HSSJ RESEARCH June 2025, Volume: 5, Number: 1 ISSN: 2792-0135

Health Sciences Student Journal healthssj.com

C6 Glioma Hücrelerinde Mentha Longifolia ve Dosataksel ile Kombine Tedavisinin Histon Deasetilazlar Üzerine Etkisi

The Effect of Combined Treatment with Mentha longifolia and Docetaxel on Histone Deacetylases in C6 Glioma Cells

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Conflict of interest: There is not a conflict of interest.

How to Cite
Benli, N., Keşkek, D. B., Kabak, G., Siliğ, Y. (2025). The Effect of Combined Treatment with Mentha longifolia and Docetaxel on Histone Deacetylases in C6 Glioma Cells. *Health Sciences Student Journal*, 5(1), 12-18.

ÖZET

Amaç: Gliomalar, tedaviye dirençli yapıları nedeniyle etkin tedavi stratejileri geliştirilmesi gereken agresif tümörlerdir. Bu çalışmanın amacı, Mentha longifolia (yabani nane) ekstresinin kemoterapötik ajan dozotaksel ile kombinasyonunun C6 glioma hücre hattı üzerindeki sitotoksik etkisini ve histon deasetilaz (HDAC) gen ekspresyonları üzerindeki düzenleyici rolünü araştırmaktır.

Yöntem: C6 glioma hücrelerine 250 – 0.5 µM konsantrasyonlarda Mentha longifolia ekstresi, dozotaksel ve her ikisinin kombinasyonu uygulandı ve 24, 48, ve 72 saatlerde inkübasyona bırakılmıştır. Uygulama sonrası hücre canlılığı MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür) testi ile değerlendirilmiştir. HDAC1, HDAC2 ve HDAC3 gen ekspresyon düzeyleri ise kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile analiz edilmiştir.

Bulgular: Kombine tedavi grubunda hücre canlılığında anlamlı bir azalma gözlemlenmiştir. Ayrıca, HDAC1, HDAC2 ve HDAC3 genlerinin ekspresyon seviyelerinde - özellikle HDAC1'de olmak üzere - tekli tedavi gruplarına kıyasla belirgin bir düşüş saptanmıştır.

Sonuç: Elde edilen veriler, Mentha longifolia ekstresinin dozotaksel ile birlikte uygulanmasının C6 glioma hücrelerinde HDAC genlerinin ekspresyonunu baskılayarak sitotoksik etkiyi artırdığını göstermektedir. Bu sonuçlar, bitkisel bileşiklerin kemoterapötik ajanlarla kombinasyonunun, glioma gibi dirençli tümörlerde epigenetik hedefli tedavilere katkı sağlayabileceğini ortaya koymaktadır.

Anahtar kelimeler: Mentha longifolia, Dosetaksel, Glioma, HDAC genleri, epigenetik düzenleme.

ABSTRACT

Aim: Gliomas are aggressive tumors that require effective treatment strategies due to their resistance to therapy. This study aimed to investigate the cytotoxic effects of Mentha longifolia (wild mint) extract in combination with the chemotherapeutic agent docetaxel on the C6 glioma cell line, as well as its regulatory role on histone deacetylase (HDAC) gene expression.

Methods: C6 glioma cells were treated with specific concentrations of Mentha longifolia extract, docetaxel, and their combination. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Expression levels of HDAC1, HDAC2, and HDAC3 genes were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: A significant decrease in cell viability was observed in the combination treatment group. Additionally, expression levels of HDAC1, HDAC2, and HDAC3 genes, particularly HDAC1, showed a marked reduction compared to single treatment groups.

Conclusion: The findings indicate that combined treatment with Mentha longifolia extract and docetaxel enhances cytotoxic effects in C6 glioma cells by suppressing HDAC gene expression. These results suggest that the use of herbal compounds in combination with chemotherapeutic agents may contribute to epigenetically targeted therapies in resistant tumors such as gliomas.

Keywords: Mentha longifolia, Docetaxel, Glioma, HDAC, epigenetic regulation.

INTRODUCTION

Cancer is a malignancy characterized by changes occurring in abnormal cells, leading to uncontrolled growth and proliferation.[1] Cancer is classified into different types based on the tissue of origin and the regions to which it has spread. Among cancer types, glioblastoma multiforme (GBM) stands out as one of the most aggressive brain tumors. GBM is known for its rapid cell proliferation, widespread infiltration into brain tissue, and resistance to standard treatments. These characteristics contribute to a shortened average life expectancy in patients.[2]

C6-CCL-107 ™ (C6) glioma cells are spindle-shaped cells that, when injected into the mouse brain, exhibit behaviors similar to those of human GBM.[3] This cell line, which is widely used in both in vitro and in vivo models, provides a valuable model system for studying various aspects of glioma biology, including invasion, proliferation, angiogenesis, and response to chemotherapy.[4,5] In particular, C6 cells are critical tools in investigating resistance mechanisms to chemotherapeutic agents and developing new treatment strategies. In cancer treatment, surgery, radiotherapy, and chemotherapy are the main therapeutic approaches.[6] Surgical resection in GBM may extend survival by removing the tumor, but complete resection is often not possible due to the tumor's invasive nature.[7] Radiotherapy following surgery increases local tumor control, but it can damage healthy brain tissue, leading to severe side effects such as cognitive disorders and radiation necrosis.[8] Chemotherapy, while targeting cell proliferation, may also cause toxic effects in healthy cells. Side effects of chemotherapy on the central nervous system include chemobrain, acute encephalopathy, leukoencephalopathy, cerebellar dysfunction, and spinal cord toxicity. Additionally, chemotherapy can affect the peripheral nervous system, leading to chemotherapy-induced peripheral neuropathic pain [9]. These serious side effects have driven scientists to develop safer and more effective alternative treatment methods. Alternative approaches involving natural sources - plants, animals, and minerals - have gained importance in medical applications.[10] Methods such as prolotherapy, mesotherapy, and phytotherapy are among these approaches; particularly, phytotherapy is based on the use of plant-derived pharmaceuticals.[11,12]

One of the widely used plants in phytotherapy belongs to the Mentha genus from the Lamiaceae family. Mentha species hold a significant place among essential oil sources and are extensively utilized in the pharmaceutical industry with an annual production of approximately 2,000 tons of essential oil.[13] With more than 25 species composed of perennial herbaceous plants that grow in humid and temperate climates, a notable species in this genus is Mentha longifolia. Native to northeastern Tunisia, this fast-growing plant's leaves, flowers, stems, bark, and seeds are known for their antimicrobial, antioxidant, anti-inflammatory, carminative, and antispasmodic properties.[14–16] The antioxidant effects of Mentha longifolia are attributed to its phenolic compounds - particularly rosmarinic acid, flavonoids, and tannins - which neutralize free radicals.[17] Its anti-inflammatory effects stem from natural compounds that suppress prostaglandin synthesis, making it a potential treatment alternative in inflammation-related diseases. In vitro studies have shown that Mentha longifolia

extracts can inhibit the proliferation of cancer cells.[18]

Figure 1. Mentha Longifolia L.

One plant-derived anticancer agent is Docetaxel (DTX), an effective drug used in chemotherapy. DTX is a semisynthetic derivative of 10-deacetylbaccatin III obtained from the European yew tree (Taxus baccata) and belongs to the taxoid class.[19] Its plant origin supports the idea that plant-based compounds may possess antitumor properties. The anticancer effect of DTX occurs by binding to the β-subunit of tubulin, disrupting microtubule polymerization, and thereby inhibiting the cell division processes of cancer cells. Furthermore, DTX has been shown to interact with the Bcl-2 protein, thereby triggering apoptosis.[20,21]

Histone deacetylases (HDACs), which play a crucial role in the regulation of gene expression within the cell, function by removing acetyl groups from histone proteins. This leads to tighter binding of DNA to histones and prevents transcription factors from accessing DNA.[22,23] This mechanism is critically important in suppressing the expression of proteins involved in cell differentiation, cell cycle control, tumor immunity, and apoptosis.[24,25]

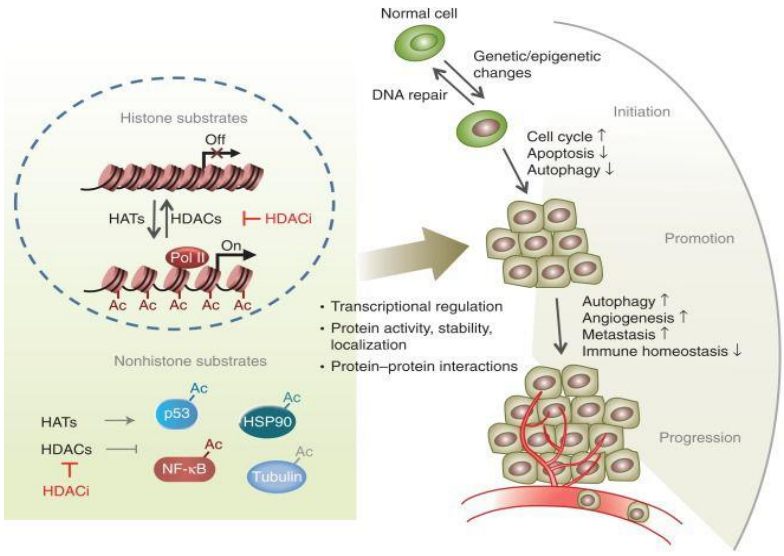

Figure 2. Mechanism of action of HDACs in various stages of cancer.[25]

Enzymes belonging to the HDAC I family - namely HDAC1, HDAC2, HDAC3, and HDAC8 - play significant roles in suppressing tumor cell migration, invasion, and angiogenesis.[26] HDAC1, in particular, acts as a core deacetylase in glioma stem cells and is associated with poor prognosis due to its high expression in GBM tissue.[27,28] While HDAC2 is structurally similar to HDAC1, it has roles in modulating immune responses and is considered a potential target in the treatment of inflammatory diseases and cancer.[29] HDAC3 also contributes to gene regulation in GBM, promoting cell proliferation and inhibiting tumor progression.[30]

MATERIALS AND METHODS

Plant material

Mentha longifolia L. used in the study was collected in two different periods, in May and September of 2023, from the villages of Tepeköy and Ütük, located in the Zara district of Sivas province.

Preparation of the extracts

The essential oils of the plants were extracted separately from the stem without

flowers and from the combination of flowers and stem. The essential oils were obtained by hydrodistillation of the specified parts of the plants using a Clevenger apparatus for 3 hours. The condenser part of the Clevenger apparatus was connected to a micro chiller device to maintain the cooling water at 4°C. The yield of the obtained oil was determined as v/w (mL oil/g plant). The isolated essential oil was dehydrated over Na_2SO_4 and transferred to amber-colored bottles for storage until analysis. The samples were stored at -20°C[31]

Cytotoxicity activities

The cytotoxic activity of Mentha longifolia was analyzed on C6 and L929 cell lines using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. For the cell lines, DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine was used. The cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. Cells (1×10^5/well) were seeded into 96-well plates, and after 24 hours of growth, the indicated compounds were applied to the cells at concentrations of 250, 100, 50, 25, 10, 5, and 0.5 µM. The cells were exposed to Mentha longifolia for incubation periods of 24, 48, and 72 hours. At the end of each incubation period, the MTT assay was performed. Accordingly, 10 µL of MTT solution prepared in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL was added to each well, followed by incubation at 37 °C in a 5% CO_2 environment for 3 hours. After the MTT-containing medium was aspirated, dimethyl sulfoxide (DMSO) was used to dissolve the resulting formazan crystals. Then, 100 µL of DMSO was added to each well, incubated at room temperature for 15 minutes, and the absorbance values were read at 540 nm using a microplate reader UV spectrophotometer. These measurements were performed in triplicate for each sample and repeated using at least three different passage numbers. IC_{50} values were calculated using GraphPad Prism, and graphs were generated accordingly.

Cell culture

Cell lines including C6 and L929 cells were maintained in DMEM medium, containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 mg/L). Cells were grown in at 37°C, 5% CO2 and 95 % air in a humidified incubator. For each cell line, 70-80% confluent cell culture flask was trypsinized and cells were seeded in 6 well plates.

Combined treatment application

Cells were seeded in 6-well plates at 1x105 cells/well. According to the IC_{50} values obtained as a result of statistical analysis, first Mentha longifolia alone, docetaxel alone and finally combined groups (both agents simultaneously) were applied to the cells, respectively.

Real Time PCR (RT-PCR)

RNA isolation from cells was performed according to the kit protocol. cDNA samples synthesized from RNAs were used for RT-PCR analysis according to the kit content. Each analysis set was run in 3 replicates. The average of the 3 replicate data obtained after the study was calculated. In order to determine the expression level between the study samples and the control group, the Housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as an internal control gene.

DISCUSSION

The effects of Mentha longifolia extract and docetaxel on C6 glioma cells were evaluated in terms of HDAC1, HDAC2, and HDAC3 gene expression and cytotoxic potential. The findings indicate that the combined treatment was more effective than individual applications in both suppressing gene expression and reducing cell viability. Histone deacetylases (HDACs) regulate gene expression at the epigenetic level by altering chromatin structure. Among the class I HDACs; HDAC1, HDAC2, and HDAC3 are particularly overexpressed in highly proliferative tumor cells and contribute to tumor progression.[32] Suppression of gene expression has the potential to inhibit the growth of tumor cells. In our study, a marked decrease in HDAC1 expression was detected, indicating strong inhibition against this enzyme's proliferative effect in tumor cells. Similarly, downregulation was observed in HDAC2 and HDAC3 gene expression, suggesting that multi-targeting at the epigenetic level may be achievable. In cytotoxicity analyses, the combination of Mentha longifolia and docetaxel reduced cell viability more significantly than either agent alone. This finding supports the notion that the two agents exert a synergistic effect in suppressing cell proliferation. It has previously been demonstrated in some plant species that phenolic compounds and flavonoids found in Mentha longifolia may inhibit HDAC activity.[33] Docetaxel, on the other hand, induces cell death by halting mitosis through microtubule stabilization.[34] The combination of these two distinct mechanisms may exert a dual inhibitory effect on both gene regulation and cytoskeletal structure in cancer cells. The literature suggests that combining HDAC inhibitors with conventional chemotherapeutic agents can reduce resistance to treatment and enhance cell death.[35] The observed correlation between decreased HDAC gene expression and cytotoxic efficacy in our study supports this synergistic interaction. Furthermore, this combination may disrupt the life cycle of glioma cells through epigenetic reprogramming mediated by HDACs. However, the fact that this study was conducted at the in vitro level limits the direct applicability of the results to clinical settings. It was found that the combined application of *Mentha longifolia* and docetaxel on C6 glioma cells caused a significant decrease in *HDAC1, HDAC2* and *HDAC6* gene expression levels. The obtained results are parallel to previous studies indicating that overexpression of *HDAC* genes in particular increases cellular proliferation and suppresses apoptosis in many tumor types.[36.] Histone deacetylases (HDACs) suppress gene transcription by increasing chromatin density. This is associated with the silencing of tumor suppressor genes in many cancer types, especially gliomas.[37] In this context, the significant decrease in *HDAC* gene expression observed in our study ($p<0.05$) suggests that the combination treatment may trigger epigenetic reprogramming in tumor cells. Similarly reported that knockdown of HDAC1 and HDAC2 expression in glioblastoma cells led to an increase in apoptosis and a decrease in cell viability.[38] The significant decrease in cell viability observed after combination treatment in our study supports this result. The effect of docetaxel on HDAC expression has been previously evaluated in breast cancer cells, and docetaxel alone has been shown to reduce HDAC1 and HDAC6 expression.[39] However, some studies have

emphasized that this effect becomes more pronounced when combined with plant-derived compounds. For example, reported that the combination of Curcuma longa extract and paclitaxel provided a synergistic decrease in *HDAC* activity.[40] From this perspective, the fact that the application of Mentha longifolia together with docetaxel in our study suppressed HDAC expression levels more reveals the synergistic effect of phytotherapeutic agents with chemotherapeutic drugs. In particular, the decrease in expression of 65% in HDAC1, 52% in HDAC2 and 48% in HDAC6 shows that this effect is statistically significant ($p<0.01$). In addition, it has been reported in the literature that *Mentha longifolia* exhibits anti-proliferative and antioxidant effects with its phenolic compounds and can induce apoptosis in cancer cells.[41]

However, there is limited information in the literature regarding its effect on *HDAC* gene expression, and it is thought that this study makes an important contribution to the field.

CONCLUSION

In order to validate the findings, further analyses should be performed using in vivo experimental models, including evaluations of HDAC protein activity and apoptotic markers. In conclusion, the combination of Mentha longifolia extract and docetaxel was found to exert a significant cytotoxic effect on C6 glioma cells by suppressing the expression of HDAC1, HDAC2, and HDAC3 genes. This study suggests that the combination of plant-derived compounds with chemotherapeutic agents may offer a promising approach in glioma treatment through targeting epigenetic mechanisms.

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