Antiproliferation and Antiactivity of proanthocyanidins Against Colorectal Cancer Cells (Caco-2) Line Through Mitochondrial Pathway

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ABSTRACT

Proanthocyanidins, natural polyphenolic compounds found in plants, are known to have antioxidant and anti-cancer effects. We investigated whether the anti-cancer effects of proanthocyanidins are induced by apoptosis on human colorectal cancer cell line, Caco-2. The anticancer effect of proanthocyanidins, *via* apoptosis induction resulting from mitochondrial dysfunction, was assessed in colorectal cancer (Caco-2) cells *in vitro* with an IC_{50} of 120µg/mL. Treatment of Caco-2 cells with proanthocyanidins encouraged apoptosis and antiproliferative effect with cell death-transducing signals by a down-regulation of Bcl-2, up-regulation of p53 and caspases-3 expression were also observed in treated cells. Additionally, a typical characteristics of apoptosis including DNA fragmentation and cytological alterations was observed. Future study will may deal with further investigations of proanthocyanidins as possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colorectal cancer type.

Key Words: proanthocyanidins, apoptosis, mitochondrial pathway.

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INTRODUCTION

Proanthocyanidins are some of the most abundant polyphenolic substances in the plant kingdom. Proanthocyanidins are an integral part of the human diet, found in high concentration in fruits, vegetables and seeds as well as in most types of tea [1]. Numerous studies have reported that flavonoids have potent antioxidant effects through scavenging of superoxide and hydroxyl radicals [2] and anti-proliferative actions via inhibition of metabolic pathways and inhibition of intra-cellular signal transduction [3]. In addition, a variety of proanthocyanidins have been shown to be anti-bacterial, anti-viral, anti-carcinogenic, anti-inflammatory, anti-allergic and consequently reduce the concentration of reactive oxygen species and cardioprotective effects in human beings [4].

Apoptosis, a programmed cell death, also plays an essential role as a protective mechanism against cancer cells [5]. Induction of apoptosis is a highly desirable mode as a therapeutic

strategy for cancer control. In fact, many chemopreventive agents act through the induction of
apoptosis as a mechanism to suppress carcinogenesis [6]. Recently, cancer chemotherapy has gradually improved with the development and discovery of novel anti-tumor drugs, but sometimes these drugs have been limited in clinical application by drug resistance of tumor and by serious damage to the normal tissues and cells.
The major apoptotic pathways can be divided into caspase- and mitochondria-dependent pathways, according to caspase-3 activation which is generally considered to be a key hallmark of apoptosis [7]. Apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspase-3 [8]. Tumor suppressor protein p53 is a principal factor in regulation of growth arrest as well as apoptosis. Indeed, in response to various types of stress, p53 becomes activated as a consequence, cells can undergo marked phenotype changes, ranging from increased DNA repair to senescence and apoptosis [9]. Mitochondria are involved in a variety of key events, including release of caspase-3 activators, changes in electron transport, loss of mitochondrial membrane potential, and participation of both pro- and anti-apoptotic Bcl-2 protein [10]. Alterations in mitochondrial structure and function have been shown to play a crucial role in caspase-3-dependent apoptosis and Bcl-2 expression [11]. Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis. Bcl-2 itself is an anti-apoptotic gene that prevents initiation steps of apoptosis and programmed cell death [12].
Proanthocyanidins incubated with several human cancer cell lines (breast, lung, gastric, and skin) revealed a selective cytotoxicity for the cancerous cells [13]. However proanthocyanidins has not been tested for colorectal cancer which is one of the major cause of cancer-related mortality. Over the past decade, there is continuous increase in colorectal carcinoma in the world as the most common malignant diseases. 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 [14]. 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 [15]. In this study, we examined the inhibitory effects of proanthocyanidins on cancer cell proliferation, for anti-tumor effects on human colorectal cancer cell line (Caco-2).

MATERIALS AND METHODS

Chemicals: Proanthocyanidins, MTT salt [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], dimethylsulfoxide (DMSO), commercial methanol, ethanol and 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, rabbit polyclonal antibodies against cleaved caspase-3, primary monoclonal antibody against Bcl-2 and against p53, AB reagent, biotinylated immunoglobulin secondary antibody and Tween 20 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 mono-layer according to the 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 was 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 [16]. Viable cell number/well is directly proportional to formazans production. 8.25×10^3 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing proanthocyanidins at concentrations: 80µM/L, 100µM/L, 120µM/L and 140µM/L for 72h at 37ºC in an incubator with 5%CO_2. After incubation, proanthocyanidins modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5%CO_2). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage=[1–(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against proanthocyanidins concentrations.

2.Determination of DNA fragmentation by DNA laddering assay: cells were seeded in 60-mm petri dishes at density $4x10^5$ cells/plate (treated cells by IC_{50} concentration of proanthocyanidins or 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, Triton-X100 0.5%) 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 pH 8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide [17].

3.Cytological changes investigation: detached and trypsinized cells (IC_{50} concentration of proanthocyanidins treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 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 stain and examined under light microscope [18].

4.Immunocytochemical investigations: by detection of Bcl-2, p53 and Caspase-3 by immunocytochemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC_{50} concentration of proanthocyanidins treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with rabbit polyclonal antibodies against cleaved caspase-3 at 4°C, then AB reagent and substrate-chromogen mixture (30min). Between each step, the slide was washed with washing buffer (PBS) with 0.1% Tween 20). Cells were incubated overnight with primary monoclonal antibody against Bcl-2 and against p53 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used [19]. The slides were then mounted and examined under light microscope.

5.Statistical analysis: results were presented as mean±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. Statistical significance was set at P<0.05 and high significance was set at P≤ 0.01 [20].

RESULTS

1.Cell viability assay: *In vitro* evaluation of antiproliferation effect.

Cytotoxic effect of proanthocyanidins concentrations (80µM, 100µM, 120µM and 140µM)/72h on Caco-2 cells was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with proanthocyanidins concentrations in a dose dependent manner. All concentrations were found to be high significantly different (P≤0.01) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with proanthocyanidins concentration increasing and 90% of cell inhibition was observed in treated cells with 140µM/72h. Cell proliferation reduced about 20% and 25% when cells were treated with 80µM and 100µM for 72h, respectively. Cells proliferation decreased to 50% as treated with concentration 120µM/72h.

2.Determination of DNA fragmentation by DNA laddering assay.

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with proanthocyanidins concentration at 120µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that proanthocyanidins induced DNA fragmentation which was caused by apoptosis.

3.Cytological changes investigation.

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by proanthocyanidins concentration at 120µM/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 granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

4.Immunocytochemical investigation.

After Coca-2 cells treatment by proanthocyanidins concentration at 120µM/72h, the reaction of caspase-3 protein was considered positive (over expression of caspase-3 protein) when over 50% of treated tumor cells had a clear brown cytoplasm staining, with slight degrading in the intensity in the same field (Figures 3f). Specially those fields that had necrotic or apoptotic nucleus as sign for proanthocyanidins treatment effect, but fields of positive control cells have negative reaction of caspase-3 (cytoplasm did not show the brownish reaction stain) (Figure 3e). On the other hand regarding to the positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 55% of cells had nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane brown staining, with slight intensity degrading in the same field (Figures 3g). After Coca-2 cells treatment by proanthocyanidins concentration at 7µM/72h, those fields that had necrotic or apoptotic nucleus as sign for proanthocyanidins apoptotic effect with Bcl-2 negative reaction (faint to non-brown stain) (Figure 3h). Also, when applying p53 stain, p53 protein reaction in the positive control Coca-2 cells, was showed negative reaction (no brown stain) (Figure 3j). Treated Coca-2 cells, those fields had necrotic or apoptotic nucleus for proanthocyanidins effect showed p53 positive reaction (over expression of p53 protein)

when over 55% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3i).

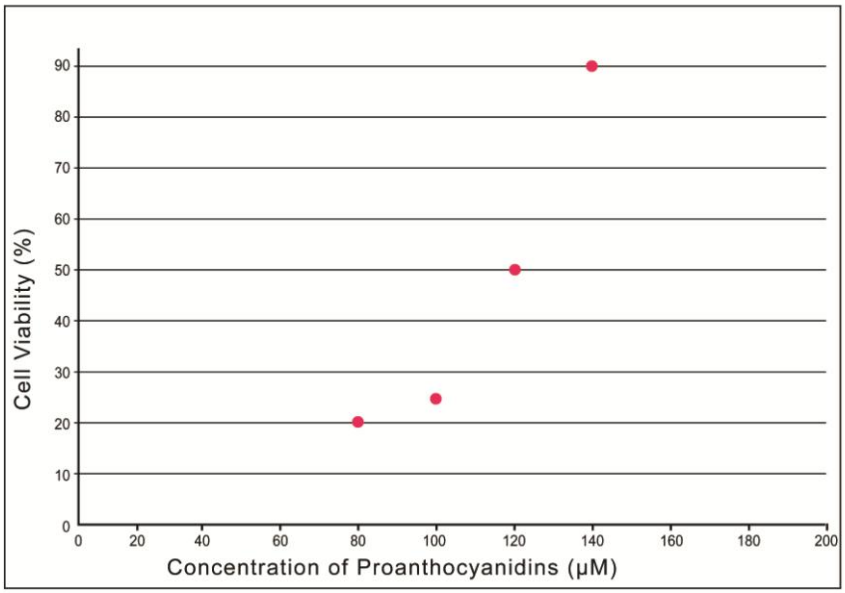

Figure 1: Effect of proanthocyanidins with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean±SD, n (for each concentration)=4].

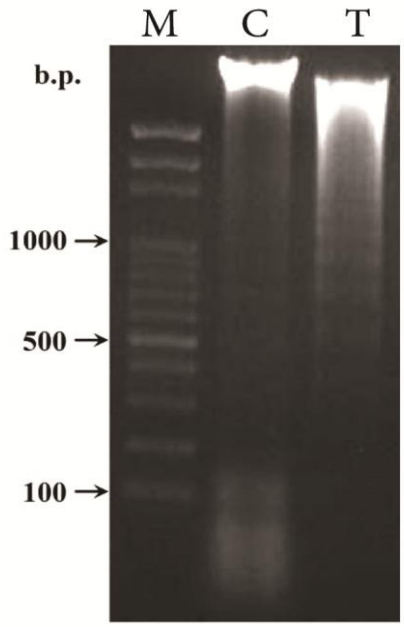

Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from proanthocyanidins treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C)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

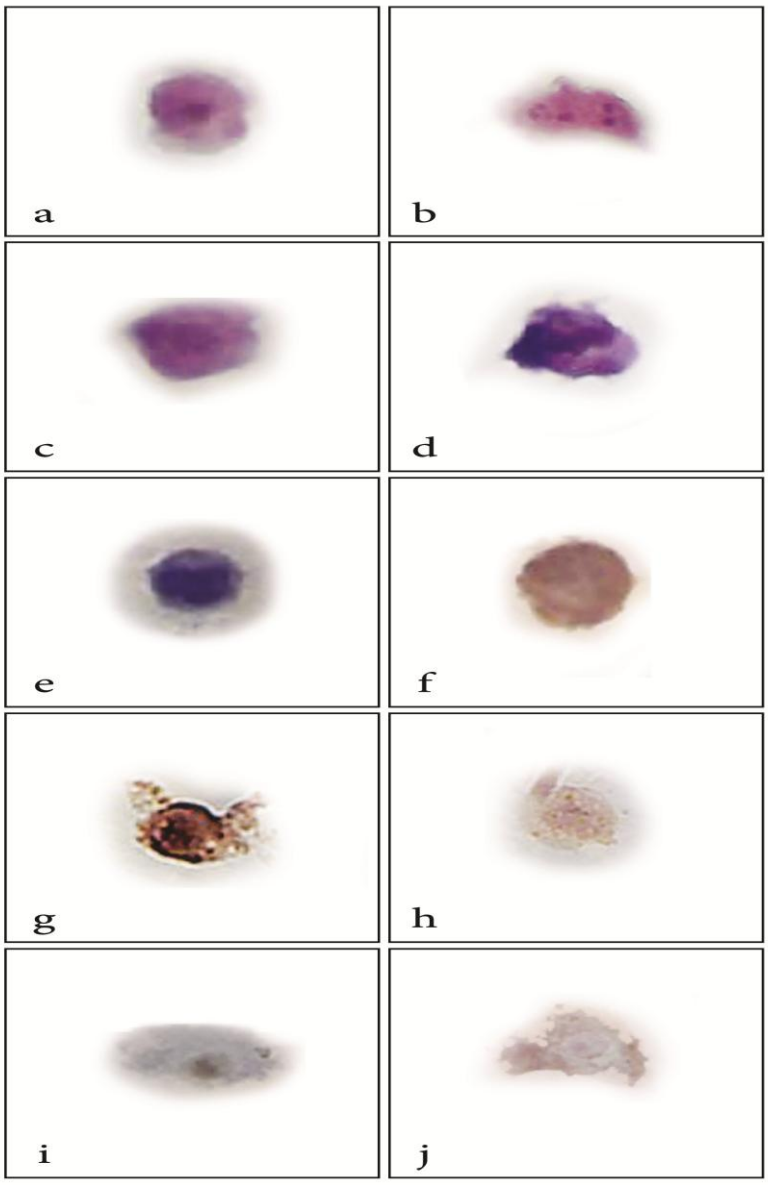

Figure 3: Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (b). Complete apoptotic cell (c). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Immunocytochemistry of caspase-3 protein. Control positive cell showing cytoplasm negative reaction for caspase-3 protein (e). Treated cell showing cytoplasm positive reaction for caspase-3 protein (f). Control positive cell showing Bcl-2 protein nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane showing brownish positive reaction (g). Treated cell showing negative reaction of apoptotic cell (h). Treated cell showing p53 protein nuclear positive reaction (i). Control positive cell showing nuclear negative reaction (j).

DISSCUTION

Colorectal cancer is the most common of visceral malignancies with the third most common cause of cancer-related mortality [21]. Despite improvements in the management of the colon cancer patient, there is little change in survival rates over the past 50 years [22]. Tumor cells

differ from normal cells in their non-responsiveness to normal growth-controlling mechanisms.

We investigated the effect of proanthocyanidins induced apoptosis on human colorectal cancer cell line Caco-2 to define the pharmacological basis for anticancer effects and its mechanism of proanthocyanidins. The results of present study demonstrated that proanthocyanidins induce cytotoxic effect measured by the cell viability on Caco-2 cells in a dose-dependent manner. Caco-2 cells also showed apoptotic morphological change of nuclear shrinkage, chromatin condensation, irregularity in shape and retraction by proanthocyanidins treatment. At the execution phase of apoptosis, a series of morphological and biochemical changes appear to have resulted from the action of caspase [23]. In this study, we observed the apoptotic morphology of cellular bodies. It is known that DNA strand breaks occur during the process of apoptosis [24]. DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor [25]. In our data, it is shown that the Caspase-3 gene expression and its activity were increased after proanthocyanidins treatment. Caspase-3, in particular, is believed to be most commonly involved in the execution of apoptosis in various cell types [26].

Apoptosis has 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 [27]. Therefore, we may presume that as primary mechanism involved in proanthocyanidins growth-inhibitory effects as it considered main apoptotic signals.

In a number of studies, it has been documented that the progress of apoptosis is regulated by the expression of several genes, one of these genes is a member of BCL-2 family [28]. BCL-2, an anti-apoptotic gene, is known for regulating the apoptotic pathways and protecting cell death [29]. Our data showed that proanthocyanidins altered BCL-2 gene expression as it decreased after proanthocyanidins-treatment.

P53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest and differentiation responses that prevent further proliferation of stressed or damaged cells and so protect from outgrowth of cells harboring malignant alterations [30]. P53 role in the repair of DNA damage has also been described and its ability to induce reversible cell-cycle arrest may contribute to the cells ability to repair and recover from damage before reentering a normal proliferative state [31]. Caco-2 cells which were treated with proanthocyanidins exhibited increased levels of p53 expression which involved in proanthocyanidins-induced Caco-2 cell death. These results suggested that the mitochondrial pathway was involved in proanthocyanidins-induced Caco-2 cell death. So, the treatment with proanthocyanidins show low toxicity on normal cells, and it might have a novel anti-tumor effect on human colorectal cancer cells.

CONCLUSION

In summary, we demonstrated that proanthocyanidins caused an inhibition of cell growth with apoptosis induction by DNA fragmentation and p53 activation in human colon (Caco-2) cancer cell line. Moreover, a large part of our study essentially focused on the mitochondrial pathway and we investigated that proanthocyanidins's action was caspase-3 dependent according to its inhibitory effect on Bcl-2 expression. There was also noticeable cytological alterations. These new findings suggest that proanthocyanidins-induced effects may have novel therapeutic applications for the treatment of different cancer type as previously described for breast cancer. Future *in vitro* and *in vivo* study will may deal with further investigations of the possible usages of proanthocyanidins as a new alternative chemotherapeutic agent.

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