www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online; ISSN 1308-5301 Print 5/1 (2012) 1-4

Research article/Araştırma makalesi

Time dependent cytotoxic role of *Homalothecium sericeum* extracts on glioma

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Abstract

Bryophytes have been used as medicinal plants for more than 400 years in China, Europe and North America. There is also evidence confirming the antibiotic and anticancer activity of Bryophytes against, prokaryotes, fungi and different cancer cells. The purpose of the current study was to investigate cytotoxic property of *Homalothecium sericeum* (hedw.) schimp., which is a bryophyte, extracts on rat glioma (C6) cells for 48 hrs, *in vitro*. We first collected two different (acetone and A) extracts from *H. sericeum* by two different extraction processes. C6 cells were seeded in 96 well plates ($2x10^4$ cells/well) and incubated for 24 hrs. Following this incubation period, the medium was replaced with only medium (control) or medium with extracts at concentrations of 0.17, 1.7, 17, 85 or 170 µg/mL for 48 hrs. Cytotoxicity was determined by using 3-(4,5-dimethylthiazol-2yl)- 2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Acetone extract of *H. sericeum* at 0.17, 1.7 and 17 µg/mL concentrations did not change the survival rate of C6, but 85 and 170 µg/mL inhibited about 16 % and 36 % after 48 hr ($p<0.001$), respectively. Extract A at concentration of 0.17 µg/mL did not also affect C6 viability, but 1.7, 17 ($p<0.01$), 85 and 170 ($p<0.001$) µg/mL decreased C6 cell viability by 6, 8, 24 and 33 % for 48 hr, respectively. Acetone and A extracts of *H. sericeum* showed a moderate but similar dose dependent cytotoxicity on C6. Further studies are needed to clarify the content of these extracts.

Key words: *Homalothecium sericeum*, Bryophyta, Glioma, MTT, Cytotoxicity

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Homalothecium sericeum özütlerinin *zamana bağlı olarak* glioma hücrelerinin çoğalması üzerindeki etkileri

Özet

Bryofitler 400 yıldan fazla bir süredir Çin, Avrupa ve Kuzey Amerika'da bitkisel ilaç olarak kullanılmaktadır. Bryofitlerin prokaryot, mantar türleri üzerinde antibiyotik ve farklı kanser hücreleri üzerinde antikanser etkinlik gösterdiği konusunda veriler bulunmaktadır. Bu çalışmanın amacı, bir Bryofit olan *Homalothecium sericeum*'un sıçan glioma (C6) hücreleri üzerinde 48 saatlik sitotoksik etkinliğini araştırmaktır. İlk olarak, iki farklı özüt elde etme yöntemiyle aseton ve A özütlerini elde ettik. C6 hücreleri 96 kuyucuklu kültür kaplarına ekilerek ($2x10^4$ hücre/kuyucuk) 24 saat inkübe edildi. İnkübasyon süresinin ardından kuyucuklara özütlerin 0.17, 1.7, 17, 85 ve 170 µg/mL konsantrasyonları eklenerek 48 saat muamele edildi. Sitotoksisite, MTT [3-(4,5-dimetiltiazol-2yl)- 2,5-difeniltetrazolyum bromid] yöntemi ile belirlendi. 48 saat sonunda aseton özütünün 0.17, 1.7 ve 17 µg/mL konsantrasyonları C6 hücre canlılığını değiştirmezken, 85 ve 170 µg/mL konsantrasyonları hücre canlılığını sırası ile %16 ve %36 oranında azalttı ($p<0.001$). A özütünün 0.17 µg/mL konsantrasyonu hücre canlılığını 48 saat sonunda değiştirmezken, 1.7, 17 ($p<0.01$), 85 and 170 ($p<0.001$) µg/mL konsantrasyonları sırası ile % 6, 8, 24 ve 33 oranında azaltmıştır. *H. sericeum'un* aseton ve A özütü glioma hücreleri üzerinde orta ve benzer bir şekilde doza bağımlı sitotoksik etki göstermiştir. Bu özütlerin içeriği hakkında yapılacak daha kapsamlı çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: *Homalothecium sericeum,* Bryofit, Glioma, MTT, Sitotoksisite

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 BioDiCon. 230-0112

1. . Introduction

Glial tumors are the largest group of central nervous system tumors and glioblastoma multiforme (GBM) is the most anaplastic type of gliomas. GBM is the most aggressive and difficult to treat between neoplasms arising from the brain (Collet et al., 2011). The median survival of patients with glioblastoma is between 12 and 24 months (Salford et al., 2002; Liu et al., 2011). Despite all the efforts of neurosurgeons, oncologists, radiotherapists, biologists and other scientists, there has been almost no change in the prognosis of primary malignant brain tumors in last 30 years. Therefore researchers try to find and improve more natural, much less dangerous, equally or more effective new drugs.

Bryophyta is a division of photosynthetic, chiefly terrestrial and nonvascular plants that includes the mosses, liverworts, and hornworts (Matsuo and Sato, 1991). More than 22,000 members of the mosses (Bryophyta), represents about 5.5% of plant species spreading throughout the world (Zinsmeister and Mues, 1987). Even though few reviews concerning the biologically active chemical constituent of bryophytes have been published, (Zinsmeister and Mues, 1987; Zinsmeister et al., 1991; Asakawa, 2001) the chemistry of bryophytes has been neglected for a long time. The reasons for this neglect are as follows: morphologically very small and difficult to collect in large amounts as pure samples, identification is difficult, and considered to be nutritionally useless to humans. Meanwhile, studies on Bryophytes are insufficient in Turkey and these studies are just focused on location and systematic botany (Ezer et al., 2010). However, the bryophytes have been used as medicinal plants for more than 400 years in China, Europe and North America to cure cuts, burns, external wounds, bacteriosis, pulmonary tuberculosis, neurasthenia, fractures, convulsions, scalds, uropathy, pneumonia etc. (Basile et al., 1998; Asakawa, 2001; Singh et al., 2007)., Isoflavonoids, flavonoids and biflavonoids content of bryophytes extracts have been reported to be possible chemical barriers against microorganisms (Hahn et al., 1995; Basile et al., 1999). Terpenoids, phenolic and volatile constituents were also investigated in some species of mosses (Zinsmeister and Mues, 1987; Saritas et al., 2001). There is evidence about the antibiotic activity of bryophytes against fungi and prokaryotic cells (Singh et al., 2007; Sabovljević et al., 2006; Savaroglu et al., 2011). Furthermore, our previous study indicated that extracts of *Homalothecium sericeum* (hedw.) schimp. have both antimicrobial and cytotoxic activity on rat glioma (C6) cells for 24 hrs (Oztopcu-Vatan et al., 2011).

The purpose of the current study was to investigate the cytotoxic property of *H. sericeum* extracts on C6 cells for 48 hrs. In addition, our aim is to determine whether this effect was time dependent.

2. . Material and method

2.1. Plant material

Plant material was collected from Sundiken Mountains (Eskisehir, Turkey), at a height of 1420 m, in May 2006, and identified in the Department of Biology of Eskisehir Osmangazi University. A voucher specimen was deposited at the Herbarium of the department. We collected two different (acetone and A) extracts from *H. sericeum* by two different extraction processes as described previously (Oztopcu-Vatan et al., 2011).

2.2. Cell culture and viability

All the chemicals were purchased from Sigma for glioma cell culture experiments. The cultures were maintained in 75 cm^2 flasks, and incubated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% penicilin-streptomycin solution at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. When confluence was achieved, the glioma cells were incubated with trypsin-EDTA (0.25%) solution for 5 min at 37 °C. After the cells were dispersed, trypsin activity was inhibited by adding growth medium, and then the cells were centrifuged at 1000 rpm for 5 min at 4 °C, and counted with a Coulter counter. Cell viability was accessed by trypan blue dye exclusion and found to be higher than 98%.

The cells at exponential growth phase were seeded in $2x10^4$ cells/well in 96 wells microtiter plates. 8 wells for the control (included growth medium only) and 8 wells for each dose of extracts. Concentrations of 0.17, 1.7, 17, 85 or 170 µg/mL acetone and A extracts were added to the growth medium for 48 hr. All the test compounds were prepared immediately prior to use and protected from light. Extracts was dissolved in dimethyl sulfoxide (DMSO). Further dilutions were made at ratio of 1:10 in DMEM and the maximum concentration of DMSO was adjusted to be 0.01%. This amount of DMSO had no effect on cell viability when used alone.

2.3. The anticancer activity

After 48 hrs., drug cytotoxicity was determined by using 3-(4,5-dimethylthiazol-2yl)- 2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mossmann, 1983). 25 µL MTT solution was added to the each well and incubated for 4 hrs at 37°C. The MTT solution is converted into blue formazan by mitochondrial dehydrogenase activity of the viable cells. The amount of formazan produced is proportional to the number of living cells (Abe and Matsuki, 2000). After the medium was removed from the wells, 100 µL DMSO was added to each well and the crystal formazan particles produced in viable cells was dissolved for 5 min at room temperature with a shaker. The absorbance of formazan dye was read at 550 nm using a microplate reader (Bio-Tek Instruments), and cell survival percentages were

calculated according to the following formula; absorbance of treated cells in each well x 100 / mean absorbance of control cells. The dose response curves were calculated for extract C at the above-mentioned concentrations and expressed as the mean percent fraction of control ± SEM.

All statistical analyses were performed using one-way analysis of variance (ANOVA) and followed by Tukey's multiple comparison tests. The results are the means of at least three independent assays and a p value less than 0.05 was considered to be significant.

3. Results

Acetone extract of *H. sericeum* at 0.17, 1.7 and 17 µg/mL concentrations did not change the survival rate of C6, but inhibited at 85 and 170 µg/mL concentrations in 48 hrs, 16 % and 36 % ($p<0.001$) respectively, when compared to the control. Extract A at concentration of 0.17 µg/mL did not also affect C6 viability, but 1.7, 17 ($p<0.01$), 85 and 170 ($p<0.001$) µg/mL decreased C6 cell viability in 48 hrs, 6,% 8%, 24% and 33 % respectively (Figure 1).

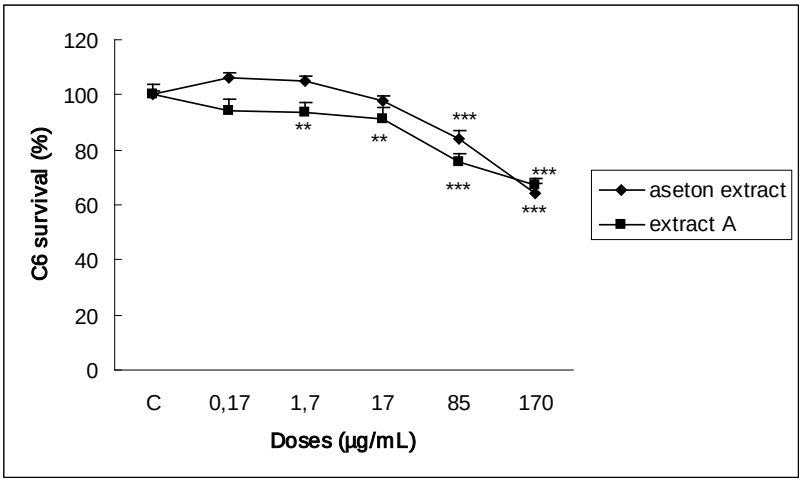

Figure 1. The effect of acetone and A extracts on C6 cell survival (C: control, **.$p<0.01$, ***: $p<0.001$).

4. Conclusions and discussion

In the present study, extracts of acetone and A of *H. sericeum* showed a moderate but similar dose dependent cytotoxicity on C6. Acetone extract of *H. sericeum* at 85 and 170 µg/mL inhibited about 16% and 36 % after 48 hrs. The highest concentration of the extract A decreased C6 cell viability by 33 % for 48 hrs. Our previous study demonstrated that acetone extract of *H. sericeum* of 170 µg/mL inhibited about 16 % , but the highest concentration of extract A decreased C6 cell viability by 13 % in 24 hrs (Oztopcu-Vatan et al., 2011). When compared to our previous results in 24 hrs, in this study growth inhibition rates in 48 hrs increased approximately by 20 % time dependently. Similarly, Savaroglu et al. (2011) found that extract C of aquatic moss *Fontinalis antipyretica* at high doses, possess a dose and time dependent anticancer activity against glioma cells. In support of our data, Krzaczkowski et al. (2009) determined that some bryophytes extracts from different species leading to cytotoxic effects in human HeLa cancer cells. In addition, Ivanova et al (2007) demonstrated that Sanionin A and B, from the moss *Sanionia georgico-uncinata*, have anti-proliferative action on human leukemia cells, mouse fibroblast cells, and human cervix carcinoma cells for 72 hrs. On the other hand, Yamada et al (2007) studied the cytotoxicity of fulvic acid (0.001-100 µg/ml) extracted from Canadian Sphagnum peat on rat basophilic leukemia by MTT assay and found that only 100 µg/ml show decreasing effect by 10 % after 48 hrs.

Further studies are needed to clarify the content of these extracts. The present results, indicate that *H. sericeum* extracts may be new source of cytotoxic agents.

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(Received for publication 12 January 2012; The date of publication 15 April 2012)