IN VITRO CHEMOPROTECTIVE AND ANTICANCER ACTIVITIES OF PROPOLIS IN HUMAN LYMPHOCYTES AND BREAST CANCER CELLS

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Abstract: Propolis has been used in folk medicine for centuries due to its healing properties. Ethanolic extracts of propolis (EEP) are rich sources of phenolic acid and flavonoids. Natural phenolic compounds may exert chemoprotective activity in cancer cells due to their ability to scavenge free radicals. The aim of this in vitro study was to investigate the genotoxic and anti-mutagenic effects of the EEP on human peripheral blood lymphocytes (PBLs) and their cytotoxic potential on the human breast cancer cell line (MDA-MB-231 cells). Both cell cultures were treated with six concentrations (1, 10, 50, 100, 250 and 500 µg/ml) of EEP1 and EEP2, separately and in combination with mitomycin C (MMC). Our results show that the EEP1 and EEP2 samples of propolis after separate and combined treatments with MMC did not influence the nuclear division index (NDI). In the combined treatment, both tested EEPs significantly reduced MMC-induced micronuclei (MN) in PBLs. At 48 h after exposure of the MDA-MB-231 cell line to a combined treatment of EEP samples with MMC, the IC₅₀ values were significantly reduced (23.79 and 19.13 µg/ml, for EEP1+MMC and EEP2+MMC, respectively, in comparison to the single treatment. In conclusion, the tested ethanolic extracts of propolis exhibited a certain level of in vitro antimutagenic activity in PBLs from healthy subjects, and anticancer activity in breast cancer cell line. The presented findings suggest that the ethanolic extracts of propolis show potential in anticancer therapeutic strategy.

Key words: propolis; human lymphocytes; genotoxicity; cytotoxicity; MDA-MB-231 cell line

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INTRODUCTION

Propolis, which is produced by honeybees (Apis mellifera), is a resinous mixture derived from various plant sources. Propolis is used as a bee glue to fix holes and as protection from external intruders. Different constituents of propolis have been identified such as, polyphenols, sesquiterpenoids, coumarins, steroids and amino acids (Khalil, 2006; Park et al., 2002). Propolis has been used in folk medicine from antiquity due to its healing properties. Many studies and research groups have confirmed that propolis possesses numerous biological properties, such as antibacterial, antioxidant, anti-inflammatory, antitumoral, immunomodulatory and anti-HIV-1
Propolis has traditionally been used in the treatment of different human disorders, but there are no current data in the literature on the biological activities of Serbian propolis extracts on human health and genetic material. The aim of this in vitro study was to explore the genotoxic and anti-mutagenic potential of EEP on human peripheral lymphocytes (PBLs) of healthy donors, as well as anticancer activities on the MDA-MB-231 breast cancer cell line.

The micronucleus (MN) is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments. The MN test has been used for screening populations under the risk of mutagenic agents, especially for the identification

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentrations EEP1</th>
<th>Total of analyzed BN cells</th>
<th>MN/1000BN cells (X±S.D.)</th>
<th>BN with MN (%)</th>
<th>Distribution of MN</th>
<th>NDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3000</td>
<td>5.00 ± 1.73</td>
<td>15 (0.50)</td>
<td>15 (0.50)</td>
<td></td>
<td>1.58 ± 0.29</td>
</tr>
<tr>
<td>Positive Control cells</td>
<td>0 + MMC</td>
<td>3000</td>
<td>52.00 ± 15.72</td>
<td>138 (4.60)</td>
<td>124 (4.14)</td>
<td>11 (0.37)</td>
</tr>
<tr>
<td>Separate treatments</td>
<td>1 µg/ml</td>
<td>3000</td>
<td>5.67 ± 2.52</td>
<td>17 (0.56)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>3000</td>
<td>7.67 ± 0.58</td>
<td>22 (0.73)</td>
<td>21 (0.70)</td>
<td>1 (0.03)</td>
</tr>
<tr>
<td></td>
<td>50 µg/ml</td>
<td>3000</td>
<td>7.33 ± 0.58</td>
<td>22 (0.73)</td>
<td>22 (0.73)</td>
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<tr>
<td></td>
<td>100 µg/ml</td>
<td>3000</td>
<td>7.33 ± 1.53</td>
<td>22 (0.73)</td>
<td>22 (0.73)</td>
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<tr>
<td></td>
<td>250 µg/ml</td>
<td>3000</td>
<td>7.33 ± 1.53</td>
<td>20 (0.66)</td>
<td>18 (0.60)</td>
<td>2 (0.06)</td>
</tr>
<tr>
<td></td>
<td>500 µg/ml</td>
<td>3000</td>
<td>10.00 ± 2.65</td>
<td>28 (0.93)</td>
<td>26 (0.87)</td>
<td>2 (0.06)</td>
</tr>
<tr>
<td>Combined treatments</td>
<td>1 µg/ml + MMC</td>
<td>3000</td>
<td>47.00 ± 7.00</td>
<td>134 (4.46)</td>
<td>127 (4.23)</td>
<td>7 (0.23)</td>
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<tr>
<td></td>
<td>10 µg/ml + MMC</td>
<td>3000</td>
<td>42.00 ± 7.55</td>
<td>117 (3.90)</td>
<td>108 (3.60)</td>
<td>9 (0.30)</td>
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<tr>
<td></td>
<td>50 µg/ml + MMC</td>
<td>3000</td>
<td>38.33 ± 10.69</td>
<td>108 (3.60)</td>
<td>102 (3.40)</td>
<td>5 (0.17)</td>
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<tr>
<td></td>
<td>100 µg/ml + MMC</td>
<td>3000</td>
<td>32.00 ± 13.89*</td>
<td>88 (2.93)</td>
<td>80 (2.67)</td>
<td>8 (0.26)</td>
</tr>
<tr>
<td></td>
<td>250 µg/ml + MMC</td>
<td>3000</td>
<td>26.00 ± 11.27*</td>
<td>71 (2.37)</td>
<td>65 (2.17)</td>
<td>5 (0.17)</td>
</tr>
<tr>
<td></td>
<td>500 µg/ml + MMC</td>
<td>3000</td>
<td>23.00 ± 10.44*</td>
<td>60 (2.00)</td>
<td>54 (1.80)</td>
<td>5 (0.17)</td>
</tr>
</tbody>
</table>

Percentage of cells with MN in relation to total number of analyzed cells; *statistically significant difference in the MN frequency between cells treated with MMC alone (positive control) and cells treated with MMC and EEP1 in co-treatments.
of preclinical steps of the carcinogenic process (Rueff, et al., 2009). On the other hand, the nuclear division index (NDI) is used to characterize proliferating cells and to identify compounds that inhibit or induce mitotic progression.

**MATERIALS AND METHODS**

**Sample collection and propolis extract preparation**

Two distinct propolis samples (EEP1 and EEP2) were collected in the summer of 2011 from *Apis mellifera* hives located in different apiaries in the southwest of Serbia (locations of samples: EEP1: Jabuka (42° 54' 09'' N, 20° 39' 37'' E), EEP2: Velika Župa (43° 19' 55'' N, 19° 39' 5'' E). Raw propolis samples were obtained by scraping the frames of beehives, and stored at 4°C until analysis. Prior to the extraction, the samples of propolis (10 g) were ground and homogenized. The samples were extracted in the dark with 96% ethanol (1:20 w/v), and mixed with a magnetic stirrer at room temperature for 24 h. The resulting mixtures were filtered and stored overnight at 4°C to induce the crystallization of dissolved waxes. The resultant solutions were filtered and concentrated on a rotary evaporator under reduced pressure at 40°C, giving resinous red-to-brown products (EEP). The extracts were stored at 4°C and protected from light until use.

**In vitro cytokinesis-block micronucleus test (CBMN test)**

Cell cultures from three healthy donors aged 26, nonsmokers, who had not been exposed to drugs or other xenobiotics for at least 3 months, were cultivated in RPMI-1640 medium containing 10% fetal bovine serum and 2% L-glutamine in a humidified atmosphere of 5% CO₂ at 37°C. Cytokinesis-block micronucleus test (CBMN test) was performed on peripheral blood lymphocytes (PBLs) according to the protocol used in our previous studies (Gvozdenovic et al., 2013).

The frequency of micronuclei (MN) and nuclear division index (NDI) values in peripheral blood lymphocytes (PBLs) of healthy donors after the separate treatment with six concentration EEP2 and its combined treatments and with mitomycin C (MMC=0.5 µg/ml) in vitro are shown in Table 2.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentrations EEP2</th>
<th>Total of analyzed BN cells (X±S.D.)</th>
<th>BN with MN (%)</th>
<th>Distribution of MN</th>
<th>NDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MN/1000BN cells</td>
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<tr>
<td></td>
<td></td>
<td>BN with MN (%)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1MN (%)</td>
<td>2MN (%)</td>
<td>3MN (%)</td>
<td>≥4 MN (%)</td>
</tr>
<tr>
<td>Control untreated cells</td>
<td>0</td>
<td>3000</td>
<td>5.00 ± 1.73</td>
<td>15 (0.50)</td>
<td>1.58 ± 0.29</td>
</tr>
<tr>
<td>Positive Control cells</td>
<td>0 + MMC</td>
<td>3000</td>
<td>52.00 ± 15.72</td>
<td>138 (4.60)</td>
<td>1.40 ± 0.15</td>
</tr>
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<td>Separate treatments:</td>
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<td></td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>3000</td>
<td>4.33 ± 1.16</td>
<td>12 (0.40)</td>
<td>11 (0.37)</td>
<td>1.59 ± 0.44</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>3000</td>
<td>5.67 ± 0.58</td>
<td>16 (0.53)</td>
<td>15 (0.50)</td>
<td>1.71 ± 0.25</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>3000</td>
<td>6.33 ± 0.58</td>
<td>19 (0.63)</td>
<td>19 (0.63)</td>
<td>1.66 ± 0.26</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>3000</td>
<td>6.67 ± 0.58</td>
<td>14 (0.46)</td>
<td>11 (0.37)</td>
<td>1.54 ± 0.30</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>3000</td>
<td>8.33 ± 1.53</td>
<td>25 (0.83)</td>
<td>25 (0.83)</td>
<td>1.52 ± 0.22</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>3000</td>
<td>12.33 ± 0.58</td>
<td>36 (1.20)</td>
<td>35 (1.17)</td>
<td>1.59 ± 0.34</td>
</tr>
<tr>
<td>Combined treatments:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg/ml + MMC</td>
<td>3000</td>
<td>40.67 ± 15.14</td>
<td>108 (3.60)</td>
<td>2 (0.06)</td>
<td>1.37 ± 0.17</td>
</tr>
<tr>
<td>10 µg/ml + MMC</td>
<td>3000</td>
<td>16.37 ± 13.32</td>
<td>88 (2.93)</td>
<td>5 (0.17)</td>
<td>1.64 ± 0.20</td>
</tr>
<tr>
<td>100 µg/ml + MMC</td>
<td>3000</td>
<td>26.33 ± 6.81</td>
<td>76 (2.53)</td>
<td>3 (0.10)</td>
<td>1.61 ± 0.34</td>
</tr>
<tr>
<td>250 µg/ml + MMC</td>
<td>3000</td>
<td>19.33 ± 4.04</td>
<td>54 (1.80)</td>
<td>4 (0.13)</td>
<td>1.67 ± 0.33</td>
</tr>
<tr>
<td>500 µg/ml + MMC</td>
<td>3000</td>
<td>14.00 ± 1.00</td>
<td>40 (1.33)</td>
<td>38 (1.27)</td>
<td>1.53 ± 0.30</td>
</tr>
</tbody>
</table>

Percentage of cells with MN in relation to total number of analyzed cells; *statistically significant difference in the MN frequency between control untreated and PBL treated with EEP2; **statistically significant difference in the MN frequency between cells treated with MMC alone (positive control) and cells treated with MMC and EEP2 in co-treatments.
to known mutagen agents were used in the investigation of the in vitro effects of EEP1 and EEP2 by cytokinesis-block micronucleus (CBMN) test. Informed consent was obtained from all donors and experiments conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki).

Micronuclei were prepared using the method described by Fenech (2000). Whole heparinized blood (0.5 ml) was added to 5 ml of PBMax Karyotyping (Invitrogen, California, USA), the complete medium for lymphocyte culture. Cultures were incubated at 37°C for 72 h. Forty-four hours after the beginning of incubation, cytochalasin B (Sigma, St. Louis, MO, USA) was added in the final concentration of 4 μg/ml. Cultures were harvested 28 h later. The cells were collected by centrifugation and treated with cold (+4°C) hypotonic solution (0.56% KCl). Then the cells were fixed with fixative methanol:glacial acetic acid = 3:1, three times. The cell suspensions were dropped onto clean slides, air-dried and stained with 2% Giemsa (Alfapanon, Novi Sad, Serbia).

MN scoring was performed using a light microscope (Nikon E50i) at 400 x magnification and following the criteria for MN scoring in binucleated (BN) cells only, as described by Fenech (2007). MN frequencies were scored in one thousand binucleated cells (BN) from each donor (3 000 BN cells per concentration). Five hundred cells from each donor were scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and to calculate the nuclear division index (NDI) using the formula NDI = ((1 x M1 + (2 x M2) + (3 x M3) + (4 x M4))/N, where M1-M4 represent the number of cells with 1 to 4 nuclei, and N is the total number of the cells scored (Fenech, 2000).

Treatment of PBL cultures with EEPs

Ethanolic extracts of propolis at six concentrations (1, 10, 50, 100, 250 and 500 µg/ml) in a small volume (0.1 ml) were added to lymphocyte cultures 24 h after the beginning of incubation. To determine the comutagenic/antimutagenic effect, mitomycin C (MMC, Sigma, St. Louis, MO, USA) at a concentration of 0.5 µg/ml and EEP were concomitantly added to the cell culture. MMC was concurrently used as a positive control, while untreated cell cultures were used as the negative control.

Cell preparation and culturing

The human breast cancer MDA-MB-231 cell line was obtained from American Type Culture Collection. Cells were maintained in DMEM medium, supplemented with 100 g/l heat-inactivated FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. Cells were grown in 75-cm² culture bottles supplied with 15 ml of DMEM.

Treatment of MDA-MB-231 cell line with EEPs

EEP samples (EEP1 and EEP2) were prepared as stock solutions (1000 µg/ml) in 0.1% DMSO. MMC was prepared as a stock solution (1 µg/ml) in 0.1% DMSO. Working solutions were prepared prior to testing. MDA-MB-231 cells (10⁴ cells per well) were seeded in 96-well microtiter plates (exponentially growing viable cells were used throughout the assay) and 24 h later, after cell adherence, the culturing medium was replaced with a) 100 µl of medium containing various doses of EEP at different concentrations (1, 10, 50, 100, 250 and 500 µg/ml), b) 100 µl of medium containing 0.5 µg/ml MMC, and c) 100 µl of medium containing various doses of ethanolic propolis extracts at different concentrations (1, 10, 50, 100, 250 and 500 µg/ml).
with 0.5 μg/ml MMC. Cells were incubated with single and combined drug treatments for 48 h prior to testing.

**Cell Viability Assay (MTT Assay)**

Cell viability was determined by MTT assay (Mosmann, 1983). At the end of the treatment period, 25 μl of MTT solution (final concentration 5 mg/ml PBS) was added to each well and incubated at 37°C in 5% CO₂ for 3 h. The colored crystals of produced formazan were dissolved in 150 μl DMSO. The absorbance was measured at 570 nm on a microplate reader (ELISA 2100C). To determine cell viability (%), the absorbance (A) of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided by the control (the A of control cells grown only in culturing medium) and multiplied by 100. The A of the blank was always subtracted from the A of the corresponding sample with target cells. We also calculated the half maximal inhibitory concentration (IC₅₀) delineated as the concentration of substance eliciting inhibition of cell growth by 50% compared with a vehicle-treated control. A DMSO solution was used as a negative control. All experiments were done in triplicate.

Drug interaction between EEP samples and MMC was assessed using the combination index (CI) where CI<1, CI>1 and C=1 indicate synergistic, additive and antagonistic effects, respectively (Chou et al., 1994). On the basis of the isobologram analysis for mutually exclusive effects, the CI value was calculated as follows:

\[
CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}
\]

where (Dx)₁ and (Dx)₂ are the concentrations of EEP samples and mitomycin, respectively, required to inhibit cell growth by 50%, and (D)₁ and (D)₂ are the drug concentrations in combined treatments that also inhibit cell growth by 50% (isoeffective as compared with the single drugs).

**Statistical analyses**

The results are shown as mean ± standard deviation (S.D). Statistically significant differences between the mean baseline and induced MN frequencies and NDI values were determined using the Student’s t-test. Levels of significance were p <0.05 (SPSS for Windows, version 17, 2008). The relationship between the tested concentrations of extract and MN and NDI was determined by Pearson's correlation coefficient. The magnitude of correlation between variables and the IC₅₀ values was calculated from the dose curves.
RESULTS

Genotoxic potential of ethanolic extracts of propolis

The results of the genotoxic and antimutagenic effects of both EEP1 and EEP2 on genomic damage in the PBLs of healthy donors are shown in Tables 1 and 2. Table 1 shows the effects of six different concentrations of EEP1 on MN frequency per 1 000 BN cells (± S.D.) and NDI values (± S.D.) obtained after separate and combined treatments with MMC. Our results show that after separate treatments of PBLs with EEP1, the extract did not significantly affect either the mean MN frequency or NDI in any of the tested concentrations (1-500 µg/ml), in comparison to the control untreated PBLs (for MN p = 0.423; p = 0.157; p = 0.118; p = 0.073; p = 0.192; p = 0.082; for NDI p = 0.644; p = 0.623; p = 0.094; p = 0.922; p = 0.508; p = 0.787). Analysis of the distribution of MN revealed that the tested concentrations of EEP1 in cultured PBLs did not change the distribution of BN cells with MN in comparison to control untreated cells. The most abundant BN cells were those with one MN, while the BN cells with more than one MN were significantly less represented. The tested concentrations of EEP1 did not change NDI values, in comparison to NDI values in the control untreated cells.

The same Table shows the results of MN frequencies and NDI values after combined treatment of EEP1 and MMC. MMC alone (positive control) significantly increased the MN frequency in PBLs in comparison to untreated cells (p = 0.043). Our results showed that EEP1 significantly decreased MN frequency in a dose-dependent manner (r = -0.890; p = 0.017), in higher tested concentrations (100, 250, 500 µg/ml) with the following probabilities p = 0.034; p = 0.023; p = 0.023. The analysis of MN distributions revealed that EEP1 at concentrations 100-500 µg/ml significantly reduced both the number of BN cells containing MN and number of MN in BN cells compared with positive control cells (MMC alone). The most represented were BN cells with one MN, BN cells with 2 and 3 MN were less present while BN cells with 4 and more MN were not found. All tested concentrations of EEP1 administered in combination with MMC did not change NDI values in comparison to NDI values in positive control cells (MMC alone).

The results of the genotoxic and comutagenic/antimutagenic effects of EEP2 on chromosome damage in the PBLs from healthy donors are shown in Table 2. The treatments with different tested concentrations of EEP2 on PBLs showed increases in MN frequency, but were only significant after the highest concentrations treatments (250 and 500 µg/ml) in comparison to untreated PBLs (p = 0.038; p = 0.014). Analysis of the distribution of MN (Table 2) revealed that the BN cells with 1 MN were mostly present, and that an increased number of BN cells containing MN was present only in the two highest concentrations of EEP2 treatments. No significant differences were observed between NDI values in any treatment with EEP2 in comparison to NDI values of untreated control PBLs (p = 0.991; p = 0.382; p = 0.792; p = 0.865; p = 0.730; p = 0.965).

The results of combined treatments of tested concentrations of EEP2 and MMC on MN frequency in PBLs showed a significant reduction in MMC-induced MN frequency after treatment with 10, 50, 250 and 500 µg/ml concen-
trations of EEP2 compared to MMC alone (p = 0.034; p = 0.031; p = 0.042; p = 0.049) in a clearly dose-dependent manner (r = -0.905; p = 0.013). Analysis of the distribution of MN revealed that all tested concentrations of EEP2 significantly decreased both the number of MMC-induced BN cells containing MN and the number of induced MN in BN cells. As the concentration of extract increased, the number of MN in BN cells decreased, as well as the number of BN cells with MN. There were no significant differences in NDI values between combined treatments of EEP2 with MMC and MMC alone (p = 0.903; p = 0.321; p = 0.605; p = 489; p = 0.426; p = 0.644).

Table 3 Growth inhibitory effects-IC$_{50}$ values (μg/ml) of EEP1 and EEP2 on MDA-MB-231 cell line after 48h of exposure. IC$_{50}$ values were determined by linear regression analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ values (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>EEP1</td>
<td>96.57±4.38</td>
</tr>
<tr>
<td>EEP2</td>
<td>81.65±3.56</td>
</tr>
<tr>
<td>EEP1 + 0.5 μg/ml MMC</td>
<td>23.79±1.58</td>
</tr>
<tr>
<td>EEP2 + 0.5 μg/ml MMC</td>
<td>19.13±1.26</td>
</tr>
</tbody>
</table>

Results are mean values ± SD from at least three experiments.

Cytotoxicity of ethanolic extracts of propolis

Cytotoxic effects of the two EEP samples were assessed on an MDA-MB-231 cell line. Results are presented as a percentage of cell viability compared to untreated control cells (Fig. 1). Dose- and time-dependent inhibition of cell growth was observed in all cells treated with both propolis samples within the tested concentration range.

The results of in vitro cytotoxic activity of the two investigated EEPs were also expressed by IC$_{50}$ values, presented in Table 3, with IC$_{50}$ values 96.57 μg/ml and 81.65 μg/ml for samples EEP1 and EEP2, respectively, after 48 h of exposure. Treatment of MDA-MB-231 cells with 0.5 μg/ml of MMC induced a low cytotoxic effect, since 79.75% of the cells were viable, compared to untreated cells, 48 h after the treatment. Exposure of the MDA-MB-231 cell line to a combined treatment of EEP samples with MMC reduced the IC$_{50}$ values significantly to 23.79 μg/ml for EEP1+MMC treatment and 19.13 μg/ml for EEP2+MMC treatment, after 48 h of exposure. Calculation of the CI showed synergism at effect levels >30% (fraction of cells affected by the treatments) for both EEP samples in combination with MMC (Fig. 2), but the degree of synergism obtained with the EEP1/MMC combination was considerably greater than with EEP2/MMC combination.

DISCUSSION

Propolis has been used in traditional medicine for centuries. Phenolic compounds of EEP have shown various biological activities, including immunomodulatory, chemopreventive and antitumor effects (Sforcin 2007). Previous studies have demonstrated that propolis preparations from different geographic regions exposed a direct inhibitory effect on different cultured tumor cell lines (Eroglu et al., 2008; Kouidhi et al., 2010; Pratsinis et al., 2010), as well as direct and indirect effects on the animal model (Oršolić et al., 2005).

Benković et al. (2009) demonstrated that the water extracts of propolis (WEP) were highly effective in radioprotection in vitro in PBLs. Spigoti et al. (2009) showed the radioprotective effects of EEP on radiation-induced chromosomal damage in Chinese hamster ovary cells (CHO), as well as in vitro radioprotective effects against radiation-induced chromosomal damage in PBLs (Montoro et al., 2011).

The MN assay is one of the most sensitive markers of chromosomal damage and has been
Milošević-Đorđević et al. used to investigate the genotoxicity of a variety of chemicals in vivo or in vitro (Milošević-Djordjević et al., 2007; 2011; Vrndić et al., 2013). MN presents an index of cytogenetic damage in analyzed cells while the nuclear division index (NDI) is the parameter that shows the number of cell cycles through which the treated cells have passed in culture (Surralles et al., 1995; Fenech, 2000).

Studies have shown that high concentrations of EEP had a genotoxic effect in PBLs and CHO cells in vitro (Ozkul et al., 2005; Tavares et al., 2006). Similarly, Montoro et al. (2012) noted that the highest concentrations of EEP induced a significant increase in chromosome aberrations in PBLs. Our results show that the ethanolic extracts of EEP1 and EEP2 did not influence cell cycle kinetics (NDI), neither separately nor in combination with MMC. MMC is an alkylating agent that induces covalent DNA cross-links, generates free radicals when metabolized and produces oxidative DNA damage (Liao et al., 2012; Rencuzogullari et al., 2012). The results of the present study showed that MMC was genotoxic in human lymphocytes. In the combined treatment, both EEPs significantly reduced MMC-induced MN in PBLs. This effect can be explained by the fact that the flavonoids from propolis are scavengers of free radicals that are induced by MMC. In this way, EEP reduces MMC-oxidative DNA damage. The analysis of MN distributions showed that all tested concentrations of both EEPs in combined treatments with MMC decreased not only the number of BN cells with MN but also the number of MN in the BN cells (2 and 3MN/cells) in comparison to positive control cells (MMC alone). Similar results were obtained by other authors (Valadares et al., 2008; Oršolić et al., 2010) who have shown the chemoprotective role of propolis in combination with several chemotherapeutic drugs (irinotecan, doxorubicin, MMC).

The different cytogenetic effects of both propolis extracts (EEP1 and EEP2) may be explained by the fact that the tested extracts have different chemical contents. There are numerous studies showing that propolis collected from different localities has different chemical compositions and different biological activities (Watanabe et al., 2011; Sfocin and Bankova 2011).

In a previous study (Žižić et al., 2013), on the basis of HPLC-DAD analysis it was demonstrated that both tested propolis samples contained high concentrations of flavonoids (chrysin, pinocembrin and galangin), phenolic acids (cafeic acid and isofurulic acid) and CAPE. In comparison to sample EEP 1, sample EEP 2 contains slightly higher amounts of all identified constituents. In addition, the concentrations of two other identified flavanones (hesperetin and naringenin) were slightly higher in sample EEP 2 than in sample EEP 1. Our propolis samples showed similarities with analyzed samples from Europe (Balkan region, Italy and Switzerland) (Bankova et al., 2002). Recent studies have shown that chrysin induces strong cytotoxic effects on various breast cancer cell lines (Chang et al., 2008; Hong et al., 2010).

The genotoxic effects induced by higher concentrations of EEP2 can be explained by the fact that higher concentrations of flavonoids can have pro-oxidative effects that induce DNA damage. On the other hand, EEP1’s absence of genotoxic effects in human PBLs can be attributed to its powerful scavenging of free radicals (Rice-Evans, 2001; Nijveldt et al., 2001; Cao et al., 1997).

Fig. 2. Combination index (CI) plots of a) EEP1 and MMC and b) EEP2 and MMC combination in MDA-MB-231 cells. Point 1 – 50 μg/ml EEP+ 0.5μg/ml MMC; 2 – 100 μg/ml EEP+ 0.5μg/ml MMC; 3 – 250 μg/ml EEP+ 0.5μg/ml MMC; point 4 – 500 μg/ml EEP+ 0.5μg/ml MMC.
In MDA-MB-231 cells, EEP2 had a stronger cytotoxic effect in both treatments, separately or combined with MMC than EEP1, which is in accordance with its higher flavonoid content. However, it had a less pronounced synergistic effect with MMC than EEP1.

Based on our results, we conclude that both tested propolis samples from Serbia had different *in vitro* responses on MDA-MB-231 cells and cultured PBLs from healthy subjects. EEP1 in PBLs has antigenotoxic and anti-mutagenic effects, while in MDA-MB-231 cells, in combined treatments with MMC, it has strong antioxidative protection, lower cytotoxic effects, and more pronounced synergistic effects when compared with EEP2.

Our results show that the tested propolis had different mechanisms of actions depending of the health status of cells (healthy/malignant). Our results are closely related to those from Najafi et al. (2007), who showed that the water extract of propolis could inhibit cell growth of different cell lines (McCoy, HeLa, SP20, HEP-2 and BHK21). Similarly, other authors have demonstrated that different samples of propolis displayed effective antiproliferative and cytotoxic activities against different human cancer cells (Eroglu et al., 2008; Kouidhi et al., 2010). Also, the *in vitro* response of normal and cancer cells to propolis extract showed growth inhibitions only in cancer cells, without affecting normal human cells (Oršolić et al., 2005; 2010; Valente et al., 2011).

Our recommendation is that all ethanolic extracts of propolis should be tested for antioxidant properties if used with other drugs. Propolis samples, with high antioxidative properties, have a strong synergistic effect on cancer cells in combined treatment with standard chemotherapeutics, such as MMC, while at the same time they can protect normal cells from the high DNA damage incurred by chemotherapy. The results showed that EEP1 did not induce any genotoxic effects and that, on the other hand, only higher concentrations of EEP2, after separate treatment, induced genotoxic effects in human PBLs.

Consequently, the tested ethanolic extracts of propolis exhibited a certain level of *in vitro* antimutagenic activity in lymphocytes from healthy subjects, and anticancer activity in breast cancer cell lines and may be considered as safe and healthy food supplements in cancer therapy. The mechanism of action should be investigated in future studies.

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**Authors’ contributions:** Olivera Milošević-Djordjević designed the study and interpreted the results of MN frequencies and NDI values; Darko Grujičić performed the treatment of PBL cultures and analysis of MN frequencies and NDI values and interpreted results; Marina Radović – treatment of PBL cultures and analysis of MN frequencies and NDI values; Nenad Vuković performed propolis sampling and extraction; Jovana Žižić – treatment and cytotoxic evaluation on MDA-MB-231 cell line and interpretation of results; Snežana Marković designed the study and interpreted the results of the cytotoxic evaluation on MDA-MB-231 cell line.

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