

RESEARCH ARTICLE

In vitro Investigation of Cytotoxic and Apoptotic Effects of *Cynara L.* Species in Colorectal Cancer Cells

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Abstract

Apoptotic and cytotoxic activity of plant extracts obtaining from naturally growing *Cynara syriaca* in Turkey and cultivated *C. cardunculus* against DLD1 colorectal cancer cells was determined. Extracts from wild and cultivated *Cynara* species were obtained from their vegetative parts and receptacles using hexane and applied with five different dose (0.1-1 mg/ml) as well as apigenin for MTT tests for three time periods (24, 48 and 72 hours). After cells were treated with IC₅₀ doses for each extract total DNA and RNA were isolated for determination of the cause of cell death. From isolated RNAs, cDNA were synthesized and amplification of p21, BCL-2 and BAX gene regions was carried out. Consequently, we found that pro-apoptotic (BAX) gene expression and a cell cycle inhibitor (p21) were induced in the presence of our artichoke extracts. In contrast, anti-apoptotic BCL-2 gene expression was reduced compared to the control group. In addition DNA fragmentation results demonstrated DLD1 cell death via apoptosis.

Keywords: Artichoke - cDNA - colorectal cancer - DLD1 - DNA fragmentation - anti-cancer agent

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Introduction

Diet plays an important role on cancer prevention. The best diet for preventing cancer is a largely plant-based diet that includes generally vegetables and fruits.

Although inconsistencies exist across studies that have investigated the relationship between diet and cancer, the basic assertion that dietary factors influence cancer risk is not really a matter of debate. Nevertheless, many questions remain to be resolved, including exactly which specific dietary factors are most closely linked to cancer prevention, by what mechanisms food components exert their putative effects, how dietary factors might interact to affect cancer risk and what preventive steps can be taken to minimize adverse effects of factors that appear to increase disease risk (Greenwald et al., 2001).

Artichoke (*Cynara*) is a vegetable which especially a part of Mediterranean diet. Traditionally, the usage of plants has been commonly preferred for a long time and the conclusion to modern medical research from ethno botanical studies have rated day to day and very important conclusions have been obtained from them. And also artichoke has been used as a medicinal plant for more than hundred years. Artichoke has been used in traditional medicine for centuries as a specific liver and gallbladder remedy. In addition an artichoke, anti choleric, anti-fungal, antioxidant, anti-carcinogen effects has been known up-to-now. In all herbal medicine systems where it is employed, artichoke is used to increase bile production in the liver, increase the flow of bile from the gallbladder,

and to increases the contractive power of the bile duct.

In addition artichoke has a large amount of phenolic compounds. Natural phenolic compounds play an important role in cancer prevention and treatment. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways. (Yang et al., 2009).

Artichoke extracts hepatoprotective, cholagogic, and hypolipemic properties have been documented in a wide range of experimental and clinical studies. Modern medical findings have confirmed the traditional medical applications of the extracts (Adzet et al., 1987; Gebhardt, 1998; Lupatelli et al., 2004). The extract also displays a potent antioxidative activity, which was demonstrated in numerous *in vitro* studies (Jimenez-Escrig et al., 2003; Wang et al., 2003).

The anticarcinogenic effects of artichoke extract or one of its phytochemicals has been known. So artichoke extracts possesses the ability to inhibit the angiogenesis related to cancer. Other studies demonstrated anti-proliferation and apoptotic properties and also inhibit

inflammation (Miccadei et al., 2008; Nadova et al., 2008). Extract from artichoke *C cardunculus* has been shown also to exhibit antigenotoxic, and antiproliferative properties on leukemia cells and to induce apoptosis of these cells through a mitochondrial/caspase dependent pathway (Nadova et al., 2008). In addition artichoke extracts have strong antiproliferative activity on cancer cell lines, amelanotic melanoma C32 and renal adenocarcinoma ACHN has been reported (Conforti et al., 2008). The flavinoids at artichoke extract apigenin and luteolin have also anticarcinogenic effect on cancer cells (Chowdhury et al., 2002; Zhong et al., 2010). The aim of our study was evaluate the cytotoxic and apoptotic effects of different artichoke extracts on human colorectal DLD1 cell line.

Materials and Methods

Plant material

In our study, the kinds of cultural types of artichoke were obtained from local markets and wild type artichoke were collected from natural population of Kayseri, Yahyalı – Çamlıca village, Dönberi plateau, 1550 meters high.

Cell line

DLD1 a human colorectal cancer cell line was obtained from American Type Culture Collection via Gülhane Military Medical Academy. DLD1 cell line was routinely cultured in the media RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin. Cells were grown under the conditions of 5% CO₂ at 37°C.

Preparation of plant extracts

The plants were dried without sun light and broken into small pieces under sterile conditions. Firstly plant powders put on Pasteur oven at 110°C for 5h. Following, the prepared samples of 25g of *Cynara cardunculus* and *C syriaca* were extracted with n-hexane for 6h by using a Soxhlet apparatus. The plant hexane extracts were obtained after the solvent was evaporated at 40°C by rotary evaporator. By this way we gained three different extracts (E1, E2, and E3). Whereas, E1 was extracted from species heads and vegetative parts of cultivated *Cynara*, E2 was extracted from the same parts of wild artichokes. Lastly, E3 was extracted from only eatable parts of cultivated *Cynara* species.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The cytotoxicity of the extracts was tested upon DLD1 cell line. Cells were harvested, counted and transferred to 96-well plates and incubated for 24h. The artichoke extracts and apigenin were prepared and applied in various concentrations, and the treated cells were incubated 24h-48h-72h. Cell proliferation assay was performed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (0.5mg/ml) was prepared and MTT solution was added to each of the 96 wells. Viable tumor cells were counted by the ability to reduce the yellow dye (MTT) to a blue formazan product (Mossman, 1983). Four hours later, the formazan product of MTT reduction was

dissolved in isopropanol and optical density of plates was measured using a Elisa micro plate reader at 540nm. Each experiment was performed in triplicate.

DNA isolation and fragmentation

After MTT assay, each extracts IC₅₀ dose was calculated and treated to cells. We preferred 48 hours as optimized time. 48h later, DNA isolation was carried out via Fermentas Genomic DNA purification kit according to manufacturer's procedure. Isolated DNA's were loaded to 1.5% agarose gel and run via electrophoreses systems and visualized via UVP Gel Doc It Imaging system.

RNA isolation and RT-PCR

Total RNA was extracted with Axygen RNA isolation kit and 0, 5µg RNA was turned to cDNA by using a Fermentas First strand cDNA synthesis kit during to RT-PCR process. Total PCR mixture was prepared as 25microliter (25mM MgCl₂, 10mM primers, 10mM Dntps, 5× buffer) One micro liter of cDNA obtained was used for PCR amplifications and specific primers belonging to targeted gene regions (GAPDH, p21, BAX, BCL-2) were designed to amplify and determine expression level of targeted gen regions. Our PCR amplification was performed under the following conditions: 2 min initial denaturation at 94°C; 30 cycles of denaturation (30 s at 94°C), annealing (45s at 55°C), and extension (45s at 72°C); a final extension at 72°C for 8 min. PCR products were run in 1% agarose gel and visualized via UVP Gel Doc It Imaging system. Housekeeping gene GAPDH was used to critic and evaluates expression levels of PCR products.

Statistical analysis

To calculate viability %, absorbency value of each group was divided to absorbency value of control group. Graph Prism 5 (5.04 Version) program was used to evaluate of MTT results. In addition, Microsoft Excel (Office 2007) program was used to give the meaning of raw data and converting of statistical data to computer graphics. Finally, a one-way analysis of variance (One-way ANOVA) and Dunnett's test were used to determine the differences between the experimental groups, apart from statistical analyses. In case p<0.05, our results was accepted meaningful as statistically.

Results

MTT assay results

MTT assay shows that the DLD1 cells were significantly decreased with the application of artichoke extracts and apigenin at different time intervals. The results indicate that all extracts and apigenin showed a dose dependent but only E2 extract showed time and dose dependent inhibitory effect on the viability of DLD1 cells (p<0.05).

After our artichoke extracts (E1, E2, and E3) and apigenin were treated on DLD1 cell line, the graphic of dose dependent viability % at 24, 48, 72 hour time intervals were generated by the data obtained from MTT assay and it is shown in the Figure 1.

Mitochondrial activity of cancer cells was evaluated at the end of the time intervals as % viability. So, % viability values inferred that DLD1 cells exposed to E2 and E3 extracts were arrested effectively to cell death. Cytotoxic activity of apigenin upon DLD1 cells was relatively lower than cytotoxic effect of our extract used (Figure 1).

Morphological observations

During cell proliferation, when we treated the extracts of artichokes upon DLD1 cancer cells in different time intervals changed from 24-72 hours, we preferred 48 hours as optimized time. When the images taken from DLD1 cells before DNA and RNA isolation which are exposed to several artichoke extracts during to 48 hours, a significant reduction was seen in the number of cells compared to the control group. Moreover, cell integrity not be destroyed and a reduction were determined in cell volume as well as apoptotic bodies (Figure 2). Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste et al., 2000). All of these morphologic findings demonstrate clearly cell death via apoptosis as reported before many studies (Janicke et al., 1998; Elmore, 2007).

DNA fragmentation results

The progression of apoptosis is known to bring along fragmentation of the cellular DNA through activation of various endonucleases. Endonucleases occurs regular oligonucleosomal breaks in DNA so it shows cell death via apoptosis. The isolated DNA from DLD1 colorectal cancer cells of which were exposed to E1, E2, E3 extracts and apigenin after than loading to gel shows a certain fragmentation changed from 1000-10000 bp. DNA fragmentation of DLD1 cell lines which treated with E1, E2, E3 and apigenin were seen respectively (Figure 3).

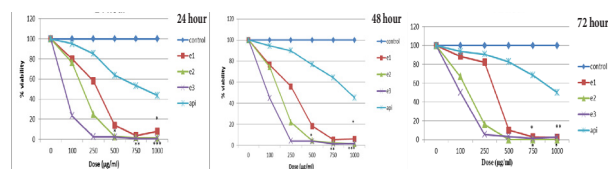


Figure 1. MTT Assay Results for Different Time Intervals. Data presented are the mean±SD of results in one of three independent experiments with similar results. Statistically meaningful results are shown with signs, (** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$)

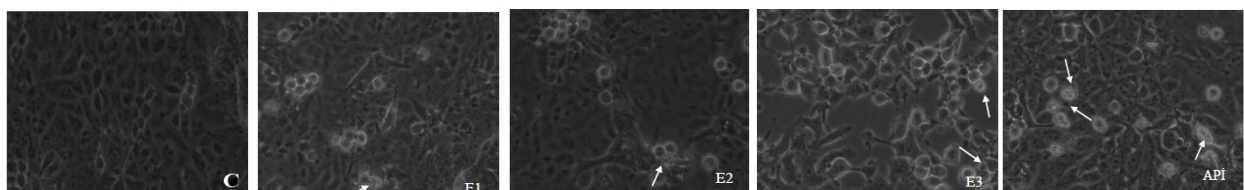


Figure 2. Morphological Changes at DLD-1 Cells 48 Hours after Extracts Application. Apoptotic bodies are shown by arrows

RT-PCR results

In this study, RT-PCR was utilized to analyze the mRNA levels of apoptotic markers (p21, BAX, BCL-2) in DLD1 cells exposed to artichoke extracts and apigenin at IC₅₀ dose for 48 h. We also performed reverse-transcription polymerase chain reaction (RT-PCR) and found that the mRNA levels of these apoptotic markers were significantly changed in DLD1 cells due to extracts exposure. Housekeeping genes has been used for the normalization of target gene expression data. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene is expressed in all cells and is one of the most commonly used in comparisons of gene expression data. Our GAPDH gene expression results are same in all samples and these results permitted us to comment other gene expression values (Figure 4).

p21 plays a critical role in inducing growth arrest after DNA damage. We observed morphologically that cell proliferation of DLD1 lines was blocked by treatment of artichoke extracts during applications. The mRNA levels of cyclin dependent kinase inhibitor gene p21 increasing from 1.6-2.3 fold were measured for Apigenin, E3, E1 and E2, respectively. E1 and E2 extracts had been more effective than particularly commercial product apigenin in point of rising expression level of p21 gene. The results show that both artichoke extracts and apigenin inhibit the cell proliferation of human colorectal cancer DLD1.

We also performed the effect of artichoke extracts and apigenin on the mRNA expression of BAX and BCL-2. BAX and BCL-2 have been known as playing a major role in determining cellular fate under injured conditions. Increased expression of BAX can induce programmed

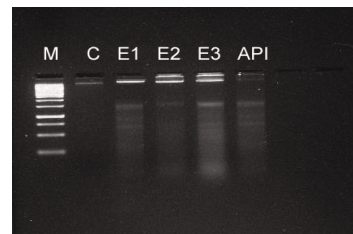


Figure 3. The Effects of Artichoke Extracts and Apigenin on the Formation of DNA Fragmentation. C: control, E1: E1 extract treated group, E2: E2 extract treated group, E3: E3 extract treated group, API: Apigenin treated group

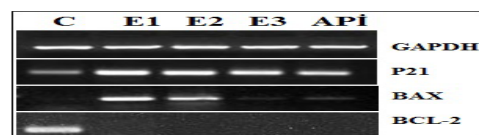


Figure 4. Expression Levels of Performed Gene Regions on Agarose Gel after PCR Amplification. C: control, E1: E1 extract treated group, E2: E2 extract treated group, E3: E3 extract treated group, API: Apigenin treated group

cell death, while up regulation of BCL-2 protein protects cells from apoptosis (Salomons et al., 1997; Tallman et al., 2005).

In our study the expression pro-apoptotic BAX gene compared with control group had a significant increase at treatment group's BAX gene expression. Level of BAX gene expression was raised importantly around cells 5 fold by E1 and E2 extract, while it was affected temporarily in level of 1.6-2.9 fold respectively by E3 and apigenin extracts in DLD1 cells. Increase in the BAX gene expression in cancer cells causes increase the membrane permeability and it leads to release of cytochrome c. By this way, artichoke extracts led to apoptotic death DLD1 cells. On the other hand anti-apoptotic BCL-2 gene expression was significantly decreased compared to the control group. Therefore, we said from these results that our extracts may be activated programmed cell death (apoptosis) pathway by removing barriers of BCL-2 gene. The balance between BAX and BCL-2 expression levels help to determine the susceptibility of a cell to apoptotic stimuli (Oltvai et al., 1993; Eliopoulos et al., 1995; Chresta et al., 1996). In the end of our assays, we also determined that BAX/BCL-2 ratio increased significantly in treatment groups as compared to control. Detected increase of the rate of BAX/BCL-2 ratio shows occurrence of cell death by way of apoptosis.

According to these data, our results suggest that our artichoke extracts causes a consistent up-regulation of BAX and down-regulation of BCL-2 may be one of the molecular mechanism through which our all extracts induce the intrinsic apoptotic pathway. At least, an increase of the BAX/BCL-2 ratio and up-regulation of cyclin-dependent kinase inhibitor, p21, crucial apoptotic players, were documented at this study.

Discussion

Cancer is a leading cause of death all over the world. Breast, prostate and colon cancer are three of the most common cancers. Today, a wide spectrum of chemotherapeutic drugs used in the treatment of cancer constant given in the relevant field continues to explore new opportunities for therapeutic purposes because of chemotherapeutic side effects and damage to the immune system. There is growing interest in the potential health beneficial properties of the constituents of natural products, among them those containing polyphenol antioxidants, artichoke extracts being a rich source (Wegener et al., 1999; Wang et al., 2003). For this goal many of the plant-derived components used in recent times. One of the most effective strategies is using of plant extracts or natural products like artichokes against to carcinogenic agents and cancer risks and primarily this must be preferred to instead of struggle with several cancer types.

Artichoke extracts showed antiproliferative activity on human hepatocellular carcinoma HEP2B cells and reduction in tumor cell viability (Menghini et al., 2010). Atasever et al. (2003) investigated effects of caffeic acid, apigenin, apigenin-7-glycosid, luteolin, luteolin-7-glycosid, cynarin, which are obtained from *Cynara syriaca*, on leukemic cell line (K562, DG75, BB58, B95)

so they reported especially apigenin and luteolin shows antiproliferative and cytotoxic activity on cancer cells. In addition one report showed that apigenin has a positive effect on lung cancer (Liu et al., 2005).

So far, such as artichokes or tea extract, the filtrate obtained in various ways in different tissues and organs, both for healing practices and ethno botany many positive effects from clinical trials have been reported (Gebhardt, 1997; Saenz Rodriguez et al., 2002; Speroni et al., 2003; Conforti et al., 2008; Juzyszyn et al., 2010; Mileo et al., 2012).

This paper is a first report, concerning with, positive effects of plant extracts obtained from two different artichoke species against to colorectal cancer cell line. Additionally, it is fairly remarkable that is to be more effective of extracts obtaining from wild *Cynara* compared to cultivars species on DLD1 cells in view of cytotoxic activity and apoptosis. Many studies have demonstrated that plant extracts provide anti-tumorigenic activity; in fact, this paper prompted extensive studies in our country that have defined a key role for tumor suppressor genes and proteins.

In this study, our results demonstrated that artichoke extracts had inhibitory effects on the proliferation of human colorectal cancer DLD1 cells. Artichoke extracts not only inhibit cell proliferation but also induce apoptotic pathway on DLD1 cells.

Atasever et al. (2003) investigated the effect of flavonoids belonging to artichokes on leukemic cancer. When they applied in different doses of flavonoid compounds changed from 0.005 to 500 $\mu\text{g/ml}$, they found a cytotoxic effect in varying intervals 8 and 20% upon cancer cells. In spite of these results compliance with our results in terms of positive cytotoxic effect, there are a proportionally differences among them. Our results indicated that cytotoxic effects of apigenin changed between 5 and 50%, respectively. Conforti et al. (2008) reported that *Cynara* extracts had a strong cytotoxic effect at C32 and ACHN cancer cell lines. They calculated IC_{50} dose was 18 $\mu\text{g/ml}$ for C32 cells and 21 $\mu\text{g/ml}$ for ACHN cells. Our extracts IC_{50} doses were higher than this study. In our opinion this difference may be caused from our extracts biochemical content, preferred solvent for extraction or the fact that used a different cell line. In general, we look at the data of MTT, our results are correlated with previous relevant reports except from higher application doses.

Zhong et al. (2010) studied molecular targets of apigenin in colorectal cancer cells (HCT-116, HT-29, SW480 and LoVo cells) and they were reported apigenin caused induction p21 and NAG-1 expression levels on cancer cells. When we compared our apigenin results with relevant previous reports with regards to p21 gene expression levels of DLD1 cells, we saw that all of our extracts had been more effective than apigenin as well as similar positive effects including tumor suppressor and regulatory responsibilities (Figure 4).

We also performed mRNA expression levels of apoptotic control point genes which p21, BAX and BCL-2. Tumor suppressor p53 gene controlled growth inhibition is dependent on induction of p21, which is an inhibitor of

cyclin-dependent kinases that are required for cell cycle progression. To shut down cell cycle at G1/S control point, activated p53 protein, induced gene transcription which encodes p21 protein. p21 protein inhibits CDK4/cyclin D1 complex and cell cycle arrest at G1 phase. So in our study, induction of p21 gene expression is important for uncontrolled cell proliferation and cancer therapy. In addition increase of BAX/BCL-2 ratio shows that artichoke extracts are effective to induce intrinsic apoptotic pathway.

As a result, we suggest that artichoke species especially wild artichokes would be an important natural source including beneficial chemical products against colorectal cancer in the future and it has majorly potential in development of related anticancer drugs.

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