

## Full Length Research Paper

# Induction of apoptosis by Rhizoma Paridis saponins in MCF-7 human breast cancer cells

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This study investigates the relationship between the induction of MCF-7 human breast cancer cell apoptosis by Rhizoma Paridis saponins (RPS) and the mitochondrial apoptotic pathway. We treated MCF-7 cells with RPS at various concentrations and examined the inhibitory effect of RPS on the proliferation of MCF-7 cells using the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the change in mitochondrial membrane potential (MMP) using JC-1 staining, and the expression levels of related proteins using western blot. The results showed that the inhibitory effect of RPS against the growth of MCF-7 human breast cancer cells might be related to the apoptosis induced caspase family and the caspase-3-dependent pathway. The results suggest that RPS has the potential to be a valuable anticancer agent.

**Key words:** Rhizoma paridis saponins, MCF-7 cells, apoptosis, mitochondria

## INTRODUCTION

Breast cancer is the second most serious malignant tumor in women worldwide, especially in Europe and America (Parkin et al., 2005). The treatment of breast cancer has usually included surgery, radiotherapy, chemotherapy, immunotherapy, and traditional Chinese medicine therapy. However, a significant number of patients experience severe side effects (Group EBCTC, 1998). Thus, it is important to find a new alternative for breast cancer treatment that has high efficiency and low toxicity.

Rhizoma Paridis refers to the roots and rhizomes of *Paris polyphylla* var. *yunnanensis*. It has been used widely in traditional Chinese medicine for cancer treatment for a thousand years. Recent studies have shown that Rhizoma Paridis has significant antipyretic, alexipharmic, detumescent, demulcent, and hemostatic effects and is useful in the treatment of hepatopathy

(Chen et al., 1995; Li, 1984; Matsuda et al., 2003; Wang et al., 1990, 1996). Studies have also shown that steroid saponins are the main components in Rhizoma Paridis (Cheunga et al., 2005; Lee et al., 2005; Wu et al., 2004).

Although many studies have shown that Rhizoma Paridis saponins (RPS) have strong antitumor activity, few studies have reported on the mechanism of RPS against human cancer cell lines. In this study, we treated MCF-7 cells with RPS at various concentrations. The potential of RPS to inhibit cell proliferation and induce apoptosis in MCF-7 cells was evaluated by measuring cytotoxicity using the MTT assay, cell apoptosis, the change in MMP, and expression levels of Bax, Bcl-2, cytochrome C, caspase-3/9, cleaved caspase-3/9, poly ADP-ribose polymerase (PARP), and cleaved PARP associated with the apoptosis signaling pathway.

## MATERIALS AND METHODS

### Chemicals and reagents

Growth medium RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Annexin V-FITC/PI Apoptosis Assay Kit and JC-1 dye were purchased from Molecular Probes, Inc. (Eugene, OR). Antibodies of Bax, Bcl-2, caspase-3/9, cleaved caspase-3/9, PARP cleaved PARP -actin and cytochrome C were purchased from Cell Signaling Technology Inc.

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**Abbreviations:** RPS, Rhizoma Paridis saponins; PARP, poly ADP-ribose polymerase; FBS, fetal bovine serum; MMP, mitochondrial membrane potential; MTT, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(Danvers, MA). The ECL Western Blotting kit was purchased from Pierce (Rockford, IL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). Cell Mitochondria Isolation Kit was purchased from Beyotime (Suzhou, China).

### Cell lines

Human breast cancer cell lines MCF-7 (ER+, HER2/neu-) was purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences.

### Preparation of RPS

The crushed Rhizoma Paradis (100 g) were extracted with 70% ethanol (800 ml) for 3 times, 2 h under reflux. The combined 70% ethanol extracts were filtered and centrifuged after being concentrated. The supernatant dissolved in water was then eluted by 65% ethanol on macroporous adsorptive resin D101. The eluent was finally condensed with a vacuum rotary evaporator to give a gray, viscous extract, which was RPS. Then RPS was lyophilized and stored at -20°C for further studies.

### Cell culture and cytotoxicity assay

Human breast cancer MCF-7 cells were cultured in RPMI -1640 medium supplemented with 10% FBS under the condition of 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The effect of RPS on MCF-7 cells viability was determined using the MTT assay. MCF-7 cells were treated with RPS at various concentrations (0, 20, 40, 80, and 160 g/ml). 48 h later, 50 l of MTT stock solution (2 mg/ml) was added and after incubation for 4 h, the absorbance at 490 nm was then measured on a scanning multi-well spectrophotometer. The cytotoxicity was evaluated with reference to the IC<sub>50</sub> value. The tests were performed 3 independent times.

### Apoptosis assay

Apoptosis induced by RPS treatment for 48 h were determined with an Annexin V-FITC/PI Apoptosis Assay Kit according to manufacturer's instructions. Analyses were performed on a FACSCalibur flow cytometer (BD Biosciences; Mountain View, CA). The cells in the FITC-positive and PI-negative fraction were regarded as apoptotic cells.

### MMP assay

Treated MCF-7 cells were washed twice and incubated in medium containing 10 g JC-1 dye for 30 min at 37°C in the dark. Stained cells were harvested, washed, resuspended, and subjected to flow cytometry analysis according to manufacturer's instructions. JC-1 was selectively accumulated in intact mitochondria and formed multimer J-aggregates that fluoresced red (590 nm) at a high membrane potential. Monomeric JC-1 fluoresced green (527 nm) at a low membrane potential. Thus, the different colors of fluorescence of JC-1 represented different MMP, which could be analyzed by flow cytometer.

### Western blot assay

Treated cells were lysed and then total protein was extracted.

Samples were separated in a 10% polyacrylamide gel and then transferred onto polyvinylidene fluoride (PVDF). The membranes were blocked in 5% nonfat milk in Tris Buffer Saline Tween20 (TBST) buffer for 1 h and hybridized with antibodies specific for Bax, Bcl-2, caspase-3/9, cleaved caspase-3/9 and -actin respectively, and then with appropriate HRP-conjugated secondary antibodies. Signal was developed using an ECL kit, and the relative photographic density was analysed by Quantity one software.

### Cytosolic cytochrome C assay

Treated MCF-7 cells were processed by Cell Mitochondria Isolation Kit according to manufacturer's instructions. Cytosolic extracts were resolved in a 12.5% polyacrylamide gel and transferred, and the blot was hybridized with cytochrome C antibody. Signal was developed using an ECL kit, and the relative photographic density was analysed by Quantity one software.

### Statistical analysis

The data were presented as mean ± SD ( $\bar{x} \pm s$ ). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at  $p < 0.05$ . The statistical software package SPSS11.5 was used for the analysis.

## RESULTS

### Cytotoxic effects of RPS in MCF-7 cells

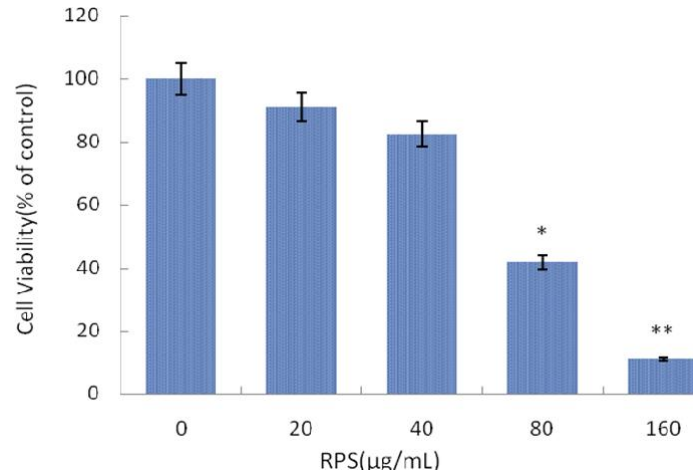
After the treatment of the cells with various concentrations of RPS for 48 h, the cytotoxicity was examined using the MTT assay. We found that cell viability was decreased when RPS concentration was raised in a dose-dependent manner (Figure 1). The IC<sub>50</sub> of RPS in MCF-7 cells for 48-h treatment is  $71.2 \pm 2.3$  g/ml.

### Apoptosis assay

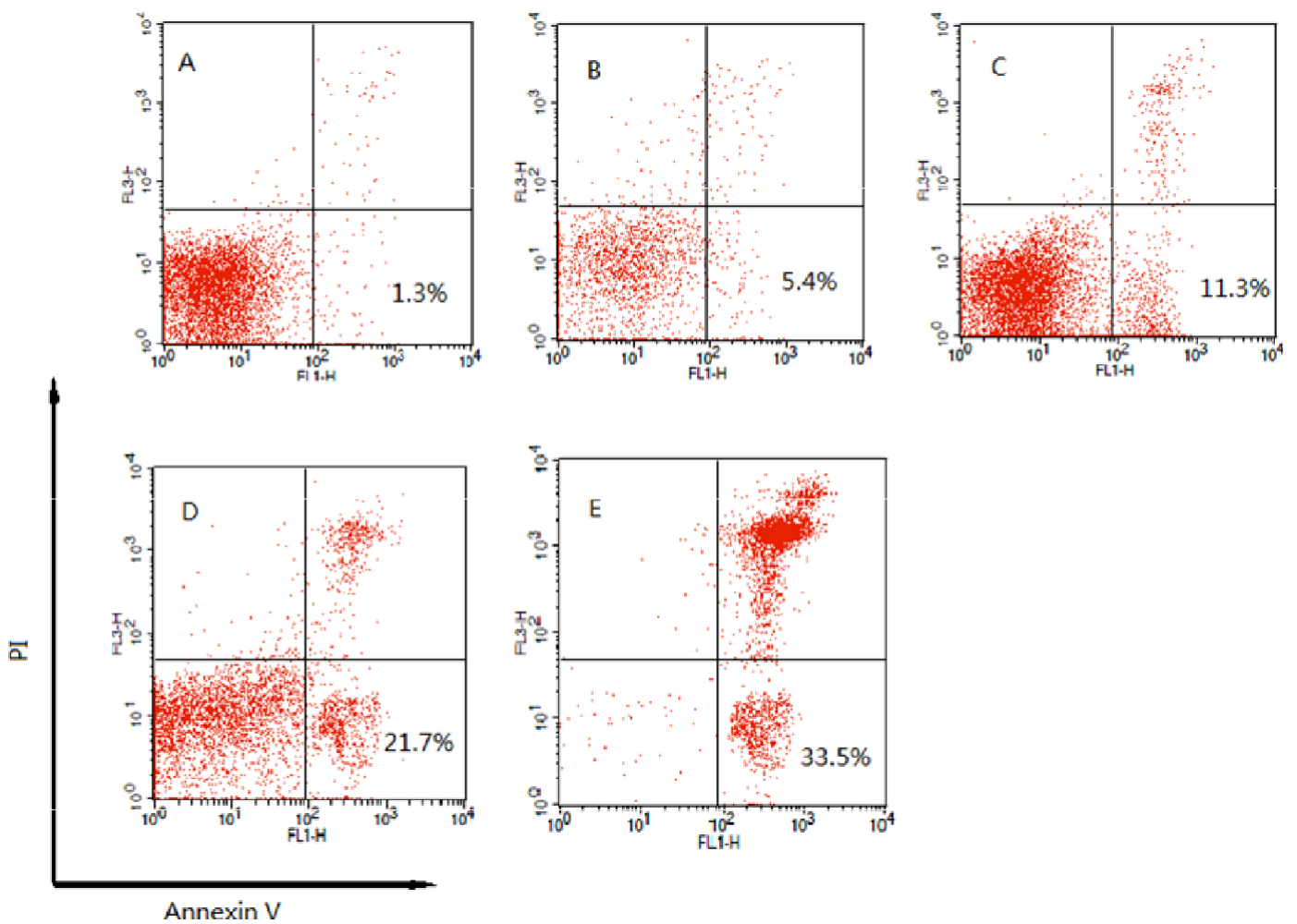
MCF-7 cells were treated with various concentrations of RPS for 48 h, then processed with Annexin V-FITC/PI Apoptosis Assay Kit and examined by flow cytometry. The results indicated that the apoptosis rate of MCF-7 cells was higher with RPS treatment than without RPS treatment (Figure 2).

### RPS induced the loss of MMP

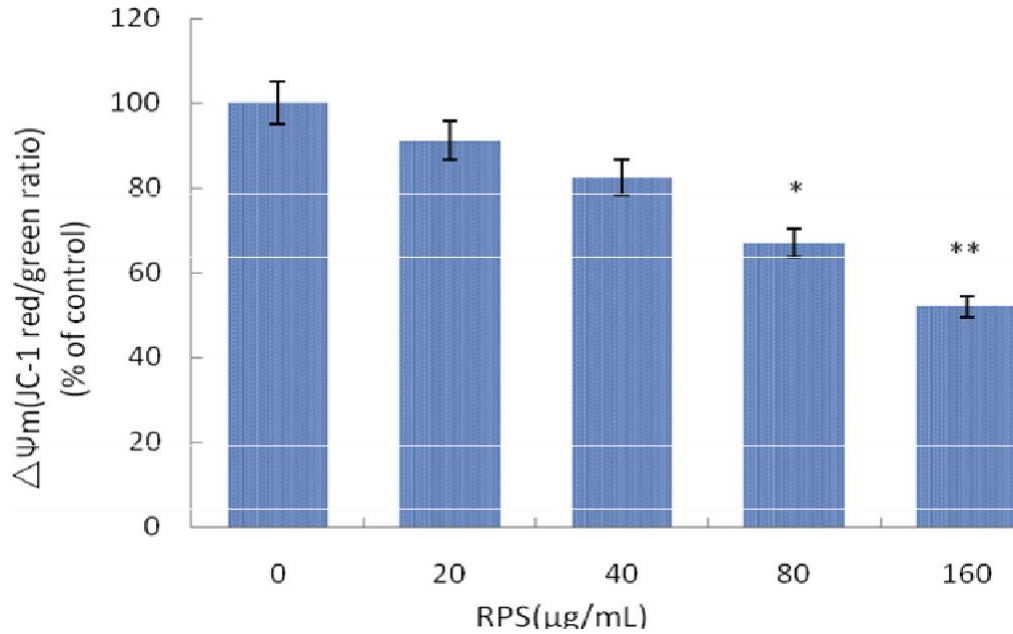
To characterize the upstream factors involved in the intrinsic apoptotic pathway, the mitochondrial permeability of cells was measured by JC-1 staining-based flow cytometry. As shown in Figure 3, the significant decrease in the ratio of JC-1 red/green fluorescence indicated a loss of MMP (Figure 3).



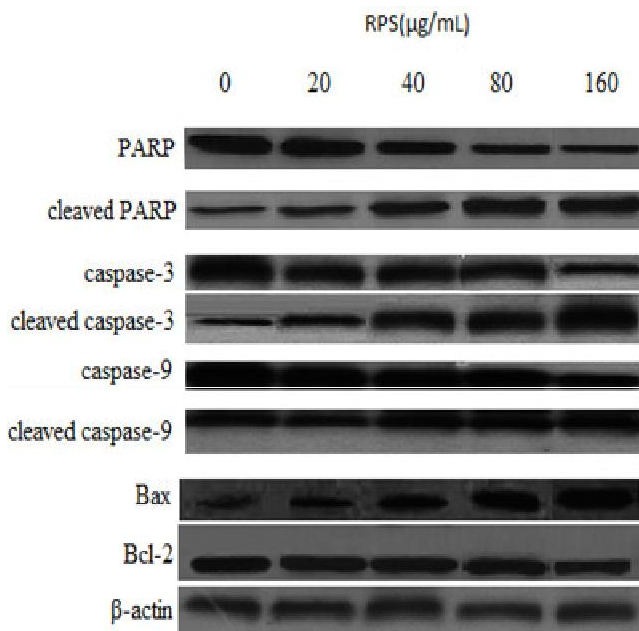
**Figure 1.** Cytotoxic effects of RPS in MCF-7 cells. Cell viability was assessed by MTT assay. Data are expressed as mean  $\pm$  SD (n = 3). Triplicate measurements were performed. \* P < 0.05. \*\* P < 0.01 compared with control.



**Figure 2.** Flow cytometer analysis of apoptotic cells by annexin V-FITC/PI expression. RPS induced apoptosis in MCF-7 cells: (A) MCF-7 cells not treated with RPS; (B) MCF-7 cells treated with 20 g/ml RPS; (C) MCF-7 cells treated with 40 g/ml RPS; (D) MCF-7 cells treated with 80 g/ml RPS; (E) MCF-7 cells treated with 160 g/ml RPS. Early apoptotic cells were defined as annexin V-positive, PI- negative cells.



**Figure 3.** RPS induced the loss of MMP in MCF-7 cells. Cells were treated with various concentrations of RPS for 48 h. MMP was measured by flow cytometry after JC-1 staining. Triplicate measurements were performed. \* P < 0.05. \*\* P < 0.01 compared with control.



**Figure 4.** Bax, Bcl-2, caspase-3/9, cleaved caspase-3/9, PARP, and cleaved PARP were determined by western blot analysis. -Actin was used as an internal control.

### Western blot analysis

To investigate the change in protein levels involved in RPS-induced apoptosis, total cell lysates from treated

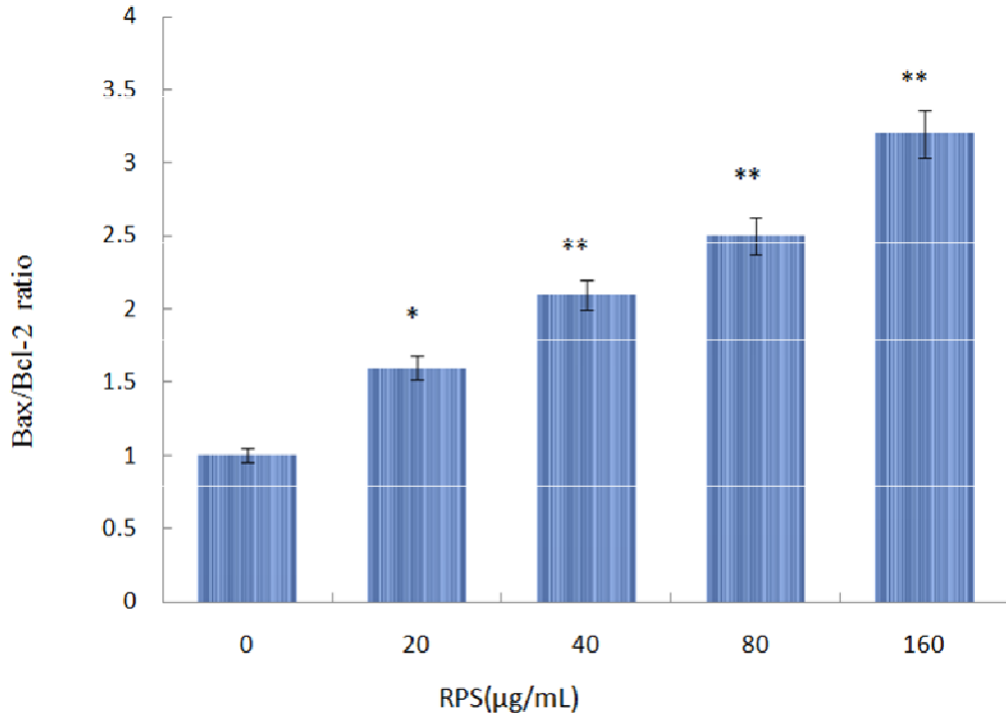
cells were prepared, and Bax, Bcl-2, caspase-3/9, cleaved caspase-3/9, PARP, and cleaved PARP levels were determined by western blot analysis. Results showed that the 48-h RPS treatment activated PARP, caspase-3, and caspase-9 and increased the ratio of Bax and Bcl-2 (Figures 4 and 5).

### RPS induced the release of cytochrome C in MCF-7 cells

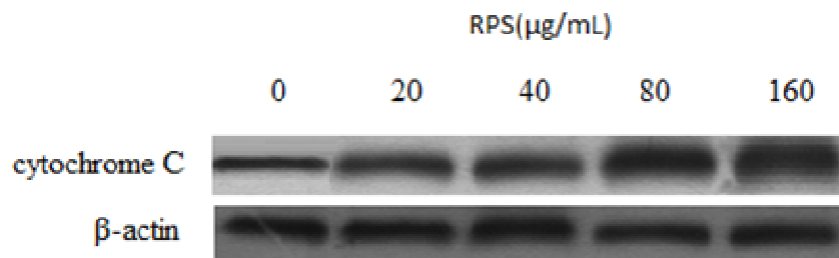
The disruption of the mitochondrial membrane is known to result in the release of cytochrome C into the cytosol, and this was detected by western blot analysis (Figure 6). These results showed that the treatment of MCF-7 cells with RPS induced the release of cytochrome C from the mitochondria in a dose-dependent manner.

### DISCUSSION

In recent years, natural compounds have shown significant antitumor effects. To balance cell proliferation and cell death to maintain homeostasis in normal tissues is an important mechanism. Cell death occurs via 2 pathways; apoptosis and necrosis. Apoptosis, also called programmed cell death, is characterized by the maintenance of intact cell membranes during the cell suicide process (Hetz et al., 2005). Therefore, apoptosis is considered to play a role in cancer prevention. Some studies have shown that RPS has the ability to reduce



**Figure 5.** The change in Bax/Bcl-2 levels. The relative photographic density was analyzed by Quantity One software. Triplicate measurements were performed. \*  $P < 0.05$ . \*\*  $P < 0.01$  compared with control.



**Figure 6.** The release of cytochrome C from mitochondria was detected by western blot analysis. -Actin was used as an internal control.

the viability ratio of cancer cells and induce apoptosis and that it has a strongly cytotoxic effect on MCF-7 human breast cancer cells, but the molecular mechanisms underlying these effects are not clear.

Apoptosis is the most potent defense mechanism against cancer (Sun et al., 2004). Mitochondrial apoptosis pathway is the main procedure in cell apoptosis. MMP is important in maintaining the stability of the environment of the inner mitochondria and the oxidative phosphorylation pathway (Jack et al., 2005).

The role of the Bcl-2 family proteins in apoptosis regulation has been extensively studied. These proteins are widely distributed in the mitochondrial outer membrane, nuclear membrane, and endoplasm. The

family is divided into anti-apoptosis proteins like Bcl-2 and Bcl-xL, and apoptosis-promoting proteins like Bax, which are the most important regulators for characterizing apoptosis (Deveraux and Reed, 1999; Roy et al., 1997). Bcl-2 is a kind of mitochondrial membrane protein that can inhibit apoptosis and increase cell viability. Bax is an apoptosis-promoting protein in the mitochondrial apoptosis pathway. In the mitochondrial-dependent pathway, the major function of the Bcl-2 family proteins is to regulate the permeabilization of the mitochondrial membrane. The ratio of Bax/Bcl-2 is the key factor in determining whether apoptosis occurs after cells receive the apoptotic signal. When the ratio of Bax/Bcl-2 increased, the loss of MMP was promoted, the

permeabilization of mitochondrial membrane was increased, and more cytochrome C was released (Kakkar and Singh, 2007; Sheridan et al., 2008). As an apoptosis induction factor, cytochrome C mediates the binding of Apaf-1 to pro-caspase-9, resulting in the activation of caspase-9 and initiation of the caspase cascade (Katoh et al., 2008; Zhao et al., 2007), and then it cleaves PARP and induces apoptosis (Egger et al., 2007). Cleavage of caspase-3 and caspase-9 are characteristics of apoptosis (Kok et al., 2005; Sunaga et al., 2004).

In the present study, RPS treatment resulted in the loss of MMP and induced the down-regulation of anti-apoptotic Bcl-2 levels as well as the up-regulation of pro-apoptotic Bax levels. The expression level of cleaved caspase-3/9 and cleaved PARP and the concentration of cytochrome C in the cytoplasm increased. These results demonstrated that RPS induced apoptosis in MCF-7 human breast cancer cells via the mitochondrial apoptosis pathway, involving the release of cytochrome C, activation of Bax, inhibition of Bcl-2, activation of the caspase cascade, and activation of PARP.

## Conclusion

This study demonstrated that RPS has a potent inhibitory effect on the proliferation of MCF-7 human breast cancer cells. Induction of apoptosis by RPS is confirmed via the loss of MMP, release of cytochrome C, increase of Bax/Bcl-2 ratio, activation of the caspase cascade, and cleavage of PARP. These results suggest that the RPS-induced loss of MMP has an important role in the apoptosis mechanism of MCF-7 human breast cancer cells and that RPS may be potentially used as an anticancer agent against human breast cancer.

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†Chuan Lu and Chunjiang Li contributed equally to this work as co-first authors

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