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Polyphenol-rich extract of Aronia melanocarpa inhibits TNF-α induced apoptosis in H9c2 cells

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ABSTRACT

Introduction. In certain pathological states within cardiovascular system, cardiomyocytes may exhibit overexpression of TNF-α that results in induction of myocardial oxidative stress and cardiac cells apoptosis. Thus, they may participate in development and progression of post-infarct congestive heart failure. The aim of this study was to evaluate the effect of polyphenol-rich *Aronia melanocarpa* extract (AME) on TNF-α induced apoptosis in cardiomyoblast H9c2 cells.
Material and Methods. Apoptosis was measured in H9c2 cells preincubated with increasing concentrations of commercial extract of aronia – Aronox (10–50 μg/mL) for 24h and then treated with TNF-α: 100 ng/mL for 24h as well. The MTT assay was used to determine cardiomyoblasts viability. Alteration of the mitochondrial membrane potential, specific for apoptotic cells, was evaluated with caspase-3 activity assay kit.
Results. Our results showed that AME significantly inhibited TNF-α induced apoptosis (IC_{50} = 55.84 μg/mL) and cytotoxicity in H9c2 cells. Significant inhibition of apoptosis was observed in all tested concentrations of AME. The highest anti-apoptotic and cytoprotective effect was observed at the highest concentration (50 μg/mL), while in lower the concentrations cytoprotective effect was statistically insignificant.
Conclusions. Polyphenol-rich AME exhibits anti-apoptotic and cytoprotective effect in H9c2 cardiomyoblasts treated with TNF-α. Further studies are required in context of its possible application in prevention and/or therapy of cardiovascular diseases.

Keywords: *Aronia melanocarpa*, plant extract, H9c2 cardiomyoblasts, oxidative stress, apoptosis, cardiovascular disease.

Introduction

The cardiovascular diseases (CVD) are a major cause of death worldwide (World Health Organization, 2014). The prevailing view is that oxidative stress may play a crucial role in CVD development and progression [1]. It is postulated that increased expression of proinflammatory cytokine TNF-α in cardiomyocytes can have an important impact on induction of oxidative stress in myocardium. Hence, it may lead to increased apoptosis of cardiomyocytes and endothelial cells, further ventricular remodeling, down-regulation of myocardial contractility, that results in chronic heart failure, wherein the intensity of those alterations depends on TNF-α expression level [2, 3]. Therefore, researchers are looking for a new compounds that can be used in prevention or/and treatment of cardiovascular diseases associated with oxidative stress.

Polyphenols are naturally occurring antioxidants highly effective in scavenging of free radicals (FR) and reactive oxygen species (ROS) [4, 5]. They are commonly found in food products like: fruits, vegetables, legumes or red wine, green and black tea [5]. One of the richest source of plant-derived polyphenols are berries of *Aronia melanocarpa* (Michx.) Elliott (Black Chokeberry).

Researchers reported numerous beneficial health properties of aronia products including anti-inflam-

matory and cardio-protective activity [4, 6 ,7]. Several studies have also indicated cytoprotective and anti-apoptotic activity of aronia extract, but the exact mechanism is still unclear [8–10]. The reliable experimental in vitro model of human myocardial cells widely used for evaluation of antioxidant and anti-apoptotic effect of a number of plant extracts and plant-derived antioxidants in conditions of induced apoptosis is the cardiomyoblasts cell line – H9c2 [10–14]. Therefore, in our study, we treated H9c2 cells with TNF-α to induce oxidative stress-mediated apoptosis in vitro. The impact of polyphenol-rich AME – Aronox on TNF-α induced apoptosis was investigated.

Material and Methods

We used a natural, polyphenol-rich plant extract derived from berries of *Aronia melanocarpa* – Aronox (Adamed Ltd, Czosnów, Poland) containing polyphenols 60% w/w, including at least 20% w/w of anthocyanins, according to manufacturer's data. High-performance liquid chromatography (HPLC) analysis showed that total concentration of phenolics in the Aronox extract was 309.6 mg/g wherein concentration of phenolic acids (isomers of chlorogenic acid) was 149.2 mg/g, anthocyanins (anthocyanin glycosides: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xyloside) was 110.7 mg/g and flavonoids (quercetin glycosides) – 49.7 mg/g of extract [15].

The adherent cardiomyoblast cell line H9c2 primarily derived from embrionic rat heart (Sigma Aldrich, St. Louis, MO, USA) was cultured in Dulbecco Modified Eagle Medium (DMEM, Sigma Aldrich, St. Louis, MO, USA), containing 4500 mg/L glucose and L-glutamine, supplemented with 10% v/v fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) and antibiotic-antimycotic solution (AAS, Sigma Aldrich, St. Louis, MO, USA). The cell culture was maintained in monolayer on standard Petri dishes (Becton Dickinson, East Rutherford, NJ, USA) at 37°C, 5% CO_2 in a fully humidified atmosphere. The culture medium was replaced by fresh medium every 2 days. In our experiment we used cells from passages 4 ± 2. Cardiomyoblasts were preincubated with 10, 20, 40, 50 µg/mL of Aronox dissolved in Phosphate Buffered Saline (PBS, Biomed-Lublin, Lublin, Poland) for 24h, and subsequently treated with 100 ng/mL of TNF-α for 24h. Three control groups have been performed (including negative control group): after 24h of incubation without additives cells were treated with 50 µg/mL of Aronox,100 ng/mL of TNF-α for 24h, and a negative control group was incubated without any additives for 48h.

Cells viability was evaluated with the MTT Cell Proliferation Assay Kit (Biotium, Hayward, CA, USA) based on their metabolic activity status, while alterations of the mitochondrial membrane potential related with cells apoptosis were evaluated using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Mountain View, CA, USA).

Statistical analysis

All experiments were repeated at least three times. Data were analyzed using R Project software version 2.15.1 (www.r-project.org). The continuous variables were expressed as means ± SD. Data comparison was executed by the one sample Student's T-test, one-way ANOVA and post-hoc Tukey HSD test for one-way ANOVA. The statistical significance was adopted at p-value < 0.05.

RESULTS

Inhibition of H9c2 cells cytotoxicity induced by TNF-α

Data collected from the MTT cell viability assay are shown as means ± SD, lower absorbance values are related with reduced metabolitic activity of H9c2 cells (**Figure 1**). Viability of H9c2 cells preincubated with 10–50 µg/mL AME and treated with TNF-α were evaluated in comparison to 50 µg/mL AME control group.

Pretreatment with AME significantly inhibited cytotoxicity induced by TNF-α ($p = 0.04652$, T-test; $p = 0.00454$, ANOVA). The strongest cytoprotective effect of AME was observed in its highest concentration of 50 µg/mL ($p = 0.00630$). There was no significant difference in viability of cells preincubated with 10, 20 µg/mL of AME and and AME control samples. In concentration 40 µg/mL, cytoprotective effect of AME was low and also statistically insignificant. Surprisingly, we have found that H9c2 cells viability was decreased in control samples treated with 50µg/mL of AME only, that may be caused by the lower initial H9c2 cell count in some evaluated samples.

The obtained values of relative cell viability, presented in **Figure 2** as a percentage relative to the untreated control cells, showed protective effect of AME in H9c2 cells in conditions of induced apoptosis. Obviously, cytoprotective effect was observed in samples preincubated with AME in concentration higher than 20 µg/mL.

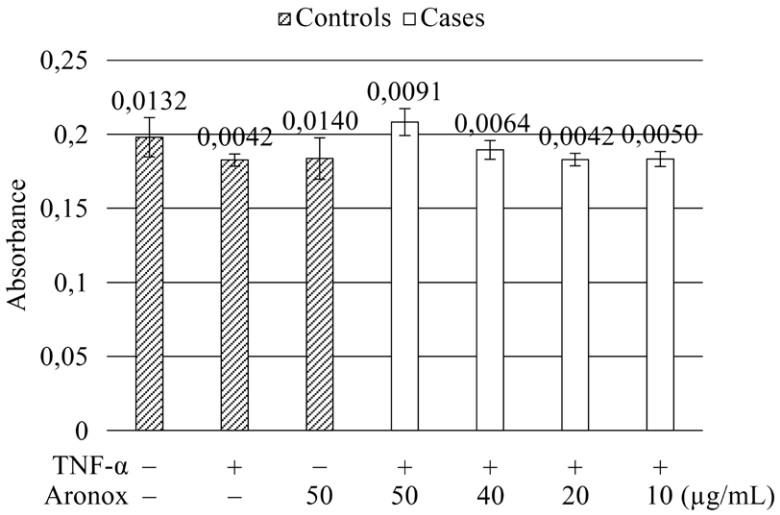

Figure 1. Cytoprotective effect of Aronia melanocarpa extract (Aronox: 10, 20, 40, 50 µg/mL) in H9c2 cells treated with 100 ng/mL TNF-α

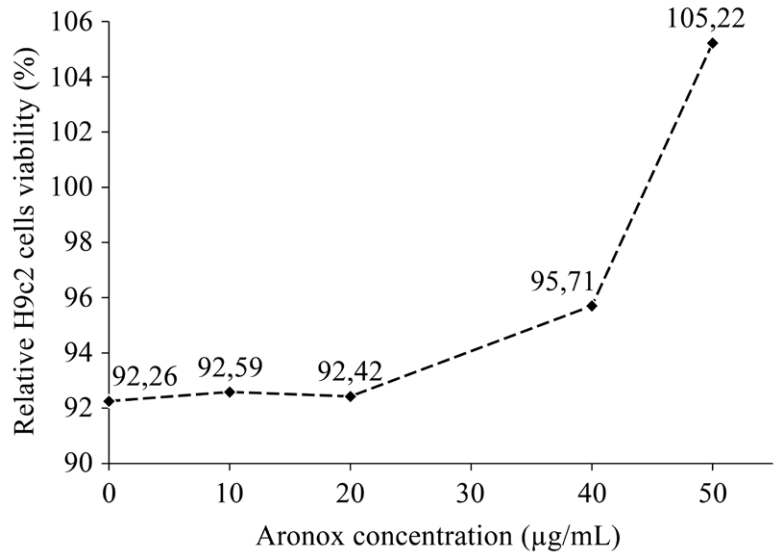

Figure 2. Concentration-dependent cytoprotective effect of Aronia melanocarpa extract (Aronox) in H9c2 cells treated with 100 ng/mL TNF-α

Anti-apoptotic activity of AME extract in H9c2 cells induced by TNF-α

Analysis of the caspase-3 activity in H9c2 cells preincubated with AME and treated with TNF-α. showed anti-apoptotic activity of AME, regarding to control samples Results shown in **Figure 3** are presented as mean absorbance value ± SD, where lower absorbance values were related with decreased apoptotic cells ratio. Apoptotic cells count was significantly lower in case samples (10–50 µg/mL AME; 100 ng/mL TNF-α) compared to AME treated controls ($p = 4.146x10^{-14}$, T-test; p = 0.0012, ANOVA). Statistical analysis revealed that AME in all tested concentrations significantly inhibited TNF-α induced H9c2 cells apoptosis, wherein the strongest anti-apoptotic effect was observed in the highest applied concentration of AME 50 µg/mL (p = 0.00049, Tukey HSD). The caspase-3 activity was comparable between control negative samples and controls incubated with 50 µg/mL of extract, thus AME in its maximal concentration did not induce H9c2 cells apoptosis.

The inhibition of apoptosis (in %) was presented on the basis of caspase-3 activity measurements. We found that AME inhibited apoptosis induced by TNF-α starting with its lowest tested concentration (10 µg/mL) (**Figure 4**).

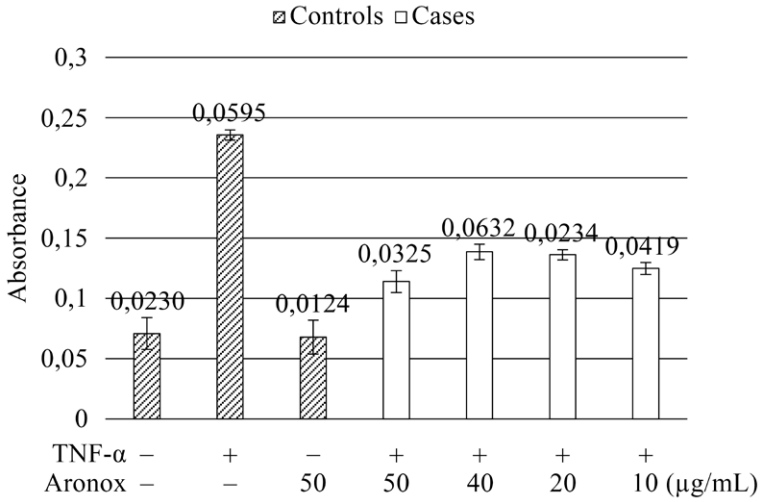

Figure 3. Anti-apoptotic effect of Aronia melanocarpa extract (10, 20, 40, 50 µg/mL) in H9c2 cells treated with 100 ng/mL TNF-α

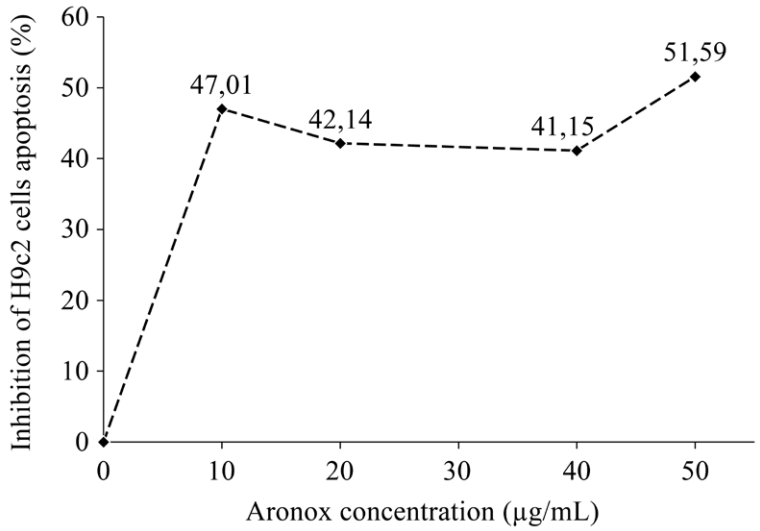

Figure 4. Inhibition of TNF-α (100 ng/mL) induced apoptosis in H9c2 cells preincubated with Aronia melanocarpa extract (10, 20, 40, 50 µg/mL)

The estimated value of half maximal inhibitory concentration (IC50) for AME in conditions of TNF-α induced H9c2 cell apoptosis was 55.84 µg/mL.

Discussion

According to the epidemiological data in group of non-communicable diseases, the cardiovascular diseases (CVD) are the major cause of death in developed countries [16, 17]. It is estimated that annual CVD mortality will rise rapidly from 17.5 mln in 2008 to 22.2 mln in 2030 [16]. These facts contributed to intense research for a new compounds that could be used in prevention and treatment of CVD. Ones of the most promising group of nutrients identified as possibly cardioprotective are plant derived antioxidants – polyphenols, commonly found in *Aronia melanocarpa* berries [4, 7].

Study performed by Olivetti *et al.* on postmortem samples of human hearts revealed the presence of apoptotic cells in „border zone" of myocardium during acute myocardial infarction. Therefore, apoptosis appears to be a significant factor involved in cardiomyocytes loss in the post-infarcted heart [18]. Additionally, it is suggested that apoptosis may lead to post-infarct ventricular remodeling and development of heart failure [19]. Thereby, a crucial issue in myocardial infarction treatment is prevention of myocardial cell loss as it is an important determinant of patients morbidity and mortality. In most cases of chronic heart failure the cardiomyocytes apoptosis incidence is low, however its

long-term deleterious effects on myocardium and participation in the loss of cardiac cells may be a clinically significant factor for these patients [20].

As was already mentioned, important role in both: induction of cardiomyocytes and endothelial cells apoptosis, and development of myocardium structural-functional alterations plays an increased expression of TNF-α in cardiac myocytes in certain pathological conditions [2, 3]. The key role in programmed cell death induced by TNF-α plays the increased mitochondrial ROS generation. This specific mechanism is still not fully understood, however, Kim *et al.* suggested that mitochondrial ROS modulator 1 (Romo1) may be involved in TNF-α dependent induction of mitochondrial ROS generation [21]. Thus, it can be considered that inhibition of TNF-α induced intracellular oxidative stress and following cardiomyocytes apoptosis by antioxidant compounds (polyphenols) found e.g. in the AME could find a practical application in prevention and treatment of heart failure in a group of patients with CVD.

We studied the effect of polyphenols from AME on TNF-α induced apoptosis in H9c2 cardiomyoblasts. In our study the cytoprotective and anti-apoptotic effect of AME (Aronox) on H9c2 cells under exposure to TNF-α was observed. This result confirmes other reports about AME and plant-derived polyphenolic compound effect on a different cell lines under conditions of induced oxidative stress. In 2012, Zapolska-Downar *et al.* reported dose-dependent cytoprotective effect of Aronox in TNF-a treated human aortic endothelial cells (HAEC), associated with inhibition of intracellular ROS production [8]. It confirms our observations of increased viability of H9c2 cells preincubated with AME compared to untreated controls under conditions of TNF-α induced apoptosis and its dependence on the AME dosage. Moreover, earlier study by Zapolska-Downar *et al.* also confirms our observation of AME anti-apoptotic activity based on reduced caspase-3 activity in case samples regarding to controls [9]. Our findings are consistent with results by Angeloni *et al.* as well. Their study showed that quercetin, the glycosides of which are present in AME, reduces intracellular ROS synthesis, prevents oxidative cell damage and inhibits caspase-3 activation in cardiomyoblasts treated with H_2O_2. On the other hand, O-methylated quercetin metabolites appear to inhibit intracellular generation of ROS but do not affect cells viability nor caspase-3 activation [10]. Recently, the anti-apoptotic activity of polyphenols (epigallocatechin gallate) in H9c2 cells treated with H_2O_2 has also been reported [14]. Other researchers also show the protective effect of plant polyphenols (cyanidin-3-O-β-glucoside) on oxidative stress-induced apoptosis in different cell lines: human umbilical vein endothelial cell (HUVEC), liver hepatocellular carcinoma (HepG2) and mouse insulin-producing pancreatic β-cells (MIN6N) [22–24].

Additionally, existing reports from clinical trials prove the beneficial effects of polyphenols from *Aronia melanocarpa* on the overall condition of the cardiovascular system in vivo. Naruszewicz *et al.* evaluated the clinical utility of statins-flavonoids combined therapy for patients with hypercholesterolemia following acute myocardial infarction. Their study revealed several beneficial effect of flavonoids supplementation e.g. reduced blood pressure, decreased serum levels of C-reactive protein (CRP), cell adhesion molecules – glycoprotein VCAM and immunoglobulin ICAM and oxidized low density lipoprotein (ox-LDL) [6]. In later work, Naruszewicz *et al.* described the decrease in the serum level of interleukin 6 (IL-6) and monocyte chemoattractant protein (MCP-1) and an increase in adiponectin level in a group of patients following myocardial infarction after 6-week supplementation with Aronox regarding to placebo group [7].

However, considering the possible clinical applications of plant polyphenols it should be mentioned that they may also exhibit pro-oxidative and pro-apoptotic activities. It was reported that it may depends e.g. on their applied concentration. According to Watjen *et al.*, some polyphenols (flavonoids) may be cytotoxic or cytoprotective to the hepatoma cells (H4IIE) treated with H_2O_2 depending on their concentration in culture medium [25]. It is reported that compounds contained in *Aronia melanocarpa* berries show selective pro-apoptotic activity in leukemic cells, leaving regular T-cells unaffected [26]. Thus, the cited researches confirm the general view of the selective cytotoxic activity of polyphenolic compounds that do not affect the regular cells viability. Yet, in our study we observed reduction of H9c2 cells viability in control samples incubated with AME at its highest tested concentration.

In fact, this study seems to confirm anti-apoptotic activity of polyphenol-rich AME in H9c2 cardiomyoblast treated with TNF-α. However, it should be considered that in vivo animal/human studies revealed low bioavailability and decline in antioxidant activity of polyphenol compounds after oral ingestion [4, 27–30]. For this reason, it is postulated that in vitro studies metabolites of these compounds should be applied, rather than their native forms present in plant products [29]. It also should be noted that despite the generally low bioavailability of polyphenols, anthocya-

nins are determined in the peripheral blood plasma in the native form (as glycosides) [30]. Regarding to low bioavailability of orally ingested polyphenols, and the fact that in cardiovascular system they are circulating generally in metabolized form additional in vitro studies of polyphenol metabolites are required.

Our results confirm the available literature data and provide evidence on anti-apoptotic activity of *Aronia melanocarpa* derived polyphenols on H9c2 cardiomyoblasts in conditions of TNF-α induced apoptosis**.** However, further studies with higher concentrations of aronia extract are needed to evaluate its possible cytotoxic effect. An in-depth understanding of molecular mechanism underlying the anti-apoptotic activity of polyphenols in cells like i.a. cardiomyocytes as well as further in vivo studies would be useful in context of their possible application to clinical practice for patients with cardiovascular system diseases.

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Conflict of interest statement
The authors declare no conflict of interest.

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