RESEARCH ARTICLE

Cytotoxicity of flavonoid glycosides, flavonoid aglycones and phenolic acids from *Inula oculus-christi* L. on mammalian cell lines

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ABSTRACT

Herbs of the genus *Inula* are well known in traditional medicine. Their extracts are used as expectorants, antitussives, bactericides as well as for the treatment of lung inflammation and have shown to possess anti-inflammatory and secretolytic activity. Experimental research findings indicate the anti-tumor effect of certain components of extracts from *Inula cappa* and *Inula britannica* and those from *Inula racemosa* have antimicrobial and antidiabetic activity.

We have directed our efforts on investigating the effects of different extracts from *Inula oculus-christi* L., enriched with certain groups of biologically active substances - flavonoid glycosides (*A*), phenolic acids (*B*), flavonoid glycosides and phenolic acids (*C*), flavonoid aglycones and phenolic acids (*D*). The effect of these extracts on normal (MDCK II and RPE1) and carcinoma cell lines (A549 and HepG2) was evaluated. We have performed cytotoxicity study (crystal violet assay) as well as morphological analysis of changes induced by the extracts. Among the tumor cell lines HepG2 show greater sensitivity. Surprisingly extract C has no significant influence on both cancer cell lines.

Key words: *Inula oculus-christi* L., cytotoxicity, phenolic compounds, plant extracts.

Introduction

Center of research interest in modern pharmacological therapy is increasingly shifting on drugs of plant origin. This statement largely relates to anti-tumor therapy. Natural bioactive molecules or their derivatives could serve as prototypes of final formulations for anticancer drugs (Rocha et al., 2001, Gordaliza, 2007). Multidrug resistance (MDR) is a critical problem in cancer chemotherapy. Cancer cells can develop resistance not only to a single cytotoxic drug, but also to entire classes of structurally and functionally unrelated compounds. Certain natural products obtained from medicinal plants may serve as potent drugs for reversing multidrug resistance in cancer cells due to their ability to inhibit ATP-dependent protein transporters, metabolic enzymes, and to induce apoptosis (Eid et al., 2015).

It is essential to extend the investigations on medicinal plants, used in traditional medicine in order to reveal the natural remedies' potential. Plants of the genus *Inula* are widely used in folk medicine as expectorants, antitussives, bactericides as well as for the treatment of lung inflammation and have shown to possess anti-inflammatory and secretolytic activity (Seca et al., 2014). Experimental research findings indicate anti-tumor, antimicrobial and antidiabetic activity for some of them (Tripathi & Chaturvedi, 1995, Lokhande et al., 2007, Lee et al., 2016). It is difficult to assess which compounds of the extracts are accountable for the beneficial impact. We have directed our efforts on investigating the effects of different extracts from *I. oculus-christi*, enriched with certain groups of biologically active substances – flavonoids, flavonoid glycosides, phenolic acids. It is shown that certain flavonoids from *I. britannica* protect primary cultured neurons against oxidative stress (Kim et al., 2002), and reduce the levels of histamine and bronchoconstriction in cases of asthma (Das et al., 2003). Other reported biological activities of flavonoids are: anti-inflammatory, antibacterial, antiviral, anticancer, hepatoprotective. They are capable to inhibit lipid-peroxidation, platelet aggregation, capillary permeability (Cushnie & Lamb, 2005). Epidemiological studies show an inverse correlation between dietary flavonoid intake and mortality from coronary heart disease (Cook & Samman, 1996).

Flavonoids occur as aglycones, glycosides, and their methylated derivatives. The basic flavonoid structure is aglycone. Many flavonoids are shown to have antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides (Ratty & Das, 1988, Kumar & Pandey, 2013), but bioavailability of the latter is sometimes enhanced by a glucose moiety (Hollman et al., 1999). After oral administration of flavonoid glycosides, they keep higher plasma levels and have a longer mean residence time than those of aglycones (Xiao, 2015).

The extracts we have used are enriched with various biologically active substances and combinations of them, comprising flavonoid glycosides, phenolic acids and flavonoid aglycones. This leads to diversity in their biological activity. Our study is aimed to investigate the activity of the chosen extracts from *I. oculus-christi* on the cell viability of two normal (MDCK II and RPE1) and two carcinoma cell lines (A549 and HepG2). We evaluated IC_{50} in the cases where such concentration was reached within the investigation. We applied morphological analysis of changes induced by the extracts.

Materials and Methods

All reagents and chemicals were supplied by Sigma-Aldrich (FOT, Sofia, Bulgaria) unless otherwise stated.

IC_{50} values were determined with Microsoft Excel, using mean values from triplicate experiments.

Plant material and plant extracts

Wild growing *I. oculus-christi* was collected in full flowering stage in July 2013 from western Rhodope Mts in Bulgaria. The plant was identified by Dr. Ina Aneva (Institute of Biodiversity and Ecosystem Research, BAS, Sofia). A voucher specimen (SOM 1360) has been deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, BAS, Sofia, Bulgaria. Air dried leaves and flowers from *I. oculus-christi* separately were successively extracted with chloroform and methanol to obtain crude extracts. The methanol extracts after evaporation of the solvent were fractioned by column chromatography on Sephadex LH-20 using methanol as a solvent. Dominant components in the respective fractions were determined by TLC comparison with previously isolated compounds as standards.

We used deliberately enriched in certain secondary metabolites fractions. The leaf extract, comprising flavonoid glycosides (labeled as ***A***); leaf extract enriched in phenolic acids (***B***); flower extract containing mainly flavonoid glycosides and phenolic acids (***C***), and flower extract enriched in flavonoid aglycones and phenolic acids (***D***). The extracts were dissolved in 100 µL dimethylsulfoxide (DMSO). Final concentration of extracts in medium was presented in µg / mL.

Cell cultures

We have used normal canine kidney epithelial cells (MDCK II), normal human retinal pigment epithelial cell line (RPE 1), human lung carcinoma cell line (A549), and human liver carcinoma cells (Hep G2) for this research.

Cells were grown in 25cm^2 'CELLSTAR®' flasks, at standard conditions in humidified atmosphere with 5% CO_2, at 37°C, in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic solution (penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 0.25 µg/mL). For assessment of cytotoxicity cells were plated on Nunk™ 96-well plates.

Morphological and cell viability tests

Crystal violet assay.

For determination of cytotoxicity we have treated cells for 24 and 48 hours with extracts at five different concentrations (10 µg/mL; 50 µg/mL; 100 µg/mL; 250 µg/mL; 300 µg/mL). The cytotoxic activity of the extracts was determined by crystal violet assay. On the 24th/48th hour after treatment the cell monolayers were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde solution in PBS for 20 min. Plates were washed with distilled water and 1% crystal violet solution was added to every well for 20 min. at room temperature. The samples were washed, air dried and the protein-bound dye (which is corresponding to the cell number) was solubilized with 10 % acetic acid.

The optical density of each sample was measured spectrophotometrically at λ = 570nm, by Epoch Microplate Spectrophotometer with the Gen5 Data Analysis software. We have performed two types of controls: 1) cells incubated at the same conditions without extracts and 2) with added DMSO at the highest concentration we have used to dissolve the extracts. The values were calculated as per cent of control 1 (cells incubated at the same conditions, but without extracts) and IC_{50} was determined where it is applicable.

The assessment of cell morphology was obtained through crystal violet staining. Prior to staining, the cells were cultured on sterile cover slips, treated with the plant extracts and fixed with 4% formaldehyde solution. Plates were washed with distilled water and 1% crystal violet solution was added to every well for 20 min. at room temperature. After washing, the cells were observed under inverted microscope (XDS-2A, China) supplied with a digital camera (DV-130, China) and micrographs were taken.

Results

Cytotoxicity of plant extracts

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Leaf extracts enriched with flavonoid glycosides (***A***) or phenolic acids (***B***) alone affected mostly the cancer cells. Generally, HepG2 cells were slightly more sensitive, in comparison to the other carcinoma cell line. IC_{50} for A549 cells of both extracts were over 250 µg/mL, whereas for HepG2 cells extract B reached IC_{50} at 137.5 µg/mL after 24 h treatment (Table 1). Similar biological activity was detected affected in general (Fig. 3). After application of extract ***B*** both cell lines shown cytoplasmic changes - transparent vesicles in MDCK II cells and dense aggregations and paled sections of the cytoplasm in RPE1 (Fig. 3).

Extract ***D***, which is most enriched with antioxidants, surprisingly was most toxic for all tested cells. The morphology of cell line MDCK II was significantly disturbed

Table 1. *Estimated IC50 (at µg/mL) of the plant extract for tested cell lines. N.A. – non applicable, the cases where IC50 concentration was not reached within the investigated interval.*

Cell line	MDCK II		RPE1		A549		HepG2	
Extract	24 h	48 h	24 h	48 h	24h	48h	24h	48h
A flavonoid glycosides	N.A.	N.A.	N.A.	N.A.	295	256	207,5	256
B phenolic acids	N.A.	N.A.	N.A.	N.A.	N.A.	280	137,5	133
C flavonoid glycosides and phenolic acids	N.A.	N.A.	N.A.	N.A.	280	262	N.A.	292
D Flavonoid aglycones and phenolic acids	40	27	148	114	92	45	50	40

using extract ***C*** (flavonoid glycosides and phenolic acids). The most surprising finding was that this extract has no significant influence on HepG2 cell line, whereas flavonoid glycosides alone and phenolic acids alone showed some cytotoxicity on these cells. In this case, viability of HepG2 cells was slightly affected – over 70% viable cells, compared to the control (Table 1; Fig. 1,2,3).

Extract ***D*** (flavonoid aglycones and phenolic acids) showed most potent cytotoxic activity among all tested extracts. There was no significant distinction in the sensibility of cancerous and non-cancerous cells (Table 1; Fig. 2, 3). Most sensitive were kidney epithelial cells MDCK II and most resistant were RPE1.

The controls with DMSO didn't show any significant effect. It was observed reduction in cell growth less than 10 %. Estimated IC_{50} are summarized at Table 1.

Analysis of cell morphology

The morphology of the cell lines used for this study is generally epithelial. Untreated MDCK II cells are organized in tightly formed monolayer, which corresponds to the presence of many adherent junctions and tight junctions, specific for them. RPE1 cells are more loosely spread, larger and more flattened compared to the kidney epithelial cells. A549 cells have a cobblestone epithelial morphology. HepG2 are growing in small aggregates when they are at low confluency and confluent monolayer has similar appearance to A549 (Fig. 3).

Morphology of the noncancerous cells was not considerably impaired of extracts ***A*** and ***C***. Although some altered cells had emerged, the cultures remained slightly with atypical, irregular cell shape, vesiculated and granular cytoplasm, in some of the nuclei central clustering of chromatin was observed. There was dense substance in the vesicles found in RPE1 cells' cytoplasm.

Alterations in A549 cells, induced by the extracts are as follows: extracts ***C*** and ***D*** provoked formation of long and thin cytoplasmic outgrowths (Fig 3.); treatment with extract ***A*** led to appearance of many vesicles in light, transparent cytoplasm (Fig. 3) and extract ***B*** provoke formation of large transparent vesicles with dense substance in them (Fig. 3).

HepG2 cell line was most impaired after application of extract ***B*** – shrunk cells and extended cytoplasmic outgrowths were observed. There were recorded similar deviations, in milder form caused by the other extracts (Fig. 3).

On the basis of our results we were able to define certain generalization:

Among the extracts tested, extract ***A*** (flavonoid glycosides) gained the most significant distinction in the way it affected cancerous and non-cancerous cells.

Extract ***C*** (flavonoid glycosides and phenolic acids) has no significant influence on HepG2 cell line, whereas flavonoid glycosides alone (***A***) and phenolic acids alone (***B***) showed some cytotoxicity on these cells, which was confirmed with the assessment of cell morphology.

Because extract ***D*** (flavonoid aglycones and phenolic acids) showed most potent cytotoxic activity among all tested extracts it could be suggested that flavonoids and phenolic acids potentiate each other's activity, but the presence of

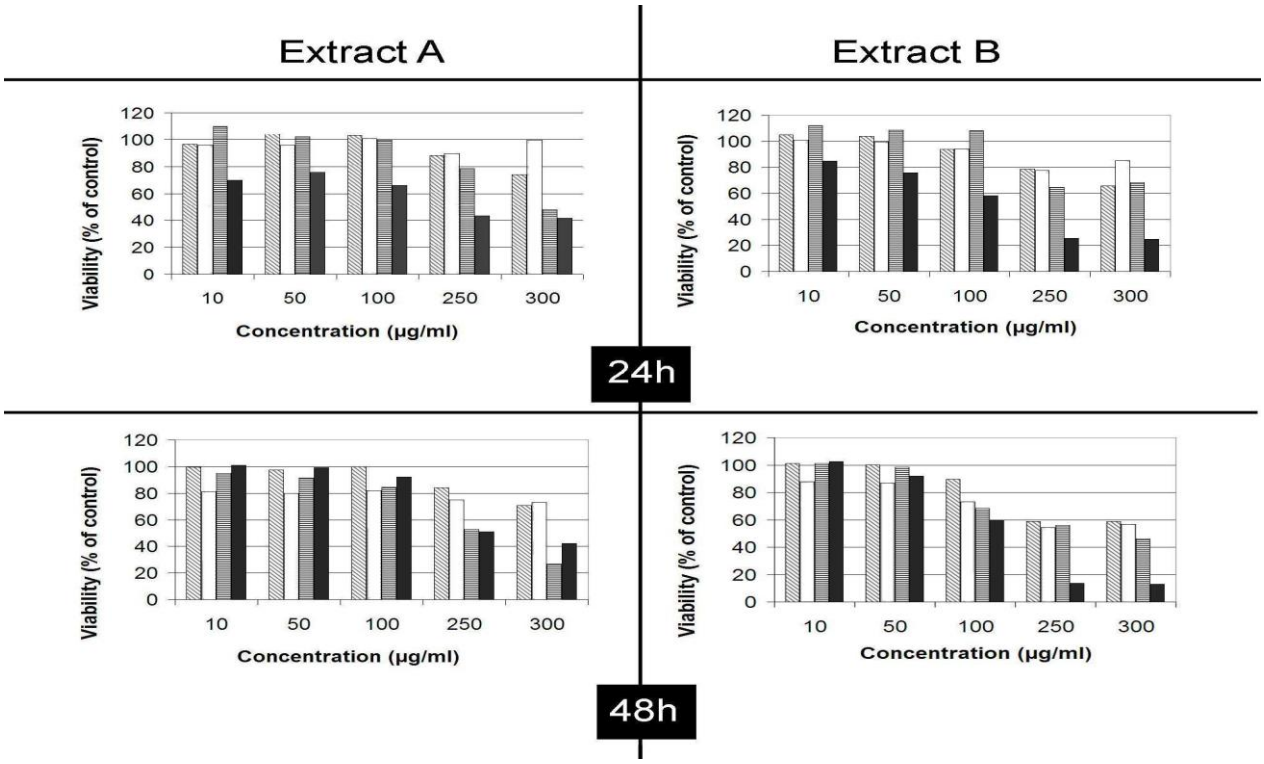

Figure 1. *Cytotoxicity of methanol extracts from leafs: extract **A** (flavonoid glycosides) and extract **B** (phenolic acids) on MDCK II cells (diagonals); RPE 1 (white); A 549 (horizontals); HepG2 (black). Cytotoxicity was estimated by crystal violet assay and measured at OD 570nm.*

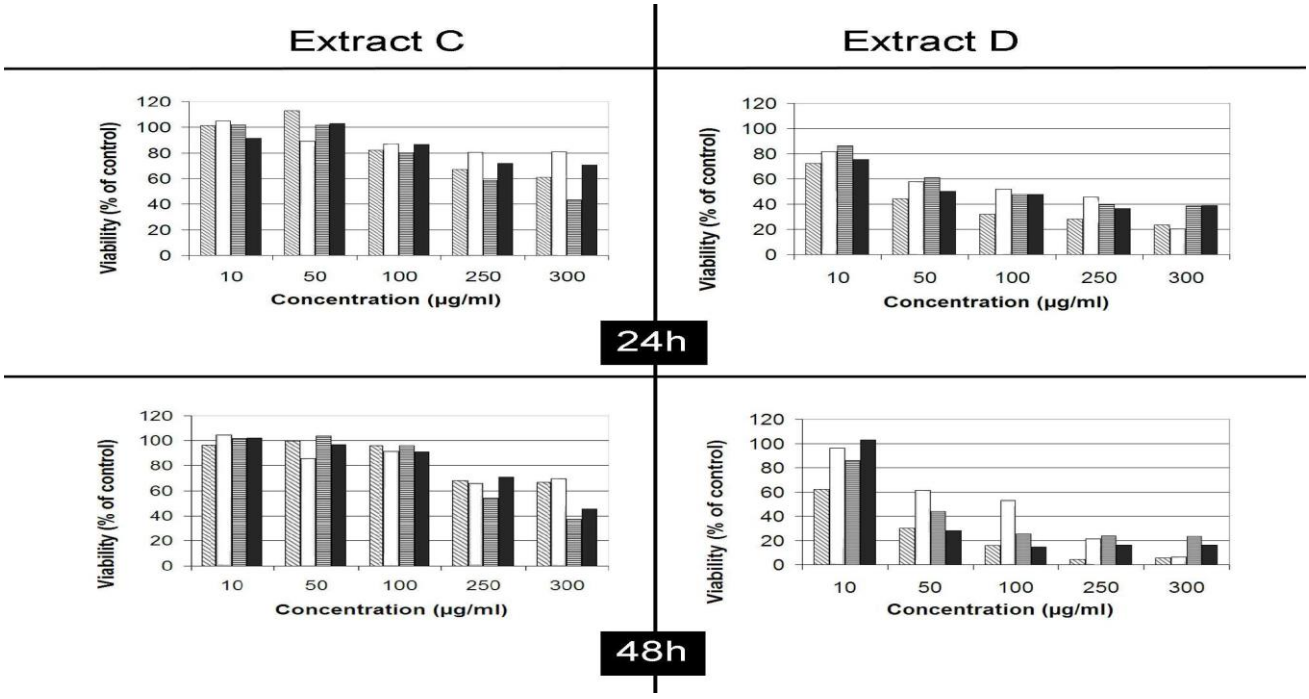

Figure 2. *Cytotoxicity of methanol flower extracts: extract **C** (flavonoid glycosides and phenolic acids) and extract **D** (flavonoid aglycones and phenolic acids) on MDCK II cells (diagonals); RPE 1 (white); A 549 (horizontals); HepG2 (black). Cytotoxicity was estimated by crystal violet assay and measured at OD 570nm.*

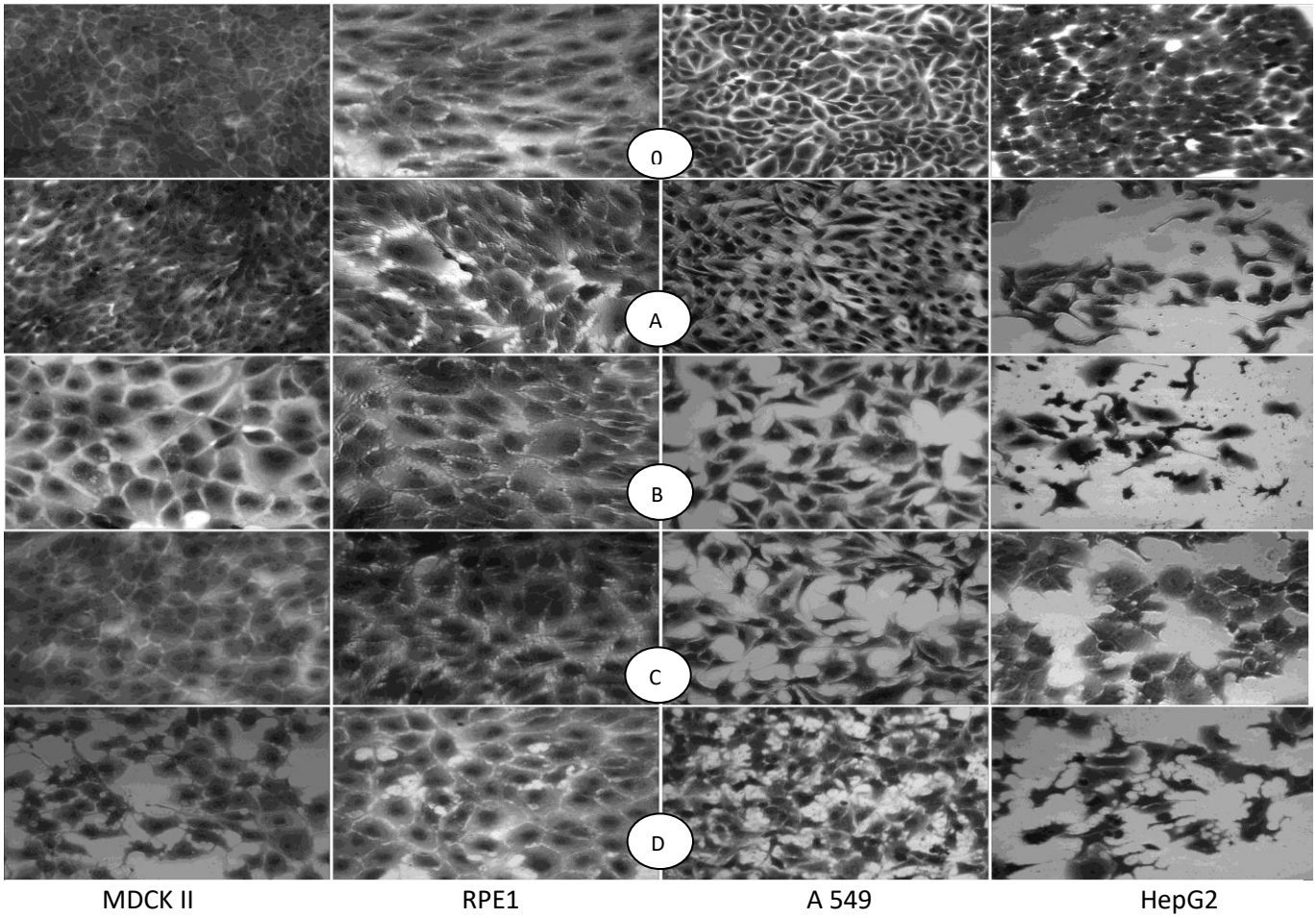

Figure 3. *Morphology of MDCK II, RPE1, A 549 and HepG2 cells. Crystal violet staining, magnification 100x. Row 0 – untreated controls; Treatments with extracts: extract A – row A; B – row B; C – row C; D – row D respectively*

flavonoid glycosides compensate and diminish the effect of phenolic acids.

The overall observed tendency is that cytotoxicity of the investigated extracts increases with time of application. There is interesting exception – HepG2 cells underwent distinct adaptation toward extract ***A***. Within concentrations up to 100µg/ml the survival rate is ~ 30% higher on 48th hour compared to 24th hour of treatment. This is evident from the values of IC_{50} as well (24h – 207.5 µg/ml; 48h – 256 µg/ml). The same, but to a lesser extent also applies to the activity of extract ***B*** on HepG2 cells.

Discussion

Flavonoid aglycones, flavonoid glycosides and phenolic acids as antioxidants are commonly known for their beneficial effects on human health and their antitumor properties (Singhal et al., 1995; Middleton et al., 2000; Kim et al., 2002; Das et al., 2003; Ren et al., 2003; Cushnie & Lamb, 2005; Kandaswami et al., 2005; Seelinger et al., 2008; Ferrazzano et al., 2011). In the present study we found that extract ***A*** (flavonoid glycosides) faintly affected the normal cell lines, while it was detected considerable cytotoxicity on the cancerous ones when treated with high concentrations. Extract ***B*** (phenolic acids) was distinctly most toxic towards HepG2 cells. Our present results are consistent with the previously mentioned reports.

Nakamura et al. have reported that topical application of a protocatechuic acid (phenolic acid) exerts contrasting effects on tumor promotion in mouse skin. As expected, low dose of this compound has inhibited the tumor progression. In our study we also detected comparable results for extracts ***A*** and ***B***. Nakamura's study also revealed that a high dose of the same phenolic acid causes enhancement of the skin tumor promotion and enhances the rate of an overall oxidative damage biomarkers. There is increasing number of evidences indicating important roles of oxidative stress in tumor promotion. Y. Nakamura et al. demonstrated increased oxidative damage, caused by high doses of certain potent antioxidant (Nakamura et al., 2000). Radical scavengers are known to have pro-oxidative potential because of their conversion to more reactive or stable radicals after they react directly with ROS, which may contribute to the induction of secondary oxidative damage on the target organs. It is shown that there is generation of hydrogen peroxide in cell culture

media as a result of flavonoid autooxidation and this is suggested to underlie its prooxidant action (Stanojevic et al., 2004). In the present investigation we revealed that extract ***D***, which is most enriched with antioxidants had most potent cytotoxic activity among all tested extracts. We can only speculate whether this cytotoxicity is due to the paradoxal prooxidative effect of antioxidants.

Yang Y et al. have performed a study on the cytotoxicity of eight different flavonoids. Their results indicated that the flavonoids with the high lipophilicity had high cytotoxicity (Yang et al., 2014). These results showed that the cytotoxicity of the flavonoids decreased directly with the numbers of glycoside and hydroxyl groups among the flavonoid compounds. This is one of the possible reasons for the lower toxicity of extract ***C***, comprising flavonoid glycosides as compared with extract ***D***.

Conclusions

Considerable negative effect on cell viability of the noncancerous cell lines after administration of extracts ***A***, ***B*** and ***C*** was not observed. Although these extracts didn't show significant cytotoxicity there were some alterations in cell morphology observed. The fact that influence on MDCK II cells was detected is alarming and such substances must be applied cautiously with regard to kidney function.

The reported alterations in morphology induced by the extracts are indicative for impaired cell periphery and disrupted intercellular contacts and mechanisms of this activity need further investigations.

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