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Original Investigation

The Effect of *Rosmarinus Officinalis* and Chemotherapeutic Etoposide on Glioblastoma (U87 MG) Cell Culture

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ABSTRACT

AIM: Glioblastoma (GBM) is the most invasive and common type of brain cancer with very poor prognosis. One of the drugs administered for GBM pharmacotherapy is etoposide (VP-16), which belongs to the topoisomerase inhibitor family. It can be used in combination with other drugs or chemicals to avoid high dose toxicities or augment its effect at lower doses. In this study, we aimed to investigate whether high dose toxicities of etoposide can be overcome when used in combination with a natural compound named *Rosmarinus Officinalis*.

MATERIAL and METHODS: The impact of *Rosmarinus Officinalis* in combination with etoposide on GBM U87 MG cells and Mouse Embryonic Fibroblast (MEF) cells was investigated. Both neutral red and 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assays were employed to gauge cell viability.

RESULTS: We observed that increased quantities of *Rosmarinus Officinalis* induced MEF cell proliferation while it inhibited the survival of GBM cells. Our results indicate that *Rosmarinus Officinalis* did not affect the cytotoxicity of etoposide on GBM cell cultures. In contrast, in the MEF cell cultures, *Rosmarinus Officinalis* induced proliferation and diminished the impact of etoposide.

CONCLUSION: *Rosmarinus Officinalis* offers hope for developing new cancer treatment strategies. However, further studies are needed to verify these results.

KEYWORDS: Etoposide, Glioblastoma, *Rosmarinus Officinalis*

■ INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive type of primary brain cancer with very poor prognosis (5,24). GBM is a highly invasive tumor with prominent vascular involvement, characterized by twisted blood vessels and infiltration into external vessel walls, which make it resistant to treatment (4,10). Patients with GBM are usually treated with surgical resection, radiation, and chemotherapy. However, following diagnosis, the life expectancy of patients is usually no longer than 6-12 months (9,18,25,31). Etoposide, which belongs to the topoisomerase inhibitor family, is appropriate

for use in GBM pharmacotherapy while also being an effective medium in GBM therapy (17,19). It is a widely used cancer drug that provides good results in the treatment of various types of cancer, such as small-cell lung cancer, ovarian cancer, testicular cancer, glioblastoma, and lymphoma (7,27).

Etoposide (VP-16) is a semi-synthetic podophyllotoxin derivative that functions as a natural antibiotic. It inhibits topoisomerase II and prevents the accumulation of double-strand deoxyribonucleic acid (DNA) breaks, which is toxic for the cell (6,29). Although high doses of etoposide are effective on GBM cells, they often cause similar toxic side effects. In vari-



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ous studies, etoposide is being tested for its efficiency against GBMs, along with myriads of drugs such as Bevacizumab and Vandetanib, to avoid high dose toxicities or increase its effect at lower doses (6,10,16). In this study, we aimed to test etoposide in combination with a natural compound named *Rosmarinus Officinalis*, to overcome high dose toxicities and increase the effectiveness of etoposide.

Rosmarinus Officinalis is an aromatic, perennial plant that belongs to the Lamiaceae mint family. Although it originates from the Mediterranean region, *Rosmarinus Officinalis* is now being grown in various parts of the world owing to its several beneficial properties (14,22). *Rosmarinus Officinalis* is used fresh, dried, as essential oil or tea infusion. It contains phenolic diterpenes such as carnosol, carnosic acid, rosmarinic acid, rosmanol, and iso- and epi-rosmanol, as well as many active components including caffeic acid and ursolic acid (1,23,26,30). From past centuries to the present day, *Rosmarinus Officinalis*, has also been used in traditional medicine, has been employed in the food industry as an antioxidant and a flavoring agent, and has various uses in the cosmetic industry. In traditional medicine, *Rosmarinus Officinalis* has been used to cure many diseases such as diabetes, inflammatory diseases, cancer etc. (3,22,28). Furthermore, many studies have reported that *Rosmarinus Officinalis* possesses antimicrobial, antioxidant, anti-inflammatory, and anti-cancer properties (11,13,15,20). In addition, *Rosmarinus Officinalis* has been demonstrated to have anti-proliferative effects on numerous cancer types such as leukemia, breast cancer, and prostate cancer besides having repressive effects on melanoma and glioma (8,12). Berrington and Lall have shown that *Rosmarinus Officinalis* has high antioxidant content. Because of its high content of polyphenolic compounds, it has potentially chemo-preventive properties (2). Moreover, prostate cancer represents an example of the potential uses of *Rosmarinus Officinalis* for chemoprevention and tumor reduction. It has been shown that *Rosmarinus Officinalis* extract reduces cellular viability and induces apoptosis in prostate cancer cells (21). Based on these findings, in the current study, we aimed to investigate the effects of *Rosmarinus Officinalis* on the high dose toxicities of etoposide when used in combination in the GBM cell culture.

■ MATERIAL and METHODS

Cells and Culture Conditions

The study was conducted using GBM (U87 MG (ATCC® HTB-14™)) and Mouse Embryonic Fibroblast (MEF) cell lines. The cells were seeded in 24 well plates (40000 GBM cells and 10000 MEF cells for each well), cultured using Dulbecco's Modified Eagle's medium (DMEM, Lonza, Belgium), and supplemented with 10% fetal bovine serum (FBS, South America).

Preparation of the *Rosmarinus Officinalis* Extract and Dose Optimization

Rosmarinus Officinalis has been used for traditional medicine to cure many diseases alone or in a mixture with various other plants (up to 350 mg/g tea)(13). Plant materials were harvested from Adana, Turkey and registered in the Herb.Reg. Turc.Medit. (herb no: 5263). *Rosmarinus Officinalis* extract

was prepared as follows: Two grams of herbal extract were added to 100 ml of double distilled, boiling water and waited for 10 minutes. The mixture was sterilized using a 22 µm pore-size filter. *Rosmarinus Officinalis* extract was administered to cell cultures, except the control group, in the concentrations equivalent to 1/1000, 1/100, and 1/75 (v/v) and incubated for 1 day. Subsequently, cell viabilities were measured by neutral red assay and optimum doses of *Rosmarinus Officinalis* for GBM and MEF cells were determined.

Cytotoxic Effect of *Rosmarinus Officinalis* and etoposide on GBM and MEF cells

Rosmarinus Officinalis extract at 1/75 (v/v), 40 µM etoposide, and 1/75 (v/v) *Rosmarinus Officinalis* extract with 40 µM of etoposide were administered to the GBM and MEF cell cultures. Cell cultures were incubated for 1, 3, and 5 days respectively and the medium was refreshed once every two days. At the end of the incubation period, cytotoxic effects were measured by neutral red and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays.

Cytotoxicity Assay

Cell viabilities were tested using neutral red and MTT assays. As regards the neutral red assay, firstly the growth medium was removed after the incubation period, and 500 µL of neutral red solution was added to each well. Cells were incubated for nearly 2 hours until the red precipitates became visible. Following the growth of visible red precipitates, 1000 µL of neutral red solubilization solution (50% absolute ethanol, 1% acetic acid, and 49% ddH₂O mixture) was added to each well. Plates were incubated for nearly 1 hour until the red precipitate completely dissolved. Absorbance values of each well were measured at 540 nm using a spectrophotometer (Shimadzu UV-VIS, UVmini-1240, Japan).

For the MTT assay, after the incubation period, the growth medium was removed and 500 µL of MTT solution was added to each well. Cells were incubated for nearly 2 hours until the purple MTT formazan crystals became visible. Following the growth of visible purple precipitates, 1000 µL of MTT solubilization solution (isopropanol) was added to each well. Plates were incubated for nearly 1 hour until the purple precipitate completely dissolved. Absorbance values of each well were measured at 570 nm using a spectrophotometer (Shimadzu UV-VIS, UVmini-1240, Japan). Assays were performed in triplicate. Cell viabilities were calculated with respect to control groups and the absorbance value of the control group was selected as 100% cell viability.

■ RESULTS

According to dose optimization results it was indicated that while increased quantities of *Rosmarinus Officinalis* induced MEF cell proliferation, it inhibited the survival of GBM cells (Figure 1). A dilution of 1/75 (v/v) was selected as the optimum dose at which *Rosmarinus Officinalis* increased the viability of healthy MEF cells by nearly 9.5% and reduced the viability of GBM cells by nearly 42%.

The results of cell viability assays are shown in Figures 2A, B and 3. According to the neutral red assay, while etoposide

reduced the viability of GBM cells, cultured for one and three days by 50% and 73% respectively, *Rosmarinus Officinalis* reduced the viability of GBM cells, cultured for one and three days, by 38% and 57% respectively. Etoposide, when combined with *Rosmarinus Officinalis*, reduced the viability of GBM cells cultured for one and three days by 50% and 75% respectively. Thus, *Rosmarinus Officinalis* does not seem to decrease or increase the cytotoxicity of etoposide. The MTT assay results confirmed this data. According the MTT assay, for three- and five-day cultures, etoposide reduced the viability of GBM cells by 71% and 96% respectively, *Rosmarinus Officinalis* reduced the viability of cells by 54% and 65% respectively and when etoposide was combined with *Rosmarinus Officinalis*, reduced the viability of GBM cells by 79% and 99% (Figure 2A, B).

According to results of the neutral red assays performed with MEF cell cultures, it was observed that etoposide reduced the

viability of cells by nearly 84% in the 24 hours, while *Rosmarinus Officinalis* diminished the effectiveness of etoposide. The neutral red cell viability assay, on the other hand, showed that *Rosmarinus Officinalis*, when used together with etoposide, reduced the number of MEF cells by nearly 57%. The MTT assay results also confirm the data. According to the MTT assays, etoposide reduced the viability of the cells by 86%, and when combined with *Rosmarinus Officinalis*, reduced the viability of MEF cells by 61% (Figure 3).

DISCUSSION

In our study, it was found on the basis of neutral red assays, which were performed after the first, third and fifth day of the cultures, that etoposide (40 μM), *Rosmarinus Officinalis* (1/75 v/v) and etoposide and *Rosmarinus Officinalis* combinations were successful in eliminating GBM cells (Figure 2A, B).

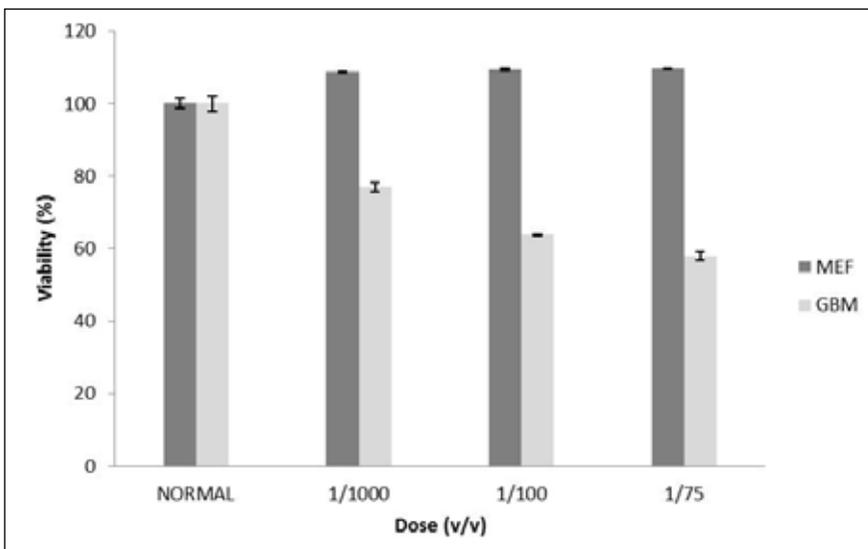


Figure 1: Neutral red assay results of dose optimization of *Rosmarinus Officinalis*.

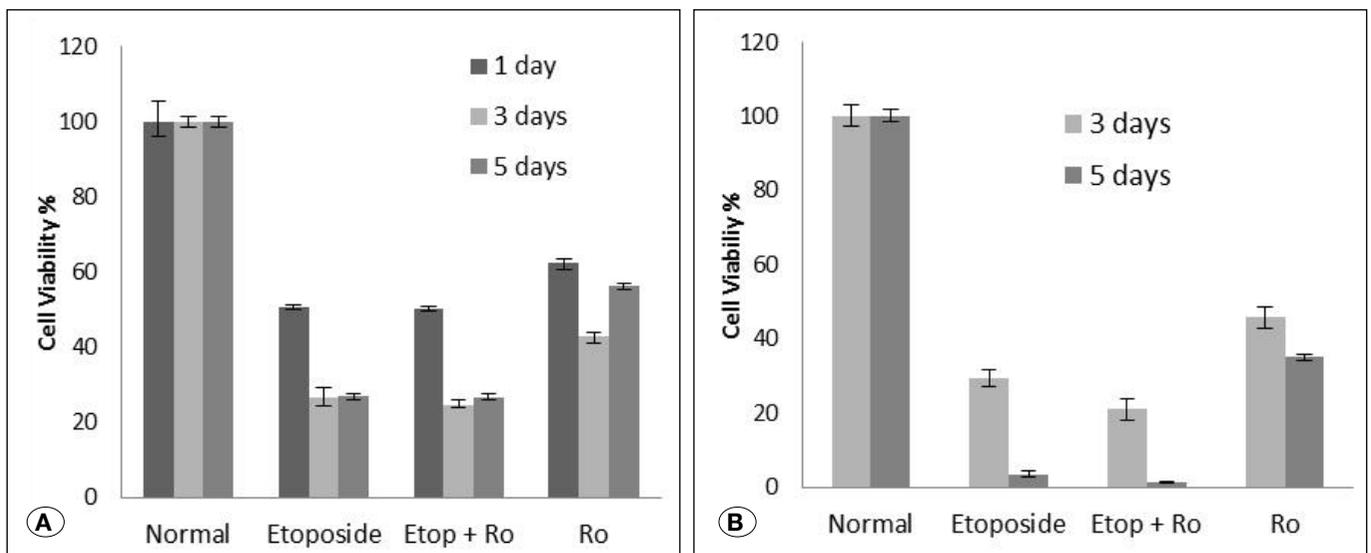


Figure 2: Cytotoxic Effect of the *Rosmarinus Officinalis* and etoposide on GBM cells (etoposide (40 μM), *Rosmarinus Officinalis* (1/75 v/v) (RO), etoposide and *Rosmarinus Officinalis* combination (Etop + RO)). **A)** Results of the neutral red assay. **B)** Results of the MTT assay.

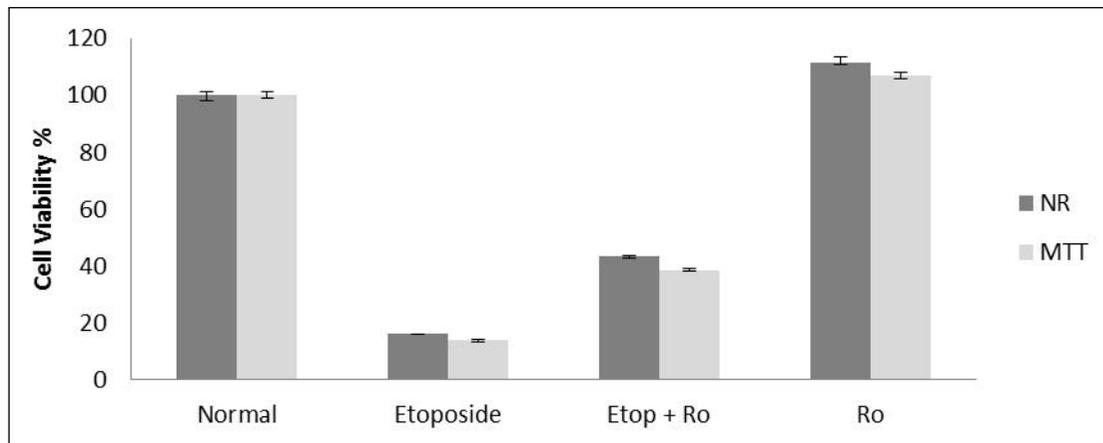


Figure 3: Neutral red assay (NR) and MTT assay results of MEF cells (etoposide (40 μ M), *Rosmarinus Officinalis* (1/75 v/v) (RO), etoposide and *Rosmarinus Officinalis* combination (Etop + RO)).

The results showed that *Rosmarinus Officinalis*, when given together with etoposide, increased the cytotoxicity of etoposide by some measure rather than inhibit it. In addition, *Rosmarinus Officinalis* by itself induced the proliferation of MEF cells while it diminished the impact of etoposide on MEF cells (Figure 3) when used together with etoposide. It can be assumed that *Rosmarinus Officinalis* protects the healthy cells against the side effects of etoposide without diminishing its effect in the GBM cells. It has also been observed that *Rosmarinus Officinalis* and etoposide, when used together, are effective on GBM.

CONCLUSION

Rosmarinus Officinalis offers hope for developing new cancer treatment strategies. However, further studies are needed to examine the effects of *Rosmarinus Officinalis* with other therapeutics on various cancer types.

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