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Protective Effect on Human Lymphocytes of Some Flavonoids Isolated from Two *Achillea* Species

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Dedicated to Professor Werner Herz on the occasion of his 90th birthday.

This study was conducted to elucidate the *in vitro* protective effect of five flavonoids [apigenin (**1**), apigenin-7-*O*-glucoside (**2**), centaureidin (**3**), jaceidin (**4**) and quercetin (**5**)] against chromosomal damage in mitogen-induced human lymphocytes. Using the Cytochalasin-B blocked micronucleus (CBMN) assay, in which the biomarker of chromosome breakage and/or chromosome loss is the elevated frequency of micronucleus (MN) in binucleated (BN) cells, the presence of flavonoid **2** in minimal concentration (3 μg/mL) gave a 35.5% decrease in the frequency of MN when compared with control human lymphocytes. The same concentration of flavonoids **1**, **3** and **4**, reduced the MN frequency by 24.4%, 28.0% and 28.0%, respectively. Higher concentrations (6 μg/mL and 10 μg/mL) seemed less effective. Flavonoid **5** (3 μg/mL) induced a slight decrease in MN frequency (5%), while higher doses (6 μg/mL and 10 μg/mL) provoked an increase of DNA damage. The comparable values for the cytokinesis-block proliferation index (CBPI) of the tested flavonoids and positive control suggested an inhibitory effect on lymphocyte proliferation. In the DPPH˙ scavenging assay, flavonoids **1**-**4** demonstrated modest activity, in a dose-dependent manner, compared with the synthetic antioxidants BHT and Trolox, while **5** exhibited comparably high antioxidative activity.

Keywords: Flavonoids, CBMN, DPPH, BHT.

Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known. Many studies suggest that flavonoids exhibit biological activities, including antiallergenic, antiviral, antiinflammatory and vasodilatory. Most interest has been devoted to the antioxidant activity of flavonoids, due to their ability to reduce free radical formation and to scavenge free radicals [1a,1b].

Increased DNA damage is associated with illness such as Alzheimer's and Parkinson's disease. Micronuclei (MNs), which are biomarkers of chromosome breakage and/or whole chromosome loss, were significantly higher within peripheral blood lymphocytes of Alzheimer's [1c] and Parkinson's [1d] patients. Werner syndrome, often followed by osteoporosis, arteriosclerosis, diabetes and cancer is associated with increase of MNs in fibroblasts culture [1e]. The extent of DNA damage is successfully measured using the cytokinesis-block micronucleus (CBMN) assay. Micronuclei are efficiently expressed in dividing cells when chromosome breaks are induced by hydroxyl radicals [1f]. The CBMN technique enables micronuclei to be specifically scored in cells that have completed nuclear division and is, therefore, not influenced by variations in cell division kinetics [1g]. Currently, the CBMN assay is being used extensively to evaluate the presence and extent of chromosome damage (both clastogenic and aneuploid) in human populations that are exposed to genotoxic agents in different occupational settings, environments and lifestyles.

We employed the CBMN assay in normal human peripheral lymphocytes to evaluate the ability of apigenin (**1**), apigenin-7-*O*-glucoside (**2**), centaureidin

(**3**) and jaceidin (**4**), isolated from two *Achillea* species [2a,2b], along with commercially available quercetin (**5**), to protect human lymphocyte cells against chromosomal damage. The results were compared with a positive control (Amifostine) and control cell culture. Flavonoids **1-5** were also subjected to the DPPH$^{\bullet}$ scavenging assay and the results compared with the literature data for the synthetic antioxidants BHT and Trolox. In this study we found the lowest concentration (3 μg/mL) at which each of the tested flavonoids exerted a beneficial effect on control lymphocyte cells by decreasing the frequency of MN in comparison with the positive control, Amifostin WR 2721 (A-WR). Among the tested flavonoids, apigenin-7-*O*-glucoside (**2**) exibited the most prominent effect, decreasing the frequency of MN by 35.5%. The reductions of the MN frequency for flavonoids **1**, **3** and **4** were 24.4%, 28.0% and 28.0%, respectively, when compared with control cell cultures (calculated from Table 1).

The cell culture treated with amifostin WR-2721 (positive control) at a concentration of 1 μg/mL gave a decrease in the frequency of MN by 19.5% when compared with the control cell culture. Treatment of the cell culture with quercetin (**5**) at concentration of 3 μg/mL produced a decrease in the frequency of MN by 5.5% compared with control human lymphocytes, while 6 μg/mL and 10 μg/mL increased the MN frequency by 11% and 72%, respectively, compared with control human lymphocytes (Table 1).

Higher concentrations (6 and 10 μg/mL) of flavonoids **1-4** were less effective, compared with control human cells (Table 1). The reduction of the MN frequency for flavonoids **1**, **3** and **4** were 18.7%, 12.0%, and 16.3%, when 6 μg/mL were applied, and 4%, 4%, and 0.6%, for 10 μg/mL concentrations. Apigenin-7-*O*-glucoside (**2**), at concentrations of 6 and 10 μg/mL, decreased the MN frequency by 24.3% and 9.1%, respectively, compared with control human lymphocytes (Table 1). The number of micronuclei (MN) scored per 1000 binuclear (BN) cells increased in a dose dependant manner in experiments with 6 μg/mL and 10 μg/mL of flavonoids **1-4** (Table 2). These results are in accordance with a previous study of apigenin (**1**) in which lower doses (2,5-10 μg/mL) decreased the frequency of MN and thus protected human lymphocytes if added to cell cultures, while higher doses (25 μg/mL) increased the MN frequency exerting a possible toxic effect [2c].

The effect of flavonoids **1-5** on cell proliferation was investigated by determining the cytokinesis-block proliferation index (CBPI). Table 1 shows the mean CBPI values and the standard errors calculated for different concentrations of flavonoids. No significant difference in CBPI was observed between different concentrations of apigenin, apigenin-7-*O*-glucoside, centaureidin, jaceidin and quercetin (Figure 1). A similar conclusion had been drawn for three larger concentrations of apigenin (10 μM, 33 μM and 100 μM) [2d]. The comparable CBPI values for the compounds and positive control suggested an inhibitory effect on lymphocyte proliferation of the tested flavonoids. Furthermore, no signs of cytotoxicity of the flavonoids at the doses used in this study could be observed after trypan blue staining.

In this study we found that a protective effect against chromosomal damage in the CBMN assay was achieved with the lowest concentration used (3 μg/mL) of the tested flavonoids **1-4**, while higher concentrations (6 μg/mL and 10 μg/mL) were less effective. Since the number of micronuclei serves as an indicator of DNA damage, these results indicate that flavonoids isolated

Table 1: Cytokinesis-block proliferation index (CBPI) and micronuclei (MN) per 1000 binucleated (BN) cells for three different concentrations of flavonoids (**1-5**).

compd	Conc. (μg/mL)	CBPI	MN (Mean ± S.E.)
control	0	1.68 ± 0.05	25.43 ± 3.0
positive control	1	1.62 ± 0.01	20.46 ± 0.55
	3	1.59 ± 0.02	19.21 ± 0.52
1	6	1.63 ± 0.03	20.67 ± 1.73
	10	1.66 ± 0.01	24.42 ± 0.22 [b]
	3	1.58 ± 0.01	16.31 ± 0.86 [a; b]
2	6	1.64 ± 0.01	19.23 ± 1.34
	10	1.66 ± 0.02	23.12 ± 0.88 [b]
	3	1.59 ± 0.03	18.03 ± 2.07
3	6	1.65 ± 0.03	22.37 ± 0.70
	10	1.68 ± 0.05	24.41 ± 0.43 [b]
	3	1.60 ± 0.01	18.31 ± 0.83
4	6	1.66 ± 0.03	21.28 ± 0.54
	10	1.68 ± 0.02	25.27 ± 0.35 [b]
	3	1.71 ± 0.01	24.04 ± 2.04
5	6	1.70 ± 0.02	28.06 ± 2.63 [b*]
	10	1.64 ± 0.02	43.82 ± 2.36 [a*; b]

The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Difference was considered significant at $p < 0.05$.
a → compared with control groups, statistically significant difference p<0.05; a* → compared with control groups, statistically significant difference p<0.01; b→ compared with amifostine – WR 2721, statistically significant difference p<0.01; b* → compared with amifostine – WR 2721, statistically significant difference p<0.05

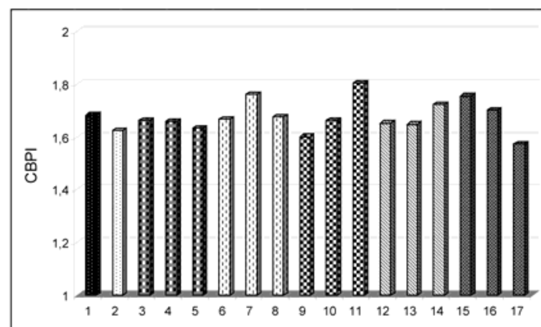

Figure 1: Cytokinesis-block proliferation index (CBPI) in cultured human lymphocytes: control (1); positive control amifostine WR-2721 (2). Flavonoids in concentrations of 3 μg/mL, 6 μg/mL and 10 μg/mL: apigenin (3-5); apigenin-7-*O*-glucoside (6-8); centaureidin (9-11); jaceidin (12-14); quercetin (15-17).

Table 2: Distribution of micronuclei (MN) in binucleated (BN) human lymphocytes for three different concentrations of flavonoids (**1**-**5**).

compd	conc. (μg/mL)	DISTRIBUTION 0	1	2	3	4
control	0	4079	81	11	1	0
Positive control	1	4031	67	7	1	0
	3	4043	60	8	1	0
1	6	4045	56	9	1	2
	10	4066	51	13	4	3
	3	4030	46	9	1	0
2	6	4045	60	13	1	0
	10	4032	63	10	4	0
	3	4037	58	8	0	0
3	6	4055	58	11	2	1
	10	4054	70	9	3	1
	3	4028	61	5	0	1
4	6	4053	70	9	0	0
	10	4034	63	12	3	2
	3	4031	75	6	4	0
5	6	4099	81	12	3	1
	10	4090	159	14	0	0

from extracts of *Achillea* species, especially apigenin-7-*O*-glucoside, protected DNA in human lymphocytes. We observed that quercetin (**5**) exerted an opposite effect, inducing an increase of DNA damage at higher concentration. It has already been shown that quercetin induces chromosomal damage in the CBMN assay using a lymphoblastoid cell line [3a]. In previous studies, the CBMN assay using a lymphoblastoid cell line has been established as an effective system to detect ROS-induced chromosomal damage [3b].

The ability of flavonoids **1-5** to scavenge the DPPH radical was tested, and the concentrations of the flavonoids varied between 0.25-5.5 mM. The scavenging effect of **1-4** on the DPPH radical increased linearly with concentration, reaching EC_{50} values at 1.88 ± 0.02 mM, 4.69 ± 0.03 mM, 2.17 ± 0.02 mM and 1.13 ± 0.01 mM, respectively. Quercetin (**5**) demonstrated a strong scavenging effect with an EC_{50} value of 0.012 ± 0.001 mM. These values were also compared with literature data for the synthetic antioxidants BHT (0.028 ± 0.001 mM) and Trolox (0.027 ± 0.001mM) [3c]. The strong scavenging effect of **5** is probably due to the presence of 3'-OH attached to the 2',3'-double bond with a carbonyl in the 4' position, as well as the 4,5-diphenolic structure of ring B [3d]. If the hydroxy groups at positions 3',4,5- were either blocked or not present in the molecule, the scavenging activity would be weakened, as in the case of flavonoids **1-4**.

Flavonoids **1-4** isolated from *Achillea* species showed a protective effect against chromosomal damage in the CBMN assay in diverse proportion to the tested concentrations, while their mild $DPPH^{\bullet}$ scavenging capacity was directly proportional to the tested concentrations. Strong $DPPH^{\bullet}$ scavenging capacity was observed for **5**, while higher concentrations induced an increase of DNA damage in the CBMN assay. No signs of cytotoxicity of flavonoids **1-5** at the doses used in this study could be observed, but an inhibitory effect on lymphocyte proliferation was noticed. The present results demonstrate that flavonoids **1-4** could have potential pharmacological importance.

Experimental

Subjects: Venus blood samples were withdrawn into sterile heparinized vacutainer tubes (Becton Dickinson, Bradford, MA) from 6 healthy, non-smoking, male volunteers who had not been exposed to chemicals, drugs or other substances. A safety protocol concerning blood-born pathogen/biohazard was applied. The volunteers gave their permission for using their blood for the experiment. From each subject, 2 aliquots of blood, each of 5 mL, were obtained. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). The blood samples were obtained at the Medical Unit in accordance with current Health and Ethical regulations in Serbia (Law on Health Care, 2005).

Cytokinesis-block micronucleus (CBMN) assay: The culture lymphocytes were treated with flavonoids **1-4**, isolated and purified, as described [2a,2b], and purchased quercetin (**5**) (Sigma-Aldrich, Vienna, Austria). Three concentrations of flavonoids were used, i.e. 3 μg/mL, 6 μg/mL and 10 μg/mL. One cell culture served as the control and, to this, flavonoid was not added. Amifostine WR- 2721 (S-2[3-aminopropylamino]-ethylphosphothioic acid; Marligen-Biosciences, USA) was added to one culture tube at a concentration of 1 μg/mL; this served as a positive control. All cultures were incubated at 37ºC. Treatment with flavonoids **1-5** lasted for 19 h, because it has been calculated that this is the time duration necessary for the flavonoids ingested by the organism *per se* to be metabolised and excreted. Consequently, all cultures were rinsed with pure medium, transferred into 5 mL fresh RPMI 1640 medium (RPMI 1640 Medium + GlutaMAX + 25mM HEPES; Invitrogen- Gibco-BRL, Vienna, Austria) and incubated for an additional 72 h.

The CBMN test was performed according to a described procedure [1f]. Lymphocytes were stimulated with 2.4 μg/mL of phytohemaglutinin (Invitrogen- Gibco-BRL, Vienna, Austria). Briefly, 44 h affter culture initiation, Cytochalasin B (Cyt-B; Invitrogen-Gibco-BRL, Vienna, Austria) was added to each culture tube, resulting in a final concentration of 6 μg/mL. Cells were harvested 28 h after the addition of Cyt-B. The total culture time was 72 h. This resulted in the formation of many first division binucleated cells that were scored for the induction of MN. After incubation for 72 h, the cultures were harvested and fixed in 3 changes of

methanol/acetic acid (3:1 v/v). The cells were spread onto glass slides (2 slides for each culture), dried and stained with alkaline Giemsa (Sigma-Aldrich, Vienna, Austria). Experiments were repeated on the samples of blood obtained from each donor and the results were summarized. MN were scored in 1000 binucleated cells per slide, registering MN according to established criteria [3e]

The effects of flavonoids **1-5** on cell proliferation were determining from the cytokinesis-block proliferation index (CBPI). The CBPI was calculated as suggested by Surralles [3f], i.e. CBPI = [(*M*I +2*M*II + 3(*M*III + *M*IV)] / *N*, where *M*I-MIV represents the number of cells with 1-4 nuclei, and *N* is the number of cells scored. For the analysis of MN, only binucleated cells with well-preserved cytoplasm were scored for MN (under a light microscope with a 40 x 10 magnification). The criteria for selection of binucleated cells and identification of MN given in the HUMAN project website [http://www.humn.org] were followed. The numbers of binucleated cells with 1, 2, 3 and more MN were then tabulated. The data for each treatment were expressed as the frequency of MN per 1000 binucleated cells.

DPPH• scavenging assay: Flavonoids **1-5** were subjected to the DPPH• scavenging assay according to the described procedure [3g]. The samples were prepared by addition of various concentrations of flavonoid in MeOH (300 μL) to 0.1 mg/mL methanolic solution of DPPH (300 μL). Absorbance at 517 nm was measured after 30 min. The inhibition percentage was calculated using the following equation:

$$I = [(Ac - As)/Ac]x100$$

where *I* is the inhibition percentage, *Ac* is the absorbance of the negative control (flavonoid was omitted in the sample), and *As* is the absorbance of the sample. Trolox and *tert*-butyl hydroxytoluene (BHT) were used as positive controls [3c]. The inhibition percentage was plotted against concentration of the samples, and EC_{50} values were determined by linear regression analysis. The results are expressed as means ± standard deviation of three determinations.

Statistical analysis: The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA), followed by the Tukey test. Statistical difference was considered significant at $p < 0.05$. The index calculating is presented as the % of change comparing different groups.

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References

[1] (a) Saija A, Scalese M, Lanza M, Marzullo D, Bonina F, Castelli F. (**1995**) Flavonoid as antioxidant agents: importance of their interaction with biomembranes**.** *Free Radical Biology & Medicine*, ***19***, 481-486; (b) Nijveldt JR, van Nood E, van Hoorn ECD, Boelens GP, van Norren K, van Leeuwen AMP. (**2001**) Flavonoids: a review of probable mechanisms of action and potential applications. *American Journal of Clinical Nutrition*, ***74***, 418-425; (c) Migliore L, Testa A, Scarpato R, Pavese N, Petrozzi L, Bonuccelli U. (**1997**) Spontaneous and induced aneuploidy in peripheral blood lymphocytes of patients with Alzheimer's disease. *Human Genetics*, ***101***, 299–305; (d) Petrozzi L, Lucetti C, Gambaccini G, Bernardini S, Del Dotto P, Migliore L, Scarpato R, Bonuccelli U. (**2001**) Cytogenetic analysis oxidative damage in lymphocytes of Parkinson's disease patients. *Neurological Science*, ***22***, 83-84; (e) Scappaticci S, Forabosco A, Borroni G, Orecchia G, Fraccaro M. (**1990**) Clonal structural chromosomal rearrangements in lymphocytes of four patients with Werner's syndrome. *Annales de genetique*, ***33***, 5-8; (f) Fenech M, Morley AA**.** (**1985**) Measurement of micronuclei in lymphocytes. *Mutation Research*, ***147***, 29-36; (g) Fenech M, Morley AA. (**1991**) Optimization of micronucleus assay for biological dosimetry. *Progress in Clinical Biological Research*, ***372***, 373-386.

[2] (a) Aljančić, I., Vajs, V, Menković N, Karadžić I, Juranić N, Milosavljević S, Macura S. (**1999**) Flavones and sesquiterpene lactones from *Achillea atrata* subsp. *multifida*: antimicrobial activity. *Journal of Natural Products*, ***62***, 909-911; (b) Milosavljević S, Aljančić I, Macura S, Milinković D, Stefanović, M. (**1991**) Sesquiterpene lactones from *Achillea crithmifolia*. *Phytochemistr*y, ***30***, 3464-3466; (c) Rithidech KN, Tungjai M, Whorton EB. (**2005**) Protective effect of apigenin on radiation-induced chromosomal damage in human lymphocytes. *Mutation Research*, ***585***, 96-104; (d) Noel S, Kasinathan M, Rath KS. (**2006**) Evaluation of apigenin using *in vitro* cytochalasin blocked micronucleus assay. *Toxicology in Vitro*, ***20***, 1168-1172.

[3] (a) Saito A, Sugisawa A, Umegaki K, Sunagawa. (**2004**) .Protective effects of quercetin and its metabolites on H_2O_2-induced chromosomal damage to WIL2-NS cells**.** *Bioscience, Biotechnology, and Biochemistry*, ***68***, 271-276; (b) Umegaki K, Fenech M. (**2000**) Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis*, ***15***, 261-269; (c) Gođevac D, Vujisić Lj, Mojović M, Ignjatović A, Spasojević I, Vajs V. (**2008**) Evaluation of antioxidant capacity of *Allium ursinum* L. volatile oil and its effect on membrane fluidity. *Food Chemistry*, ***107***, 1692-1700; (d) Rice-Evans CA, Miller NJ. (**1996**) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, ***20***, 933-56; (e) Fenech M, Morley AA**.** (**1993**) The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutation Research*, ***285***, 35-44; (f) Surralles J, Xamena N, Creus A, Catalan J, Norppa H, Marcos R. (**1995**) Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. *Mutation Research*,***341***, 169-184; (g) Blois MS. (**1958**) Antioxidant determinations by the use of a stable free radical. *Nature*, ***181***, 119-1200.