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Anticarcinogenic Activity of Diallyl Disulfide Against Colorectal Cancer Cells

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ABSTRACT

Garlic (allium sativum) possesses anti-proliferative properties that can be used in anticancer intervention as promising cancer chemo-preventive constituent. Diallyl disulfide (DADS) is an oil soluble sulfur organic compound. We investigated DADS inhibitory activity against colon cancer (Caco-2) cells viability or proliferation. Cells were incubated with DADS at concentrations, 400µM, 500µM, 600µM and 700µM for 72h at 37°C and 5% CO2. DADS effect was dose-dependently. IC50 concentration of DADS, inhibited the viability and proliferation of treated Caco-2 cells in vitro and induced apoptosis even by DNA fragmentation or Bcl-2 reaction expression inhibition. Future study will may deal with further investigations of DADS possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colon cancer type.

KEYWORDS DADS, apoptosis, Bcl-2, Caco-2 cells.

INTRODUCTION

Recently, there has been encouraging progress, from a western perspective, in cancer research field regarding Chinese Herbal Medicines (CHM) as an effective therapeutic method to improve cancer survival scavenge, increase tumor response or reduce chemotherapy toxicity and keep cancer from recurring. For example, it has been demonstrated that garlic (*allium sativum*) possesses anti-mutagenic or anti-proliferative properties that can be used in anticancer interventions[1]. Thus, garlic derivatives are believed to be a promising a cancer chemo-preventive constituent for its anticancer role by inducing apoptosis, inhibiting differentiation, inhibiting tumor angiogenesis and reversing multidrug resistance[2]. Lipid-soluble organic compounds from garlic that possess the most effective antiproliferative agents warranting further study as antitumorigenic agents. Diallyl disulfide (DADS) is an oil soluble sulfur organic compound has recently become more appealing as an anticarcinogenic agent due to its ability to induce apoptosis *in vitro* and inhibit the growth of tumors in animal studies *in vivo*[3]. Further elucidation of apoptosis associated to cellular proteins which regulated by DADS and other natural products that are pro-apoptotic, is important because of their anticarcinogenic agents[4].

Apoptosis, a programmed cell death, occurs under a variety of physiological and pathological conditions that control development and homeostasis of multicellular organisms[5]. Apoptosis induction was recognized to be the best strategy for agents to kill cancer cells. Bcl-2 protein action mechanism has not been fully defined but may involve oxidative phosphorylation and/or mitochondrial electron and metabolite transport, and its main effect is to prolong cell survival by avoidance of apoptosis[6]. Bcl-2 expression confers resistance to a variety of chemotherapeutic agents *in vitro*[7], but any deregulation of its expression lead to accelerate the apoptotic progress of cancer cells.

Colorectal cancer is the cause of more than 1/2 million deaths worldwide, and it was ranked as the third leading cause of cancer-related death after lung cancer and stomach cancer[8]. Epidemiological studies have shown strong evidence that diet and lifestyle play an important role in preventing cancer. In particular, an increased consumption of fruits and vegetables is associated with decreasing in cancer onset and mortality[9]. Therefore, in this study we focused on DADS role as natural bioactive compound in the induction of cytotoxicity and apoptosis in human colorectal carcinoma cell line (Caco-2) *in vitro*.

MATERIALS AND METHODS

Chemical reagents: MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx&E) stain, Bcl-2 monoclonal antibody, biotinylated immunoglobulin secondary antibody and diallyl disulfide (DADS) were purchased from Sigma-Aldrich, Egypt.

Cell line and cell culture: Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as monolayer according to instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5%CO_2. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media and supplements were purchased from Invitrogen (Carlsbad, CA).

Methods: 1.Cell Viability Assay: *In vitro* evaluation of antiproliferation effect: growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans[10]. Viable cell number/well was directly proportional to formazans production. 25 × 10^3cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing DADS at concentrations of 400µM, 500µM, 600µM and 700µM for 72h at 37ºC in an incubator with 5%CO_2. After incubation, DADS modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5%CO_2) with DMSO and left for 10min on a platform shaker to solubilize converted formazan. Absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as positive control cells and all values were correlated with this set of data. Experiment was performed in triplicates. Inhibition Percentage=[1–(net Absorbance of treated well/net Absorbance of

positive control well)]x100% was plotted against DADS concentrations.

2.Determination of DNA fragmentation: cells were seeded in 60-mm petri dishes at density $4x10^5$cells/plate (treated cells by IC_{50} concentration of DADS and positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, 0.5%Triton-X100) and kept at 4ºC/10min then, lysate was centrifuged at 25.000×g/20min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15min. After drying, DNA was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide[11].

3.Cytological changes investigation: detached and trypsinized cells (IC_{50} concentration of DADS treated cells and positive control cells) were collected and centrifuged at 2000rpm for 5min. Cell pellet was resuspended with 100µL of PBS (pH 7.3). 10µL of the suspension were smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5min before proceeding by Hx&E and examined under light microscope[12].

4.Immunocytochemical investigations: by detection of Bcl-2 by immunocytochemistry staining kits. The procedure was done according to manufacturer's instructions, simplified as: 1-2 drops of Peroxidase was applied to cells (IC_{50} concentration of DADS treated cells and positive control cells) on slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed with cold PBS at room temperature. Cells were incubated overnight with primary monoclonal antibody against Bcl-2 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody[13]. Between each step, slide was washed with washing buffer (PBS) with 0.1%Tween 20). Slides were mounted and examined under light microscope.

5.Statistical analysis: results were presented as means±-standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc and was set at $P<0.05$ and high significance at $P\leq0.01$[14].

RESULTS

1.Cell Viability Assay: *In vitro* evaluation of antiproliferation effect.

Cytotoxic effect of different concentrations of DADS (400µM, 500µM, 600µM and 700µM) for 72h on Caco-2 cell line was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with DADS concentrations in a dose dependent manner. All concentrations were found to be high significantly different ($P\leq0.01$) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with DADS concentration increasing and 96% of cell inhibition was observed when cells were treated with 700µM/72h. Cell proliferation reduced about 22% and 40% when cells were treated with 400µM and 500µM for 72h, respectively. Cells proliferation decreased to 55% when treated with concentration of 600µM/72h.

2.Determination of DNA fragmentation by DNA laddering assay.

DNA degradation into multiple internucleosomal fragments is apoptosis distinct biochemical hallmark. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis, stained with ethidium bromide and a typical ladder formation was observed upon 72h when treated with DADS concentration at 600µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that DADS induced DNA fragmentation which was caused by apoptosis.

3.Cytological changes investigation.

Positive control cells had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by DADS concentration at 600µM/72h, apoptotic cells were identified by a series morphological changes as an important experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation as granules, scant vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

4.Immunocytochemical investigations.

Positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 50% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3e). After Coca-2 cells treatment by DADS concentration at 600µM/72h, those fields that had necrotic or apoptotic nucleus as sign for DADS apoptotic effect with Bcl-2 negative reaction (no nuclear brown stain) (Figure 3f).

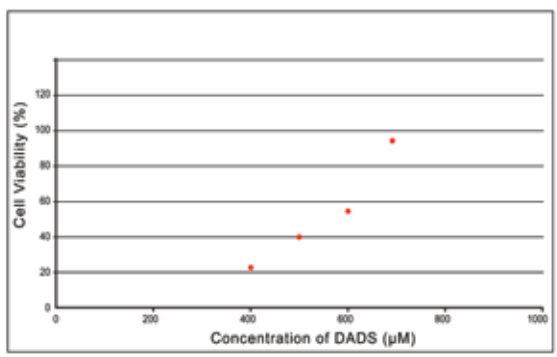

Figure 1: DADS effect with different concentrations on Caco-2 cells viability. Values means were calculated [mean±SD, n (for each concentration)=4].

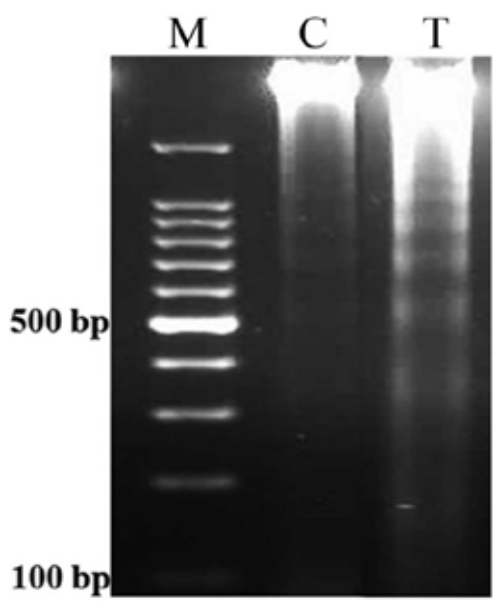

Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from DADS treated cells and positive control cells. DNA laddering, typical for apoptotic cells, in treated Coca-2 cells (T), and there was no any apoptotic features in positive untreated cells (C). M indicating to marker.

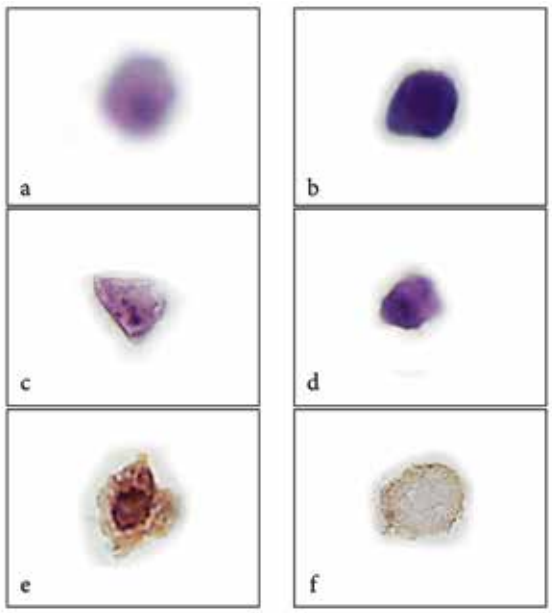

Figure 3: Cells in different stages of apoptosis in treated cells. Cell with normal morphology (a). Complete apoptotic cell (b). Nucleus, vacuolated cytoplasm with apoptotic bodies (c). Nuclear condensation (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Bcl-2 protein immunocytochemistry: control positive cell showing Bcl-2 protein nuclear positive reaction (e) and treated cell showing nuclear negative reaction indicating cell apoptosis incidence (f).

DISSCUTION

In Taiwan, about 15.03 persons/100 thousand people die/ year in colon cancer from reports of "People Health Bureau of Taiwan"[15]. Surgery, radiotherapy and chemotherapy are used for clinical therapy in human colon cancer. Currently the strategies for treatment of human colon cancer are not yet satisfactory[16]. Chemoprevention of colorectal cancer has become essential in the modern industrialized world[17].

Garlic is widely consumed worldwide. Fresh garlic contains water, carbohydrates, proteins, fiber, fat, various amino acids, minerals, and vitamins[18]. Additionally, garlic includes sulfur compounds, specially allicin, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and ajoene. When garlic is cut or crushed, the clove's membrane is disrupted, and S-allylcysteine (SAC) sulfoxide is converted enzymatically into allicin by allinase[19]. Allicin is responsible for the typical odor of garlic, but is unstable and converts readily into mono-, di-, and trisulfides. Total allicin yield has been determined as 2.5 mg/g of fresh crushed garlic, or about 5-20 mg/clove[20]. Experimental and epidemiological studies provided evidence the association between garlic intake and a reduced cancer risk, for example, esophageal, mammary, skin, pulmonary, stomach and lung tumors[21].

Diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoene, allicin, Sallyl cysteine (SAC), and Sallylmercaptocysteine (SAMC), lead to apoptosis by upregulation of p53, Bax, activation of Caspase-3, -8, and -9, JNK, p38, downregulation of Bcl2, and the production of reactive oxygen species (ROS)[22].

Diallyl disulfide (DADS) is a major constituent of garlic as it is an important oil-soluble organo-sulfur component of garlic (*Allium sativum*), has been reported to inhibit human cancer cells growth as lung, skin and breast[23]. It was investigated its effects in human gastric cancer MGC803 cells. They had shown that the growth of MGC803 cells was inhibited by DADS[24]. Although DADS as an anti-tumor agent has been established, the exact mechanism of cytotoxic effect in apoptotic pathways is not completely clear and apoptotic potential mechanism of signal pathway in the cell remains to be evaluated.

Apoptosis is a kind of cell death upon receiving specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process[25]. That was agreed with the results of recent study after treatment by DADS. DADS decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells via inhibiting Bcl-2 activity. Therefore, we may presume that as primary mechanism involved in DADS growth-inhibitory effects as it considered main apoptotic signals[26].

CONCLUSION

Epidemiological studies have shown strong evidence that diet is an important role in preventing cancer. In this study, we have demonstrated that DADS inhibited proliferation and induced apoptosis in colon cancer (Caco-2) cells. Future study will may deal with further investigations of the possible usages of DADS as an alternative and complementary chemotherapeutic agent for human colon cancer.

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REFERENCES

1.Kwon, K.; Fadeel, B.; Orrenius, S.; Zhivotovsky, B.; Yang, J. and Park, B. (2002). Induction of apoptosis by diallyl disulfide through activation of caspase-3 in HL-60 cells. Biochem. Pharmacol., 63:41-47. | 2.Liao, Q.; Yu, C.; Zhou, X. and Song, Y. (2007). Inhibitory effect of DADS on proliferation of human colon cancer cell line SW480 in nude mice. Ai Zheng., 26:828-832. | 3.Zhao, J.; Liao, Q.; Kluck, R.; Wolf, B. and Su, Q. (2006). Diallyl disulfide suppresses growth of HL-60 cell through increasing histone acetylation and p21WAF1 expression in vivo and in vitro. Acta Pharmacol. Sin., 27:1459-1466. | 4.Xiang, S.; Ling, H. and Su, Q. (2005). Antitumor effect of diallyl disulfide on human gastric cancer MGC803 cells xenograft in nude mice. Ai Zheng., 24:940-944. | 5.Evan, G. and Vousden, K. (2005). Proliferation, cell cycle and apoptosis in cancer. Nature., 411:342-348. | 6.Ruvolo, P.; Vrbacky, M.; Drahota, Z.; Melkova, Z.; Deng, X. and May, W. (2001). Phosphorylation of Bcl-2 and regulation of apoptosis. Leukemia., 15:515-522. | 7.Rudner, J.; Jendrossek, V. and Huber, S. (2010). Anti-apoptotic Bcl-2 fails to form efficient complexes with pro-apoptotic Bax to protect from Celecoxib-induced apoptosis. Biochem. Pharmacol., 250:125-129. | 8.Mayer, R. (2009). Targeted therapy for advanced colorectal cancer-more is not better. J. Med., 360:623-625. | 9.Murillo, G.; Hoard, M.; Naithani, R. and Mehta, R. (2008). Efficacy of herbal products in colorectal cancer prevention. Current Colorectal Cancer Reports., 4:34-42. | 10.Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods., 65:55-63. | 11.Zhang, Y.; Liu, J. and Wong, T. (2003). DNA excision repair system of highly radioresistant bacterium Deinococcus radiodurans is facilitated by pentose phosphate pathway. Mol. Microbiol., 48:1317-1323. | 12.John, A. and Abraham, S. (1991). Cytological changes produced by red pepper in mitotic cells of Vicia faba L. Caryologia., 44:325-331. | 13.Yoon, K.; Nakamura, Y.; Doubek, D. and Arakawa, H. (2004). Identification of ALDH4 as Bcl-2-inhibitor gene and its protective role in cellular stresses. J. Hum. Genet., 49:134-140. | 14.Snedecor, G. and Cochran, W. (1980). Statistical Methods, 7th ed Iows State unive. Press, Iowa, U.S.A. | 15.Ooi, B.; Lee, J.; Ho, Y. and Seow Choen, F. (2001). Primary colorectal signet-ring cell carcinoma in Singapore. ANZ. J. Surg., 71:703-706. | 16.Boffetta, P.; Ito, N.; Aagness, B. and Andersen, A. (2005). Smokeless tobacco use and risk of cancer of pancreas and other organs. Int. J. Cancer., 10:992-995. | 17.Takahashi, S.; Hakoi, K.; Yada, H.; Hirose, M. and Fukushima, S. (1992). Enhancing effects of diallyl sulfide on hepatocarcinogenesis and inhibitory actions of the related diallyl disulfide on colon and renal carcinogenesis in rats. Carcinogenesis., 13:1513-1518. | 18.Augusti, K. (1996). Therapeutic values of onion (Allium cepa L.) and garlic (Allium sativum L.), Ind. J. Exp. Biol., 34:634-640. | 19.Block, E. (1985). The chemistry of garlic and onions, Sci. Am., 252:94-99. | 20.Abdullah, T.; Elkadi, A. and Carter, J. (1988). Garlic revisited: therapeutic for major diseases of our time. J. Nat. Med. Assoc., 25:439-445. | 21.Ameen, M.; Su, J.; Zhou, X.; Tang, H.; Song, Y.; Musthapa, M. and Rahman, Q. (2003). Garlic attenuates chrysotile-mediated pulmonary toxicity in rats by altering the phasel and phasell drug metabolizing enzyme system., J. Biochem. Mol. Toxicol., 17:366-371. | 22. Munday, R. and Munday, C. (1999). Low doses of DADS, a compound derived from garlic, increase tissue activities of quinone reductase and glutathione transferase in gastrointestinal tract of rat., Nutr. Cancer., 34:2-8. | 23.Ling, H.; Yang, J.; Rho, H.; Song, Y. and Zhou, X. (2006). Erk is involved in differentiation induced by diallyl disulfide in human gastric cancer cell line MGC803. Cell. Mol. Biol. Lett., 11:408-23. | 24.Wu, X.; Gilmartin, A.; Imburgia, C.; Kassie, F. and Mersch-Sundermann, V. (2005). Role of reactive oxygen species production on diallyl disulfide (DADS) induced apoptosis and cell cycle arrest in human A549 lung carcinoma cells. Mutat. Res., 579:115-124. | 25.Fadeel, B.; Orrenius, S. and Zhivotovsky, B. (1999). Apoptosis in human disease: a new skin for the old ceremony. Biochem. Biophys. Res. Commun., 266:699-717. | 26.Hong, Y.; Ham, Y.; Choi, J. and Kim, J. (2000). Effects of allyl sulfur compounds and garlic extract on the expression of Bcl-2, Bax, and p53 in non small cell lung cancer cell lines. Exp. Mol. Med., 32:127-134. |