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Inhibition of growth of U87MG human glioblastoma cells by *Usnea longissima* Ach.

BUGRAHAN EMSEN[1], OZLEM OZDEMIR[2], TUBANUR ENGIN[3], BASAK TOGAR[4], SEYDA CAVUSOGLU[5] and HASAN TURKEZ[2,6]

[1]Department of Biology, Kamil Özdağ Faculty of Science, Karamanoğlu Mehmetbey University, İbrahim Öktem Street, 70200, Center, Karaman, Turkey
[2]Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Airport Road Street, 25050, Yakutiye, Erzurum, Turkey
[3]Department of Physiology, Faculty of Veterinary Medicine, Kafkas University, Fevziçakmak, Paşaçayırı Campus, 36100, Center, Kars, Turkey
[4]Department of Medical Services and Techniques, Vocational School of Health Services, Bayburt University, 21 February Street, Dede Korkut Campus, 69000, Center, Bayburt, Turkey
[5]Department of Horticulture, Faculty of Agriculture, Van Yüzüncü Yıl University, Bardakçı, 65090 Tuşba, Van, Turkey
[6]Department of Pharmacy, University "G. d'Annunzio" Chieti-Pescara, Via dei Vestini, 31, 66100, Chieti, Italy

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Abstract: Herbal medicines are efficient to reduce side effects in the fight against glioblastoma, which plays a critical role within brain cancer species. The recent studies designated for testing the effects of lichens that have shown numerous anticancer activities on glioblastoma so far. In the present study, different concentrations of water extract obtained from *Usnea longissima* Ach. were used in order to determine cytotoxic (via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase tests), antioxidant (via total antioxidant capacity test), pro-oxidant (via total oxidant status test) and genotoxic (via 8-hydroxy-2′-deoxyguanosine test) effects of them on human U87MG-glioblastoma cancer cell lines. Primary mixed glial-neuronal non-cancerous cells from Sprague-Dawley rats were also utilized to measure the effects of treatments on non-cancerous cells. Based on median inhibitory concentration values, the data belonged to non-cancerous cells (2486.71 mg/L) showed distinct towering compared to U87MG (80.93 mg/L) cells. The viability of non-cancerous and U87MG cells exposed to extract is decreased in a dose dependent manner. It was also showed that low concentrations of extract notably increased total antioxidant capacity on non-cancerous cells. In addition, various phenolic compounds in extract were detected through high-performance liquid chromatography. The recent results encourage that extract will be able to have therapeutic potential against glioblastoma.

Key words: cytotoxicity, genotoxicity, HPLC, total antioxidant capacity, total oxidative stress.

Correspondence to: Bugrahan Emsen
E-mail: bugrahanemsen@gmail.com
ORCid: http://orcid.org/0000-0002-9636-2596

INTRODUCTION

Primary brain cancer (PBC) is responsible from 1.4% of all cancers and 2.4% of deaths associated with cancer when cancer patients and related patients are searched completely (Greenlee et al. 2000). PBC, which is the most common solid tumor in children, is the second largest cause of cancer deaths among children (Chamberlain and Kormanik 1998). Although PBC is responsible for only a small percentage of all cancers and cancer-related deaths in adults, quality of life of patients is severely affected negatively due to PBC (Chow et al. 2014). Approximately 60% of all PBC is glioma (Chamberlain and Kormanik 1998). Gliomas in adults are histologically classified to the degree of malignancy according to their degree of differentiation (Levin et al. 1999). In classification of tumors of the central nervous system determined by the 2016 World Health Organization (WHO), glioblastoma (GBM) is included in high-grade gliomas (Louis et al. 2016).

GBM is the most aggressive type of glioma and the progression of the disease in the first year following diagnosis and death in these patients is generally observed (Luwor et al. 2013). GBM seeing frequently primary brain tumor in adults can be surgically excised if the tumor is small. However, in the most cases, it has been already widely infiltrated before first diagnosing. Therefore, cyto-reduction is targeted through radiotherapy and chemotherapy combinations (Neagu and Reardon 2015, Rasul and Watts 2015). On the other hand, side effects of radiotherapy and chemotherapy on human must not be ignored. Negative effects on bone marrow, gastrointestinal tract (Giglio 2010), musculoskeletal (Mavrogenis et al. 2010) cardio (Perrino et al. 2014) and physiology (Chan and Ismail 2014) caused by chemotherapy were determined. Likewise, side effect factors originating from radiotherapy on various tissues and organs were investigated in many research results. (Pettersson et al. 2014, Gunjal et al. 2015, Smolska-Ciszewska et al. 2015, Taylor and Kirby 2015). All these results have led scientists to look for safer alternative routes. In the last decade, herbal products combined treatment processes have been developed. In this way, very low levels of side effects were observed against different cancer types (Shen et al. 2007, Russo et al. 2010). In these studies, plant extracts and components had an important role (Sadi et al. 2016, Turkez et al. 2015).

Usnea longissima Ach. is an effective lichen species using in biological researches. Numerous studies about extracts and compounds obtained from *U. longissima* emphasize the importance of this lichen species. Antioxidative (Halici et al. 2005), insecticidal (Yildirim et al. 2012a) and antifungal (Goel et al. 2011) properties of *U. longissima* extracts were reported. Likewise, insecticidal (Emsen et al. 2012, Yildirim et al. 2012b), antioxidant (Atalay et al. 2011, Choudhary 2011), antifungal (Goel et al. 2011), acaricidal (Shang et al. 2014) and anticancer (Odabasoglu et al. 2012, Karagoz et al. 2014, Singh et al. 2015) activities by some isolated *U. longissima* compounds were shown. Nevertheless, there is blank of knowledge in literature about studies exploring anti-proliferative, oxidative and genotoxic effects of *U. longissima* water extract (ULE) on primary mixed glial-neuronal (PMGN) non-cancerous and human GBM U87MG cells. From the point of view of this fact, in this study, we aimed to elucidate anti-proliferative activities with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) analyses, oxidative effects with total antioxidant capacity (TAC) and total oxidant status (TOS) degrees and genotoxicity with 8-hydroxy-2′-deoxyguanosine (8-OH-dG) level on PMGN and U87MG cells by using ULE. Furthermore, we detected main phenolic compounds of ULE by high-performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION OF LICHEN SAMPLE

U. longissima was collected from different localities within Trabzon province of Black Sea region of Turkey. The samples noted morphological and ecological properties were photographed in their natural habitats (Figure 1). Afterwards, the samples were exposed to dry in room conditions. Macroscopic and microscopic findings were compared with literature (Purvis et al. 1992, Wirth 1995) and lichen sample was identified as *U. longissima* (KKEF-803). Voucher specimens are kept in the herbarium of Kazım Karabekir Education Faculty, Atatürk University, Erzurum, Turkey.

EXTRACTION PROCESS OF *U. longissima*

Process the extraction of *U. longissima* was performed according to previously study (Yildirim et al. 2012a). *U. longissima* sample was powdered through liquid nitrogen. It was used 150 g of *U. longissima* and the extraction process was performed with 250 mL of water solvent using a Soxhlet extraction apparatus throughout three days. After extraction, solvents were evaporated with rotary evaporator (IKA, Staufen Germany) under vacuum to dryness and lyophilized to get ultra dry powders that were solubilized with minimum amount of sterile distilled water. Extraction of *U. longissima* yielded 12.25% (w/w) of lichen substances. The crude extract of lichen sample was filtered and stored at 4°C, for 24 h. ULE was diluted to different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/L) before the experimental setup. Dimethyl sulfoxide (DMSO) + relevant cell culture medium (2% DMSO) was used as negative control (control$^-$).

IDENTIFICATION AND QUANTIFICATION OF MAIN PHENOLIC COMPOUNDS BY HPLC

The method determined by Rodríguez-Delgado et al. (2001) was used to separate the phenolic compounds by HPLC. The obtained samples after the extraction were centrifuged at 25200 *g* for 15 min. The supernatant was filtered through a 0.45 μm millipore filter and collected in the vials. The vials were placed in HPLC autosamplers. Chromotographic separation was carried out on an Agilent 1100 HPLC system using a diode array detector and a 250×4.6 mm, 4 μm octadecyl-silica column. Methanol-acetic acid-water (10:2:88) and methanol-acetic acid-water (90:2:8) solvents were used as mobile phase. HPLC conditions for the separation of phenolic compounds were set at 254 and 280 nm wavelength. Flow rate and injection volume were determined 1 mL/min and 10 μL, respectively. Finally, eleven phenolic compounds (chlorogenic acid, p-cumaric acid, q-cumaric acid, ferulic acid, gallic acid, phlorodizin, protocatechuic acid, resveratrol, rutin, syringic acid and valinic) in the extracts were detected.

Figure 1 - *U. longissima* species in their natural habitat.

PRIMARY MIXED GLIAL-NEURONAL CELL CULTURES

This study organized at the Medical Experimental Research Center was approved by Ethical

Committee (protocol number 42190979-01-02/705). Six newborn Sprague-Dawley rats were used to obtain PMGN cultures. This treatment process was performed according to previously study (Emsen et al. 2016). The cerebral cortices were dissociated with Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, Germany) + trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 0.02% EDTA; Sigma-Aldrich), treated with DNAse type 1 (Sigma-Aldrich) and centrifuged. After having thrown away the supernatant, fresh medium containing neurobasal (Gibco, Germany) 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2% B-27 (Gibco) and 0.1% penicillin-streptomycin (PAN Biotech, Germany) were added to the residue. Finally, mixed glial-neuronal cells were seeded at the density of 1×10^5 cells/well in 96 well plates and incubated at 37°C in 5% CO_2.

U87MG-GBM CELL CULTURES

Human glioblastoma (GBM) cell line U87MG was obtained from ATCC (Rockville, MD, USA) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) containing 15% FBS, 1% L-glutamine (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich). After reaching to proper volume, cells were harvested with 0.25% trypsin-EDTA and seeded in 96 well plates.

MTT ASSAY

The cells seeded in 96-well plates were incubated at 37°C in a humidified 5% CO_2/95% air mixture and exposed to ULE at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/L) for 48 h. MTT assay was performed by using a commercially available kit (Cayman Chemical Company, USA), following the providers' instructions. The intensity of the formazan was measured at 570 nm wavelengths with Multiscan Go microplate reader (Thermo Scientific, USA). Miyomycin C, a chemotherapeutic agent, was used as a positive control.

LDH RELEASE ASSAY

Commercially available kit (Cayman Chemical Company, USA) was used to perform LDH assay from culture medium. LDH is an enzyme that released to the cell culture medium as a result of rapid cell damage occurred during apoptosis or necrosis events. In kit assay, primarily, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. Afterwards, diaphorase uses the newly formed NADH and H^+ to catalyze the reduction of a tetrazolium salt to highly colored formazan. Created Formazan amount is proportional to the amount of LDH released into the culture medium by reason of cytotoxicity. LDH activity in the supernatant of culture increases via rising of dead cells. Miyomycin C, a chemotherapeutic agent, was used as a positive control.

TAC ASSAY

Commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey) was used for TAC assay on PMGN and U87MG-GBM cell cultures for 48 h. The aim of kit assay is to reveal antioxidant levels of samples by inhibiting formation of a free radical, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) compound. Positive control was ascorbic acid from organic antioxidant compounds for TAC assays.

TOS ASSAY

Commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey) was used for TOS assay on PMGN and U87MG-GBM cell cultures for 48 h. In kit assay, complexes with ferric ion are oxidized to ferrous ion by oxidants presented in the sample. The oxidation reaction is performed via strengthening molecules in the reaction medium. Ferrous ions form a colored structure with chromogen in the

acidic environment. The color intensity measured spectrophotometrically is related to total amount of oxidant molecules in the sample. Hydrogen peroxide (H_2O_2) was used for calibration. Positive control was H_2O_2, reactive oxygen species for TOS assays.

OXIDATIVE DNA DAMAGE ASSAY

Commercially available DNA/RNA oxidative damage kit (Cayman Chemical Company, USA) was used for oxidative DNA damage assay in the culture medium. Miyomycin C, a chemotherapeutic agent, was used as a positive control.

STATISTICAL ANALYSES

All the experiments were run in triplicate. Diverse activities of ULE, positive and negative controls were elucidated with analysis of variance followed by appropriate post-hoc test (Duncan test) and differences were accepted as statistically significant at $p < 0.05$. Probit regression analysis was used for calculating median inhibitory concentration (IC_{50}) values. Relations between the variables were tested by bivariate correlation analysis. Statistical Package for Social Sciences (SPSS, version 21.0, IBM Corporation, Armonk, NY, USA) software was used for the calculations.

RESULTS

HPLC ANALYSES OF ULE

HPLC analysis was used to detect the active substances in ULE, which had an antioxidant effect. Antioxidants in ULE were the most important compounds causing the increase of antioxidant capacity of the cells. Therefore, the phenolics determined by HPLC analysis are of great importance. In the recent study, ten components were determined in ULE and they were identified as chlorogenic acid, p-cumaric acid, q-cumaric acid, ferulic acid, gallic acid, phlorodizin, protocatechuic acid, rutin, syringic acid and valinic (Table I). Among these components, chlorogenic and gallic acid were the most numerous in terms of quantity. Phenols (protocatechuic acid and valinic) that were not statistically ($p > 0.05$) different from each other in the extract were in low quantities.

ANTI-PROLIFERATIVE ACTIVITIES

In this analysis, the cytotoxic effects of ULE on the cells were determined. In the present study, in particular, the toxic effect on cancer cells has led to different studies in the future. Viability rate in PMGN and U87MG cells exposed to different concentrations of ULE was measured with MTT test. The lowest survival rate for PMGN and U87MG cells belonged to positive control, mitomycin-C (41.36 and 38.32%, respectively). It was revealed that high concentrations of ULE such as 100 and 200 mg/L caused high cytotoxicity on the tested cells. Moreover, there was significantly ($p > 0.05$) no difference between these concentrations (Figure 2).

LDH analysis was performed in addition to MTT test in order to determine anti-proliferative activities on tested cells. In this analysis carried out on PMGN and U87MG cells, while the control group had the lowest LDH release (12.32 and

TABLE I
Different phenolic compounds of ULE.

Phenolic compound	Quantity (mg/kg)
Chlorogenic acid	$226.25^{a} \pm 0.09$
p-Cumaric acid	$4.84^{f} \pm 0.05$
q-Cumaric acid	$21.16^{e} \pm 1.77$
Ferulic acid	$23.14^{d} \pm 0.31$
Gallic acid	$123.79^{b} \pm 0.65$
Phlorodizin	$4.36^{f} \pm 0.03$
Protocatechuic acid	$1.87^{g} \pm 0.03$
Rutin	$56.11^{c} \pm 1.56$
Syringic acid	$4.26^{f} \pm 0.02$
Valinic	$0.78^{g} \pm 0.09$

Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different superscript letters in the same column differ significantly at $p < 0.05$.

13.21 µU/mL, respectively), mitomycin-C treated cells exhibited the highest value (23.30 and 24.40 µU/mL, respectively). The lowest concentration (3.125 mg/L) of ULE caused the maximum LDH release (19.29 µU/mL) (Figure 3) on cancer cells (U87MG).

ANTI-OXIDATIVE ACTIVITIES

Increasing the antioxidant capacity of healthy cells is very important for the functioning of the metabolism. The antioxidant capacity in the cells can minimize the damage to the body. For this, it is important to take the components with antioxidant content as a supplement to our body. In the present study, antioxidant capacities of different concentrations of ULE on PMGN and U87MG cells were detected with TAC analysis. Control$^-$ group had minimum TAC level on both cells and its data (18.28 mmol Trolox equivalent/L) on PMGN cells was not distinctly ($p > 0.05$) different from concentrations of 100 and 200 mg/L of ULE. TAC levels of ascorbic acid as a positive control, on both cells were statistically ($p < 0.05$) different from all other values. Among all concentrations tested on PMGN cells, concentrations of 6.25 and 3.125 mg/L of ULE had higher TAC levels (Figure 4).

As shown in Figure 4b, TAC values of U87MG cells treated with ULE were very close. Except for its concentrations of 3.125 and 6.25 mg/L, there were no statistically ($p > 0.05$) significant differences among all other experiments. Among all treatments of ULE, concentration of 100 mg/L had maximum TAC level (18.51 Trolox equivalent/L).

PRO-OXIDATIVE ACTIVITIES

Oxidative stress is caused by the imbalance between the antioxidants and the prooxidants created by the reactive oxygen/nitrogen species. Oxidative stress in the body can cause many damages, especially on the immune system. This may lead to processes that can lead to cancer. In the present study, oxidative stress levels caused by ULE on PMGN and

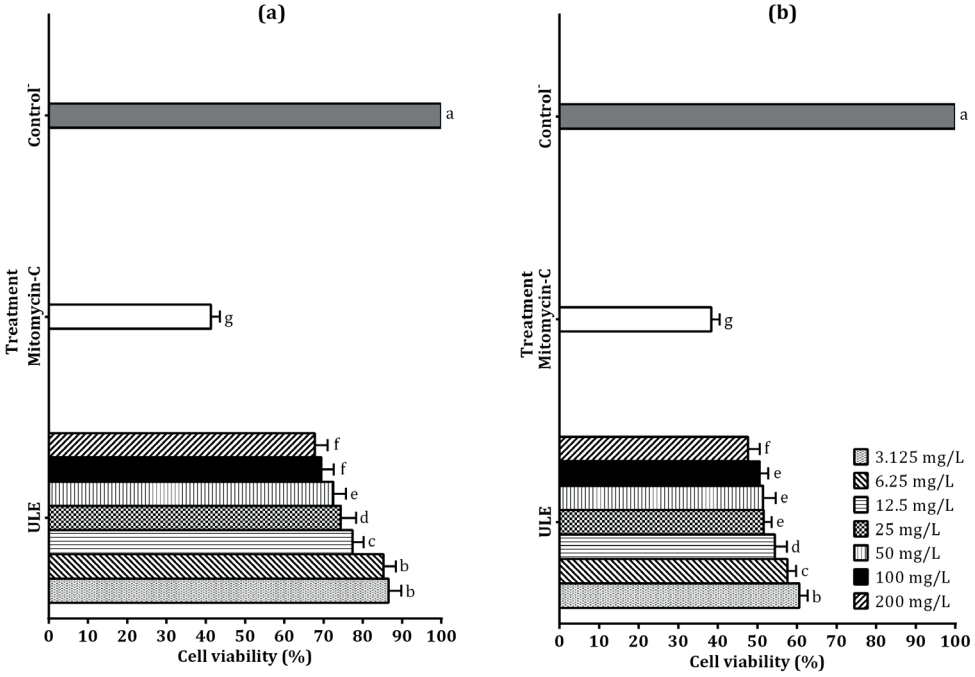

Figure 2 - Viability rates in the cells exposed to ULE **(a)** for PMGN cells **(b)** for U87MG cells. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$.

U87MG cells were investigated via TOS analysis. TOS level of control$^-$ group was the lowest value of this study (2.14 µmol H_2O_2 equivalent/L) and there was no statistically ($p > 0.05$) significant difference between TOS levels of control$^-$ and other tested experiments. Positive control, H_2O_2, displayed maximum TOS activity (4.77 µmol H_2O_2 equivalent/L) on healthy PMGN cells (Figure 5a).

According to the results of TOS assay performed on cancerous U87MG cells, it was showed that oxidative stress levels occurred by all concentrations of ULE were not significantly ($p > 0.05$) different as compared with control$^-$ value. However, ULE had statistically ($p > 0.05$) close values to H_2O_2 (5.28 µmol H_2O_2 equivalent/L) due to treatments with its 6.25, 12.5, 25, 100 and 200 mg/L concentrations. Among aforementioned five treatments, concentration of 100 mg/L represented the highest TOS activity after H_2O_2 (3.80 µmol H_2O_2 equivalent/L) (Figure 5b).

GENOTOXICITY ACTIVITIES

Generally, genotoxicity defined as damage to the genetic material of the cell, can disrupt the metabolic balance. In the event of genetic damage, the treatment methods should be directed to the damaged tissue or cell. Healthy cells should not be adversely affected. In the present study, oxidative stress induced DNA damage arose in PMGN and U87MG cells by ULE was measured with 8-OH-dG level occurring in the cells. Based on PMGN cells, only minimum concentration (3.125 mg/L) of ULE had 8-OH-dG level (3.16 pg/mL) that was not significantly ($p > 0.05$) different from control$^-$ group. Other treatments of ULE significantly ($p < 0.05$) increased oxidative DNA damage level, but none of them come near to 8-OH-dG level (8.82 pg/mL) caused by positive control, mitomycin-C and all concentrations of ULE displayed statistically ($p < 0.05$) different values compared to mitomycin-C (Figure 6a).

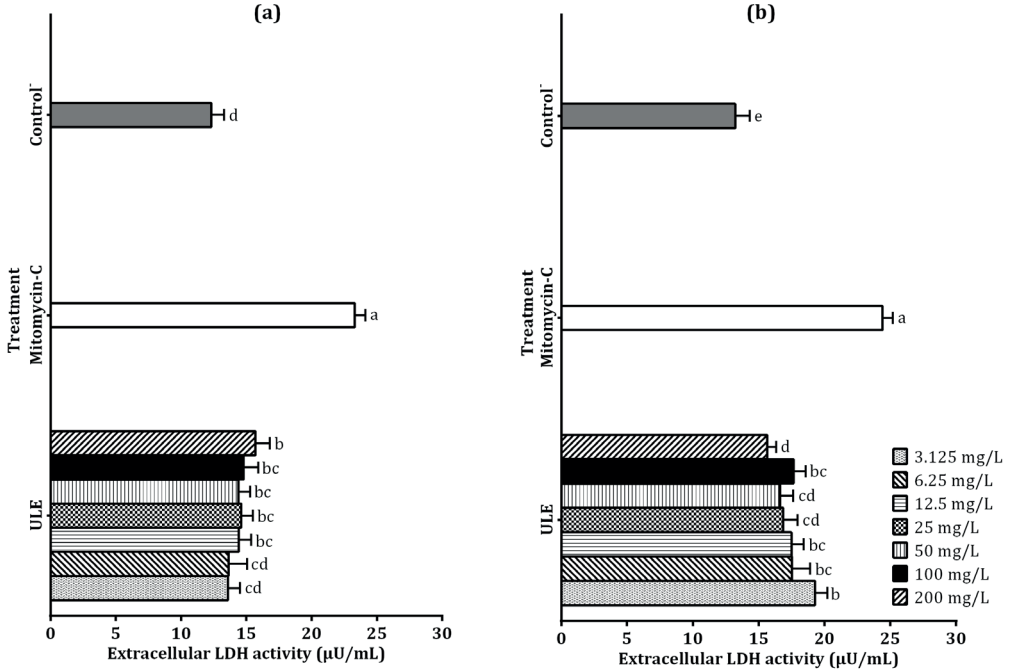

Figure 3 - LDH release levels in the cells exposed to ULE **(a)** for PMGN cells **(b)** for U87MG cells. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$.

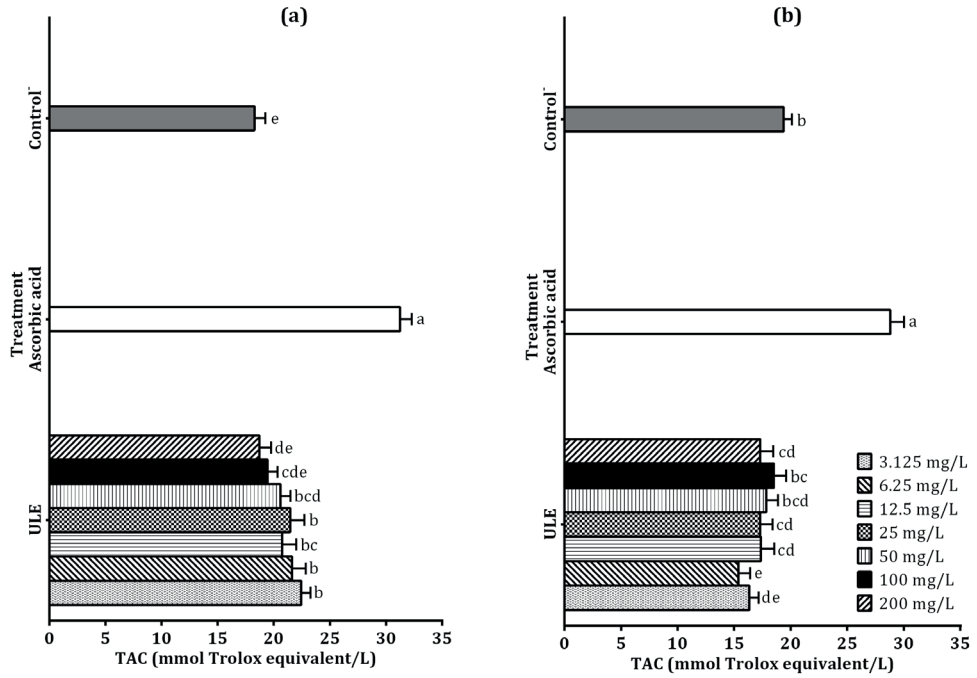

Figure 4 - TAC levels in the cells in the presence of ULE **(a)** for PMGN cells **(b)** for U87MG cells. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$.

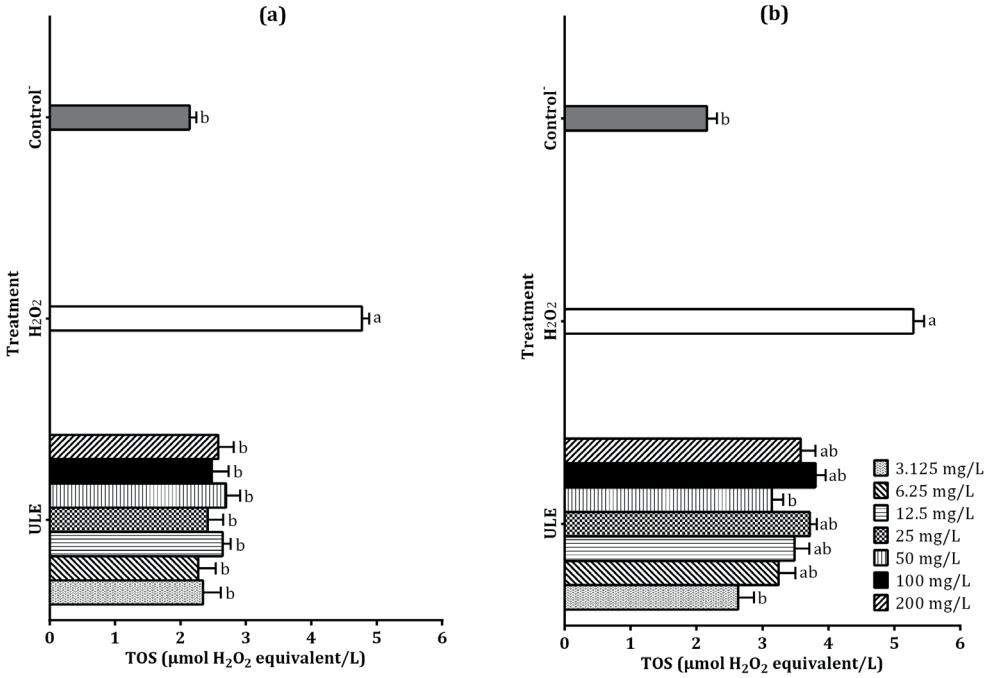

Figure 5 - TOS levels in the cells in the presence of ULE **(a)** for PMGN cells **(b)** for U87MG cells. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$.

As shown in Figure 6b, ULE caused close 8-OH-dG levels in U87MG cells. Findings occurred by all treatments of them were ($p < 0.05$) significantly different from positive and negative control. Based on U87MG cells, the closest 8-OH-dG level to control$^-$ belonged to concentration of 3.125 mg/L of ULE (5.53 pg/mL).

DISCUSSION

GBM defined as high-grade tumor by WHO has the most dangerous process within brain cancer types (Zhang et al. 2012). This situation has caused numerous investigations on GBM. Surgery treatment, radiotherapy and chemotherapy are at the core of the studies performed (Gauden et al. 2009, Wang et al. 2010). However, since side effects of these methods are critical factors in treatments, researchers have turned to more natural and safe methods such as using plant-based products (Deng et al. 2009, Hahm et al. 2010, Jung and Ghil 2010, Jeong et al. 2011, Markiewicz-Żukowska et al. 2013, Wang et al. 2013).

U. longissima lichen utilized in the recent studies has not been used treatment of GBM according to literature. On the other hand, determining of anticancer activities of this lichen (Odabasoglu et al. 2012, Karagoz et al. 2014, Koçer et al. 2014, Singh et al. 2015) on various cancer types has prompted us to perform this research.

Cytotoxic activities of ULE on PMGN and U87MG cells were positively correlated with concentration. Correlations between cell viability and concentration are significant at the 0.01 level (Tables III and IV). Similarly, in another anticancer study occurred with *U. longissima*, cytotoxicity of diffractaic acid compound obtained from *U. longissima* depending on concentration was defined on Ehrlich ascites carcinoma cells (Karagoz et al. 2014). Many studies of concentration-dependent cytotoxic effect about lichens were pronounced by a lot scientist (Koparal et al. 2006, Brandao et al. 2013, Brisdelli et al. 2013, Shrestha et al. 2015).

While it was detected lower cytotoxic effect non-cancerous PMGN cells (Figure 2a), ULE was more effective against cancerous U87MG cells (Figure 2b). Towering IC_{50} values calculated for PMGN also showed that ULE had minimal cytotoxic effect on healthy cells. (Table II). Bézivin et al. (2003) evaluated cytotoxicity of some lichen extracts on murine (lymphocytic leukaemia, and Lewis lung carcinoma) and chronic myelogenous leukaemia (K- 562), glioblastoma (U251), prostate carcinoma (DU145), and breast adenocarcinoma (MCF7) cell lines and their data were in agreement with present studies. They reported that some lichens caused low levels of side effects in GBM treatment. Correlation analysis showed that anti-proliferative effect caused by ULE on PMGN cells might result from LDH release. Because, we detected significant correlation ($p < 0.05$) between cell viability and extracellular LDH activity (Table III). However, same relation was not found for U87MG cells based on results of correlation analysis (Table IV). In addition to that, it was observed that LDH activity of ULE increased in a concentration-dependent manner on PMGN cells (Table III). Another study about LDH induced cell death caused by usnic acid a lichen compound was performed on breast and pancreatic cancer cells (Einarsdóttir et al. 2010). Similarly, it was determined that while cytotoxic effect was increasing on human keratinocyte cells, LDH activity also showed a rise owing to treatments of atranorin and usnic acid isolated from different lichens (Varol et al. 2015).

When investigated antioxidant properties of ULE, it was determined that they had higher TAC on healthy PMGN cells than cancerous U87MG cells. TOS levels of some concentrations of ULE on U87MG cells were found higher compared to PMGN cells. When we compared to control$^-$ group, it was indicated that ULE did not significantly cause oxidative stress ($p > 0.05$) on PMGN cells.

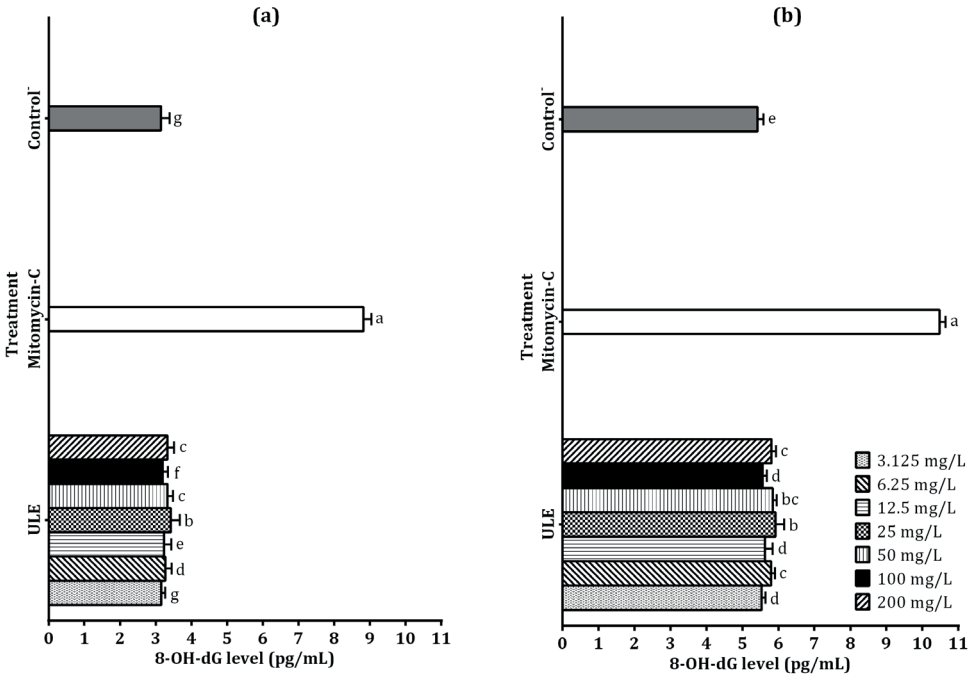

Figure 6 - 8-OH-dG adducts in the cells in the presence of ULE **(a)** for PMGN cells **(b)** for U87MG cells. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$.

Furthermore, while positive Pearson correlation coefficient (Pcc = 0.75) between TAC and cell viability was great for PMGN cells (Table III), this coefficient was significantly negative for U87MG cells (Table IV). It was not observed any significant correlation related with TOS activity on PMGN cells (Table III). Thus, it was proposed that once ULE was used in certain concentrations, they did not cause oxidative stress on healthy cells. Moreover, low concentrations of them showed antioxidative feature.

In many previous studies performed in regard to oxidative capacities of lichens, similar results appeared with the current studies. Thadhani et al. (2011) measured 2,2-diphenyl-1-picrylhydrazil (DPPH), superoxide and nitric oxide radicals scavenging capacities of two lichen compounds, namely sekikaic and lecanoric acid and reported that these compounds had high antioxidant capacities. Grujičić et al. (2014) pronounced that methanol extract obtained from *Cetraria islandica* lichen had antioxidant components and so it showed antioxidant activities such as reducing power, superoxide anion and DPPH radicals scavenging effects. Another study about suppression of oxidative stress caused by mitomycin-C in human lymphocytes by *Xanthoria elegans* lichen extract revealed again protective properties of lichens (Turkez et al. 2012). Otherwise, in the last decade, there have been many publications about oxidative capacities of lichens (Manojlović et al. 2012, Paudel et al. 2012, Ghate et al. 2013, Kosanić et al. 2013, Kumar et al. 2014, Mitrović et al. 2014). Aforementioned anti-oxidative and oxidative stress studies of the present study related with lichens suggested that if the lichens were used at certain concentrations, they were useful for health. Conversely, it was emerged that high doses of the lichens might cause oxidative stress and toxicity on some cells.

Oxidative DNA damage test used in order to genotoxicity degree of ULE on PMGN and U87MG

TABLE II
IC_{50} values for tested cells exposed to different treatments (mg/L).

Cell type	IC_{50} (limits)	Slope ± standard error (limits)	X^2
PMGN	2486.71[b] (904.42-14086.31)	0.37 ± 0.05 (0.27-0.47)	5.36
U87MG	80.93[a] (37.59-438.09)	0.17 ± 0.05 (0.08-0.26)	1.58

Values followed by different superscript letters in the same column differ significantly at $p < 0.05$.

TABLE III
Correlation between different variables for PMGN cells exposed to ULE.

	Cell viability	Concentration	LDH activity	TAC	TOS	8-OH-dG level
Cell viability	1[a]					
Concentration	-0.77**	1				
LDH activity	-0.51*	0.54*	1			
TAC	0.75**	-0.73**	-0.32	1		
TOS	-0.08	0.05	0.10	-0.01	1	
8-OH-dG level	-0.41	0.19	0.26	-0.12	0.01	1

[a]Pearson correlation coefficient; *correlation is significant at the 0.05 level; **correlation is significant at the 0.01 level.

TABLE IV
Correlation between different variables for U87MG cells exposed to ULE.

	Cell viability	Concentration	LDH activity	TAC	TOS	8-OH-dG level
Cell viability	1[a]					
Concentration	-0.77**	1				
LDH activity	0.38	-0.53*	1			
TAC	-0.48*	0.32	0.20	1		
TOS	-0.18	0.16	-0.09	0.25	1	
8-OH-dG level	-0.44*	0.14	-0.39	-0.08	0.09	1

[a]Pearson correlation coefficient; *correlation is significant at the 0.05 level; **correlation is significant at the 0.01 level.

cells revealed that 8-OH-dG level statistically decreased compared to mitomycin-C for the both cells. Presence of higher 8-OH-dG level on U87MG cells as compared with PMGN cells indicated that ULE had poor genotoxic properties on healthy cells. Correlation analysis carried out on U87MG cells also supported these results. Significant negative correlation between 8-OH-dG level and cell viability was reflected in analysis results (Table IV).

There are many past studies about genotoxic and non-genotoxic effects of many lichens. Grujičić et al. (2014) exhibited that genotoxic effect of methanol extract of *C. islandica* increased depending on dose on peripheral blood lymphocytes. In analogy to previous study, Ari et al. (2014) found that the experiments with higher concentrations of *Hypogymnia physodes* lichen species caused genotoxic activity on human breast cancer cell lines. In addition, non-genotoxic properties of many lichen species such as *Rhizoplaca chrysoleuca*, *Lecanora muralis* (Alpsoy et al. 2011) *C. islandica* (Kotan et al. 2011), *Cetraria aculeata* (Zeytinoglu et al. 2008) and *Pseudevernia furfuracea* (Emsen et al. 2018) on human lymphocytes were also elucidated.

Consequently, it was revealed that ULE inhibited the growth of glioblastoma *in vitro* and when ULE were used at the concentrations ranged from 3.125 to 25 mg/L, they will be able to become alternative treatments that in GBM therapy process (Emsen et al. 2016). In addition, information about the substances that cause the increase of the antioxidant capacity in healthy cells was obtained thanks to the determination of some of the antioxidant components in the extract. Overall, the obtained data indicate that *U. longissima* may act as a novel bio resource for naturally occurring antioxidant therapies.

AUTHOR CONTRIBUTIONS

BE, OO, TE and BT carried out the experiments. SC carried out HPLC analyses. BE and HT analysed the data and wrote the manuscript.

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