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# *Tegillarca granosa* extract Haishengsu (HSS) suppresses expression of mdr1, BCR/ABL and sorcin in drug-resistant K562/ADM tumors in mice

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## ABSTRACT

**Purpose:** To evaluate the effect of Haishengsu (HSS), a protein extract from *Tegillarca granosa*, on multidrug-resistance genes mdr1, BCR/ABL and sorcin in transplanted tumors.
**Material/Methods:** Mice were inoculated subcutaneously with a drug resistant leukemia cell line K562/ADM. Tumor-bearing animals were divided into control, adriamycin, HSS and combination therapy (adriamycin plus HSS) groups. Flow cytometry was used to detect apoptosis of tumor cells, and RT-PCR was used to evaluate the expression of mdr1, BCR/ABL and sorcin.
**Results:** The apoptosis rate in the high (71.8%), medium (72.3%) and low doses HSS group (72.4%) was higher than in control (1.2%, $p<0.01$), adriamycin (34.4%, $p<0.05$) or combination therapy group (46.4%, $p<0.05$). The mean optical density of mdr1, BCR/ABL and sorcin in HSS groups was lower than in control, adriamycin and combination therapy group ($p<0.01$). The optical density of the three genes in high HSS group was lower than in medium and low HSS group ($p<0.01$).
**Conclusions:** Haishengsu promotes apoptosis of drug-resistant K562/ADM tumors in mice in a dose-dependent manner. The pro-apoptotic effect of Haishengsu may be related to a reduced expression of multidrug-resistance genes mdr1, BCR/ABL and sorcin.

**Key words:** Tegillarca granosa; K562/ADM tumor; multidrug resistance genes; apoptosis; mice.

## INTRODUCTION

*Tegillarca granosa L.* (order Arcoida) is a sea creature that contains high concentration of proteins and vitamin B12. It has been used as a traditional Chinese medicine to treat cancer and other chronic illnesses for more than a century [1]. *Haishengsu* (HSS) is a purified protein from *Tegillarca granosa* with a molecular weight of approximately 15 KDa [2]. HSS has been found to have suppressive effects on leukemia K562 cells and transplanted tumor cells in animals [3, 4]. When used as an adjuvant therapy, HSS seems to improve clinical outcomes of patients who underwent conventional chemotherapy for lung or renal cancer [5,6]. Furthermore, *in vitro* studies showed that HSS induces apoptosis in leukemia K562 cells by reducing the expression of apoptosis suppressor bcl-2, and increasing the expression of apoptosis promoting bax [2]. K562 cell line is a blast crisis cell line of chronic myeloid leukemia. Adriamycin (ADM)

has been used to induce drug resistance strains of K562 cells known as K562/ADM cells [4]. HSS was found to inhibit the growth of drug-resistant K562/ADM tumors in mice [4]. The primary purpose of the present study was to investigate the molecular mechanisms by which HSS inhibited transplanted drug-resistant K562/ADM tumors.

## MATERIALS AND METHODS

This study was approved by the institution review board of Liaocheng People's Hospital. HSS was obtained from Qingdao Haisheng Oncology Hospital (Shandong, China, batch number 990211). Adriamycin was purchased from Pharmacia Cor. (New Jersey, USA). K562 and K562/ADM cell lines were provided by the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). Annexin-V-Fluorescein isothiocyanate (FITC) and Pyridine iodide (PI) were purchased from Jingmei Biological (Shengzhen, China). Nude mice (female, 15-18g) were obtained from Beijing Vital River Laboratory Animal Co., Ltd (Beijing, China).

### Cell culture

Drug-resistant leukemia cell line K562/ADM was derived from the parental K562 cells by continuous exposure to increasing concentrations of adriamycin (up to 20 μg/ml). K562 and K562/ADM cells were cultured in RPMI1640 culture medium containing 10% fetal calf serum (Gibco BRL, Gaithersburg, USA) at 37°C in an incubator (SANYO, Tokyo, Japan) of saturated humidity.

### Mice model and drug administration

Twenty-four hours after receiving radiation treatment (4 Gy), nude mice were inoculated subcutaneously with$1\times10^7$ K562/ADM cells (n=18) in the buttocks. Five days after inoculation, K562/ADM tumor-bearing mice were randomly divided into control, three HSS groups, adriamycin and combination therapy (adriamycin + HSS) groups. Each group was comprised of 3 animals. HSS and adriamycin were intravenously administered via the tail vein of the mice. The dosing regimens and duration of treatment are listed in *Table 1*. In control group, normal saline was injected to the tail vein of the mice *(Tab. 1)*. On the 21st day of the treatment, the mice were euthanized and the tumor tissues were dissected under sterile condition. The tumor was weighed and the fresh tumor tissues were treated with liquid nitrogen for 4 hours and then were stored at -80°C in refrigerator.

### Assessment of apoptosis by Annexin V-FITC

Tumor tissues were harvested after the conclusion of treatment. Apoptotic cell death was measured using a fluorescing isothiocynate (FITC)-conjugated Annexin V/PI assay kit by flow cytometry. Briefly, $5\times10^5$ cells were washed with ice–cold Phosphate Buffered Saline (PBS), resuspended in 100 μl binding buffer, and stained with 5 μl of FITC-conjugated Annexin V (10mg/ml) and 10μl of PI (50mg/ml). The cells were incubated for 15 min at room temperature in the dark, and 400 μl of binding buffer was added. The processed ells were analyzed with FACScan (Becton-Dickinson, USA). The PC-3 cells were gated separately according to their granularity and size on forward scatter (FSC) versus Side Scatter (SSC) plots. Early and late apoptosis was evaluated on fluorescence 2 (FL2 for propidium iodide) versus fluorescence 1 (FL1 for Annexin) plots. Cells stained with only annexin V were defined as being in early apoptosis; cells stained with both annexin V and propidium iodide were defined as being in late apoptosis or in a necrotic stage. Cells were analyzed with a flow cytometer (FACSCalibur, BD, Vienna, Austria).

### RT-PCR detection of multidrug resistance gene mdr1, sorcin, BCR/ABL gene expression

To investigate the expression level of multidrug-resistance genes in transplanted tumor tissues, we employed a reverse transcriptase-polymerase chain reaction (RT-PCR) assay using mdr1, BCR/ABL and sorcin primers. Total RNA was extracted from the frozen tumor tissues. Integrity of RNA was demonstrated by a high-resolution gel method. After the reverse transcription, mdr1, BCR/ABL, sorcin and GAPDH primers were used for cDNA amplification. PCR products were electrophoresed on agarose gels containing ethidium bromide and visualized by UV photography. Primer sequences for mdr1, BCR/ABL, sorcin and GAPDH are shown in *Table 2*.

To assess the role of mdr1, sorcin, BCR/ABL genes in drug resistant K562/ADM tumor cells, mdr1, sorcin, BCR/ABL gene expression was also evaluated in non-drug resistant K562 cells in the same manner as in K562/ADM tumor cells.

### Western blot assays detection of products of multidrug resistance gene mdr1, sorcin, BCR/ABL and caspase-3

Cells of the control group and three HSS groups were lysed by boiling in 2×SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Western blots were serially probed with combinations of antibodies to mdr1, sorcin, BCR/ABL and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), β-catenin (to control for loading). Each experiment was repeated several times with different batches of proteins, and representative blots are shown.

### Statistical Analysis

Data are expressed as means ± SD. SAS6.12 software was used for data analysis. Numerical data were analyzed with one-way ANOVA. Categorical data were analyzed with Chi-square test. $P<0.05$ was considered statistically significant.

*Table 1.* **Intravenous administration of drugs (3 animals in each group).**

| Groups | Drugs | Dosages |
|---|---|---|
| Placebo | Normal saline | 0.5ml, q3d×4d |
| Adriamycin | Adriamycin | 2mg/kg, q3d×4d |
| Low HSS | HSS | 450mg/kg, qd×10d |
| Medium HSS | HSS | 900mg/kg, qd×10d |
| High HSS | HSS | 1800mg/kg, qd×10d |
| Combination therapy | ADR +HSS | ADR 2mg/kg, q3d×4d; HSS 900mg/kg, qd×10d |

*ADR - Adriamycin; qd - once daily; q3d - three times a day*

# RESULTS

## Apoptosis by flow cytometry

All animals survived the treatment and completed the study. Apoptosis in each group is shown in *Figure 1.* The apoptosis rate in the high (71.8%), medium (72.3%) and low doses HSS group (72.4%) was higher than in control group (1.2%, $p<0.01$), adriamycin (34.4%, $p<0.05$), or combination therapy group (46.4%, $p<0.05$). As shown in *Table 3*, the mean weight of the tumor in the medium and high HSS group was less than in the control, adriamycin and low HSS group ($p<0.01$).

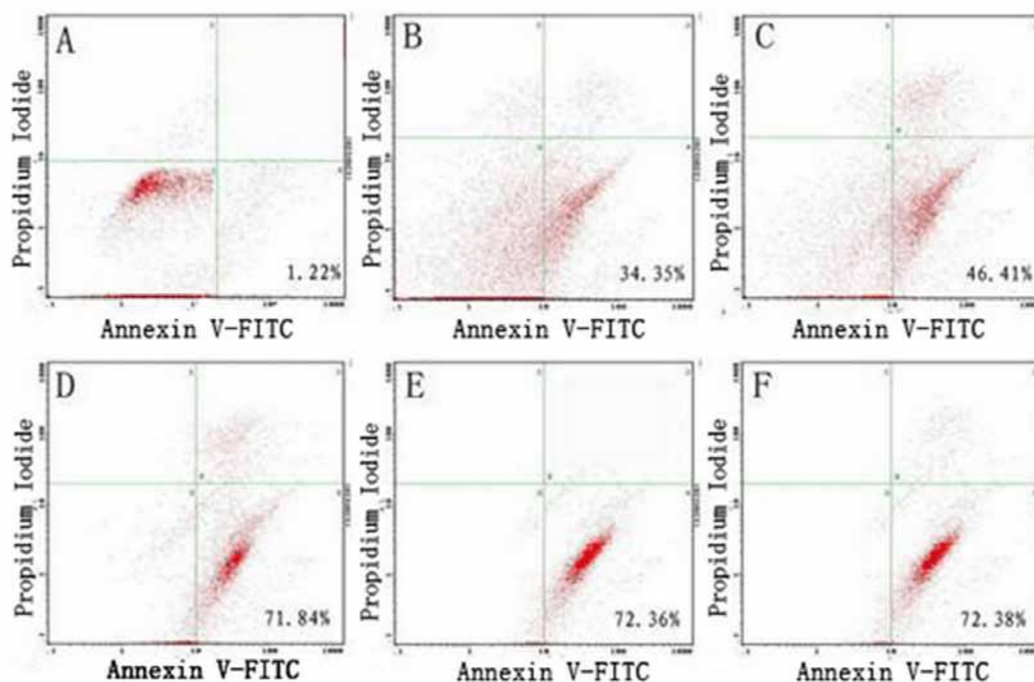
## RT-PCR detection of mdr1, BCR/ABL, and sorcin expression

Expression of drug-resistance-related genes of each group is shown in *Figure 2.* There was almost no expression of these

*Table 2.* **Mdr1, sorcin, BCR/ABL, GAPDH primer.**

| | | |
|---|---|---|
| BCR/ABL | Forward primer | 5’ CTC CAG ACT GTC CAC AGC ATT CCG 3’ |
| | Reverse primer | 5’ CAG ACC CTG AGG CTC AAA GTC AGA 3’ |
| sorcin | Forward primer | 5’ GGT GAT CTT TCC ATT GGT G 3’ |
| | Reverse primer | 5’ TCC GCT GTA TGG TTA CTT T 3’ |
| mdr1 | Forward primer | 5’ AAG CCT AGT ACC AAA GAG GCT CTG 3’ |
| | Reverse primer | 5’ GGC TAG AAA CAA TAG TFA AAA CAA 3’ |
| GAPDH | Forward primer | 5’ CTT AGC ACC CCT GGC CAA G 3’ |
| | Reverse primer | 5’ GAT GTT CTG GAG AGC CCC G 3’ |

*Figure 1.* **The apoptosis rate in each group.**



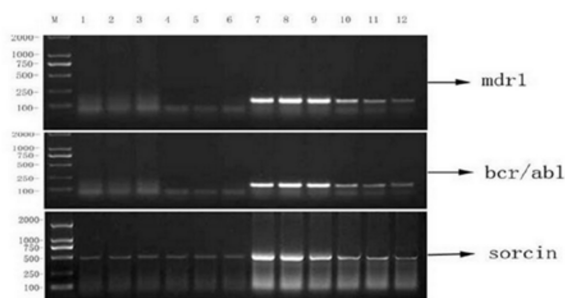

*A - control; B - Adriamycin; C - combination therapy; D - low dose HSS; E - medium dose HSS; F - high dose HSS.*

genes in non-drug resistant K562 cells, but the expression was noticeable in the drug-resistant K562/ADM cells. Optical density of mdr1, BCR/ABL, and sorcin are listed in *Table 3.* The mean optical density of mdr1, BCR/ABL and sorcin in control group was lower than in all other groups ($p$<0.01). The mean optical density of mdr1, BCR/ABL and sorcin in HSS groups was lower than in control, adriamycin and combination therapy group ($p$<0.01). The optical density of the three genes in high HSS group was lower than in medium and low HSS group ($p$<0.01).

## Western blot assays detection of products of multidrug resistance gene mdr1, sorcin, BCR/ABL and caspase-3

Expression of products of multidrug resistance gene mdr1, sorcin, BCR/ABL and caspase-3 of each group is shown in *Table 4*. The mean optical density of *mdr1, BCR/ABL and sorcin* in HSS group was lower than in control groups, especially in the high dose group ($p$<0.01). The mean optical density of *caspase-3* of the three HSS group was higher than in control group ($p$<0.01).

***Figure 2.*** **Expression of resistance-related genes of each group.**




*M - Maker; Non-drug resistant K562 cells (zone 1-6): zone 1: control; zone 2: ADR; zone 3: ADR + HSS; zone 4: low-HSS; zone 5: Mid-HSS; zone 6: High-HSS.*
*Drug-resistant K562/ADM cells (zone 7-12): zone 7: control; zone 8: ADR; zone 9: ADR + HSS; zone 10: low-HSS; zone 11: Mid-HSS; zone 12: High-HSS.*

***Table 3.*** **Weight of the tumors following Haishengsu (HSS) treatment.**

| Groups | Weight of the tumors (g) |
|---|---|
| Control | 32.8±1.8 |
| Adriamycin | 29.2±0.9 |
| Adriamycin + HSS | 29.6±1.2 |
| Low HSS | 28.3±0.7 |
| Medium HSS | 24.5±0.3* |
| High HSS | 22.6±1.3* |

**p <0.01 compared with control, low or medium HSS group*

***Table 4.*** **Optical density values of mdr1, BCR/ABL and sorcin.**

| Groups | Optical density values | | |
|---|---|---|---|
| | mdr1 | BCR/ABL | sorcin |
| K562 cells | 83.5±11.5** | 85.4±13.3** | 73.6±12.5** |
| Control | 982.8±16.5 | 213.8±18.3 | 1211.5±21.4 |
| ADR | 1125.1±15.8 | 356.8±19.1 | 1136.8.3±11.6 |
| ADR+HSS | 1028.6±19.2 | 214.4±16.7 | 810.6±13.8 |
| Low HSS | 589.3±15.4* | 166.2±14.2* | 566.5±18.4* |
| Medium HSS | 422.6±19.4* | 124.3.±15.8* | 402.9±15.3* |
| High HSS | 289.2±14.7*Δ | 101.2±17.5*Δ | 326.3±14.7*Δ |

***p <0.01 compared with all other groups; *p <0.001, compared with PBS (phosphate-buffered saline), ADR (adriamycin) and ADR+HSS group. Δ p <0.01 compared with low dose HSS group.*

***Table 5.*** **Optical density values of protein of mdr1, BCR/ABL, sorcin and caspase-3.**

| Groups | Optical density values | | | |
|---|---|---|---|---|
| | mdr1 | BCR/ABL | sorcin | Caspase-3 |
| Control | 967.8±11.3 | 934.8±10.3 | 1101.5±11.4 | 202.1±14.4 |
| Low HSS | 563.3±12.3* | 566.2±17.2* | 766.4±13.1* | 314±14.7* |
| Medium HSS | 402.6±15.1* | 324.3.±14.6* | 302.4±13.3* | 557.2±14.2* |
| High HSS | 232.2±12.7* | 154.2±12.5* | 222.3±15.4* | 918.8±10.8* |

**p <0.01 compared with all other groups*

## DISCUSSION

Overexpression of the multidrug resistance gene has been associated with treatment failure in leukemia and other malignancies [7]. Co-administration of conventional chemotherapy and multidrug-resistant modulators that block *mdr*-mediated drug efflux may reduce drug-resistance and enhance therapeutic effects of conventional chemotherapy for relapsed or refractory leukemia. Several pharmacological modulators of *mdr* have been tested, such as calcium channel blockers, steroid hormones (tamoxifen and progesterone), antibiotics (ceftriaxone), protein kinase inhibitors (staurosporine, imatinib mesulate), or monoclonal antibodies, but the effect of these modulators have been variable [4].

Philadelphia chromosome, a molecular hallmark of chronic myeloid leukemia and acute lymphoblastic leukemia, contains a BCR-ABL hybrid gene. BCR-ABL encodes an oncogenic fusion protein of 190, 210, or 230 kDa, depending on the breakpoint on the BCR gene. The unifying feature of all these BCR-ABL fusion proteins is their deregulated protein tyrosine kinase activity that is responsible for leukemogenesis *in vitro* and *in vivo* [8]. Targeting tyrosine kinase activity of BCR-ABL is an attractive therapeutic strategy in chronic myeloid leukemia and in BCR-ABL-positive acute lymphoblastic leukemia. Whereas imatinib, a selective inhibitor of BCR-ABL tyrosine kinase, is now used in frontline therapy for chronic myeloid leukemia. Second-generation inhibitors of BCR-ABL tyrosine kinase such as nilotinib or dasatinib have been developed for the treatment of imatinib-resistant or imatinib-intolerant disease [8]. Overexpression of BCR-ABL and multidrug resistance gene mdr-1 were found among the investigated mechanisms [9]. Sorcin overexpression has been found to be associated with gemcitabine resistance and a poor prognosis in patients with lung cancer [10]. In human cervical carcinoma cell line HeLa-Ohio, sorcin repressed apoptosis via dysfunction of caspase-3 [11]. However, the role of sorcin in mediating multi-drug resistance in transplanted K562/ADM tumors is unclear.

Apoptosis, in which cells grow and die in a controlled way, is the single most important factor in determining whether chemotherapy is successful. Adriamycin in one of the many therapeutic agents that can induce apoptosis in cancer cells such as K562 cell lines [12]. In the current study on drug-resistant K562/ADM cancer cells, the apoptosis rate in adriamycin treated tumors was higher than in the control, but it was less than half of the animals treated with HSS. The apoptosis rate in the high dose (1800 mg/kg) HSS group was greater than in the low dose (450 mg/kg) HSS group. These results suggest that adriamycin has only limited effect on apoptosis in drug-resistant K562 tumors, whereas HSS induces apoptosis in these transplanted tumors in a dose-dependent manner. Furthermore, the apoptosis rate in animals treated with both adriamycin and medium dose of HSS (900 mg/kg) was lower than in animals treated with HSS alone, suggesting that the concurrent use of adriamycin has diminished the pro-apoptotic effects of HSS in this animal model.

How HSS induces apoptosis in the drug-resistant K562/ADM cells is not entirely clear, as apoptosis is a complex process, involving a cascade of reactions and multiple genes. Our previous *in vitro* study on K562/ADM cells showed that HSS suppressed expression of *p*-glycoprotein, a drug-resistance inducing protein [13]. Multidrug-resistant genes, mdr1, BCR/ABL and sorcin, appear to play a role in drug-resistant K562/ADM tumor, as the mean optical density of these genes in the control or adriamycin group was significantly higher than in the non-drug resistant K562 cells *(Tab. 3).* The mean optical density of mdr1, BCR/ABL and sorcin in HSS groups was lower than in control, adriamycin and combination therapy groups, suggesting that HSS lowered expression of these multidrug-resistance genes in the transplanted tumors, and this reduced expression of these genes may have contributed to the enhanced apoptosis rates in the three HSS groups.

The translation of the molecular and cellular actions of HSS to the effects on tumor growth is worth noting. In the present study, all 18 animals survived after the three-week experiment. There was no statistically significant difference in the mean weight of tumors between the low dose HSS and control group, but the tumor weight in the medium and high dose HSS group was lower than in the low dose HSS and the control group. These results suggest that medium and high dose HSS is able to suppress tumor growth after three weeks of therapy. However, the medium to long term effect of HSS on tumor growth and animal survival remains unclear.

## CONCLUSIONS

In conclusions, Haishengsu promotes apoptosis of transplanted drug-resistant K562/ADM tumors in mice. The pro-apoptotic effects of Haishengsu may be related to a reduced expression of multidrug-resistance genes mdr1, BCR/ABL and sorcin in the tumor cells. Further studies are required to specify the role of mdr1, BCR/ABL and sorcin reduction in the HSS-induced apoptosis.

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**Conflict of interest:** Authors have no conflict of interest to declare.

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