FU. Unfortunately, chemotherapy can't discriminate between normal and cancer cells. Therefore, it always targets areas where cells are replaced at a high rate, such as in the mouth and gut [29], which leads to the development of mucositis (gastrointestinal toxicity). Current mucositis treatments are largely ineffective as they target only the symptoms, but not the pathogenesis of the condition. Thus, it is important to seek new alternative treatments to enhance chemotherapeutic action without compromising the well-being of patients [30]. Nowadays, combing natural products with chemotherapy drugs become more and more popular. Many reports have shown natural products could enhance the anticancer properties of chemotherapy drugs through decreasing the functional time and dose of the drugs. In fact, combing therapy has been applied in clinical in China and India, and most patients react to it very well. What's more, the present and previous study in our lab has proved Gyp is effective at inhibiting the proliferation and migration of SW-620 cells with little damage on normal cells. Therefore, in order to develop new therapeutic options with high efficacy and low toxicity, the present study was trying to investigate the effectiveness of combining a conventional antineoplastic drug, 5-FU, with Gyp in the treatment of SW-620 cells.

Through MTT approach in vitro, Gyp has been found to have the ability to enhance the cytotoxicity of 5-FU by declining its dosage and treatment time in SW-620 cells. A few studies have shown the synergistic effects of combinations of 5-FU with botanical medicines or components. For instance, PekLeng NG has found Piper betle leaf extract synergistically enhances the cytotoxicity effect of 5-fluorouracil [8]. To further confirm whether the combined effect of Gyp and 5-FU were synergistic or not, CI value was evaluated in our hands, CI value was calculated as $0.414<1$, which provide the evidence Gyp administration could synergistically cooperate with 5-FU to inhibit colon cancer proliferation.

Accumulating evidence have shown that Chinese herbal medicines have collaborative effects in combination with traditional chemotherapy, controlling tumor progression [31]. And the synergistic effect might target on different mechanisms [32]. What's more, it has been well documented the combination effect is always related to apoptosis. For example, synergistic effects of 5-Fluorouracil and Gambogenic Acid could activate cell death through the apoptotic and necrotic mechanisms [33]. The above data have demonstrated that Gyp alone could induce SW-620 cells apoptosis. Therefore, investigations related to apoptosis, such as Annexin V/PE and morphological observation, were performed in the combination treated group. Our study further confirmed the cell death observed in the combination treatment group was likely to be triggered by apoptosis pathway.

In summary, these findings together underline Gyp could effectively reduce SW-620 cells adhesion and migration, and the F-actins network disorder after treatment may contribute to the disability of cell migration. Our study found Gyp could synergistically enhance the anticancer effect of 5-FU, and this function might be related to apoptosis. To put forward the basic research of Gyp into clinical application, further investigations both in vitro and in vivo are required.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (NSFC81972883), Natural Science Basic Research Project of Shaanxi Province (2017JQ8040; 2020JM-617; 2020JM-620; 2020JQ888); Shaanxi Provincial Department of Education" Special Project for Emergent Public Health Safety" (20JG025); Shanxi Province University Student Innovation and Entrepreneurship Training Program (S202011080054; S202111080037).

Conflicts of interest

The authors have declared that no conflict of interest exists.

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