REPORT

Investigation of anti-cancer effects of cherry *in vitro*

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Abstract: Cherry (*Prunus Cerasus*) is still one of the most popular preserve in Turkish cuisine. Cherry has been traditionally used for the treatment of inflammatory-related symptoms. Recent researches have proved that cherry is a valuable natural source of some important bioactive compounds in human health preservation. Evidence suggests that, cherry consumption may decrease the risk of chronic diseases and cancer. The aim of the present study was to determine the effects of cherry on breast cancer cells lines, asymmetric dimethylarginine (ADMA) level and certain multidrug-resistant bacteria. The cancer cell proliferation activity and analysis of apoptotic-necrotic cells was evaluated by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and scoring of apoptotic cell nuclei. Measurement of ADMA and the minimum inhibitory concentration was accomplished by HPLC and the micro dilution broth method. The results showed that, extracts of cherry exhibit anti-proliferative activity in mammary adenocarcinoma (MCF-7) & mouse mammary tumor cell (4T1) breast cancer cells lines as well as induction of apoptosis, lower ADMA concentrations in cell cultures treated with cherry extract and antibacterial effects against certain multidrug-resistant bacteria in vitro. These findings may open new horizons for traditional anti-inflammatory product as prophylactic-therapeutic agent from cancer, cardiovascular diseases and multidrug-resistant infections.

Keywords: Cherry, Breast Cancer Cells, Anticancer Effect, Minimum Inhibitory Concentration, ADMA.

INTRODUCTION

Cherry (*Prunus cerasus*) is a sour fruit strain that is like berry from the rosaceae family. The region between the Caspian Sea and North Anatolia is regarded as the possible homeland of cherry in most of the resources. Cherry especially is quite rich in terms of mineral matter. By the reason of fruit juice's efficiency's (70-75%) and total acidity's (3%) being high cherry is very convenient to be processed as fruit juice (Turkey Fruit Juice Industry 2011; Krishkov 2009). In Turkey the production of cherry that is 24.000 ton in 1965 reached 85.000 ton in 1985 and 120.000 ton in 1991. In this aspect Turkey ranks as the fifth country in cherry production. Differently from other countries, in our country the mostly consumed fruit juices are peach and cherry. The consumption of fruit juice that is 0,4 liter per person in the beginnings of 1970s increasing 10 times reached 3,9 liter per person in 1996 and today the rate is around 4,5 liter (Turkey Fruit Juice Industry 2011).

According to the researches, antioxidant capacity of the cherry is quite high and can be defined as nutriceuticals. It has been denoted that anthocyanins in cherry have the effects of decreasing rheumatism, the risk of colon cancer and inhibiting fever, inflammation and tumor progression. Cherry being rich in terms of melatonin that is a powerful antioxidant and well sleeping regulator produced in the brain backs up growth and cell renewal (Jafari *et al.,* 2008; Kim *et al.,* 2005; Burkhardt *et al.,* 2001; Wang *et al.,* 1999).

Nitric oxide (NO) is the most important endothelium borne vasodilator. In addition to NO's vascular smooth muscle proliferation's positive effects on thrombocyte aggregation and vascular superoxide productions, it has anti-atherosclerotic features. ADMA is the major inhibitor of NO biosynthesis in human body. It is thought that the increase of ADMA leading endothelial dysfunction has a significant role in atherogenesis and cardiovascular diseases (Vallance *et al.,* 1992; Boger 2004).

Some bacteria like methicillin-resistant *Staphylococcus aureus*, carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* developed resistance ways against drugs' effects. Along with surveying resistance mechanisms and new drugs, these resistant microorganisms, pose serious problems for health care workers especially nosocomial infection at the top (Jeong *et al.,* 2006; Chastre and Trouillet, 2000).

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The purpose of this study is to investigate the potential anticancer effects of cherry on human breast mammary adenocarcinoma (MCF-7) and mouse mammary tumor cell (4T1). To evaluate ADMA concentrations in cell cultures treated with cherry extract and to examine whether cherry has an antibacterial effect against certain multidrug-resistant bacteria *in vitro.*

MATERIALS AND METHODS

Reagents

Maltodextrin Non-GMO was purchased from Tate & Lyle Corporate and Investor Relations, Company, Slovakia. Chemical and reagents; Folin-Ciocalteu reagent 2N, DPPH (1,1-diphenyl-2-picrylhydrazyl), gallic acid, alpha tocopherol, BHA (3-tert-butyl-4-hydroxyanisole) were purchased from Sigma-Aldrich (St. Louis, MO) and sodium carbonate was from Alfa Aesar GmbH & Co, KG. All other chemicals were of food grade.

Plant material and preparation of extracts

Extracts of cherry pulps were used in the study. Cherry pulp concentrations were provided by an international aroma producer (Aromsa, Turkey). Cherry concentrate was macerated with water and ethanol (1:1 w/w) for 4 times at room temperature. Evaporation of the solvent under vacuum yielded 40 BX cherry extract, which used in spray dry. Another concentrates from cherry concentrates were used as a concentrate at 65 BX in all tests.

Preparation of microcapsules by spray drying

Microencapsulation of flavors in carrier matrices can provide protection prevent loss of volatile flavors and enhance stability of the flavor core materials. The most common and economical way to apply microencap-sulation is spray drying. There are many papers were published about the encapsulation of flavors in liquid by spray drying. In this study, microencap-sulation was performed by Aromsa Co. Inc and maltodextrin Non-GMO used as a wall material. The total concentration of cherry extract was 40% (w/w) and that was blended of maltodextrin 30% (w/w; MD:Water). The mixture was emulsified in a Sylverson, (Sylverson, Chesham, England) for 5 min at 4000 rpm until complete dispersion of the extract. The resulting slurry was spray dried in APV-Anhydro, Denmark. The microcapsules prepared were collected from the collecting chamber and filled in air tight self-sealable polyethylene pouches.

Studied activity

Total phenolic content was measured by using the Folin-Ciocalteu's reagent. Results are expressed as microgram of gallic acid equivalents per mg of extract. Free radical scavenging activity was determined by 1,1-diphenyl-2-picryl-hydrazil (DPPH) assay. The data on all antioxidant activity tests were triplicated.

Cancer cell lines

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) was purchased from Invitrogen Corporation (Carlsbad, CA 92008 USA). Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS solution) and MTT were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). MCF-7 and 4T1 cell lines were used, in order to determine antitumoral activity,.

Cancer Cell proliferation inhibitory assay

The cancer cell proliferation activity of the cherry extraction was tested by MTT colorimetric assay. Cancer cell cultures (MCF-7 & 4T1) were maintained at the Public Health Laboratory at Gulhane Medicine Faculty Ankara, Turkey. MCF-7 & 4T1 were cultured in 10% FBS, 2 µM L-glutamine, 100 µg/ml streptomycin and 100 µg/ml penicillin were added in DMEM medium in a humidified incubator at 37°C under 5% CO_2. MCF-7 & 4T1 cell lines were harvested, counted and moved into 96-well plates and incubated for 24 hours before treatment. By melting away the cherry extracts in DMSO the samples were made ready. Samples at 50 and 100µg/mL, were supplemented to DMEM medium. The ending concentration of DMSO in the assay was 0.1%. Samples at 50 and 100µg/mL concentrations were supplemented to the wells. Into each well 25/µL MTT solution was added after 48 hours incubation of samples with cancer cells. Plates were incubated for 3 h at 37°C. For dissolving the formed formazan crystals, content of each well removed and 200/µL DMSO was added to each well, and then the plates were shaken. Plates were incubated for 5 min at room temperature, and finally the optical density (OD) of the wells was ascertained using a micro-plate reader at 570 nm. Samples' cell viability at each concentration was calculated related to solvent control. At each concentration, cell viability was evaluated by dividing the optical density of samples with the optical density of solvent control.

Analysis of apoptotic and necrotic cells

After treating cell lines with different amounts cherry extract for 72 hours, all of the cells (attached and detached cells) in the flask were brought together. After this step, cells were washed with PBS for three times and stained with Hoechst dye (2mg/ml), propodium iodide (PI) (1mg/ml) and DNAse free-RNAse (100mg/ml) at the 25°C for 15 minutes and analyzed under a confocal fluorescence microscope (LSM-GB200: Olympus, Tokyo, Japan). The nuclei of apoptotic cells were marked blue by Hoechst dye where necrotic cells were marked red by PI. Ten areas in microscope, chosen by chance, were counted and the result presented as a ratio of apoptotic and necrotic to normal cells.

Measurement of ADMA

Measurement of ADMA was performed by HPLC (high pressure liquid chromatography), refer to the method described by Chen *et al.,* (Chen *et al.,* 1997). In short, 1

ml cell culture medium and 20/mg 5-sulfosalicylic acid was mixed and the mixture was kept waiting in an ice bath for 10 min. After centrifugation for 10 min at 2000 rpm the precipitated protein was removed. 10/µl of the supernatant that was filtered and mixed with 100/µl of extraction reagent (10/mg o-phthaldialdehyde in 0.5/ml of methanol and 2/ml of 0.4/m borate buffer) then injected into HPLC.

The minimum inhibitory concentration

Antibacterial characteristic of cherry evaluated on two standard (*Staphylococcus aureus* ATCC 29123, *Pseudomonas aeruginosa* ATCC 27853) and three clinical (MRSA, Carbapenem-resistant *Acinetobacter baumannii*, Carbapenem-resistant *Pseudomonas aeruginosa*) strains of bacteria. The micro dilution broth method was used for carrying out the minimum inhibitory concentration (MIC). By using Mueller Hinton Broth sequential two times dilutions of cherry extract were done in sterile 96-well micro-plates with concentrations between ½ and 1/1024. Bacterial suspensions were regulated to 0.5 Mc Farland standards. From an agar plate culture, at least three well-separated colonies were chosen and they were incubated at 37°C for 48 hr. Bacterial growth was analyzed by 625 nm. absorbance measurement after incubation. DMSO was used as a control. Ascertain the late antimicrobial activity of cherry all assays were also incubated for 48 hr at 37°C aerobically. Bacterial growth was analyzed by 625 nm. absorbance measurement after incubation. Lowest concentration of cherry which limited bacterial growth to a <0.05 level at 625 nm was accepted as MIC.

STATISTICAL ANALYSIS

Statistical analysis of variance was determinate by ANOVA, significant differences between means were determined by Student's-t test, P<0.05 were noted as significant.

RESULTS

Activity result

The cherry extract encapsulated was found to be the most active extract in tests. Previously, the cherry extract encapsulated was found to be rich in phenolic compounds. Total phenolic content of cherry encapsulated extracts were varied between 93.7 mg and 115.6 mg (mg GEs/100 g extract). Total phenolic content of the other cherry concentrates were varied between 54.3 mg and 79.0/mg (mg GEs/100 g extract). As far as DPPH assay, the cherry extract encapsulated was showed higher antioxidant activity than Ascorbic acid and all tested extracts of cherry (table 1). Konya and Tokat Cherry concentrate were showed similar activity in DPPH assay and all other extracts showed higher activity of Ascorbic acid (fig. 1).

Results of MTT

The cell proliferation inhibitory effects of encapsulated cherry extracts that have 50 and 100ug/ml concentration respectively has been examined in the MCF-7 and 4T1 cancerous cell series. It has been concluded that the extracts that are added to cell culture not only decreased the cell proliferation in both MCF-7 and 4T1 cells but also decreased in the concentration. It has been stated that above mentioned extracts the one that has 50 ug/ml concentrations inhibited cell growth 37% in MCF-7 cell series and 48% in 4T1 series. Similarly, it has been stated that the extract that has 100 ug/ml concentration inhibited cell proliferation 54% in MCF-7 series and 53% in 4T1 series. It has been specified that the decrease ascertained in the proliferation in cell series were significance statistically (fig. 2).

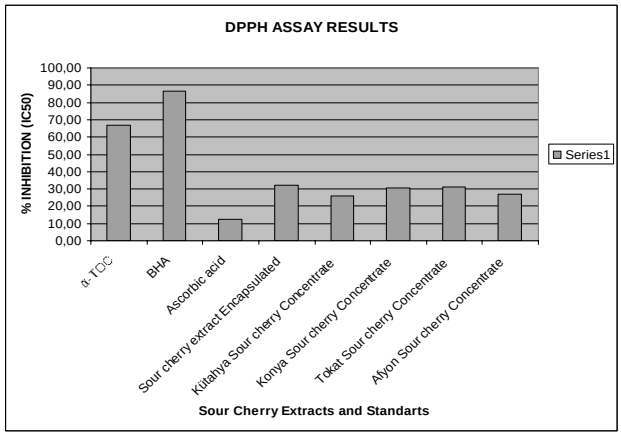

Fig. 1: DPPH assay results of cherry extracts.

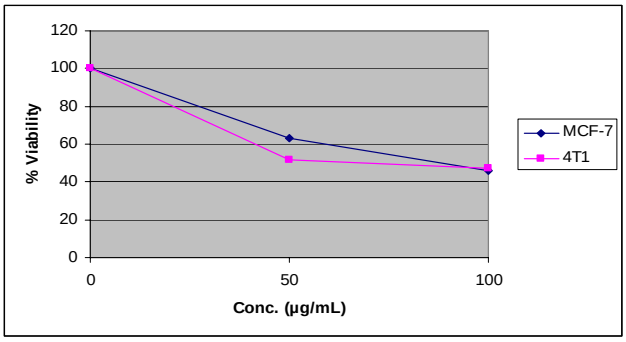

Fig. 2: *In vitro* cell proliferation inhibitory results of cherry against cancer cell lines

Results of apoptotic and necrotic cells analysis

The effects of encapsulated cherry extracts on cell development and its survival have been analyzed by staining the MCF-7 and 4T1 cells that were treated with cherry extract with Hoechst and Propodium Iodid. In consequence of microscopic examination, it has been stated that the greater part of examined cells cannot be stained by PI and they are at late apoptosis phase that is characterized by cells whose nucleuses are separated into smaller organisms in cytoplasmic membrane. In addition to these analyzed fields, the existence of red painted early

and late necrotic cells have been specified. In the light of findings above, it has been resulted that cherry extracts induced anticancer activity through apoptosis (fig. 3).

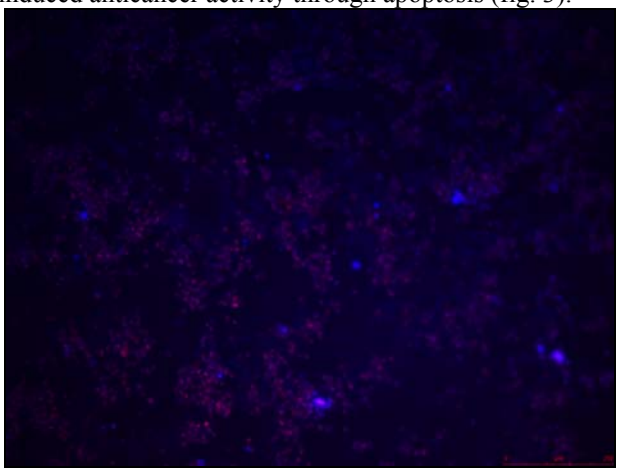

Fig. 3: A general view of apoptotic and necrotic cells by fluorescence microscopy. Blue points represent the apoptotic cells, red points represent necrotic cells.

Table 1: Minimal inhibitory concentration (µL/mL) results of cherry against clinical and standards strains of bacteria.

	24 hr of incubation (µL/mL)	48 hr of incubation (µL/mL)
Clinical isolates	200	100
Methicillin-resistant *Staphylococcus aureus*	100	12.25
Carbapenem-resistant *A. baumannii*	100	12.25
Carbapenem-resistant *Ps.aeruginosa*	100	12.25
Standard strains		
S. aureus ATCC 29123	200	100
Ps. aeruginosa ATCC 27853	100	12.25

Results of ADMA

ADMA levels of cell cultures supplemented with cherry extract were significantly lower than the Ø control and control cell culture values. ADMA levels of cell cultures supplemented with cherry extract, Ø control and control cell culture are 1.21, 2.54 and 2.79µmol/l respectively.

Minimal inhibitory concentration (µL/mL)

The results obtained from the MIC examination show that cherry extract is effective on three clinical (MRSA, Carbapenem-resistant *Acinetobacter baumannii*, Carbapenem-resistant *Pseudomonas aeruginosa*) and two standard (*Staphylococcus aureus* ATCC 29123, *Pseudomonas Aeruginosa* ATCC 27853) examined strains of bacteria. Following 24 hours incubation, MIC concentration has been found 200ug/ml for gram-positive microorganisms and 100ug/ml for gram-negative microorganisms. Similarly, following 48 hours incubation it has been stated that MIC rate fell 100ug/mg for gram-positive microorganism and 12.25ug/ml for gram-negative microorganism. At the same period there has been obtained increase in the number of microorganisms at the control wells.

DMSO, used as negative control, its effect on bacteria proliferation has been researched. At the ½ diluted well of *Carbapenem-resistant Pseudomonas aeruginosa* except detected inhibition at the end of 48 hours incubation there aren't detected any inhibition in other wells. Including positive controls, all of the results obtained from MIC examination are in the border of CLSI quality control parameter.

DISCUSSION

This is the first study in which the effect of cherry on MCF-7 and 4T1 human and mouse breast cancer cell lines was surveyed. The results obtained from this study is in accordance with the results of a study in which the effects of cyanine, giving the bright color of cherry and classified as subgroup of anthocyanins, on human colon cancer was surveyed. Furthermore, it has been stated that cherry inhibits the formation and development of intestinal cancer on ApcMin mice that are developed as human cancer model. In the stated study, the number and size of adenoma have been stated respectively less and small in the mice whose diet cherry is added compared to other mice (Kamei *et al.,* 1998; Kang *et al.,* 2003).

NAG-1 has been ascertained to be a gene that shows anti-tumorigenic activity by triggering the apoptosis in the cell lines. Studies proved that NAG-1 is a significant promising target gene in inhibiting the cancer development. The "wild-cherry" extract sometimes called as "black-cherry" has been stated to increase NAG-1 expression and inhibit cell reproduction in the cancer cell lines by increasing apoptosis as well (Baek *et al.,* 2001; Liu *et al.,* 2003).

The surveys conducted revealed that wild-cherry extract respectively suppresses B-catenin/TCF signal. It has been stated that in the colorectal adenokarsinoms cyclin D1 protein expression increases. Similarly, the results of wild cherry treatment made in the colorectal adenokarsinom cell lines verify the decrease of cyclin D1 protein expression with treatment. In the light of this information it has been assessed that wild cherry suppresses B-catenin signal in cancer cells and in consequence of this suppression cyclin D1 protein expression decreases and cancer cell development regresses (Yamaguchi *et al.,* 2006; Fu *et al.,* 2004).

Genetic surveys states that genetic features of different cherry kinds resemble each other very much. NAG-1

gene's and cyclin D1 protein's being covalent in the analyzed kinds is possible. It has been thought that anti-tumoral effect stated in this survey occurred by gene and proteins in question (Mariette *et al.,* 2010; Stockinge *et al.,* 1996).

When we consider the results of the survey, cherry is specified to have shown antimicrobial action against the gram-negative and gram positive bacteria examined in the cope of survey. Examined bacteria's causing nosocomial infection and their being antibiotic resistant increases importance of obtained results of the survey. Cherry along with showing antimicrobial action on both gram-negative and gram-positive bacteria, its effect on gram-negative bacteria is stronger. In the previous studies cherry has been mentioned to shown antibacterial action by inhibiting the adhesion of bacteria. Mechanism of action of the cherry is a subject that has not been sufficiently researched yet. It is possible that the difference of the action that cherry showed towards gram-negative and gram-positive bacteria kind results from the difference of the protein and lipid layer in the membrane structure of these bacteria kinds. There is needed further study on this subject (Klevens *et al.,* 2007; Hebeler *et al.,* 1973; Rothfield *et al.,* 1964).

In a recent study it is stated that much of fruit juices and extracts especially those that are rich of anthocyanins and acidic show antibacterial effect. It is ascertained that the action in question disappears with the disappearance of the acidic structure. However, in the same study it is also ascertained that cherries and raspberries extracted with water and blackcurrant extracted with methanol show highly antibacterial effect. Effect showed in cherry, raspberry and blackcurrant is stated to have been independent from low acidic level and resulted from anthocyanins and ellagitannin that are intensive in these fruits (Galgóczy *et al.,* 2009).

In this study ethanol was used in the preparation of cherry extract. This situation made us think that the strong antibacterial effect ascertained in cherry extracts, as showed in some other studies, depends on both used extract's being acidic and pigments included in cherry's ingredient like anthocyanins and etc (Lee *et al.,* 2003; Harborne and Williams 2000).

This is the first study in which ADMA level increase observed in MCF-7 and 4T1 breast cancer lines treated with cherry extract. After showing its inhibiting NO synthesis, in a short time lots of study was carried out about ADMA's pathophysiology (Valkonen *et al.,* 2001; Böger at al 2003). Increased ADMA levels by inhibiting NO synthesis activity, inhibits NO formation and vascular structure is preserved. In recent studies it is stated that anthocyanins in cherry have positive effects on vascular endothelium cells. It is expressed that mentioned effects were controlled by plenty factors and enzymes partaking in ADMA catabolism like Dimetilarginin dimetil amimo hidrolaz are among these factors (Ding *et al.,* 2000; Mc Carty 2004).

The effects of cherry on cancer development, antibiotic-resistant gram-positive and gram-negative pathogen microorganisms and ADMA levels is of great concern to human health because it is an ingredient in foods consumed daily. Whereas, little is known about the metabolic outcome of ingested cherry and its compounds in humans in order to correlate its health benefits *in vivo* as compared to in vitro studies.

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