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In vitro Evaluation of Antigenotoxic Effects of Phloridzin

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ABSTRACT

Phytochemicals have a vast number of properties contributing to human health by acting on numerous different mechanisms. Phloridzin, a phytochemical mainly found in *Malus* species, possesses diverse biological activities including anti-diabetic and antioxidative activities. Here, our aim is to explore antigenotoxic potential and proliferative effects of phloridzin on human lymphocytes *in vitro* by employing chromosome aberration, micronucleus and comet assays. Mitomycin C, both an anticancer and genotoxic agent, was utilized to induce genotoxicity. Phloridzin significantly suppressed the genotoxic effects of mitomycin C at 125-500 µg/mL concentrations in all assays used *($p < 0.05$)*. We also revealed that phloridzin and mitomycin C combination had a significantly negative effect on mitotic index *($p < 0.05$)*, whereas in general, gender differences did not play a role in manifestation of neither antigenotoxic nor antiproliferative activities of the combination.These results suggest that phloridzin is an antigenotoxic compound and its consumption may interfere with the activity of anticancer drugs that exert their effects based on genotoxic mechanisms.

Keywords: Phlorizin, Genotoxicity, Mitomycin C, Chromosome aberration.

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Introduction

Numerous plants have been used in the field of medicine for therapeutic purposes. For many decades, phytochemicals, bioactive components of plant origin, have been under investigation because of the beneficial effects in maintenance of human health [1]. Many studies report that a diet rich in phytochemicals is directly associated with reduced risk of chronic diseases including cancer [2-4].

Phloridzin, a phytochemical belonging to the group of dihydrochalcones, is the predominant phenolic compound in *Malus* species [5]. Phloridzin is formed by the glycosylation of phloretin and was detected in several other plants such as Australian native sarsaparilla (*Smilax glyciphylla*), sweet tea (*Lithocarpus polystachyus*), and at trace amounts in strawberry fruit [6-8]. Phloridzin is found at highest levels in seeds, moderate levels in core and the skin and lowest level in the cortex of apple fruits [9]. Phloridzin and its derivatives are renowned for their activity to inhibit sodium-linked glucose transporters which in turn lowers blood glucose levels [9]. Accordingly, numerous studies have been conducted to understand the significance of phloridzin in treating type 2 diabetes [10,11]. Moreover, phloridzin has been studied for antioxidative, anti-inflammatory, hepatoprotective and cardioprotective activities [12-15]. Although phloridzin has been examined for its biological activities from various aspects, antigenotoxic properties of this molecule have not been studied so far. Our aim is to determine the potential genotoxic and antigenotoxic effects of phloridzin *in vitro* in human lymphocytes by employing chromosome aberration test, micronucleus and comet assays.

Materials and Methods

Chemicals and Lymphocyte Culture

Phloridzin (catalogue number PHL80513) was purchased from Merck, Germany. Phloridzin was dissolved in ethanol and diluted with distilled H_2O. The stock solution was kept at -20°C for further use. Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo, Japan and its stock solution was freshly prepared at 250 µg/mL concentration before each experiment. MMC was diluted 1:1000 upon addition to lymphocyte culture to a final concentration of 0.25 µg/mL.

Lymphocyte culture was set up by adding 0.5 mL of heparinized blood to 5 mL of RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum (Biochrom, AG), 1% penicillin/streptomycin (Merck, Darmstadt, Germany), 2% L-Glutamine (Merck, Darmstadt, Germany) and 2 to 10 µg/mL concentration of phytohemagglutinin (Biochrom AG, Germany). The culture media was stored at -20°C.

Human peripheral blood lymphocytes were obtained from 2 female and 2 male healthy, non-smoking

volunteers with no recent history of medication. Peripheral blood samples (5 mL) were collected under sterile conditions by venipuncture into heparinized tubes. Whole blood (0.5 mL) was then added to 5 mL culture medium and incubated at 37°C.

Ethanol was used as solvent control (1:1000). Phloridzin concentrations were determined as 125, 250 and 500 µg/mL after a preliminary study. All phloridzin doses were used in simultaneous combinations with a single dose of MMC (0.25 µg/mL) [16]. Treatments started 48 h (24 h for comet assay) after the beginning of incubation at 37°C. Lymphocyte cultures were treated with phloridzin and/or MMC for 24 h before harvest in all assays.

Chromosome Aberration (CA) Test

Briefly, 2 h prior to harvesting, 50 µl of demecolcine (10 µg/mL) was added to each culture medium. The cells were incubated with a hypotonic solution (0.075 M KCl) and then fixed in cold methanol:glacial acetic acid (3:1). Following a series of 3 washing steps with the fixative, metaphase spreads were prepared by dropping the concentrated cell suspension onto ice-cold slides. Air-dried slides were stained with Giemsa (pH 6.8) stain for 15 min and examined under a light microscope. A total of well-spread, 100 metaphases were scored in each group for CA analysis and the frequency of CA per cell was recorded. The CAs were scored according to Environmental Health Criteria 51 for short-term tests for mutagenic and carcinogenic chemicals [17]. Mitotic index (MI) was calculated according to following formula:

MI = (number of cells in mitosis / total number of cells) × 100.

Micronucleus (MN) Assay

The assay was carried out as described by Fenech and Morley [18] with minor modifications. Cytochalasin B (6 µg/mL) was added at 44 h to block cytokinesis. Lymphocyte cultures were harvested after 72 h. MN value was scored as the frequency of binucleated lymphocytes containing MN per 1000 binucleated cells. Nuclear division index (NDI) was calculated among the 500 lymphocytes that were scored in MN assay according to the following equation:

NDI = (MONO + (2 × BN) + (3 × TRI) + (4 × TETRA)) / 500.

Here, MONO, BN, TRI and TETRA depict the number of mononucleated, binucleated, trinucleated and tetranucleated lymphocytes, respectively.

Comet Assay

Alkaline single cell gel electrophoresis was carried out according to the procedure originally developed by Singh et al. [19] with minor modifications. Shortly, the cells were centrifuged at 500 rpm for 4 min at the end of treatment. Subsequently, the cells were suspended with pre-warmed 100 µl low melting agarose and immediately pipetted onto slides precoated with normal melting agarose. Then the slides were immersed in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO) and kept overnight in the dark at 4°C. The slides were then placed in an alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA; pH > 13) and the embedded cells were exposed to this alkali solution for 15 min to allow for DNA unwinding.

Electrophoresis was performed at 0.75 V/cm (25 V, 300 mA) for 25 min. After electrophoresis, the slides were neutralized with 0.4 M Tris at pH 7.5. The slides were stained with ethidium bromide (2 µg/mL) and 100 cells per group were examined under a microscope with fluorescence attachment (Novel N-800M). Imaging was performed by using software (Kameram 21, Argenit, Istanbul). Comet tail length, tail DNA %, Olive tail moment, percentage of damaged cells (PDC) and DNA damage index (DDI) parameters were evaluated for each cell. DDI was calculated according to the following formula [20]:

DDI = (1 × ΣType1) + (2 × ΣType2) + (3 × ΣType3) + (4 × ΣType4) / (ΣType0 + ΣType1 + ΣType2 + ΣType3 + ΣType4)

PDC was calculated according to the following formula [20]:

%Damaged Cells = ΣType2 + ΣType3 + ΣType4

Statistical Analysis

All statistical analyses were performed using the SPSS 23.0 statistical software for Windows. Results were expressed as mean ± standard deviation. Kolmogorov-Smirnov normality test was applied to evaluate the distribution characteristics of the variables. Parametric one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis analyses were selected throughout the study depending on the normality test results. Intergroup differences were determined by Tukey HSD or Tamhane's T2 post hoc tests following one-way ANOVA analysis. Paired sample *t*-tests were employed to determine whether there was a significant difference between genders in each treatment group in all assays. Two-sided $p < 0.05$ was considered to be statistically significant.

Results

Potential Genotoxic and Antigenotoxic Effects of Phloridzin

Mean frequencies of observed chromosome gaps, breaks, rings, exchanges and the total number of CAs per cell were given in Table 1. Phloridzin alone caused a statistically significant increase in total CA frequencies compared to solvent control group at 500 µg/mL concentration ($p < 0.05$). Mean value for total CA aberration per cell in MMC treatment group was slightly higher than that of control groups, but the difference was not significant ($p > 0.05$). On the other hand, significantly elevated total CA frequencies were detected in all combination treatments in contrast to negative and solvent controls, and also compared to MMC treatment alone ($p < 0.05$). Fig. 1A displays the results of total CA frequencies by gender. In general, there were no significant differences between genders across all treatment groups ($p > 0.05$) except MMC and 500 µg/mL phloridzin combination ($p < 0.05$).

Table 1. CA and MI values of lymphocyte cultures treated with phloridzin and/or MMC.

Dose (µg/mL)	N [b]	Chromosome Gap (%)	Chromosome Breakage (%)	Chromosome Ring (%)	Chromosome Exchange (%)	Total Aberration / Cell	MI (%)
NC	4	0.56±0.06	0.45±0.09	0.03±0.01	1.24±0.43	0.57±0.12	5.91±1.26
SC	4	0.63±0.18	0.58±0.04	0.11±0.02[c]	1.47±0.60	0.70±0.17	6.55±0.04
125 µg/mL Phl[a]	4	1.09±0.18	0.45±0.04	0.13±0.01[c]	2.63±0.87	1.08±0.24	6.80±0.83
250 µg/mL Phl	4	1.33±0.04[c]	0.75±0.06	0.05±0.02	2.97±1.26	1.28±0.32[c]	7.12±0.93
500 µg/mL Phl	4	1.39±0.28	0.87±0.23[c]	0.12±0.04	4.39±1.08[cf]	1.70±0.38[cd]	7.73±0.85
MMC	4	1.03±0.56	0.81±0.25	0.07±0.01[c]	1.89±1.03	0.95±0.45	5.26±0.81
MMC +125 µg/mL Phl	4	1.49±0.14[cd]	1.10±0.26[cd]	0.15±0.03	5.55±0.92[cde]	2.07±0.26[cde]	2.40±0.74[cde]
MMC + 250 µg/mL Phl	4	1.73±0.18[cd]	1.11±0.03[cd]	0.13±0.06	5.67±0.7[cde]	2.16±0.19[cde]	2.60±1.27[cde]
MMC + 500 µg/mL Phl	4	1.65±0.14[cd]	0.91±0.15[c]	0.13±0.03	5.99±0.59[cde]	2.17±0.16[cde]	2.23±0.98[cde]

MI: Mitotic index; NC: Negative control; SC: Solvent control; a: Phloridzin; b: Number of donors; c: $p < 0.05$ compared to negative control; d: $p < 0.05$ compared to solvent control; e: $p < 0.05$ compared to MMC alone.

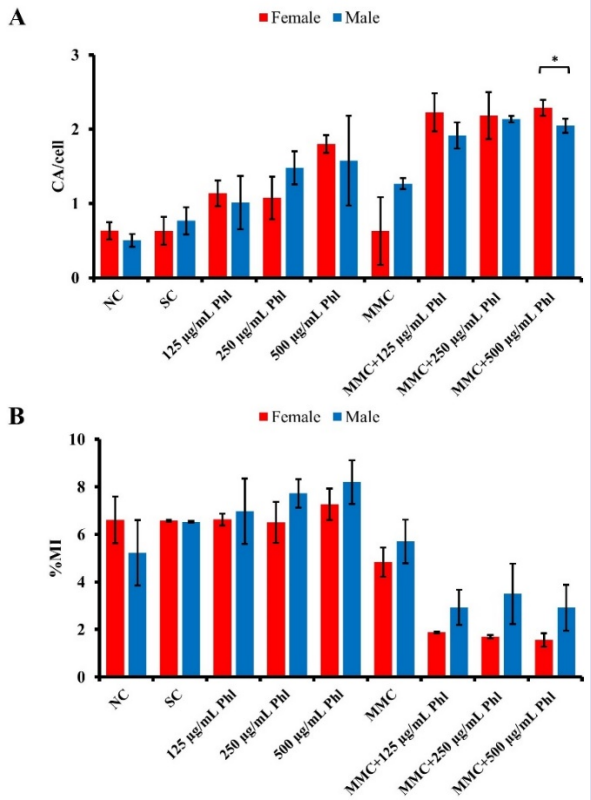

Figure 1. CA (A) and MI (B) values by genders of lymphocyte cultures treated with phloridzin and/or MMC. CA: Chromosome aberration; MI: Mitotic index; NC: Negative control; SC: Solvent control; Phl: Phloridzin; *: p < 0.05.

The mean MN (‰) frequencies scored in four donors were given in Table 2. Phloridzin treatments alone did not cause a significant MN formation compared to solvent control at all doses (p > 0.05). MMC treatment alone induced MN induction approximately 4 to 7-fold compared to control groups (p < 0.05, Table 2). In contrast, MMC combination with phloridzin (125-500 µg/mL) resulted in significantly diminished MN frequencies (p < 0.05, Table 2 and Fig. 2A). The effectiveness of phloridzin in MMC induced MN reduction was more evident at higher doses. MN frequencies by genders in response to treatments were presented in Fig. 2A. The findings reveal that there is no significant difference in MN frequencies across genders in treatment groups (p > 0.05). A significant difference between genders was detected in negative control group (p < 0.05); however, that difference did not appear in solvent control group.

Table 2. MN and NDI values of lymphocyte cultures treated with phloridzin and/or MMC.

Dose (µg/mL)	N[b]	NDI	MN (‰)
NC	4	2.72±0.61	4.08±3.42
SC	4	2.79±0.64	7.87±2.17
125 µg/mL Phl[a]	4	2.77±0.93	10.07±0.59
250 µg/mL Phl	4	3.65±0.24	13.59±2.60[c]
500 µg/mL Phl	4	3.19±0.70	11.34±1.07
MMC	4	2.55±0.19	28.85±7.72[cd]
MMC + 125 µg/mL Phl	4	3.31±0.09	9.30±1.81[e]
MMC + 250 µg/mL Phl	4	2.52±0.21	4.44±5.42[e]
MMC + 500 µg/mL Phl	4	2.95±0.26	5.54±1.92[e]

NDI: Nuclear division index; MN: Micronucleus; NC: Negative control; SC: Solvent control; a: Phloridzin; b: Number of donors; c: $p < 0.05$ compared to negative control; d: $p < 0.05$ compared to solvent control; e: $p < 0.05$ compared to MMC alone.

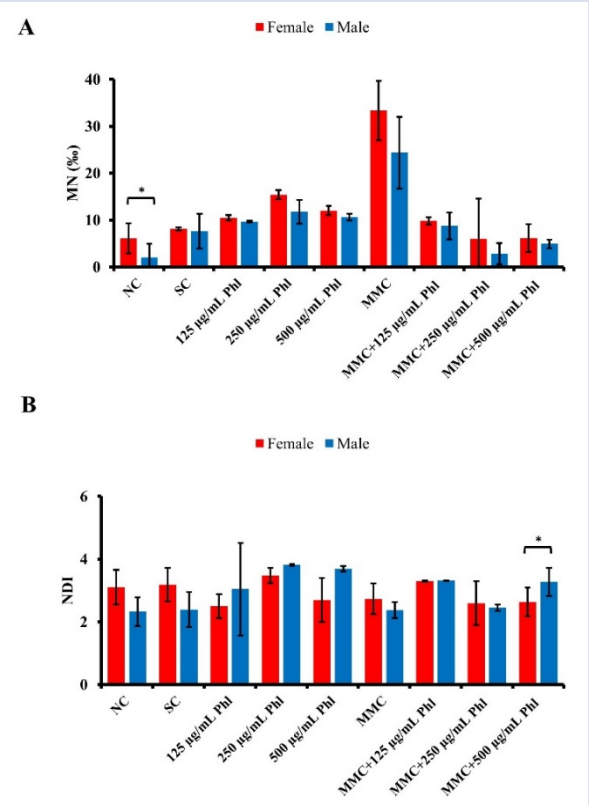

Figure 2. MN (A) and NDI (B) values by genders of lymphocyte cultures treated with phloridzin and/or MMC. MN: Micronucleus; NDI: Nuclear division index; NC: Negative control; SC: Solvent control; Phl: Phloridzin; *: $p < 0.05$.

The findings of comet assay were expressed as the changes in the comet tail length, percentage of DNA in tail, Olive tail moment, DDI and PDC (Table 3). There were no

significant changes detected among any of the comet parameters compared to solvent control ($p > 0.05$) after phloridzin treatment except an increase in tail DNA percentage at 500 µg/mL concentration ($p < 0.05$). MMC dramatically raised the values of all DNA damage associated comet parameters ($p < 0.05$). On the other hand, when phloridzin was used in combination with MMC, it significantly suppressed the DNA damage induced by MMC at all doses ($p < 0.05$). Moreover, both 125 and 250 µg/mL concentrations were effective in suppressing the DNA damage to the level observed in the control groups. Only combination treatment with 500 µg/mL phloridzin induced DNA damage at a level that is both significantly higher than solvent control and lower than MMC alone according tail DNA % and PDC values ($p < 0.05$). The effects of gender differences on DDI and PDC were represented in Fig. 3A and B, respectively. In the context of gender differences in response to treatments, there was no significant change between genders in DDI and PDC values ($p > 0.05$) apart from the DDI values after 500 µg/mL phloridzin treatment ($p < 0.05$).

Table 3. DDI and PDC values after comet assay of lymphocyte cultures treated with phloridzin and/or MMC.

Dose (µg/mL)	N[b]	Tail length (µm)	Tail DNA (%)	Olive tail moment	DDI	PDC
NC	4	3.13± 0.49	2.09±0.28	0.20±0.03	0.12±0.03	0.01±0.01
SC	4	3.30±0.21	1.56±0.68	0.20±0.11	0.11±0.06	0.01±0.01
125 µg/mL Phl[a]	4	3.21±0.35	2.46±0.28	0.37±0.09	0.21±0.04	0.03±0.01
250 µg/mL Phl	4	3.34±1.48	2.07±0.51	0.29±0.23	0.16±0.08	0.01±0.01
500 µg/mL Phl	4	4.23±0.45	3.37±0.14[d]	0.38±0.18	0.18±0.07	0.02±0.02
MMC	4	5.30±1.09[cd]	4.92±1.13[cd]	0.70±0.20[cd]	0.55±0.16[cd]	0.13±0.04[cd]
MMC + 125 µg/mL Phl	4	2.65±0.97[e]	2.33±0.70[e]	0.23±0.04[e]	0.15±0.04[e]	0.01±0.01[e]
MMC + 250 µg/mL Phl	4	3.09±0.54[e]	2.58±0.48[e]	0.26±0.08[e]	0.17±0.08[e]	0.03±0.03[e]
MMC + 500 µg/mL Phl	4	2.67±0.25[e]	3.00±0.42[de]	0.36±0.12[e]	0.25±0.05[e]	0.05±0.01[cde]

DDI: DNA damage index; PDC: percentage of damaged cells; NC: Negative control; SC: Solvent control; a: Phloridzin; b: Number of donors; c: $p < 0.05$ compared to negative control; d: $p < 0.05$ compared to solvent control; e: $p < 0.05$ compared to MMC alone.

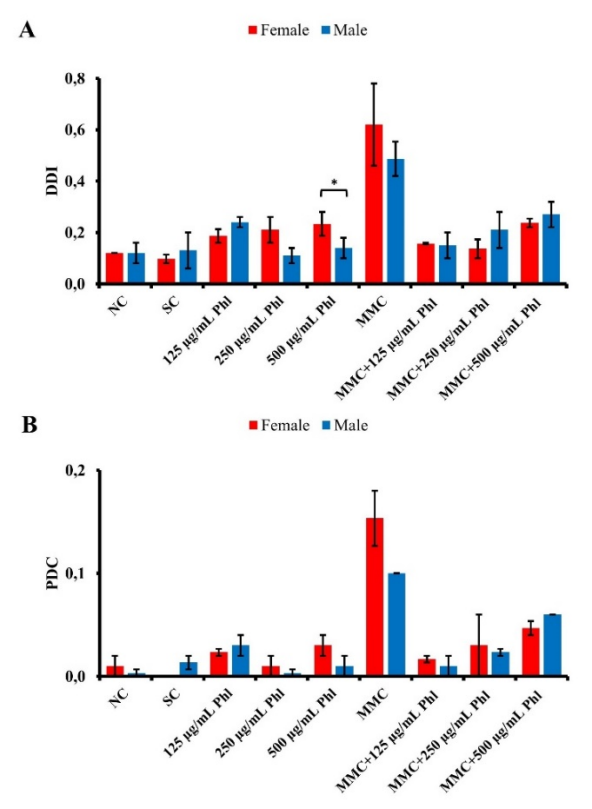

Figure 3. DDI (A) and PDC (B) values by genders after comet assay of lymphocyte cultures treated with phloridzin and/or MMC. DDI: DNA damage index; PDC: percentage of damaged cells; NC: Negative control; SC: Solvent control; Phl: Phloridzin; *: $p < 0.05$.

Effect of Phloridzin on Proliferative Activity

Change in MI values is presented in Table 1 and Fig. 1B. Phloridzin alone heightened MI values compared to control groups at all concentrations ($p > 0.05$). However, MMC alone decreased MI value compared to control groups ($p > 0.05$). Interestingly, all of the MMC and phloridzin combination treatments resulted in a remarkable decrease in MI values compared to both control groups and MMC treatment alone ($p < 0.05$, Table 1). When gender differences in response to treatments were examined, there were no significant differences throughout all of the treatments, but of note, female group suffered a steeper decline in MI after combination treatments ($p > 0.05$, Fig. 1B).

NDI remained more or less the same in all treatment groups compared to solvent control (Table 2). Data displayed in Fig. 2B on gender differences in response to treatments of phloridzin and/or MMC were analogous to those presented in Table 2. There was a statistically significant difference between male and female groups only in MMC and 500 µg/mL phloridzin combination ($p < 0.05$, Fig. 2B).

Discussion

Phytochemicals have been utilized for both their therapeutic and preventive characteristics in traditional and modern medicine [21]. Also, research suggests that apple consumption is involved in lowering the risk of severe chronic diseases in general [22]. Thus, it becomes

meaningful to intensify the research on elucidating the phytochemicals responsible for the aforementioned effects in order to maximize health benefits. High phytochemical content provides apple with its antioxidant properties and the consumption of apple juice is reported to have antigenotoxic potential [23]. Apple is rich in phloridzin that is mainly found in *Malus* species [24]. Therefore, we investigated the potential genotoxic and antigenotoxic effects of phloridzin *in vitro* in human lymphocytes by employing several genotoxicity assays.

Slightly increased values of total CA, MN, DDI and PDC in almost all selected doses of phloridzin suggest that this molecule might have genotoxic effects above a certain threshold concentration. This observation becomes more pronounced when 500 µg/mL phloridzin treatment is considered since it led to significant increase in total CA per cell and tail DNA % parameters compared to solvent control ($p < 0.05$). Phenolic compounds such as curcumin, vanillic acid and vitamin C at certain concentrations might be regarded as other examples for a phytochemical to exert genotoxic effects alone [25-27]. This phenomenon was also observed when Fox et al. [28] employed a novel cell based ATAD-5 luciferase assay to detect genotoxic compounds and identified resveratrol, genistein and baicalein as DNA damage inducers. Moreover, Lu [29] et al. showed that epigallocatechin gallate, an exemplary antioxidant found in green tea, induced DNA damage via interaction of its weakly-bound electrons with DNA bases (especially guanine) through a reductive mechanism. Therefore, it is crucial to acknowledge that while antioxidants can minimize the detrimental effects of ROS, they alone may have the capability to induce DNA damage at certain concentrations.

In our study, MMC was selected as a genotoxic agent as it generates oxidative stress and induces DNA damage via DNA alkylation [30]. As expected, MMC treatment resulted in increased genotoxic effects in all genotoxicity assays (Tables 1-3). Combination of phloridzin with MMC mitigated the genotoxic activity of this agent significantly in all assays regardless of the selected phloridzin concentration ($p < 0.05$, Tables 1-3). The extent to which phloridzin mitigated genotoxic effects caused by MMC varied between the assays used. In both MN and comet assays, DNA damage was suppressed to the level observed in the control groups (Tables 2 and 3). However, phloridzin was not as effective in mitigating the genotoxic effects as it was in MN and comet assays at reducing CAs. The resulting total CAs per cell after MMC and phloridzin combinations were significantly higher than that of solvent control ($p < 0.05$, Table 1). This difference can be examined by looking further into the applied methods. CA presence is acknowledged as an evidence of major structural and/or numerical aberrations at chromosomal level [31]. Other types of CAs—chromosome or chromatid gaps and breaks arise from unrepaired double strand DNA damage whereas chromosome rings and exchanges originate from mis-repaired DNA damage [32]. In contrast, MN originates from whole or acentric chromosome fragments which manifests genomic instability at chromosomal level caused by not only DNA damage, but also distortions in components of mitotic apparatus such as mitotic spindle or centrosomes [33]. Comet assay focuses on analysis of repairable DNA damage whether it is a single- or double-strand breakage, but this test is unable to give information at chromosomal level such as in CA and MN [34]. Therefore, the differences in our results can be explained by the fact that CA, MN and comet assays measure the genotoxic effect of an agent indirectly in different manners and due to the different evaluation sensitivity of the assays. Limited observation of significant change involving gender differences in response to treatments suggests that phloridzin genotoxicity or antigenotoxicity is not dependent upon gender.

Antigenotoxic effects of phloridzin can be attributed to its antioxidative properties of harboring free radical scavenging activities and inhibitory action against lipid peroxidation [35]. Furthermore, similar studies have demonstrated that phloridzin was responsible in reducing the amount of potentially genotoxic substances such as heterocyclic amines found in processed foods and reactive carbonyl species generated under oxidative stress [36,37].

From MI and NDI analyses, it seems in general that phloridzin alone has a positive impact on cell proliferation in an insignificant manner. In addition, proliferative effects of phloridzin on keratinocyte cultures was demonstrated in another study [38]. Phloridzin and MMC combination led to a significant decrease in MI values ($p < 0.05$, Table 1) and somewhat increased NDI values (Table 2). There are several studies in the literature exemplifying that MI and NDI values were not affected in the same manner. For instance, acacia honey and food additives monopotassium glutamate and magnesium diglutamate were found to decrease MI values, but not significantly affect NDI values which are in correlation with our findings [39,40]. MI and NDI values following phloridzin and its combination treatments did not differ between genders considerably except at 500 µg/mL phloridzin and MMC combination (Fig. 2B). Overall, the lack of gender differences in response to treatments suggests that proliferative effects of phloridzin is independent from gender.

To conclude, our study has demonstrated that phloridzin is able to inhibit genotoxic effects of antineoplastic agent, MMC. It is also noticeable that phloridzin has reduced the proliferation of lymphocytes when used in combination with MMC. In general, gender differences did not take part in how phloridzin induced genotoxic and proliferative effects whether it is used alone or in combination with MMC. As a result, the ability of phloridzin to interfere with the action of MMC and possibly with other DNA damaging agents suggests that caution should be exercised when phloridzin is consumed together with chemotherapeutic agents.

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Conflicts of interest

The authors report no conflict of interest.

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