

Exploration for the multi-effect of cardamom in's resistance to multiple myeloma

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Abstract: This paper aimed to probe the cardamom in effect on the viability, proliferation, apoptosis and periodic function of the multiple myeloma, and explore its mechanism. We used CCK-8 method to evaluate the effect of cardamom in on the viability of PBMNCs (Persom Blood Mononuclear Normal Cells). EdU can test the influence of small cell proliferation. We used the method of PI single-staining flow cytometry, in order to test the influence of tumor cell cycle. AO (Acridine Orange), EB (Ethidium Bromide) double staining fluorescence microscope was applied to observe the influence of tumor apoptotic morphology. It can be concluded that cardamom in can inhibit the viability and proliferation of MM (Multiple Myeloma) cells and cardamom in is the anti-myeloma drug with strong viability.

Keywords: Cardamom in; Multiple Myeloma; Natural medicine.

INTRODUCTOIN

Multiple myeloma (short for MM) is a kind of malignant tumor with clonal proliferation of abnormal plasma cells in the bone marrow (Palumbo and Anderson, 2011). The incidence increases year by year, and currently the incidence of multiple myeloma exceeds the incidence of acute myeloid leukemia, second only to non-Hodgkin's lymphoma. MM has poor treatment response, the conventional chemotherapy efficiency is about 40%-60%, and the complete remission rate is less than 5%, the median survival is less than 3-4 years (Ahmedin *et al.*, 2010). In recent years, the high-dose chemotherapy, hematopoietic stem cell transplantation and molecular targeted drugs, have improved the overall survival and disease-free survival of the young patients, but the recurrence is still inevitable (Pappa *et al.*, 2013). Therefore, to find a new effective treatments and therapies is the focus of the study.

MM is considered to be an inflammatory disease, because this kind of disease has various common pathological features with inflammation. MM cells and various stromal cells in bone marrow microenvironment secrete the various inflammatory mediators abnormally; the most important inflammatory mediator is interleukin-6 (IL-6). Nuclear factor- kappa B (NF-κB) exists and plays a key role in the regulation of inflammatory response genes (Yee Mon and Ann, 2010). Under the condition of inflammation, NF-κB is activated, which launches a variety of gene expressions, so as to promote the secretion of inflammatory cytokines. NF-κB is one of the most important nuclear transcription factors, MM cells also in the activated state. Moreover, according to the report, MM cell surface also expresses various Tolls like receptors (TLR). TLR was used to simulate the MM cells, which can promote the proliferation and inhibit the apoptosis (Yang *et al.*, 2010).

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Therefore, some anti-inflammatory drugs have the potential function to cure MM, Feng R (Lentzsch *et al.*, 2007) found that, new non-steroidal anti-inflammatory drugs SDX-308 can lead to the apoptosis of MM cells and inhibit the formation and viability of the osteoclast through inhibiting the viability of NF-κB. The recent report shows that some natural anti-inflammatory drugs have the potential function to cure MM. Resveratrol, a natural anti-inflammatory drug, shows the good anti-MM effects. According to the report, Huanglian jiedu soup, a kind of Chinese medicine prescriptions, this has obvious anti-inflammatory effects (Panisinee *et al.*, 2012), Ma reported that Huanglian jiedu soup can inhibit the cell proliferation, induce the apoptosis, and inhibit the viability of NF-κB. The main active ingredient in the prescription is scutellarein, which not only has the anti-inflammatory effect, but also can inhibit the IL-6 signal transduction pathway in the MM cells (Shangqin *et al.*, 2010). Cantharidin, cantharidin extract, can induce the apoptosis of MM cells through inhibiting the JAK/STAT pathway. At present, the new targeted drugs, such as thalidomide and bortezomib, also have the anti-inflammatory effects.

Thus, in this paper, we can infer that the anti-MM medicine can be selected in the anti-inflammatory drugs. Many natural drugs have the anti-inflammatory effects, such as sinomenine, daphnetin, scutellarein, resveratrol, geniposide and cardamom in, etc (Quanxing and Xiaokang, 2011). Many people are interested in the anti-MM drugs, which select from the anti-inflammatory drugs. In this paper, we focused on the study of cardamom in.

MATERIALS AND METHODS

Experimental materials

Cell lines

Human MM cell lines RPMI 8226 and U266 were preserved in the laboratory, ARH-77 was purchased in America ATCC.

Main reagents and instruments

The formula of cardamom in is $C_{16}H_{14}O_4$, the molecular weight is 270.28 (molecular structure shown in fig. 1). We took cardamom in (20mg) and added dimethyl sulfoxide (DMSO) 482 μ L to dissolve completely, then getting the liquor that the concentration is 200mM, packing at the same amount under the condition of -20 °C before the usage, and applying the medium to dissolve the experiment desired concentration. In the dissolved cardamom in solution, the maximum concentration of DMSO is less than 1%, in the all experimental negative control group, 0.1% DMOS was used to collate, so as to eliminate the interference caused by DMSO.

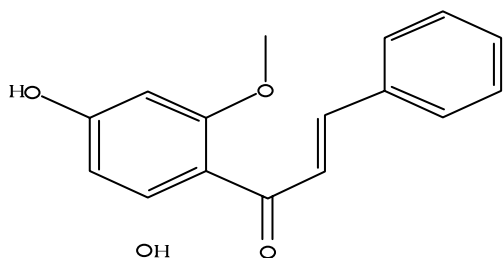


Fig. 1: The chemical structure of Cardamom in

Experimental methods

Human MM cell culture

Culturing Human MM cell lines RPMI 8226, ARH-77 and U266 based on the conventional suspension cell culture methods. RPMI 1640 complete medium was used, which includes 10% FBS, 100U/ml penicillin and 100mg/L streptomycin, to culture the cells under 37°C, 5% CO₂ and humidity conditions. PRMI 8226 and ARH-77 cell changed the liquid every 1 to 2 days. Three kinds of MM cells changed the half liquid before 24 hours of the experiment. Trypan blue staining was used to test the cell viability, which was more than 98%.

Observation of MM cells morphology after the drug intervention

- (1) RPMI 8226 cell was collected by centrifugation; the serum medium was used to count;
- (2) We inoculated the cells of 100 μ L into 96-well plate, and the density was about 105/ml in every well, under the condition of 37°C, 5% CO₂;
- (3) 50 μ M cardamom in was added to the growing well cell culture plate, so as to establish the negative control (0.1% DMSO) at the same time;
- (4) We observed the changes in cell morphology in inverted microscope after 24 hours;
- (5) Then we took parts of cells to do Hoechst 33342 coloring, and observed the changes in nuclear morphology in Fluorescence microscopy.

STATISTICAL ANALYSIS

Statistical analysis was performed in SPSS 17.0 software. The general test included homogeneity of variance test and normality test. The experimental data of measurement data used mean \pm standard deviation (m \pm SD) to express. The comparison between the two

set of data of univariate adopted t test or one factor analysis of variance, adopted non-parametric test to examine the heterogeneity of variance. P<0.05 had the statistical significance.

RESULTS

Effects of cardamom in on the viability of RPMI 8226 cell

Cardamom in inhibited the viability of RPMI 8226 cell in 12.5 μ M after 24 hours and 48 hours, while cardamom in inhibited the viability of RPMI 8226 cell in 3.125 μ M after 72 hours and 96 hours. With the increasing of drug concentration, inhibition has become more and more obvious; it was the dose-dependent trend. Cardamom in with the concentration of 100 μ M, almost completely inhibited the viability of RPMI 8226 in 24 hours (fig. 2). From 24 hours, smaller concentration (10 μ M) of cardamom in had the obvious inhibition on the viability of PRMI 8226 cell, initially showed the characteristics of anti-MM cell.

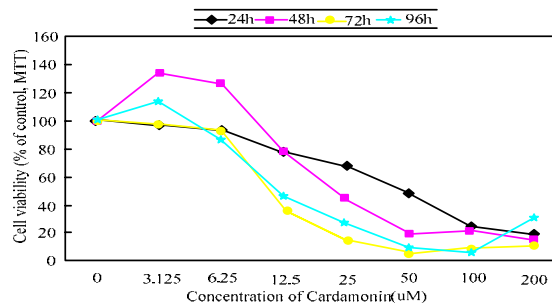


Fig. 2: Effects of cardamom in on the viability of RPMI 8226 cell

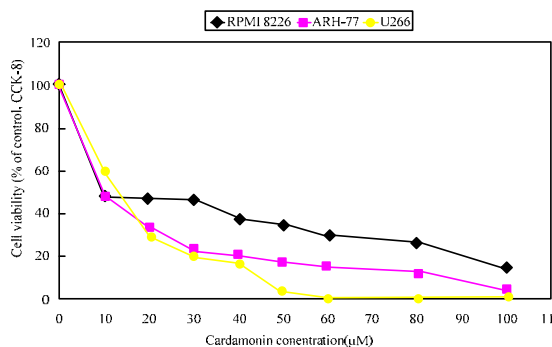


Fig. 3: Effect of different dose of cardamom in on MM cells activity

Effects of cardamom in on human MM cell viability

In this paper, different dose (0, 10, 20, 30, 40, 50, 60, 80 and 100 μ M) of cardamom in was used to interfere three kinds of cells (RPMI 8226, ARH-77 and U266). 48 hours later, the method of CCK-8 was used to test the viability of three kinds of cells. The comparison between the result and control group, the cell viability of every treatment group reduced significantly. With the increasing of the drug concentration, inhibition has become more and more obvious; it was the dose-dependent trend. After 48 hours, 10 μ M cardamom in affected RPMI 8226, ARH-77 and U266 cells, the

survival rates were $47.30 \pm 14.11\%$, $48.74 \pm 0.76\%$ and $59.05 \pm 1.77\%$; after 48 hours, $100 \mu\text{M}$ cardamom in affected RPMI 8226, ARH-77 and U266 cells, the survival rates were $14.00 \pm 3.88\%$, $3.96 \pm 0.23\%$ and $1.40 \pm 0.75\%$ (fig. 3). The comparison between the control group and processing inhibition rate shown us that, they all had the statistical meaning ($P < 0.001$). During the 48 hours, the IC_{50} value of cardamom in on the three kinds of cells, RPMI 8226, ARH-77 and U266, were about 14.69, 10.82 and $13.06 \mu\text{M}$ (table 2).

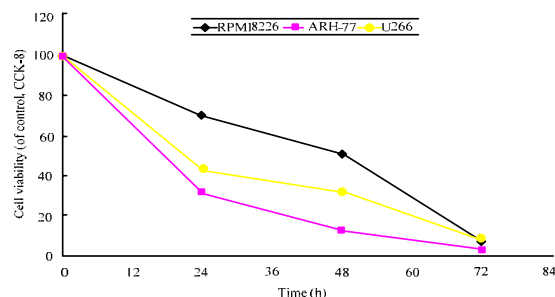


Fig. 4: Effect of cardamom in on the viability of MM cells at different time.

Effect of cardamom in on the MM cell cycle

In order to observe the effect of cardamom in on the MM cell cycle, different dose (0, 10 and $20 \mu\text{M}$) was used to intervene RPMI 8226, U266 and ARH-773. 24 hours later, we used PI sing-straining flow cytometry to test the changes of MM cell cycle distribution. In fig. 4 and table 3, with the increasing of drug concentration, among these three kinds of cells, in G2/M phase cells increased, while in S period cells reduced. Among them, in G2/M phase, the ratio of RPMI 8226 cell increased from 14.62% to 81.89%, the growth rate was about 6 times; in G2/M phase, the ratio of U266 cell increased from 13.39% to 50.00%, the growth rate was about 4 times; in G2/M phase, the ratio of ARH-77 cell increased from 11.13% to 39.26%, the growth rate was about 3 times. The experimental results showed that, the small dose of cardamom in can inhibit the MM cells in G2/M phase and showed the dose-dependent rule

Effect of cardamom in on human MM cell apoptosis

In order to observe the effect of cardamom in on human MM cell apoptosis, different dose (0, 25, 50, $100 \mu\text{M}$) was used to intervene RPMI 8226, U266 and ARH-773. Twenty-four hours later, we used Annexin V FITC/PI double staining and flow cytometry to test the apoptosis of cells under the different intervention conditions. With the increasing of drug dose, the ratio of Annexin V positive cells (early apoptosis cells and late apoptosis cells) also increased gradually. RPMI 8226 cell increased from 5.3% (in the cardamom in intervention period) to 91.14% (in $100 \mu\text{M}$), the growth rate was about 18 times; U266 cells increased from 9.5% to 83.6%, the growth rate was about 8 times; ARH-77 cell increased from 25.6% to 95.6%, the growth rate was about 4 times. And, each treatment group had less necrotic cells, there was no significant changes compared with the untreated group. The experimental results showed that, cardamom in can dose-independent

induce the MM cell apoptosis, and had no significant effects on necrosis. Among three kinds of cells, the majority of cells were Annexin V+PI cells, which showed that the cell apoptosis was mainly the early apoptosis.

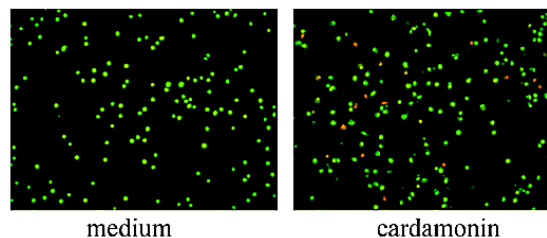


Fig. 5: Effect of cardamom in on cell apoptosis morphology

In order to further improve the effect of cardamom in on MM cell apoptosis, RPMI 8226 cells were selected as the research object, $25 \mu\text{M}$ was used to intervene the cells 24 hours, double staining AO and EB, then observed the cell apoptosis morphology in the fluorescence microscope. In fig. 5, the normal RPMI 8226 cells were green, round and oval with large, round nucleus and chromatin evenly. After the cardamom in invention, early and late apoptosis cells appeared. The early apoptosis cells were green, smaller cell morphology, and had the obvious karyopyknosis, which gathered to the cytoplasm surrounding, like water-drop and beads. The late apoptosis cells were orange; the obvious apoptosis cell morphology appeared, that is, karyopyknosis. This experiment further improved that the cardamom in can induce MM apoptosis cells.

Table 1: Main reagents and instruments

Cardamom in, dimethyl sulfoxide	Simga company, America
IL-6, VEGF, Cocktail protease inhibitors	Pepro Tech company, America
CCK-8 kit, BCA protein assay reagent kit	PIERCE company, America
Pro-apoptotic/existence Bcl-2 family antibody kit	CST company, America
Annexin V-FITC/PI apoptosis kit	Nanjing KeyGEN Biotech. Co., Ltd
Carbinol, ethanol	Shanghai Institute of Chemical Reagent
CO2 ice maker, SIM-F140	SANYO company, Japan
Cell culture tools	Corning company, Japan
Fluorescence microscopy DP72	Olympus company, Japan

DISCUSSION

There are a variety of active ingredients with ant-tumor effects in natural herbs, it is a viable idea that selecting the high efficiency and low toxicity anti-MM medicine in the natural herbs (Gordon *et al.*, 2009). In the past few decades, many scholars have successfully screened

out various drugs to cure the blood cancer, such as arsenite, all-trans retinoic acid, indirubin and other drugs. These natural medicines are widely used in clinical practice, and also bring the gospel to the patients with hematologic malignancies. Nuclear transcription NF- κ B and cytokine IL-6 all play an important role in MM and inflammation, it also shows that MM and inflammation have the common characteristics in some pathological aspects. Moreover, some anti-inflammation drugs, such as SDX-101, SDX-108, honokiol and Huanglian Jiedu soup, have the effects of anti-inflammation (Chunyan *et al.*, 2007). These shows some potential application prospects, and the results are confirmed by multiple laboratories internationally. Therefore, it is possible to select the anti-MM medicine from the anti-inflammatory drugs.

Table 2: Effect of cardamom in on the IC50value of MM cell 48 hour later

	RPMI 8226	ARH-77	U266
IC ₅₀ (μ M)	14.26	10.82	13.06

Cardamom in shows the stronger inhibitory activity on RPMI 8226, which also shows that cardamom in may be a potential anti-MM drug. Cardamom in comes from the chalcone of different Chinese medicine, and also has the various biological activities, including vasodilatation, anti-platelet aggregation, inhibition of melanin formation, the promotion of glucose metabolism, etc. (Yadav *et al.*, 2011). More research shows that cardamom in has significant anti-inflammatory effects *in vitro* and *in vivo*. Cardamom in can inhibit MAPK and NF- κ B pathway in monocyte-macrophages, reduce the expression of cyclooxygenase -2 and nitric oxide synthase, inhibit the secretion of pro-inflammatory cytokine and prevent the death of lipopolysaccharide induced rats. In terms of anti-MM, there is no report at home and abroad at present. Therefore, the research should focus on the cardamom in. In this part, we observed the multiple effects of cardamom in on anti-MM and its preliminary molecular mechanisms, so as to lay a good foundation on further study of cardamom in. Firstly, the experiment applied CCK-8, whose repeatability was better than MTT, RPMI 8226 cells, ARH-77 cells and U266 cells were the research object, in order to verify the inhibition of cardamom in on MM cell viability. The experiment showed that cardamom in can inhibit the MM cell viability in dose-independent and time-independent. Moreover, it was established on the basis of smaller IC50. The further research showed that, 24 hours later, under the effects of cardamom in, we observed the large number of typical apoptotic morphology cells in common microscope and Hoechst 33342 staining fluorescence microscopy. In order to confirm whether the cardamom in effect has a specific performance and observe the toxic effects of cardamom in. Isolated experiment cultured the normal human PBMNCs and then used cardamom in to interfere PBMNCs and the results showed that cardamom in was within 100 μ M anti-MM efficacy range, and had not the obvious effects

on PBMNCs viability, as long as the concentrations increased to 200 μ M, then the toxicity would be tested 48 hours later. Taking into account the bioavailability of cardamom in *in vivo* could not be so high, but it also shows that cardamom in has minimal toxicity. The inhibition of medicine on MM cell viability usually shows in two aspects: the first one is inhibit MM cell proliferation, block the cell cycle; the second is inducing MM cell apoptosis or necrosis.

Therefore, we need to use further experiment to observe the effect of cardamom in on anti-MM cells. Firstly, we need to use new experimental methods: EdU method (Fabio *et al.*, 2010). EdU is a nucleotide analogue, which can be synthesized into DNA, in the cell proliferation, so EdU positive cells are new cell proliferation. Through observing the proportion of EdU positive cells in former and later drug intervention, then we can explain the inhibition strength on cell proliferation. As can be seen from the experimental results, 24 hours later, cardamom in can inhibit RPMI 8226 cell proliferation obviously. Cell cycle refers to a dynamic process that a continuous normal cell suffers from the end of mitosis to the completion of mitosis. Cell cycle is a precise and orderly regulatory process that many multiple factors participate; this process can be divided into five phases, including: G0 phase (quiescent phase), G1 phase (DNA pre-synthesis phase), S phase (DNA synthesis phase), G2 phase (DNA post-synthesis phase) and M phase (mitosis phase). Throughout the cell cycle, there are two major limit points, G1/S phase (G1/S phase mainly controls the cell from G1 phase to S phase, repairs the chromosome mutation) and G2/M phase (G2/M phase is the control point of cell division, and repairs the damage in the duplicated DNA before the cells enter into the cell division). Cell cycle regulation plays an important role in cell proliferation, cell differentiation and cell apoptosis. Once the cell cycle regulation disorders, then it may lead to the malignant tumor. In this paper, we observed the effect of cardamom in on the cell cycle. The results showed that, small dose of cardamom in was used to intervene the MM cells, then MM cells would be blocked in G2/M phase and with the increasing of the drug concentration, the proportion in S phase will reduce. The reasons of cell block effect include the following two parts: the result of cardamom in effect and adaptive process of cells on exogenous factors. The nature of cell cycle block leads to the cell apoptosis is, increasing the injury frequency or degree of cell DNA, reducing the repair ability of anti-body injury.

We need to use Annexin V/PI double staining to test the effect of cardamom in on MM cell apoptosis in this experiment (Guanhua *et al.*, 2012). The result shows that, cardamom in induces three cells apoptosis in dose-independent manner, while necrosis rate is very low, mainly is the early apoptosis. But, the concentration of induced MM cell apoptosis is higher than drug concentration of cell cycle block. The result shows that under the effect of small dose cardamom in, MM cells

Table 3: Effect of cardamom in on the MM cell cycle distribution

Cardamom in (μ M)	RPMI 8226			U266			ARH-77		
	G1	G2/M	S	G1	G2/M	S	G1	G2/M	S
0	31.34%	14.62%	54.05%	48.52%	13.39%	38.09%	43.95%	11.13%	44.92%
10	31.25%	51.53%	17.21%	44.94%	20.86%	34.19%	31.84%	19.93%	48.24%
20	4.12%	81.89%	14.00%	14.29%	50.00%	35.71%	32.23%	39.26%	28.24%

will be blocked, and the cell proliferation will slow down; under the effect of large dose cardamom in, MM cells apoptosis will appear. AO/EB double staining was used to further test the effect of cardamom in induces MM cell apoptosis in this experiment. Cell apoptosis is the body active death under procedural regulation of self genes; it plays an important role in tumor, including the MM pathogenesis process. Now, many scholars believe that the cell apoptosis will suffer the following phases: initiation, regulation, execution and final death, in regulation and execution phase, cascade in the caspase family plays a crucial role (Wei *et al.*, 2010). At present, we have found dozens of mammalian caspase, including two categories, one is the executor, and the other is starter. Under the effect of foreign protein signals, the initial caspase was be cut and activated, then the executor caspase was be cut and activated, lastly, the activated executor caspase lead to the programmed cell death through hydrolyzing of caspase target protein. Among them, caspase-3 (also known as CPP32, YAMA, Apopain), the executor, is the core members of the cascade. The substrates of caspase -3 include poly-ADP-ribose polymerase (PARP), by cutting the downstream substrates through the enzyme, so as to initiate apoptotic events. Therefore, in this paper, we observed whether the effect of cardamom in on MM cell apoptosis was dependent on activated caspase. Then we can select the caspase downstream effectors molecules, that is, caspase-3 and PARP. In the invention of cardamom in, caspase-3 and PARP were be activated, and shown the time-dependent, which shown that the effect of cardamom in on MM cell apoptosis was dependent on the kinase pathway of activated caspase. In recent years, more and more evidence shows that the occurrence and development of MM cells have a close contact with bone marrow microenvironment (Anna 2011); the neovascularization will promote MM progression disease in the bone marrow microenvironment. Bone marrow micro vessel density has become the progress and prognosis index of MM patients. Bone marrow micro vessel density has the positive correlation with MM activity level, and is related to the proliferation of malignant plasma cells. Moreover, bone marrow micro vessel density has close relation with the prognosis and overall survival of MM patients. The overall survival of bone marrow micro vessel density patient is lower than low bone marrow micro vessel density.

CONCLUSION

Cardamom in is an anti-MM drug with strong viability. We researched the multiple effects of cardamom in on anti-MM. The results showed that cardamom in can

inhibit the viability and proliferation of MM cells effectively, block the MM cell cycle in G2/M and induce the MM cell apoptosis.

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