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Effects of Diallyl Disulfide in Elephant Garlic Extract on Breast Cancer Cell Apoptosis in Mitochondrial Pathway

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Abstract As a close relative of garlic, elephant garlic contains abundant allicin, which means the extract of elephant garlic might induce apoptosis in breast cancer cells. The elephant garlic was extracted in alcohol with Solvent - Microwave Extraction. The main component was analyzed by liquid chromatography. The MCF-7 (michigan cancer foundation -7) cells were cultured in vitro. The apoptosis rate was determined with Annexin V-FITC/PI staining based flow cytometry (FCM). Mitochondrial transmembrane potential variation in the cells was observed with JC-1 probe and fluorescence confocal microscopy. Caspase-3 was determined with fluorescence Western Blot. The main component of alcohol extract of elephant garlic was Diallyl Disulfide (DADS), and its relative content was 17.04 %. The early apoptosis of MCF-7 cells could be induced by the alcohol extract solutions and it involved the activation of mitochondrial pathway. The results indicated that the extract and DADS standards exerted pro-apoptotic effects on breast cancer cells in a dose-response manner. From the results of fluorescence confocal microscopy, it showed that the Red/Green fluorescence ratio of mitochondrial membrane potential in MCF-7 cells could be significantly decreased after inducing with DADS reference substances for 24 h. In addition, mitochondrial depolarization could be observed as adding the extract. The activated Caspase-3 was detected with Western Blot. It was demonstrated that the expression level of procaspase-3 had gone up with increased concentration of alcohol extract. The expression level was also increased with the treatment of DADS and the protein band was clear.

Keywords: *caspase-3, cell apoptosis, Diallyl Disulfide (DADS), elephant garlic, mitochondrial transmembrane potential*

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1. Introduction

Garlic (*Allium sativum* L.) is one of the best-researched herbal remedies. It exhibits the capability of protecting the human body against a wide variety of diseases. Much health benefit related biological effects have been found in garlic. It has proved that the special effects of garlic were attributed to organosulfur compounds rich in garlic. Among these compounds, allicin has been best known and most extensively studied. In 1944, CJ Cavallito and co-workers have observed the extract of fresh garlic cloves could be antibacterial. They proved the antibacterial effects were resulted from the allicin in garlic. Compared with widely applied phytoncides, allicin was more stable and less volatile. [1] In following studies, Cavallito' s group has firstly determined the chemical structures of allicin. The allicin has been proved to be a mixture of many allyl sulfides. It was composed of 50 % to 80 % diallyl trisulfide (DATS), 20 % to 50 % diallyl disulfide (DADS) and 8 % of other impurities (such as acetone, ethanol and so on).

Many health related effects have been observed in allicin, including antibacterial, antiseptic, antioxidant, lowering the blood pressure and blood fat, as well as preventing from cardiovascular diseases such as atherosclerosis. [2,3] Specifically, allicin in its pure form has been observed to exhibit i) antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria; ii) antifungal activity, particularly against *Candida albicans*; iii) antiparasitic activity, including some major human intestinal protozoan parasites; and iv) antiviral activity.

Therefore, a plenty of studies have been performed to explore the specific biological activities and mechanism of these effects, including anti-microbial, anti-tumor, antifungal and immune-modulatory. [4,5,6,7] The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes. Using PC-3 (human prostate cancer cell lines) and DU145 (human prostate cancer cell lines) as a model, Dong Xiao et al. found that DATS was a significantly more potent apoptosis inducer than diallyl sulfide (DAS) or diallyl disulfide (DADS). Tooba Ghazanfari has showed that peritoneal macrophage phagocytic activity against

Leishmania major could be enhanced by garlic (*Allium sativum*) treatment [8].

As the main effective ingredient in garlic, allyl sulfides have attracted more attentions from scientists. The researches have mainly focused on the anti-tumor activities and related mechanism. Epidemiological and experimental studies at home and abroad showed that a variety of tumors could be significantly inhibited by allyl sulfides, including stomach cancer, colon cancer, liver cancer, lung cancer, prostate cancer, breast cancer and leukemia etc. [9,10] Oommen S has explored that Caspase-3, Caspase-8 and Caspase-9 in tumor cells could be activated by allicin and poly (ADP-ri2bose) polymerase could be degraded with the effects of allicin. [11] There was also study on apoptotic response of human breast cancer cells to allicin. For human breast cancer cells, cell proliferation in MDA-MB-231 (human breast cancer cell lines) cells could be decreased by allicin in concentration-dependent manner. In MDA-MB-231 cells, cell growth was inhibited and apoptosis was induced through MAPKs (mitogen-activated protein kinases) and ER (estrogen receptor) signaling pathways. [12] In their further study on the apoptotic effect of allicin in MCF-7 cells, another human breast cancer cells. The data suggested that apoptosis was induced by allicin through MAPKs and ATF3 (cyclic AMP-dependent transcription factor) signaling pathways in MCF-7 cells [13].

However, elephant garlic (*Allium ampeloprasum*), a close relative of garlic with very large cloves, tender flavor and abundant allicin. [14,15] Although the elephant garlic has been larger than garlic in volume, its flavor is tender and more acceptable for some people not like garlic. The allicin content was also varied in different species. Analysis of thiosulfinates from various Allium sp. revealed a 3-fold order of magnitude variation among species. Common garlic (*A. sativum*) and wild garlic had the highest levels, while elephant garlic (*A. ampeloprasum*) and Chinese chives have intermediary levels. Environmental conditions were also found to affect the total thiosulfinate levels. As an important specie of garlic, much less attentions have been paid on the anti-tumor effects of elephant garlic. In this study, it mainly explored the effects of alcohol extract of elephant garlic on MCF-7 tumor cells. It aimed to reveal the drug induced early apoptosis in breast cancer cells, as well as the mitochondrial pathway in apoptosis induction mechanism.

2. Materials and Methods

2.1. Subjects

Elephant garlic was provided by Agricultural Biotechnology Center, Beijing Academy of Agriculture and Forestry Sciences. MCF-7 breast cancer cells were purchased from cell resource center in School of Basic Medicine, Peking Union Medical College. MCF-7 cells were cultured in 1640 medium containing 10 % fetal bovine serum, 1 % penicillin and 1 % streptomycin. The cells were incubated in 37°C, 5 % carbon dioxide incubator. Annexin V-FITC/PI kit, JC-1 kit and Protein cell lysis solution were purchased from Applygen Technologies Inc. 2* reaction solution, 6* Protein Loading Buffer, molecular markers marker, NC membrane (nitrocellulose filter membrane, 0.45 nm 15* 25 cm), 30 % polyacrylamide solution, Tris HCl 8.8, APS ammonium persulfate solution, Tris HCl 6.8, ECL substrate, electrical transferring solution, 10* blocking washing buffer, separating gel buffer, spacer gel buffer, TEMED, imported skimmed milk powder, DADS reference substance, methanol were all obtained from Applygen Technologies Inc. The water was sterile distilled water. Other involved instruments were aseptic clean biological safety cabinet, automatic electric pressure steam sterilizer, cell incubator (BC-J80S), centrifuges, BD flow cytometery (BD Vantage SE), electrophoresis, electrophoresis tank, confocal microscopy (XSP-63XDV), vortex shaker, ZQ24000/2695 quadrupole LC / MS spectrometer (Waters, USA) and 2695 high-performance liquid chromatography.

2.2. Preparation of Crude Elephant Garlic Extract and Standard Solutions

The elephant garlic was extracted with alcohol by solvent-microwave extraction. The method was previously reported by Qiao et al. and He et al. [16,17] The elephant garlic was peeled and mashed with Plant tissue triturator for 20 min. The mashed garlic was prepared. 2 g, 4 g and 8 g mashed garlic was weighed (with the accuracy of 0.001 g). 3 samples were placed in water bath of 40°C and kept still for 0.5 h. 95 % alcohol was added in mashed garlic samples with the solid-liquid ratio of 1:4 (g ml^{-1}). The mixture was extracted for 2 h in microwave oscillator at 30°C. The supernatant was separated and concentrated with rotary evaporation at 50°C with speed of 75 r min^{-1}. After ethanol evaporated, the crude extract of elephant garlic was collected. The allicin content was determined with spectrophotometric method. [18] The solutions of allicin extract was filtered by sterilized filter. After filtration, the solution was diluted with 1640 medium with a final concentration of 100 ul 10 ml^{-1}. The solutions were stored at 4°C. Three parallel experiment analyses are carried out.

7.62 mg DADS reference substance was accurately weighed and dissolved with methanol (high performance liquid chromatography/HPLC grade). The methanol was added to volume of 25 mL volumetric flask. After shaking, the uniform solution was taken as stock solution of reference substance.

2.3. Chromatographic Conditions

The gradient elution was carried out with methanol and water. The column was Waters Symmetry C18 (3.9 mm × 150 mm, 5 μm) column. The flow rate of mobile phase was 0.5 mL min^{-1}. The good separation efficient could be realized at 30 min. Three parallel experiment analyses are carried out.

Column: Waters Symmetry C18 column (3.9 mm*150 mm, 5 μm). Mobile phase: methanol, water. Elution gradient of methanol: 60 % to 100 % (0 to 10 min); 100 % (10 to 20 min); 100 % to 60 % (20 to 25 min); 60 % (25 to 30 min). Flow rate: 0.5 mL min^{-1}. Column temperature: 30°C. Detection wavelength: 254 nm.

2.4. Apoptosis Analysis with Annexin V-FITC/PI Staining Based Flow Cytometry

The MCF-7 cells in logarithmic phase were passaged. The cells were counted and seeded in 6-well cell culture plate. The cell concentration was 10^5 mL^{-1} with 2 mL cells per well and cultured overnight for cell adhesion. There were blank control group and experiment groups. Three parallel experiment analyses are carried out. In blank control group, 1640 medium was added. In experiment groups, 1640 medium with different concentrations of allicin was added. After incubating for 24 h, following procedures were carried out according to the instruction of Annexin V-FITC/PI kit. The culture medium was discarded and the cells were washed twice with PBS (phosphate buffered saline) at 4°C. The cells were trypsinized to prepare single cell suspension. After centrifuging and washing, the cells were re-suspended with blind buffer, with a final concentration of 10^6 per ml. 100 ul cells were suspended in 5 mL streaming tube and 5ul Annexin V-FITC solution was added and kept still for 5 min. 10 μl PI (propidium iodide) with the concentration of 20 μg ml^{-1} was mixed with above solutions and stayed at room temperature away from light for 15 min. 500 μL blinding buffer was added before detecting cell apoptosis with flow cytometry.

2.5. Determination of Mitochondrial Membrane Potential

The MCF-7 cells in logarithmic phase were passaged and then plated in confocal Petri dish with concentration of 10^5 per mL. The cells were cultured overnight. There were blank control group and experiment groups. Three parallel experiment analyses are carried out. In blank control group, 1640 medium was added. In experiment groups, 1640 medium with different concentrations of allicin was added. After 24 h, following procedures were carried out according to the instruction of JC-1 kit. The culture medium was discarded and the cells were washed twice with PBS at 4°C. JC-1 solution with final concentration of 2.5 μg ml^{-1} was added and incubated for 30 min in incubator. The cells were stained and washed with PBS. After discarding PBS washing buffer, the cell culture medium was added. The results were observed with confocal microscopy.

2.6. Expression Analysis of Caspase-3 Protein with Western Blot

The MCF-7 cells in logarithmic phase were passaged. The cells were counted and seeded in 6-well cell culture plate. The cell concentration was 10^5 mL^{-1} with 5 mL cells per well and cultured overnight for cell adhesion. There were blank control group and experiment groups. Three parallel experiment analyses are carried out. In blank control group, 1640 medium was added. In experiment groups, 1640 medium with different concentrations of allicin was added. After incubating for 24 h, the culture medium was discarded and the cells were washed twice with PBS at 4°C. The cells were trypsinized to prepare single cell suspension. The cells were washed once with PBS. After discarding the supernatant, lysis solution was added (50 μL lysis solution per 2 million cells). The deposition was re-suspended and lysed for 30 min in ice bath. During this period, the solutions were vortexed for 3-4 times, 10 s per time. Then the solutions were centrifuged for 10 min at 12000 r min^{-1}. The supernatant was carefully disposed and placed in 1 ml centrifuge tube. 6 * loading buffer was added. The solution was boiled at 100°C and stored at -20°C for use.

The separation gel and spacer gel for 12 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) were prepared according to the ratio of the table. The gel was stored at 4°C for further uses. In the electrophoresis, the marker and different samples of protein solutions were injected into the slots of gel. After accessing to power, the voltage was adjusted. After electrophoresis for 2 h, the glass plates were removed. The spacer gel was disposed and the separation gel was cut into appropriate size. The NC membrane and sponge was added and wet-transferred for 2 h. The 1st antibody was added and then the membrane was blocked overnight at 4°C. The NC membrane was washed three times, 5 min each time. The NC membrane was incubated with 2nd antibody at room temperature for 2h. The substrate was added after washing.

3. Results and Analysis

3.1. Maximum Absorption Peak and Chromatogram of Allicin in Elephant Garlic

The crude extract solution of elephant garlic was analyzed with liquid chromatography. The main component in crude extract of elephant garlic was diallyl disulfide (DADS) (Table 1 and Figure 1). The maximum adsorption peak was at 254 nm. As the main component, DADS was separated from crude extract of elephant garlic with liquid chromatography. The peak area was 17.03516.

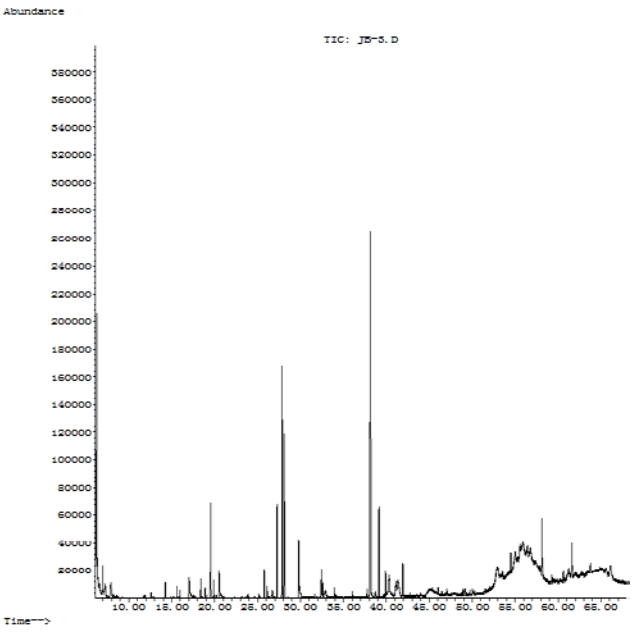

Figure 1. The chromatogram of crude extract solution of elephant garlic

3.2. The Effects of DADS and Elephant Garlic Crude Extract On Early Apoptosis Rate of MCF-7

Both the DADS reference substance and elephant garlic crude extract have made effects on the apoptosis of MCF-7. The effects were detected by flow cytometry with

Annexin V-FITC/PI double staining flow cytometry. In the analysis of apoptosis, there was apoptosis happened to MCF-7 breast cancer cells after treatment for 24 h with different concentrations of crude extract solutions. It suggested the pro-apoptotic effect of crude extract solution was made in a dose-response manner. In the treatment of DADS, the early apoptosis could also happen to MCF-7 cell in an identical proportion. With the increased concentration of crude extracts, the early apoptosis rates were 11.57 %, 18.69 % and 26.98 %, respectively (Figure 2). The results demonstrated that breast cancer cells could be significantly induced by crude extract solution of elephant garlic. The diallyl disulfide, as the main component of crude extract solution, could perform anti-tumor effects.

Table 1. Components analysis of crude extract solution of elephant garlic

PK	RT	Area Pct	Library/ID
1	6.24	6.36±0.72	Cyclohexane, methyl-
2	6.30	0.14±0.04	Cyclopentane, ethyl-
3	6.97	1.05±0.13	Norbornane
4	7.92	1.83±0.09	Thiirane, methyl-
5	14.25	0.60±0.02	1-Propene, 3,3'-thiobis-
6	15.61	0.44±0.07	1-Propene, 1,1'-thiobis-
7	17.03	1.15±0.12	Thiacyclobutane-3-ol
8	18.38	0.87±0.09	3,4-Dimethylthiophene
9	18.88	0.40±0.05	Thiophene, 2,5-difluoro-
10	19.53	4.63±0.56	Disulfide, methyl 2-propenyl
11	19.89	0.76±0.08	1,3-Dithiane
12	20.53	1.68±0.19	N-Methyl-7-azabicyclo(2,2,1)hept-2-ene
13	25.76	1.31±0.14	Ethene, 1,2-bis(ethylthio)-
14	26.11	0.50±0.06	Cyclohexanone, 2,2,5,5-tetramethyl-3-methylene-
15	27.26	4.66±0.51	Diallyl disulphide
16	27.86	12.38±1.32	Diallyl disulphide
17	28.10	8.48±0.92	1-Oxa-4,6-diazacyclooctane-5-thione
18	29.79	2.75±0.53	Thiourea, ethyl-
19	32.44	1.52±0.18	Methyl(methyl 4-O-methyl-.alpha.-d-mannopyranoside)uronate
20	32.59	0.81±0.12	Thiophene, 2-chloro-
21	38.14	26.01±1.94	3-Vinyl-1,2-dithiocyclohex-4-ene
22	39.13	5.12±0.66	Crotonic acid, 4-mercapto-3-(methylthio)-, gamma.-(thio lactone)
23	39.91	1.27±0.22	Trisulfide, di-2-propenyl
24	40.31	2.85±0.19	Anhydro-1,6-dimethyl-5-hydroxyimidazo-[2,1-b][1,3]-thiazine-7-one hydroxide
25	41.10	0.91±0.11	Ethanone, 1-(3-methylenecyclopentyl)-
26	41.35	0.90±0.08	Benzene, diethenyl-
27	41.92	1.81±0.24	3-Vinyl-1,2-dithiocyclohex-5-ene
28	52.84	0.11±0.02	n-Hexadecanoic acid
29	52.88	0.02±0.01	n-Hexadecanoic acid
30	54.50	0.95±0.16	Hexadecanoic acid, ethyl ester
31	54.99	0.90±0.15	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-
32	55.03	0.19±0.03	2H-Cyclopropa[a]naphthalen-2-one, 1,1a,4,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, (1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)-
33	55.54	0.17±0.02	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-
34	55.56	0.20±0.05	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-
35	58.11	3.26±0.38	Naphthalene, 2,3,6-trimethyl-
36	60.63	0.66±0.09	2-Nonadecanol
37	61.62	1.92±0.24	Linoleic acid ethyl ester

All values of Area Pct are expressed as means±SE.

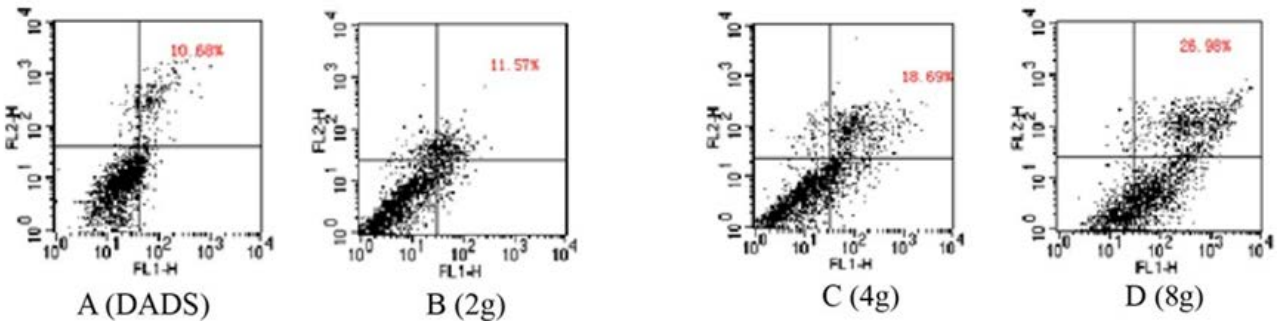

Figure 2. Early apoptosis analysis with flow cytometry

3.3. The Mitochondrial Depolarization Induced by DADS and Elephant Garlic Crude Extract

The early events in pro-apoptosis effects of DADS on breast cancer cells have been explored. The effects of crude extract on mitochondrial depolarization in MCF-7 were determined with JC-1 probe and fluorescence confocal microscopy. The results showed that the depolarization was observed in cells treated with DADS. For crude extract of elephant garlic, the mitochondrial membrane potential would be decreased after treating for 24 h and the depolarization was observed. It performed as decreased ratio of red/green fluorescence (Figure 3). There was also a dose-response relationship. The results suggested that mitochondrial depolarization could be

induced by DADS in breast cancer cells. Thus, the cell apoptosis could be triggered.

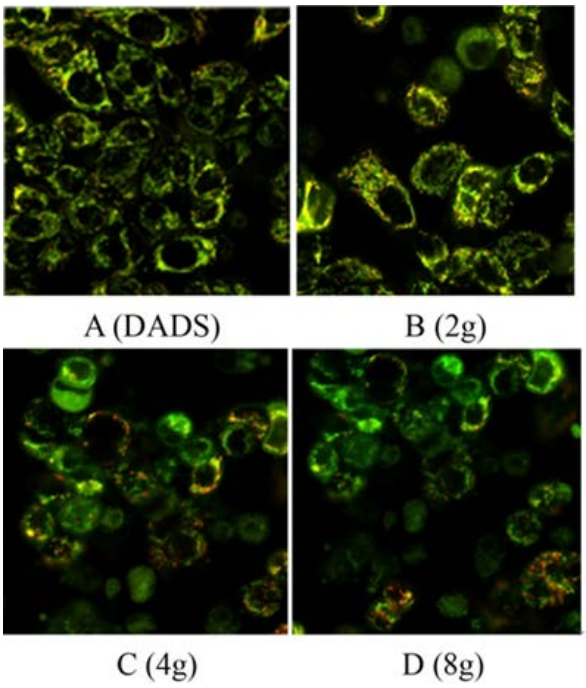

Figure 3. The effects of DADS on the mitochondrial depolarization in breast cancer cells

3.4. The Expression Level of Procaspase-3 in MCF-7 after Induction

The MCF-7 cells were treated with different concentrations of elephant garlic crude extract and DADS, respectively. After 24 h, the total cell protein was lysed for performing Western Blot. From the results, the activated procaspase was observed in DADS treated cells. For crude extract treated group, the bands of procaspase (32 KD) and large subunit (17 KD) could be clearly observed, indicating apoptosis in cells. The results indicated that cell apoptosis could be promoted by DADS, the main component of elephant garlic crude extract. The protein expression of apoptotic gene caspase-3 could be clearly observed (Figure 4).

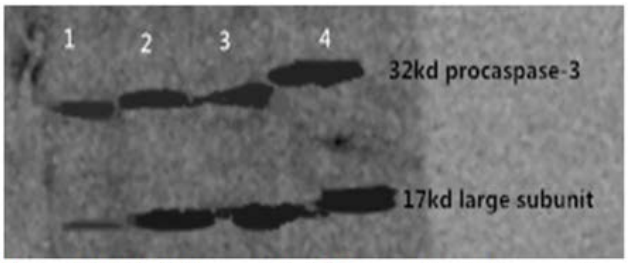

Figure 4. The Western Blot analysis of activated procaspase-3

4. Discussion

Apoptosis is triggered by environment changes or death signals. It has been an active cell death program under the gene regulation. Most of people believed that cancer was due to excessive cell proliferation and insufficient cell apoptosis. In the treatment or induction of tumor cells, the cell proliferation has been generally inhibited, as well as inducing early cell apoptosis [19,20].

Three major pathways of cell apoptosis are death receptor mediated apoptosis pathway or extrinsic pathways, mitochondrial apoptotic pathway or the intrinsic pathway and the endoplasmic reticulum pathway. Caspase family plays vital roles in mediating apoptosis. Wherein, Caspase-3 has been a key implementation molecule. It has made effects on many pathways related to apoptosis signal transduction. Generally, Caspase-3 existed in cytoplasm in the form of plasminogen (32 KD). In early stage of apoptosis, it was activated. The activated Caspase-3 is composed of two large subunits (17 KD) and two small subunits (12 KD). The corresponding cytoplasmic and nuclear substrates would be lysed, resulting in cell apoptosis. In this study, it focused on the mitochondrial pathway. The results have proved that the early apoptosis in MCF-7 cells would be induced by DADS. The mitochondrial depolarization was observed and it was an important performance of early apoptosis. The MCF-7 cells were activated by procaspse-3 in dose dependent manner.

Mitochondrial permeability transition pore (PT) is multi-protein complex located between inner and outer membrane of mitochondria. It plays an important role in regulating apoptosis. When the cell apoptosis is stimulated by various factors, the permeability of mitochondrial membrane would be increased. Many proteins in mitochondria would be released, including Cytochrome C. The Cytochrome C would be translocated into cytoplasm. The apoptotic body is formed by Cytochrome C, apoptosis activating factor Apaf-1 and Caspase-9 precursor. With the co-action of apoptotic body and dATP in cytoplasm, the Caspase-9 would be activated. Other caspase including Caspase-3 etc could be activated by activated Caspase-9. Then the DNA fragmentation factor would also be activated by Caspase-3, resulting in the activation of nucleic acid enzyme in resting state. It finally causes DNA breakage and damage [21].

The function integrity of mitochondrial membrane would be damaged in the high metabolic tumor cells. Thus, these cells would be more sensitive to apoptotic stimuli of mitochondrial apoptotic pathway. There have been drugs for cancers specific to the effective targets in mitochondria. [10] In this study, it proposed a target specific to mitochondria in MCF-7 tumor cell for cancer drugs. The results provided laboratory basis and reference for developing and studying drugs with relatively selectivity in cancers treatment.

5. Conclusions

The main component of alcohol extract of elephant garlic was Diallyl Disulfide (DADS). The early apoptosis of MCF-7 cells could be induced by the alcohol extract solutions and it involved the activation of mitochondrial pathway.

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