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Analysis of delphinidin and luteolin genotoxicity in human lymphocyte culture

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ABSTRACT

Introduction: Bioflavonoids delphinidin (2-(3,4,5-Trihydroxyphenyl)chromenylium-3,5,7-triol) and luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone) have been recognized as promising antioxidants and anticancer substances. Due to their extensive use, the goal of the research was to determine whether they have any genotoxic potential *in vitro*.

Methods: Analysis of genotoxic potential was performed applying chromosome aberrations test in human lymphocyte culture, as this kind of research was not conducted abundantly for these two bioflavonoids. Delphinidin and luteolin were dissolved in DMSO and added to cultures in final concentrations of 25, 50 and 100 μM.

Results: In human lymphocytes cultures Delphinidin induced PCDs in all treatments, potentially affecting the cell cycle and topoisomerase II activity. In concentration of 50 μM luteolin showed strong genotoxic effects and caused significant reduction of cell proliferation.

Conclusion: Luteolin exhibited certain genotoxic and cytostatic potential. Delphinidin was not considered genotoxic, however its impact on mitosis, especially topoisomerase II activity, was revealed.

Keywords: chromosome aberrations; cell proliferation; bioflavonoids

INTRODUCTION

Delphinidin and luteolin belong to the group of flavonoids. They are plant pigments mostly found in flowers and fruits, consumed on a daily basis. Antioxidative effects of bioflavonoids have been proved in many biological studies, meaning that they are recognized as very efficient natural protectants. Delphinidin is abundantly present in the flowers and fruits of the following: plum, grapes, currant, blueberry, cranberry, strawberry, raspberry, blackberry, elderberry (1, 2). It has been proven that delphinidin has a protective role and it decreases the micronuclei frequency *in vivo* (3). It also shows cytostatic effects in a concentration-dependent gradient (4), antiangiogenic effects in tumor tissue (5), and the ability to induce apoptosis in cancer cells (6). The genotoxic effects of delphinidin have not been completely explored. Results of the Ames test suggest a general flavonoid genotoxicity (7), but specific analysis of delphinidin show that it does not manifest genotoxic effects (3). It is also been noticed that

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delphinidin can inhibit the activity of topoisomerase II, that plays a role in chromosome segregation during mitosis (2).

Luteolin is a plant flavonoid from flavone class. It is a polyphenolic compound, with certain pharmaceutical characteristics, found in various fruits, vegetables, seeds. The clinical studies aiming to assay the anticancer effects of different bioflavonoids according to their antioxidant potential, suggest luteolin as a potential inhibitor of cell proliferation (8). In the cells of various cancer types, luteolin is proven to be an effective inhibitor of cell proliferation in the average range of concentrations from 3-50 microns (9). Luteolin, like some other flavonoids, can stop the cell cycle of cancer cells at G1/S or G2/M checkpoints (10). It has been proven that luteolin can stop the cell cycle in G1 phase in the cells of human melanoma by inhibiting the activity of CDK2 (cyclin-dependent kinase 2), the enzyme that participates in progression of the cell cycle (11). Luteolin poses the potential to inhibit an angiogenesis by the suppression of the angiogenic factor VEGF (vascular endothelial factor) expression in the cancer cells. The antimetastatic effects of luteolin can be attributed to the suppression of the cytokines synthesis, such as TNFα (tumor necrosis factor α) and IL-6 (interleukin 6) involved in tumor cells migration and metastasis (10).

Regarding presented flavonoids bioactivity, the goal of this research was to determine the genotoxicity of delphinidin and luteolin in human lymphocyte cultures of peripheral blood using chromosome aberrations analysis.

METHODS

Tested substances

Delphinidin is an anthocyanin, and has the molecular mass of 338.69664 g/mol, with the molecular formula $C_{15}H_{11}O_7^+$. Delphinidin is a pigment, whose color varies from a purple-blue shade (pH 6-7) to a bright red shade (pH 1-3).

Luteolin is a flavone, and has the molecular mass of 286.2363 g/mol, with the molecular formula $C_{15}H_{10}O_6$. Luteolin is a common plant pigment, whose color is yellow.

Purple delphinidin powder (96.7% HPLC), in the form of delphinidin chloride (PhytoLab GmbH et Co. KG) and yellow luteolin powder (98.34% HPLC) (PhytoLab GmbH & Co. KG) were separately dissolved in DMSO (dimethyilsulphoxide) (Panreac Quimica, Barcelona, Spain). After initial 24 hours of the cultivation, prepared solutions were added in the proper separate cultures to the final concentrations of 25, 50 and 100 μM, determined according to the relevant literature (6,9,12,13), while negative controls were incubated with the same volume (10 μl) of the DMSO.

Chromosome aberrations analysis

Human lymphocyte cultures of 4 donors (2 ♂ i 2 ♀), healthy non-smokers of approximately the same age, were established immediately upon venipuncture of the cubital vein, in sterile vacutainers containing sodium heparin (BD Vacutainer Systems, Plymonth, UK). All participants in the study had signed the informed consent.

Cultures were set up by addition of 400 μl of whole blood in 5 ml of PBMAX™ Karyotyping Medium (GIBCO-Invitrogen, Carlsbad, CA, USA). Incubation lasted for 72h on 37°C (Cytoperm 8080, Heraeus, Germany). The cell division was blocked in metaphase by the colcemid treatment in the concentration of 0.18 μg/ml 90 minutes before the cell harvesting. Cell harvesting included hypotonic (0.75% KCl) treatment followed by centrifugation (1000 rpm for 10 minutes) and tripled of ice-cold acetic-alcohol fixative treatments and centrifugations. Cell suspension was dropped on ice-cold coded slides. Air-dried microscopic preparations were stained in 5% Giemsa stain in Gurr buffer (GIBCO-Invitrogen, Carlsbad, CA, USA).

Slides were analyzed on an Olympus BX51 microscope, on 1000x magnification. Analysis included observation of structural and numerical chromosome aberrations according to the International System for Human Cytogenetic Nomenclature. Structural aberrations were classified as: aberrations of chromosomal (chr) type (chrb-chromosome breaks, ace-acentric fragments), and aberrations of chromatid (cht) type (chtb-chromatid breaks) (14).

Since the reduction in metaphase spreads of cultures treated with luteolin was noticed, these slides

were additionally used to determine mitotic activity expressed as mitotic index (MI).

Statistical analysis

The mean, standard deviation, standard error of the mean, and variability coefficient were calculated using *Microsoft Excel 2007*. Proportion comparison (Z-test), using *Winks 4.5 Professional edition* (TexaSoft, Cedar Hill, Texas) was applied to determine significance of differences between treatments and controls.

RESULTS

The most common of the registered aberrations in delphinidin treated cultures were PCD (premature centromere division). At 100 μM, there was a PCD registered in each of 4 samples with the significant difference in comparison against controls (z=-2.005; p=0.045).

Summarized results of chromosome aberrations analysis in 400 metaphases (4 lymphocyte sample cultures) of controls and delphinidin treated cultures are presented in Table 1. Relative frequencies of observed chromosome aberrations in controls and lymphocytes cultures treated with tested concentrations of delphinidin are shown in Figure 1.

The most common of the registered aberrations in luteolin treated cultures were chromatid breaks. At 50 μM, significant increase in chromatid-type (cht) (z=-7.557; p=0.0), chromosome-type (chr) (z=-4.172; p=0.0) aberrations, hypodiploidies (2n-1) (z=-5.027; p=0.0) and hyperdiploidies (2n+1) (z=-2.711; p=0.007) was registered. Parallel, decrease in mitotic activity of lymphocytes was observed in concentration dependant manner. In untreated cultures mitotic index was 10.075%; 8.875% in cultures treated with 25 μM and 1.9% in cultures treated with 50 μM of luteolin while mitotic activity was completely inhibited in cultures treated with luteolin in concentration of 100 μM. Significantly decreased MI in cultures treated with 50 μM of luteolin caused poor slides quality and impossibility to analyze adequate number of metaphases.

Summarized results of chromosome aberrations analysis of controls and luteolin treated cultures are presented in Table 2. Relative frequencies of observed chromosome aberrations in controls and lymphocytes cultures treated with tested concentrations of luteolin are shown in Figure 2.

Discussion

The results of the chromosome aberration analysis and the associated statistical analysis have shown that delphinidin in tested concentrations does not significantly increase observed categories of aberrations, except PCD. These results are completely concordant with the previous research on delphinidin genotoxicity confirming that delphinidin is not genotoxic, even in extremely high concentrations

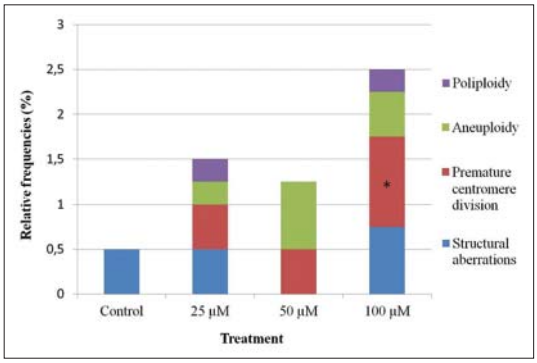

FIGURE 1. Relative frequencies of observed chromosome aberrations upon delphinidin treatment (* significantly different against the control, p < 0.05)

TABLE 1. Results of chromosome aberrations analysis upon delphinidin treatment

Treatment	N	Structural aberrations				Numerical aberrations			
		cht	chr	Σ	PCD	2n-1	2n+1	Σ	Polyploidy
Control	400	2	0	2	0	0	0	0	0
25 μM	400	0	2	2	2	0	1	1	1
50 μM	400	0	0	0	2	3	0	3	0
100 μM	400	1	2	3	4*	1	1	2	1

*Significantly different against the controls, p<0.05. Note: cht: Aberrations of chromatid type; chr: Aberrations of chromosomal type; 2n-1: Hypodiploidies; 2n+1: Hyperdiploidies

TABLE 2. Results of chromosome aberrations analysis upon luteolin treatment

Treatment	N	Structural aberrations				Numerical aberrations			
		cht	chr	chre	Σ	2n-1	2n+1	Σ	Polyploidy
Control	400	4	9	0	13	6	1	7	0
25 μM	400	65	8	0	73	8	2	10	1
50 μM	188	32*	19*	1	52*	20*	4*	24*	0

*Significantly different against the controls, p<0.05. Note: cht: Aberrations of chromatid type; chr: Aberrations of chromosomal type; chre: Chromosome exchanges; 2n-1: Hypodiploidies; 2n+1: Hyperdiploidies

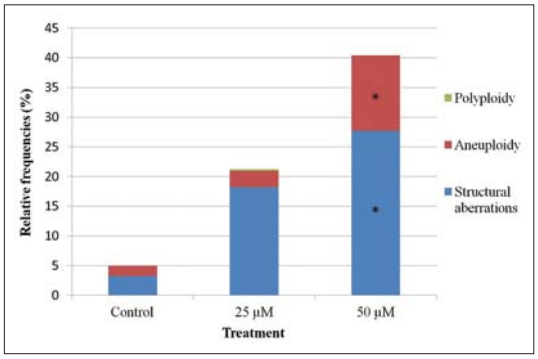

FIGURE 2. Relative frequencies of observed chromosome aberrations upon luteolin treatment (* significantly different against the control, p < 0.05)

(3,15,16). Opposing, it has been reported that delphinidin has a strong cytotoxic and cytostatic effects, especially in cancer cells (3-6). Although the mechanism of PCD has not been completely described, it is considered that the inhibition of topoisomerase II may be the basis and cytostatics are recognized as the main cause of PCD (17). Also, the significant increase of PCD frequencies is being associated with cytotoxic effect of delphinidin, assuming that delphinidin induces premature centromere division by inhibiting topoisomerase II. Playing the significant role in chromosome segregation during mitosis, topoisomerase II induces endoreduplication. Luteolin and delphinidin treatments of human lymphocyte cultures were previously reported to induce endoreduplications in the presence of halogenated boroxine (18).

Also, the potential to inhibit the topoisomerase II activity was previously confirmed for luteolin (12). However, in the presented research, the most significant effect of luteolin in human lymphocytes culture was inhibition of cell proliferation. It is known that luteolin is an effective inhibitor of some cancer cell proliferation and is also able to arrest the cell cycle in G1/S and G2/M checkpoints (10). In the concentration of 50 μM luteolin inhibits genotoxic effects induced by halogenated boroxine and reduce cell proliferation *in vitro* (18). Determined significant increase of structural chromosome aberrations as well as aneuploidies for lymphocytes cultures treated with luteolin in concentration of 50 μM, presents the important finding as chromosome aberrations are the primary genotoxicity biomarker associated with the increased cancer risk (19). The reduction of the mitotic activity could be the consequence of DNA synthesis inhibition or blocking of the cell cycle in G phase (20, 21).

CONCLUSION

Chromosome aberrations analysis of selected bioflavonoids in tested concentrations applied in human lymphocyte cultures has revealed that delphinidin is neither clastogenic nor aneugenic but the incidence of PCDs may indicate its impact on mitosis and especially topoisomerase II activity. However, luteolin exhibits genotoxic effects in concentration of 50 μM while the most considerable effect of luteolin is the reduction of cell proliferation revealing its remarkable cytostatic potential.

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