Synergetic effects of aqueous extracts of Fuzi (*Radix Aconiti Lateralis Preparata*) and Tubeimu (*Rhizoma Bolbostemmatis*) on MDA-MB-231 and SKBR3 cells

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**OBJECTIVE:** To test the synergistic effects of the aqueous extracts of Tubeimu (*Rhizoma Bolbostemmatis*) and Fuzi (*Radix Aconiti Lateralis Preparata*) on MDA-MB-231 and SKBR3 breast cancer cells.

**METHODS:** A combined index was created for the effects of Tubeimu (*Rhizoma Bolbostemmatis*) and Fuzi (*Radix Aconiti Lateralis Preparata*) extracts. Cell proliferation was performed by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays. Flow cytometry was used to assess cell cycle distribution and apoptosis. Cell migration was determined by wound-healing and transwell assays. Confocal microscopy was used to detect E-cadherin and actin filaments.

**RESULTS:** The aqueous extract from Tubeimu (*Rhizoma Bolbostemmatis*) and Fuzi (*Radix Aconiti Lateralis Preparata*) exerted synergetic effects on the growth of MDA-MB-231 cells and G1 phase arrest. When exposed to extracts at concentrations of 62.5 µg/mL and 62.5:31.3 µg/mL, the combination index was 0.83 and 0.74, respectively. Interestingly, 62.5:31.3 µg/mL of combined drugs enhanced the inhibitory effect of Tubeimu (*Rhizoma Bolbostemmatis*) on the migration of SKBR3 cells and reduced the stimulative effect of Fuzi (*Radix Aconiti Lateralis Preparata*) in which cells showed an increased expression of E-cadherin and reorganization of actin filaments.

**CONCLUSION:** Tubeimu (*Rhizoma Bolbostemmatis*) and Fuzi (*Radix Aconiti Lateralis Preparata*) extracts...
had synergic effects on MDA-MB-231 and SKBR3 cells.

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**Key words:** Radix Aconiti Lateralis Preparata; Bolbostemma Paniculatum; Drugs, Chinese Herbal; Breast neoplasms; MDA-MB-231; SKBR3

### INTRODUCTION

Traditional Chinese Medicine (TCM) has been used to treat various diseases for more than 2500 years. Based on TCM theory, we designed a warming and relieving cold phlegm formula (WRCP), composed of Fuzi (Radix Aconiti Lateralis Preparata), Tubeimu (Rhizoma Bolbostemma), Shanglu (Radix Phytolaccae), Sanqi (Radix Notoginseng), and Bihu (Gekko Swinhonis). WRCP was shown to have direct anti-cancer effects in vitro and in vivo. In a human breast MDA-MB-231 xenograft model, WRCP could effectively inhibit tumor growth and induce tumor necrosis without changing body weight or organ indexes.\(^5\) A recent study indicated that Tubeimu (Rhizoma Bolbostemma) has been used to treat breast cancer for more than 270 years in TCM.\(^6\) A study indicated that Tubeimu (Rhizoma Bolbostemma) could induce apoptosis in the human breast cancer cell line MDA-MB-231. Tubeimoside I and acetylbenzoyloacine, the respective major active ingredients of Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata), also showed antitumor activities in several cancer cell lines.\(^7\) These results suggest possible synergistic effects between Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata). Here, we aimed to explore the effect of Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata) on MDA-MB-231 and SKBR3 breast cancer cells.

### MATERIALS AND METHODS

**Herbs and preparation of aqueous extracts**

Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata) were obtained from the Manufacturing and Distributing Branch of the Sichuan Chinese Herb Company on January 8, 2012 (Xi-er-duan, Yi-an-lu, Chengdu, China). The herbs were authenticated by Dr. Wu Jun-Qing at Si-Yang Medical Institute of Meishan. A voucher specimen (No. 20120108) was deposited in the Zhong-Shan-Men Inpatient Department of the Tianjin Medical University Cancer Institute and Hospital. Whole herbs of Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata) (100 g each) were ground into powder and extracted twice with hot distilled water (1000 and 800 mL, respectively) at 100 °C for 30 min. The solutions were combined and centrifuged at 2.2 g for 10 min. The resulting supernatant was filtered, concentrated, and dried to obtain residue (Fuzi: 36.01 g and Tubeimu: 34.03 g). The residue was dissolved in phosphate buffered saline at a concentration of 10 mg/mL and stored at −20 °C as the stock extract solution.

**Cell culture and reagents**

MDA-MB-231 and SKBR3 breast cancer cell lines were obtained from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) in a free gas exchange with atmospheric air. SKBR3 cells were cultured in DMEM medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO\(_2\) incubator.

**Trypan blue exclusion assay**

MDA-MB-231 cells were seeded at 1 × 10\(^4\) cells/well in 24-well plates (Corning, NY, USA). Cells were collected 48 h after treatment with Tubeimu (Rhizoma Bolbostemma) at 15.6-500 µg/mL, Fuzi (Radix Aconiti Lateralis Preparata) at 15.6-500 µg/mL or the combination of Tubeimu (Rhizoma Bolbostemma) and Fuzi (Radix Aconiti Lateralis Preparata) at the weight ratio of 1:1, 2:1, or 1:2. The trypan blue exclusion assay was used to determine viable and total cell numbers. This method offers quantitative definitions for antagonism, additive effect and synergism in drug combinations and is widely used in drug combination studies.\(^1\) The fraction affected, where 1 is equivalent to 100% inhibition. Combination index (CI) was calculated using the CI-isobologram method based on data derived from cells treated with single drugs and drug combinations at the indicated weight ratios and plotted versus Fa. A CI value of less than, equal to, or more than 1 indicates synergistic, additive, and antagonistic effects, respectively. CI values were calculated using the equation: CI = (D1)/(Dx1 + (D2)/(Dx2). (Dx1 and (Dx2 are the concentrations of Fuzi (Radix Aconiti Lateralis Preparata) and Tubeimu (Rhizoma Bolbostemma) alone at x% growth inhibition. (D1 and (D2 are the concentrations of the drugs in combination at x% growth inhibition. (Dx1 and (Dx2 were calculated using the median-effect equation: Dx = Dm(Fa/[1 − Fa]) 1/m. Dm is the median-effect dose and m is the slope of the median-effect plot.

The percentage of cell survival was calculated as follows: percentage of cell survival (%) = (mean viable cell number/mean total cell number) × 100%. Morphological variations of the cells were observed by optical microscopy at 48 h after drug treatment.
MTS assay
1×10^4 SKBR3 cells in 100 µL of culture medium were seeded in each well of a 96-well plate. Cells were then divided into tubeimose I (Bellancom, Beijing, China) groups (3-10 µg/mL), acetylbenzoylalacrine (Bellancom) groups (1.5-20 µg/mL), and combination groups at weight ratios of 1:1, 2:1, or 1:2. Tubeimose I and acetylbenzoylalacrine are the respective major ingredients of Tubeimu (Rhizoma Bolbostemmatiss) and Fuzi (Radix Aconiti Lateralis Preparata) (Figure 1). At 44 h after drug treatment, 20-µL MTS reagent (Promega, Madison, WI, USA) was pipetted into each well of the 96-well plate. The plate was then incubated at 37 °C for 4 h and the optical density (OD) values were recorded at 490 nm.

Flow cytometry analysis
MDA-MB-231 and SKBR3 cells were seeded at 3×10^4 cells/well in 6-well plates. Cells were collected 48 h after adding Tubeimu (Rhizoma Bolbostemmatiss) (62.5 µg/mL) and/or Fuzi (Radix Aconiti Lateralis Preparata) (62.5 µg/mL). AsO3 was used as a positive control. To detect apoptosis in early and later stages, cells were stained with FITC Annexin V and PI (BD Biosciences, Franklin Lakes, NJ, USA), respectively. For cell cycle analysis, cells were fixed with 75% ethanol and stained with PI alone (50 µg/mL). Apoptosis and DNA distribution were analyzed by flow cytometry (BD FACSCalibur, Franklin Lakes, NJ, USA).

Enzyme-linked immunosorbent assay
MDA-MB-231 cells were seeded at 3×10^4 cells/well in 12-well plates. After 24 h, the culture medium was replaced with medium containing Tubeimu (Rhizoma Bolbostemmatiss) (62.5 µg/mL) and/or Fuzi (Radix Aconiti Lateralis Preparata) (62.5 µg/mL). Cells were then incubated an additional 72 h. The cell monolayers were then lysed with 100 µL of RIPA buffer for 30 min, collected, and centrifuged at 9.0×g for 5 min at 4 °C. The supernatants were collected and the concentration of aquaporin (AQP) 1 was measured using AQP1 enzyme-linked immunosorbent assay kit (Dongge, Beijing, China) according to the manufacturer’s instructions.

Wound-healing assay
MDA-MB-231 and SKBR3 cells were serum-starved for 24 h then seeded at 1×10^5 cells/well in 24-well plate. After 24 h, a linear wound approximately 1 mm in width was made by scratching the monolayer cell culture with a pipette tip. For cell growth, 2% FBS of culture medium containing Tubeimu (Rhizoma Bolbostemmatiss) (62.5 µg/mL), Fuzi (Radix Aconiti Lateralis Preparata) (62.5, 31.3 µg/mL), or combined drugs (62.5:62.5 or 62.5:31.3 µg/mL) were added. Cell migration was observed under a phase-contrast microscope and expressed as the spacing of scarification at 0 and 12 h after exposure to drugs.

Transwell assays
MDA-MB-231 and SKBR3 cells were serum-starved for 24 h and resuspended in serum-free culture medium. Approximately 5×10^5 cells were seeded into each upper transwell chamber (Millipore, Boston, MA, USA), while the lower chambers were supplemented with 10% FBS of culture medium as well as Tubeimu (Rhizoma Bolbostemmatiss) (62.5 µg/mL) and/or Fuzi (Radix Aconiti Lateralis Preparata) (62.5, 31.3 µg/mL). To determine the effect of the active ingredients of Tubeimu (Rhizoma Bolbostemmatiss) and Fuzi (Radix Aconiti Lateralis Preparata) on cell migration, lower chambers were supplemented with tubeimose I (3-10 µg/mL) and/or acetylbenzoylacrine (3-10 µg/mL). Cells were then incubated at 37 °C for 12 h, at which point cells on the inner surface of the upper chamber were removed. Cells that penetrated through the chamber were fixed, stained with crystal violet (0.1%), and counted using a light microscope (Olympus, Tokyo, Japan).

Figure 1 Chemical structure of acetylbenzoylacrine and tubeimose I
A: Acetylbenzoylacrine, formula: C_{18}H_{22}NO_{11}, molecular weight: 645.74; B: tubeimose I, formula: C_{18}H_{16}O_{10}, molecular weight: 1319.46.
Confocal microscopy
SKBR3 cells were serum-starved for 24 h and seeded at 1 × 10^5 cells/well in 6-well plates. Cells were then divided into the Tubeimu (Rhizoma Bolbostemmatis) group at 62.5 µg/mL, Fuzi (Radix Aconiti Lateralis Preparata) group at 62.5 µg/mL, and combination groups of Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata) at 62.5:62.5 and 62.5:31.3 µg/mL. Twelve hours after drug treatment, cells were first incubated with an antibody against E-cadherin (Abcam, Cambridge, UK) at 1:100 for 16 h at 4 ℃, followed by incubation with a fluorescence-conjugated secondary antibody (1:500) at room temperature for 1 h. To determine the localization and configuration of actin filaments, the cells cultured on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with 1% bovine serum albumin and 2-µg/mL phalloidin rhodamine (Enzo Life Sciences, New York, NY, USA) at 4 ℃ overnight, followed by staining with 4',6-diamidino-2-phenylindole (1 µg/mL). Immunostained cells were mounted using antifade mounting medium (Leica, Dresden, Germany). The fluorescence of E-cadherin was quantified as integral optical density.

Statistical analysis
Single-factor analysis of variance was used for data analysis. P < 0.05 was considered statistically significant. Statistical calculations were performed by statistical software SPSS 16.0 (SPSS, Chicago, IL, USA). All values are expressed as the mean ± standard deviation (x ± s).

RESULTS
Effect of the extract on cell proliferation and cell cycle distribution
Our data showed that Tubeimu (Rhizoma Bolbostemmatis) extract at 15.6-500 µg/mL effectively inhibited the proliferation of MDA-MB-231 cells, while Fuzi (Radix Aconiti Lateralis Preparata) extract at 15.6-500 µg/mL had no influence on proliferation. The CIs of the combined treatments of the two extracts at 1:1 and 2:1 weight ratios were less than 1 at every Fa level ranging from 0.1 to 0.9, indicating synergic effects. However, the CI of the combined extracts at 1:2 was more than 1, indicating antagonistic effects. The dose of combined treatment of the two extracts was less than those of either individual extract while reaching the same Fa (Table 1). Furthermore, after exposure to combined treatment of the two extract concentrations, 62.5:62.5 µg/mL and 62.5:31.3 µg/mL, the Fa values were 79.72% and 82.23% and the CI values were 0.83 and 0.74, respectively (Figure 2A).

In the MTS assay, tubeimoside I at 3-7 µg/mL, acetylbenzoylconitine at 1.5-20 µg/mL, and control had no effects on the proliferation of SKBR3 cells. Tubeimoside I at 10 µg/mL effectively inhibited cell proliferation compared with the control group (P < 0.05). The inhibitory effects of combined tubeimoside I and acetylbenzoylconitine treatment on cell proliferation at 10:10 µg/mL and 10:20 µg/mL were superior to that of tubeimoside I treatment at 10 µg/mL (P < 0.01, P < 0.05, respectively). Furthermore, the inhibitory effect of combined treatment at 10:10 µg/mL was greater than that of 10:20 µg/mL (P < 0.01) (Figure 2B).

Fuzi (Radix Aconiti Lateralis Preparata) at 62.5 µg/mL did not influence the cell cycle distribution in MDA-MB-231 cells, while Tubeimu (Rhizoma Bolbostemmatis) at 62.5 µg/mL increased the percentage of G1-phase cells, compared with the control group (P < 0.05). The percentage of G1-phase cells in the Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata) combined treatment group at 62.5:62.5 µg/mL was much higher than those in the groups treated with the two extracts alone (Figure 3). An obvious apoptosis peak before G1 in the combined treatment group was observed, which is consistent with that observed in the apoptosis assay (Figure 5A).

Effects of both extractions on cell viability
The Fuzi (Radix Aconiti Lateralis Preparata) extraction had no effect on cell survival rate, cell damage, or AQP1 expression in MDA-MB-231 cells. Meanwhile, the MDA-MB-231 cells became tumid, accompanied by abundant cytoplasmic vacuoles in the cytoplasm,
Figure 2 Effect of Tubeimu (Rhizoma Bolbostemmati) and/or Fuzi (Radix Aconiti Lateralis Preparata) on cell proliferation
A: Effect of [Tubeimu (Rhizoma Bolbostemmati), RB] and [Fuzi (Radix Aconiti Lateralis Preparata), RALP] on MDA-MB-231 cells. A1: Fa treated with RB or RALP alone. MDA-MB-231 cells were collected 48 h after treatment with RB at 15.6-500 µg/mL, RALP at 15.6-500 µg/mL. The trypan blue exclusion assay was used to determine viable and total cell numbers. Fa is the fraction affected, where 1 is equivalent to 100% inhibition. A2: Fa treated with the combination of RB and RALP. The method was same as that of A1. The administration of drugs was combination of RB and RALP at the weight ratio of 1:1, 2:1, or 1:2. A3: CIs plotted versus Fa. CIs was calculated using the CI-isobologram method based on data derived from cells treated with single drugs and drug combinations at the indicated weight ratios and plotted versus Fa. B: Effects of tubeimoside I (T) and acetylbenzoyleaconine (A) on SKBR3 cells. B1: SKBR3 cells were seeded and were treated with drugs of T at 3µg/mL and/or A at 1.5-6 µg/mL for 48 h respectively. MTS reagent was used to detect optical density at 490 nm. B2-B4: the method was same as that of B1. B2: the administration of drugs was T at 5µg/mL and/or A at 2.5-10 µg/mL. B3: the administration of drugs was T at 7 µg/mL and/or A at 3.5-14 µg/mL. B4: the administration of drugs was T at 10µg/mL and/or A at 5-20 µg/mL. Compared with the control group, $P < 0.05$; compared with A (5, 10, 20) group, $P < 0.01$; compared with A (5, 10, 20) group, $P < 0.001$; compared with T (10) group, $P < 0.01$; compared with T: A (10: 20), $P < 0.01$; compared with T (10) group, $P < 0.05$. 

Fa: Fa treated with RB or RALP alone. MDA-MB-231 cells were collected 48 h after treatment with RB at 15.6-500 µg/mL, RALP at 15.6-500 µg/mL. The trypan blue exclusion assay was used to determine viable and total cell numbers. Fa is the fraction affected, where 1 is equivalent to 100% inhibition. A2: Fa treated with the combination of RB and RALP. The method was same as that of A1. The administration of drugs was combination of RB and RALP at the weight ratio of 1:1, 2:1, or 1:2. A3: CIs plotted versus Fa. CIs was calculated using the CI-isobologram method based on data derived from cells treated with single drugs and drug combinations at the indicated weight ratios and plotted versus Fa. B: Effects of tubeimoside I (T) and acetylbenzoyleaconine (A) on SKBR3 cells. B1: SKBR3 cells were seeded and were treated with drugs of T at 3µg/mL and/or A at 1.5-6 µg/mL for 48 h respectively. MTS reagent was used to detect optical density at 490 nm. B2-B4: the method was same as that of B1. B2: the administration of drugs was T at 5µg/mL and/or A at 2.5-10 µg/mL. B3: the administration of drugs was T at 7 µg/mL and/or A at 3.5-14 µg/mL. B4: the administration of drugs was T at 10µg/mL and/or A at 5-20 µg/mL. Compared with the control group, $P < 0.05$; compared with A (5, 10, 20) group, $P < 0.01$; compared with A (5, 10, 20) group, $P < 0.001$; compared with T (10) group, $P < 0.01$; compared with T: A (10: 20), $P < 0.01$; compared with T (10) group, $P < 0.05$. 

Fa: Fa treated with RB or RALP alone. MDA-MB-231 cells were collected 48 h after treatment with RB at 15.6-500 µg/mL, RALP at 15.6-500 µg/mL. The trypan blue exclusion assay was used to determine viable and total cell numbers. Fa is the fraction affected, where 1 is equivalent to 100% inhibition. A2: Fa treated with the combination of RB and RALP. The method was same as that of A1. The administration of drugs was combination of RB and RALP at the weight ratio of 1:1, 2:1, or 1:2. A3: CIs plotted versus Fa. CIs was calculated using the CI-isobologram method based on data derived from cells treated with single drugs and drug combinations at the indicated weight ratios and plotted versus Fa. B: Effects of tubeimoside I (T) and acetylbenzoyleaconine (A) on SKBR3 cells. B1: SKBR3 cells were seeded and were treated with drugs of T at 3µg/mL and/or A at 1.5-6 µg/mL for 48 h respectively. MTS reagent was used to detect optical density at 490 nm. B2-B4: the method was same as that of B1. B2: the administration of drugs was T at 5µg/mL and/or A at 2.5-10 µg/mL. B3: the administration of drugs was T at 7 µg/mL and/or A at 3.5-14 µg/mL. B4: the administration of drugs was T at 10µg/mL and/or A at 5-20 µg/mL. Compared with the control group, $P < 0.05$; compared with A (5, 10, 20) group, $P < 0.01$; compared with A (5, 10, 20) group, $P < 0.001$; compared with T (10) group, $P < 0.01$; compared with T: A (10: 20), $P < 0.01$; compared with T (10) group, $P < 0.05$.
and increased expression of AQP1 in the Tubeimu (Rhizoma Bolbostemmatis) extract group and the group treated with both extracts (Figure 4A, C). However, there was no difference in viability of MDA-MB-231 cells among all groups (P > 0.05) (Figure 4B). Similar results were observed in SKBR3 cells.

**Effects of the two extracts on apoptosis**

Although the Fuzi (Radix Aconiti Lateralis Preparata) extract at 62.5 and 31.3 µg/mL did not induce apoptosis of MDA-MB-231 cells, Tubeimu (Rhizoma Bolbostemmatis) extract at 62.5 µg/mL effectively induced apoptosis compared with untreated controls and the As2O3 positive control. The apoptotic rate following treatment with both extracts at 62.5: 62.5 µg/mL was much higher than that in the Tubeimu (Rhizoma Bolbostemmatis) extract group, which indicated synergistic induction of apoptosis (P < 0.05). Though the apoptosis rate of SKBR3 cells increased from 3.12 ± 0.69 [Tubeimu (Rhizoma Bolbostemmatis) extract-treated group] to 8.33 ± 1.64 or 5.63 ± 1.76 [Fuzi (Radix Aconiti Lateralis Preparata) group treated with both extracts], no significance was observed (Figure 5).

**Effects of the two extracts on cell migration**

The Tubeimu (Rhizoma Bolbostemmatis) extract at 62.5 µg/mL down-regulated the migration of MDA-MB-231 and SKBR3 cells, while the Fuzi (Radix Aconiti Lateralis Preparata) extract at 62.5 and 31.3 µg/mL up-regulated migration, compared with the control. Treatment with both Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata) at 62.5: 62.5 µg/mL and 62.5: 31.3 µg/mL down-regulated the migration of MDA-MB-231 and SKBR3 cells, compared with the group only treated with Fuzi (Radix Aconiti Lateralis Preparata) extract (Figure 6). There was no difference in cell migration between the combined Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata) groups and the Tubeimu (Rhizoma Bolbostemmatis) group in MDA-MB-231 cells. Interestingly, concomitant treatment with Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata) (62.5: 31.3 µg/mL) exerted a synergistic effect that effectively enhanced the inhibitory effect of Tubeimu (Rhizoma Bolbostemmatis) on SKBR3 cell migration (Figure 6M right, P < 0.001; Figure 7A left, P < 0.01).

In the study of main active ingredients of Tubeimu (Rhizoma Bolbostemmatis) and Fuzi (Radix Aconiti Lateralis Preparata), combined tubeimoside I and acetylbenzoylaconine treatment at 3: 1.5 µg/mL and 5: 2.5 µg/mL in SKBR3 cells and 5: 2.5 µg/mL in MDA-30MB-231 cells, also exerted synergistic inhibitory ef-
Effects of Tubeimu (Rhizoma Bolbostemmatis) and/or Fuzi (Radix Aconiti Lateralis Praeparata) treatment on cell survival

A: cell microphotography (×100). MDA-MB-231 cells were treated with different drugs for 48 h. A1: L-15 culture medium; A2: RALP at 62.5 μg/mL; A3: RALP at 31.3 μg/mL; A4: RB at 62.5 μg/mL; A5: RALP∶RB at 62.5∶62.5 μg/mL; A6: RALP∶RB at 62.5∶31.3 μg/mL. Cell morphology was observed using a light microscope. B: Survival rate (P > 0.05). MDA-MB-231 cells were collected 48 h after adding RALP at 31.3, 62.5 μg/mL and/or RB at 62.5 μg/mL respectively. The trypan blue exclusion assay was used to determine viable and total cell numbers. C: Secretion of AQP1. MDA-MB-231 cells were collected 72 h after the administration of RALP at 62.5 μg/mL and/or RB at 62.5 μg/mL. The concentration of AQP1 was measured using AQP1 enzyme-linked immunosorbent assay kit and expressed as the secretion of AQP1 every 1×10⁶ cells. Compared with the control group, *P < 0.001; compared with RALP 62.5 groups, †P < 0.001. RB: Tubeimu (Rhizoma Bolbostemmatis); RALP: Fuzi (Radix Aconiti Lateralis Praeparata); AQP: aquaporin.

Effects of Tubeimu (Rhizoma Bolbostemmatis) and/or Fuzi (Radix Aconiti Lateralis Praeparata) treatment on apoptosis and cell cycle distribution

A: MDA-MB-231 cells were seeded and collected 48 h after adding RB at 31.3, 62.5 μg/mL and/or RALP at 62.5 μg/mL. As2O3 was used as a positive control. Cells were stained with FITC Annexin V and PI respectively. Apoptosis were analyzed by flow cytomet. B: SKBR3 cells were seeded and collected 48 h after adding RB at 31.3, 62.5 μg/mL and/or RALP at 62.5 μg/mL. Cells were stained with FITC Annexin V and PI respectively. Apoptosis were analyzed by flow cytomet. Compared with the control group, *P < 0.001; compared with AS2O3 group, †P < 0.05; compared with AS2O3 group, ‡P < 0.01; compared with RALP (62.5, 31.3) groups, ‡P < 0.001; compared with RB (62.5) group, ‡P < 0.05; compared with RB (62.5) group, ‡P < 0.001. RB: Tubeimu (Rhizoma Bolbostemmatis), RALP: Fuzi (Radix Aconiti Lateralis Praeparata).
Tubeimu (Rhizoma Bolbostemmatidis) showed polymerization and reorganization of actin filaments along the cell membrane and in the cytoplasm, accompanied by an increased expression of E-cadherin (P < 0.01). After combined treatment with Tubeimu (Rhizoma Bolbostemmatidis) and Fuzi (Radix Aconiti Lateralis Preparata) at concentrations of 62.5: 62.5 µg/mL and 62.5: 31.3 µg/mL, cells changed into a polygonal shape and displayed decreased membrane ruffles. Actin filaments were mainly localized in the cytoplasm and an additional increase in E-cadherin expression level was observed (P < 0.001) (Figure 8).

**DISCUSSION**

TCM has advocated combinatory therapeutic strategies for more than 2500 years. Guided by the theories of TCM, formulae are designed with a combination of different plants or minerals to increase therapeutic efficacy and decrease adverse effects. Examples include Re-algar-Indigo naturalis formula and PHY906, whose efficacies in treating cancer have been established.1,2 WRCP, a Chinese medical formula that we previously

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**Figure 6 Effects of Tubeimu (Rhizoma Bolbostemmatidis) and/or Fuzi (Radix Aconiti Lateralis Preparata) treatment on cell migration by Wound-healing assay**

A–L: The photos of Wound-healing assay (400×). SKBR3 cells were treated with different drugs of DMEM medium (A and C), RB at 62.5 µg/mL (B and D), RALP at 62.5 µg/mL (E and G), RALP: RB at 62.5: 62.5 µg/mL (F and H), RALP at 31.3 µg/mL (I and K), RALP: RB 62.5: 31.3 µg/mL (J and L) respectively. Cell migration was observed under a phase-contrast microscope at 0 h (A, B, E, F, I, J) and 12 h (C, D, G, H, K, L) after exposure to drugs. M: The statistical results of wound-healing assay were expressed as the spacing of scarification. Compared with the control group, 2P < 0.01; compared with RALP (62.5, 31.3) groups, 3P < 0.001; compared with the RB (62.5) group, 4P < 0.001; compared with the RALP:RB (62.5: 62.5) group, 5P < 0.001.

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**Figure 7 E-cadherin expression in SKBR3 cells**

Cells treated with Fuzi (Radix Aconiti Lateralis Preparata) displayed pseudopodia formation in which actin filaments preferentially accumulated at the leading edge of ruffled cell membranes to form lamellipodia and the expression of E-cadherin was less than that in control cells. However, cells treated with Tubeimu (Rhizoma Bolbostemmatidis) showed polymerization and reorganization of actin filaments along the cell membrane and in the cytoplasm, accompanied by an increased expression of E-cadherin (P < 0.01). After combined treatment with Tubeimu (Rhizoma Bolbostemmatidis) and Fuzi (Radix Aconiti Lateralis Preparata) at concentrations of 62.5: 62.5 µg/mL and 62.5: 31.3 µg/mL, cells changed into a polygonal shape and displayed decreased membrane ruffles. Actin filaments were mainly localized in the cytoplasm and an additional increase in E-cadherin expression level was observed (P < 0.001) (Figure 8).
Rhizoma Bolbostemmathas designed, has demonstrated direct anti-cancer effects in vitro and breast cancer cells in vivo. In this study, we used Fuzi (Radix Aconiti Lateralis Praeparata) and Tubeimu (Rhzma Bolbostemmathis) to investigate the synergistic effects of herbs and the rationale of the formula on breast cancer cells. The median effect principle can quantitatively define antagonism, additive effects, and synergism in drug...
combinations and is widely used in drug combination studies. Therefore, we used the median effect principle to test the possible synergistic effects of Fuzi (Radix Aconiti Lateralis Preparata) and Tubeimu (Rhizoma Bolbostemmatis) on breast cancer cell proliferation. Assessment of the CI value by the median-effect method directly demonstrates the synergistic inhibitory effect of Fuzi (Radix Aconiti Lateralis Preparata) and Tubeimu (Rhizoma Bolbostemmatis) on the proliferation of breast cancer cell lines MDA-MB-231 and SKBR3. We further tested whether Tubeimu (Rhizoma Bolbostemmatis) plus Fuzi (Radix Aconiti Lateralis Preparata) could act on cell cycle progression and found that their combination caused G1 blockage of MDA-MB-231 cells. Tubeimu (Rhizoma Bolbostemmatis) down-regulated cell migration, while Fuzi (Radix Aconiti Lateralis Preparata) up-regulated migration. Comitant treatment with Fuzi (Radix Aconiti Lateralis Preparata) and Tubeimu (Rhizoma Bolbostemmatis) down-regulated cell migration. These observations are in agreement with the rationality of the formula, which is mutual reinforcement of the compounds and reduction in adverse effects. Moreover, cell migration was detected at 12 h after drug treatment at the time at which cytotoxicity or inhibitory effects on cell proliferation was not observed (data not shown). Therefore, the synergistic
inhibitory effect on cell migration was not because of the cytotoxicity or proliferation inhibition effect of drugs.

Cell adhesion and migration are interdependent cellular processes whose alterations lead to the acquisition of a more motile phenotype by cancer cells, allowing for tumor migration. Decreased expression of the epithelial adhesion molecule E-cadherin plays a key role in the acquisition of the invasive phenotype of many tumors. The cytoskeleton provides the basic infrastructure for the maintenance of cell motility. Furthermore, the promotion effect of Fuzi (Radix Aconiti Lateralis Preparata) on the proliferation and migration of cell line MDA-MB-231 and SKBR3 is enhanced when used with Tubeimu (Rhzizoma Bolbostemma) extract. Furthermore, the promotion effect of Fuzi (Radix Aconiti Lateralis Preparata) extract on the migration of MDA-MB-231 and SKBR3 cells was reduced when combined with Tubeimu (Rhzizoma Bolbostemma). We further demonstrated that acetylbolbostemol and tuberimside I acted as the essential components in the extracts of Fuzi (Radix Aconiti Lateralis Preparata) and Tubeimu (Rhzizoma Bolbostemma), exerting synergistic effects on MDA-MB-231 and SKBR3 cells.

REFERENCES


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