In vitro antitumor activities of the lichen compounds olivetoric, physodic and psoromic acid in rat neuron and glioblastoma cells

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ABSTRACT

Context Since methods utilised in the treatment of glioblastoma multiforme (GBM) are inadequate and have too many side effects, usage of herbal products in the treatment process comes into prominence. Lichens are symbiotic organisms used for medicinal purposes for many years. There are various anticancer treatments and components of two lichen species used in the present study.

Objective Antitumor potential of three lichen secondary metabolites including olivetoric acid (OLA) and physodic acid (PHA) isolated from Pseudevernia furfuracea (L.) Zopf (Parmeliaceae) and psoromic acid (PSA) isolated from Rhizoplaca melanophthalma (DC.) Leuckert (Lecanoraceae) were investigated on human U87MG-GBM cell lines and primary rat cerebral cortex (PRCC) cells for the first time.

Materials and methods PRCC cells used as healthy brain cells were obtained from Sprague-Dawley rats. The treatments were carried out on the cells cultured for 48 h. Cytotoxic effects of different concentrations (2.5, 5, 10, 20 and 40 mg/L) of metabolites on the cells were determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) analyses. Total antioxidant capacity (TAC) and total oxidant status (TOS) parameters were used for assessing oxidative alterations. Oxidative DNA damage potentials of metabolites were investigated via evaluating 8-hydroxy-2'-deoxyguanosine (8-OH-dG) levels.

Results Median inhibitory concentration (IC50) values of OLA, PHA and PSA were 125.71, 698.19 and 79.40 mg/L for PRCC cells and 17.55, 410.72 and 56.22 mg/L for U87MG cells, respectively. It was revealed that cytotoxic effects of these metabolites showed positive correlation with concentration, LDH activity and oxidative DNA damage.

Discussion and conclusion The present findings obtained in this study revealed that primarily OLA and then PSA had high potential for use in the treatment of GBM.

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Introduction

Glioblastoma multiforme (GBM), the most dangerous form of astrocytomas, is the highest-grade (grade IV) glioma tumour. Since GBM always grows rapidly and shows highly malignant properties, it is known as grade IV tumour (Zhang et al. 2012). GBM occurs in the nervous system and, therefore, it is difficult to treat this disease. As central nervous system does not have regenerative capabilities in contrast to other organ systems and if it is damaged, it can be devastating (Goldlust et al. 2008).

Normal function of the brain is disrupted by the growth of brain tumors. Pressure tumour growth-dependent in the brain increases in GBM. The first symptoms resulting from this pressure are headache and dizziness. Loss of sensation and movement occurring on one side of the body, speech disorders, visual disturbances (blurred vision, double vision) and cognitive disorders are other GBM symptoms (Goldlust et al. 2008; Urbanska et al. 2014).

GBM treatment methods vary according to types, dissemination, size and region of the tumour. Surgical treatment comes at the beginning of the main treatment methods. Radiotherapy and chemotherapy performed in a combined manner with surgery can increase the survival time of patients (Combs et al. 2005; Gauden et al. 2009; Wang et al. 2010). However, based on side effects of radiotherapy and chemotherapy, successful
Materials and methods
Collection and identification of lichen samples
Samples were collected in 2014 from different localities within Erzurum province of Eastern Anatolian region of Turkey. Necessary morphological and ecological characteristics of the samples were recorded and they were photographed in their natural habitats. After collecting these materials were exposed to dry room conditions. Herbaria of these lichens were made. Comparing the obtained macroscopic and microscopic data with literature (Purvis et al. 1992; Wirth 1995), they were identified as P. furfuracea (KKEF-801) and R. melanophthalma (KKEF-802). Voucher specimens are kept in the herbarium of Kazım Karabekir Education Faculty, Atatürk University, Erzurum, Turkey.

Isolation of lichen secondary metabolites
After air-drying lichen samples were powdered with liquid nitrogen. Then, 150 g of P. furfuracea and R. melanophthalma was extracted by 250 mL of ethanol and acetone solvents using a Soxhlet extraction apparatus throughout five days at 80°C, respectively. After extraction, solvents were evaporated with rotary evaporator (IKA, Staufen, Germany) under vacuum to dryness and lyophilised to get ultra-dry powders that were solubilised with minimum amount of sterile distilled water. Extraction of P. furfuracea and R. melanophthalma yielded 20.26% and 17.46% (w/w) of lichen substances, respectively. The crude extract of lichen sample was filtered and stored at 4°C, for 24 h.

The crude extract of P. furfuracea was collected and subjected to silica gel (70–230 mesh) column chromatography (CC) by eluting it with n-hexane–ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) and ethyl acetate–methyl alcohol (90:10, 80:20, 60:40, 40:60) solvent systems. At the end of this process, OLA and PHA were obtained with yields of 11.25% and 13.88% (w/w), respectively.

The crude extract of R. melanophthalma was collected and subjected to silica gel (70–230 mesh) CC by eluting it with dichloromethane–ethyl acetate (90:10, 80:20, 70:30, 50:50, 0:100) and ethyl acetate–methyl alcohol (90:10, 80:20, 60:40, 40:60) solvent systems. At the end of this process, PSA was obtained with a yield of 21.45% (w/w).

Secondary metabolites obtained from lichens were diluted to different concentrations (2.5, 5, 10, 20 and 40 mg/L) before the experimental setup. Dimethyl sulphoxide (DMSO) + relevant cell culture medium (2% DMSO) was used as a negative control (control−).
Chemical structures of secondary metabolites have been previously reported by Culberson (1969) and Asahina and Shibata (1971). $^1$H NMR and $^{13}$C NMR spectra utilised to determine the chemical structures (Figure 1) of the compounds were obtained in CDCl$_3$. Tetramethylsilane was used as standard and the chemical shift values ($\delta$) were expressed as ppm (Tables 1–3).

**Neuron cell cultures**

This study was conducted at the Medical Experimental Research Center in Atatürk University (Erzurum, Turkey). The Ethical Committee of Atatürk University approved the study protocol (Date: 13.02.2015, Decision number: 42190979-01-02/705).

PRCC cultures were obtained from six newborn Sprague-Dawley rats. The cerebral cortices were dissociated with Hank’s balanced salt solution (Sigma-Aldrich, Germany) + trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 0.02% EDTA; Sigma-Aldrich, Darmstadt, Germany), treated with DNAse type 1 (Sigma-Aldrich) and centrifuged. After having thrown away the supernatant, fresh medium containing neurobasal (Gibco, Dreieich, Germany) 10% foetal bovine serum (FBS) (Sigma-Aldrich, Darmstadt, Germany), 2% B-27 (Gibco, Dreieich, Germany) and 0.1% penicillin-streptomycin (PAN Biotech, Aidenbach, Germany) were added to the residue. Finally, neurons were seeded in 96-well plates and incubated at 37°C in 5% CO$_2$. Thus, each well contained 150 μL medium and $1 \times 10^5$ cells.

**Table 1. $^1$H NMR and $^{13}$C NMR spectral data of OLA.**

| Position | $\delta_\text{H (ppm)}$ | $\delta_\text{C (ppm)}$ |
|----------|-----------------|-----------------|
| 1        | 167.95          | 166.95          |
| 2        | 110.52          | 110.52          |
| 3        | 150.23          | 150.23          |
| 4        | 7.23            | 118.48          |
| 5        | –               | 156.64          |
| 6        | 6.96            | 111.92          |
| 7        | 2.65            | 39.32           |
| 8        | 1.61            | 33.92           |
| 9        | 1.32            | 34.48           |
| 10       | 1.35            | 25.74           |
| 11       | 0.93            | 17.15           |
| 12       | –               | 174.42          |
| 13       | –               | 108.93          |
| 14       | –               | 168.29          |
| 15       | 6.18            | 104.92          |
| 16       | –               | 166.87          |
| 17       | 6.71            | 115.71          |
| 18       | –               | 144.23          |
| 19       | 3.46            | 50.19           |
| 20       | –               | 211.16          |
| 21       | 2.42            | 45.26           |
| 22       | 1.55            | 26.29           |
| 23       | 1.32            | 34.36           |
| 24       | 1.35            | 25.41           |
| 25       | 0.93            | 17.15           |

**Figure 1.** The chemical structures of lichen secondary metabolites: (a) olivetoric acid, (b) physodic acid and (c) psoromic acid.
U87MG-GBM cell cultures

We employed human GBM U87MG cell line used widely as a model for brain cancer. The human brain GBM cell line U87MG was obtained from Ataturk University, Faculty of Medicine, Erzurum, Turkey. Cells were harvested with 0.25% trypsin–EDTA and suspended with Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Germany) containing 15% FBS, 1% L-glutamine (Sigma-Aldrich, Germany) and 1% penicillin–streptomycin. Cells were seeded in 25 mL flasks. After reaching to proper volume, they were seeded in 96-well plates. Thus, each well contained 100 μL medium with 1 × 10^5 cells.

MTT assay

The cells were seeded in 96-well plates. Cells were incubated at 37 °C in a humidified 5% CO₂/95% air mixture and treated with secondary metabolites at different concentrations (2.5, 5, 10, 20 and 40 mg/L) for 48 h. MTT assay was carried out with a commercially available kit (Cayman Chemical Company, Ann Arbor, MI). In this assay, MTT moves into the cell due to owned net positive charge and plasma membrane potential and it is reduced a purple formazan intracellular by NAD(P)H oxidoreductases in the cell (Berridge et al. 2005).

MTT reagent (10 μL) was added to the cell cultures. The plate was incubated in CO₂ incubator at 37 °C for 4 h and it was centrifuged at 400 g for 10 min. One hundred microlitres of crystalline solvent solution was added to each well. Crystals of formazan were dissolved through this solution. The intensity of the formazan was measured at 570 nm with a Multiscan Go microplate reader (Thermo Scientific, Landsmeer, Netherlands).

LDH release assay

LDH assay was carried out in the culture medium with a commercially available kit (Cayman Chemical Company, Ann Arbor, MI). 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 the first step of the assay, LDH catalyzes the reduction of NAD⁺ to NADH and H⁺ by oxidation of lactate to pyruvate. In the second step, diaphorase uses the newly formed NADH and H⁺ to catalyze the reduction of a tetrazolium salt to highly colored formazan. The amount of formazan produced is proportional to the amount of LDH released into the culture medium as a result of cytotoxicity. Increase in the number of dead or cells damaged plasma membrane leads to increasing of LDH activity in the culture supernatant (Haslam et al. 2000; Wolterbeek & van der Meer 2005).

One hundred microlitres of LDH standard was added relevant wells and 100 μL of medium on cells incubated for 48 h was added to other wells. One hundred microlitres of LDH reaction solution was added to each well. The plate was incubated gently for 30 min via orbital shaker (Labnet, Edison, NJ) at room temperature. Spectrophotometric reading was carried out at 490 nm. In LDH assays, mitomycin-C chemotherapeutic agent was used as a positive control.

TAC assay

TAC assay was carried out with a commercially available kit (Rel Assay Diagnostics, Turkey) on PRCC and

| Table 2. ¹H NMR and ¹³C NMR spectral data of PHA. |
|-----------------------------------------------|
| Position | δ_H (ppm) | δ_C (ppm) |
| 1        | 6.94      | 108.14    |
| 2        | –         | 164.42    |
| 3        | –         | 110.78    |
| 4        | –         | 142.16    |
| 5        | –         | 143.91    |
| 6        | –         | 150.12    |
| 7        | –         | 177.23    |
| 8        | 2.65      | 30.61     |
| 9        | 1.61      | 33.28     |
| 10       | 1.32      | 33.44     |
| 11       | 1.35      | 24.78     |
| 12       | 0.93      | 16.22     |
| 13       | –         | 162.74    |
| 14       | –         | 116.16    |
| 15       | –         | 162.58    |
| 16       | 6.66      | 102.26    |
| 17       | –         | 164.19    |
| 18       | 6.86      | 115.29    |
| 19       | –         | 141.59    |
| 20       | 3.46      | 50.16     |
| 21       | –         | 209.32    |
| 22       | 2.42      | 44.26     |
| 23       | 1.56      | 25.29     |
| 24       | 1.32      | 33.35     |
| 25       | 1.35      | 24.46     |
| 26       | 0.93      | 16.22     |

| Table 3. ¹H NMR and ¹³C NMR spectral data of PSA. |
|-----------------------------------------------|
| Position | δ_H (ppm) | δ_C (ppm) |
| 1        | 6.78      | 108.14    |
| 2        | –         | 164.42    |
| 3        | –         | 110.78    |
| 4        | –         | 142.16    |
| 5        | –         | 143.91    |
| 6        | –         | 150.12    |
| 7        | –         | 177.23    |
| 8        | 2.65      | 30.61     |
| 9        | 1.61      | 33.28     |
| 10       | 1.32      | 33.44     |
| 11       | 1.35      | 24.78     |
| 12       | 0.93      | 16.22     |
| 13       | –         | 162.74    |
| 14       | –         | 116.16    |
| 15       | –         | 162.58    |
| 16       | 6.66      | 102.26    |
| 17       | –         | 164.19    |
| 18       | 6.86      | 115.29    |
| 19       | –         | 141.59    |
| 20       | 3.46      | 50.16     |
| 21       | –         | 209.32    |
| 22       | 2.42      | 44.26     |
| 23       | 1.56      | 25.29     |
| 24       | 1.32      | 33.35     |
| 25       | 1.35      | 24.46     |
| 26       | 0.93      | 16.22     |
U87MG-GBM cell cultures for 48 h. The purpose of the kit assay is to determine antioxidant levels of samples by inhibiting formation of a free radical, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) compound (Erel 2004). The assay is calibrated with a stable antioxidant of vitamin E analogue called Trolox equivalent.

Cells incubated for 48 h were removed from the incubator. Medium on the precipitated cells was added to relevant wells. Standard solutions in the kit were added to relevant wells. Reagent 1 solution was added to each well. First spectrophotometric reading was carried out at 660 nm. After the first reading, reagent 2 solution was added to each well and the plate was incubated at room temperature for 10 min. Second spectrophotometric reading was carried out at 660 nm. In TAC assays, ascorbic acid from organic antioxidant compounds was used as a positive control.

**TOS assay**

The TOS assay was carried out with a commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey) on PRCC and U87MG-GBM cell cultures for 48 h. With this assay, complexes formed with the ferric ion are oxidised to ferrous ion by oxidants presented in the sample. Ferrous ions form a colored structure with chromogen in the acidic environment. The color intensity measured spectrophotometrically is related to the total amount of oxidant molecules in the sample (Erel 2005). The assay is calibrated with hydrogen peroxide (H2O2).

Cells incubated for 48 h were removed from the incubator. Medium on the precipitated cells was added to relevant wells. Standard solutions in the kit were added to relevant wells. Reagent 1 solution was added to each well. First spectrophotometric reading was carried out at 530 nm. After the first reading, reagent 2 solution was added to each well and the plate was incubated at room temperature for 10 min. Second spectrophotometric reading was carried out at 530 nm. In TOS assays, H2O2, reactive oxygen species was used as a positive control.

**Oxidative DNA damage assay**

Oxidative DNA damage assay was carried out in the culture medium by commercially available DNA/RNA Oxidative Damage kit (Cayman Chemical Company, Ann Arbor, MI). The purpose of this assay is determining of oxidative DNA damage in the cells via calculation of 8-OH-dG level. 8-OH-dG is the form of oxidised guanine (Gan et al. 2012). Experimental steps were performed in accordance with the kit procedure. In oxidative DNA damage assays, mitomycin-C chemotherapeutic agent was used as a positive control.

**Statistical analyses**

All the assays were carried out at least in triplicate measurements. Activities of secondary metabolites were analysed using variance (ANOVA) test followed by an appropriate post hoc test (Duncan test) and values with \( p < 0.05 \) were considered as significantly different. IC\(_{50}\) values were calculated with Probit regression analysis and associated 95\% confidence limits for each treatment. Relations among the variables were tested by bivariate correlation analysis. These calculations were carried out using Statistical Package for Social Sciences (SPSS, version 21.0, IBM Corporation, Armonk, NY).

**Results**

**Antiproliferative activities**

Cell viabilities of PRCC and U87MG cells exposed to different concentrations of OLA, PHA and PSA metabolites were determined by MTT analysis. The results showed that solutions with the highest concentration (40 mg/L) highly inhibited cell proliferation. OLA was the most potent cytotoxic agent for PRCC and U87MG cells. While cell viability decreased to 59.09\% in PRCC cells exposed to maximum concentration of OLA, this rate was 38.36\% in U87MG cells. PHA metabolite showed the lowest cytotoxic effect for both cell types (Figure 2). Forty-eight hours median inhibitory concentration (IC\(_{50}\)) values for tested metabolites indicated the toxic concentrations of the metabolites. According to IC\(_{50}\) values, secondary metabolites were in the ascending order of PSA < OLA < PHA for PRCC cells and OLA < PSA < PHA for U87MG cells. It was determined that IC\(_{50}\) values calculated for both cell types were statistically (\( p < 0.05 \)) different from each other (Tables 4 and 5).

In addition, in order to determine cytotoxic effects of OLA, PHA and PSA on PRCC and U87MG cells, LDH release test was also used. The present study revealed that metabolite solutions with the highest concentration caused maximum LDH release. Maximum concentration of LDH in PRCC and U87MG cell mediums was detected at treatment with mitomycin-C (positive control) (100.32 and 94.95 \( \mu \)U/mL, respectively). In metabolite treatments, for PRCC and U87MG cells, the closest values to LDH activity of mitomycin-C were associated with the solution with a concentration of 40 mg/L of OLA (48.61 and 72.00 \( \mu \)U/mL, respectively). LDH activities of solutions with concentrations of 2.5 and
5 mg/L of PHA and PSA were not statistically \( (p > 0.05) \) different from activities of control having minimum LDH activity for both cell types (Figure 3).

**Antioxidative activities**

TAC analysis was carried out to determine total antioxidant capacities of different concentrations of OLA, PHA and PSA metabolites on PRCC and U87MG cells. It was shown that positive control, ascorbic acid, had the highest TAC on both cells. When the TAC of metabolites was investigated on PRCC cells, it was determined that maximum concentrations of PHA and PSA showed greater TAC (43.13 and 42.34 mmol Trolox equivalent/L, respectively) in comparison with other solutions. Furthermore, there was no statistically \( (p > 0.05) \) significant difference between TAC level of concentration of 20 mg/L of PHA and TAC levels possessed by maximum concentrations of PHA and PSA. In addition, it was demonstrated that OLA metabolite had the lowest TAC on PRCC cells (Figure 4).

As for TAC activities of metabolites on U87MG cells, it was observed that TAC values for all metabolite concentrations were close to each other. A concentration of 40 mg/L of PSA had the highest TAC (7.63 mmol Trolox equivalent/L) among all the metabolite treatments. TAC rates (4.60, 4.65 and 4.06 mmol Trolox equivalent/L, respectively) of solutions at concentration of 2.5, 20 and 40 mg/L of OLA were the lowest values and there was no statistically \( (p > 0.05) \) significant difference between these values and TAC value of control^− (Figure 4).
TOS analysis was carried out to determine TOS of different concentrations of OLA, PHA and PSA metabolites on PRCC and U87MG cells. Based on TOS levels on PRCC cells, maximum concentrations of tested metabolites, except for PSA, showed the highest TOS activity. Among treatment groups, positive control, H$_2$O$_2$ and then solution at concentration of 40 mg/L of OLA caused high degree of oxidative stress. Considering all concentrations of the compounds, it was determined that PSA caused oxidative stress in low rates (Figure 5).

TOS data revealed for U87MG cells clearly indicated that the OLA caused high TOS activity after H$_2$O$_2$. TOS levels of concentrations of 20 and 40 mg/L of OLA were 49.52 and 57.77 μmol H$_2$O$_2$ equivalent/L, respectively. However, remarkably, a concentration of 5 mg/L of PSA elevated oxidative stress level (46.66 μmol H$_2$O$_2$ equivalent/L). In this study, it was demonstrated that PHA had lower TOS activity on U87MG cells. Moreover, as a result of performed statistical analyses, it was detected that TOS levels occurred by tested lichen secondary metabolites on both cell types were significant at the 0.05 level as compared with control treatments (Figure 5).

8-OH-dG activities increased in a concentration-dependent manner for both cells. Based on 8-OH-dG levels on U87MG cells, it was shown that 8-OH-dG activity of OLA was higher and statistically different from all other values. By comparison with 8-OH-dG activities of the control, activities caused by concentrations of 20 and 40 mg/L of OLA; 40 mg/L of PHA; 10, 20 and 40 mg/L of PSA were higher and statistically different from control values (Figure 6).

When levels of oxidative DNA damage occurred by tested lichen secondary metabolites on U87MG cells were examined, it was revealed that 8-OH-dG activity was higher and statistically different than the other metabolites. While treatment having maximum 8-OH-dG level (12.33 pg/mL) belonged to mitomycin-C, values of all metabolite treatments were found in lower
amounts (3.88–6.06 pg/mL). Difference among the 8-OH-dG levels of concentrations of 20 and 40 mg/L of OLA showing the highest genotoxic effect (6.01 and 6.06 pg/mL, respectively) among the tested metabolites was not significant at the 0.05 level.

Discussion

Since GBM is very common in brain tumours and shows dangerous malignant characteristics, many therapeutic experiments have been carried out. In addition to surgery, radiotherapy or chemotherapy methods performed for the treatment of the disease, herbal treatment methods cannot be ignored. Many researchers aimed to contribute to treatment process of GBM by using herbal products (Kim et al. 2008; Deng et al. 2009; Hahm et al. 2010; Jung & Ghil 2010; Jeong et al. 2011; Markiewicz-Zukowska, Borawska, et al. 2013; Markiewicz-Zukowska, Naliwajko, et al. 2013; Wang et al. 2013). However, to the best of our knowledge, studies have not yet been conducted to evaluate the activities of lichens from herbal products on GBM treatment.

It was reported that lichen extracts and metabolites have been so far used for many studies and positive results were obtained. In the present study, cytotoxic effects of OLA, PHA and PSA isolated from lichens on U87MG cells and whether these effects are oxidative stress-induced were investigated. Additionally, it was analysed whether LDH and 8-OH-dG levels occurred by tested secondary metabolites affect inhibition of cell proliferation. Furthermore, cytotoxic effects of the metabolites were investigated on healthy cells of brain neurons by using PRCC cells and suitable metabolite concentrations possessing antioxidant capacity and reducing oxidative stress on healthy cells were determined by measuring TAC levels of solutions at different concentrations of the metabolites.

Binary correlation analyses showed that all metabolite treatments decreased viable cell numbers and increased LDH and 8-OH-dG level in a concentration-dependent manner in both PRCC and U87MG cells (Tables 6–11). Also, calculated IC50 values gave insight about proliferation inhibitory concentrations on cells (Tables 4 and 5).

Among the tested metabolites, OLA was compound possessing low levels of side effects and showing higher

Figure 4. TAC levels of different lichen secondary metabolites on cells (a) for PRCC cells and (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.
cytotoxic activity on U87MG cancer cells compared to PRCC cells. As shown in Table 4, IC_{50} value of OLA on PRCC cells was 125.71 mg/L. OLA showed strong cytotoxic activity on U87MG cancer cell line that was reflected by its low IC_{50} value (17.55 mg/L) (Table 5). As can be seen clearly in Table 7, there were high negative correlations between cell viability and concentration; cell viability and LDH activity; cell viability and oxidative DNA damage; cell viability and TOS for OLA treatment on U87MG cells [Pearson correlation coefficient (Pcc) ≤ -0.90]. Similarly based on OLA treatment on PRCC cells, it was detected that there were negative correlations between previously mentioned variables, but coefficients were lower. When TAC level of OLA was investigated, it was determined that negative correlations between TAC and concentration on PRCC and U87MG cells were significant at 0.01 and 0.05 levels, respectively. This result revealed that there was a higher level of negative correlation between antioxidant capacity and concentration of OLA on PRCC cells in comparison with U87MG cells (Tables 6 and 7).

While concentrations of 20 and 40 mg/L of OLA significantly caused oxidative DNA damage on PRCC cells, all concentrations of OLA significantly increased 8-OH-dG level on U87MG cells compared to control (Figure 6). The results obtained OLA treatment revealed that although low concentrations of OLA showed cytotoxic effect on U87MG cancer cells, they did not greatly damage healthy PRCC cells.

Many researchers have previously examined activities of OLA. The latest ones in these activities have been anticancer, antimicrobial and antioxidant. Koparal et al. (2010) investigated antiangiogenic activity of OLA isolated from P. furfuracea and reported that reduction in endothelial cells occurred as OLA broke structure of the actin cytoskeleton. Inhibitory activities of bacteria growth of OLA were reported by some researchers (Tu¨rk et al. 2006; Mitrovic ´ et al. 2014). In addition to these activities, it was determined that OLA had antioxidant capacity containing considerable amount of antioxidant components and having 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Mitrović et al. 2014).
Figure 6. 8-OH-dG level in cells exposed to different lichen secondary metabolites (a) for PRCC cells and (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.

Table 6. Correlation between different variables for PRCC cells exposed to OLA.

|                      | Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
|----------------------|----------------|---------------|--------------|----------------------|-----|-----|
| Cell viability       | 1.00*          | -0.90**       | -0.88**      | -0.85**              | 0.37| -0.78**|
| Concentration        | -0.90**        | 1.00          | 0.91**       | 0.95**               | -0.70**| 0.54** |
| LDH activity         | -0.88**        | 0.91**        | 1.00         | 0.97**               | -0.51| 0.85** |
| Oxidative DNA damage | -0.85**        | 0.95**        | 0.97**       | 1.00                 | -0.64*| 0.92** |
| TAC                  | 0.37           | -0.70**       | -0.51        | -0.64*               | 1.00| -0.79**|
| TOS                  | -0.78**        | 0.94**        | 0.85**       | 0.92**               | -0.79**| 1.00 |

*Pearson correlation coefficient.
*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.

Table 7. Correlation between different variables for U87MG cells exposed to OLA.

|                      | Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
|----------------------|----------------|---------------|--------------|----------------------|-----|-----|
| Cell viability       | 1.00*          | -0.92**       | -0.97**      | -0.98**              | 0.48| -0.95**|
| Concentration        | -0.92**        | 1.00          | 0.98**       | 0.85**               | -0.59*| 0.95** |
| LDH activity         | -0.97**        | 0.98**        | 1.00         | 0.93**               | -0.54*| 0.97** |
| Oxidative DNA damage | -0.98**        | 0.85**        | 0.93**       | 1.00                 | -0.45| 0.93** |
| TAC                  | 0.48           | -0.59*        | -0.54*       | -0.45                | 1.00| -0.63**|
| TOS                  | -0.95**        | 0.95**        | 0.97**       | 0.93**               | -0.63*| 1.00 |

*Pearson correlation coefficient.
*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.
Among the IC\textsubscript{50} values, the highest values belonged to PHA. IC\textsubscript{50} values of OLA for PRCC and U87MG cells were 698.19 and 410.72 mg/L, respectively (Tables 4 and 5). High IC\textsubscript{50} values represented low cytotoxicity of PHA on the cells.

LDH release and 8-OH-dG level in low rates were observed in PRCC and U87MG cells treated with PHA metabolite. The amount of LDH in the cell culture medium can give idea about the integrity of the cell membrane. Therefore, cell medium that have a damaged cell membrane contains greater proportion of LDH enzyme (Haslam et al. 2000; Wolterbeek & van der Meer 2005). In light of this information, it was showed that PHA produced less damage with PRCC and U87MG cell membranes than the other metabolite treatments. In U87MG cells, while concentration of 40 mg/L of PHA

| Table 8. Correlation between different variables for PRCC cells exposed to PHA. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
| 1.00* | -0.97** | -0.98** | -0.96** | -0.90** | -0.40 |
| -0.97** | 1.00 | 0.97** | 0.95** | 0.89** | 0.50 |
| -0.98** | 0.97** | 1.00 | 0.98** | 0.92** | 0.32 |
| -0.96** | 0.95** | 0.98** | 1.00 | 0.94** | 0.28 |
| -0.90** | 0.89** | 0.92** | 0.94** | 1.00 | 0.22 |
| -0.40 | 0.50 | 0.32 | 0.28 | 0.22 | 1.00 |

*Pearson correlation coefficient.
**Correlation is significant at the 0.01 level.

| Table 9. Correlation between different variables for U87MG cells exposed to PHA. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
| 1.00* | -0.97** | -0.97** | -0.93** | 0.38 | 0.42 |
| -0.97** | 1.00 | 0.95** | 0.89** | -0.42 | -0.32 |
| -0.97** | 0.95** | 1.00 | 0.97** | -0.28 | -0.55* |
| -0.93** | 0.89** | 0.97** | 1.00 | -0.18 | -0.56* |
| 0.38 | -0.42 | -0.28 | -0.18 | 1.00 | -0.02 |
| 0.42 | -0.32 | -0.55* | -0.56* | -0.02 | 1.00 |

*Pearson correlation coefficient.
*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.

| Table 10. Correlation between different variables for PRCC cells exposed to PSA. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
| 1.00* | -0.90** | -0.91** | -0.94** | -0.95** | -0.02 |
| -0.90** | 1.00 | 0.99** | 0.97** | 0.94** | 0.30 |
| -0.91** | 0.99** | 1.00 | 0.97** | 0.95** | 0.27 |
| -0.94** | 0.97** | 0.97** | 1.00 | 0.97** | 0.25 |
| -0.95** | 0.94** | 0.95** | 0.97** | 1.00 | 0.20 |
| -0.02 | 0.30 | 0.27 | 0.25 | 0.20 | 1.00 |

*Pearson correlation coefficient.
**Correlation is significant at the 0.01 level.

| Table 11. Correlation between different variables for U87MG cells exposed to PSA. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Cell viability | Concentration | LDH activity | Oxidative DNA damage | TAC | TOS |
| 1.00* | -0.94** | -0.96** | -0.93** | -0.93** | 0.30 |
| -0.94** | 1.00 | 0.99** | 0.99** | 0.93** | -0.34 |
| -0.96** | 0.99** | 1.00 | 0.99** | 0.95** | -0.35 |
| -0.93** | 0.99** | 0.99** | 1.00 | 0.92** | -0.33 |
| -0.93** | 0.93** | 0.95** | 0.92** | 1.00 | -0.45 |
| 0.30 | -0.34 | -0.35 | -0.33 | -0.45 | 1.00 |

*Pearson correlation coefficient.
**Correlation is significant at the 0.01 level.
had higher values in LDH activity treatment (Figure 3), all its concentrations showed 8-OH-dG activity (Figure 6) compared to PRCC cells.

High concentrations of PHA showed strong TOS activity when compared with low concentrations for U87MG cells, whereas the maximum concentration of PHA caused high oxidative stress on PRCC cells (Figure 5). Meanwhile, average TAC level of PHA on PRCC cells represented the highest value of metabolite treatments. The highest value after ascorbic acid-induced TAC level belonged to maximum concentration of PHA. For PRCC cells, PHA significantly increased TAC level compared to the control (Figure 4) and it was showed high positive correlation at 0.01 level between concentration and TAC level (Table 8). The results obtained for PHA metabolite indicated that present metabolite did not show a high degree of cytotoxicity on both tested cells. However, U87MG cancer cells were more affected than healthy PRCC cells with PHA treatment. Considering high antioxidant capacity of PHA on PRCC cells, it was expected that using certain concentrations of PHA could reduce oxidative stress on healthy cells.

Many researchers carried out studies about antioxidant capacity of PHA in previous years. As a result of examination of DPPH and superoxide anion capture and reducing power activities of PHA, it was detected that PHA metabolite had high antioxidant capacity (Kosanić & Ranković 2011; Kosanić et al. 2013; Ranković et al. 2014). Furthermore, it was reported that PHA that showed antimutagenic effect inhibiting the formation of reactive metabolites (Osawa et al. 1991) possessed antimicrobial activity (Yilmaz et al. 2005; Kosanić & Ranković 2011; Kosanić et al. 2013; Ranković et al. 2014) if it was used at certain concentrations. In recent years, cytotoxic effects of PHA tested toxicity on microorganisms were evaluated on different cells. Proliferation inhibitory activities on rat thymocytes (Pavlovic et al. 2013), human melanoma and colon cancer cells (Kosanić et al. 2013; Ranković et al. 2014) and cervical cancer cells (Stojanović et al. 2014) of PHA metabolite were investigated.

As shown in Tables 4 and 5, presented IC50 values, PSA-induced cytotoxic activities on PRCC and U87MG cells were close to each other. Among tested concentrations of PSA, ones over 2.5 mg/L significantly exhibited higher LDH activity in comparison with control (Figure 3). There was very high level of positive correlation between concentration and LDH activity for PRCC and U87MG cells (Pcc = 0.99). Similarly, positive correlation at 0.01 level was detected in concentration-oxidative DNA damage and LDH activity-oxidative DNA damage binary correlation analyses for both tested cells. Any significant correlation could not found between TOD and another variable (Tables 10 and 11). While concentration of 5 mg/L of PSA caused high oxidative stress on U87MG cells, it showed low TOD activity on PRCC cells. Hence, especially the aforementioned concentration of PSA has become important in terms of GBM cancer treatment (Figure 5). TAC level of PSA on PRCC cells were higher than U87MG cells (Figure 4) and this level increased in a concentration-dependent manner (Tables 10 and 11). When overall data about PSA examined, it was indicated that PSA was a compound possessed high antioxidant capacity. If this compound was used at certain concentrations, it showed cytotoxic effect on the cancer cell lines and did not arise an oxidative stress on healthy neuron cells.

It was recently observed in many studies that PSA carried protective property due to its antioxidant capacity as well as toxic effect when used in high concentrations. Behera et al. (2012) found that limited concentrations of PSA increased activities of free radicals and nitric oxide radicals scavenging and lipid peroxidation inhibition level proportionally with increasing concentration. Thus, cardiovascular protective activity of PSA detected presence of the antioxidant capacity was also included in the results of the same study. Another study on the protective effect of PSA was carried out on mice and the gastric protective effect of PSA was determined (Sepulveda et al. 2013). In the studies related with toxic concentrations of PSA, inhibition of growth as determined in different bacteria species (Celenza et al. 2013; Mitrovic et al. 2014), liver parasites (Lauinger et al. 2013) and some cancer cells (Correcché et al. 2004; Brandao et al. 2013) exposed to PSA. In addition, Correcché et al. (2004) revealed that PSA significantly showed apoptotic activity on hepatocytes.

Conclusions

In the present study evaluating cytotoxicity, antioxidative, prooxidative and genotoxicity potentials of OLA, PHA and PSA isolated from lichens on healthy (PRCC) and cancerous (U87MG) brain cells, the findings revealed that there was concentration-dependent cytotoxicity and genotoxicity for all metabolite treatments. While high antioxidant capacity was noted at low concentrations, it was determined that high concentrations caused oxidative stress-induced cytotoxic effect on the tested cells. Considering effects (side effects) of tested lichen secondary metabolites on PRCC cells, primarily OLA and then PSA had high potential for use in the treatment of GBM. In addition, it was demonstrated that since PHA had high antioxidant capacity, it would be able to use as a natural antioxidant.
Declaration of interest

There is no conflict of interest in any form between the authors. This work was supported by Karmano glu Mehmetbey University Scientific Research Projects Commission with 01-D-13 project number.

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