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U373, HUVEC VE RN-33B HÜCRE HATLARIYLA ÜÇLÜ KO-KÜLTÜR YAPILMASINA OLANAK VEREN TRANSWELL YÖNTEMİ İLE NÖROTOKSİSİTENİN BELİRLENMESİ VE MORİNGA BİTKİSİ İLE NÖROTOKSİK ETKİNİN TEDAVİ EDİLMESİ: İN VİTRO (1)

DETERMINATION OF NEUROTOXICITY BY TRANSWELL METHOD THAT ALLOWS TRIPLE CO-CULTURE OF U373, HUVEC AND RN-33B CELL LINES AND TREATMENT OF NEUROTOXIC EFFECT WITH MORINGA PLANT: IN VITRO

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Öz: Amaç: Glioblastoma en yaygın ve agresif majör beyin tümörüdür. Tedaviyi iyileştirme çabalarına ve ilerlemelere rağmen, hastaların ortalama sağkalımı tanıdan sonra 14 ay ile sınırlı kalmaktadır. Son araştırmalar, Moringa'nın geleneksel tıpta yaygın olarak kullanıldığını göstermiştir. Çalışmamızın amacı, tedavide kullanacağımız Moringa'nın transwell ko-kültür yöntemi ile oluşturulan nörotoksisite üzerindeki nöroprotektif etkisini ortaya koymaktır. **Yöntem:** Öncelikle RN-33B, U373 ve HUVEC hücre hatları hazırlanmış besiyerlerinde büyütüldü. Ardından, %85 konflense ulaşan HUVEC ve U373 hücre hatları, tripsin EDTA ile çıkarıldı. U373, transwell membranın üst tabanına ve HUVEC alt tabanına ekildi. Transwell membranlar nöron kültürünün olduğu kuyucuklara yerleştirildi ve 24 saat boyunca inkübasyona bırakıldı. Sonrasında kuyucuklara Moringa ekstraktının 25-50-100-200 ve 400 ug/ml dozları eklenerek 24 saat boyunca inkübe edildi. Çalışma sonunda MTT, TAC, TOS, GSH ve LDH testleri yapıldı ve sonuçlar kontrol grupları ile karşılaştırılarak istatistiksel olarak değerlendirildi. **Bulgular:** Canlılık oranında Moringa'nın doza bağlı olarak bir artış gösterdiği ve en yüksek dozunda hücre canlılığı % 90 oranında bulundu. Ayrıca antioksidan parametreleri (TAC, GSH) MTT analizi ile korelasyon gösterdi. TOS ve LDH seviyelerinde ise doza bağlı olarak oksidan seviyesinin düştüğü gözlendi. **Sonuç:** Nöronların U373 kanser hücrelerine kısa süreli maruz kalmasının ardından nörotoksisiteyi önleyerek Moringa'nın nöroprotektif etkisini ortaya koydu.

Anahtar Kelimeler: U373, Moringa, Transwell

Abstract: Aim: Glioblastoma is the best widespread and aggressive major brain tumor. Despite efforts to improve treatment and advances, the average survival of patients remains limited to 14 months after diagnosis. Recent research has shown that Moringa is widely used in traditional medicine. The purpose of our study is to show the neuroprotective effect of Moringa, which we will use in the treatment, on the neurotoxicity induced by the transwell co-culture method. **Method:** First of all, RN-33B, U373 and HUVEC cell lines were grown in prepared media. Then, HUVEC and U373 cell lines reaching 85% confluence were removed with trypsin EDTA. U373 was seeded on the top base of the transwell membrane and the lower base of the HUVEC. Transwell membranes were placed in the wells with neuron culture and incubated for 1 day. Afterward, 25-50-100-200 and 400 µg/ml doses of Moringa extract were added to the wells and incubated for 1 day. After 1 day, MTT, TAC, TOS, GSH, and LDH tests were performed and the results were compared with the control groups and statistically evaluated. **Results:** Moringa showed a dose-dependent increase in viability and cell viability was found to be 90% at the highest dose. In addition, antioxidant parameters (TAC, GSH) correlated with MTT analysis. In TOS and LDH levels, it was observed that the oxidant level decreased depending on the dose. **Conclusion:** It demonstrated the neuroprotective effect of Moringa by preventing neurotoxicity following short-term exposure of neurons to U373 cancer cells.

Keywords: U373, Moringa, Transwell

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor and is classified as grade IV astrocytoma (Wirsching et.al., 2016: 381). Despite efforts to improve treatment and advances in microsurgery, radiotherapy, and chemotherapy in the last two decades, the average survival of patients remains limited to 14 months after diagnosis (Huber et.al., 2013: e57793). It has been reported that GBMs are highly vascular tumors type. Glioblastoma stem cells (GSCs) secrete angiogenic factors and trigger neovascularization formation. It has also been found that GSCs can differentiate into endothelial cells to promote tumor vascularization (Petropoulos et.al., 2018: 20640).

Moringa is a type belonging to the Moringaceae family and has 13 types of shrubs and trees outspread in many countries (Martínez-González et.al., 2017: 87). All parts of the Moringa tree are appropriate for the animal and human consumption. The leaves, which are rich in protein, β carotene, quercetin, kaempferol, vitamins, caffeoylquinic acid, minerals and β sitosterol particularly essential amino acids, and antioxidants compounds, are used not only in human and animal nutrition but also in traditional medicine (Leone et.al., 2016: 17). Although whole parts of the Moringa herb are traditionally used, Moringa seeds especially have anticancer, antioxidant, anti-inflammatory, antibacterial, hypotensive, antifungal, antibiotic, and hepatoprotective properties (Jaja Chimedza et.al., 2017: e0182658, Giacoppo., 2017: 362).

Traditional 2D cell cultures have long been used to evaluate the impacts of drugs on tumor cell growth. However, the 2D culture medium does not ensure knowledge about the complicated interactions between the physicochemical microenvironment that exists within viable tumors formed in human organs. More recently, more sophisticated transwell-based tests have been used to research cancer cell migration and invasion through their microscale pores (Sontheimer-Phelps et.al., 2019: 81). Based on this information, a co-culture model was constructed in this study that allows direct interaction between human endothelial cell (HUVEC) and the U373 cancer line, thus facilitating the investigation of the signaling pathways that govern blood vessel formation in GBM cancer and increased the proliferation of neuron cells to increase tumorigenesis in neuronal cells. As a result, the therapeutic effect of the Moringa plant on neurotoxicity caused by the transwell system was determined.

MATERIAL METOD

RN-33B, HUVEC and U373 Cell Culture

For our study, RN-33B (ATCC® CRL-2825), HUVEC (ATCC® CRL-1730) and U373

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(ATCC® HTB-17) cell lines in the medical pharmacology department of Atatürk University were used. The cell suspension was re-suspended with fresh medium (87% dulbecco-modified eagle medium (DMEM), 15% fetal bovine solution (FBS), and 1% antibiotic (streptomycin, penicillin and amphotericin B) (Thermo Fisher, Germany) by centrifugation at 1200 rpm for 5 minutes. The suspended cells were then collected in a 25cm^2 flask and incubated (5% CO_2; 37°C). When 85% of the flask is filled with cells, HUVEC was lifted with EDTA and centrifuged, and cells were seeded at the bottom of the transwell membrane and kept in matrigel medium for 24 hours. Then, the U373 cell was lifted with EDTA and centrifuged and inoculated on the upper part of the transwell membrane, and kept in matrigel medium for 24 hours.

Transwell

HUVEC cells were seeded on the bottom of the 24-well transwell polyethylene-coated membrane filter and after 24 hours in basal medium, U373-line cancer cells were seeded on the top bottom of the membrane filter. Separately, neuron cells were seeded into a polyethylene-coated 24-well plate. HUVEC-U373 and neuron cells were incubated in a medium for 1 day before co-culture, and transwell membranes containing HUVEC-U373 were placed in the wells of the neuron-containing plate. After 1 day, the co-cultured cells were separated and cultured in fresh culture media for an additional 24 hours.

Moringa Application

Different concentrations of Moringa (25, 50, 100, 200 and 400 µg/ml) were added to the wells ready for drug application and the plates were incubated for 1 day.

MTT Assay

After treatment, 20 µL of MTT solution was added to each well plate. The playlets were then incubated in a CO_2 incubator at 37 °C for 4 hours. At the end of the period, Formazan crystals were dissolved by adding 100 µL of Dimethyl sulfoxide solution to each well. The density of the formazan crystals was read by a Spectrophotometer reader at a wavelength of 570 nm.

LDH Assay

Lactate dehydrogenase (LDH) activity was determined by colorimetric technique by performing the LDH kit (Elabscience, Texas, USA) procedure. The values obtained were measured at a wavelength of 450 nm.

Determination of Oxidative Stress

Cell media were collected 24 hours after drug administration and measurements were made according to the manufacturer's instructions to determine total oxidant status (TOS), total antioxidant capacity (TAC) (Rel Assay Diag-

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nostics, Gaziantep, Turkey), and glutathione (GSH) (Elabscience, Texas, USA) levels.

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's LSD was used to make post hoc comparisons using SPSS 22.0 software. $p<0.05$ and $p<0.001$ were accepted as the statistical threshold for each analysis.

RESULTS

MTT and LDH Assay

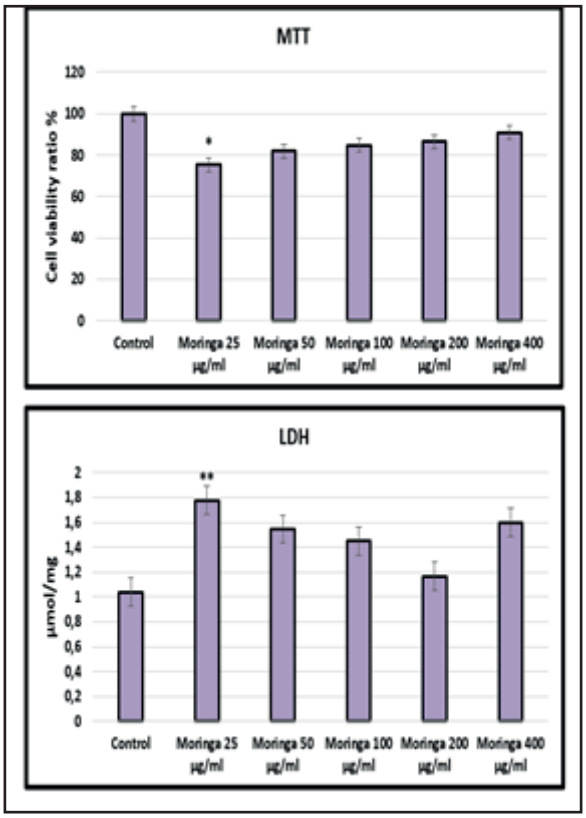

Figure 1. Effects of Moringa on the Cell Viability (MTT and LDH). *$p < 0.05$, **$p < 0.001$ Control Group

The results from the cell viability assay are shown in Figure 1. The viability of the Moringa groups was graded accordingly to the control group and showed significantly lower cell viability. Our results showed that the Moringa (400 µg/ml) group had the highest viability compared to other treatments. In addition, Moringa (25 µg/ml) group had the lowest survival rate compared to other treatments and showed statistical was difference ($p<0.05$). In LDH activity, an increase in LDH level was observed in the Moringa

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(25 µg/ml) group. In addition, this group was found to be statistically very significant compared to the control group ($p<0.001$).

Oxidative Stress Markers

According to the TAC test, among the groups, the Moringa (400 µg/ml) group showed the highest antioxidant capacity compared to the control. There was no statistical difference in this group compared to the control group ($p>0.05$). In addition, Moringa 25 and 50 µg/ml groups were statistically significant compared to the control group ($p<0.05$). In GSH activity, while Moringa (25 µg/ml) group showed the lowest GSH level compared to the control group, 400 µg/ml dose reached the highest level ($p<0.05$). Also, looking at the oxidant levels, the Moringa (25 µg/ml) group showed the highest oxidant level compared to the control. This group was found to be statistically very significant compared to the control group ($p<0.001$) (Figure 2).

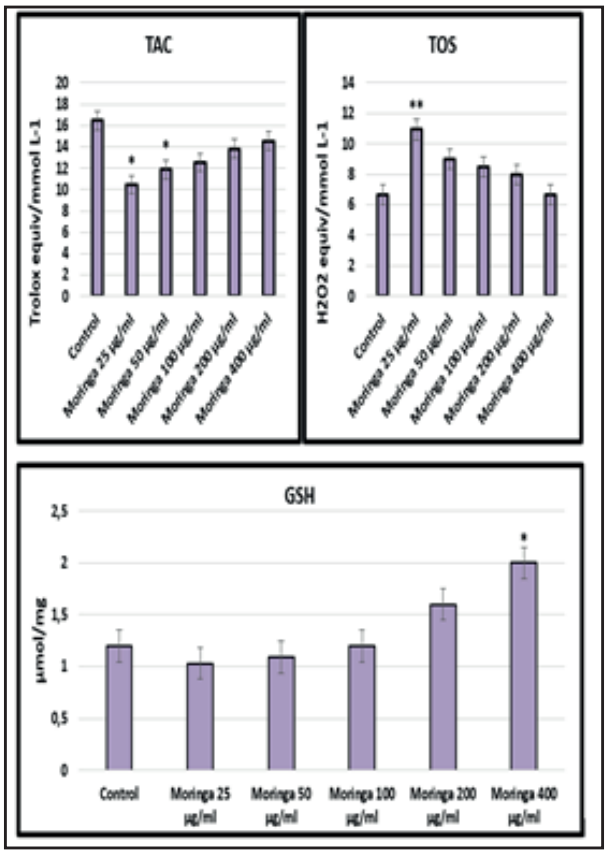

Figure 2. Effects of Moringa on the Oxidative Stress Markers (TAC, TOS, and GSH). *$p < 0.05$, **$p < 0.001$ Control Group

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DISCUSSION

The challenge in GBM cure is tumor-derived angiogenesis, which leads to the uncontrolled generation of vessels and a dysfunctional blood tumor barrier. Abnormal vascularization caused by GBM metastasis disrupts the blood-brain barrier, preventing drug penetration. Therefore, the recognition of novel molecular targets controlling angiogenesis will provide a novel focus for more effective therapeutic strategies. In current studies, it is reported that Moringa has a protective effect on various cancers. In recent studies, it has been determined that Moringa has strong antiproliferative activity on pancreatic cancer. Moringa oleifera leaf extract targets the NF-κB signaling pathway by reducing the expression of p-IκBa, and its proteins and inhibits pancreatic cancer cells, thereby increasing the effect of chemotherapeutic agents on human pancreatic cancer cells (Berkovich et.al., 2013: 212). In addition, in a study on neuroblastoma (SH-SY5Y), it was shown that Moringa extract obstructs the proliferation of malign cell lines by apoptosis activation of programmed cell death in SH-SY5Y cells. Specifically, the antiproliferative activity of Moringa is attributed to its capability to inhibit the MAPKs and PI3K/Akt/mTOR pathways, ultimately resulting in cell death (Sontheimer-Phelps et.al., 2019: 81).

In a study on the retina, dilated arterioles and venules of diabetic patients compared to healthy ones were observed. However, the group treated with Moringa appeared to be less dilated in arterioles and venules than the diabetic group. Recent experimental works propose that excessive ROS production and low antioxidant may contribute to retinal oxidative stress (Arden et.al., 2011: 291, Kumar-Gupta et. al., 2013: 419). However, it turns out that uncontrolled chronic diabetes is associated with impaired retinal antioxidant levels. As a result, it has been shown that low levels of GSH, SOD and CAT are present in the diabetic retina. Different polyphenolic compounds have proven to be a powerful antioxidants (Kamalakkannan et.al., 2006: 97). Moringa forms some of such polyphenolic compounds, and in this study, we found the plus modulatory impact of Moringa, which has antioxidant properties, on neurotoxicity. It was observed that the antioxidant level of Moringa increased in a dose-dependent in TAC and GSH levels, while the oxidant level decreased in TOS level.

CONCLUSION

This study, in line with the data obtained using the co-culture model with different cerebral cell types separated by a semi-permeable membrane, reveals the neuroprotective effect of Moringa by preventing neurotoxicity following a short-term exposure of neurons to U373 cancer cells.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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