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# Regulation of p21, MMP-1, and MDR-1 Expression in Human Colon Carcinoma HT29 Cells by Tian Xian Liquid, a Chinese Medicinal Formula, In Vitro and In Vivo

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## Abstract

*Ethnopharmacological relevance*. Tian-Xian liquid (TXL), a commercially available Chinese medicine decoction, has been used as an anticancer dietary agent for more than 10 years without reported side effects. *Aim of the study*. The safety and quality consistency of TXL and its mechanisms of action on antiproliferation, antimetastasis, and reversion of multidrug resistance (MDR) regimens were explored. *Materials and methods*. In this study, an atomic absorption spectrophotometer and reversed phase high performance liquid chromatography with photodiode array detection (HPLC-DAD) were used to evaluate the main toxic elements and the quality consistency among different batches of TXL extracts, respectively. HT29 human colon cancer cell line and tumor-bearing nude mice were used. TXL was provided by China-Japan Feida Union Company Limited. The effect of TXL on in vitro proliferation of HT29 human colon cancer cell line was examined. The percentages of treated cells distributed in different phases of the cell cycles were analyzed by flow cytometry. Antiproliferative effect after treatment with TXL was assessed by determination of the protein levels of p21, cyclinD1, PCNA, and cdk-2, which are the key regulators for cell cycle progression. Meanwhile, the protein levels of MMP-1 and MDR-1 (multidrug resistance protein-1) were also determined to assess the effect of TXL on antimetastasis and reversion of MDR regimen, respectively. *Results*. The contents of main toxic elements were lower in TXL extract compared with the standard set by the Department of Health of the Government of Hong Kong Special Administrative Region (SAR). Our HPLC results showed that the relative standard deviations of the amount of the 5 standards were less than 5% in different batches of TXL. Immunoblotting analysis revealed a dramatic induction of cyclin kinase inhibitor p21 as well as an inhibition of cyclinD1, PCNA, and cdk-2 in the TXL-treated in vitro models, thereby, impeding cell progression from G1/S phase. Results obtained from the in vivo study also demonstrated that TXL upregulated the protein level of p21 and downregulated the protein levels of MMP-1 and MDR-1. *Conclusions*. Results obtained from the present investigation not only demonstrate the safety and quality of TXL extract but also demonstrate that TXL possesses antiproliferative and antimetastatic activities and brings about reversion of MDR on HT29 cell and on xenografted tissue in tumor-implanted nude mice.

## Keywords

proliferation, metastasis and multidrug resistance, Chinese medicine decoction, Tian-Xian liquid, colon cancer

## Introduction

Colorectal cancer is one of the most common malignancies and the third leading cause of cancer mortality in the world. Similar to people in Western countries, Asians also have a high incidence rate of colon cancer.[1] According to the Hong Kong Cancer Registry, colon cancer is the second most common cancer, and more than 3000 new cases are diagnosed every year.[2] Despite years of intensive research and development, conventional approaches, including surgery, radiotherapy, and chemotherapy, have not been able ensure a cure. Cancer cells display the characteristics of uncontrolled

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growth, invasion, metastasis, and multidrug resistance (MDR). Thus, it is not surprising that scientists are seeking more effective anticancer agents from natural products, which reverse these aberrant characteristics of carcinomas. Chinese Medicine has been widely used for treatment or/and adjuvant treatment of various cancers in Chinese communities. There is considerable interest among oncologists to find anticancer drugs in Chinese Medicine.

Tian-Xian liquid (TXL) has been commercially used as an anticancer dietary supplement for more than 10 years with no known adverse effects.[3] It is an aqueous extract of a Chinese herbal mixture that consists mainly of 10 Chinese medicinal herbs: Radix Ginseng, Cordyceps, Radix Astragali, Radix Glycyrrhizae, Rhizoma Dioscorea, Margarita, Fructus Lycii, Ganoderma, Fructus Ligustri Lucidi, and herba scutellariae barbatae. Previous experiments reported that TXL had inhibitory effects on different carcinomas.[3,4] However, the mechanisms of action of TXL on antiproliferation, antimetastasis, and reversion of MDR on HT29 colon cancer have not been reported so far.

Understanding the control of cell cycle regulators, matrix metalloproteinase, and MDR has the potential to gain widespread significance in cancer therapy. $p21^{CIP1/WAF1}$ is a key cell cycle regulator, which contributes to the arrest of the cell in the G1 phase by inhibiting the activity of the cyclin–cyclin-dependent kinase (cdk) complex, which can also interact with proliferating cell nuclear antigen (PCNA) and inhibit DNA replication.[5] Matrix metalloproteinase-1 (MMP-1) is an enzyme for the degradation of the extracellular matrix and, therefore, considered to be important in facilitating tumor invasion and metastasis.[6] MDR transporter is one of the key mechanisms of cancer drug resistance for different tumor cell types, which impedes effective and successful chemotherapy.[7]

We, therefore, hypothesized that TXL is effective in reversion of the aberrant characteristics of carcinomas through regulating the cell cycle regulators, matrix metalloproteinase, and MDR. In this study, we proposed (1) to evaluate the antiproliferative effect of TXL and its underlying mechanisms using the MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide) assay by analyzing the percentages of treated cells distributed in different phases of the cell cycle using flow cytometry; measuring the translational level of cell cycle regulatory proteins, such as p21, cyclinD1, PCNA and cdk-2; and measuring the tumor size in tumor-bearing nude mice after TXL treatment and (2) to evaluate the antimetastasis and reversion of MDR properties of TXL by measuring the translational levels of MMP-1 and MDR-1, respectively.

Besides, the levels of main elements with toxicity, including arsenic, cadmium, mercury, and lead, were assessed using an atomic absorption spectrophotometer (AAS) to ensure the safety of TXL. A simple, rapid, and valid chromatographic fingerprint with marker standards was developed using high-performance liquid chromatography with photodiode array detection (HPLC-DAD) for assessing quality consistency among the TXL products.

## Materials and Methods

### Herbal Materials

The sample of TXL, a commercially available Chinese medicine decoction, was provided by China-Japan Feida Union Company Limited. It is an aqueous extract of a Chinese herbal mixture that consists mainly of 10 Chinese medicinal herbs: 12.5% Radix Ginseng, 24% Cordyceps, 15% Radix Astragali, 5% Radix Glycyrrhizae, 11% Rhizoma Dioscorea, 4% Margarita, 9% Fructus Lycii, 17% Ganoderma, 0.5% Fructus Ligustri Lucidi, and 2% Herba Scutellariae Barbatae. The sample was kept in the School of Chinese Medicine, the University of Hong Kong.

### Quality Analysis

For detection of toxic elements in the TXL aqueous extract, all standards, including arsenic, cadmium, mercury and lead, were diluted to working concentrations ranging from 1 part per billion to 10 parts per billion in milli-Q water. TXL (500 μL) was added in 50 mL 17% nitric acid to dissolve the metal constituents in the samples and filtered for AAS furnace analysis.

For evaluation of the quality consistency among TXL extracts, 2 batches of TXL (10 mL) were added in 50 mL of methanol, followed by ultrasonication for 30 minutes. After centrifugation, the supernatant was filtered by a 0.45-μm Millex syringe filter unit and then injected in a volume of 10 μL in HPLC. We used 5 standard chemicals that were conspicuous in the HPLC chromatograms of TXL—namely, uracil, adenine, adenosine, liquiritin, and glycyrrhetinic acid—for fingerprint analysis in the present study. The experiment was repeated 3 times for each batch.

Reproducibility and linearity were estimated by performing repetitive injections. The external standard method using a series of mixed standard solutions with concentrations ranging from 5 to 2000 μg/mL was examined. A reversed-phase Waters Bondapak C18 column (3.9 mm × 300 $mm^2$, 5.0 μm, NO. P33081B) connected with a XBridge C18 guard column (4.6 mm × 7.5, 5.0 m) was used; the mobile phase consisted of 0.2% acetic acid (A) and acetonitrile (B) using a gradient program of 0 to 15 minutes, 1% to 5% B; 15 to 35 minutes, 5% to 30% B; 35 to 60 minutes, 30% to 60% B; 60 to 80 minutes, 60% to 80% B. A pre-equilibration period of 15 minutes was used between

individual runs. The flow rate was 1.0 mL/min. The DAD detector was set at 275 nm for obtaining chromatograms with the maximum number of peaks. UV spectra were acquired from 200 to 400 nm. Chromatogram and peak integration were analyzed using the Waters Empower 2 software.

### Cell Culture

Human colon carcinoma HT29 cell line (HTB-38, ATCC, Rockville, MD) was cultured in RPMI-1640 medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotic (100 IU/mL penicillin and 100 mg/mL streptomycin sulfate) (Hyclone, Logan, UT). Cells were incubated at 37°C in a humidified 5% $CO_2$ incubator.

### Cell Proliferation Assay (MTT Assay)

HT29 cells were cultured in 96-well plates at a density of $1 \times 10^4$ cells/0.1 mL/well. Then, 24 hours later, cells were serum starved for 24 hours, and the cells were incubated with serial concentrations of TXL for an additional 48 hours. Inhibition of cell proliferation was assessed by MTT assay as described in a previous article.[8] All reported values are the means of triplicate samples. $IC_{50}$ was calculated using SigmaStat statistical analysis software.

### Cell Cycle Distribution Analysis of TXL-Treated HT29 Cells

HT29 cells were seeded at subconfluent densities and left to adhere for 24 hours. The cells were then serum starved for 24 hours[9] in serum-free RPMI medium, which was then replaced with supplemented RPMI medium containing 1% TXL for 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 48, and 54 hours. Supplemented RPMI medium without TXL treatment served as a negative control. Following the treatment, the cells were fixed with ice-cold 70% ethanol overnight at 4°C. The cells were then washed with cold Dulbecco's PBS (phosphate-buffered saline) twice and stained in cold PBS containing 1 mg/mL propidium iodide solution, 2% RNase A, and 1% Triton X-100 at room temperature for 30 minutes. Cell cycle distribution was then analyzed by flow cytometry using a Beckman Coulter EPICS ALTRA flow cytometer (Beckman Coulter, Inc, Miami, FL).

### Tumor Xenografts in Nude Mice, Drug Administration, and Tumor Specimen Collection

The experiment was approved by the Department of Health, Hong Kong SAR, and Committee on the Use of Live Animals in Teaching and Research (CULATR) of Li Ka Shing Faculty of Medicine, the University of Hong Kong. For the experiment, 5-week-old female nude mice were purchased from the Animal Laboratory Unit, the University of Hong Kong. The mice were kept under sterile conditions in isolated pathogen-free ventilation chambers under an ambient temperature of 22°C to 24C and 50% to 65% relative humidity, with automatic 12-hour light:dark illumination cycles. Cell suspension was obtained by trypsinization of confluent HT29 cells. The HT29 carcinoma was established subcutaneously in nude mice by injecting $1 \times 10^5$ cells into the right thigh of each animal. When the tumors became palpable (size 18 $mm^3$) after xenografting, mice were randomly divided into 2 groups of 6 animals each. TXL (200 μL), human equivalent dose, was administered orally every day for 16 days. The control group received an equal volume of water orally instead of TXL. Tumor volume was measured using a digital caliper every day and calculated using the following formula: length in mm × width in mm × height in mm. The body weights of all animals were recorded throughout the whole experiment as an assessment of drug toxicity. At the end of the experiment, all animals were killed by cervical dislocation. Their tumor specimens were collected and stored at −80°C for further analysis.

### Western Blotting Analysis of TXL-Treated HT29 Cells and Excised Tumor Specimens

HT29 cells that had been treated with 1% TXL ($IC_{50}$) for various time periods, ranging from 6, 12, 18, 24, 36, and 48 hours were lysed, and protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Richmond, CA). About 50 μg of the denatured cellular protein content and excised tumor specimens' protein content were separated on a 12% sodium dodecyl sulfate polyacrylamide gel, and the resolved proteins were transferred to a polyvinylene difluoride membrane (Roche Diagnostics Corporation, Indianapolis, IN). The membrane was blocked in 5% nonfat milk for 1 hour at room temperature, then incubated overnight with a primary antibody specifically recognizing p21 (sc-756), PCNA (sc-56), cyclin D1 (sc-8396), cdk-2 (2B6+8D4), MMP-1 (sc-21731), and MDR-1 (sc-1517). All antibodies, except cdk-2 (2B6+8D4, Neomarker), were purchased from Santa Cruz Biotechnology. The membranes were then washed with Tris-buffered saline Tween-20 thrice and were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology and Dako Cytomation) in Tris-buffered saline Tween-20 for 1 hour. Chemiluminescence detection (GE Bio-health) was accomplished with HRP-conjugated secondary antibody (Santa-Cruz Biotech). The detection was performed using the advanced chemiluminescence Western blotting detection system (GE Bio-health). The band intensities

**Table 1.** Analysis of Main Toxic Elements in TXL Extracts Using AAS, n = 3

| Heavy Metals in TXL | TXL Sample (ppm), Mean ± SD (%RSD) | Calibration Correlation Coefficient (%) | Safety Standard Set by the Hong Kong Department of Health (ppm) |
|---|---|---|---|
| Arsenic | 0.023 ± 0.021 (8.18%)[a] | 99.46 | <2.0 |
| Cadimum | 0.170 ± 0.103 (6.05%)[a] | 99.06 | <0.3 |
| Lead | 0.359 ± 0.257 (7.17%)[a] | 99.94 | <5.0 |
| Mercury | 0.008 ± 0.155 (18.43%)[a] | 99.41 | <0.2 |

NOTES: TXL = Tian-Xian liquid; AAS = atomic absorption spectrophotometer; SD = standard deviation; RSD = relative standard deviation.
[a]The individual toxic element content in TXL extracts is below the safety standard levels set by the Department of Health of the Government of Hong Kong Special Administrative Region.

were quantified by a Bio-Rad Chemi Doc EQ densitometer and Bio-Rad Quantity One software (Bio-Rad laboratories, Hercules, USA) and normalized by anti-GADPH (MAB374, Millipore). Antibodies were stripped by the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc, USA) and reprobed with other antibodies as described above.

### *Immunohistochemical Analysis of p21 Protein Levels in Tumor Tissues*

The excised tumor specimens were fixed for paraffin embedding. Sections of tumor samples of thickness 5 μm were prepared on slides. After blocking with 0.3% hydrogen peroxide in methanol, the sections were further blocked for 30 minutes with 10% normal horse serum to minimize nonspecific background and then incubated overnight with anti-p21 antibody (working dilution: 1:100A) at 4°C. Subsequent steps were performed with the UltraVision LP Value Large Volume Detection System HRP Polymer (Thermo Fisher, CA) and DAB Plus Chromogen (Lab Vision Corp, Fremont, CA). The positively stained cells were visualized by incubating the sections with 3, 3′-diaminobenzidine and Mayer's hematoxylin counterstain at a magnification of 400×.

### *Statistical Analysis*

Data are reported as mean ± standard deviation (SD). All comparisons for statistical significance were made with Student's *t* test, except for immune-blotting analysis in vitro, for which 2-way ANOVA was used. $P < .05$ was considered to be statistically significant. Calculations were made with the commercially available software SPSS (SPSS Inc, Chicago, IL).

## Results

### *The Quality Control*

The proportions of the main toxic elements, including arsenic, cadmium, mercury, and lead, were lower in TXL formulations as compared with the standard in the Department of Health of the Government of Hong Kong SAR. The results are summarized in Table 1.

To explore most of the detectable peaks in the HPLC chromatogram, the spectra of all eluted peaks found in the chromatogram of TXL were investigated using DAD. The chromatograms were generated under the detection wavelengths of 258 nm, 275 nm, and 295 nm. A chromatographic fingerprint showing the elution peaks of 5 standard compounds and other common peaks is shown in Figure 1. Two batches (n = 3 per each) of TXL preparations were examined by HPLC using optimum running conditions. The results of their contents are shown in Table 2. Interassay relative SD (RSD) values were less than 5%.

### *Cytotoxicity Assay*

MTT reduction, a common assay for evaluation of cell viability, was used to assess the cytotoxicity of TXL on HT29 cells in this study. A dose-dependent decrease in MTT reduction activity was observed after treatment with TXL from 0.0781% to 5% (V/V; Figure 2), and the $IC_{50}$ value was 1% (V/V) for TXL. The cytotoxicity of TXL on HT29 cells increased in a dose-dependent manner.

### *Cell Cycle Distribution Analysis of TXL-Treated HT29 Cells*

To examine the mechanism responsible for antiproliferation of TXL-treated HT29 cells, the distribution of cell cycle phases was evaluated using flow cytometry. The results show that TXL treatment triggered a cell cycle arrest in the G1 phase (Figure 3A). From 21 hours, TXL-treated cells were delayed from progressing from the G1 phase to the S phase of the cell cycle, whereas the untreated cells progressed smoothly from the G1 phase to the S phase of the cell cycle (Figure 3B). From 21 hours to 36 hours, the increase in the distribution in the S phase was linked to a simultaneous decrease in the number of cells in the G1 phase, most noticeably for the untreated cells. No significant increase of S and G2/M cells could be demonstrated in

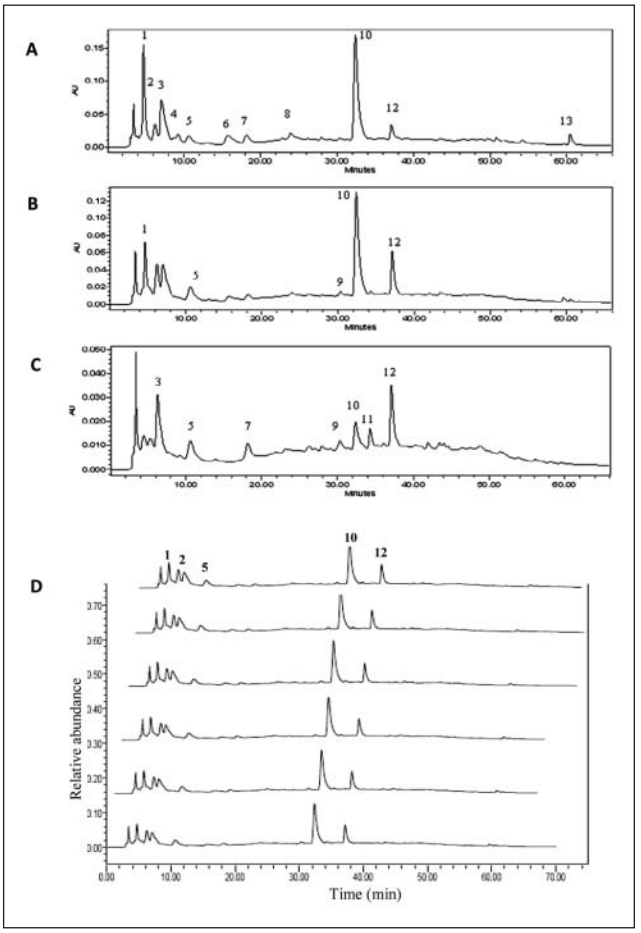


**Figure 1.** Typical HPLC chromatograms of TXL: A. 258 nm. B. 275 nm. C. 295 nm. 1, uracil; 2, adenine; 5, adenosine; 10, liquiritin; 12, glycyrrhetinic acid. D. Representative HPLC chromatograms of batches of TXL monitored at 275 nm; 5 standard chemicals—namely, uracil, adenine, adenosine, liquiritin, and glycyrrhetinic acid—and their respective retention times are shown
NOTES: TXL = Tian-Xian liquid; HPLC = high performance liquid chromatography.

the TXL-treated samples with a significant decrease in G1 cells from 21 hours to 48 hours (Figure 3B), indicating that the TXL-treated cells could not pass through both the S and G2/M phases and were then arrested in the G1 phase of the cell cycle.

### *Tumor Xenografts in Nude Mice*

The body weights of tumor-implanted nude mice with and without TXL treatment were measured. There were significant changes in body weight between treatment groups and the control group for days 11 to 13 (Figure 4A). TXL treatment could significantly diminish the tumor size of xenografted HT29 cells starting from day 4 of the treatment (Figure 4B).

### *Western Blotting Analysis of TXL-Treated HT29 Cells and Excised Tumor Specimens*

To elucidate the mechanisms by which TXL exerted its antiproliferative effect and brought about growth arrest in the G1 phase (Figure 3B), we studied the expression of cell-cycle-related proteins (p21, cyclin D1, PCNA, and cdk-2) by Western blot analysis in HT29 cells and the excised tumor specimens (Figure 5A). With respect to the corresponding controls, TXL treatment upregulated the cyclin kinase inhibitor p21 and downregulated the G1-phase cell cycle regulatory proteins, such as cyclin D1 and cdk-2 in vitro (Figure 5B), which may contribute to the antiproliferative activity of TXL, as demonstrated by the MTT assay (Figure 2) and cell cycle distribution analysis (Figure 3B). Overexpression of MMP-1 in cancer cells will facilitate tumor invasion and metastasis. We have found that TXL treatment could significantly increase the expression of p21 and decrease the expression of MMP-1 both in vitro (Figure 5) and in vivo (Figure 6). Overexpression of MDR-1 in cancer cells could decreased the efficacy of antitumor agents in cancer patients.[10] In this study, we have found that TXL treatment can significantly decrease the expression of MDR-1 in vivo (Figure 6).

### *Immunohistochemical Analysis of p21 Protein Levels in Tumor Tissues*

Immunohistochemical analysis of p21 protein levels in tumor tissues demonstrated that the treatment of TXL for 14 days upregulated the translational expression of p21 protein (Figure 7). Expression of p21 protein was at an undetectable level in the control group.

## Discussion

In this study, an AAS and a reversed phase HPLC-DAD assay were used to evaluate the main toxic elements and the quality consistency among different batches of TXL, respectively. The contents of the main elements in Chinese herbs, toxic to the mammalian reproductive system including arsenic, cadmium, mercury, and lead, have been assessed in this study. Usually, higher contents of these toxic elements in Chinese herbs may be attributed to the uptake of these elements from polluted soil because of industrial and anthropogenic activities.[11] Exposure to arsenic has been associated with carcinogenesis.[12] Exposure to cadmium is associated with activation of some proto-oncogenes that have been associated with enhancement of cell proliferation with damaged DNA and blockage of

**Table 2.** The Contents of 5 Marker Compounds in 2 Batches of TXL (n = 3 Each)

| TXL Batch No. | Uracil (μg/mg)[a] | Adenine (μg/mg)[a] | Adenosine (μg/mg)[a] | Liquiritin (μg/mg)[a] | Glycyrrhetinic Acid (μg/mg)[a] |
|---|---|---|---|---|---|
| TXL1 | 886.74 | 631.96 | 445.89 | 99.81 | 83.32 |
| TXL2 | 892.04 | 648.74 | 452.78 | 100.84 | 83.74 |
| TXL3 | 884.01 | 629.79 | 462.75 | 97.50 | 83.63 |
| TXL4 | 886.18 | 632.48 | 451.72 | 101.38 | 78.20 |
| TXL5 | 893.92 | 630.91 | 451.00 | 101.17 | 83.81 |
| TXL6 | 902.58 | 655.21 | 455.12 | 98.28 | 85.56 |
| Mean | 890.91 | 638.18 | 453.21 | 99.83 | 83.04 |
| SD | 6.84 | 10.92 | 5.58 | 1.62 | 2.50 |
| RSD | 0.77 | 1.71 | 1.23 | 1.62 | 3.01 |

NOTES: TXL = Tian-Xian liquid; SD = standard deviation; RSD = relative standard deviation.
[a]The weight ratio of compound (in μg) to TXL (in mL); HPLC chromatograms for 6 batches of TXL samples.

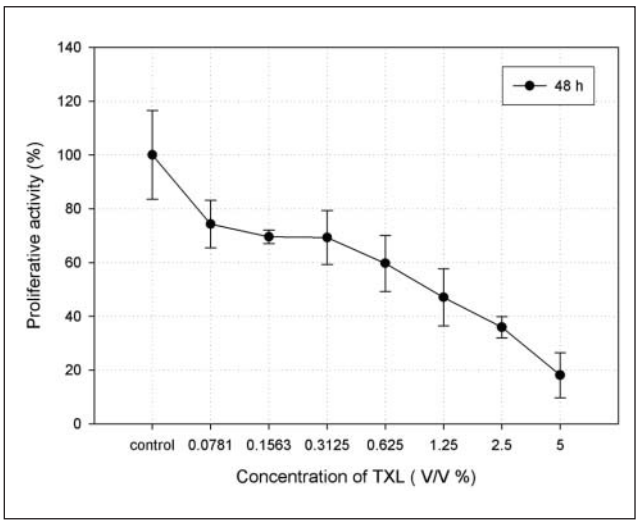


**Figure 2.** Cytotoxicity of TXL on HT29 cells as determined by MTT assay. Cells were serum starved for 24 hours and then treated with TXL at doses of 0.0781%, 0.1563%, 0.3125%, 0.625%, 1.25%, 2.5%, and 5% for 48 hours. The percentage of proliferative activity was calculated as a ratio of $Ab_{595nm}$ of treated cells versus that of control cells[a]
NOTES: TXL = Tian-Xian liquid; MTT = 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide.
[a]Data presented are mean ± standard deviation of 3 independent experiments.

apoptosis.[13] Lead is only weakly mutagenic, but it inhibits DNA repair and acts synergistically with other mutagens and carcinogens.[14] Exposure to mercury and its compounds can be associated with carcinogenicity.[15] Our results demonstrated that the levels of these 4 main carcinogenic toxic elements in TXL were lower compared with the permitted level set by the Department of Health of the Government of Hong Kong SAR.[16] This evaluation ensures the safety of the TXL extract.

Also, a novel, simple, accurate, reliable, and reproducible method to evaluate the quality consistency of TXL among different batches was developed by using the 5 separated compounds—namely, uracil, adenine, adenosine, liquiritin, and glycyrrhetinic acid—as markers in a single chromatographic run at the detection wavelength of 275 nm (Figure 1D). Anticancer therapy with uracil, adenine, adenosine, liquiritin, and glycyrrhetinic acid has been reported. Uracil combined with tegafur has been used in the treatment of metastatic colorectal cancer.[17] Adenine complexes also have an anticancer effect.[18] Adenosine can act as an anticancer agent in lymphoma.[19] The anti–tumor promoting effect of glycyrrhetinic acid has been reported.[20] Liquiritin has the maximum peak area of the TXL chromatographic fingerprint (Figure 1). Therefore, it was selected as an important marker compound. This chromatographic fingerprint with 5 marker compounds is used as a reference standard, indicating the purity, identity, and quality consistency among the TXL extracts. Our results showed that the relative standard deviations of the amount of the 5 standards were less than 5% in our different batches of TXL (Table 2). These results indicated quality consistency among different batches of TXL extracts and also excluded the influence of any unknown variability or instability found in the composition of the active constituents in the molecular investigation of the TXL extract.

This is the first study that clearly characterizes the antiproliferative, antimetastatic, and reversion of MDR properties of TXL in HT29 colon cancer cells and the tumor xenograft. To unveil the mechanism of action involved in TXL-induced growth arrest, antimetastasis, and reversion of MDR, we first inspected the events where TXL inhibits the proliferation of HT29 colon cells via blocking the cell cycle in the G1 phase under serum-stimulation condition after synchronizing HT29 cells; the underlying mechanism of G1 arrest is accompanied by the downregulation of the positive cell cycle regulators, cyclin D1 and cdk-2, and the upregulation of the negative cell cycle regulators, the cdk inhibitor p21.

Cyclins, essential components of the cell cycle machinery, function to bind and activate their specific cdk partners.

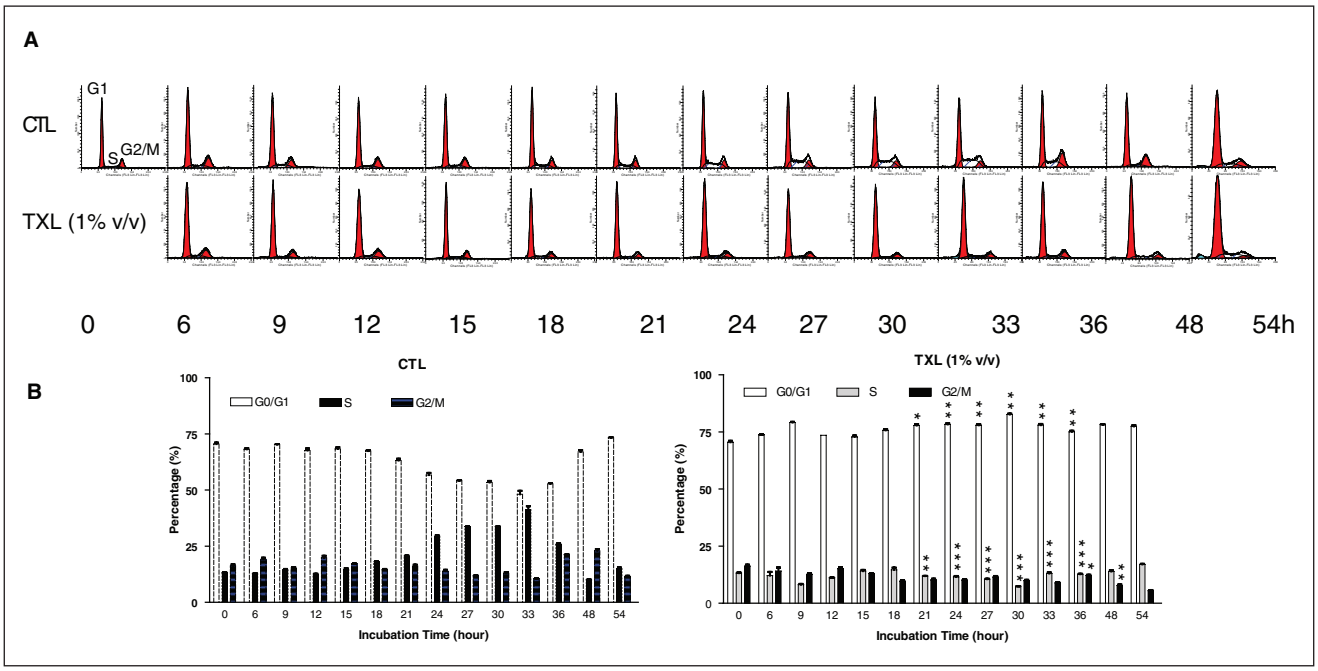


**Figure 3.** Kinetic effects of TXL (1% v/v) on phase distribution of cell cycle in HT29 cells: A. DNA histograms show the phase distribution of cell cycle in HT29 cells treated with1% TXL or Control = CTL (vehicle, 0% TXL) at different time points. B. Distribution of HT29 cells at different phases of the cell cycle with or without TXL treatment for 0, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 48, and 54 hours was analyzed by flow cytometry using ModFit 3.0 LTsoftware. Results are expressed as the percentage of cells in each phase ± standard deviation of 3 independent experiments[a]

NOTES: TXL = Tian-Xian liquid.

[a]Significant difference from the corresponding control (2-way ANOVA): *$P < .05$; **$P < .01$; ***$P < .001$.

D-type cyclins and E-type cyclins are attributed to the progression through the G1 phase of the cell cycle.[21] D-type cyclins activate cdk-4 and -6 specifically, whereas E-type cyclins activate cdk-2. During the progression of the cell cycle, D-type cyclins are active at mid-G1, whereas E-type cyclins are activated at late-G1, just prior to the G1/S transition.[22]

During G1 phase arrest, cyclin-dependent kinase inhibitors (ie, p21) bind the cdk/cyclin complexes,[23,24] thereby, inhibiting their kinase activity on retinoblastoma protein that inhibits the expression of E2F-regulated genes, resulting in blocking of the cell cycle transition from the G1 phase to the S phase.[23,25,26] In the present study, results obtained from flow cytometry demonstrated that TXL causes HT29 cells to accumulate and arrest at the G1 phase (Figure 3). With reference to the findings from our Western blotting analysis, our results revealed that a 3-fold increase of universal cdk inhibitor p21 was detected in HT29 cells after treatment with TXL for 6 to 12 hours (Figure 5). p21 is a key element in regulating cell cycle progression and is associated with inhibition of cyclin D and cdk-2.[27] Cyclin D is a well-known positive regulator of cell proliferation and is also essential for the initiation and progression through the G1 phase,[28] therefore, decreasing the level of cyclin D starting from 36 hours after TXL treatment. It could also in turn prevent the TXL-treated colon cancer cells from entering into the S phase, causing G1 phases (Figure 5B). Interestingly, the protein level of cyclin D1 in TXL-treated HT29 is accumulated and increased significantly (Figure 5B) from 12 to 18 hours compared with their corresponding controls. A previous study reported that an excessive level of cyclin D1 is responsible for the constitutive growth arrest by inhibiting DNA replication and cdk-2 activity through the binding of cyclin D1 to PCNA and cdk-2.[29] p21 also inhibits the cell proliferation promoted by the cyclin D1–cdk-2 complex.[27] Therefore, TXL-induced cell accumulation in G1 phases (Figure 3B) could be mediated at least in part by upregulation of p21 and cyclin D1 after TXL treatment from 6 to 12 hours and downregulation of cyclin D1 from 12 to 18 hours, respectively; the protein levels of PCNA and cdk-2 both decrease thereafter (Figure 5B).

MMP-1, one of the enzyme family members for degrading major constituents of the extracellular matrix barriers, has been associated with cancer cell invasion and metastasis. Therefore, the prognosis for cancer patients with high expression of MMP-1 is poorer than for those with low

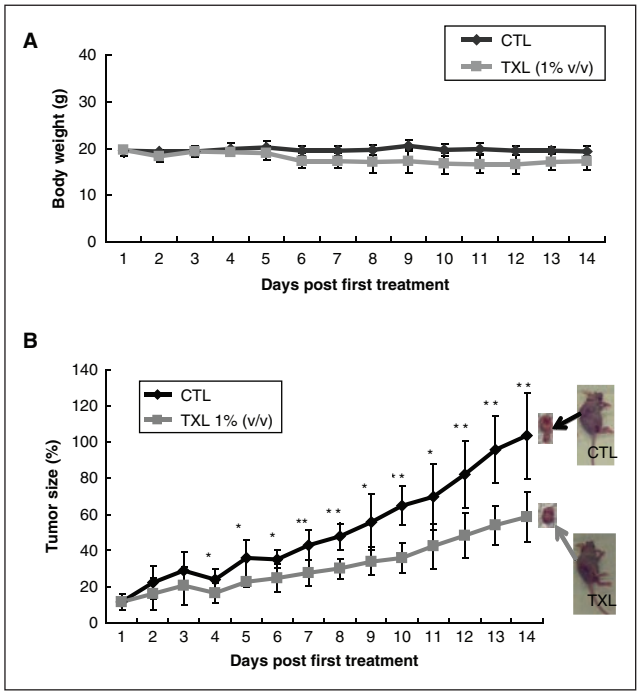


**Figure 4.** The body weight and tumor size analysis of nude mice with HT29 cells implanted and treated with TXL: A. The xenografted nude mice implanted with human colon carcinoma HT29 cells line were treated with TXL for 14 consecutive days. Vehicle controls were fed with Dulbecco's phosphate-buffered saline. The body weight of nude mice was measured. B. The tumor size in xenografted nude mice was also measured (inset, representative images of tumor xenografts and the corresponding nude mice)[a]

NOTES: TXL = Tian-Xian liquid.

Data represent means ± standard deviations; n = 6, *$P < .05$, ** $P < .01$ compared with vehicle control group by independent samples test.

expression of MMP-1.[30,31] Our results demonstrate that the MMP-1 protein level in TXL-treated HT29 cells is lower than that in control cells (Figure 5), which revealed that TXL treatment could reduce cancer invasion and metastasis.

The antiproliferative, antimetastatic effects and reversion of MDR of TXL were further evaluated in HT29 xenografted athymic nude mice. TXL is capable of inducing tumor growth inhibition after 4 days of TXL treatment in vivo (Figure 4B). The body weight of nude mice showed significant differences from day 11 to 13, but no significant difference was found on day 14 (Figure 4A). This could be because the Chinese medicine affected their food consumption behaviour. To unveil the mechanism of action in vivo, we measured the G1 phase regulator proteins, such as p21, cyclin D1, cdk-2 and PCNA, metastasis-related protein MMP-1, and MDR-related protein MDR-1. Results obtained from immune-blotting analysis and diaminobenzidine immunostaining microscopy indicated that TXL inhibited tumor growth at least by upregulation of p21 expression, which is in line with our in vitro results. The protein levels of cyclin D1, cdk-2, and PCNA in TXL-treated xenografts seem to be decreased, but the reduction was not statistically significant. The results obtained from this study suggested that the mechanism of action through which TXL upregulates p21 expression is through a p53-independent pathway. Because of p53 mutation on HT29 cells, upregulation of p21 after TXL treatment was independent of the status of its upstream cyclin kinase inhibitor p53.[23,32,33] Mostly, p53 influences the effectiveness of anticancer chemotherapy, which has played an important role in drug resistance that leads to low efficacy.[34,35] Therefore, seeking the p53-independent anticancer agent will be crucial. Together, these observations suggested that the successful promotion of antiproliferation by TXL treatment in HT29 colon cancer cells and tumor xenograft with nonfunctional p53 has indeed proved that TXL possesses strong antitumorigenic effects irrespective of p53 status of the tumor.

MMP-1 protein level after TXL treatment is lower than that in the control group in vivo (Figure 4). The findings in vivo are line with those in vitro, which reveal that TXL treatment could reduce cancer invasion and metastasis, at least through the downregulation of MMP-1 protein.

MDR has been mostly studied as a major obstacle to successful cancer chemotherapy. MDR in cancer is the ability of cancer cells to become cross-resistant to structurally and functionally unrelated anticancer drugs.[36] MDR-1 encoded by the ATP-binding cassette superfamily B1 (ABCB1) gene is the most typical ATP-dependent drug transporter contributing to drug resistance in cancer cells.[37] Therefore, MDR-1 protein is one of the most noteworthy biomarkers for evaluation of the MDR in cancer cells. Our results demonstrated the significant downregulation of MDR-1 protein expression in TXL-treated colon cancer xenografts without affecting GAPDH (the housekeeping protein); indicating that TXL has a higher potency to reverse in MDR in MDR-1-related cancer drug resistance. Also, because of the limited impact on GAPDH housekeeping protein, TXL could give a higher safety margin at clinical or subclinical concentrations.[38] Although the question of whether herbal medicines affect drug transporters, leading to pharmacokinetic interactions, is still unresolved,[39] our study provided valuable information for further mechanistic investigations of TXL decoction on cancer drug resistance in vivo.

In the present study, the mechanism of action of TXL responsible for antiproliferation, antimetastasis, and reversion of MDR has been elucidated by using HT29 cells and its tumor xenografts in nude mice, which entailed, at least, the p21, MMP-1, and MDR-1-related pathways, respectively.

This study may shed new light on establishing a simple and rapid molecular-mechanism-driven pharmacological discovery platform for screening more effective antitumorigenic drugs and active constituents derived from TXL, which

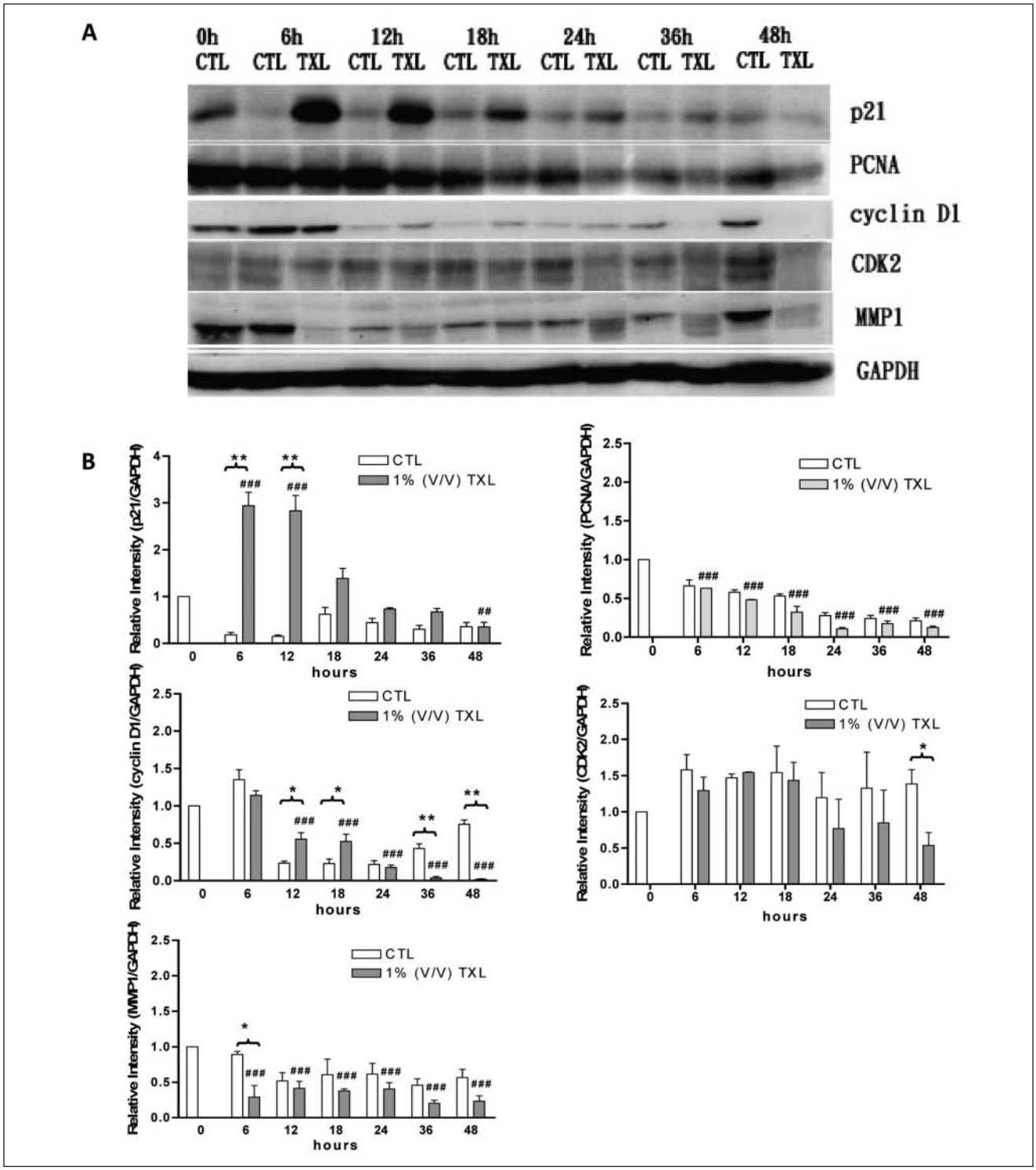


**Figure 5.** Expression of proteins associated with cell-cycle regulatory proteins and MMP-1 by Western blotting analysis in vitro. A. HT29 cells were treated with 1% (v/v) TXL for the different time courses. Total cell lysates were incubated with antibodies against p21, PCNA, cyclin D1, cdk-2, MMP-1, and GAPDH. Photographs of ECL detection shown here represent 3 independent experiments in each case. B. Intensity of the target bands was normalized with GADPH reprobed on the same immunoblot. The relative expressions of the proteins were quantified using Bio-Rad Quatity-One software[a]

NOTES: MMP-1 = matrix metalloproteinase-1; TXL, = Tian-Xian liquid; PCNA = proliferating cell nuclear antigen; cdk = cyclin-dependent kinase.

[a]Results are expressed as means ± standard deviations; n = 3, $^{*}P < .05$, $^{**}P < .01$ compared with corresponding control group at the same time points by an independent samples test, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ compared with the 0-hour control group by an independent samples test.

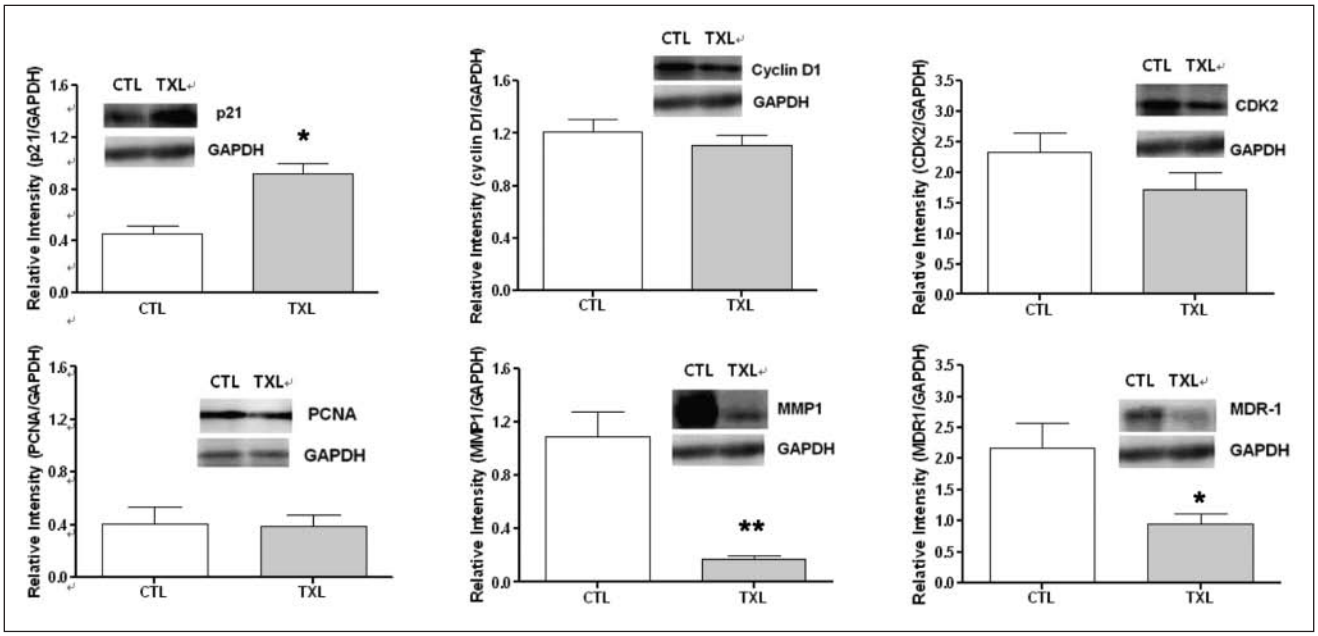


**Figure 6.** Expression of proteins associated with cell cycle regulatory proteins, MMP-1 and MDR-1, by Western blotting analysis in vivo. The xenografted nude mice implanted with human colon carcinoma HT29 cells were treated with TXL for 14 consecutive days. Vehicle controls were fed with Dulbecco's phosphate-buffered saline. On the 15th day, total tissue lysates from tumor xenografts were incubated with antibodies against p21, PCNA, cyclin D1, cdk-2, MMP-1, MDR-1, and GAPDH (inset, representative images of photographs of ECL detection shown here represent 3 to 5 independent experiments in each case. Intensity of the target bands was normalized with GADPH reprobed on the same immunoblot. The relative expressions of proteins were quantified using Bio-Rad Quatity-One software[a]

NOTES: MMP-1 = matrix metalloproteinase-1; MDR-1 = multidrug resistance protein-1; TXL = Tian-Xian liquid; PCNA, = proliferating cell nuclear antigen; cdk = cyclin-dependent kinase.

[a]Results are expressed as means ± standard deviation; n ≥ 3, $*P < .05$, $**P < .01$ compared with the control group by the independent samples test.

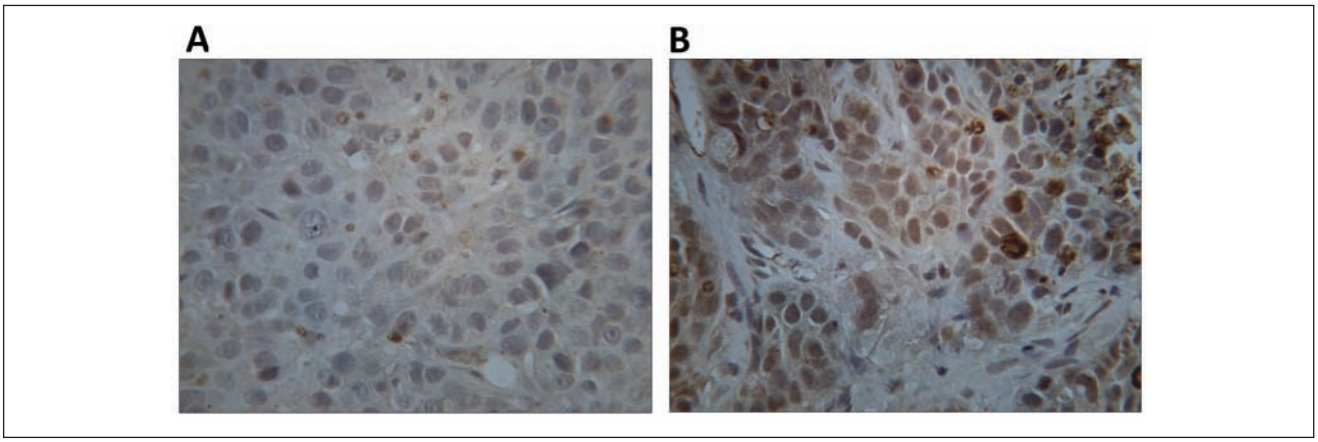


**Figure 7.** The brown color in immunohistochemical staining of tumor xenografts indicating positive signals of p21 by diaminobenzidine stain: A. p21 staining of xenograft tissue section without TXL treatment at 400×. B. Positive p21 staining of xenograft tissue section with TXL treatment at 400×.

NOTES: TXL = Tian-Xian liquid.

will be conducted in our future studies. At this moment, we are performing fractionation of TXL and seeking the bioactive constituents responsible for the antitumorigenic effects. In summary, we have demonstrated that TXL possesses the antitumorigenic effects with a known mechanism of action.

## Conclusion

In this study, we developed comprehensive quality control methods to examine the main elements toxic to the reproductive system, including arsenic, cadmium, mercury, and lead, and to evaluate the quality consistency of TXL among different batches of TXL extract. These ensure the safety and quality of TXL extract. Furthermore, the novel mechanism of TXL for antitumorigencity was explored. TXL treatment upregulated the protein levels of p21 and downregulated the protein level of MMP-1 and MDR-1 and displayed antiproliferative activity. These findings also confirmed that TXL exhibited in antitumorigencity.

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### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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