Afyon Kocatepe Üniversitesi Fen ve Mühendislik Bilimleri Dergisi

Afyon Kocatepe University Journal of Science and Engineering

AKÜ FEMÜBİD **18** (2018) **011003** (763-773) AKU J. Sci. Eng. **18** (2018) **011003** (763-773)

DOİ: 10.5578/fmbd.67733

Araştırma Makalesi / Research Article

In Vitro Investigation of the Anticancer Activity of Friedelin in Glioblastoma Multiforme

Bugrahan Emsen[1]*, Tubanur Engin[2], Hasan Turkez[3]

[1]*Department of Biology, Kamil Özdağ Faculty of Science, Karamanoğlu Mehmetbey University, Karaman, Turkey*

[2]*Department of Physiology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey*

[3]*Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey*

e-posta: bugrahanemsen@gmail.com

Geliş Tarihi: 27.01.2018 ; Kabul Tarihi: 28.11.2018

Anahtar kelimeler
Cytotoxicity; Genotoxicity; Total Antioxidant Capacity; Total Oxidative Stress

Abstract

There are different herbal methods used for support in many cancer diseases. Lichens are important organisms containing unique herbal compounds and it is known that they have different anticancer activities. Starting from these features, the present study was aimed to investigate anticancer activity of friedelin (FRI), a lichen compound against glioblastoma multiforme (GBM) showed dangerous malignant properties within brain cancer species. It was used human U87MG-GBM cancer cell lines and primary rat cerebral cortex (PRCC) non-cancerous cells isolated from Sprague-Dawley rats in order to determine side effect level of FRI. In the experiments, cytotoxic (via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) tests), antioxidant (via total antioxidant capacity (TAC) test), pro-oxidant (via total oxidative stress (TOS) test) and genotoxic (via 8-hydroxy-2′-deoxyguanosine (8-OH-dG) test) activities of different concentrations of FRI were tested. As a result of the study, MTT assay revealed that FRI showed higher cytotoxic activity on U87MG cells compared to PRCC cells (median inhibitory concentration (IC_{50}): 46.38 and 1271.77 mg/L, respectively). Based on U87MG cells, it was determined a significant positive correlation between LDH and TOS activities. High positive correlation between TAC and cell viability on healthy PRCC cells exhibited antioxidant capacity of FRI. Consequently, the results obtained from the present study proved the potential of natural product with an antioxidant capacity as a source for anticancer compound against GBM.

Glioblastoma Multiformede Friedelinin Antikanser Aktivitesinin *In Vitro* İncelenmesi

Keywords
Sitotoksisite; Genotoksisite; Toplam Antioksidan Kapasite; Toplam Oksidatif Stres

Özet

Pek çok kanser hastalığında destek için kullanılan farklı bitkisel yöntemler vardır. Likenler benzersiz bitkisel bileşikler içeren önemli organizmalardır ve farklı antikanser aktivitelerine sahip oldukları bilinmektedir. Bu özelliklerden yola çıkarak, mevcut çalışma, beyin kanseri türleri içerisinde tehlikeli habis özellikler gösteren glioblastoma multiformeye (GBM) karşı bir liken bileşiği olan friedelinin (FRI) antikanser aktivitesini araştırmayı amaçlamıştır. FRI'nın yan etki düzeyini belirlemek için insan U87MG-GBM kanser hücre hatları ve Sprague-Dawley sıçanlarından izole edilen primer sıçan serebral korteks (PSSK) kanserli olmayan hücreler kullanılmıştır. Denemelerde, FRI'nın farklı konsantrasyonlarının sitotoksik (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) ve laktat dehidrogenaz (LDH) testleri ile), antioksidan (toplam antioksidan kapasite (TAK) testi ile), pro-oksidan (toplam oksidatif stres (TOS) ile) ve genotoksik (8-hidroksi-2′-deoksiguanozin (8-OH-dG) testi ile) aktiviteleri test edilmiştir. Çalışma sonucunda MTT uygulaması, FRI'nın PSSK hücrelerine kıyasla U87MG hücreleri üzerinde daha yüksek sitotoksik aktivite gösterdiğini ortaya koymuştur (sırasıyla medyan inhibitör konsantrasyonu (IC_{50}): 1271.77 ve 46.38 mg/L). U87MG hücreleri temel alındığında, LDH ve TOS aktiviteleri arasında anlamlı bir pozitif korelasyon olduğu saptanmıştır. Sağlıklı PSSK hücrelerinde, TAK ve hücre canlılığı arasındaki yüksek pozitif korelasyon FRI'nın antioksidan kapasitesini sergilemiştir. Sonuç olarak, mevcut çalışmadan elde edilen sonuçlar, GBM'ye karşı antikanser bileşiği kaynağı olarak antioksidan kapasiteye sahip doğal ürün potansiyelini ortaya koymuştur.

1. Introduction

Astrocytoma is the most common glioma and constitutes half of primary brain and spinal tumors. It is usually located in the brain, although it is all over the central nervous system (Somasundaram *et al*. 2005). The astrocytoma seen at all ages is common in adults and more commonly in middle-aged men. Otherwise, astrocytomas located in the brain stem are often seen in children and young adults and cause a large proportion of childhood tumors (Omar and Mason 2012). Among astrocytomas classified as low-grade astrocytoma, glioblastoma and anaplastic astrocytoma, glioblastoma known also as glioblastoma multiforme (GBM) is the most aggressive and common tumor type (Rao *et al*. 2014). GBM, the fastest growing astrocytoma type is most commonly found in brain, frontal and temporal lobes and there are dead tumor cells seen as necrosis in the field of GBM. GBM patients generally lose their lives in the first fifteen months (Ohgaki and Kleihues 2007; Smoll *et al*. 2013). Among the main treatment methods, chemotherapy, radiotherapy and surgery are the most important ones (Clarke *et al*. 2013). With surgery, it is essential to remove as much tumor as possible without damaging the patient, but it is not possible to completely clean the tumor (Reed 2009). The aim of radiotherapy and chemotherapy is to kill the proliferating tumor tissue and these methods are generally used as combined (Bosset *et al*. 2006). Scientists have been searching for different ways for years because of the fact that radiotherapy and chemotherapy treat to a certain level and later causes side effects on different tissue and organs such as musculoskeletal (Mavrogenis *et al*. 2010), cardio (Perrino *et al*. 2014) bone marrow and gastrointestinal tract (Giglio 2010).

In recent years, combined treatment methods created by adding natural products to basic treatment processes as well as many different activities (Emsen *et al*. 2016b; Dogan *et al*. 2017) of herbal products have been gaining importance (Karatas *et al*. 2015). Many studies have shown that the side effects of plant components used on different cancer cells are low (Kour 2014; Emsen *et al*. 2016a). Because of their characteristic biochemical and ecological characteristics resulting from their symbiotic systems, lichens are one of the most important potential organisms for which plant-based drug substances can be obtained (Brodo *et al*. 2001). One of the most interesting aspects of lichen biology is that lichens produce hundreds of secondary compounds, and almost all of them are specific to lichens (Boustie and Grube 2005). These special ingredients add many different biological activity properties such as antibacterial (Abuiraq *et al*. 2015), antioxidant (Sundararaj *et al*. 2015), antimutagenic (Koçer *et al*. 2014), antiviral (Odimegwu *et al*. 2015) and insecticidal (Emsen *et al*. 2012, 2015) to the lichens. Furthermore, there are a lot of studies on the anticancer activity of lichens while looking for alternative treatment methods and medicines against various cancer diseases such as human melanoma, colon carcinoma (Manojlović *et al*. 2012; Grujičić *et al*. 2014), breast, pancreatic cancer (Einarsdóttir *et al*. 2010), Burkitt's lymphoma (Shrestha *et al*. 2015), lung and prostate carcinoma (Bézivin *et al*. 2004). However, no report so far has found anticancer activity of friedelin (FRI) (Feng *et al*. 2009; Laxinamu *et al*. 2013), one of the compounds of *Usnea longissima* Ach., an important medicinal lichen (Bai *et al*. 2014), against human GBM U87MG cells. Thus, the aim of the present study was to evaluate cytotoxic, oxidative and genotoxic effects of FRI on cancerous U87MG as well as healthy primary rat cerebral cortex (PRCC) cells.

2. Materials and Methods

2.1 Test compound

FRI (Cas: 559-74-0, $C_{30}H_{50}O$) was purchased from Sigma-Aldrich Group, Germany. The compound was diluted to different concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/L) with 2% DMSO.

2.2. Neuron cell cultures

The present research was organized at the Medical Experimental Research Center was approved by Ethical Committee (reference number 42190979-01-02/705). Six newborn Sprague-Dawley rats were used obtaining PRCC cultures. It was used Hank's Balanced Salt Solution + trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Germany) in order to detach cerebral cortices. Supernatant was removed and medium with neurobasal (Gibco, Germany) were added to test tube. The cells were seeded in the plates and were incubated at 37°C in 5% CO_2. In this way, each well contained 150 µL medium and 1×10^5 cells.

2.3. U87MG-GBM cell cultures

We utilized human U87MG-GBM cell line used generally as a model for brain cancer. The cells were cultured with RPMI 1640 medium containing 15% FBS, 1% L-glutamine, 1% penicillin-streptomycin (Sigma-Aldrich) with 0.25% trypsin-EDTA. The cells were cultured and then reaching to proper volume and seeded in the plates. Thus, each well contained 100 µL medium with 1×10^5 cells.

2.4. 3- (4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) assay

The cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture for 48 h and exposed to FRI with various concentration (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/L). Commercially MTT kit (Cayman Chemical Company, USA) was used for this assay. The cell cultures were exposed to MTT reagent and incubated at 37°C in CO_2 incubator for 4 h. Crystals of formazan were dissolved with this solution. Formazan intensity was measured at 570 nm. Positive control was mitomycin-C chemotherapeutic agent for MTT assays.

2.5. Lactate dehydrogenase (LDH) release assay

Commercially LDH kit (Cayman Chemical Company, USA) was used for this assay. The relevant wells were treated with LDH standard and medium. Then, LDH reaction solution was used. The plate with the cells was incubated at room temperature. The plate was read at 490 nm. Positive control was mitomycin-C chemotherapeutic agent for LDH assays.

2.6. Total antioxidant capacity (TAC) assay

TAC levels in the cells for 48 h were measured by commercially TAC kit (Rel Assay Diagnostics, Gaziantep, Turkey). 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. Medium on the cells was used. First spectrophotometric reading was performed adding standard solutions and Reagent 1 solution at 660 nm. Next reading was performed at 660 nm. Positive control was ascorbic acid from organic antioxidant compounds for TAC assays. The assay is calibrated with a stable antioxidant of vitamin E analogue called Trolox equivalent. The calculations were performed using the formulas included in the kit.

2.7. Total oxidative stress (TOS) assay

TOS levels in the cells for 48 h were measured by commercially TOS kit (Rel Assay Diagnostics, Gaziantep, Turkey). Complexes with ferric ion are oxidized to ferrous ion by oxidants presented in the sample. The oxidation reaction is performed by 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. First spectrophotometric reading was performed adding standard solutions and Reagent 1 solution at 530 nm. Next reading was performed at 530 nm. Positive control was hydrogen peroxide (H_2O_2), reactive oxygen species for TOS assays. H_2O_2 was used for calibration. The calculations were performed using the formulas included in the kit.

2.8. Oxidative DNA damage assay

Commercially 8-hydroxy-2′-deoxyguanosine (8-OH-dG) kit (Cayman Chemical Company, USA) was used for DNA damage in the cells. 8-OH-dG is the form of oxidized guanine (Gan *et al*. 2012). Experimental stages were carried out considering the kit

procedure. Positive control was mitomycin-C chemotherapeutic agent for oxidative DNA damage assays. The purpose of this assay is determining of oxidative DNA damage in the cells via calculation of 8-OH-dG level. The calculations were performed using the formulas included in the kit.

2.9. Statistical analyses

All the experiments were run in triplicate. Diverse activities of FRI, 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 in order to calculate median inhibitory concentration (IC_{50}) values. Relations among 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.

3. Results

3.1. Cytotoxic activities

Growth inhibitory potentials of FRI on PRCC and U87MG cells were measured with MTT test. It was revealed that control$^-$ did not cause cytotoxicity on neither PRCC nor U87MG cells. Positive control, mitomycin-C had the lowest survival rate for both cells. Although maximum concentration (200 mg/L) of FRI statistically ($p < 0.05$) showed different effect compared with mitomycin-C on PRCC and U87MG cells, its values were the closest to positive control (61.66 and 40.36%, respectively) (Figure 1). As shown in Table 1, the resulting IC_{50} value for U87MG is much lower than PRCC (46.38 and 1271.77 mg/L, respectively). When the cells were damaged, the LDH analysis was carried out to understand whether LDH enzyme was released. While control$^-$ showed the lowest LDH release rate on PRCC and U87MG cells, mitomycin-C presented the highest values. There was statistically ($p > 0.05$) no difference between LDH activities of FRI treatments except for 200 mg/L and control$^-$ for PRCC cells. As for U87MG cells, treatment with 12.5 mg/L statistically ($p > 0.05$) showed LDH activity similar to control$^-$ (Figure 2).

3.2. Antioxidative activities

Antioxidant properties of FRI on PRCC and U87MG cells were determined with TAC analysis. There were no treatments reducing TAC in PRCC cells. Moreover, based on control$^-$, the concentrations of 3.125-25 mg/L statistically ($p < 0.05$) enhanced TAC level in PRCC cells (Figure 3a). As shown in Figure 3b, while the concentrations of 3.125 and 6.25 mg/L of FRI took attention with their poor TAC (14.34 and 15.51 Trolox equivalent/L, respectively) for U87MG cells, TAC level did not statistically ($p > 0.05$) rise with high concentrations of FRI compared with control$^-$ except for 100 mg/L.

3.3. Total oxidative stress activities

Based on TOS levels on both PRCC and U87MG cells, it was measured that positive control, H_2O_2 had maximum TOS activity while control$^-$ showed the lowest TOS level. Additionally, TOS levels in the cells were not significantly ($p > 0.05$) increased by FRI treatments. The closest values to that of H_2O_2 for PRCC and U87MG cells belonged to the concentrations of 100 (2.56 µmol H_2O_2 equivalent/L) and 12.5 (2.66 µmol H_2O_2 equivalent/L) mg/L, respectively (Figure 4).

Table 1. IC_{50} values for PRCC and U87MG cells exposed to FRI (mg/L)

Cell type	IC_{50} (limits)	Slope ± standard error (limits)
PRCC	1271.77^b (565.34-4708.20)	0.40 ± 0.05 (0.30-0.50)
U87MG	46.38^a (35.47-63.36)	0.48 ± 0.05 (0.39-0.57)

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

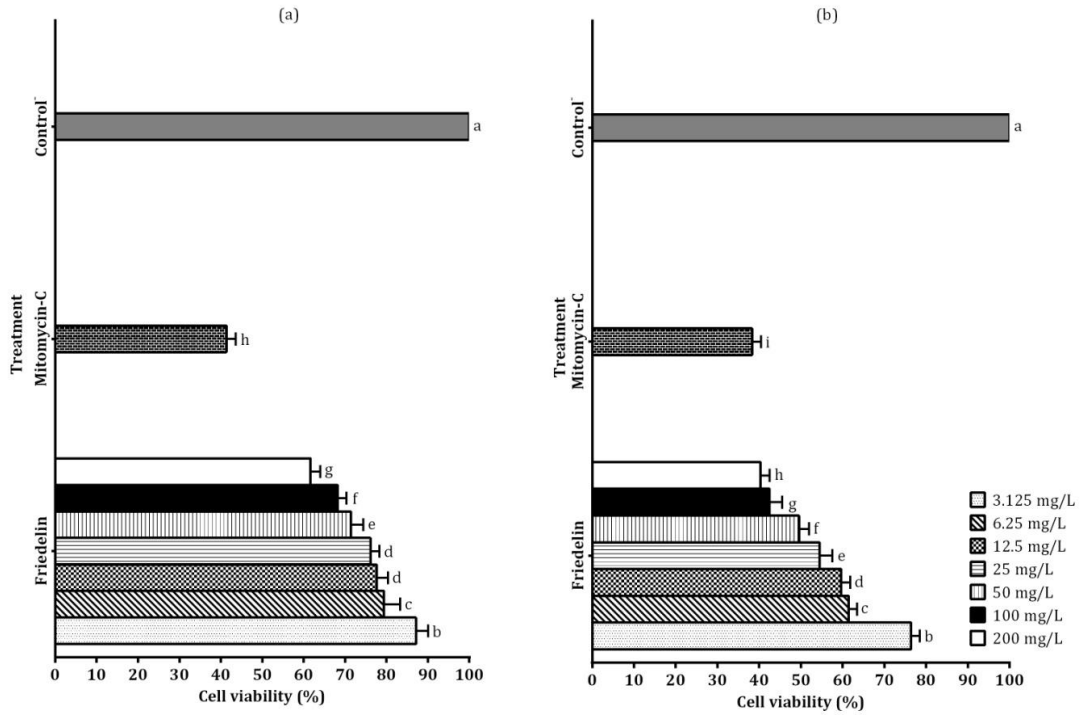

Figure 1. Viability rates in the cells [(a) PRCC, (b) U87MG] exposed to FRI. Each value represents the mean standard deviation of three experiments. Different small letters indicate significant differences among treatments at $p < 0.05$

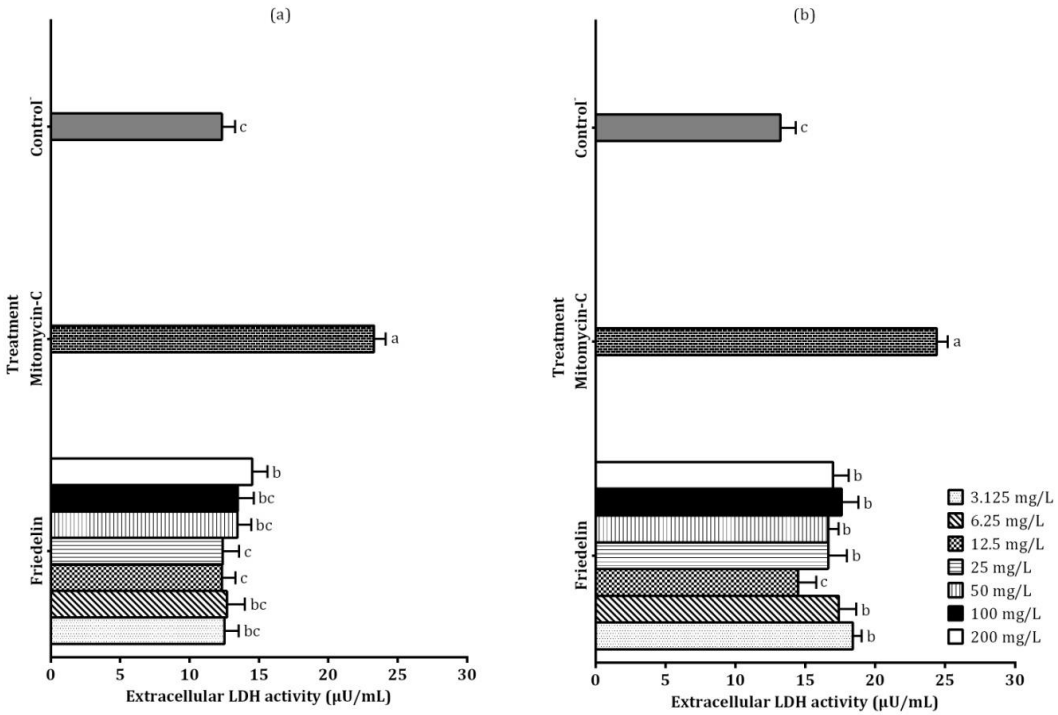

Figure 2. LDH release levels in the cells [(a) PRCC, (b) U87MG] exposed to FRI. Each value represents the mean standard deviation of three experiments. Different small letters indicate significant differences among treatments at $p < 0.05$

3.4. Genotoxic activities

DNA damage caused by oxidative stress in PRCC and U87MG cells by FRI was determined with measuring 8-OH-dG rate. When investigated the findings in Figure 5, wavy appearances drew attention. There was not any correlation between concentration and 8-OH-dG level. Based on oxidative DNA damage on the both cells, it was

identified that all experiments of FRI were significantly (p < 0.05) enhanced 8-OH-dG level compared to control$^-$ and the treatment with concentration of 25 mg/L had the highest 8-OH-dG level (3.87 and 6.11 pg/mL, respectively) on PRCC and U87MG cells after that of mitomycin-C. Even so, there was a huge difference between 8-OH-dG level arose by mitomycin-C and the other treatments.

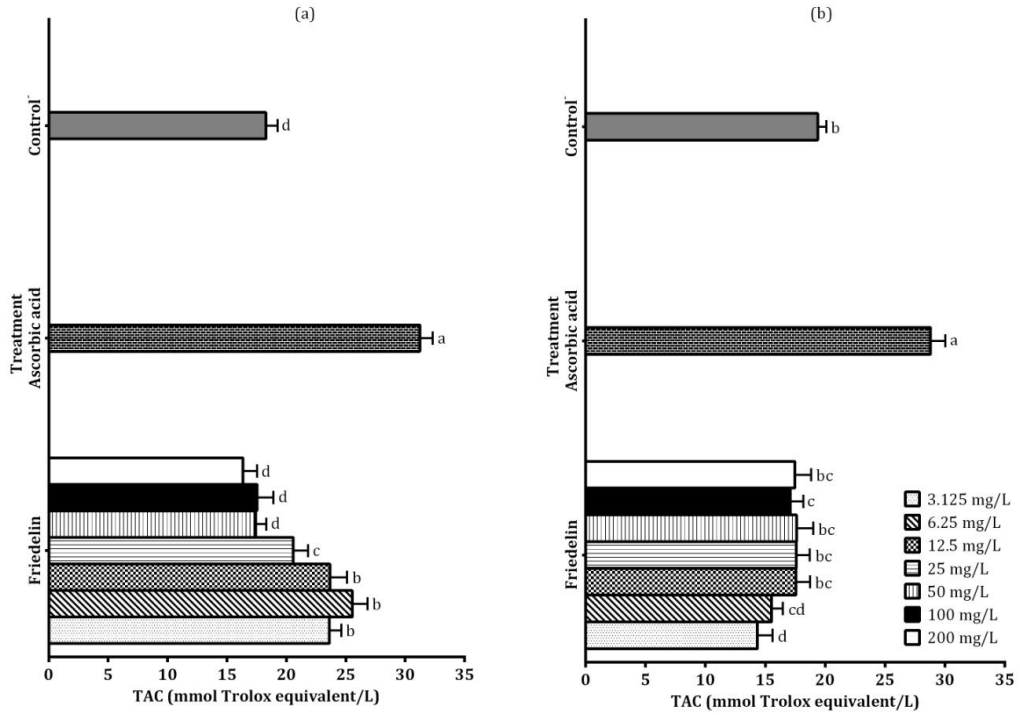

Figure 3. TAC levels in the cells [(a) PRCC, (b) U87MG] in the presence of FRI. Each value represents the mean standard deviation of three experiments. Different small letters indicate significant differences among treatments at p < 0.05

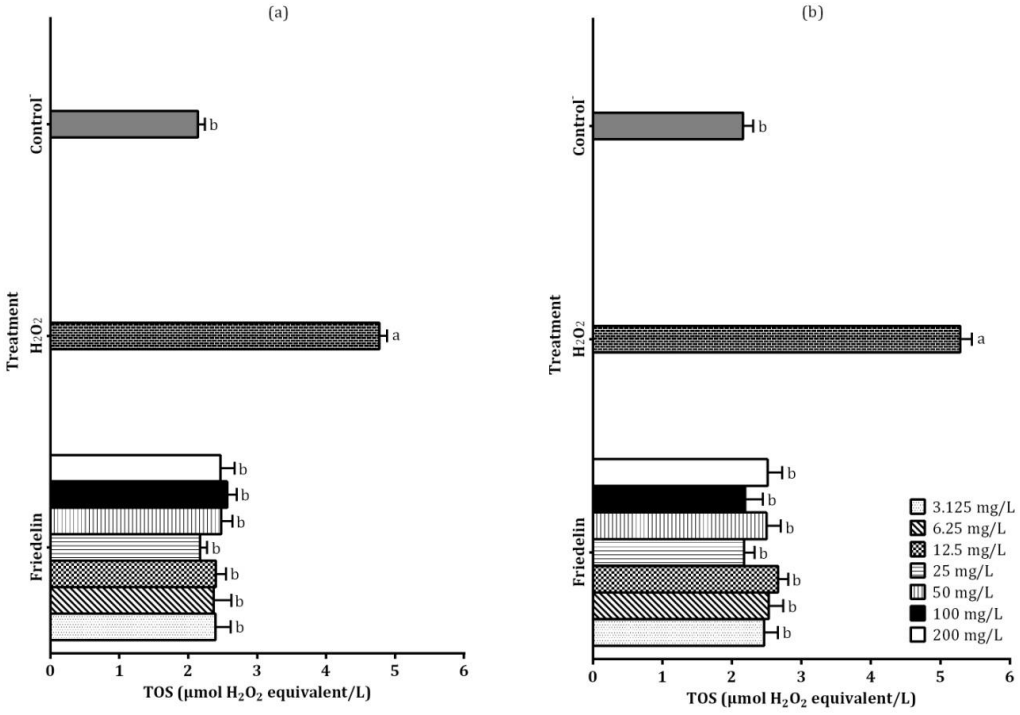

Figure 4. TOS levels in the cells [(a) PRCC, (b) U87MG] in the presence of FRI. Each value represents the mean standard deviation of three experiments. Different small letters indicate significant differences among treatments at p < 0.05

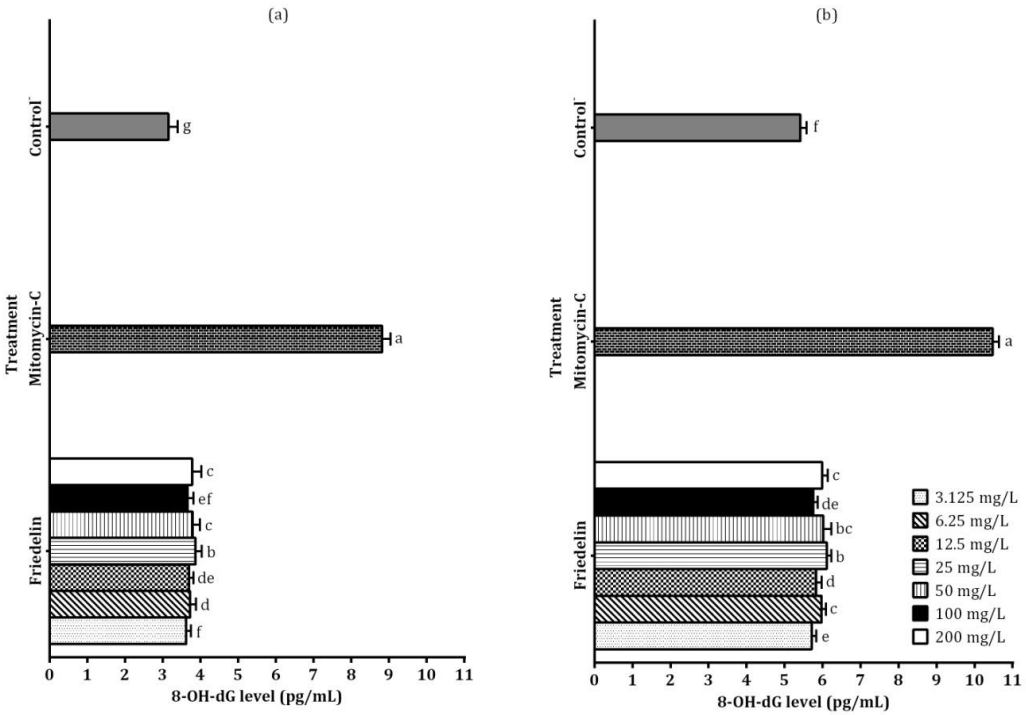

Figure 5. 8-OH-dG rates in the cells [(a) PRCC, (b) U87MG] in the presence of OA and PA. Each value represents the mean standard deviation of three experiments. Different small letters indicate significant differences among treatments at $p < 0.05$

4. Discussion

Although surgical intervention, chemotherapy and radiotherapy are the most important methods in the treatment of GBM, the most common and severe of brain tumors, the side-effect levels of these treatment methods are critical (Zhang *et al*. 2012). Treatment methods that cannot be achieved with high results when used alone are more effective in combination (Gauden *et al*. 2009). In recent years, herbal products have also been included in the preferred combined treatment process (Markiewicz-Żukowska *et al*. 2013; Wang *et al*. 2013). The importance of secondary metabolites in these products is great. FRI, a lichen secondary metabolite, utilized in the present study has not been used treatment of GBM according to literature reports.

Viability rates of PRCC and U87MG cells exposed to FRI were negatively correlated with concentration at the 0.01 level (Tables 2-3). Similarly, concentration-dependent cytotoxic activities of different lichen metabolites on various cells were identified in several studies (Brisdelli *et al*. 2013; Shrestha *et al*. 2015). Lower cytotoxic activity on non-cancerous PRCC cells compared with U87MG cells (Figure 1) revealed minimum side effect of FRI. Towering IC_{50} values calculated for PRCC also supported this suggestion (Table 1). Furthermore, cytotoxic activity caused by maximum concentration of FRI on U87MG cells was very close to that of mitomycin-C. The data in agreement with the present study belonged to Bézivin *et al*. (2003). They reported that different lichen extracts caused low levels of side effects on U251 GBM cell line.

Performed correlation analyses gave an idea of the mechanism that could lead to cytotoxic activity and Pearson correlation coefficients (PCCs) showed that cytotoxic effect caused by FRI on PRCC cells might result from LDH release. There was a significantly ($p < 0.01$) negative correlation between LDH activity and cell viability (Table 2). However, same relation was not detected for U87MG cells based on PCCs (Table 3). In addition, it was observed that LDH release rose depending the concentration on PRCC cells.

Table 2. Correlation between different treatments for PRCC cells exposed to FRI

	Cell viability	Concentration	LDH activity	TAC	TOS	8-OH-dG level
Cell viability	1[a]					
Concentration	-0.89**	1				
LDH activity	-0.56**	0.59**	1			
TAC	0.81**	-0.77**	-0.44*	1		
TOS	-0.01	0.05	0.36	0.01	1	
8-OH-dG level	-0.37	0.18	0.07	-0.32	-0.03	1

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

Table 3. Correlation between different treatments for U87MG cells exposed to FRI

	Cell viability	Concentration	LDH activity	TAC	TOS	8-OH-dG level
Cell viability	1[a]					
Concentration	-0.79**	1				
LDH activity	0.10	0.07	1			
TAC	-0.60**	0.33	-0.57**	1		
TOS	-0.04	0.03	0.42*	0.11	1	
8-OH-dG level	-0.47*	0.13	-0.14	0.17	0.01	1

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

In other studies carried out with various lichen compounds such as atranorin and usnic acid, LDH induced cytotoxicity was determined on breast, pancreatic cancer cells (Einarsdóttir *et al*. 2010) and human keratinocyte cells (Varol *et al*. 2015). When investigated antioxidant properties of FRI, it was assessed that it increased TAC on healthy PRCC cells. Based on TAC level of control$^-$ on PRCC cells, the most effective treatments having significantly (p < 0.05) high antioxidant capacity were concentrations of 3.125-25 mg/L (Figure 3). Additionally, TOS levels of some concentrations of FRI on U87MG cells were found higher compared to PRCC cells. Once compared to control$^-$, it was indicated that FRI did not statistically (p > 0.05) increase oxidative stress on both cells. Furthermore, while positive PCC (0.81) between TAC and cell viability were great for PRCC cells exposed to FRI (Table 2), PCC for U87MG cells was significantly (p < 0.01) negative (Table 3). These results proposed that the viability rate of healthy PRCC cells would also increase due to the increased antioxidant capacity of FRI.

In correlation analyses carried out for TOS, it was determined that there was only a significant correlation (PCC = 0.42) between TOS and LDH activity on U87MG cells exposed to FRI (Table 3). This result suggested that LDH releasing caused cytotoxicity on cancerous cells could associate with oxidative stress. It was not measured any significant correlation related with TOS activity on PRCC cells (Table 2). Therefore, it was advised that when FRI was used in certain concentrations, it did not cause oxidative stress on healthy cells. Moreover, low concentrations of FRI caused an increase in antioxidant capacity in PRCC cells.

Many studies on the oxidative capacities of the lichens have been carried out in recent years. The studies about scavenging of oxidative radicals such as 2,2-diphenyl-1-picrylhydrazil, superoxide and nitric oxide by different lichen compound and extracts took the lead. While Thadhani *et al*. (2011) used sekikaic and lecanoric acid in these treatments, Grujičić *et al*. (2014) revealed antioxidant capacity of methanol extract obtained from *Cetraria islandica*. Another lichen-antioxidant related study was carried out by testing physodic and olivetoric acid obtained from *Pseudevernia furfuracea* on human amnion fibroblasts and it was detected that these metabolites increased the antioxidant capacity of the cells (Emsen *et al*. 2017). Otherwise, studies on the oxidant properties of lichens are also common (Paudel *et al*. 2012; Ghate *et al*. 2013; Kumar *et al*. 2014). Aforementioned oxidative capacity results of the lichens suggested that many lichen components could increase the antioxidant capacity of various cells when used in certain doses. In order to genotoxic effect of FRI on PRCC and U87MG cells, 8-OH-dG rate performing in the cells was

determined. It was found that FRI statistically ($p < 0.05$) increased the level of 8-OH-dG on both cells compared to control$^-$ but these values were far from that of mitomycin-C (Figure 5). Taking into account the PCCs calculated for 8-OH-dG adducts in U87MG cells, only a significant negative correlation ($p < 0.05$) was found between cell viability and 8-OH-dG level (Table 2). Therefore, cytotoxicity in cancerous cells was predicted to be caused by genetic damage.

Previous studies about genotoxic and non-genotoxic effects of different lichen species support the current work. In a study on the biological activities of *C. islandica* on lymphocytes *in vitro*, the genotoxic effect due to the dose was revealed (Grujičić *et al*. 2014). Another lichen-genotoxic effect study was performed on human breast cancer cell lines and it was defined that *Hypogymnia physodes* increased genotoxic activity depending on the concentration (Ari *et al*. 2014). There are also studies that some lichens do not show genotoxic effects. These studies performed with different lichen species such as *Usnea filipendula* (Çelikler Kasımoğulları *et al*. 2014), *Lecanora muralis* (Alpsoy *et al*. 2011) and *Cetraria aculeata* (Zeytinoglu *et al*. 2008) usually are focused on human lymphocytes.

5. Conclusions

Taken together, the results suggested that FRI had anticancer activity *in vitro* against U87MG-GBM cells. On the other hand, side effects of FRI were also tested on healthy PRCC cells and low rates were encountered. Cytotoxic activity caused by FRI on U87MG cells rose depending the concentration and FRI was found to be a compound that increased antioxidant capacity in PRCC cells when used at certain concentrations. In summary, it could be stated that tested lichen compound, FRI had a strong antioxidant and anticancer activity *in vitro*, which advised that lichens could be good natural antioxidant and anticancer agents.

6. References

Abuiraq, L., Kanan, G., Wedyan, M. and El-Oqlah, A., 2015. Efficacy of extracts of some lichens for potential antibacterial activity. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, **6**, 318–331.

Alpsoy, L., Aslan, A., Kotan, E., Agar, G. and Anar, M., 2011. Protective role of two lichens in human lymphocytes *in vitro*. *Fresenius Environmental Bulletin*, **20**, 1661–1666.

Ari, F., Celikler, S., Oran, S., Balikci, N., Ozturk, S., Ozel, M.Z., Ozyurt, D. and Ulukaya, E., 2014. Genotoxic, cytotoxic, and apoptotic effects of *Hypogymnia physodes* (L.) Nyl. on breast cancer cells. *Environmental Toxicology*, **29**, 804–813.

Bai, L., Bao, H.Y. and Bau, T., 2014. Isolation and identification of a new benzofuranone derivative from *Usnea longissima*. *Natural Product Research*, **28**, 534–538.

Bézivin, C., Tomasi, S., Lohézic-Le Dévéhat, F. and Boustie, J., 2003. Cytotoxic activity of some lichen extracts on murine and human cancer cell lines. *Phytomedicine*, **10**, 499–503.

Bézivin, C., Tomasi, S., Rouaud, I., Delcros, J.G. and Boustie, J., 2004. Cytotoxic activity of compounds from the lichen: *Cladonia convoluta*. *Planta Medica*, **70**, 874–877.

Bosset, J.-F., Collette, L., Calais, G., Mineur, L., Maingon, P., Radosevic-Jelic, L., Daban, A., Bardet, E., Beny, A., Ollier, J.-C. and EORTC Radiotherapy Group Trial 22921, 2006. Chemotherapy with preoperative radiotherapy in rectal cancer. *The New England Journal of Medicine*, **355**, 1114–1123.

Boustie, J. and Grube, M., 2005. Lichens-A promising source of bioactive secondary metabolites. *Plant Genetic Resources*, **3**, 273–287.

Brisdelli, F., Perilli, M., Sellitri, D., Piovano, M., Garbarino, J.A., Nicoletti, M., Bozzi, A., Amicosante, G. and Celenza, G., 2013. Cytotoxic activity and antioxidant capacity of purified lichen metabolites: an *in vitro* study. *Phytotherapy Research*, **27**, 431–437.

Brodo, I.M., Sharnoff, S.D. and Sharnoff, S., 2001. About the Lichens, in: Lichens of North America. Yale University Press, New Haven & London, 3–113.

Clarke, J., Penas, C., Pastori, C., Komotar, R.J., Bregy, A., Shah, A.H., Wahlestedt, C. and Ayad, N.G., 2013. Epigenetic pathways and glioblastoma treatment. *Epigenetics*, **8**, 785–795.

Çelikler Kasımoğulları, S., Oran, S., Ari, F., Ulukaya, E., Aztopal, N., Sarimahmut, M. and Öztürk, Ş., 2014. Genotoxic, cytotoxic, and apoptotic effects of crude extract of *Usnea filipendula* Stirt. *in vitro*. *Turkish Journal of Biology*, **38**, 940–947.

Dogan, M., Emsen, B., Aasim, M. and Yildirim, E., 2017. *Ceratophyllum demersum* L. extract as a botanical insecticide for controlling the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae). *Egyptian Journal of Biological Pest Control*, **27**, 11–15.

Einarsdóttir, E., Groeneweg, J., Björnsdóttir, G.G., Harethardottir, G., Omarsdóttir, S., Ingólfsdóttir, K. and Ogmundsdóttir, H.M., 2010. Cellular mechanisms of the anticancer effects of the lichen compound usnic acid. *Planta Medica*, **76**, 969–974.

Emsen, B., Aslan, A., Togar, B. and Turkez, H., 2016a. *In vitro* antitumor activities of the lichen compounds olivetoric, physodic and psoromic acid in rat neuron and glioblastoma cells. *Pharmaceutical Biology*, **54**, 1748–1762.

Emsen, B., Dogan, M., Aasim, M. and Yildirim, E., 2016b. Insecticidal activity of *in vitro* propagated aquatic plant *Ceratophyllum demersum* L. against granary weevil *Sitophilus granarius* L. (Coleoptera: Curculionidae). *Egyptian Journal of Biological Pest Control*, **26**, 619–624.

Emsen, B., Turkez, H., Togar, B. and Aslan, A., 2017. Evaluation of antioxidant and cytotoxic effects of olivetoric and physodic acid in cultured human amnion fibroblasts. *Human & Experimental Toxicology*, **36**, 376–385.

Emsen, B., Yildirim, E. and Aslan, A., 2015. Insecticidal activities of extracts of three lichen species on *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). *Plant Protection Science*, **51**, 156–161.

Emsen, B., Yildirim, E., Aslan, A., Anar, M. and Ercisli, S., 2012. Insecticidal effect of the extracts of *Cladonia foliacea* (Huds.) Willd. and *Flavoparmelia caperata* (L.) Hale against adults of the grain weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). *Egyptian Journal of Biological Pest Control*, **22**, 145–149.

Feng, J., Yang, X., Su, S. and He, C., 2009. Studies on chemical constituents from herbs of *Usnea longissima*. *Zhongguo Zhongyao Zazhi*, **34**, 708–711.

Gan, W., Nie, B., Shi, F., Xu, X.M., Qian, J.C., Takagi, Y., Hayakawa, H., Sekiguchi, M. and Cai, J.P., 2012. Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescence-accelerated mice analyzed by LC-MS/MS: urinary 8-oxoguanosine as a novel biomarker of aging. *Free Radical Biology & Medicine*, **52**, 1700–1707.

Gauden, A.J., Hunn, A., Erasmus, A., Waites, P., Dubey, A. and Gauden, S.J., 2009. Combined modality treatment of newly diagnosed glioblastoma multiforme in a regional neurosurgical centre. *Journal of Clinical Neuroscience*, **16**, 1174–1179.

Ghate, N.B., Chaudhuri, D., Sarkar, R., Sajem, A.L., Panja, S., Rout, J. and Mandal, N., 2013. An antioxidant extract of tropical lichen, *Parmotrema reticulatum*, induces cell cycle arrest and apoptosis in breast carcinoma cell line MCF-7. *Plos One*, **8**, e82293.

Giglio, P., 2010. Chemotherapy for glioblastoma: Past, present, and future, in: Ray, S.K. (Ed.), Glioblastoma: Molecular Mechanisms of Pathogenesis and Current Therapeutic Strategies. Springer New York, New York, NY, 203–216.

Grujičić, D., Stošić, I., Kosanić, M., Stanojković, T., Ranković, B. and Milošević-Djordjević, O., 2014. Evaluation of *in vitro* antioxidant, antimicrobial, genotoxic and anticancer activities of lichen *Cetraria islandica*. *Cytotechnology*, **66**, 803–813.

Karatas, M., Dogan, M., Emsen, B. and Aasim, M., 2015. Determination of *in vitro* free radical scavenging activities of various extracts from *in vitro* propagated *Ceratophyllum demersum* L. *Fresenius Environmental Bulletin*, **24**, 2946–2952.

Koçer, S., Uruş, S., Çakır, A., Güllüce, M., Dığrak, M., Alan, Y., Aslan, A., Tümer, M., Karadayı, M., Kazaz, C. and Dal, H., 2014. The synthesis, characterization, antimicrobial and antimutagenic activities of hydroxyphenylimino ligands and their metal complexes of usnic acid isolated from *Usnea longissima*. *Dalton Transactions*, **43**, 6148–6164.

Kour, A., 2014. Plants exhibiting potential for cancer treatment. *International Journal of Pharmaceutical Sciences Review and Research*, **27**, 23–53.

Kumar, J., Dhar, P., Tayade, A.B., Gupta, D., Chaurasia, O.P., Upreti, D.K., Arora, R. and Srivastava, R.B., 2014. Antioxidant capacities, phenolic profile and cytotoxic effects of saxicolous lichens from trans-Himalayan cold desert of Ladakh. *Plos One*, **9**, e98696.

Laxinamu, J., Tang, Y.X., Bao, H.Y. and Bau, T., 2013. Chemical constituents from *Usnea longgisima*, a

traditional mongolian medicine. *Zhongguo Zhongyao Zazhi*, **38**, 2125–2128.

Manojlović, N., Ranković, B., Kosanić, M., Vasiljević, P. and Stanojković, T., 2012. Chemical composition of three *Parmelia* lichens and antioxidant, antimicrobial and cytotoxic activities of some their major metabolites. *Phytomedicine*, **19**, 1166–1172.

Markiewicz-Żukowska, R., Naliwajko, S.K., Bartosiuk, E., Moskwa, J., Isidorov, V., Soroczyńska, J. and Borawska, M.H., 2013. Chemical composition and antioxidant activity of beebread, and its influence on the glioblastoma cell line (U87MG). *Journal of Apicultural Science*, **57**, 147–157.

Mavrogenis, A.F., Papagelopoulos, P.J., Romantini, M., Angelini, A. and Ruggieri, P., 2010. Side effects of chemotherapy in musculoskeletal oncology. *Journal of Long-Term Effects of Medical Implants*, **20**, 1–12.

Odimegwu, D.C., Ejikeugwu, C. and Esimone, C.C., 2015. Lichen Secondary Metabolites, in: Ranković, B. (Ed.), Lichen Secondary Metabolites: Bioactive Properties and Pharmaceutical Potential. Springer International Publishing, Cham, 165–177.

Ohgaki, H. and Kleihues, P., 2007. Genetic pathways to primary and secondary glioblastoma. *The American Journal of Pathology*, **170**, 1445–1153.

Omar, A.I. and Mason, W.P., 2012. Anaplastic astrocytomas. *Handbook of Clinical Neurology*, **105**, 451–466.

Paudel, B., Bhattarai, H.D., Pandey, D.P., Hur, J.S., Hong, S.G., Kim, I. and Yim, J.H., 2012. Antioxidant, antibacterial activity and brine shrimp toxicity test of some mountainous lichens from Nepal. *Biological Research*, **45**, 387–391.

Perrino, C., Schiattarella, G.G., Magliulo, F., Ilardi, F., Carotenuto, G., Gargiulo, G., Serino, F., Ferrone, M., Scudiero, F., Carbone, A., Trimarco, B. and Esposito, G., 2014. Cardiac side effects of chemotherapy: State of art and strategies for a correct management. *Current Vascular Pharmacology*, **12**, 106–116.

Rao, S.A.M., Srinivasan, S., Patric, I.R.P., Hegde, A.S., Chandramouli, B.A., Arimappamagan, A., Santosh, V., Kondaiah, P., Sathyanarayana Rao, M.R. and Somasundaram, K., 2014. A 16-gene signature distinguishes anaplastic astrocytoma from glioblastoma. *Plos One*, **9**, e85200.

Reed, M., 2009. Principles of cancer treatment by surgery. *Surgery*, **27**, 178–181.

Shrestha, G., El-naggar, A.M., Clair, L.L.S. and Neill, K.L.O., 2015. Anticancer activities of selected species of North American lichen extracts. *Phytotherapy Research*, **29**, 100–107.

Smoll, N.R., Schaller, K. and Gautschi, O.P., 2013. Long-term survival of patients with glioblastoma multiforme (GBM). *Journal of Clinical Neuroscience*, **20**, 670–675.

Somasundaram, K., Reddy, S.P., Vinnakota, K., Britto, R., Subbarayan, M., Nambiar, S., Hebbar, A., Samuel, C., Shetty, M., Sreepathi, H.K., Santosh, V., Hegde, A.S., Hegde, S., Kondaiah, P. and Rao, M., 2005. Upregulation of ASCL1 and inhibition of Notch signaling pathway characterize progressive astrocytoma. *Oncogene*, **24**, 7073–7083.

Sundararaj, J.P., Kuppuraj, S., Ganesan, A., Ponnusamy, P. and Nayaka, S., 2015. *In vitro* assesssment of antioxidant and antimicrobial activities of different solvent extracts from lichen *Ramalina nervulosa*. *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**, 200–204.

Thadhani, V.M., Choudhary, M.I., Ali, S., Omar, I., Siddique, H. and Karunaratne, V., 2011. Antioxidant activity of some lichen metabolites. *Natural Product Research*, **25**, 1827–1837.

Varol, M., Tay, T., Candan, M., Türk, A. and Koparal, A.T., 2015. Evaluation of the sunscreen lichen substances usnic acid and atranorin. *Biocell*, **39**, 25–31.

Wang, S., Huang, M., Li, J., Lai, F., Lee, H. and Hsu, Y., 2013. Punicalagin induces apoptotic and autophagic cell death in human U87MG glioma cells. *Acta Pharmacologica Sinica*, **34**, 1411–1419.

Zeytinoglu, H., Incesu, Z., Tuylu, B.A., Turk, A.O. and Barutca, B., 2008. Determination of genotoxic, antigenotoxic and cytotoxic potential of the extract from lichen *Cetraria aculeata* (Schreb.) Fr. *in vitro*. *Phytotherapy Research*, **22**, 118–123.

Zhang, X., Zhang, W., Cao, W.D., Cheng, G. and Zhang, Y.Q., 2012. Glioblastoma multiforme: Molecular characterization and current treatment strategy (Review). *Experimental and Therapeutic Medicine*, **3**, 9–14.