Redox-modulation, Suppression of "Oncogenic" Superoxide and Induction of Apoptosis in Burkitt's Lymphoma Cells Using *Geum urbanum* L. Extracts

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Abstract: *Burkitt's lymphoma is a highly aggressive type of non-Hodgkin's lymphoma, linked to the Epstein-Barr virus, which induces oxidative stress and DNA damage in the infected cells. We investigated the cytotoxicity and redox-modulating ability of ethyl acetate (EtOAc) and n-butanol (n-BuOH) extracts from Geum urbanum L. roots and aerial parts on Burkitt`s lymphoma cells (BLC), to elucidate their impact on oxidative stress and cell survival. BLC Raji was treated with EtOAc and n-BuOH extracts to analyze: cell viability; induction of apoptosis; hydroperoxides and reactive nitrogen species (RNS) by 2',7'-dichlorodihydrofluorescein assay; superoxide by dihydroethidium assay; total antioxidant capacity by TAC assay. All extracts suppressed cell growth and induce apoptosis. n-BuOH extracts possessed higher cytotoxicity and pro-apoptotic activity compared to EtOAc. The fractions decreased the hydroperoxides and RNS levels.*

There was no correlation between the DCF fluorescence in the treated cells and their viability (R = –0.3722; p > 0.05). Root extracts decreased the superoxide level, while the leaf extracts did not. There was a good correlation between the dihydroethidium fluorescence in the treated cells and their viability (R = 0.9843; p < 0.01). All extracts increased the TAC of BLC. G. urbanum extracts serve as redox-modulators and anti-inflammatory compounds, decreasing the intracellular level of "oncogenic" superoxide and cell proliferation.

Keywords: *Burkitt's lymphoma, Total antioxidant capacity, Reactive oxygen species, Apoptosis, Geum urbanum* L.

Introduction

Burkitt's lymphoma is a highly aggressive type of non-Hodgkin's lymphoma. It is linked to the Epstein-Barr virus (EBV), which contributes to about 1.5% of all cases of human cancer worldwide [6, 8]. EBV infects up to 90% of the human population and the primary infection is not malignant [5]. It is a herpes virus responsible for a lifelong latent infection in almost every adult. The primary infection usually occurs in early childhood. Most cases are mild, with symptoms similar to a cold, but other cases are more severe and can lead to infectious mononucleosis, characterized by a high fever, swollen lymph nodes and an enlarged spleen. Prolonged infection can cause nasopharyngeal carcinoma and lymphomas [5]. The diet is essential in the treatment of EBV infections. Up to 80% of the diet should come from foods rich in anti-inflammatory ingredients: fresh fruits and vegetables, herbs and seeds. It has been reported that EBV induces oxidative stress and subsequent DNA damage in the infected cells, resulting in a variety of biochemical events that cause cell proliferation: telomere abnormalities, nitrosilation, methylation, up-regulation of transcription factors, etc. [12-13, 20]. It is generally accepted that persistent moderate oxidative stress is a trigger of genomic instability and carcinogenesis. Oxidative stress plays a significant but paradoxical role, acting as a „double-edged sword" in regulating cellular response to radiation, which is the main therapeutic strategy for EBV-related cancers [8].

It was found that antioxidants of synthetic and natural origin, such as resveratrol, quercetin, and other polyphenols, inhibit the lytic cycle of EBV and induce apoptosis in the infected cells [10, 17, 22, 35]. A large number of terpenoids also induce a strong cytotoxicity in Burkitt`s lymphoma cells (BLC) and other carcinoma cell lines by inhibiting the transcription factor NF-kB, anti-apoptotic protein Bcl-2 and proteasomes – factors that play a substantial role in genomic instability and cell proliferation [9, 18, 21, 26, 33]. It has been shown that tannins of natural origin inhibit DNA polymerase of EBV and induce apoptosis in BLC [2, 16]. The efforts are directed to the screening of new generation drugs that are characterized by strong selective cytotoxicity towards EBV-infected cells, but harmless to normal cells and tissues. Medicinal herbs, containing large amounts of polyphenols, terpenoids and tannins are one of the major sources of such substances [7, 27].

Geum urbanum L., commonly known as wood avens or St. Benedict's herb, is a widespread plant species over the territory of Bulgaria [1]. In traditional herbal medicine, it is applied for treatment of gastrointestinal diseases, accompanied by high temperature, cough, bleeding gums, flatulence, vomiting, intestinal colic, disorders of liver and biliary tract, etc. [27]. Phytochemical screening of *G. urbanum*, *G. japonicum*, *G. rivale* and *G. aleppicum* has demonstrated that species of genus *Geum* are rich of compounds with potential anticancer activity: flavonoids, monoterpenoids, sesquiterpenes, triterpenoids, ellagitannins, gallotannins, phenylpropanoids and phenolic acids [25, 34]. Thus, the extracts and ingredients of *G. urbanum* have a great potential to suppress proliferative activity and induce apoptosis in EBV-positive cancer cells.

In this study, we analysed the cytotoxicity and redox-modulating ability of *G. urbanum* extracts on BLC, to elucidate their impact on oxidative stress and cell survival and to assess their potential as supplements in anticancer therapy.

Materials and methods

Preparation of extracts

Roots and aerial parts from *G. urbanum* were collected in April 2014 from a district of Stara Zagora, Bulgaria and provided by Sunny-Yambol Ltd. (Bulgaria). The extracts were prepared as described previously [4]. Briefly, 500 g roots and 500 g aerial parts from the plant were macerated in 3 l methanol (MeOH) for two days at room temperature (two times). The total MeOH extract was concentrated in rotative evaporator under reduced pressure and the residue was successively extracted with petroleum ether, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). Solvent solutions were evaporated to obtain dried fractions.

Quantification of total phenolic compounds

The total phenolic content (%) was determined quantitatively by the method described previously [4]. Briefly, 50 mg of dry EtOAc and 200 mg of *n*-BuOH extracts were dissolved in MeOH. Aliquots of 3 ml were diluted with MeOH in 25 ml volumetric flask. The solutions were subjected to spectrophotometric analysis. 0.5 ml of the solutions were added to 2 ml of Folin-Ciocalteu's phenol reagent, 3 ml of 20% Na_2CO_3 and distilled water to a final volume 25 ml. The experiments were performed in triplicate. Incubation was carried out for 2 h, after which the absorbance was measured at 760 nm. Gallic acid was used as a standard.

Cells and treatment protocol

BLC *Raji* were cultured in RPMI-1640 medium, supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, at humidified atmosphere (37 °C, 5% CO_2). Twenty-four hours before the experiment, the cells were collected by centrifugation (1000×g, 10 min) and transferred in a fresh medium without antibiotics. Cells (1×10^6 cells/ml) were placed in 96-well plates and incubated with the respective fraction in different concentrations (from 50 μg/ml to 1 mg/ml). The samples were analysed after 24-, 48- and 72-hours incubation.

Cytotoxicity assay

The cell viability was analysed by trypan blue staining and automated cell counting (Countess™ Automated Cell Counter, Invitrogen, OR, US) with precise standardization of the measurements. Three independent tests (with two repetitive measurements) for each experiment were performed for each sample. Non-treated cells were used as controls.

Apoptosis assay

The induction of apoptosis was analysed by the expression of phosphatidylserine (PSer) on the cell surface, using FITC-Annexin V Apoptosis Detection Kit (BioVision). Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation (1000×g, 10 min), washed twice with Phosphate Buffer Solution (PBS), containing 2.5 mM of $CaCl_2$ (annexin V-binding buffer) and re-suspended in the same buffer. One hundred μl of the suspension were incubated with 5 μl of FITC-annexin V for 10 min at room temperature, in a dark. The cells were washed three times with Annexin V-binding buffer and finally re-suspended in 500 μl of the same buffer. FITC-Annexin V, bound to PSer on the cell surface, was detected spectrofluorimetrically at λ_{ex} = 488 nm and λ_{em} = 535 nm, using microplate reader (Tecan Infinite F200 PRO, Austria).

Total antioxidant capacity (TAC) assay

The TAC assay was performed using an OxiSelect™ Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs, Inc., US). The method is based on the reduction of Cu^{2+} to Cu^{+} by antioxidants and other reducing equivalents in the biological sample. Cu^{+} interacts with a chromophore to obtain a color product with an absorption maximum at 490 nm. The value of the absorption is proportional to the total antioxidant, respectively reduction capacity of the biological object. Briefly, cells were incubated with the respective extract and cell lysates were prepared as it was described in the manufacturer's instruction. Aliquots of the cell lysates were placed in a 96-well plate. Each cell lysate was incubated with cupper ion reagent and chromophore as it was described in the instruction. The absorption of the product at 490 nm was detected by a microplate reader (Tecan Infinite F200 PRO, Austria). Three independent experiments were performed for each sample, with two parallel sample measurements for each experiment. The antioxidant capacity of the samples was determined by a calibration curve using uric acid as a standard. The results are presented as TAC, which is equivalent to "Total Reduction Capacity" in "mM Uric Acid Equivalents". One mM of uric acid corresponds to 2189 μM of Cu^{2+}-reducing equivalents.

ROS (reactive oxygen species)/RNS (reactive nitrogen species) assay

The amount of ROS was analyzed using OxiSelect™ *in vitro* ROS/RNS Assay Kit – Green Flourescence (Cell Biolabs., Inc.). The method is based on the use of fluorogenic probe – 2',7'-dichlorodihydrofluorescin DiOxyQ (DCFH – DiOxyQ). In the cytosol, the probe is deacetylate to the non-fluorescent 2',7'-dichlorodihydrofluorescin (DCFH). DCHF reacts with ROS and RNS (predominantly H_2O_2, ROO, NO, $ONOO^-$) with formation of fluorescent product – 2',7'-dichlorodihydrofluorescein (DCF). The intensity of DCF fluorescence is proportional of the amount of ROS/RNS in the biological sample. Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation (1000×g, 10 min) and lysed by using 300 μl of 0.1% sodium dodecylsulfate (SDS; dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to ROS/RNS assay, according to the manufacturer's instruction. The fluorescence of the product was detected by a microplate reader (Tecan Infinite F200 PRO, Austria) at λ_{ex} = 480 nm and λ_{em} = 530 nm. Three independent experiments were performed for each sample, with two parallel sample measurements for each experiment. The level of ROS in each sample was determined by a calibration curve using DCF solution as a standard.

Dihydroethidium (DHE) assay

DHE is a cell-penetrating fluorogenic probe, interacting predominantly with superoxide [3]. It allows distinguishing between superoxide and hydrogen peroxide and analyzing the level of intracellular superoxide. Briefly, DHE was dissolved in dimethyl sulfoxide (DMSO) to 65 mM stock solution (kept at -40 °C), which was diluted with PBS to prepare 50 mM of DHE working solution in the day of experiment. Ten 50 μl of DHA (50 mM) were added to 1 ml of each cell suspension (1×10^6 cells/ml). The samples were incubated within 15 min at room temperature, washed three times with PBS, and finally re-suspended in 500 μl of PBS. The fluorescence intensity was detected immediately at λ_{ex} = 518 nm and λ_{em} = 605 nm, using microplate reader (TECAN Infinite® M1000, Austria).

Statistical analysis

The data were statistically analysed by Excel using Student's *t*-test.

Results and discussion

Four *Geum urbanum* L. extracts were investigated: *n*-BuOH extract from roots (*n*-BuOHR) and aerial parts (*n*-BuOHAP), EtOAc extract from roots (EtOAcR) and aerial parts (EtOAcAP). The EtOAc extracts contained almost two-fold higher levels of polyphenols than those of the *n*-BuOH extracts (Table 1).

Table 1. Qualitative determination of the presence of tannins, terpenoids and flavonoids in extracts

Extracts	Tannins	Terpenoids	Flavonoids
EtOAcAP	++	+	+
EtOAcR	++	++	++
n-BuOHAP	++	+	+
n-BuOHR	++	+	+

In our previous studies, it was found that the EtOAc extracts possessed the highest potential as a free radical scavenger [4]. All extracts suppressed the cell growth in a dose-dependent and time-dependent manner (Figs. 1 and 2).

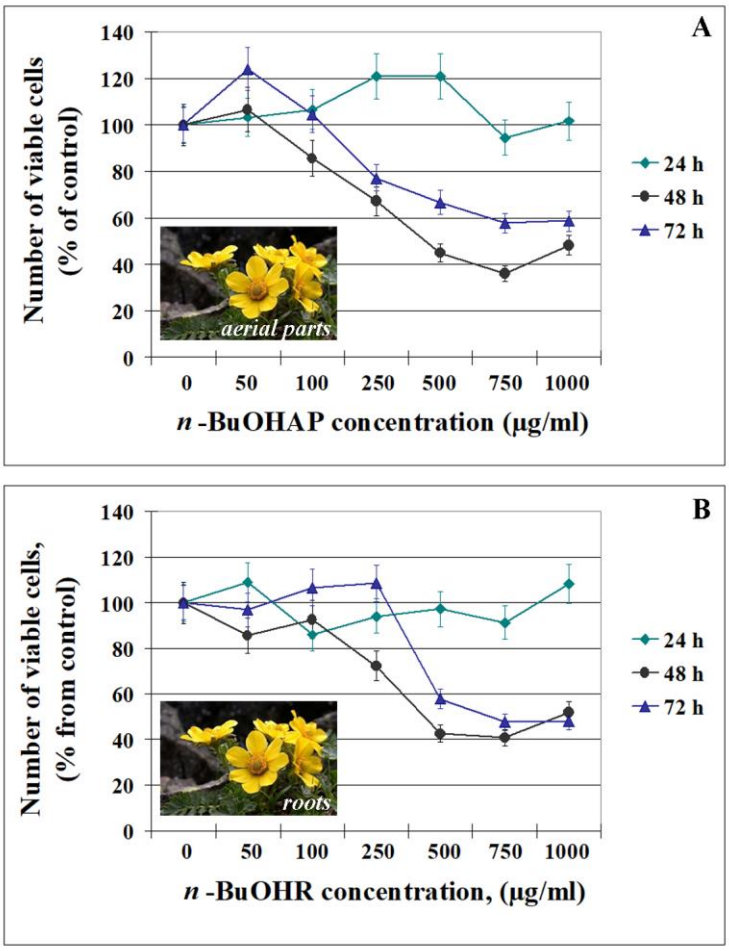

Fig. 1 Effects of *Geum urbanum* L. *n*-BuOH extracts on viability of BLC *Raji* at different incubation times – concentration dependent curves. The data are means ± SD from 3 independent experiments with 3 parallel measurements for each experiment.

The best cytotoxic effect of *n*-BuOHR, *n*-BuOHAP and EtOAcR was detected at 48 h incubation, while EtOAcAP showed highest cytotoxicity after 72 h. Both *n*-BuOH extracts caused a significant decrease (~40-60%) of cell viability at concentration interval between 500-1000 μg/ml. EtOAcAP extract inhibited cell growth (~35-40%) in the same concentration interval. The lowest cytotoxicity was recorded for EtOAcR extract – about 50 %, but only at 1000 μg/ml, after 48 h incubation. Compared to conventional anticancer drugs, the cytotoxicity of *G. urbanum* extracts was about 2 times lower. For example, the IC_{50} values for doxorubicin, cisplatin, and lomustine, calculated at the same experimental conditions (same leukemia cells and 48 h incubation) are ~275, ~200, and ~200 μg/ml, respectively. However, it should be noted that conventional chemotherapeutics are characterized by harmful side-effects on normal cells and tissues [11], whereas the side-effects of natural products (such as *G. urbanum* extracts) should be less and more tolerant. In addition, many natural products sensitize cancer cells to conventional drugs and their combinations exhibit synergistic cytotoxicity or eliminate multidrug resistance [14, 19, 36].

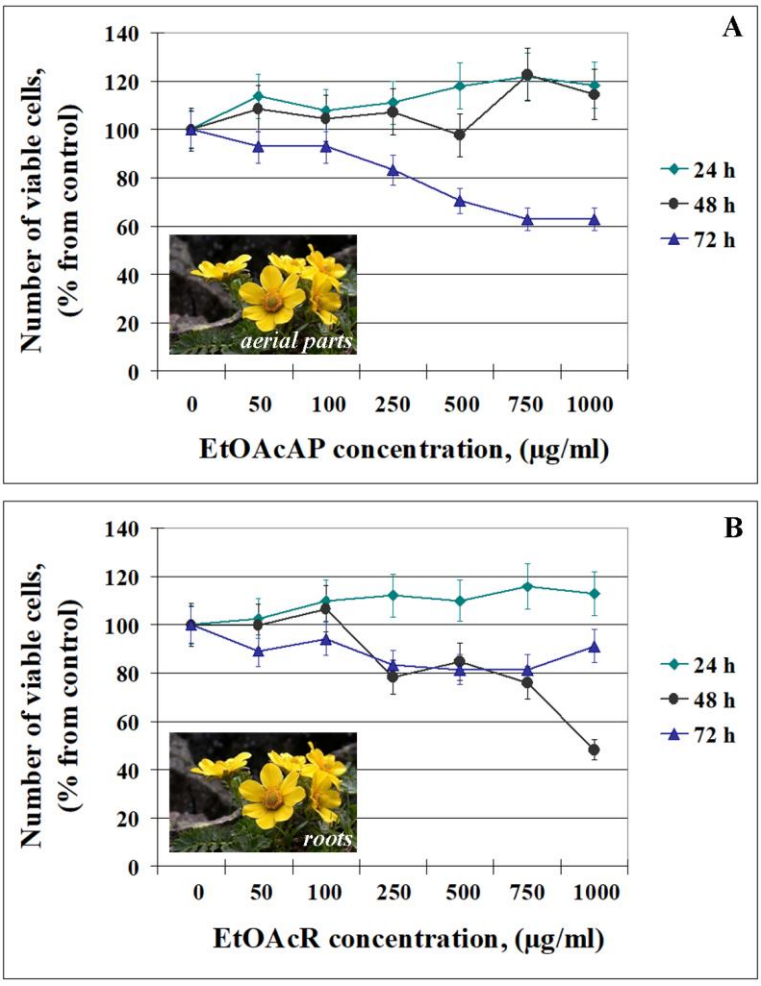

Fig. 2 Effects of *Geum urbanum* L. EtOAc extracts on viability of BLC *Raji* at different incubation times – concentration dependent curves. The data are means ± SD from 3 independent experiments with 2 parallel measurements for each experiment.

In the next stage of the study, we analysed the effect of these extracts on the redox-status of BLC and the induction of apoptosis. Cells (1×10^6 cells/ml) were incubated with the respective extract (750 μg/ml) for 48 h and washed several times by PBS to remove the constituents of the extracts, existing in the environment. The following parameters were analysed: expression

of PSer on the cell surface as an apoptotic marker, level of intracellular hydroperoxides and reactive nitrogen species, level of intracellular superoxide and total antioxidant (reducing) cell capacity.

The data in Fig. 3 demonstrate that all extracts induce apoptosis in BLC. There was a very good negative correlation between the level of PSer on the cell surface and the cell viability (R = –0.9825; p < 0.01). *n*-BuOH extracts possess better cytotoxicity and apoptotic activity compared to EtOAc extracts.

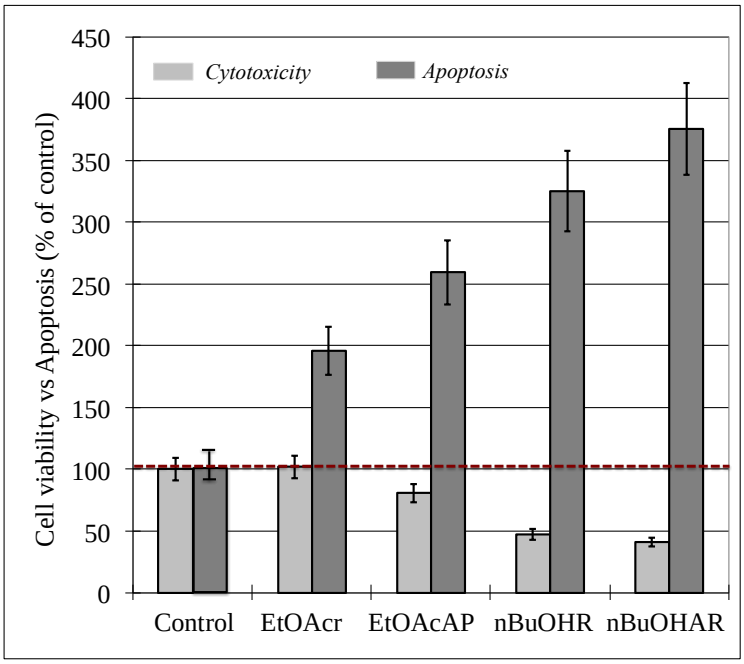

Fig. 3 Effects of *Geum urbanum* L. extracts (750 μg/ml) on cell viability and induction of apoptosis in BLC *Raji* at 48 h incubation. The data are means ± SD from 3 independent experiments with 2 parallel measurements for each experiment.
The red dotted line shows the control level.

EtOAc extracts did not affect the intracellular superoxide, analysed by DHE test (Fig. 4). In contrast, *n*-BuOH extracts significantly decreased the level of "oncogenic" superoxide. There was a good correlation between the DHE fluorescence in the treated cells and their viability – cell viability decreases with the decreasing of superoxide (R = 0.9843; p < 0.01). Authors in [22] have also reported that two polyphenols, composed of ferulic acid and gallic acid, inhibit phorbol esters-induced EBV activation via suppression of superoxide generation. These data suggest that the level of superoxide in EBV-positive BLC is essential for their survival. This is another proof that decreasing "oncogenic" superoxide is one of the strategies for destroying cancer cells. Hydrogen peroxide is considered to be "anti-oncogenic" ROS, and superoxide is considered to be "oncogenic" ROS [24, 29]. We established that all extracts decreased the level of hydroperoxides and RNS, analysed by DCF test (Fig. 4B).

All extracts increased the total reducing capacity of BLC (Fig. 4C). This is not surprising, as many of their ingredients are antioxidants. In our previous study, we found that EtOAc extracts from roots and aerial parts of *G. urbanum* contained 61% and 32% polyphenols, respectively, while *n*-BuOH extracts contained 16% and 13%, respectively [4]. The data in Fig. 4C show that cells treated with EtOAc extracts possess a higher reducing capacity than cells treated with *n*-BuOH extracts. However, the anticancer activity of the tested extracts in

BLC can be explained by the presence of other chemical substances, but not polyphenols. The cytotoxicity and pro-apoptotic potency of *n*-BuOH extracts were much stronger than those of EtOAc fractions (Fig. 3). A recent study has shown that the root extract of *G. urbanum* consists of more terpenoids and sterols than polyphenols [28]. The anticancer activity of sterols and terpenoids is well known [9, 15, 23, 26, 32, 33].

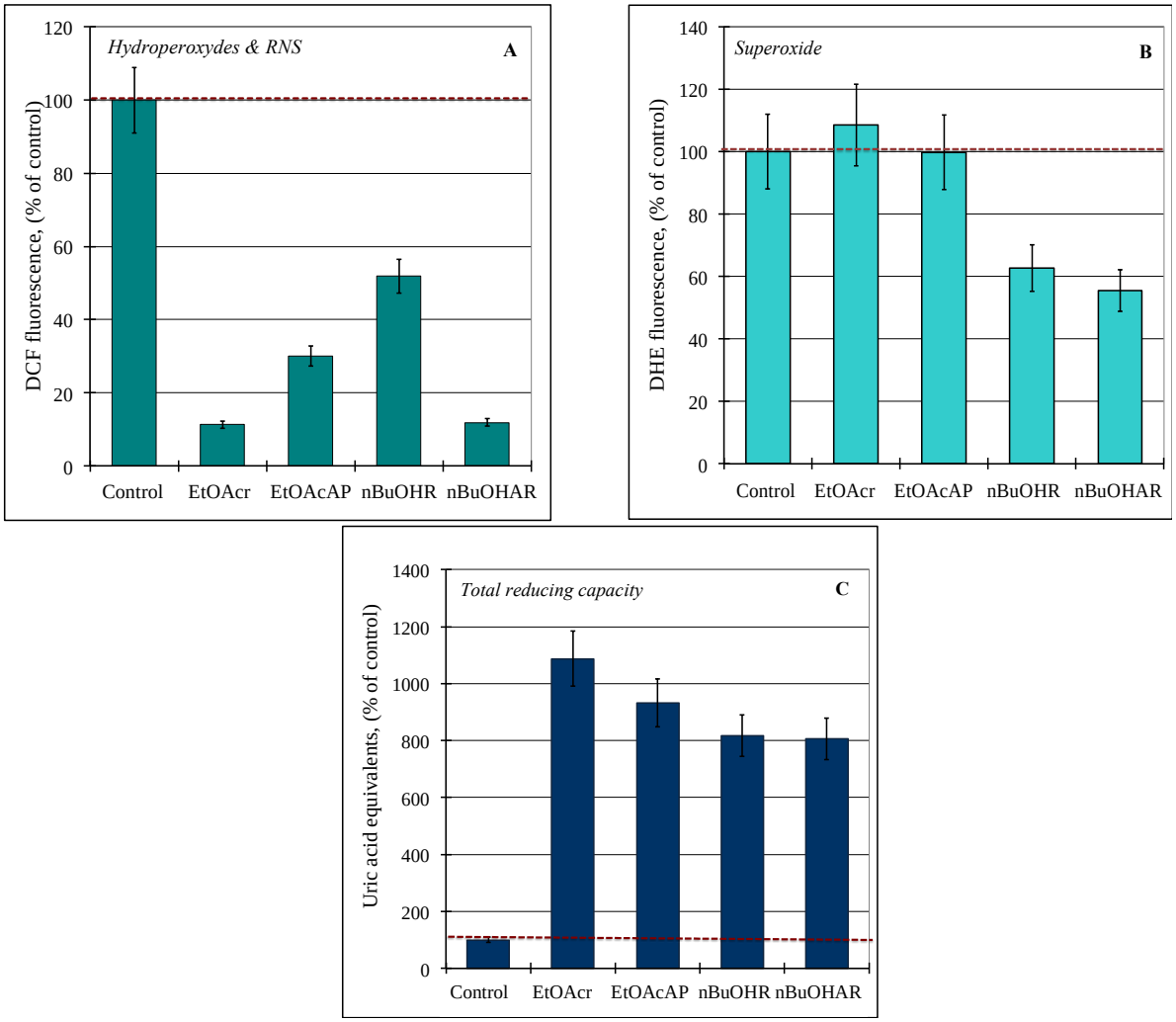

Fig. 4 Effects of *Geum urbanum* L. extracts (750 µg/ml) on the intracellular levels of superoxide (A), hydroperoxides and RNS (B) and total antioxidant capacity (C) in BLC *Raji* at 48 h incubation. The data are means ± SD from 3 independent experiments with 2 parallel measurements for each experiment. The red dotted lines show the control levels.

However, there was no correlation between the DCF fluorescence in the treated cells and their viability (R = –0.3722; p > 0.05). Presumably, this is due to the non-specificity of this analytical test. DCF-probe cannot distinguish between the "onco-suppressive" hydrogen peroxide and RNS, which have a dual role in cell proliferation and viability [31].

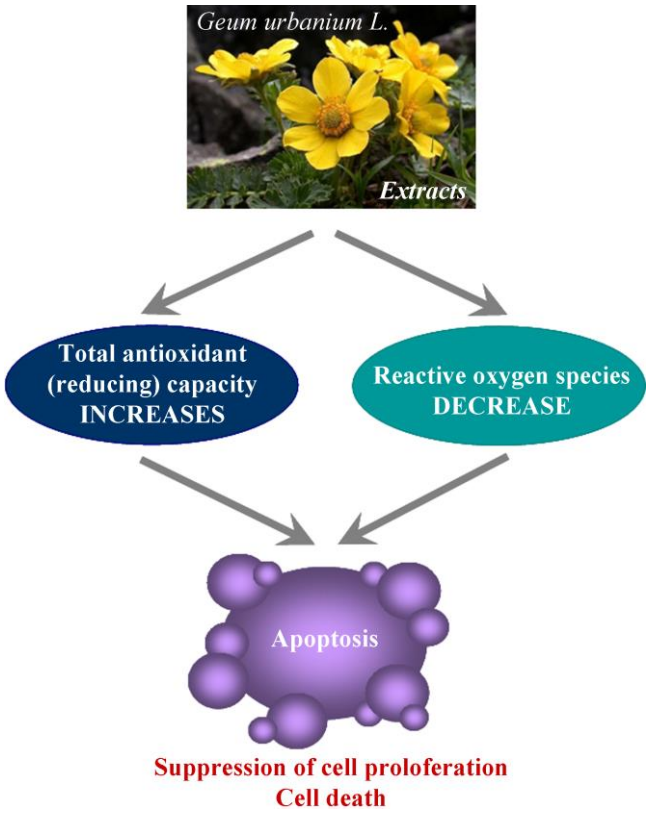

Fig. 5 Redox-modulation, suppression of inflammation and induction of apoptosis in BLC treated with *G. urbanum* extracts

Suppression of oxidative stress and enhancement of total reducing capacity in BLC are indicative for the anti-inflammatory effect of *G. urbanum* L. The domination of oxidative processes over reduction (redox-imbalance), as well as the inflammation are the most distinctive features of cancer cells [29, 30]. The anticancer effect of extracts is most likely due to a complex mixture rather than a single compound. The presence of several biologically active compounds in the extract allows regulation of different cell-signalling pathways, resulting in more effective suppression of cell proliferation and induction of apoptosis.

Conclusion

Extracts of *G. urbanum* L. have a potential as herbal supplements not only in the prophylaxis and treatment of Burkitt`s lymphoma, but for all primary EBV infections.

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