In vitro safety assessment of the strawberry tree (*Arbutus unedo* L.) water leaf extract and arbutin in human peripheral blood lymphocytes

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Received: 23 October 2017 / Accepted: 31 March 2018 / Published online: 25 April 2018
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Abstract Strawberry tree (*Arbutus unedo* L.) leaves have long been used in the traditional medicine of the Mediterranean region. One of their most bioactive constituents is the glycoside arbutin, whose presence makes *A. unedo* suitable as a potential substitute for bearberry [*Arctostaphylos uva ursi* (L.) Spreng] leaves, an herbal preparation widely used for treating urinary tract infections. The safety and biocompatibility of strawberry tree water leaf extract have not yet been documented well. This study estimated arbutin content in strawberry tree water leaf extract (STE) using high performance liquid chromatography. Furthermore, we performed an in vitro safety assessment of the 24 h exposure to three presumably non-toxic concentrations of standardized STE and arbutin in human peripheral blood lymphocytes using the apoptosis/necrosis assay, the alkaline comet assay, and the cytokinesis-block micronucleus cytome assay. The

STE was also tested for total antioxidant capacity and lipid peroxidation. At a concentration corresponding to the maximum allowable daily intake of arbutin, the tested extract was not cytotoxic, had a negligible potential for causing primary DNA damage and even hindered micronuclei formation in lymphocytes. It also showed a valuable antioxidant capacity, and did not exert marked lipid peroxidation. These promising results represent a solid frame for further development of STE-based herbal preparations. Although arbutin generally had a low DNA damaging potential, the slowing down of lymphocyte proliferation observed after 24 h of exposure points to a cytostatic effect, which merits further research.

Keywords Apoptosis · *Arbutus unedo* L. · Cytokinesis-block micronucleus “cytome” assay · Oxidative stress · Primary DNA damage

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Introduction

Extracts from medicinal plants constitute a source of various potentially therapeutic compounds and have long drawn attention within the biomedical field. Strawberry tree (*Arbutus unedo* L.) leaves have a history of medicinal use in the Mediterranean region because of their beneficial effects in the treatment of inflammation, diabetes, and hypertension (Oliveira

et al. 2011). One of the most bioactive as well as the most abundant constituents present in strawberry tree leaves is the glycoside arbutin. The presence of arbutin in *A. unedo* leaves makes them suitable as potential substitutes for bearberry [*Arctostaphylos uva ursi* (L.) Spreng] leaves, an herbal preparation widely used in treating urinary tract infections (Pavlović et al. 2009; Jurica et al. 2017). As known, arbutin is the key component responsible for the therapeutic effect of bearberry on the bladder (Schindler et al. 2002; EMA 2012).

Contrary to bearberry leaves preparations, whose biocompatibility has been extensively investigated and reviewed (EMA 2012; Garcia de Arriba et al. 2013), the safety and biocompatibility of the strawberry tree water leaf extract have not yet been documented well. The mechanisms that underline the biological effects of both the extract and arbutin, especially at cellular level, have also not been fully explained. Besides the mentioned herbal extracts, some other foods, beverages and spices are rich in arbutin, for instance pears, cranberries, blueberries, onions, broccoli, wheat, cereals, beer, red wine, tea, coffee, oregano, etc. (DeCaprio 1999; Blaut et al. 2006; EMA 2012). Also, in recent years an important source of human exposure to this compound came from cosmetic whitening treatments with arbutin preparations applied to the skin (Gillbro and Olsson 2011).

To enlarge the knowledge on the biological effects of arbutin and strawberry tree water leaf extract, we performed an in vitro safety assessment, whose purpose was two-fold: (1) to determine arbutin content in the strawberry tree water leaf extract using high performance liquid chromatography (HPLC) and (2) to evaluate the biocompatibility of the standardized strawberry tree water leaf extract (STE) and arbutin with human peripheral blood lymphocytes exposed for 24-h to their three presumably non-toxic concentrations.

Lymphocytes were chosen as a model system as they represent a common source of primary cells with a stable genome, which makes them particularly useful for genotoxicity testing, as confirmed in many contemporary studies (Aydin et al. 2015; Farhan et al. 2015; Turkez et al. 2015; Branica et al. 2016; Valencia-Quintana et al. 2016a, b; Petrović et al. 2017; Qari and El-Assouli 2017; Sreejaya et al. 2017). They are collected in a fairly non-invasive manner and do not entail special pre-treatment.

The evaluations for all of the compounds were performed using: (1) dual acridine orange/ethidium bromide fluorescent staining, (2) alkaline comet assay, and (3) cytokinesis-block micronucleus cytochrome assay (CBMN Cyt assay). The rationale behind the use of dual ethidium bromide/acridine orange staining was that this method permits a simultaneous identification of apoptotic and necrotic cells, and also offers the possibility of assessing cytotoxic effects at the single cell level. The subject method was proven useful in earlier studies with different plant extracts or their active constituents (Gasiorowski et al. 2001; Kopjar et al. 2007; Prasad et al. 2009; Rusak et al. 2010; Grace Nirmala et al. 2018). The alkaline comet assay was selected having in mind that it allows for the sensitive detection of different forms of primary DNA damage in single cells (Rojas et al. 1999; Tice et al. 2000; Collins 2004; Azqueta et al. 2014; Langie et al. 2015). Needless to say, more than three decades of use established the method as a useful endpoint in genotoxicity testing of medicinal plants as well as their constituents (Benković et al. 2008, 2009; Rusak et al. 2010; Farhan et al. 2015; Azqueta and Collins 2016; Mohamed et al. 2016; Qari and El-Assouli 2017). CBMN Cyt assay was selected to obtain more insight into the relationship between primary DNA damage and the cytogenetic outcomes observed following treatments with the tested compounds. This assay is also widely applied in toxicology, and allows for concomitant evaluation of genotoxicity and cytotoxicity, providing valuable insight into chromosome damage, mitotic spindle apparatus damage, cell cycle kinetics, along with information on apoptosis and necrosis in the treated cell cultures (Fenech 2000; 2006; Fenech et al. 2011).

Finally, we also examined the levels of two oxidative stress biomarkers (total antioxidant capacity and lipid peroxidation) in lymphocytes treated with the standardized strawberry tree water leaf extract for 24 h to test its potential antioxidant capacity.

Materials and methods

Chemicals and reagents

Arbutin (4-hydroxyphenyl- β -D-glucopyranoside; CAS Number: 497-76-7) was purchased from Sigma-Aldrich (Steinheim, Germany). If not

specified, other chemicals and reagents were purchased from the same provider.

Plant material and preparation of water extracts

Strawberry tree leaves (Fig. 1) were randomly collected in May 2013 on the island of Mali Lošinj (Croatia; GPS coordinates: 44°31′50″N; 14°28′06″E; 14 m a.s.l.), air-dried for 20 days, grinded in a laboratory mill, and stored in plastic containers in a dark place at room temperature. Powdered dried leaf samples were extracted with water in an ultrasonic bath as described previously (Jurica et al. 2017). Thereafter, the mixture was filtered through a 0.45- μ m Whatman filter paper and freeze-dried. More details regarding lyophilisation of the strawberry tree leaves extract are given in Jurica (2016).

HPLC analysis of arbutin

Five mg of lyophilisate was dissolved in 1.5 mL of ultrapure water, and centrifuged at 13,000 $\times$ g at room temperature. Clear supernatant was decanted, mixed with a standard solution of arbutin (80 μ g/mL) at a 1:1 (v/v) ratio, filtered through a syringe filter (13 mm, polytetrafluoroethylene (PTFE) membrane 0.45 μ m; Supelco, Bellefonte, PA, USA) and 20 μ L of extract was injected into the HPLC system with a diode array

Fig. 1 Strawberry tree (*Arbutus unedo* L.), a small evergreen tree or shrub native to the Mediterranean

detection (DAD) (Agilent Technologies, Santa Clara, CA, USA). Details of the method are described elsewhere (Jurica et al. 2015).

Blood sampling and isolation of lymphocytes

A peripheral blood sample was drawn from a healthy male volunteer (41 years, non-smoker) who had no diagnostic or therapeutic irradiations, and was not exposed to genotoxic agents for 1 year before the blood collection. Written informed consent was obtained from the donor. This experiment was part of a larger research, which was included in the preparation of a PhD thesis of one of the authors, and has been approved by the Faculty of Science, University of Zagreb Ethics Committee.

For the purpose of the experiment, a total of 40 mL of venous blood was collected under sterile conditions into vacutainer tubes (BD vacutainer, Becton-Dickinson, Franklin Lakes, NJ, USA) coated with lithium heparin. The blood sample was immediately divided into two portions, one subjected to lymphocyte isolation, and the other one used for CBMN Cyt assay as whole blood.

To isolate lymphocytes, whole blood was mixed with an equal volume of RPMI 1640 cell culture medium (Gibco, Invitrogen, Burlington, ON, Canada) (1:1 v/v). Then 3 mL of the diluted blood was carefully layered onto 3 mL of the Histopaque[®] 1077 reagent. Lymphocyte isolation was performed according to the manufacturer's instructions (Sigma-Aldrich). After the first centrifugation at 400 $\times$ g for 30 min, the layer containing lymphocytes was transferred into a centrifuge tube, filled with 10 mL RPMI 1640 medium. The cell suspension was then centrifuged at 250 $\times$ g for 10 min, and the procedure was repeated once more. Finally, the lymphocyte pellet was resuspended with RPMI 1640. Using trypan blue staining, cell viability was immediately checked and was over 96%. There were 2.5×10^5 cells per mL of suspension. The lymphocytes were then used in the experiments.

Experimental design

Selection of the tested concentrations

The selection of the tested concentrations was made based on available safety assessments of bearberry leaves preparations issued by the European Medicines

Agency (2012). The lowest concentration of 11.4 µg/mL was calculated on the basis of the maximum allowable daily intake of arbutin (i.e. 800 mg) into the human body average weight of 70 kg. The concentration of 200 µg/mL of arbutin (or standardized STE) was used to verify the previous observation that a matching concentration of ethanolic bearberry leaf extract did not induce micronuclei in human peripheral blood lymphocytes (EMA 2012). To look for potential cytogenetic effects we decided to study a two-fold higher arbutin (or standardized STE) concentration as well.

After having determined the content of arbutin in the strawberry tree water leaf extract using HPLC, the tested extract was standardized for arbutin and appropriate calculations for the treatments were made.

Lymphocyte treatments

Aliquots of the previously prepared lymphocyte suspension ($V = 1$ mL per each replica) were placed in sterile Falcon® tubes and mixed with different concentrations of the test compounds. Treated lymphocyte samples were incubated for 24 h at 37 °C in a humidified atmosphere with 5.0% CO₂ (Heraeus Hera Cell 240 incubator, Langenselbold, Germany). For each experimental group, duplicate samples were prepared. Negative and positive controls were kept in the same conditions. As positive control, lymphocytes treated for 24 h in vitro with 1.25 µg/mL of bleomycin were used.

After treatment, the treated and control lymphocytes were immediately subjected to viable cell counting (using acridine orange/ethidium bromide double staining), and the preparation of microgels for genotoxicity estimations by alkaline comet assay. Lymphocytes treated with the standardized strawberry tree water leaf extract were also used in the determination of oxidative stress parameters (total antioxidant capacity and lipid peroxidation). After treatment, these cells were first rinsed with fresh RPMI 1640 cell culture medium, and then concentrated in 1 mL of the same medium. They were immediately sonicated for a 1.5 min (in cycles of 30 s), centrifuged at 2460×*g* for 10 min at 4 °C and used in the above mentioned analyses, according to the proposed protocols.

For the purpose of the CBMN Cyt assay, whole blood was used. It was treated for 24 h at 37 °C with

selected compounds and then the cell cultures were established according to standard protocol (Fenech 2000), which lasted for 72 h. Then the microscopic preparations were prepared and used to establish the levels of cytogenetic change and lymphocyte proliferation.

Fluorescent viability assay with ethidium bromide and acridine orange (EtBr/AO) staining

The staining procedure was carried out according to the method by Duke and Cohen (1992), as follows: 20 µL of lymphocyte suspension was pipetted on a microscope slide and mixed with 10 µL of fluorescent dyes [100 µg/mL EtBr and 100 µg/mL AO dissolved in PBS (1:1 v/v)]. The preparations were immediately analysed under an epifluorescence microscope (Olympus BX51, Tokyo, Japan; 400× magnification). Triplicate tests with aliquots of each sample were accomplished and a total of 300 lymphocytes per sample per experiment were scored. Quantifications were made by determining the percentage of viable, apoptotic, and necrotic lymphocytes. They were morphologically discriminated based on differential DNA/cytoplasm staining. Viable cells had a bright green stained nucleus due to the intercalation of AO in their DNA. Dead cells had a dark orange/red stained nucleus due to the binding of EtBr in their DNA. Apoptotic cells showed fragmented green stained nuclei in their early phase, and orange staining of the cytoplasm in late apoptosis.

Alkaline comet assay

The alkaline comet assay was performed according to the method developed by Singh et al. (1988) with slight modifications. Briefly, 5 µL of the lymphocyte suspension was mixed with 0.5% low melting point (LMP) agarose and pipetted onto previously prepared slides with a layer of 0.6% normal melting point (NMP) agarose. This layer was covered with another layer of 0.5% LMP agarose. Microgels were allowed to lyse overnight at 4 °C immersed in freshly prepared ice-cold lysis buffer [2.5 M NaCl (Kemika, Zagreb, Croatia), 100 mM Na₂EDTA, 10 mM Tris–HCl, 1% Na-laurylsarcosinate, pH 10.0] with 1% Triton X-100 and 10% dimethyl sulfoxide (Kemika). The microgels were then subjected to denaturation in freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM

Na₂EDTA, pH 13.0). After 20 min of denaturation, the slides were randomly placed in the horizontal gel-electrophoresis tank, facing the anode. Electrophoresis was conducted at 4 °C for 20 min, at 25 V and 300 mA. To wash the slides, a neutralisation buffer (0.4 M Tris–HCl, pH 7.5) was used. Microgels were washed three times at 5-min intervals. Prior to microscopic analysis, the slides were stained with 100 µL of EtBr (20 µg/mL) for 10 min.

One well-trained scorer performed all of the comet measurements on the coded/blinded slides. The microscope image was transferred to a computer-based image analysis system using a black and white camera. The level of DNA damage in individual cells was assessed with Comet Assay IVTM software (Instem-Perceptive Instruments Ltd., Bury Saint Edmunds, UK) using an epifluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with appropriate filters, under 200× magnification. A total of 200 randomly selected comets per each tested concentration (or control) were measured on replicate slides in two independent evaluations. As indicators of DNA damage, tail length (presented in micrometres) and tail intensity (i.e. DNA % in tail) were chosen.

Cytokinesis-block micronucleus (CBMN) cytome assay

The assay was performed following the protocol developed by Fenech and Morley (1985). For each experimental group, duplicate cultures were set up by adding blood into RPMI-1640 medium supplemented with inactivated foetal calf serum (Gibco, Grand Island, NY, USA), phytohemagglutinin (Remel, Lenexa, KS, USA) and antibiotics (penicillin and streptomycin). Cultures were kept in sterile flasks (25 cm²) at 37 °C, in a humid atmosphere with 5% CO₂ (Heraeus Hera Cell 240 incubator). At the 44th hour, the cytokinesis blocking agent cytochalasin B was added (6 µg/mL).

Negative and positive control cultures were incubated simultaneously. As positive control, bleomycin (1.25 µg/mL) was used and treatment lasted for 24 h.

After 72 h of in vitro growth, cultures were harvested by standard procedures including incubation in hypotonic solution of KCl, and several repetitions of fixation with methanol/glacial acetic acid solution (3:1, v/v) and centrifugation to obtain lymphocyte suspension. It was used to prepare slides for

microscopic analysis. Air-dried slides were stained with 5% Giemsa solution for 10 min at room temperature. Microscopic analysis was carried out under a light microscope (Leitz, Oberkochen, Germany) at 1000× magnification (oil immersion).

The identification and scoring of micronuclei (MN), nuclear buds (NB), nucleoplasmic bridges (NPB), and apoptotic and necrotic cells was performed using the criteria by Fenech et al. (2003). A total of 2000 binucleated (BN) cells was examined for each treatment (1000 BN cells per each replica). The same slides were used to study the nuclear division index (NDI), by screening 1000 cells per sample (500 cells per slide). NDI was calculated by the following formula: $NDI = (M1 + 2M2 + 3M3 + 4M4)/N$, where M1–M4 represent the number of cells with 1–4 nuclei, respectively, and N is the number of cells scored (Eastmond and Tucker 1989).

Measurement of oxidative stress parameters

Total antioxidant capacity

Total antioxidant capacity (TAC) was investigated using a ferric reducing antioxidant power (FRAP) assay, based upon the reduction of the Fe³⁺-TPTZ complex under acidic conditions (Benzie and Strain 1996). The FRAP assay used in this study was slightly modified on the basis of previous reports (Kozics et al. 2013). One hundred microliters of cell suspension were added to 1.0 mL of freshly prepared FRAP reagent warmed at 37 °C. Absorbance was measured after 4 min of incubation at 593 nm using the FRAP working solution as blank. The results were calculated on the basis of a standard curve obtained using Fe₂SO₄ × 7H₂O (0.05–0.4 mM) and expressed as mM.

Lipid peroxidation

The end-products of lipid peroxidation, e.g. malondialdehyde, were measured using thiobarbituric acid reactive substance (TBARS) assay with slight modification (Ohkawa et al. 1979; Pareek et al. 2013). Briefly stated, 0.5 mL of sample was added to 0.5 mL TBA–TCA reagent and incubated in a water bath at 90 °C for 30 min. The mixture was immediately cooled in an ice bath and centrifuged. Absorbances were measured at 532 nm on a Shimadzu UV Probe

Spectrophotometer. The concentration of TBARS was calculated using a standard curve constructed with 1,1,3,3-tetramethoxypropane (0.3–6.0 $\mu\text{mol/L}$), and expressed as $\mu\text{mol/g}_{\text{protein}}$. Determination of protein concentration was carried out according to the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

The data were analysed using DellTM StatisticaTM 13.2 software (Dell Inc., Tulsa, OK, USA). Mean, standard deviation, standard error, median, minimum and maximum values were used to determine basic descriptive statistical parameters. The data obtained with alkaline comet assay were logarithmically transformed to normalise the distribution and equalise variances, and multiple comparisons between groups were tested using analysis of variance (ANOVA) on log-transformed data with the appropriate post hoc test (Scheffé's test). Comparisons between values obtained for lymphocyte viability, CBMN assay, and lymphocyte proliferation were conducted using Pearson's χ^2 -test. Since data obtained by oxidative stress measurement were not normally distributed, Kruskal–Wallis ANOVA was used to test differences between groups. The level of statistical significance was set at $P < 0.05$.

Results

Arbutin content

High performance liquid chromatography with diode array detector (HPLC–DAD) was used to determine the content of arbutin in the strawberry tree water leaf extract. Figure 2 shows the chromatogram of water leaf extract detected at a working wavelength of 290 nm. Arbutin was detected in strawberry tree water leaf extract with a concentration of 10.7 mg of arbutin

per g of lyophilisate (corresponding to 3.05 mg of arbutin per g of dry leaf weight).

Lymphocyte viability

The results obtained using dual ethidium bromide/acridine orange (EtBr/AO) staining showed a low cytotoxic potential of both of the tested compounds (Fig. 3), which was concentration-dependent in all of the tested samples. The highest percentage of dead cells (15%) was observed after treatment with standardized strawberry tree water leaf extract (STE) which contained 400 $\mu\text{g/mL}$ of arbutin. At the two higher concentrations, pure arbutin was less cytotoxic than standardized STE. It is worth mentioning that in samples treated with the lowest tested concentration of both standardized STE and arbutin, lymphocyte viability was even higher than in negative control, suggesting possible cytoprotective effects (Fig. 3).

With respect to the type of cell death, apoptosis dominated over necrosis in almost all of the tested samples. Interestingly, standardized STE which contained 200 $\mu\text{g/mL}$ of arbutin induced more apoptosis than its higher concentration (Fig. 3), which may be related to the composition of the tested extract. It is possible that some of the components at the highest investigated STE concentration had a protective effect, but that needs to be further explored and tested by other methods.

A detailed explanation regarding the statistical significance of the obtained results is indicated in Fig. 3.

Morphological differences between viable, apoptotic and dead lymphocytes observed after dual EtBr/AO staining are shown in Fig. 4.

Primary DNA damage in peripheral blood lymphocytes estimated by the alkaline comet assay

Results regarding primary DNA damage levels in peripheral blood lymphocytes measured by the

Fig. 2 HPLC–DAD chromatogram of the *Arbutus unedo* L. water leaf extract. The extract contained 10.7 mg arbutin per g of lyophilisate

Fig. 3 Results of the quantitative fluorescent assay for simultaneous identification of viable, apoptotic and necrotic cells in peripheral blood lymphocytes treated with standardized strawberry tree (STE) water leaf extract and arbutin (ARB) in vitro for 24 h and in the negative (NC) and positive control (PC) samples. Mean values $\pm$ SD of three independent evaluations are shown (3×100 cells per sample per each experimental point were analysed under epifluorescence microscope, magnification $\times 400$). Positive control—lymphocytes treated for 24 h in vitro with bleomycin at 1.25 $\mu\text{g/mL}$. Statistical significance of data was evaluated using χ^2 test. The level of statistical significance was set at $P < 0.05$. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance: *versus all other samples; nc—versus negative control; e1—versus sample treated with standardized STE which contained 11.4 $\mu\text{g/mL}$ of arbutin; e2—versus sample treated with standardized STE which contained 200 $\mu\text{g/mL}$ of arbutin; a1—versus sample treated with 11.4 $\mu\text{g/mL}$ of arbutin; a2—versus sample treated with 200 $\mu\text{g/mL}$ of arbutin

Fig. 4 Photomicrographs of lymphocyte nuclei observed under epifluorescence microscope after dual ethidium bromide/acridine orange (EtBr/AO) staining. **a** viable cell in the negative control; **b** dead cell in the sample treated with 400 $\mu\text{g/mL}$ of standardized strawberry tree water leaf extract for 24 h; **c** early apoptosis in the sample treated with 11.4 $\mu\text{g/mL}$ of standardized strawberry tree water leaf extract for 24 h; **d** late apoptosis in the positive control sample, treated with 1.25 $\mu\text{g/mL}$ of bleomycin for 24 h. Magnification $\times 1000$

alkaline comet assay, and detailed explanations of their statistical significance are shown in Fig. 5. The 24-h exposure to pure arbutin at all three of the tested concentrations was not genotoxic to lymphocytes. The standardized STE caused a statistically significant increase of mean tail length compared to negative control only at the highest tested concentration (Fig. 5a). As we expected, the highest values for comet tail length and tail intensity were measured in the positive control sample (Fig. 5a,b), which is in agreement with the mechanisms of H_2O_2 action at DNA level.

Typical photomicrographs of the lymphocyte nuclei observed after the alkaline comet assay procedure are shown in Fig. 6. The nuclei of control lymphocytes (Fig. 6a) consisted of a head with no or minimal DNA migration into the comet tail. Most of the nuclei of lymphocytes treated with arbutin (Fig. 6b) or standardized STE (Fig. 6c) appeared similar under an epifluorescence microscope. In contrast, lymphocyte nuclei in the positive control sample, due to DNA migration, had shorter or longer tails, in some cases also highly damaged DNA, as shown in Fig. 6d.

Fig. 5 Primary DNA damage in peripheral blood lymphocytes treated with standardized strawberry tree water leaf extract (STE) and arbutin (ARB) in vitro for 24 h and in the negative (NC) and positive control (PC) sample as determined by alkaline comet assay. For each sample, duplicate slides were prepared and two hundred independent comet measurements per sample per experimental point were performed. As indicators of DNA damage, tail length (**a**) and tail intensity (**b**) were chosen. Lymphocytes treated ex vivo with 100 μM hydrogen peroxide (H₂O₂) applied for 10 min on ice were used as positive control. Results are shown as the mean value/median, and the range of the measured values (min–max). Statistical significance of data was evaluated using descriptive statistics, ANOVA with post hoc Scheffé’s test (intra-group comparisons). The level of statistical significance was set at $P < 0.05$. The abbreviations above whiskers indicate which groups differ with statistical significance; from negative control (nc), or all other groups (*)

Cytokinesis-block micronucleus cytome assay (CBMN Cyt assay)

The results of the CBMN Cyt assay and detailed explanation regarding their statistical significance are reported in Table 1. Standardized STE showed a low potential towards MN induction at 400 and 200 μg/

Fig. 6 Photomicrographs of lymphocyte nuclei observed under an epifluorescence microscope after the alkaline comet assay, and captured using a black and white camera coupled with image analysis software (Comet Assay IVTM, Instem-Perceptive Instruments Ltd., Bury Saint Edmunds, UK). **a** negative control; **b** sample treated with 200 μg/mL of arbutin for 24 h; **c** sample treated with 11.4 μg/mL of standardized strawberry tree water leaf extract for 24 h. **d** positive control (100 μM hydrogen peroxide, 10 min). Stained with ethidium bromide. Magnification ×200

mL, but without statistical significance compared to negative control. The very few MN observed after treatment with the lowest concentrations of both of the tested compounds points to possible cytoprotective effects. As we expected, the highest potential towards MN induction was observed in the bleomycin-treated sample, used as positive control (Table 1).

Standardized STE showed slightly greater potential towards infliction of the nuclear buds (NBs) than arbutin (Table 2). Nucleoplasmic bridges (NPBs) were observed only in the sample treated with the highest concentration of arbutin (400 μg/mL) (Table 2), but without statistical significance with respect to negative control. Similarly, following treatments there were no significant deviations in the number of apoptotic and necrotic cells compared to negative control (Table 2). As expected, positive control (bleomycin) had the highest value for all parameters of the CBMN Cyt assay, which confirms the sensitivity and specificity of the method used (Table 2).

Table 1 Effect of standardized strawberry tree water leaf extract (STE) and arbutin on induction of micronuclei (MN) in lymphocytes treated in vitro for 24 h, as well as in the negative and positive control samples

Sample	Distribution of BN cells according to no. of micronuclei (MN)					BN _{MN} cells % ± SD	No. of MN Mean ± SD
	0	1	2	3	4		
Negative control	1991	9	0	0	0	4.5 ± 0.7	4.5 ± 0.7
STE 11.4 µg/mL	1997	3	0	0	0	1.5 ± 0.7	1.5 ± 0.7
STE 200 µg/mL	1990	9	1	0	0	5.0 ± 0.0	5.5 ± 0.7
STE 400 µg/mL	1988	10	2	0	0	6.0 ± 0.0	7.0 ± 0.7
ARB 11.4 µg/mL	1995	5	0	0	0	2.5 ± 0.7	2.5 ± 0.7
ARB 200 µg/mL	1995	5	0	0	0	2.5 ± 0.7	2.5 ± 0.7
ARB 400 µg/mL	1990	10	0	0	0	5.0 ± 0.0	5.0 ± 0.0
Positive control	1969	23	5	2	1	15.5 ± 2.1 ^a	21.5 ± 3.5

BN—binucleated cells; BN_{MN} cells—binucleated cells with micronuclei; positive control—lymphocytes treated for 24 h in vitro with bleomycin at 1.25 µg/mL

Microscopic evaluation was performed using a light microscope at 1000× magnification (oil immersion). To establish the MN frequencies, 2 × 1000 cells were scored. Data are expressed as mean ± SD of two independent evaluations

Statistical significance of the results was evaluated using χ^2 test. The level of statistical significance was set at $P < 0.05$. For each exposure time intergroup comparisons were also done. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance (^a versus all samples)

Typical photomicrographs of MNi, NBs, and NPBs, apoptotic and necrotic lymphocytes observed in the CBMN Cyt assay are shown in Fig. 7. Micronuclei were morphologically identical to the main nuclei in a binucleated cell but smaller (Fig. 7a, b). Nuclear buds had the same morphology as MNi, but were connected to the nucleus by a stalk of nucleoplasmic material (Fig. 7c). Nucleoplasmic bridges represented a continuous nucleoplasmic connection between the nuclei in a binucleated cell (Fig. 7d). In late apoptosis, cells exhibited nuclear fragmentation (Fig. 7e), while necrotic cells (Fig. 7f) had a vacuolated cytoplasm and nuclei in a state of degeneration.

Analysis of lymphocyte proliferation

Results of analysis of lymphocyte proliferation in cell cultures treated in vitro with the standardized STE and arbutin are reported in Table 3.

Based on the values of the nuclear division index (NDI), treatments with standardized STE mostly did not significantly impair lymphocyte proliferation compared to negative control, whereas treatments with pure arbutin did (Table 3). A detailed explanation regarding the statistical significance of the obtained results is indicated in Table 4. At all of the tested concentrations, pure arbutin was significantly more

efficient in inhibiting lymphocyte proliferation compared to standardized STE (Table 4).

Typical photomicrographs of lymphocytes with one to four nuclei are shown in Fig. 8.

Oxidative stress biomarkers

As displayed in Fig. 9, 24 h-treatment of lymphocytes with the highest concentration of the standardized STE resulted in a significantly higher FRAP level compared to the negative control. The dose-dependent antioxidative potential of the extract, but without statistical significance compared to negative control, was determined in samples treated with the two lower concentrations of the standardized STE.

Incubation of lymphocytes for 24 h with the standardized STE did not cause significant changes in the TBARS concentration in the treated cells compared to negative control (Fig. 10), indicating its low potential for induction of lipid peroxidation in lymphocytes.

Discussion

There is a significant gap in knowledge regarding the safety profile of the strawberry tree water leaf extract (STE). Clarification of this issue is particularly

Table 2 Effect of standardized strawberry tree water leaf extract (STE) and arbutin (ARB) on the induction of nuclear buds (NB), nucleoplasmic bridges (NPB), apoptosis (APO) and necrosis (NEC) in lymphocytes treated for 24 h, as well as in the negative and positive control samples

Sample	Distribution of BN cells according to no. of NB		BN _{NB} cells % $\pm$ SD	No. of NB		Distribution of BN cells according to no. of NPB	BN _{NPB} cells % $\pm$ SD	No. of NPB		APO % $\pm$ SD	NEC % $\pm$ SD
				Mean $\pm$ SD				Mean $\pm$ SD			
	0	1		0	1			0	1		
Negative control	1995	5	2.5 $\pm$ 0.7	2.5 $\pm$ 0.7	2000	0	0	0	1.0 $\pm$ 0.0	0	0
STE 11.4 μ g/mL	1995	5	2.5 $\pm$ 0.7	2.5 $\pm$ 0.7	2000	0	0	0	1.0 $\pm$ 0.0	0	0
STE 200 μ g/mL	1994	6	3.0 $\pm$ 1.4	3.0 $\pm$ 1.4	2000	0	0	0	1.0 $\pm$ 0.0	0	0
STE 400 μ g/mL	1986	14	7.0 $\pm$ 0.0 ^{nc}	7.0 $\pm$ 0.0	2000	0	0	0	1.0 $\pm$ 0.0	0.5 $\pm$ 0.7	0
ARB 11.4 μ g/mL	1997	3	1.5 $\pm$ 0.7	1.5 $\pm$ 0.7	2000	0	0	0	1.0 $\pm$ 0.0	0	0
ARB 200 μ g/mL	1997	3	1.5 $\pm$ 0.7	1.5 $\pm$ 0.7	2000	0	0	0	1.0 $\pm$ 0.0	1.5 $\pm$ 0.7	0
ARB 400 μ g/mL	1993	7	3.5 $\pm$ 0.7	3.5 $\pm$ 0.7	1998	2	1.0 $\pm$ 0.0	1.0 $\pm$ 0.0	2.5 $\pm$ 0.7	1.0 $\pm$ 0.0	0
Positive control	1990	10	5.0 $\pm$ 0.0	5.0 $\pm$ 0.0	1997	3	1.5 $\pm$ 0.7	1.5 $\pm$ 0.7	2.5 $\pm$ 0.7	4.0 $\pm$ 0.0	0

BN—binucleated cells; BN_{NB} cells—binucleated cells with nuclear buds; BN_{NPB} cells—binucleated cells with nucleoplasmic bridges; positive control—lymphocytes treated for 24 h in vitro with bleomycin at 1.25 μ g/mL

Microscopic evaluation was performed using a light microscope at $\times 1000$ magnification (oil immersion). To establish the frequencies of NB, NPB, APO and NEC, 2×1000 cells were scored. Data are expressed as mean $\pm$ SD of two independent evaluations

Statistical significance of the results was evaluated using a χ^2 test. The level of statistical significance was set at $P < 0.05$. For each exposure time intergroup comparisons were also done. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance (*nc* versus negative control)

Fig. 7 Photomicrographs of lymphocyte nuclei observed under a light microscope after the cytochalasin B-blocked micronucleus assay. Binucleated lymphocytes observed in the sample treated with 400 $\mu\text{g/mL}$ of standardized strawberry tree water leaf extract for 24 h: **a** one micronucleus (arrow), **b** two micronuclei (arrows). Binucleated lymphocytes observed in the sample treated with 400 $\mu\text{g/mL}$ of arbutin for 24 h: **c** nuclear bud (arrow), **d** nucleoplasmic bridge (arrow). Binucleated lymphocytes observed in the positive control sample treated with 1.25 $\mu\text{g/mL}$ of bleomycin for 24 h: **e** late apoptosis, **f** necrotic cell with a vacuolated cytoplasm (arrows). Stained with Giemsa dye. Magnification $\times 1000$

important, considering the potential of STE in phytotherapy as a source of different bioactive phenolic components. As far as is known, prior to our study, no systematic research applied the same array of endpoints and the same model, or was a similar in vitro safety assessment of the compounds conducted at cellular level. The current study intended to fill the existing lack of information by employing a comprehensive design, which coupled cell viability assessment with the alkaline comet assay and CBMN Cytome assay to evaluate whether tested substances are non-harmful, safe and biologically compatible with the used test system. In addition, to prove the beneficial effects of the STE (as indicated by the

results obtained), its total antioxidant capacity and the extent of lipid peroxidation were also evaluated.

Results obtained by HPLC suggest that the arbutin content in our study, calculated on dry leaf mass (0.31%, w/w), was in accordance with those reported in other studies, ranging from 0.03% (Fiorentino et al. 2007) to 1.25% (Pavlović et al. 2011). This is rather lower than the content of arbutin in bearberry leaves that was traditionally used for treating urinary tract infections and varied from 5 to 15% (Garcia de Arriba et al. 2013).

The most important finding we added to existing knowledge is evidence that STE shows high biological compatibility with the used test system. At a concentration corresponding to the maximum allowable daily

Table 3 Effect of standardized strawberry tree water leaf extract (STE) and arbutin (ARB) on cell proliferation kinetics and nuclear division index (NDI) in lymphocytes treated for 24 h, as well as in the negative and positive control samples

Sample	Distribution of cells according to no. of nuclei				NDI
	M1	M2	M3	M4	
Negative control	148	804	30	18	1.918
STE 11.4 µg/mL	144	819	19	18	1.911 ^{e2,e3}
STE 200 µg/mL	97	841	31	31	1.996 ^{nc}
STE 400 µg/mL	98	862	18	22	1.911 ^{nc}
ARB 11.4 µg/mL	152	829	14	5	1.872 ^{nc,a2,a3}
ARB 200 µg/mL	213	778	9	0	1.796 ^{nc,a3,e2}
ARB 400 µg/mL	269	726	4	1	1.740 ^{nc,e3}
Positive control	326	623	21	30	1.755 ^a

Positive control—lymphocytes treated for 24 h in vitro with bleomycin at 1.25 µg/mL

NDI—nuclear division index, calculated by the following formula: $NDI = (M1 + 2M2 + 3M3 + 4M4)/N$, where M1–M4 represent the number of cells with 1–4 nuclei, respectively, and N is the number of cells scored

Microscopic evaluation was performed using a light microscope at $\times 1000$ magnification (oil immersion)

NDI was determined by examining 2×1000 cells per sample

Statistical significance of the results was evaluated using χ^2 test. The level of statistical significance was set at $P < 0.05$. The abbreviations next to the means indicate from which groups the relevant group differs with statistical significance: ^aversus all other samples; ^{nc}—versus negative control; ^{e1}—versus sample treated with standardized STE which contained 11.4 µg/mL of arbutin; ^{e2}—versus sample treated with standardized STE which contained 200 µg/mL of arbutin; ^{e3}—versus sample treated with standardized STE which contained 400 µg/mL of arbutin; ^{a2}—versus sample treated with 200 µg/mL of arbutin; ^{a3}—versus sample treated with 400 µg/mL of arbutin

intake of arbutin, the extract was not cytotoxic, had a negligible potential for causing primary DNA damage and even hindered micronuclei formation in lymphocytes, as revealed by the alkaline comet and CBMN Cyt assay, respectively. Results of FRAP assay indicated a valuable antioxidant capacity of the tested strawberry tree water leaf extract. Furthermore, it did not exert marked lipid peroxidation, as established from the TBARS levels, which did not significantly differ compared to the control cells. We assumed that these beneficial effects can be related to the complex phytochemical profile of the STE. A detailed phytochemical analysis of the *A. unedo* leaves used to prepare the water extract tested in this study revealed the presence of 47 polyphenolic compounds (Gašić et al. 2015; Jurica 2016). Among them, the most worthy of mention were arbutin, along with gallocatechin, catechins, catechin 3-gallate, hyperoside, rutin, quercetin, kaempferol, and *p*-coumaric and *p*-hydroxybenzoic acid.

Pure arbutin at a concentration that corresponds to its maximum allowable daily intake also showed negligible cytotoxicity in our study. At all of the tested

concentrations, it had low potential for causing primary DNA damage, and low potential to cause cytogenetic damage in lymphocytes. It is important to stress that we also observed a mild inhibitory effect of arbutin on lymphocyte proliferation. The practical significance of results obtained using each endpoint will be briefly discussed below.

The obtained results indicate that the tested substances at the concentrations studied here posed a relatively weak cytotoxic threat to lymphocytes, which died mainly due to apoptosis. Lymphocyte viability was significantly reduced only at the highest concentration tested, more in STE-treated than in arbutin-treated cells. The onset of apoptosis following treatments indicates a controlled self-killing of cells which possibly contained unreparable DNA or cytogenetic damage. The cytotoxic and apoptotic potential of strawberry tree water leaf extract in vitro has not yet been studied and thus we do not have any comparable data to discuss our results within these lines. As the EtBr/AO double staining assay used in this study enables only the morphological discrimination of apoptotic lymphocytes, we cannot suggest the exact

Table 4 Results regarding intra- and inter-group comparisons performed using χ^2 -test on data obtained for cell proliferation kinetics and nuclear division index (NDI) in lymphocytes

treated in vitro for 24 h with standardized strawberry tree water leaf extract (STE) and arbutin (ARB), as well as in the negative and positive control samples

STE concentrations	STE		Control	
	200 µg/mL	400 µg/mL	Negative	Positive
<i>Intra-group comparisons</i>				
11.4 µg/mL	$P = 0.0013$	$P = 0.0164$	n.s.	$P < 0.0001$
200 µg/mL		n.s.	$P = 0.0019$	$P < 0.0001$
400 µg/mL			$P = 0.0014$	$P < 0.0001$
ARB concentrations	ARB		Control	
	200 µg/mL	400 µg/mL	Negative	Positive
11.4 µg/mL	$P = 0.0005$	$P < 0.0001$	$P = 0.0035$	$P < 0.0001$
200 µg/mL		$P = 0.0105$	$P < 0.0001$	$P < 0.0001$
400 µg/mL			$P < 0.0001$	$P < 0.0001$
Tested concentrations				STE versus ARB
<i>Inter-group comparisons</i>				
11.4 µg/mL				$P = 0.0388$
200 µg/mL				$P < 0.0001$
400 µg/mL				$P < 0.0001$

Positive control—lymphocytes treated for 24 h in vitro with bleomycin at 1.25 µg/mL

The level of statistical significance was set at $P < 0.05$

n.s.—statistically not significant

mechanisms behind apoptosis induced by STE, and this has to be clarified in future studies using more subtle methods. We can only assume that, due to the complex composition of the extract tested, more mechanisms might be included. As known, polyphenol compounds in general induce apoptosis by different mechanisms, which also differ between various cell types. Among them, the most important are caspase-dependent and mitochondria-dependent pathways, inhibition of DNA topoisomerase I/II activity, elevation of reactive oxygen species levels, regulation of heat shock proteins expression, modulation of signalling pathways, downregulation of Bcl-2 and Bcl-xL expression, activation of nuclear transcription factor kappaB, activation of endonuclease, suppression of Mcl-1 protein, reduction of survivin protein levels, etc. (Wang et al. 1999; Hadi et al. 2000; Ren et al. 2003; Siegelin et al. 2008; Hertzog and Tica 2012; Sak and Everaus 2015).

The available literature offers almost no data regarding the in vitro cytotoxicity of arbutin. As

known, it is easily taken up by cells as an intact molecule via a Na^+ /glucose carrier, and degradation is not required for its absorption (Garcia de Arriba et al. 2013). It has generally been recognised as a cytoprotective, rather than a cytotoxic agent. As indicated by reports from other authors, arbutin did not significantly impair the viability of treated cells, even at high concentrations, and this was consistently observed on human bladder cancer cell line TCCSUP (Li et al. 2011), HepG2 cells (Seyfizadeh et al. 2012), and in human lymphoma U937 cells (Wu et al. 2014). Thus, the potential mechanisms behind its apoptotic effects are still unexplored.

Taken together, due to methodological constraints, the present study was not able to deduce which mechanisms were responsible for arbutin-induced apoptosis in our experiment. Therefore, if the same experiment becomes more extended in the future, this has to be proven by the use of more sensitive methods for apoptosis detection/characterization.

Fig. 8 Photomicrographs of lymphocyte nuclei observed under a light microscope after the cytochalasin B-blocked micronucleus assay in the negative control sample. The number of nuclei varied depending on the number of nuclear divisions occurring since cytochalasin B addition, which was used to determine the nuclear division index. **a** mononucleated lymphocyte (M1); **b** binucleated lymphocyte (M2); **c** lymphocytes with three nuclei (M3); **d** lymphocyte with four nuclei (M4). **c** and **d** are also called multinucleated lymphocytes. Stained with Giemsa dye. Magnification $\times 1000$

Fig. 9 Effect of standardized strawberry tree water leaf extract (STE) on the extent of lipid peroxidation in lymphocytes treated in vitro for 24 h at three concentrations and in the negative control sample. Results are expressed as means $\pm$ SD of four independent measurements. The level of statistical significance was set at $P < 0.05$. No statistically significant differences were observed

To evaluate the DNA-damaging potencies of the tested compounds, we applied an alkaline version of the comet assay, which recognized the widest range of primary DNA damage (Olive 1999; Collins 2004; Azqueta et al. 2014). Genotoxicity of strawberry tree

Fig. 10 Effect of standardized strawberry tree water leaf extract (STE) on the total antioxidant capacity in lymphocytes treated in vitro for 24 h at three concentrations and in the negative control sample. Results are expressed as means $\pm$ SD of four independent measurements. The level of statistical significance was set at $P < 0.05$. *Significantly different versus negative control

water leaf extract has not been studied so far, while the existing literature offers limited data regarding arbutin. Studies focused on in vitro genotoxicity of other polyphenol constituents present in *A. unedo* leaves are more common, and most suggest a low DNA damaging potential (Benković et al. 2008, 2009; Piao et al. 2008; Rusak et al. 2010; Rhouma et al. 2013; Farhan et al. 2015).

To establish the levels of DNA damage in treated lymphocytes, we focused on two comet parameters: tail length and tail intensity. Both were simultaneously measured by the Comet Assay IVTM software. Among the studied parameters, tail intensity is deemed the most suitable indicator of DNA damage, since it points to the number of DNA breaks (Olive 1999; Collins 2004). Also, beyond a critical amount of damage, tail intensity continues to increase, in contrast to tail length.

Alkaline comet assay revealed low levels of spontaneous DNA damage in non-treated lymphocytes. As anticipated, hydrogen peroxide, used as positive control, caused a significant response in comet assay. Although treatments with all of the tested compounds led to measurable levels of primary DNA damage, the overall genotoxicity was rather low. Strawberry tree water leaf extract generally had low potential towards primary DNA infliction in lymphocytes. We only found an increased mean tail length in the sample treated with the highest STE concentration (400 μg/mL). We assume that this increase of primary DNA damage could be attributed to the complex

composition of the tested extract. Considering the different oxidative potential of polyphenol compounds (Novak Jovanović and Miličević 2017), and the fact that many phytochemicals have both pro-oxidative and antioxidative properties (Inoue et al. 1994; Azmi et al. 2006; Rusak et al. 2010; Aydin et al. 2015), it is likely that at the highest concentration tested some of the pro-oxidative constituents were present in amounts capable to induce certain quantities of DNA breaks. In other words, it seems that after exposure to the highest concentration, the oxidative potential of the various constituents overweighed their protective/antioxidative properties. Although this level of DNA damage was low, it was successfully detected using a comet assay in alkaline conditions, since this method can measure even subtle changes in the amount of DNA breaks within a biologically useful range, by permitting the detection from a few hundred to several thousand breaks per cell (Langie et al. 2015).

Based on our findings, arbutin had low DNA damaging potential at the tested concentrations. The values of comet assay parameters measured in arbutin-treated samples were similar or slightly increased compared to the strawberry tree water leaf extract at the same tested concentrations. Taken together, the results obtained by the comet assay support previous reports regarding cytoprotective and antioxidant properties of arbutin obtained on different experimental models. For instance, Dong et al. (2005) found that 12 h-pretreatment of the human ECV-304 cells line with 500 µg/L of arbutin had beneficial effects against oxidative damage by H₂O₂. Takebayashi et al. (2010) found that arbutin is a potent radical scavenger. Lee and Kim (2012) also reported the antioxidative effects of arbutin in murine microglial BV2 cells. Seyfizadeh et al. (2012) observed the antioxidant effects of arbutin on Hep G2 cells at concentrations of 100 and 150 µM (below those used in our study). Pečivová et al. (2014) reported that arbutin at 0.1–100 µmol/L inhibits the formation of oxygen free radicals in isolated human neutrophils.

Although the alkaline comet assay provides valuable insight into the nature of overall primary DNA damage in single cells, it cannot assure information regarding their proliferation status. Therefore, to find more evidence on cytogenetic consequences of primary DNA damage induced by tested compounds in vitro we extended our research to the CBMN Cyt assay. The fundamental distinction among these two

endpoints is in the types of effects measured; the comet assay primarily detects repairable DNA lesions (mostly breaks or alkali-labile sites) (Collins 2004), while the CBMN Cyt assay reveals cytogenetic damage that persists for at least one mitotic cycle (Fenech 2000). Results obtained using the CBMN Cyt assay point to the low cytogenetic risks associated with exposure to both of the tested compounds. It has to be stressed that our findings undoubtedly confirmed that at a concentration of 200 µg/mL, arbutin (or standardized STE) did not induce micronuclei in human peripheral blood lymphocytes, similarly as it was reported for ethanolic bearberry leaf extract (EMA 2012), which was one of the reasons why this specific concentration was chosen for testing in the present study. Moreover, exposure to some of the tested concentrations resulted in an even lower incidence of micronuclei than in the control sample.

Besides micronuclei, our microscopic analyses were also focused on scoring the nuclear buds (NBs) and nucleoplasmic bridges (NPBs) on slides prepared for CBMN Cyt assay. Nuclear budding is generally associated with the process of gene amplification, while NPBs originate from DNA misrepair and represent a measure of chromosome rearrangement (Fenech 2006). Our results indicate that the frequencies of cells with NBs were significantly increased only following exposure to the highest concentration of the STE. Such an excess of nuclear buds obviously depended on the types and levels of DNA damage inflicted by the treatment. As mentioned before, at the highest concentration of the extract tested, we also found slightly increased primary DNA damage, which was obviously repaired during lymphocyte growth in vitro. Nuclear budding, as known, happens during the S-phase of a cell cycle, and it is likely that DNA repair processes resulted in the formation of excessive amplified DNA, which concentrated in the peripheral part of the nucleus and budded out.

None of the tested STE concentrations caused NPB induction. Similar can be said for the two lower arbutin concentrations. However, further research has to focus on unequivocally explaining the origin of nucleoplasmic bridges that were found in the sample treated with the highest arbutin concentration. Considering that the existing literature does not offer any similar report regarding the CBMN Cyt assay with arbutin, additional research is needed using the higher concentrations and other cell types as well.

The CBMN Cyt assay also offers evidence on the frequencies of apoptosis and necrosis in lymphocyte cell cultures. The results we obtained with cell cultures indicated a lower incidence of both types of cell death than those detected with EtBr/AO staining. However, both approaches showed that apoptosis plays an important role in the removal of lymphocytes bearing DNA/cytogenetic damage induced by exposure to the tested compounds.

Among the other endpoints studied, we also evaluated how the tested compounds influence lymphocyte proliferation. Judging from the values of the nuclear division index, STE did not significantly impair lymphocyte cell kinetics compared to control. Although it increased the proportion of M2 cells at the two higher concentrations, the overall values of NDI were similar to the control ones. However, at our testing conditions a mild inhibitory effect of arbutin on lymphocyte proliferation was noted. Exposure to arbutin caused concentration-dependent changes in relative numbers of lymphocytes with one to four nuclei (M1–M4), which resulted in a decrease of NDI values. A very low number of lymphocytes with three (M3) and four nuclei (M4) in all arbutin-treated samples suggests that this compound slowed down the cell division, which points to its cytostatic effect. This is an interesting result worthy of further research, especially considering that the available literature also mentions the antitumor effects of arbutin (NTP 2006; Nawarak et al. 2009).

Conclusions and future perspectives

Even though obtained on an *in vitro* model, which means that they cannot be directly extrapolated to a real *in vivo* situation, our findings shed some light on the overall understanding of the safety profiles of the tested substances.

Bearing in mind that the existing literature offers information insufficient to discuss and compare the obtained results, this study adds new evidence, broadens existing knowledge, and proposes directions in which future investigations should continue. As is, STE and arbutin show high biocompatibility towards lymphocytes, with almost no or negligible detrimental effects at cell level. The observed antiproliferative effect of arbutin merits further research, which in general should extend to other cell types, in particular

those with metabolic competence, as well as to tumour cell lines. Future studies also have to focus on *in vivo* model systems to evaluate the safety of the tested extract after prolonged exposure, and to extrapolate doses which can be safely used in phytomedicine or for human consumption.

Acknowledgements This study was financially supported by the Mali Lošinj Tourist Board and Institutional funding provided by the Ministry of Science and Education of the Republic of Croatia.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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