

Full Length Research Paper

Agrimoniin induced SGC7901 cell apoptosis associated mitochondrial transmembrane potential and intracellular calcium concentration

Bao-qing Wang* and Zhe-xiong Jin

College of Pharmacy, Harbin University of commerce, Harbin, Heilongjiang Province, 150076, P. R. China.

Accepted 22 April, 2011

Agrimoniin, a form of tannin separated from *Agrimonia pilosa* ledeb, causes cancer cell death through induction of apoptosis. The present study was designed to investigate the effects of agrimoniin on mitochondrial transmembrane potential and the reactive oxygen species (ROS) production in human gastric cancer cell SGC-7901. In addition, the concentration of intracellular calcium was measured by laser confocal scanning microscopy (LCSM). The results showed mitochondrial transmembrane potential of treatment groups was significantly lower than that in untreated group. The concentration of calcium in cells exposed to agrimoniin for 24 h was increased in a dose dependent manner compared with unexposed group, the level of ROS in the SGC-7901 cell was treated by the different dose of agrimoniin, which were higher than that in control group. Therefore, agrimoniin can increase the concentration of calcium and the intracellular level of ROS, decrease mitochondrial transmembrane potential, and then induce the SGC-7901 cells apoptosis. These results can improve our understanding about agrimoniin induced apoptosis.

Key words: Agrimoniin, apoptosis, human gastric cancer.

INTRODUCTION

Gastric cancer is one of the most common malignancies in the world, particularly in eastern Asian countries such as China, Korea and Japan (Parkin et al., 2005). It is also the second leading cause of cancer-related death in the world, with approximately 876000 new cases diagnosed each year (Guoqing et al., 2010). In China, the incidence and mortality of gastric cancer accounts approximately for 17.2 and 20% of that of all malignant diseases, respectively (Wagner et al., 2006). The surgical treatment of gastric cancer is currently the main therapy, but the median overall survival is no more than twelve months (Zhu et al., 2009). Although chemotherapy still plays an important role in gastric cancer therapy, side effect of chemotherapy is very obvious. Therefore, anticancer research of natural products has received great attentions, specially after modern biotechnical application, a lot of natural anticancer agents are being developed to improve gastric cancer therapy. *Agrimonia*

pilosa Ledeb is a traditional medicinal plant belongs to *Rosaceae* and has been reported to possess various medicinal importance (Zhe-xiong et al., 2010). In China, this plant is traditionally used to suppress diarrhoea, reduce gastric ulcers, relieve inflammation, improve eyesight, detoxify poison and increase the flow of urine. Among the polyphenolic compounds in *A. pilosa* Ledeb, tannins are the main substances with better pharmacological activities including antiviral, anticancer and hepatoprotective activities. Agrimoniin is a form of tannin separated from *A. pilosa* ledeb. In 1985, Kenichi et al. (1985) first reported that agrimoniin is the main tannin component in several species of *A. pilosa* Ledeb, (Kenichi et al., 1985). Agrimoniin has caused much attention due to its various biological activities such as antiviral, anti-microbia and anticancer. In particular, the anticancer activity has been investigated by more people. Such as agrimoniin has been reported to inhibit the growth of MH134 and Meth-A solid type tumors, it also showed strong cytotoxicity on MM2 cells *in vitro* and might be a promising antitumor tannin agent in human cancer. Agrimoniin has been proven to inhibit HepG2 cancer cell lines.

*Corresponding author. E-mail: mrwbq@yahoo.cn Fax: 86045184806033.

Apoptosis is an important mechanism in both development and homeostasis in adult tissues for the removal of either superfluous, infected, transformed or damaged cells by activation of an intrinsic suicide program, such as tumor regression (Chun et al., 2003). Up to now, several apoptotic pathways have been identified in cells responsive to apoptotic insult, such as the apoptosis mediated by the activation of death receptors, mitochondria-dependent signaling, and endoplasmic reticulum-induced apoptotic cell death (Sun et al., 2009). Thus, initiation of apoptotic signal pathway and induction of apoptosis is considered as an effective approach for tumor treatment. Especially the mitochondria-dependent signaling is associated with ROS and intracellular Calcium. ROS is the known mediators of intracellular signaling cascades (Rakhee et al., 2008). The accumulation of ROS and the overloading of intracellular calcium have been shown to trigger apoptotic responses through affecting the change of mitochondrial membrane potential. There is increasing interest to develop natural compounds as experimental cancer therapeutics, but lack of systematic research in human gastric carcinoma SGC-7901 cells. Our study was designed to investigate the effects of agrimoniin on the mitochondrial transmembrane potential and the level of intracellular calcium in SGC-7901 cells. We attempt to demonstrate the antitumor effect is associated with the generation of ROS, the mitochondrial membrane potential and the level of intracellular calcium.

MATERIALS AND METHODS

Reagents and drug

Agrimoniin and hydroxycamptothecin injection (HCPT, 2010628) was purchased from Huangshi Hsfy Pharmaceutical Corporation. RPMI 1640 culture medium (Invitrogen Corporation); fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd); pancreatin (Gibco); reactive oxygen species assay kit (Beyotime Institute of Biotechnology); Fluo-3/AM (Molecular Probes Inc, USA); Rhodamine 123 (Sigma); Methyl thiazolyl tetrazolium MTT (Sigma).

Cell lines and cell culture

Human gastric cancer SGC-7901 cells were purchased from the Institute for Cancer Research, Heilongjiang Cancer Hospital. SGC-7901 cells culture was incubated in RPMI 1640 medium containing 10% fetal bovine serum with CO₂ and at 37°C and transfer of culture was performed once every 2 to 3 days.

Cell viability assay

The effects of agrimoniin on cell proliferation were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing human gastric cancer SGC-7901 cells in 96-well plates were treated with different concentrations of agrimoniin in complete medium or the medium alone. MTT (5 mg/ml) 20 ml was added 24 h later. After the plates

were incubated a 37°C for 4 h, the supernatant was aspirated, and 150 µl dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 570 nm by a 96 well microplate reader. The percentage of surviving cells was calculated as follows:

Cell viability rate (%) = (mean absorbency in test wells)/(mean absorbency in control wells) × 100.

Measurement of apoptosis by annexin-V fluorescein isothiocyanate and propidium iodide double staining

Apoptosis was measured by a flow cytometric analysis of cells stained with AnnexinV-fluorescein-isothiocyanate (FITC) and propidium iodide (PI). According to the manufacturer's instructions, after incubation in DMSO alone for 72 h, the cells were pelleted by centrifugation and incubated with Annexin V-FITC and PI. Single cell suspensions were analyzed by FAC scan, early apoptotic cells were scored as Annexin V⁺, PI⁻, whereas late apoptotic cells scored as Annexin V⁺, PI⁺ to exclude necrotic cells (Annexin V⁻, PI⁺).

Determination of generation of ROS in SGC-7901 cells using FCM

Levels of ROS in control and agrimoniin treated cells was determined by staining the cells with DCFDA. DCFDA is a cell permeable and is cleaved by nonspecific esterases and oxidized by peroxides produced in the cells to form fluorescent DCF. The intensity of DCF fluorescence is proportional to the amount of peroxide produced in the cells. Briefly, SGC-7901 cells were plated at in 6-well plates (3 × 10⁵ cells/well) and allowed to attach overnight. After treatment of cells with medium for 24 h, cells were further incubated with 10 µM DCFDA at 37°C for 20 min. In positive control group, 3 × 10⁵ cells labelled by DCFH-DA were treated with 1 µL Rosup for 20 min. Subsequently, cells were removed, washed and re-suspended in PBS, filtrate with 300 apertures and analyzed for DCF fluorescence by FCM. Approximately, 10000 cells were evaluated for each sample.

Detection of agrimoniin-induced change in mitochondrial transmembrane potential in the cells using FCM

Rhodamine 123 was used to evaluate perturbations in mitochondrial transmembrane potential. SGC-7901 cells were plated at in 6-well plates (3 × 10⁵ cells/well) and allowed to attach overnight. After treatment of cells with agrimoniin, HCPT or medium for 24 h, cells were collected to 10 ml centrifuge tube and re-suspended in 500 µL PBS, and then 500 µL Rhodamine 123 (20 µg/ml) was gently added to the tube, so that the final concentration was 10 µg/ml. The cells were then incubated for 30 min in the dark. Cells were centrifuged at 1500 rpm for 5 min and removed supernatant, gently rinsed one times with PBS and then re-suspended in 800 µL PBS. After filtration (300 apertures), the suspension was analyzed by FCM.

Detection of the change of the concentration of calcium in SGC-7901 cells using LCSM

The change of the concentration of calcium in SGC-7901 cells were detected by using a LCSM with Fluo-3 AM labeling method. SGC-7901 cells were plated at in 6 well plates (3 × 10⁵ cells /well) and allowed to attach overnight. After treated with ethanol, agrimoniin or HCPT for 24 h, cells were collected, re-suspended with PBS and the cell concentration was adjusted to 2 × 10⁵ cells/ml. The cell suspension was centrifuged at 1500 rpm for 10 min and the

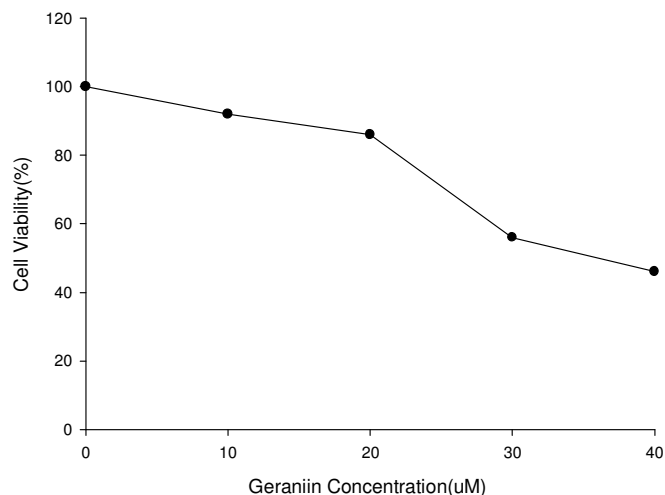


Figure 1. Effect of agrimoniin on the viability of human gastric cancer cell. SGC-7901 cells were treated with DMSO or various concentrations (10 to 40 µM) of agrimoniin for 24 h, and subsequent cell viability was measured by an MTT assay. Results from three separate experiments were averaged and are presented as mean±SE. The Student's t-test was used to determine the significance of inhibition.

Table 1. Effect of agrimoniin on the cell apoptosis of SGC-7901.

Group	Dose(µM)	Apoptosis rate (%)
Control	-	0.19±0.06
Agrimoniin	10	6.99±0.15**
	20	11.14±0.25**
	30	13.89±0.25**
	40	19.99±0.35**
HCPT	28	27.10±0.60**

*Compared with control $P < 0.05$; **Compared with control $P < 0.01$.

supernatant was removed. Then the cells were fluorescence stained with Fluo-3/AM at 37°C, avoiding light, for 60 min. Stained cells were centrifuged and the supernatant was removed. Then the cells were re-suspended with 400 µl of PBS and observed by LCSM.

Statistical analyses

All values are expressed as $\bar{x} \pm s$, and all statistical analysis was performed by analysis of variance (ANOVA). "A p" value less than 0.05 was considered as statistically significant.

RESULTS

The results of cell viability assay

First, to test the effect of agrimoniin on cell viability, SGC-

7901 cells were treated with different concentrations of agrimoniin. After 24 h of treatment, the percentage of living cells was determined, and it was shown that agrimoniin induced a dose-dependent decrease in cell viability (Figure 1).

Effect of agrimoniin on apoptosis in SGC-7901

Translocation of phosphatidylserine (PS) to the outer leaflet of the cellular membrane seems to be a key step in the early stages of apoptosis. Annexin V has a strong affinity for PS. Annexin V is conjugated to FITC, it is possible to identify and quantitate apoptotic cells on a single-cell basis by FCM. Early apoptotic and late apoptotic cells will bind Annexin V-FITC; necrotic cells have permeable membranes and will also bind Annexin V-FITC. PI is used to distinguish between viable, early apoptotic and necrotic, late apoptotic cells. PI is excluded by viable cells (FITC-negative) and early apoptotic cells (FITC-positive). Late apoptotic and necrotic cells stain with both Annexin V-FITC and PI (Lin et al., 2007). We measured the rates of apoptosis cells of SGC-7901 by FCM at 24 h after double staining with Annexin V-FITC and PI. We found that agrimoniin could induce apoptosis in SGC-7901 cells. Also, a greater number of early apoptotic cells were found after exposure to increasing concentrations of agrimoniin (Table 1 and Figure 2).

Effect of agrimoniin on the generation of ROS in SGC-7901 cells

We found that after treatment with 10, 20, 30 and 40 µM of agrimoniin, the level of ROS was 35.6, 43.6, 52.1 and 68.0%, respectively. These values are significantly higher than those of the control group (27.8%). The level of ROS in the positive control group was 72.3% (Figure 3). This result suggests that agrimoniin can raise the level of ROS in SGC-7901 cells, with the ROS concentration being positively correlated to the duration of treatment.

Mitochondrial transmembrane potential ($\Delta\psi_m$) changes in SGC-7901 cells induced by agrimoniin

From Figure 4 it can be seen that agrimoniin could decrease the mitochondrial transmembrane potential in SGC-7901 cells. There was a negative correlation between $\Delta\psi_m$ and the concentration of agrimoniin. With the increase in the concentration of agrimoniin, $\Delta\psi_m$ decreased more, or $\Delta\psi_m$ was shown to be inversely proportional to the concentration of agrimoniin administered.

Change of the concentration of calcium in SGC-7901 cells induced by agrimoniin

A sustained increase in intracellular Ca^{2+} concentrations

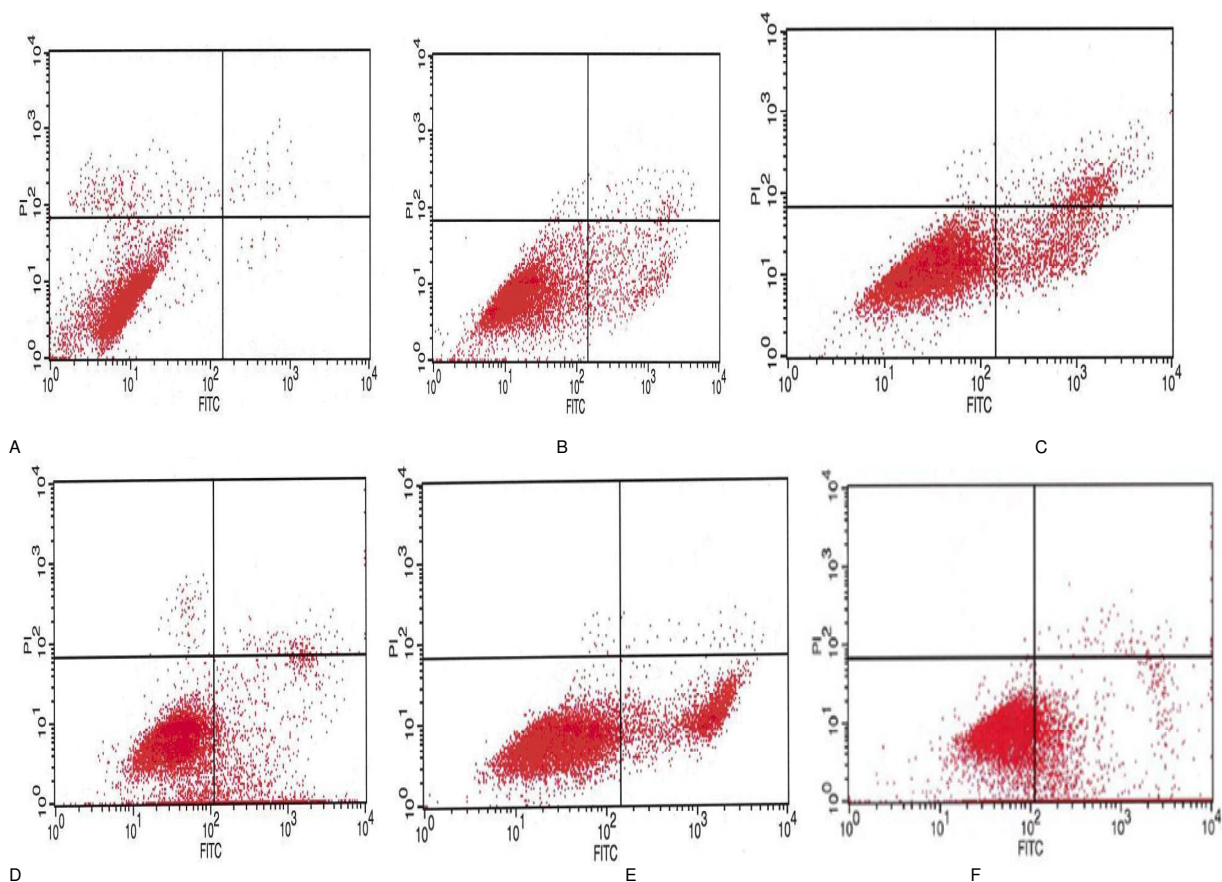


Figure 2. The apoptosis rate of SGC-7901 cells determined by FCM (A) Control ; (B) Positive of 28 μ M HCPT(C) 10 μ M agrimoniin; (D) 20 μ M agrimoniin; (E) 30 μ M agrimoniin and (F) 40 μ M agrimoniin.

is recognized to be a factor for cell death and cell injury. With this mind, we used Fluo-3/AM to examine the effect of agrimoniin on intracellular Ca^{2+} mobilizations in SGC-7901 cells. The changes of Ca^{2+} were observed by LCSM. In control group, the level of intracellular Ca^{2+} was the lowest. With the agrimoniin increased, the level of intracellular Ca^{2+} increased steadily (Table 2 and Figure 5). That was in accordance with the tendencies of mitochondrial membrane potential and cell apoptosis. The results indicated that the increase of intracellular Ca^{2+} was related with agrimoniin-induced SGC-7901 cell apoptosis.

DISCUSSION

Cancer is the leading cause of death worldwide, Gastric cancer accounts for the second highest mortality rate of all cancers worldwide (Bao-qing et al., 2010). Surgery and radiation or conventional chemotherapy treatment saved many lives, but far too many men develop metastatic gastric cancer, which is not curable with local treatment measures. Thus, present major advances in purification apparatus have led to the discovery of many natural components for cancer treatment. Agrimoniin, the

hydrolysable tannin, is decomposed to gallic acid, ellagic acid after boiling water hydrolysis. Gallic acid and ellagic acid have been reported to exhibit antioxidant activities, but relatively few reports concerning the anti-cancer activities of agrimoniin have appeared. Based on this, we have, in present study, first observed cell viability assay and then observed the effect of agrimoniin inducing apoptosis of SGC-7901 cells. We try to explicate the mechanism by which agrimoniin induces apoptosis of tumor cells by focusing on the mitochondria and by observing the effect of agrimoniin on the membrane potential, ROS and Ca^{2+} in SGC-7901 cells. ROS can induce lipid peroxidation or cross linking of thiol groups in proteins, both of which can result in opening of the mitochondrial permeability transition pore (PTP) (Kroemer et al., 1998). In this study, the results showed that after treatment of agrimoniin, the level of ROS was significantly higher than that in control group. Ca^{2+} also plays an important role during the process of apoptosis. Intracellular Ca^{2+} concentration increasing leads to mitochondrial calcium overload. Excessive Ca^{2+} within mitochondria can induce apoptosis by opening the mitochondrial PTP. Our results showed that the concentration of Ca^{2+} in the cells exposed to agrimoniin for 24 h was increased significantly in a dose dependent

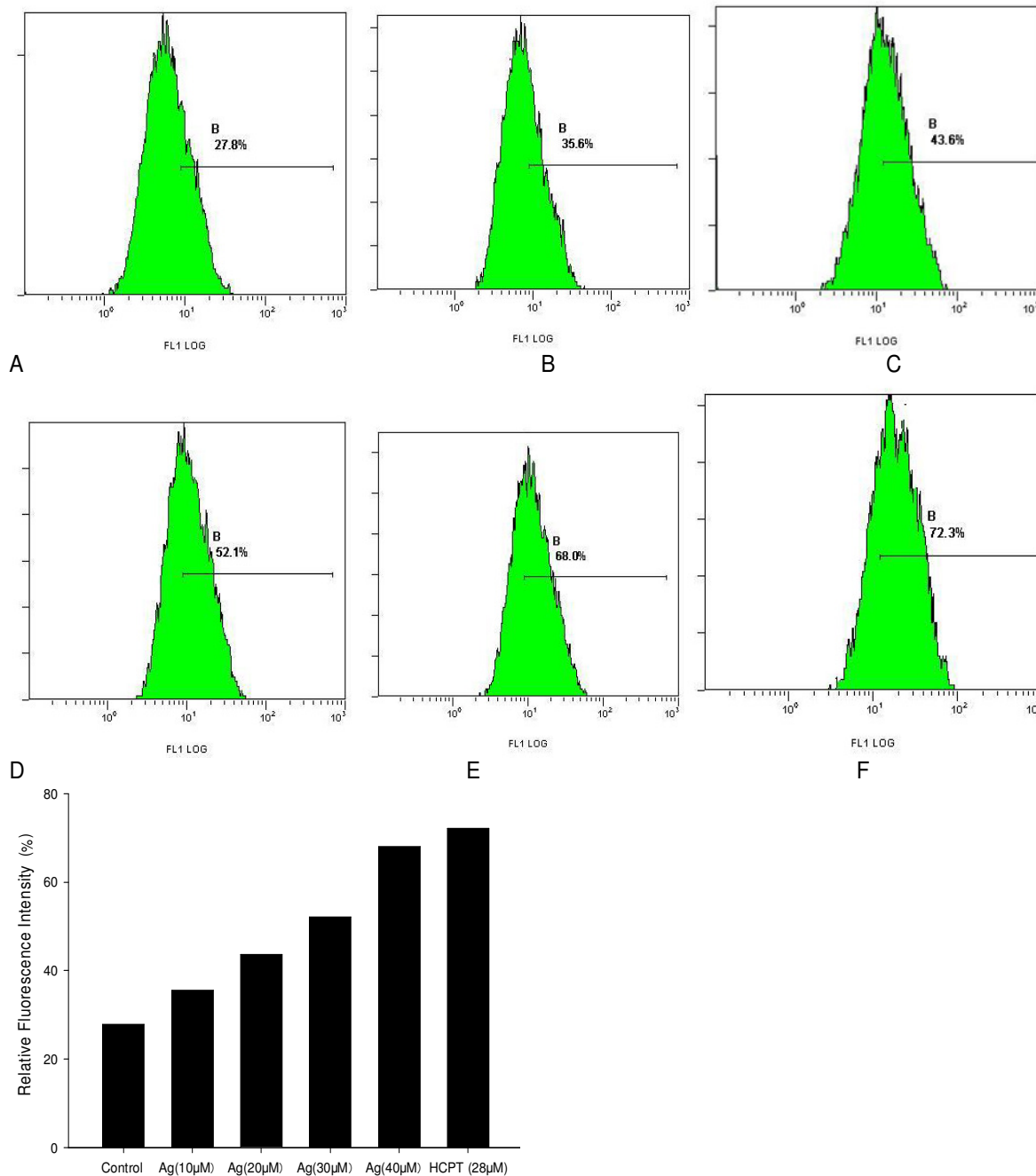


Figure 3. The levels of ROS in SGC-7901 cells exposed to agrimoniin (A) control; (B) 10 μ M agrimoniin; (C) 20 μ M agrimoniin; (D) 30 μ M agrimoniin; (E) 40 μ M agrimoniin and (F) positive of 28 μ M HCPT.

manner compared with control group. The opening of mitochondrial PTP can lead to release cytochrome c into the cytoplasm, further activates caspase-3 and perform the final steps in the apoptosis process.

Our results indicated that agrimoniin could decrease the mitochondrial transmembrane potential in SGC-7901 cells. There was a negative correlation between the mitochondrial transmembrane potential and the concentration of agrimoniin.

Conclusion

In conclusion, the present results demonstrate that agrimoniin can induce apoptosis in SGC-7901 cells through generation of ROS, increasing Ca^{2+} in the cells, decreasing mitochondrial transmembrane potential; agrimoniin can facilitate the opening of the mitochondrial permeability transition pore channels in mitochondria, releasing Ca^{2+} from these organelles. This leads to an

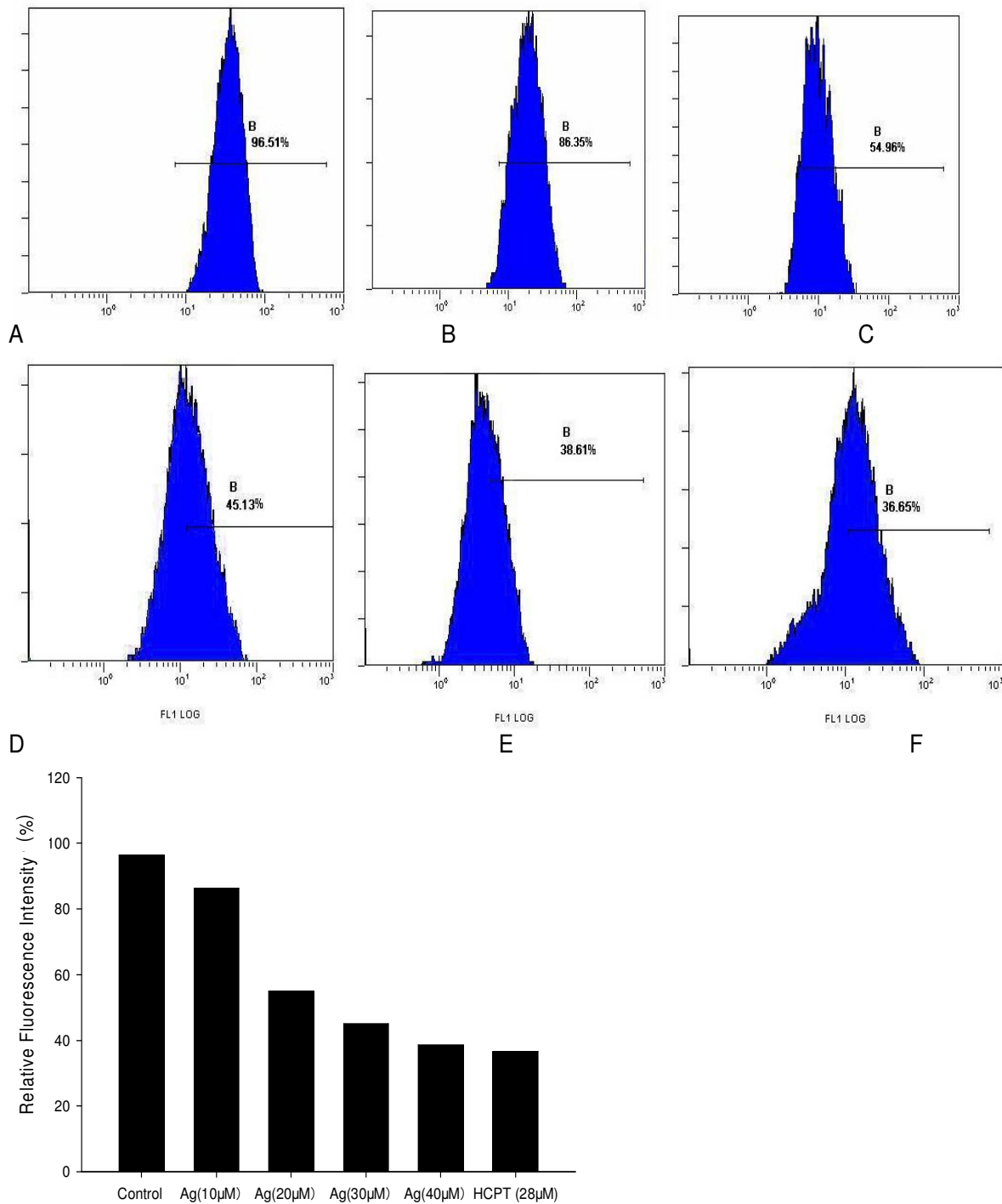


Figure 4. The mitochondrial transmembrane potential of SGC-7901 cells after incubation by with agrimoniin (A) control; (B) 10 µM agrimoniin; (C) 20 µM agrimoniin (D) 30 µM agrimoniin (E) 40 µM agrimoniin and (F) positive of 28 µM HCPT.

increase in the concentration of Ca^{2+} in the cell, thus starting the mechanism for apoptosis and inducing the occurrence of apoptosis. This research provided us an insight about agrimoniin induced SGC-7901 cell apoptosis. However, further research is needed to uncover how the channels are opened and which

enzymes and genes are involved in this process.

ACKNOWLEDGEMENT

Partial support was received with gratitude from Research

Table 2. Effects of agrimoniin on intracellular of (Ca²⁺) in SGC-7901.

Group	Concentration (μmol·L ⁻¹)	Variation of (Ca ²⁺) (fluorescent intensity)
Control		31.38±1.62
Agrimoniin	10	65.31±2.57**
	20	81.23±3.65**
	30	89.62±3.79**
	40	95.18±4.12**
HCPT	28	102.16±4.18**

*Compared with control P < 0.05. **Compared with control P < 0.01.

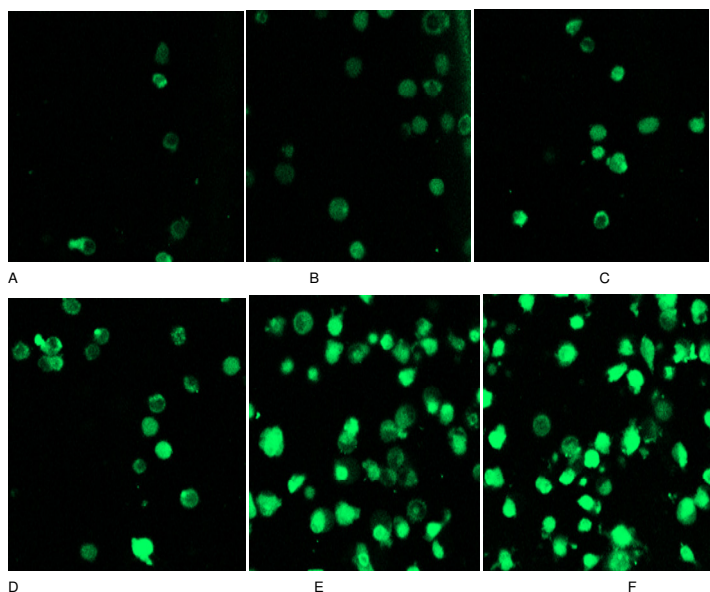


Figure 5. The concentration of calcium in SGC-7901 cells induced by agrimoniin, determined by LCSM. (A) control; (B) 10 μM agrimoniin; (C) 20 μM agrimoniin; (D) 30 μM agrimoniin; (E) 40 μM agrimoniin and (F) positive of 28 μM HCPT.

Foundation for Science and Technology Innovation Talents of Harbin (2010RFQXS072).

REFERENCES

- Baoqing W (2010). *Salvia miltiorrhiza* Chemical and pharmacological review of a medicinal plant. J. Med. Plants Res., 4(25): 2813-2820.
- Chun KH, Benbrook DM, Berlin KD (2003). The synthetic heteroarotinoid ShetA2 induces apoptosis in squamous carcinoma cells through a receptor independent and mitochondria dependent pathway. Cancer Res., 63: 3826-3832.
- Guoqing P, Hui L (2010). Elevated expression of semaphorin 5A in human gastric cancer and its implication in carcinogenesis. Life Sci., 86: 139-144.
- Kenichi M, Tyozo K, Yukinobu I, Heyhachiro T (1985). Isolation of agrimoniin an anticancer constituents from the root of *Agrimonia pilosa* Ledeb. Chem. Pharm. Bull., 33: 3977-3981.
- Kroemer G, Dallaporta B (1998). The mitochondrial death life regulator in apoptosis and necrosis. Annu. Rev. Physiol., 60: 619-642.
- Lin Xin, Wei XC (2007). Applying proteomic methodologies to analyze the effect of methionine restriction on proliferation of human gastric cancer SGC-7901 cells. Clin. Chim. Acta, 377: 206-212.
- Rakhee D, Amit R, Neeta D (2008). Reactive oxygen species and imbalance of calcium homeostasis contributes to curcumin induced programmed cell death in *Leishmania donovani*. Apoptosis, 13: 867-882.
- Parkin DM, Bray F, Ferlay J, Pisani P (2005). Global cancer statistics 2002. CA. Cancer J. Clin., 55: 74-108.
- Sun W, Xu W, Liu H (2009). Gamma-tocotrienol induces mitochondria-mediated apoptosis in human gastric adenocarcinoma SGC-7901 cells. J. Nutr. Biochem., 20: 276-284.
- Wagner AD, Grothe W, Haerting J, Kleber G (2006). Chemotherapy in advanced gastric cancer a systematic review and meta-analysis based on aggregate data. J. Clin. Oncol., 24: 2903-2909.
- Zhe XJ, Bao QW (2010). Microwave-assisted extraction of tannins from Chinese herb *Agrimonia pilosa* Ledeb. J. Med. Plants Res., 4(11): 2229-2234.
- Zhu L, Tan J (2009). Antioxidant activities of aqueous extract from *Agrimonia pilosa* Ledeb and Its fractions. Chem. Biodivers., 6: 1716-1725.