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ANTHOCYANINS FROM BERRIES FRUITS AND THEIR PROTECTIVE EFFECT IN THE PLASMID DNA DAMAGE

V. Sedlák[1], M. Konečná[1], Ľ. Tkáčiková[2], P. Kšonžeková[2], M. Mydlárová-Blaščáková[1], D. Gruľová[3], J. Gaľová[1], A. Babejová[1], Z. Gogaľová[1], A. Avuková[1], H. Vašková[1], V.S. Mirutenko[4], V.V. Mirutenko[5], T. Kimáková[6], J. Poráčová[1]

Anthocyanins from berries fruits and their protective effect in the plasmid DNA damage. – Sedlák V., Konečná M., Tkáčiková Ľ., Kšonžeková P., Mydlárová-Blaščáková M., Gruľová D.,Gaľová J., Babejová A., Gogaľová Z., Avuková A., Vašková H., MirutenkoV.V., Mirutenko V.S., Kimáková T., Poráčová J. – *Anthocyanins as secondary metabolites of berry fruit represent a large number of positive health effects. The physiological effect of polyphenols is a function of their chemical nature and bioavailability. Protective action is linked to their antioxidant, antimutagenic and anticancer activity. The aim of the studies was to determine the effect of ethanol and acetone extracts from berries of the fruits of Vaccinium myrtilus L., Sambucus nigra L., and Aronia melanocarpa L. on mitomycin C-induced plasmid DNA damage. We evaluated the protective effect of ethanol and acetone extracts of Vaccinium myrtilus, Sambucus nigra, and Aronia melanocarpa berry fruits in concentrations of 50, 25 and 10 μg.×ml^{-1} to plasmid DNA damage induced by mitomycin C, the protective effect of anthocyanins consisted in the formation of copigmentic complex. Anthocyanins inhibited the formation and release of peroxide radicals. We found that V. myrtillus ethanol extract (50 μg/ml) showed the best protective effect on plasmid DNA. The antioxidant and antimutagenic effect of anthocyanins is based on the free radical elimination. The effect was evaluated using horizontal agarose electrophoresis.*
Key words: *antimutagenic activity, anthocyanins, berry fruit, mitomycin C, plant extracts.*
Addresses: *1 - University of Presov, Faculty of Humanities and Natural Sciences, Department of Biology, ul. 17 novembra, 1, Prešov, Slovakia; e-mail: vincent.sedlak@unipo.sk, maria.konecna@unipo.sk, marta.blascakova@unipo.sk, andrea.babejova@smail.unipo.sk, zuzkagogalova@gmail.com, andrea.avukova@smail.uniposk, hedviga.vaskova@smail.uniposk, janka.poracova@unipo.sk*
2 - University of Veterinary Medicine and Pharmacy in Košice, Department of microbiology and immunology, ul. Komenského, 73, Košice, Slovakia; e-mail: ludmila.tkacikova@uvlf.sk, petra.ksonzekova@uvlf.sk
3 - University of Presov, Faculty of Humanities and Natural Sciences, Department of Ecology, ul. 17 novembra, 1, Prešov, Slovakia; e-mail: daniela.grulova@unipo.sk
4 - Uzhhorod National University, Faculty of Biology, Department of Botany, 32, A. Voloshyna str., Uzhhorod, Ukraine; e-mail: violeta.mir10@gmail.com
5 - Uzhhorod National University, Faculty of Biology, Department of Entomology and Biodiversity Conservation, 32, A. Voloshyna str., Uzhhorod, Ukraine; e-mail: vladyslav.mirutenko@uzhnu.edu.ua
6 - Pavol Jozef Šafárik University in Košice, Faculty of Medicine, Department of Public Health and Hygiene, Šrobárova 2, Košice, Slovakia; e-mail: tatiana.kimakova@upjs.sk

Антоціани ягідних плодів та їх захисний ефект в пошкодженні плазмідної ДНК. – Седлак В., Конечна М., Ткачікова Л., Кшонжекова П., Мидларова-Блашчакова М., Ґрульова Д., Ґальова Я., Бабейова А., Ґоґальова Ж., Авукова А., Вашкова Г., Мірутенко В.В., Мірутенко В.С., Кімакова Т., Порачова Я. – *Антоціани як вторинні метаболіти фруктів здатні досить позитивно впливати на стан здоров'я. Фізіологічна дія поліфенолів залежить від їх хімічної природи та біодоступності, а захисна дія пов'язана з їх антиоксидантною, антимутагенною та протипухлинною активністю. Метою даних досліджень було визначити дію екстрактів етанолу та ацетону з ягід плодів Vaccinium myrtilus L., Sambucus nigra L. та Aronia melanocarpa L. на індуковану мітоміцином С плазмідну ДНК. Ми оцінили захисну дію етанолових та ацетонових екстрактів ягід Vaccinium myrtilus, Sambucus nigra та Aronia melanocarpa у концентраціях 50, 25 та 10 мг×мл$^{-1}$ до пошкодження плазмідної ДНК, викликаного мітоміцином С. Захисний ефект антоціанів полягав у утворенні копігментного комплексу. Антоціани пригнічували утворення та вивільнення перекисних радикалів. Ми виявили, що етаноловий екстракт V. myrtillus (50 мг/мл) демонстрував найкращий захисний ефект щодо плазмідну ДНК. Антиоксидантна та антимутагенна дія антоціанів заснована на виведенні вільних радикалів. Ефект оцінювали за допомогою горизонтального агарозного електрофорезу.*

Ключові слова: *антимутагенна активність, антоціани, ягоди, мітоміцин С, рослинні екстракти.*
Адреси: *1 – Пряшівський університет, факультет гуманітарних і природничих наук, кафедра біології, вул. 17 листопада, 1, Пряшів, Словаччина; e-mail: vincent.sedlak@unipo.sk, maria.konecna@unipo.sk, marta.blascakova@unipo.sk, andrea.babejova@smail.unipo.sk, zuzkagogalova@gmail.com, andrea.avukova@smail.uniposk, hedviga.vaskova@smail.uniposk, janka.poracova@unipo.sk;*
2 – Університет ветеринарної медицини і фармації в Кошицях, кафедра мікробіології та імунології, вул. Коменського, 73, Кошице, Словаччина; e-mail: ludmila.tkacikova@uvlf.sk, petra.ksonzekova@uvlf.sk;
3 - Пряшівський університет, Факультет гуманітарних і природничих наук, кафедра екології, вул. 17 листопада, 1, Пряшів, Словаччина; e-mail: daniela.grulova@unipo.sk;
4 – Ужгородський національний університет, біологічний факультет, кафедра ботаніки, вул. Волошина, 32, Ужгород, Україна; e-mail: violeta.mir10@gmail.com;
5 - Ужгородський національний університет, біологічний факультет, кафедра ентомології та збереження біорізноманіття, вул. Волошина, 32, Ужгород, Україна; e-mail: vladyslav.mirutenko@uzhnu.edu.ua;
6 – Університет Павла Йожефа Шафарика в Кошицях, медичний факультет, кафедра громадського здоров'я і гігієни,вул. Шробарова, 2, Кошице, Словаччина; e-mail: tatiana.kimakova@upjs.sk

Introduction

Epidemiological studies have shown, that berry fruit consumption can reduce the incidence of cardiovascular disease, stroke and prevents cancer diseases. Their protective effect is attributed to their bioactive antioxidant components, including vitamins, carotenoids and polyphenols (Pandey, Rizvi 2009). Among the various antioxidants contained in berry fruits, anthocyanins have gained a lot of attention due to their positive impact on human health (Jakobek et al. 2007).

Anthocyanins belong to the group of phenolic compounds which are water-soluble glucosides and acylglycosides of anthocyanidins. The most common anthocyanins include 3-O-glycoside and 3,5-di-O glycoside from cyanidine, delfinidine, peonidine, petunidine, pelargonidine and malvidine. Studies confirm that anthocyanins are absorbed in their original glycoside form. The antimutagenic and anticarcenogenic activity of anthocyanins is generally attributed to their antioxidant activity due to the phenolic structure. Double ring bonds and hydroxyl side chains enhance their free radical scavenging effect, which are constantly produced in cells by cellular metabolism and external agents, but also allow their chelating and protein binding properties (Kong et al. 2003).Some studies provide evidence that anthocyanins can preferentially kill cancer cells with high metastatic capacity that are resistant to conventional therapies. Thus, they could set the basis for the development of new sensitizing agents in the treatment of metastatic disease (Tramer et al. 2012).

There is a number of short term, simple and cheap assays that can be used to screen for potential chemopreventive agents and their mechanisms to detect substances that protect against DNA damage. For example, reactive oxygen species can react with various biomolecules in cells such as lipids, proteins, and DNA, thereby causing damage. The imbalance between the production of reactive oxygen species and antioxidant defence subsequently leads to oxidative stress, which is associated with degenerative processes leading to the development of cancer, cardiovascular and neurodegenerative diseases. Reactive oxygen species induce numerous lesions in DNA, including deletion and cleavage of single-stranded and double-stranded DNA. Substances that reduce oxidative damage to DNA should reduce the risk and development of the disease (Wojdylo et al. 2007).

Good extraction yields for antioxidant molecules like polyphenols are often obtained with organic solvents like acetone, methanol or ethanol. Unfortunately, organic solvents are also well known to have a DNA protective effect in this kind of assay. Because the DNA backbone is negatively charged metal ions as Fe^{2+} can bind DNA and can catalyze the Fenton reaction although iron binding to DNA can also cause DNA damage. Organic solvents may impair this interaction thereby preventing the Fenton reaction from occurring (Leba et al. 2014).

Material and Methods

Acetone and ethanol extracts from selected berry fruit species blueberry (*Vaccinium myrtilus* L.), elderberry (*Sambucus nigra* L.) and aronia (*Aronia melanocarpa* L.) were used in the study. The extracts were diluted with an appropriate amount of diluted 0.01% acetic acid at concentrations of1 mg/ml, 2.5 mg/ml a 5mg/ml.Plasmid p Gem-3z (800 ng/10 μl) (2743 bp, Promega), 78 μM EDTA (10x concentrated), 143 μM $FeSO4.7H_2O$ (10x concentrated) and 0.004% mytomicin C (10x concentrated) (MMC, Sigma) were used to prepare the reaction mixture. The volume of the reaction mixture was added with distilled water as needed. The prepared samples were centrifuged for 30 seconds at 1500 rpm. The reaction mixture was

incubated at 37°C for 60 minutes. For the positive control, the reaction mixture itself without extracts was used. Negative control consisted of plasmid and distilled water without MMC. After incubation, samples were mixed with sample buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and separated by electrophoresis (5 V / cm) in agarose gel (0.9%) in electrophoretic buffer (40 mMTris-acetate and 2 mM EDTA ph 8.0) for 90 minutes. Gene Ruler 1 kb Plus DNA Ladder (Fermentas) was used as the molecular weight standard. The samples were stained with dye Gold View 9 (GoldView™ Nucleic Acid Stain, Viswagen). UV lamp was used for the visualization and photo documentation was made using a Kodak camera.

Testing of the protective effect of the extracts on DNA damage induced by mitomycin C was performed according to the method of Yamagishi et al. (2001). The protective effect of anthocyanins against DNA damage consisted in the formation of copigmentic complex. Mitomycin C transforms into a radical after reduction, which forms hydrogen peroxide $H_2O_2$when reacted with oxygen. Hydrogen peroxide produces the OH•in the Fenton reaction causing DNA breaks. Single breaks result in open circular and linear DNA form. These forms are separated from each other in an agarose gel. The supercoiled DNA migrates faster than the open ring DNA and the linear DNA migrates in an intermediate position between the supercoiled and the open ring DNA. The percent of inhibition of the MMC-induced DNA cleavage was calculated according to the formula:

$$(S - S0) / (S \text{ control} – S0) \times 100$$

- S is the percentage of supercoiled DNA in the control line (without MMC and tested compounds);
- S0 is the percentage of supercoiled DNA in the line without test compounds but with MMC;
- S control is the percentage of supercoiled DNA in the line with the tested compounds and MMC.

Data were presented of three parallel measurements. The analyses of results were performed by using the SPSS 18 Software and evaluated by t-test.

Results

We found that the ethanol extract of *Vaccinium myrtillus* at the concentration of 50 μg.ml^{-1} showed the best protective effect on plasmid DNA during the evaluating of the anthocyanins effects. A slightly lower effect was found at 25 μg.ml^{-1} and the lowest effect was at 10 μg. ml^{-1} concentration (Fig. 1).

A positive effect was also observed in *Sambucus nigra* ethanol extract at concentrations of 50 μg.ml^{-1} and 25 μg.ml^{-1}. The effect of ethanol and acetone extract was not observed at concentrations of 10 μg.ml^{-1}. Acetone extracts of *Vaccinium myrtillus*, as well as acetone and ethanol extract of *Aronia melanocarpa* did not show any positive protective effect at any investigated concentration. Total DNA cleavage was observed in a positive control containing plasmid and mitomycin C. DNA damage was not detected in the negative control, which consisted only of the plasmid.

Discussions

Based on reduction, mytomicin C is transformed into a semichinone radical which reacts with oxygen, resulting in the production of hydrogen peroxide. In the presence of Fe^{+2}, metal-catalyzing Fenton reaction generates free OH radicals, strong oxidants that cause DNA breaks.

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH\bullet + OH^-$$

In double-stranded circular DNA, single-strand breaks result in open circular DNA, and double-strands lead to the formation of linear DNA. Separation of these forms was observed by agarose gel electrophoresis. The antioxidants present in the extracts prevented DNA breaks by trapping free OH radicals, thereby blocking the course of the Fenton reaction, and thus also the conversion of super-spiral DNA into an open circular and linear form.

In our study, ethanol extract of *Vaccinium myrtillus* at concentration of 50 μg.ml^{-1} had the best protective effect on plasmid DNA. By reducing the concentration, the protective effect of the extract was also proportionally reduced from 25 μg.ml^{-1} to 10 μg.ml^{-1}. The protective effect of *Vaccinium myrtillus* extract was also confirmed by Boateng et al. (2008) investigating the protective effect of the extract in kidney damage caused by potassium bromate, a potent nephrotoxic agent. Blueberry extract reduced oxidative stress and kidney damage by attributing the ability to trap free radicals and inhibit the process of lipid peroxidation. The positive effect of *Vaccinium myrtillus* extract and mainly cyanidine derivatives was also confirmed by Lazzé et al. (2003), who examined the protective effect of the extract on DNA damage induced by tert-butyl-hydroperoxidase in the liver and muscle cells of mice. They have found that cyanidine and its glycosides are effective against cytotoxicity, inhibit single strand break DNA formation and oxidation of bases.

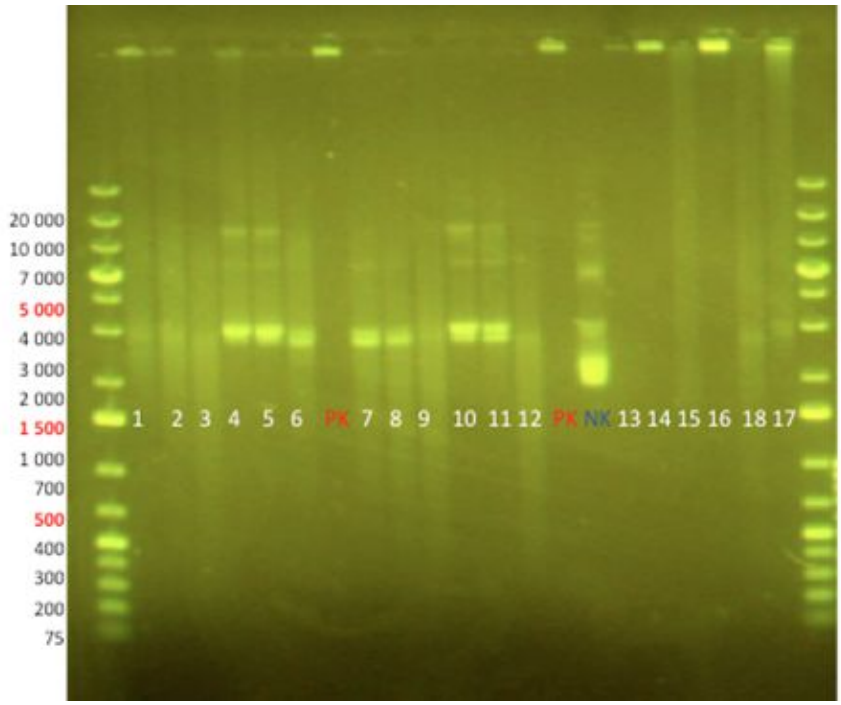

Figure 1. Evaluation of the protective effect of extracts on DNA damage induced by mitomycin C by electrophoresis

samples (1, 2, 3) acetone extracts of *Vaccinium myrtillus* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
samples (4, 5, 6) ethanol extracts of *Vaccinium myrtillus* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
samples (7, 8, 9) acetone extracts of *Sambucus nigra* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
samples (7, 8, 9) ethanol extracts of *Sambucus nigra* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
samples (10, 11, 12) acetone extracts of *Aronia melanocarpa* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
samples (10, 11, 12) ethanol extracts of *Aronia melanocarpa* with concentration 50 μg.ml^{-1}, 25 μg.ml^{-1} and 10 μg.ml^{-1};
PK - reakčná zmes bez extraktov; 20 μl;
NK - pGem, 900 ng/20 μl reakciu

The protective effect of anthocyanin extracts lies in the formation of a copigmentation complex. Sarma and Sarma (1999) confirmed the formation of a cyanidine-DNA copigmentation complex that was protected from oxidative damage by free radicals in their studies on thymus calf cells and cyanidine extract. Their results confirmed that the formation of cyanidine-DNA copigmentation has defense mechanisms against oxidative DNA damage.

A positive effect was observed in *Sambucus nigra* ethanol extract at concentrations of 50 μg.ml^{-1} and 25 μg.ml^{-1}. A concentration of 10 μg.ml^{-1} of acetone extract of *Sambucus nigra* had no positive protective effect. Lak et al. (2011) evaluated the protective effect of *Sambucus nigra* extract on albendazole-induced fetal malformations. They found that *Sambucus nigra* extract had a protective effect on albendazole-induced fetal teratogenicity.

The positive effects of the extract were also confirmed by Bratu et al. (2012), who observed *in vitro* and *in vivo* antioxidant and antimutagenic activity of *Sambucus nigra* extract in the *Allium cepa* plant model. They found that the extract showed very high antioxidant activity, even at higher concentrations it showed a cytotoxic effect along with mito-depressant activity and was able to inhibit mitosis in the pre-prophase. No mutagenic effects were shown at low concentrations.

Positive protective effect was not found in acetone extract of *Vaccinium myrtillus*, acetone extract of *Aronia melanocarpa* and also in ethanol extract of *Aronia melanocarpa*. Gasiorowski et al. (1997) isolated anthocyanins from *Aronia melanocarpa* in their experiment. They found that they significantly inhibited the mutagenic activity of benzopyrene and 2-amino fluorine in the Ames test. The frequency of sister chromatid exchange induced by benzopyrene decreased significantly in the presence of *Aronia melanocarpa* extract.

Anthocyanins significantly inhibited the formation and release of peroxide radicals. Their results confirmed that the antimutagenic effect of anthocyanins is based on the destruction of free radicals as well as the inhibition of promutagen activating enzymes and the conversion of mutagenic derivatives causing DNA changes. In the case of mitomycin C, the effect of anthocyanins on the frequency of sister chromatid exchange was lower but still visible.

It was found that ethanol extracts showed greater efficiency compared to acetone extracts, and also extracts with higher concentrations were more effective than lower concentrations. Stagos et al. (2005) presented the same experiment using *Vitis vinifera* extract and found a positive protective effect of the extracts at higher concentrations that inhibited the formation of circular and linear DNA forms. Several studies confirm that blueberry, aronia and elderberry have significant antiradical and antioxidant activity. This is due to the synergistic action of anti-radical agents, which can be used as a preventive and curative material in radical-dependent conditions and diseases.

Conclusions

The ethanol extracts of *Vaccinium myrtillus* and *Sambucus nigra* has possessed a positive DNA-damage protection ability, offered by its significant anti-oxidant potential. As compared with acetone extracts, the findings suggest that ethanol extracts has a positive anti-cancer activity against on mitomycin C-induced plasmid DNA damage at high concentration (from 25 $\mu g.ml^{-1}$ to 50 $\mu g.ml^{-1}$).

Natural plant pigments – anthocyanins are the subject of extensive research especially for their application in medicine. Currently, interest in anthocyanins is increasing in terms of their beneficial effects on the human body. Based on the obtained results we can conclude that anthocyanins do have a certain degree of positive biological activity. At the same time, there are a large number of anthocyanins nutritional supplements on the market, whose regular consumption can reduce the prevalence of civilization diseases.

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