RESEARCH COMMUNICATION

Inhibition of Tumor Growth in Vitro by a Combination of Extracts from *Rosa Roxburghii* Tratt and *Fagopyrum Cymosum*

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Abstract

Objective: Traditional Chinese herbal medicines have a very long history. *Rosa roxburghii* Tratt and *Fagopyrum cymosum* are two examples of plants which are reputed to have benefits in improving immune responses, enhancing digestive ability and demonstrating anti-aging effects. Some evidence indicates that herbal medicine soups containing extracts from the two in combination have efficacy in treating malignant tumors. However, the underlying mechanisms are far from well understood. The present study was therefore undertaken to evaluate anticancer effects and explore molecular mechanisms *in vitro*. Methods: Proliferation and apoptosis were assessed with three carcinoma cell lines (human esophageal squamous carcinoma CaEs-17, human gastric carcinoma SGC-7901 and pulmonary carcinoma A549) by MTT assay and flow cytometry, respectively, after exposure to extract from *Rosa roxburghii* Tratt (CL) and extract from *Fagopyrum cymosum* (FR). IC_{30} of CL and FR were obtained by MTT assay. Tumor cells were divided into four groups : control with no exposure to CL or FR; CL with IC_{30} CL; FR with IC_{30} FR; CL+FR group with 1/2 (IC_{30} CL + IC_{30} FR). RT-PCR and Western blot analysis were used to detect the expression of Ki-67, Bax and Bcl-2 at mRNA and protein levels. Results: Compared with the CL or FR groups, the combination of CL+FR showed significant inhibition of cell growth and increase in apoptosis; the mRNA and protein expression levels of Ki-67 and Bcl-2 in CL+FR group were all greatly decreased, while the expression of Bax was markedly increased. Conclusions: These results indicate that the synergistic antitumor effects of combination of CL and FR are related to inhibition of proliferation and induction of apoptosis.

Keywords: *Rosa roxburghii* Tratt - *Fagopyrum cymosum* - tumor - combination treatment - proliferation - apoptosis

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Introduction

Lung, stomach and esophageal cancers are the first, second and sixth common causes of death worldwide, respectively (Parkin et al., 2005). In China, 5 year survival rates for lung cancer is less than 20%, for stomach and esophageal cancers is only 5%-20% after surgery, and most patients will suffer postoperative recurrence and metastasis (Parkin et al., 2005; Jemal et al., 2010). Chemotherapy still plays an important role in this setting, although long term survival rate of reported regimens is unstatisfactory. It is imperative and critical to search for chemotherapicutic agents with high effectiveness and low adverse reactions.

Traditional Chinese herbal medicine has begun to attain great popularity for disease prevention as well as being used as complementary medicines for a variety of diseases worldwide. In Southwest regions of China, traditional herbal medicine soup which contained *Rosa roxburghii* Tratt and *Fagopyrum cymosum* was used to cure malignant tumors. However, the mechanism is not well described.

Our objectives were to evaluate the effect and possible mechanism of combination of the extract from *Rosa roxburghii* Tratt (CL) and the extract from *Fagopyrum cymosum* (FR) on proliferation inhibition and apoptosis induction of human esophageal squamous carcinoma CaEs-17, human gastric adenocarcinoma SGC-7901 and pulmonary adenocarcinoma A549 cell lines in vitro.

Materials and Methods

Drugs and chemicals

The extract from *Rosa roxburghii* Tratt (CL) and the extract from *Fagopyrum cymosum* (FR) were obtained from Tongjitang Chinese Medicine Co. (Shenzhen, China). MTT was purchased from Sigma (St. Louis, MO, USA).

Cell lines

The human esophageal squamous carcinoma CaEs-17, human gastric adenocarcinoma SGC-7901 and pulmonary adenocarcinoma A549 cell lines were obtained from Shanghai Institute of Cell Biology (Shanghai, China).

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Table 1. Primer Sequences for RT-PCR

Gene name	Forward primer	Reverse primer	size (bp)
Bax	5’-TTTGCTTCAGGGTTTCATCC-3’	5’-TGTCCCGAAGGAGGTTTATT-3’	246
Bcl-2	5’-CTTCGCCGAGATGTCCAGC-3’	5’-CCAGGAGAAATCAAACAGAGGC-3’	310
Ki-67	5’-GTGCTCTGGGTTACCTGGTC-3’	5’-CAGGTGGAGTGTGCATTACC-3’	215
β-actin	5’-AAAGACCTGTACGCCAACAC-3’	5’-GTCATACTCCTGCTTGCTGAT-3’	219

The cells were grown in RPMI1640 (GIBCO, Grand Ireland, NY, USA) supplemented with 10% fetal calf serum (GIBCO, Grand Ireland, NY, USA) and antibiotics in a 5% CO_2 humidified incubator at 37 ºC.

MTT assay

CaEs-17, SGC-7901 and A549 were seeded into 96-wells micro plate at concentrations of 5×10^4/well and incubated for 24 h in 10% FCS medium before treatment. The cells were then treated with different concentrations of CL or FR for 48 h. Cells incubated in serum-free medium were as control. After incubation for 48 hours at 37 ºC, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well for another 4 h at 37 ºC. Then, 150 μl of dimethyl sulfoxide (DMSO) was added into each well for 2 h at 37 ºC. The optical density (OD) was determined by spectrophotometer (Bio-Rad, Hercules, CA, USA) at 570 nm. Growth inhibition was calculated as percentage ratio between number of treated cells and number of untreated cells. Each assay was performed triplicate. The MTT level of cells before drug-treatment was measured as T_0, T_C and T_D represented O.D. readings of control or drug-treated cells. Inhibition rate%=$1-(T_D-T_0/T_C-T_0)\times100\%$

Experimental groups

IC_{30} (30% concentration of inhibition) of CL and FR were obtained from the previous MTT assay. Tumor cells was divided into four groups according to the treatment as follows: control group with no exposure of CL or FR; CL group with the exposure of IC_{30} CL; FR group with treatment of IC_{30} FR; CL+FR group with the treatment of 1/2(IC_{30} CL + IC_{30} FR).

Apoptosis Assays by flow cytometry

Cells were plated at 1×10^6 in 6-well dishes (Grier) and were allowed to attach after 24 h. After treated according to the experimental groups for 24 h, cells were harvested with trypsinase and washed twice with PBS. Following the manufacturer’s instruction, cells were stained with Annexin V-FITC and PI then processed by flow cytometry. Data acquisition and analysis were done on a BD (Becton Dickinson) FACSCaliber using CellQuest software (BD Biosciences).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

After treated for 24 h, the expressions of Bcl-2, Bax and Ki-67 mRNAs were assessed by RT-PCR analysis. Total RNA was extracted from different treated cells as above using Trizol Reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer recommendations. First-strand cDNA synthesis was performed on 1 μg of total RNA. PCR was performed using 1 μl cDNA as template, 10 pM of each primer (showed in Table 1), 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 U TaqDNA polymerase (Takara, Japan), 1 × reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.01% gelatin in 25 μl reaction volume. The amplification protocol involved denaturation at 94 ºC for 1min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min. This cycle was repeated for 30 times. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide (EB). The mRNA bands were then visualized by UV light.

Western blot analysis

After treated for 24 h, CaEs-17, SGC-7901 and A549 cells were rinsed twice with icecold PBS, then were extracted and the protein concentration was determined by Lowry method. Protein lysates (40 μg) from each sample were subjected to SDS-PAGE on 10% acrylamide gel and the separated proteins transferred to a PVDF membrane. Blots were incubated for 1 hour with 5% nonfat dry milk to block nonspecific binding sites and then incubated with mouse anti-human monoclonal antibody against Bax, Bcl-2, and Ki-67 (Santa Cruz Biotechnology) at 4 ºC overnight. Membranes were then incubated in horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature and washed five times in 0.1% Tween-20 in TBS before antibody binding was detected by using a SuperSignal Chemiluminescent Detection Kit (Pierce, USA). After washing with buffer, the PVDF membrane was rehybridized with a primary antibody for β-actin (Santa Cruz Biotechnology), followed by the same procedures as described above.

Data analysis and research experience

Data were shown as means ± SD and were then analyzed by one-way analysis of variance (ANOVA) by using SPSS software (version 13.0; Chicago, USA). $P<0.05$ was considered statistically significant. The study group has enough experience in conducting medical researches, and has published some results elsewhere (Huang et al., 2004; Zhou et al., 2009; Jiang et al., 2010; Yan et al., 2010; Gao et al., 2011; Huang et al., 2011; Li et al., 2011; Li et al., 2011; Li et al., 2011; Xu et al., 2011; Xu et al., 2011; Xu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Gong et al., 2012; Li et al., 2012; Yu et al., 2012).

Results

Cytotoxicity of CL and FR on the growth CaEs-17, SGC-7901 and A549

The growth curves of human cancer cells after exposures of various dosages of the extract from *Rosa roxburghii* Tratt (CL) or the extract from *Fagopyrum*

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Table 2. Inhibition Effects on the Growth of Tumor Cells. CaEs-17 (human esophageal squamous carcinoma), SGC-7901 (human gastric adenocarcinoma) and A549 (pulmonary adenocarcinoma) cell line after exposure to CL, FR or CL+FR for 48 h

	Group	Concentration (μg/ml)	Inhibition rate (%)
CaEs-17	CL	100	26.87 ± 0.29
	FR	120	25.20 ± 0.42
	CL+FR	50+60	39.58 ± 1.07*#
SGC-7901	CL	70	30.86 ± 1.17
	FR	70	33.89 ± 0.22
	CL+FR	35+35	44.25 ± 0.50*#
A549	CL	75	29.07 ± 1.41
	FR	80	30.91 ± 1.72
	CL+FR	37.5+40	43.62 ± 2.15*#

(CL, extract from Rosa roxburghii Tratt; FR, extract from Fagopyrum cymosum) (means ± SD, n=3) *P<0.05 vs CL; #P<0.05 vs FR

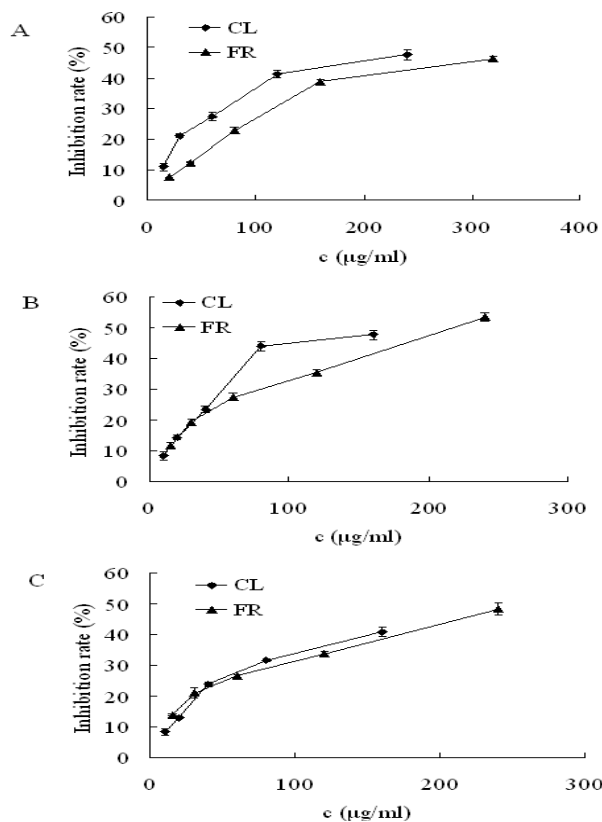

Figure 1. Growth Curves of Tumor Cells. CaEs- 17 (human esophageal squamous carcinoma), SGC-7901 (human gastric adenocarcinoma) and A549 (pulmonary adenocarcinoma) Cell Line after exposure to CL or FR for 48 h

cymosum (FR) for 48 hours were shown in Figure 1. As shown in this chart, significant inhibitions of cell-growth in the dose-dependent manner were observed in cancer cells of esophageal (CaEs-17), gastric (SGC-7901) and lung (A549) (P<0.05). The CL concentration that caused 30% inhibition of growth (IC_{30}) in CaEs-17, SGC-7901 and A549 was approximately 100 μg/ml, 70 μg/ml, and 75 μg/ml, respectively. The FR concentration that caused 30% inhibition of growth (IC_{30}) in CaEs-17, SGC-7901 and A549 was approximately 120 μg/ml, 70 μg/ml and 80 μg/ml, respectively.

The inhibition effects of cell growth treated by IC_{30} CL,

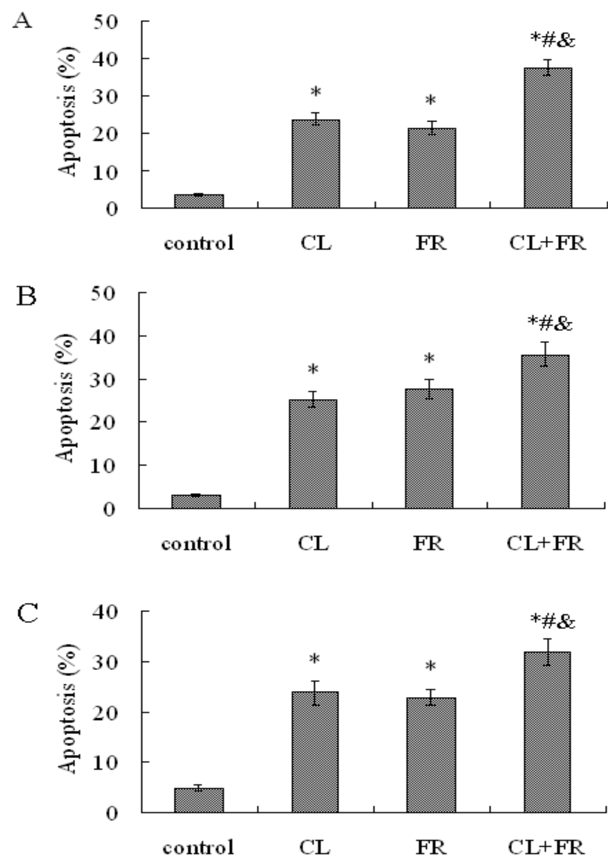

Figure 2. Effects of CL, FR and the Combination on Apoptosis of Tumor Cells. CaEs- 17 (human esophageal squamous carcinoma), SGC-7901 (human gastric adenocarcinoma) and A549 (pulmonary adenocarcinoma) Cell Line for 24 h (CL=extract from Rosa roxburghii Tratt, FR=extract from Fagopyrum cymosum). *p<0.05 vs control, #p < 0.05 vs CL, &p < 0.05 vs FR

IC_{30} FR and 1/2(IC_{30} CL + IC_{30} FR) were shown in Table 2. Compared with CL or FR group, the CL + FR group showed significant inhibition of cell growth in cancer cells of CaEs-17, SGC-7901 and A549 after exposure for 48 hours (P<0.05).

Effects of CL and FR on the cell apoptosis of CaEs-17, SGC-7901 and A549

As showed in Figure 2, compared with the control group, the CL, FR and CL+FR group all significantly increased the apoptosis of CaEs-17, SGC-7901 and A549 (P<0.05). The CL+FR group showed a significant increase on the apoptosis of CaEs-17, SGC-7901 and A549 compared with CL or FR treatment group (P<0.05).

Effects of CL and FR on the expression of Bax, Bcl-2, Ki-67 in CaEs-17, SGC-7901 and A549

RT-PCR and Western blot analysis were used to detect the expressions of Ki-67, Bax and Bcl-2 in mRNA and protein levels. As Figure 3 showed that after exposure to CL or FR alone, the mRNA expressions of anti-apoptotic gene Bcl-2 and proliferation gene Ki-67 decreased, and the expression of pro-apoptotic gene Bax increased in CaEs-17, SGC-7901 and A549.

The down-regulation of Bcl-2 and Ki-67 and the up-regulation of Bax exposed to CL or FR alone were also confirmed in protein level in CaEs-17, SGC-7901 and

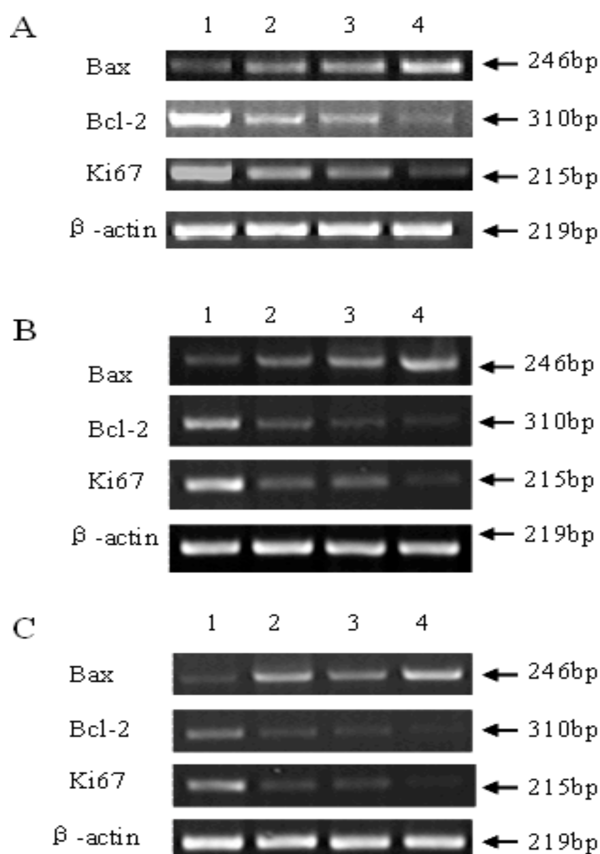

Figure 3. Expressions of Bax, Bcl-2, Ki-67 mRNA in Tumor Cells. CaEs-17 (human esophageal squamous carcinoma, A), SGC-7901 (human gastric adenocarcinoma, B) and A549 (pulmonary adenocarcinoma, C) cell line by different treatment (CL=extract from Rosa roxburghii Tratt, FR=extract from Fagopyrum cymosum). Lane 1: control group; Lane 2: CL group; Lane 3: FR group; Lane 4: CL+FR group

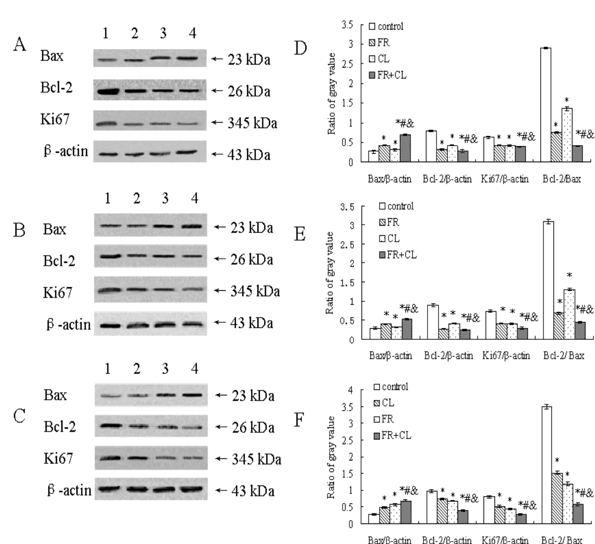

Figure 4. Expression of Bax, Bcl-2, Ki-67 Protein in Tumor Cells. CaEs-17 (human esophageal squamous carcinoma, A and D), SGC-7901 (human gastric adenocarcinoma, B and E) and A549 (pulmonary adenocarcinoma, C and F) cell line by different treatment (CL=extract from Rosa roxburghii Tratt, FR=extract from Fagopyrum cymosum). Lane 1: control group; Lane 2: CL group; Lane 3: FR group; Lane 4: CL+FR group. *p<0.05 vs control, $^{\#}p < 0.05$ vs CL, $^{\&}p < 0.05$ vs FR

A549 by Western blot analysis (Figure 4). In addition, there was significant decrease in the Bcl-2 and Ki-67 expression and significant increase in the Bax expression between CL or FR alone group and CL + FR group on CaEs-17, SGC-7901 and A549.

Discussion

Traditional Chinese herbal medicines have long been used in China in medical practice widely. *Rosa roxburghii* Tratt and *Fagopyrum cymosum* are two conventional medicinal plants which both have the functions of improving immune response, enhancing digestive ability and anti-aging effect recorded in the Compendium of Materia Medica. Current studies show that extract from *Rosa roxburghii* Tratt (CL) displays potent effects of anti-aging, deintoxication and anti-mutation (Zhang et al., 1996; Qiang et al., 2001; Xu et al., 2006) and extract from *Fagopyrum cymosum* (FR) has the activity of anti-cancer (Samel et al., 1996; Chen et al., 2001; Pui-Kwong et al., 2003). We estimated that the combination of the two active extractions would enhance the inhibition of proliferation of tumor cells.

Our experiment combined CL with FR to interfere in the growth of human esophageal squamous carcinoma CaEs-17, human gastric carcinoma SGC-7901 and pulmonary carcinoma A549 cell lines in vitro. We assessed the inhibitory rates of proliferation by MTT assay and early apoptosis rates using flow cytometry. The inhibition rates of CL or FR alone on the proliferation of CaEs-17, SGC-7901 and A549 all increased as the dosage rose and demonstrated dose-dependent manner; meanwhile, the early apoptotic rates also augmented with the rise of dosage of CL or FR alone, and the combined group showed statistically significance compared to CL or FR group alone.

We found that the induction of apoptosis of CL and FR were aimed at some specific proteins. The expressions of Bcl-2 and Bax genes are associated with the inhibition and induction of apoptosis in a variety of cancer cells (Matsumoto et al., 2004; Wang et al., 2004; Zhou et al., 2008). Bcl-2 protein involves in the inactivation of an inner mitochondrial permeability transition pore and the regulation of matrix Ca^{2+}, pH and voltage. Bax protein however acts as the pro-apoptotic regulator through the way of inducing the opening of the mitochondrial voltage-dependent channel. The quantities of mRNA or protein of Bcl-2 or Bax thus could demonstrate the apoptotic activities in CaEs-17, SGC-7901 and A549 cell lines. Ki67 protein is a cellular marker for proliferation and is strictly associated with cell proliferation (Takeuchi et al., 2003). It often correlates with the clinical course of cancer and predicts the prognosis, recurrence and metastasis for the carcinomas of the prostate and the breast. Our results showed that after exposure to CL, FR or CL+FR, they all showed the phenomena of up-regulation of the expression of Bax and down-regulation of the Bcl-2 and Ki67. Moreover, compared with CL or FR group alone, the expressions of Bax, Bcl-2 and Ki67 were significantly increased in the combined group in CaEs-17, SGC-7901 and A549 cell lines.

Dai ZK et al proposed that CL an extract of *Rosa roxburghii* Tratt had the inhibitive effect on the growth of gastric carcinoma cell line in vitro (Dai et al., 2005). Chen et al. (2006) showed that FR induced apoptosis and down-regulated the telomerase activity in HL-60 cells in vitro (Chen et al., 2006). To our knowledge, this is the first time combining CL and FR to assess their inhibitory effects on the growth of human esophageal squamous carcinoma, gastric carcinoma and pulmonary carcinoma cell lines. We found that both CL and FR had potent ability to inhibit the proliferation and induce the apoptosis of the three different cell lines in a dose-dependent manner in vitro. Our results also showed that compared with the CL or FR alone, the combined group had statistically significance on the prevention of growth of human esophageal squamous, gastric and pulmonary carcinoma cells and the synergistic effects of combination of CL and FR were identified.

In conclusion, our study showed that the extractions from traditional Chinese herbal medicines were promising alternative treatments for cancer. Our results also reached an excellent agreement on the synergistic effects of the combination of CL and FR on the growth inhibition of the three cancer cell lines on the levels of cell apoptosis rates, mRNA and protein in vitro.

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