

Apoptosis and G₂/M arrest induced by *Allium ursinum* (ramson) watery extract in an AGS gastric cancer cell line

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Background: The present study was designed to determine whether *Allium ursinum* L (ramson) could inhibit the proliferation of human AGS gastric cancer cells. Furthermore, we attempted to determine whether this inhibition could occur by targeting regulatory elements of the cell cycle.

Methods: Flow cytometry was used to observe apoptosis and the cell cycle in AGS cell lines treated or not treated with ramson watery extract. Proteins related to the cell cycle were detected by Western blotting. Caspase activity was measured using a colorimetric assay kit according to the manufacturer's instructions.

Results: Ramson watery extract induced apoptosis and G₂/M phase arrest in AGS cells. Western blotting showed that cyclin B was inhibited by ramson watery extract. However, G₁ phase-related proteins remain unchanged after treatment.

Conclusion: Our results indicate that ramson effectively suppressed proliferation and induced apoptosis and G₂/M arrest in AGS cells by regulating elements of the cell cycle.

Keywords: ramson, G₂/M phase arrest, apoptosis, Allium, gastric cancer

Introduction

Gastric cancer is one of the most common malignancies, not only in the People's Republic of China but also in the rest of the world.^{1,2} Worldwide, 989,600 new cases of stomach cancer and 738,000 deaths are estimated to have occurred in 2008, accounting for 8% of total cancer cases and 10% of total deaths.³

It has been demonstrated that dietary intake of Allium vegetables may lower the risk of several types of malignancies, including stomach, esophageal, and prostate cancer.⁴⁻⁶ Epidemiologic research has shown that the risk of prostate cancer is significantly lower in men consuming >10 g/day of Allium vegetables than in men with a total Allium vegetable intake <2.2 g/day.⁶ *Allium ursinum* L (ramson, wild garlic) is a perennial plant, and widely distributed throughout Europe.⁷ Ramson is used as a spice and as a traditional medicine, lowering blood pressure, and being effective against arteriosclerosis, diarrhea, and indigestion.⁸ Ramson leaves have been used to treat cardiovascular disease since the Middle Ages.⁹ High amounts of volatile compounds, such as sulfides and disulfides, which had been identified in ramson, have a direct impact on the quality of ramson as a medicinal plant and as a spice.¹⁰

All parts of the ramson plant are known to have antioxidant properties,⁸ but the chemical compounds in the leaves, stalks, and seeds of the plant have not been fully identified. To our knowledge, until now, no studies have investigated the effects of ramson on gastric cancer. In this study, we demonstrated that ramson significantly prevents growth and induces

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apoptosis and G₂/M arrest in AGS cells, and have confirmed the mechanism of G₂/M arrest induced by ramson.

Methods and materials

Cell culture

AGS cells were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA) and grown in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Preparation of ramson extract and spectrophotometry

Powdered samples of whole ramson plants were obtained from Fei-fei Mou, Shijiazhuang Medical College, Shijiazhuang, People's Republic of China. The powder samples (2 g) were extracted with 50 mL of methanol acidified with 1% acetic acid and then dissolved in distilled water to obtain a working solution. Using the methods described by Putnoky et al,¹¹ the ultraviolet absorption spectrum of the working solution was obtained against distilled water in a 1 cm quartz cuvette. The absorbance report measured at 240–250 nm was read according to the literature specifications to confirm the identity of the absorbing compound (Allicin; Xian Linhe Biotechnology Co. Ltd, Xi'an, People's Republic of China).

Cell viability assay

AGS cells were seeded into 96-well plates at a density of 1×10^3 cells per well, 24 hours before treatment. For the inhibition test, the cells were treated with different concentrations of ramson for 48 hours. Next, 20 µL (5 mg/mL) of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Carlsbad, CA, USA) was added to each well, and after incubation at 37°C for four hours, the MTT solution was removed and 200 µL of dimethylsulfoxide 0.2% was added to dissolve the crystals. Absorbance in each well at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The IC₅₀ value for ramson was determined and used in the subsequent experiments.

Cell apoptosis assay

Apoptosis was determined using an apoptosis detection kit (KeyGEN, Nanjing, People's Republic of China). Briefly, the cells were collected, washed twice in ice-cold phosphate-buffered solution, and then resuspended in binding buffer at a density of 1×10^6 cells/mL. The cells were incubated simultaneously with fluorescein-labeled Annexin V and propidium iodide

for 20 minutes. The mixture was then analyzed using a FACScan flow cytometer (BD Biosciences, Baltimore, MD, USA).

Detection of cell cycle changes

The cells were removed from plates by trypsinization and pooled with cell culture supernatant containing nonadherent cells. The cells were washed once with phosphate-buffered solution, fixed in cold 70% ethanol, and stored at –20°C until analysis. For staining, 1×10^6 cells were washed in phosphate-buffered solution and then stained in phosphate-buffered solution with 50 µg/mL propidium iodide (KeyGEN), 200 µg/mL RNase A, and 0.1% Triton X-100. Analyses were performed using the FACScan flow cytometer.

Western blot assays

Protein extract samples (each weighing 45 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat milk in Phosphate-Buffered Saline with Tween 20, then probed with primary antibodies at 4°C overnight. The primary antibodies used were cyclin D1, cyclin E, cyclin B, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated with the appropriate secondary antibodies for two hours at room temperature. Bound antibody was detected using an enhanced chemiluminescence kit (Amersham Biosciences, Westborough, MA, USA) according to the manufacturer's instructions.

Measurement of activity of caspases 3, 8, and 9

Caspase activity was measured using a colorimetric assay kit according to the manufacturer's instructions. After harvesting, the cells were washed in ice-cold phosphate-buffered solution and lysed. Proteins were extracted and stored at –80°C until use. Next, 20 µL of cell lysate was added to buffer containing a p-nitroaniline (pNA)-conjugated substrate (80 µL) for caspase 3 (Ac-DEVD-pNA), caspase 8 (Ac-IETD-pNA), or caspase 9 (LEHD-pNA, KeyGEN). Incubation was performed at 37°C with shaking at 500 rpm for one minute and then at room temperature for two hours. The pNA released in each well was measured using a plate-reading luminometer (Thermo Scientific, Shanghai, People's Republic of China). Data were collected from three independent experiments.

Statistical analysis

All numerical data are expressed as the mean ± standard deviation. Differences between mean values were evaluated

using the Student's *t*-test. All statistical analyses were conducted using the Statistical Package for the Social Sciences version 17.0 software (SPSS Inc, Chicago, IL, USA). *P* values < 0.05 were considered to be statistically significant.

Results

Spectrophotometric results

The ultraviolet absorption spectrum for the ramson solution, obtained against distilled water in a 1 cm quartz cuvette, is shown in Figure 1. The profile of the absorption spectrum is significant in the areas of highest and lowest absorption, and distinct profiles indicate the effects of different molecules. Using the values in the literature for specific absorption at 240 nm, the Allicin content in the fresh raw sample was found to be 0.58 $\mu\text{g/mL}$.

Effects of ramson on AGS cells

Cell viability was monitored by MTT assay. Figure 2 shows the growth inhibition curves generated. The IC_{50} value for ramson was 16.2 μM . Proliferation of the AGS gastric cancer cells was inhibited by ramson. We used double-staining with Annexin V-fluorescein isothiocyanate and propidium iodide to detect apoptotic cells. The apoptotic ratio of cells increased significantly after treatment with ramson. As shown in Figure 3, the ratio of treated cells was 3–4 times higher than that of untreated cells ($P < 0.05$). We then detected changes in the cell cycle using propidium iodide staining. As shown in Figure 4, the ratio of cells in G_0/G_1 phase was decreased in treated cells compared with untreated cells, but numbers in G_2/M phase increased ($P < 0.05$). The amount of cells in G_2 phase after treatment with ramson was 3–3.5 times higher than that of untreated cells.

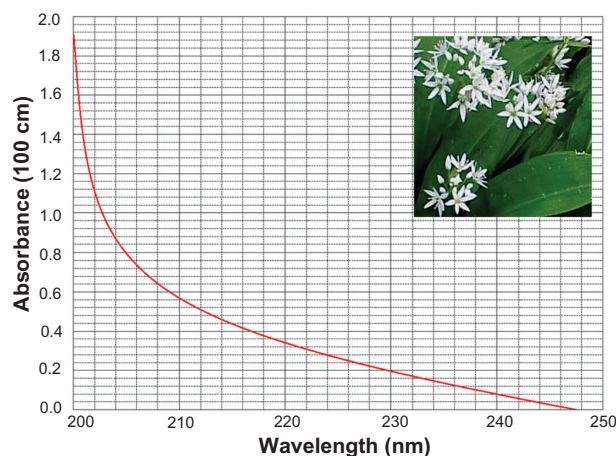


Figure 1 Ultraviolet absorption spectrum for ramson solution.

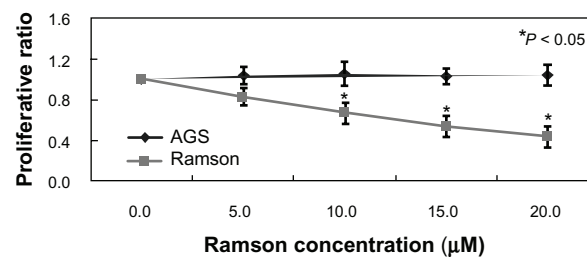


Figure 2 Growth curve for cell lines measured using the MTT assay. The experiments were repeated three times.

Abbreviations: AGS, untreated AGS cells; ramson, AGS cells treated with ramson; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Mechanism of apoptosis and G_2/M arrest in AGS cells

No changes in cyclin D1 or E levels were identified in treated cells using Western blotting (Figure 5). The main G_2 phase-related protein detected was cyclin B, which almost disappeared in treated cells (Figure 5). The results were consistent with the changes in the cell cycle reported above. Further, the activity of caspases 3, 8, and 9 was significantly increased in treated cells compared with untreated cells ($P < 0.05$, Figure 6).

Discussion

In recent decades, much research attention has been focused on the molecular basis of oncogenic formation and transformation.¹² However, drug resistance in cancer is still a challenge when attempting to cure the disease, and current therapeutic combinations used to treat gastric

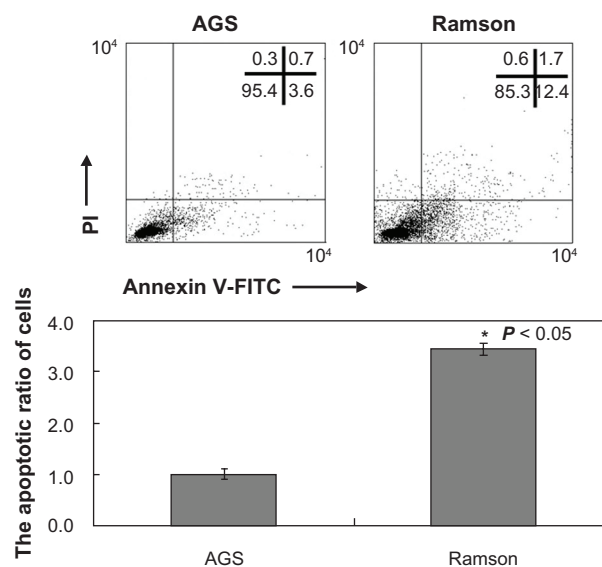


Figure 3 Apoptotic ratio of AGS cells treated with ramson analyzed by double-staining with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide. *Ramson-treated cells showed a higher apoptotic ratio than untreated cells ($P < 0.05$).

Abbreviations: AGS, untreated AGS cells; ramson, AGS cells treated with ramson.

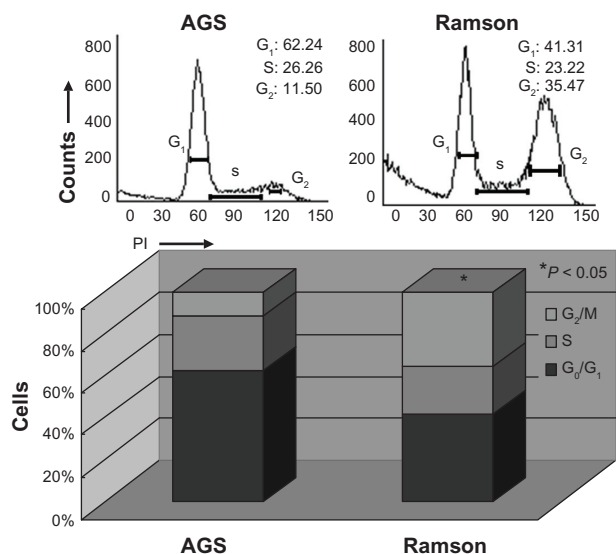


Figure 4 Propidium iodide staining highlighting changes in the cell cycle, with a marked increase in the G₂/M fraction of AGS cells treated with ramson compared with untreated cells ($P < 0.05$).
Abbreviations: AGS, untreated AGS cells; ramson, AGS cells treated with ramson.

cancer are not fully effective. The *A. ursinum* plant has been reported to have antioxidative, cytostatic, and antimicrobial activity.¹³ However, the mechanism of the antitumor activity of ramson has been unclear. In this work, we confirmed that ramson can inhibit proliferation and induce apoptosis in AGS cells. To our knowledge, limited data are available on the antitumor activity of ramson. However, diallyl disulfide, an extract of garlic, has been shown to inhibit the growth of many cancers.^{14,15} Xiao et al¹⁶ reported that diallyl trisulfide inhibited the viability of human lung cancer cells. Further, suppression of cancer cell growth in association with G₂/M phase cell cycle arrest and/or induction of apoptosis mediated by organosulfur compounds, ie, diallyl disulfide and diallyl trisulfide, has been demonstrated in human colon, neuroblastoma, and prostate cancer cells.¹⁷⁻²² Consistent with previous

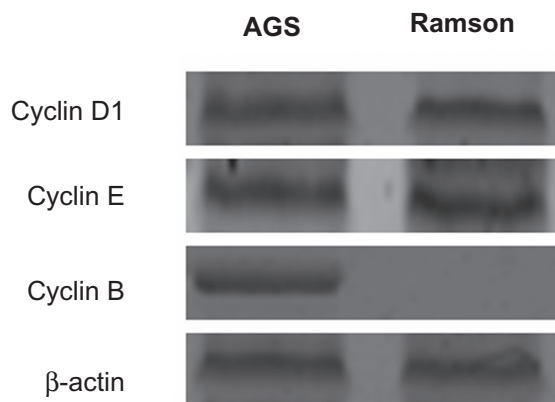


Figure 5 Western blot analysis of proteins related to the cell cycle. Cyclins D1, E, and B were detected. β -actin was used as the internal control.
Abbreviations: AGS, untreated AGS cells; ramson, AGS cells treated with ramson.

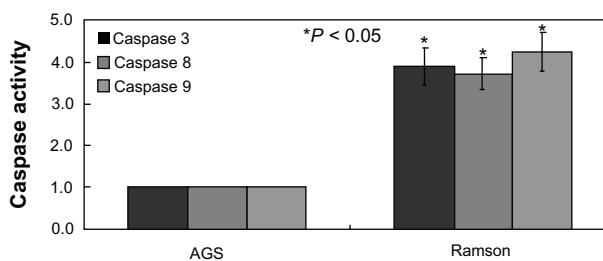


Figure 6 Activity of caspases 3, 8, and 9 was detected in each group. Ramson-treated cells showed more caspase activity than untreated cells ($P < 0.05$).
Abbreviations: AGS, untreated AGS cells; ramson, AGS cells treated with ramson.

studies, our research confirmed that ramson induced G₂/M arrest in AGS cells. These results collectively indicate that extracts from *Allium* vegetables share the common feature of antitumor activity.

To determine the mechanism(s) of action of ramson in AGS cells, we identified the proteins relevant to the cell cycle by Western blotting. There were no changes in cyclin D1 or E levels between the treated and untreated cells. Cyclins D1 and E are important factors in the conversion from G₁ phase to G₂ phase. Zurlo et al²³ also confirmed that decreased levels of cyclins D1 and E could induce arrest of G₁ phase in HT-29 human colon carcinoma cells. In subsequent experiments, we found that cyclin B, the main protein involved in G₂ phase, disappeared on treatment with ramson, which explains why the cell cycle was obstructed in G₂ phase. Similarly, Gao et al²⁴ found that downregulation of cyclin B by aspirin (a nitric oxide donor) can induce arrest of G₂/M phase.

Overall, in our experiments, we identified that ramson had an antitumor effect in AGS gastric cancer cells. Apoptosis and arrest of G₂/M phase were observed in cells after treatment with ramson. Our research offers a novel insight into the treatment of gastric cancer. In future studies, we plan to observe further the effects of ramson on gastric cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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