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ORIGINAL ARTICLE

Experimental Models for Investigation of Antioxidant and Immunomodulatory Effects *in Vitro* of *Aronia melanocarpa* Extract

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ABSTRACT
Aronia melanocarpa (Black chokeberry) has shown a high content of polyphenol compounds and possesses one of the highest antioxidant activities among fruits. On the other hand, the therapeutic value of one of the most effective the chemotherapeutic agents, Doxorubicin (DOX), is limited by its toxicity. Although the mechanisms of this toxicity are not fully elucidated, oxidative stress appears to be involved. In this aspect, the current study was directed to investigation on the possible protective effect of the total extract from this medicinal plant against DOX-induced toxicity and oxidative stress in vitro. Despite of the established decrease of the reduced Glutathione (GSH) levels in normal cells and mixed cultures in the presence of Doxorubicin, certain regeneration in them was noticed in the presence of Aronia-extract. Slight differences in cell morphology in the presence of plant extract, expressed mainly in increased size, rounded cell form and changed nuclei/cytoplasm ratio, in comparison with the untreated controls and cells, treated by Doxorubicin alone, were observed. Those changes were accepted as signs for early myeloid differentiation, probably mainly of embryonic stem cell sub-populations from 3T3 cell line. Further studies on the influence of separate plant components on the levels of intracellular GSH would be necessary.
Keywords: *Aronia melanocarpa total extract, Doxorubicin, GSH levels, normal cells, malignant cells.*

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INTRODUCTION

Medicinal plant *Aronia melanocarpa* has become popular in many countries all over the world not only with its valuable food qualities, but also as a therapeutic and prophylactic supplement [1-3]. As a rich source of polyphenols and anthocyanins, containing in it, the extract of this plant has been applied as a natural anti-hypertensive and anti-atherosclerotic drug [1], but also as anti-cancer, anti-oxidant and chemo-protective agent [4-7]. In a recent study, the protective action of chokeberry extract against oxidative stress induced by high doses of glucose in pancreatic cells has also been evaluated [8]. Their results indicated a strong scavenging effect of chokeberry anthocyanins on the intracellular ROS species and an ability to restore dose-dependently the strong decrease of GSH. According another message, the mechanism of the anthocyanin-mediated increase of GSH synthesis and protection of hepatocytes against ROS –induced injury has been proved [9].

Reduced glutathione (GSH) is a thiol-containing tripeptide (L-γglutamil-L-cysteinyl-glycine), which is ubiquitous in the cells. The activity of hydrosulfide group determines the biological significance and activity of GSH in antioxidant and detoxifying reactions [10]. This substance is responsible for keeping proper thiol-disulfide balance and related redox-potential in the cells. Moreover, the nucleophilic glutathione –SH group enters reactions with electrophilic substances, either endogenous or exogenous (xenobiotics, including drugs), yielding glutathione S-conjugates, (i. e GSH thioeters), which are then transformed to mercapturic acids and excreted [11]. Thus, the availability of GSH is crucial for antioxidant defense in a biological system. GSH deficit disrupts the redox-status and upsets the physiological cellular

balance between pro-oxidants and antioxidants [12]. Lowered cellular GSH is observed in different pathological conditions (pre-malignancies and malignancies, inflammations, Parkinson's disease, AIDS, diabetes and others), Thus, GSH modulation could represent a supportive measure to achieve a therapeutic goal.

In the current study, we hypothesized that *Aronia melanocarpa* total extract is connected with investigation on some of the mechanisms of the *in vitro*-influence of the *Aronia*-extract on laboratory-cultivated normal and malignant cells, as well as mixed cultures of both cell types.

MATERIALS AND METHODS

Normal fibroblasts from embryonic mouse Balb/c 3T3 line, malignant mouse myeloma cells, as well as mixed cultures from both cell types, were prepared. All cell cultures (1 x 10^6 cells/ml), were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in RPMI 1640, Dulbecco's Modified Minimal Essential Medium (DMEM) or a mix of both media, supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B, in 24-well plaques. In separate sub-populations from each of both cell types used, as well as in mixed cultures of them, were added total *Aronia melanocarpa* plant; 0.05M solution of Doxorubicin in distilled water, as well as with both tested substances, but respective untreated controls were also prepared. The so prepared cell cultures were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

Cells from all groups, treated with both substances, with each one of them, as well as the respective untreated controls, treated with 10% trichloroacetic acid (Cl_3CCOOH) and 0.48M solution of K_3PO_4. GSH levels in the three organs of all four groups experimental animals were determined by a spectrophotometric method and absorbances were measured at 412nm [13]. The level of GSH was defined from the standard curve with commercially available GSH and the results are expressed as milimole per 1 ml cell suspension (mM/ml cell suspension).

Fixed light microscopic slides from cultures of both normal cells 3T3 and of mixed cultures of both cell types, treated by Doxorubicin alone, by *Aronia*-extract alone, by both substances, as well as untreated controls were prepared by fixation with 95% Ethanol, washing with PBS and subsequent staining by Giemsa dye. For the same goal, suspension cell cultures used (from malignant myeloma cells and mixed of both cell types) were seeded on substrates, previously-treated by Gelatin-solution in PBS. The so prepared fixed preparations were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

RESULTS AND DISCUSSION

According the results obtained, drastic decrease in the levels of GSH in normal embryonic fibroblasts, mouse malignant myeloma cells, as well as in the mixed cultures in all cases in cultivation with the chemotherapeutic drug alone were noticed (Figure 1). The strongest influence of the chemotherapeutic agent was observed in the cultures of normal cells, which proved their highest sensitivity to it. In the cultures of malignant cells, after addition of the plant extract the strongest increase in the GSH levels was observed (Figure 1).

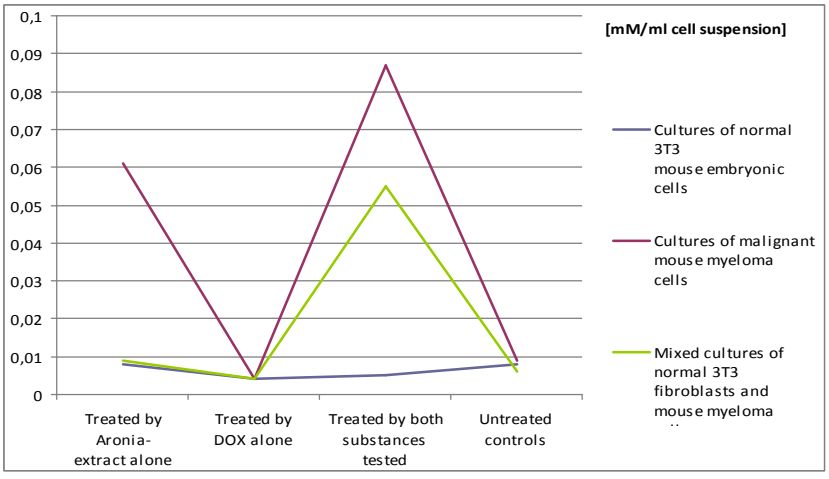

Fig. 1: Influence of Doxorubicin and *Aronia melanocarpa* total extract on the intracellular GSH levels [mM/ml cell suspension]: in cultures of normal embryonic fibroblasts; in cultures of mouse malignant myeloma cells; in mixed cultures of normal and malignant cells.

The levels observed were partially restored in the presence of *Aronia*-extract and chemotherapeutic drug. Similar effects have been observed in investigation of other plant extracts or their components [7, 15-18]. On the other hand, analogical protective action by natural plant antioxidants has been established on the toxicity, induced by Doxorubicin [19], but also by other chemical drugs [20-22].
Despite significant differences in the morphology of the untreated normal mouse embryonic cells and treated by Doxorubicin alone, by *Aronia*-extract alone and with both substances were not noticed, some changes in the normal cells were observed in the cases of chemotherapeutic drug pre-treatment (Figure 2).

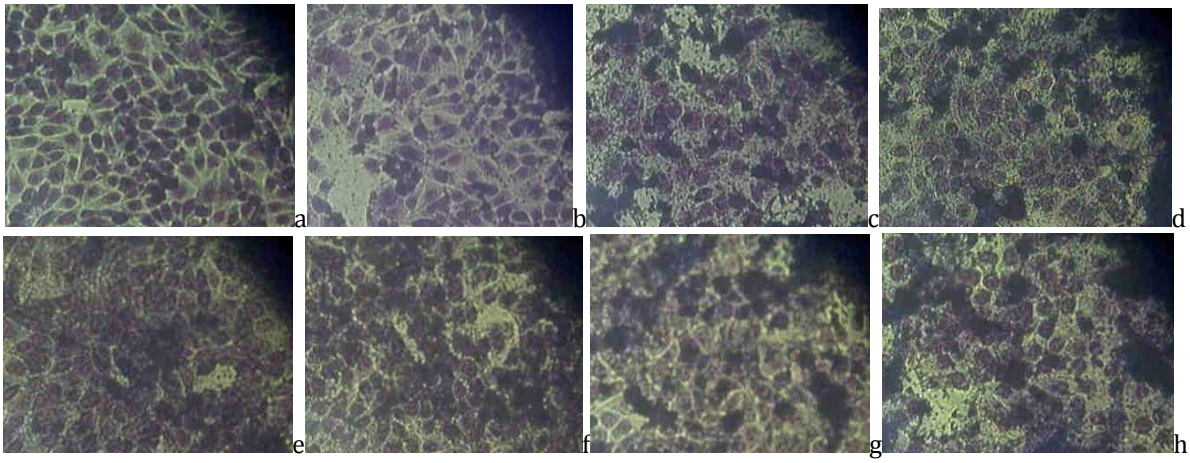

Fig. 2: Influence of Doxorubicin and *Aronia melanocarpa* total extract on the morphology, proliferation capacity and differentiation of normal and malignant cells: a) untreated control culture of normal 3T3 fibroblasts from mouse embryos; b) 3T3 cells, treated by Doxorubicin alone; c) 3T3 fibroblasts, treated by *Aronia melanocarpa* total extract alone; d) 3T3 cells, treated by both Doxorubicin and *Aronia*-extract; e) Untreated mixed culture of normal 3T3 fibroblasts and mouse myeloma cells; f) Mixed culture of normal 3T3 and mouse myeloma cells, treated by Doxorubicin alone; g) Mixed culture of normal 3T3 fibroblasts and mouse myeloma cells, treated by *Aronia melanocarpa* total extract alone; h) Mixed cell cultures, treated by both Doxorubicin and *Aronia*-extract.

Certain decrease in cell density could be noticed both in 3T3 normal embryonic cells (Figure 2 - b) and in the mixed cultures (Figure 2 - f). In the presence of plant extract (Figure 2 – c, d), of malignant cells (Figure 2 – e-h), as well as of both (Figure 2 – g, h), normal embryonic cells increase in size, acquire rounded form, but also centrally-located dark-stained nuclei and changed nuclei/cytoplasm ratio could be observed, in comparison with the untreated controls (Figure 2 - a) and with the cells, treated by Doxorubicin alone (Figure 2 - b). In the presence of *Aronia*-extract, besides the described above differences, light-stained cytoplasmic content was also noticed, which was the strongest in mixed cultures, pre-treated with the plant content (Figure 2 - g).
The data obtained were in agreement with the cited literature sources, concerning similar cultivation conditions of immature normal cells [23, 24]. In this way, the noticed morphological changes in the normal cells could be accepted as primary signs of early myeloid differentiation, probably in particular of embryonic stem cell sub-populations from the entire 3T3 cell line. In the presence of plant extract, those features could be presumably explained with GSH contribution. These results also supported literature data, connected with the influence of *Aronia* ingredients on the neutrophil differentiation, probably by intracellular antioxidant mechanisms [24-26].

CONCLUSIONS

Taking in consideration the results obtained in the current study, as well as respective literature data, further studies, directed to investigation on the immunomodulatory influence of *Aronia*-extract, but also the effects of its separate antioxidant components (polyphenols and anthocyanins) on the levels of intracellular GSH *in vitro*, should be provided. Also, future investigations on glutathione-dependent enzymes are necessary.

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