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Chemoprotective, antioxidant and immunomodulatory *in vitro* effects of *Aronia melanocarpa* total extract on laboratory-cultivated normal and malignant cells

ABSTRACT

Chemoprotective influence of *Aronia melanocarpa* total extract on the side effects of the commercial chemotherapeutic drug Doxorubicin on laboratory-cultivated normal and malignant cells was studied. For this goal, morphological changes in both used cell types separately and in mixed cultures of them in the presence and absence of Doxorubicin; of *Aronia*-extract, were investigated. One of the main mechanisms of *Aronia* polyphenols and anthocyanins action has been supposed to be by increase of the reduced Glutathione (GSH) intracellular levels. According our results, strong cytotoxic effect of Doxorubicin on *in vitro*-cultivated normal and malignant cells was observed, but in the presence of *Aronia*-extract, regeneration in the vitality and even in the proliferation capacity of both cell types was indicated. Furthermore, a strong myeloid cell differentiation of both normal and malignant cells was indicated by the *Aronia*-extract, which was strongest in normal cells, co-cultivated with myeloma cells, independently of Doxorubicin presence or absence. The observed contradictory results in GSH levels regeneration in the presence of *Aronia*-extract could be explained with a partial depletion of intracellular GSH in the concrete time of experiment, probably on the basis of feed-back principal of regulation, despite the eventual previous increase in its levels on the influence of *Aronia*-extract, in agreement with the respective literature data. Thus, further studies, directed to investigation on the influence of the total *Aronia*-extract, but also of its separate components (polyphenols and anthocyanins) on the levels of intracellular GSH, should be necessary, as well as, on the other hand, on the influence of intermediate cell components as GSH, on the processes of cell growth and proliferation by cascade regulatory pathways, are necessary.

Key words: *Aronia*-extract, Doxorubicin, normal cells, malignant cells, GSH, cascade regulatory pathways

Introduction

In XX century, *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 (Hovmaln et al., 2004; Scott et al., 2007; Kulling & Rawel, 2008; Kokotkiewicz et al., 2010; Sharif et al., 2012). It has been applied as a natural anti-hypertensive and anti-atherosclerotic drug (Domarew et al., 2002), but also as anti-cancer, anti-oxidant and

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chemoprotective agent, mainly by the polyphenols and anthocyanins, containing in it (Kähkönen et al., 1999; Zdunczyk et al., 2002; Kong et al., 2003; Malik et al., 2003; Zhao et al., 2004; Zielińska-Przyjemska et al., 2007; Wang & Stoner, 2008; Olas et al., 2010; McDonald et al., 2012). As the most important constituents in *Aronia* have been characterized polyphenols, mainly presenting pro-cyanidins (Oszmianski & Wojdyło, 2005) and anthocyanins, presented mainly of cyaniding glycosides (Jakobek et al., 2007). As one of the main mechanisms of *Aronia* polyphenols and anthocyanins action have been supposed to be by the mechanism of elevation in intracellular levels of the reduced Glutathione (GSH) (Rouse et al., 1995; Wu et al., 2004; Alexieva et al., 2009; Attia et al., 2010; Kokotkiewicz et al., 2010). GSH oxidation, in particular, of mitochondrial GSH, has been found to favor opening of the mitochondrial permeability transition pore complex, facilitating in this way the release of death-related molecular signals (Ortega et al., 2011). Moreover, GSH has also been established to be involved in regulation of different types of cancer cell death, including autophagy, besides necrosis and apoptosis. Suggestion about pro-inflammatory activation by the influence of NF- κ B on the expression of appropriate genes in neutrophils has also been obtained (El Benna et al., 2002; McDonald et al., 2002; Cloutier et al., 2007). On the other hand, however, significant inhibition of oxidative metabolism in activated by *Aronia*-extract neutrophils *in vitro* influences has been established (Zielińska-Przyjemska et al., 2007; Kokotkiewicz et al., 2010). TGF- β signals have been found to up-regulate Wnt5A expression directly through the Smad-complex, as well as through Smad-induced CUX1 and MAP3K7-mediated NF- κ B (Kohl et al., 2000; Reya & Clevers, 2005; Cloutier et al., 2007; Katoh & Katoh, 2009; Dayem et al., 2010). The established conserved NF- κ B-binding site within the Wnt5A promoter B region has elucidated the up-regulation mechanisms on Wnt via MAP3K7 by the influence of TNF- α and toll-like receptor (TLR) signals. Taken together these data have proposed eventual transcription of Wnt5A on the basis of multiple mechanisms, such as NF- κ B-, Hedgehog-, TGF- β - and Notch-signaling cascade pathways (Katoh & Katoh, 2009). These cascade regulatory pathways have been pointed as key in the mechanism of oxidative stress influence on the regulation of stem, cancer and cancer stem cells (Dayem et al., 2010). As a main pathway in the regulation of precursors processing of into active proteins has been characterized the

ubiquitin-proteasome mechanism, besides its role in the complete degradation of polypeptides (Palombella et al., 1994; Jahngen-Hodge et al., 1997). Increased levels of Cyclin D1 protein have been induced by B2-catenin over-expression and reduced - in cells, over-expressing the cadherin cytoplasmic domain, respectively (Shtutman et al., 1999). In this way, a mechanism about promoted neoplastic conversion by triggering cyclin D1 gene expression and, consequently, uncontrolled progression into the cell cycle on the influence of increased B2-catenin levels has been proposed. Wnt-cascade signaling pathway has been emerged as a critical in the regulation of self-renewal and proliferation in both normal and malignant stem and progenitor cells (Oszmianski & Wojdyło, 2005). In summarize of the link between oxidative stress, different signaling pathways, on the one hand, as well as carcinogenesis, on the other, as most important components have been characterized proteins from MAPK family and NF- κ B (Dayem et al., 2010; Sharif et al., 2012). Moreover, both MAPK- and NF- κ B-cascade pathways have been proposed to be essential in the redox-status and the development of cancer stem cells. A close link between the cellular self-catabolic process of autophagy and the programmed cell death – apoptosis, as physiological phenomena, has also been established. The relation of autophagy to tumorigenesis is complex and depends on the genetic composition of cells as well as on the extra-cellular stresses which a cell is exposed to. In this way, the relationship between oxidative stress and both apoptosis and autophagy might be crucial in cancer stem cell development, as well as in development of therapeutic approaches. Thus, the relationship between oxidative stress and both apoptosis and autophagy might be crucial in cancer stem cell development, as well as in development of therapeutic approaches. In this way, GSH has been proved to be involved in many pathological and physiological alterations (Favilli et al., 1997).

Low clinical response rates of low grade liposarcoma to the chemotherapeutic drug Doxorubicin have been shown, presented by little changes on gene expression level, as well as by divergent findings concerning the up- and down-regulation of single genes in the different malignancy samples (Sonneveld et al., 1981; Tidelfelt et al., 1991; Daigeler et al., 2010). Doxorubicin, as well as the other chemotherapeutic agents, have been found to decrease significantly the intracellular GSH levels (Khyriam & Prasad, 2001, 2003; Alexieva et al., 2009).

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In this aspect, the main goal of the current study was connected with investigation on the chemoprotective *in vitro*-influence of *Aronia melanocarpa* total extract on the side effects of the commercial chemotherapeutic drug Doxorubicin on laboratory-cultivated normal and malignant cells.

Materials and Methods

Cells

- 1) Normal cells: fibroblasts from embryonic mouse Balb/c 3T3 cell line;
- 2) Malignant cells: cells from murine myeloma line Ag853;

Chemicals

- 1) Cultivation media: Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich), RPMI1640 (Sigma-Aldrich);
- 2) Serum: 10% Fetal Calf Serum (FCS) (Sigma-Aldrich);
- 3) Antibiotics: 100 U/ml Penicillin (Sigma-Aldrich), 100 µg/ml Streptomycin (Sigma-Aldrich), 2 µg/ml Doxycyclin diluted in PBS (Sigma-Aldrich);
- 4) 0.5M and 0.1M solutions of Doxorubicin (Sigma-Aldrich) in distilled water;
- 5) Total fruit extract of *Aronia melanocarpa* – separate components:
- 6) Phosphate Buffered Solution (PBS), pH 8 (Sigma-Aldrich);
- 7) Laboratory solution of 0.02% Trypsin and 0.05% EDTA (Sigma-Aldrich);
- 8) Solution of Gelatin (Sigma-Aldrich) in PBS (Sigma-Aldrich) and subsequently sterilized by heating;

- 9) Ethanol - 95% (Sigma-Aldrich);
- 10) Haematoxylin/Eosin mixture (Sigma-Aldrich);
- 11) Giemsa stain (Sigma-Aldrich);
- 12) Tribasic potassium phosphate (K₃PO₄) - 0.48 M (Sigma-Aldrich);
- 13) Trichloroacetic acid - Cl₃CCOOH - 10% (Sigma-Aldrich);

Installations

- 1) Inverted light microscope with CCD-camera;
- 2) Eppendorf micro-centrifuge tubes.

*The data in Tables 1-4 were computed and received at Varna University of Medicine, Bulgaria, from where were kindly committed to us.

Table 1. Polyphenol content of *Aronia* juice.

	Total polyphenols, mg GAE/l
<i>Aronia</i> juice	5461

Table 2. Content of phenolic compounds in *Aronia* juice.

	Neochlorogenic acid, mg/l	Chlorogenic acid, mg/l
<i>Aronia</i> juice	830	585

Table 3. Content of anthocyanins in *Aronia* juice.

	Cyanidin-3- galactoside, mg/l	Cyanidin-3- glucoside, mg/l	Cyanidin-3- arabinoside, mg/l	Cyanidin-3- xyloside, mg/l	Total, mg/l
<i>Aronia</i> juice	143.7	4.4	61.7	11.6	221.4

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Table 4. Total proanthocyanidin content in *Aronia* juice.

	Total proanthocyanidins, mg/l
<i>Aronia</i> juice	3122.5

All cells (5×10^6 cells/ml for both cell types used) were incubated at 37°C in incubator with 5% CO_2 and 95% air humidification, in RPMI1640 (Sigma-Aldrich), Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich) or a mix of both media, supplemented in all cases with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 100 U/ml Penicillin (Sigma-Aldrich) and 100 $\mu\text{g/ml}$ Streptomycin (Sigma-Aldrich), in 24-well plaques. In separate sub-populations from each of both cell lines used, as well as in mixed cultures of them, were subsequently added *Aronia*-extract; 0.5M or 0.1M solutions of Doxorubicin, respectively, as well as both tested substances in different orders. All native cell cultures prepared were observed by inverted light microscope (Leica).

Fixed light microscopic preparations were prepared by their consequent fixation by treatment with 95% Ethanol (Sigma-Aldrich), washing with PBS (Sigma-Aldrich) and subsequent staining with Hematoxylin-Eosin (Sigma-Aldrich) technique or Giemsa stain, respectively. For the same goal, suspension cell cultures used (from Ag853 malignant cell line and mixed of both cell types) were previously treated with Gelation-solution in PBS (Sigma-Aldrich).

For determination of GSH levels, Ellman's technique was applied (Ellman, 1959). For this goal, *in vitro*-cultivated cells from all tested groups were suspended, after which the so prepared cell suspensions were subsequently treated with equal volumes 10% trichloroacetic acid and centrifuged at 3000x for 10 minutes. After pick-up of the supernatants, equal volumes of 0.48M tribasic potassium phosphate solution were added to the pellets. GSH levels were defined from the extinction capacity about each respective probe in 412 nm (in which GSH is able to absorb the light) and calculated by differences in the established values from a standard curve. Statistical significance of differences was evaluated by using of Student's test, according which $P < 0.05$ was considered as a criterion for significance.

Results

A strong cytotoxic effect Doxorubicin in both concentrations on *in vitro*-cultivated normal and malignant cells was observed (Figure 1 – c, d; Figure 2 – c, d). In the presence of *Aronia*-extract, in all cases tendency to regeneration in the vitality and even in the proliferation capacity of both cell types was indicated (Figure 1 – e, f; Figure 2 – e, f). In *in vitro*-co-cultivation of non-treated normal cells with malignant cells in equal initial concentrations of cell suspensions for both cell types (5×10^6 cells/ml), a tendency of gradual elimination of the normal cells by the malignant was observed, independently of the presence or absence of Doxorubicin (Figure 1 – n, o; Figure 2 – n, o). However, in the influence of *Aronia*-extract, a strong myeloid cell differentiation of both normal and malignant cells was indicated (Figure 1 – e-k; Figure 2 – e-k), and it was the strongest in the normal cells in their co-cultivation with myeloma cells, both in presence and absence of Doxorubicin (Figure 1 – e-m; Figure 2 – e-m). Besides that, a tendency about decrease in GSH levels in the presence of Doxorubicin was established in both cell types (Table 5). Furthermore, higher levels of GSH in the malignant cells in comparison with the normal were established, which didn't depend if both cell types were treated or non-treated with any of both substances used. On the other hand, significant differences in the morphology of both cell types, as well as in the levels of GSH in them, as a result of *Aronia*-extract addition before and after both in higher and lower concentration of Doxorubicin, were not observed.

Discussion

The observed cytotoxic *in vitro*-effect of Doxorubicin in higher and lower concentrations applied on *in vitro*-cultivated normal and malignant cells (Figure 1 – c, d; Figure 2 – c, d) was in agreement with the literature data on this problem (Al-Abt et al., 2011; Daigeler et al., 2008; Sonneveld et al., 1981; Tidefelt et al., 1991). On the other hand, the noticed influence of *Aronia*-extract on the treated and non-treated cells from both types, as well as in mixed cultures from them, were also in confirmation of many references (Al-Abt et al., 2011; Emmert et al., Hakkak et al., 2001; Olas et al., 2010) and could be accepted for a positive to its resolution in some attitudes.

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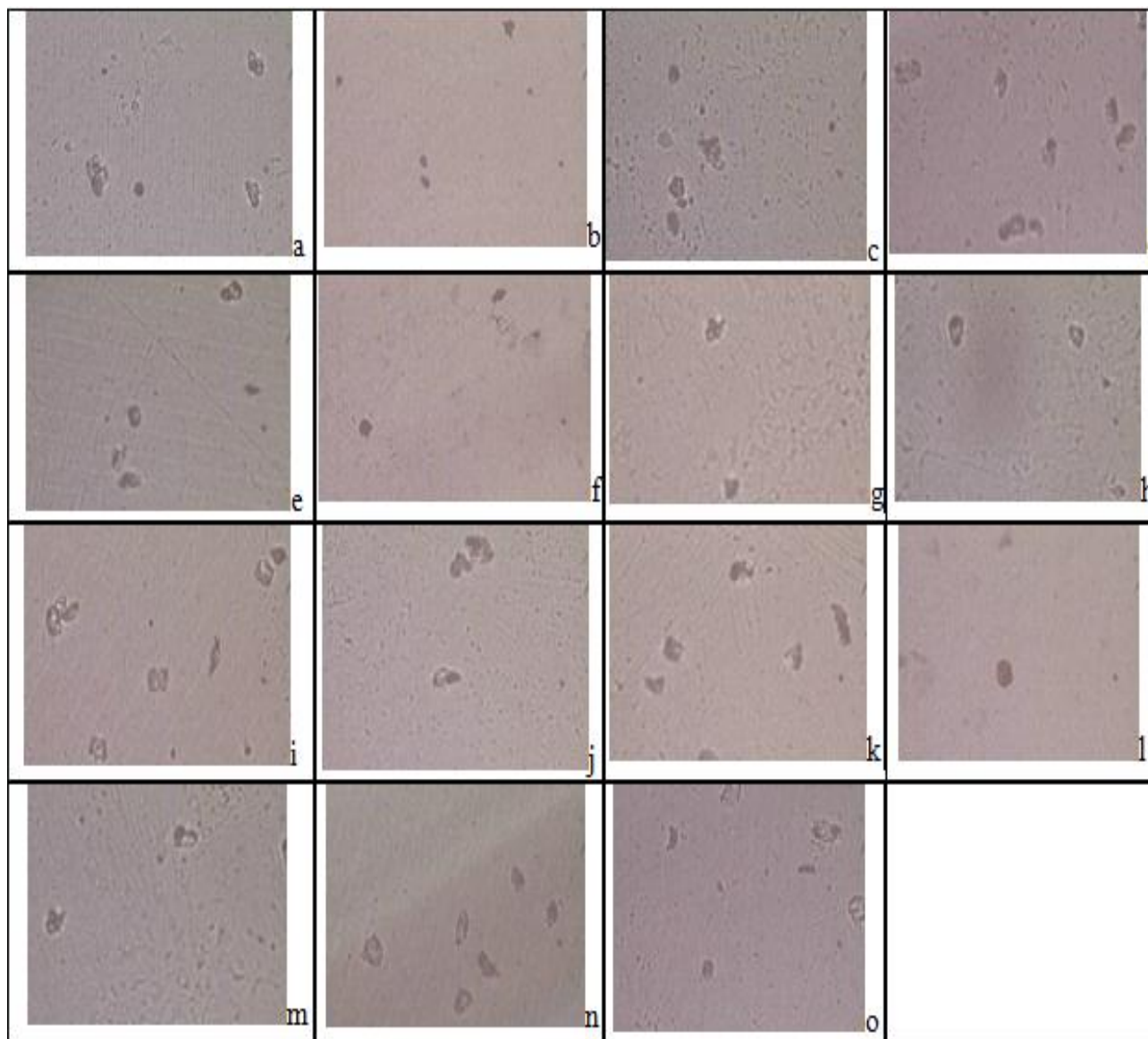


Figure 1. *In vitro*-cultivated normal Balb/c mouse embryonic fibroblasts 3T3 (a); *In vitro*-cultivated malignant cells from murine myeloma Ag853 cell line (b); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of 0.5M Doxorubicin (c); malignant Ag853 mouse myeloma cells, cultivated in the presence of 0.5M Doxorubicin (d); normal 3T3 mouse embryonic fibroblasts, cultivated in the presence of 0.5M Doxorubicin plus Aronia-extract (e); malignant mouse myeloma cells Ag853, cultivated in the presence of 0.5M Doxorubicin plus Aronia-extract (f); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of Aronia-extract plus 0.5M Doxorubicin (g); malignant Ag853 mouse myeloma cells, cultivated in the presence of Aronia-extract plus 0.5M Doxorubicin (h); co-cultivation of normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of 0.5M Doxorubicin plus Aronia-extract (i); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of Aronia-extract (j); co-cultivation normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of Aronia-extract plus 0.5M Doxorubicin (k); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of Aronia-extract (l); malignant mouse myeloma cells Ag853, cultivated in the presence of Aronia-extract (m); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 (n); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of 0.5M Doxorubicin (o), (H&E staining).

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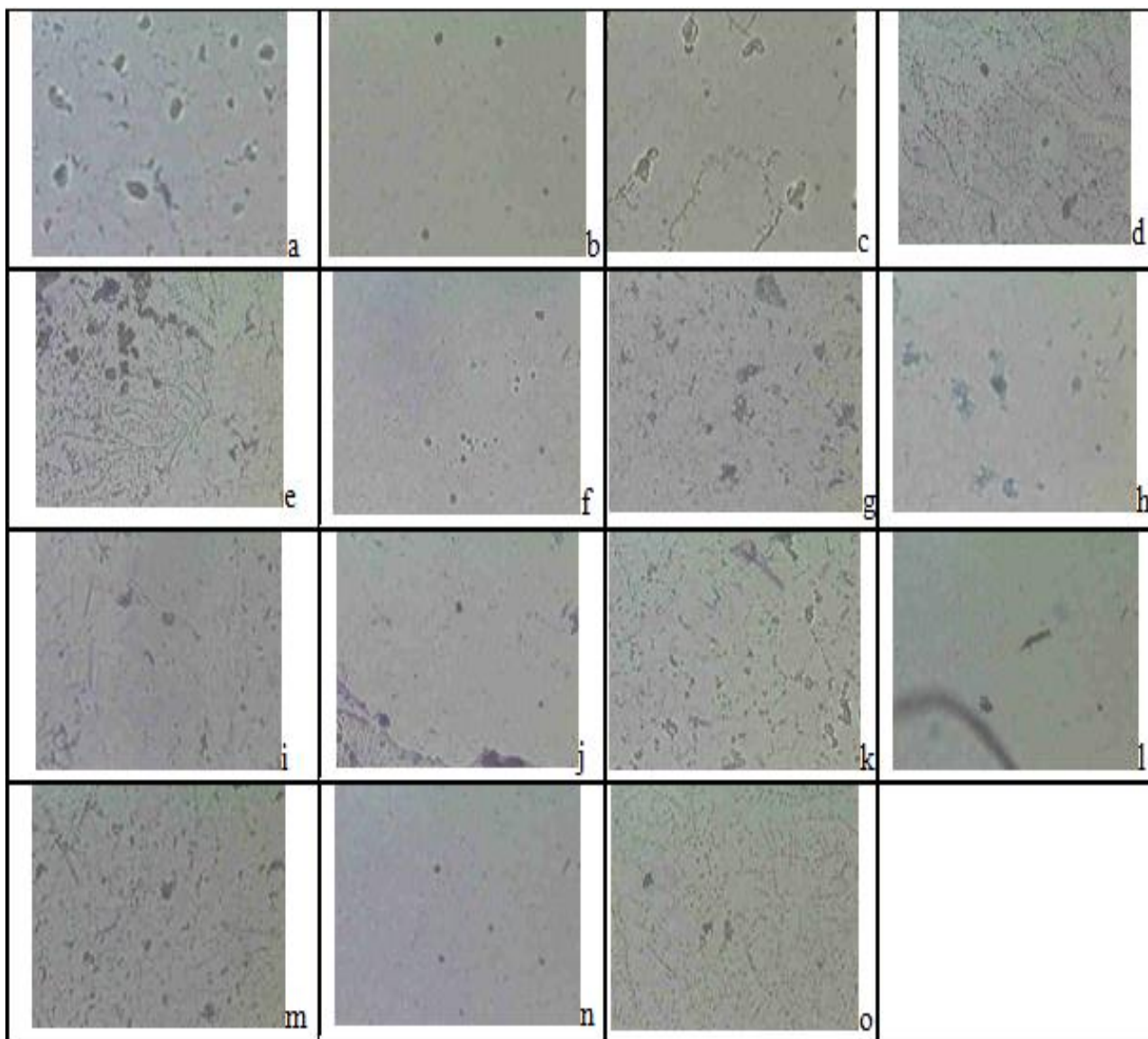


Figure 2. *In vitro*-cultivated normal Balb/c mouse embryonic fibroblasts 3T3 (a); *In vitro*-cultivated malignant cells from murine myeloma Ag853 cell line (b); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of 0.1M Doxorubicin (c); malignant Ag853 mouse myeloma cells, cultivated in the presence of Doxorubicin (d); normal 3T3 mouse embryonic fibroblasts, cultivated in the presence of 0.1M Doxorubicin plus Aronia-extract (e); malignant mouse myeloma cells Ag853, cultivated in the presence of 0.1M Doxorubicin plus Aronia-extract (f); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of Aronia-extract plus 0.1M Doxorubicin (g); malignant Ag853 mouse myeloma cells, cultivated in the presence of Aronia-extract plus 0.1M Doxorubicin (h); co-cultivation of normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of 0.1M Doxorubicin plus Aronia-extract (i); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of Aronia-extract (j); co-cultivation normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of Aronia-extract plus 0.1M Doxorubicin (k); normal mouse embryonic fibroblasts 3T3, cultivated in the presence of Aronia-extract (l); malignant mouse myeloma cells Ag853, cultivated in the presence of Aronia-extract (m); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 (n); co-cultivated normal mouse embryonic fibroblasts 3T3 and myeloma cells Ag853 in the presence of 0.1M Doxorubicin (o), (Giemsa-staining).

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Table 5. GSH levels from cultures of normal and malignant cells, as well as from mixed probes of co-cultivated cells from both types: controls; in the presence of 0.5M Doxorubicin; in the presence of Aronia-extract; in the presence of both substances.

GSH levels ($\mu\text{M}/\text{ml}$ cell lysate)/ Groups of laboratory- cultivated cells	Normal cells 3T3 Balb/c mouse embryonic fibroblasts	Malignant Ag853 mouse myeloma cells	Normal 3T3 cells, co-cultivated with Ag853 myeloma cells
Controls	0.0240	0.0280	0.0280
In the presence of Doxorubicin (0.5M)	0.0270	0.0300	0.0500
In the presence of Doxorubicin (0.5M) and Aronia-extract	0.0195	0.0375	0.0280
In the presence of Aronia-extract	0.0110	0.0620	0.0310

The results obtained also confirmed the observed strong inhibition in malignant cell growth on the influence of *Aronia melanocarpa* extract on malignant cells from HT29 line, in comparison with its action on normal colon cells (Malik et al., 2003). Similar chemoprotective effect by GSH in investigations on the protective role of Whey proteins after anti-cancer drug treatment of normal and malignant cells *in vitro* and *in vivo* (Hakkak et al., 2001; Alexieva et al., 2009). Messages about similar *in vitro*-protective action of the antioxidant substance Resveratrol against Doxorubicin unwished side effects has also been recently made (Al-Abt et al., 2011).

On the other hand, capability of Quercetin to act as antioxidant with simultaneous depletion of intracellular GSH in malignant cells has also been proved (Gibellini et al., 2010). In this way, a mechanism of induced by this compound cell death by subsequent increase of ROS levels has been supposed. Observed by us anti-malignant and antioxidant effects of *Aronia* confirmed published results from previous studies (Kong et al., 2003; Hovmaln et al., 2004; Jakobek et al., 2007; Kokotkiewicz et al., 2010; Scott & Skirvin, 2007), but they could also be explained with stimulation of immune cell differentiation, instead by induction of malignant cell cytotoxicity (El Benna et al., 1986; Khyriam & Prasad, 2001; Khyriam and Prasad, 2003; Reya & Clevers, 2005; Staal & Clevers, 2005; Cloutier et al., 2007; Katoh & Katoh, 2009; Olas et al., 2010), probably both *in vitro* and *in vivo*. These results were also in agreement with literature findings about activation of neutrophils as a consequence of increased levels of GSH, known as one of the main effects on the influence of *Aronia*-

extract (El Benna et al., 1986; Cloutier et al., 2007; McDonald et al., 2012). Our data also proposed mediation by the anti-oxidant capacity of *Aronia* polyphenols and antocyanins in regeneration of the decreased by Doxorubicin GSH intracellular levels on its influence, which was also in confirmation with recent literature sources (Kokotkiewicz et al., 2010). Despite the disparities, noticed in the assessed GSH levels in treated and non-treated by Doxorubicin cells, as well as in presence and absence of *Aronia*-extract, respectively (Table 5), the values obtained weren't statistically significant ($P > 0.001$). However, it has also been demonstrated that the intracellular GSH levels of a given cell type probably are not constant, but rather change in alteration of the conditions, in which the cells have been placed grow (Allanis-Turner et al., 1988). According many literature data, mediated activity of intermediate elements as GSH have been established as very important in the regulation of cell growth, division and proliferation, cascade regulatory pathways by of influence on nuclear DNA and proteins (Buchwitz et al., 1999; Shtutman et al., 1999; Staal & Clevers, 2005; Cloutier et al., 2007; Katoh & Katoh, 2009; Kohl et al., 2000; Ortega et al., 2011). Taking in consideration all that, one of the probable explanations of the observed unusual differences in the values of control normal cells in comparison with treated with Doxorubicin, *Aronia*-extract and both preparations (Table 5), could be connected with eventually depletion of GSH in the concrete time of measurement, as a "member" of cascade regulatory pathways, on the basis of feed-back principal of regulation, despite the eventual previous increase in its levels on the influence of *Aronia*-extract, and these results also confirmed some partly contradictory literature

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data (Gibellini et al., 2010), which have been explained with similar phenomena, probably appearing in the chain of respective regulation mechanism. Taking in consideration the established protective role of Glutathione against oxidant injury in normal tissue, on the one hand, and as a resistance mechanism against radiation- and chemotherapy-related injuries in malignant mass, on the other (Arrick & Nathan, 1984), alternative mechanisms of enhanced chemotherapeutic toxicity by alteration in GSH metabolism have also been proposed (Rouse et al., 1995; Wu et al., 2004). However, the observed by us general tendency for the higher initial levels of GSH in malignant cells in comparison with normal cells was in confirmation with cited scientific references (Berger et al., 1994). Taking in consideration that, with the agreement of the respective literature sources, further studies, directed to investigation on the influence of the separate antioxidant components from the total *Aronia*-extract (polyphenols and anthocyanins) on the levels of intracellular GSH should be necessary, but also on the mechanism of intracellular cascade regulatory pathways, in which GSH is included, would be necessary.

Conclusions

Future investigations about the influence of separate molecules, isolated from the total extract of *Aronia melanocarpa*, on the intracellular levels of GSH, as well as, on the other hand, on the influence of intermediate cell components as GSH, on the processes of cell growth and proliferation by cascade regulatory pathways, are necessary. These investigations could give possibilities about revealing of unknown regulatory mechanisms and, hence, about development of novel therapeutic strategies.

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