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Original Research

The Cytotoxic Effect of *Polygonium cognatum* and Chemotherapeutic Effect of Doxorubicin on Glioblastoma Cells

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ABSTRACT

Objective: Glioblastoma multiforme (GBM) is an aggressive malignant brain tumor common in adults. Owing to the present difficulty in treating GBM, developing alternative methods is of utmost importance. Recently, the efficacy of various plant extracts in cancer treatment have been evaluated. *Polygonum cognatum (P. cognatum)* known as 'Madımak' is used in herbal medicine in Turkey.

Methods: In this study, we investigated the cytotoxity of *P. cognatum* in the treatment of glioblastoma and its contribution to the effectiveness of doxorubicin (DXR). In the U87 cell line of the *P. cognatum* and doxorubicin administered at different doses, IC50 doses were determined using the 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) method and the effects of combined administration at these doses were examined (respectively 10–125 µg/ml and 0.1–10 µg/ml at 24, 48, and 72 h).

Results: *P. cognatum* extract decreased the cell viability of U87 cells in a time and concentration-dependent manner. It also increased the apoptotic effectiveness of DXR in U87 cells.

Conclusion: This is the first preliminary study that investigates the treatment of *P. cognatum* on glioblastoma *in vitro*. Further studies are required to investigate the effect of the extract on healthy human cells and to understand signaling pathways.

Keywords: Glioblastoma, *P. cognatum*, doxorubicin

INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most common type of brain tumor with an aggressive progression. It is noted in two to three incidents per million adults per year (1). GBM is a lethal human tumor, with a current standard-of-care therapy involving surgical resection, radiotherapy, and chemotherapy (2). The survival time after usual treatment ranges from 12 to 15 months. This is due to the limited ability of the many drugs to penetrate the blood-brain barrier. For this reason, more studies on binding the drugs on nanoparticles have been undertaken (3). However, no satisfactory evidence have been encountered.

Drugs such as vincristine, vinorelbine, vinblastindocetaxel, etoposide, paclitaxel, camptothecin, topotecan, doxorubicin (DXR) and irinotecan are used in the treatment of tumors. The basic feature of these drugs are low safety levels and many side effects (4). DXR is an anthracycline anti-cancer drug that can be used for many malignancies such as lung, breast, ovaries and multiple myeloma (5). These chemotherapeutic agents induce cell death via multiple mechanisms such as blocking nucleic acid synthesis by preventing topoisomerase II activity (6). Clinical studies involving systemic administration of the drug show that its effectiveness in the treatment of gliomas is limited (7). Very high doses of DXR are required to achieve therapeutic levels, and these doses are highly neurotoxic. This is due to its low ability to traverse the blood-brain barrier (8). In recent years, many studies to improve the efficacy of DXR in GBM are ongoing, thereby employing techniques such as local drug delivery to the tumor resection site or using DXR-loaded nanomicelles (9).

Complementary and alternative treatments commonly used in GBM include vitamin and nutritional supplements, and herbal extracts. Some studies show that treatment with herbal extracts can reduce mortality in GBM (10, 11). In order to increase the

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effectiveness of anti-cancer therapy, new approaches are being investigated for the effect of plants on cancer cells (12).

Polygonum cognatum (*P. cognatum*, Madımak) is a member of the *Polygonaceae* and is a perennial plant found in Turkey (13). Previous studies on *P. cognatum* have reported that it contains vitamin C and carotenoids. It also has antioxidant, antimicrobial, diuretic, and antidiabetic properties. It is known to be used for the treatment of various diseases for medical purposes (14).

In this study, we aimed to investigate the cytotoxic effect of *P. cognatum* (collected in the Tokat region of Turkey) on GBM cells. Moreover, we sought to investigate the combined effect of DXR and *P. cognatum* on cell proliferation in the U87 cell line.

METHODS

Plant materials

We obtained *P. cognatum* plants from the wild flora at an altitude of 800 m (40.322796 latitude 36.059895 longitude of Tokat) in North Anatolia. Plant materials were identified by Botanical and Herbarium Application and Research Center. The herbarium specimen of *P. cognatum* (EGE 43193) have been deposited at the Herbarium of Faculty of Science, Ege University.

Preparation of methanol extraction of *P. cognatum*

We crushed the samples leaves with a blender. Then, we dissolved 5 g of powder in 50 mL of methanol for 24 h while shaking intermittently. We collected the extract using a Whatman filter paper. Then, the filtrate was dried with a rotary evaporator at 80°C under reduced pressure and this procedure was repeated three times. The filtrate obtained was stored at –20°C (15). Extracts were dissolved in dimethylsulfoxide (DMSO) to prepare stock solutions as a final concentration under 0.1%. Then, we further diluted the extract in culture medium with four different concentrations.

Cell culture of U87 cell line

Human GBM cell line (U87) was obtained from Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1 µg/mL penicillin/streptomycin and 2 mM L-glutamine in 5% CO_2 saturated incubator at 37 °C. The cell medium was changed every two days when they attained enough confluence.

Cell treatment

U87 cells were seeded in 96-well plates at a density of 5,000 cells per well in 100 µL growth medium. Cells were incubated overnight before been treated with the extract and DXR. U87 cells were treated with various doses (10, 50, 100, 125 µg/mL, and 0.1, 0.2, 0.5, 1, 5, 10 µg/mL, respectively) of *P. cognatum* extract and DXR (Adrimisin, Saba Ilac, Istanbul, 2020) in triplicates. Moreover, cells were incubated for 24 h, 48 h, and 72 h and then analyzed. Median inhibitory concentration (IC50) was estimated using Microsoft excel version 14.0.4. After the IC50 dose was determined, the plant extract and DXR were applied in combination at these doses and analyzed again.

Main Points:

- *P. cognatum* extract has anti-carcinogenic effect on the U87 cell line.
- *P. cognatum* extract had a decreasing effect on cell viability on the U87 cell line.
- *P. cognatum* extract may increase the effect of DXR on the U87 cell line.

Cell viability assay (XTT)

The anti-proliferative activity of *P. cognatum* and DXR was evaluated using a commercially available proliferation kit XTT (Biological Industries, Israel). XTT reagent solution and the activation solution was prepared with a reaction solution sufficient for one plate, followed by the addition of 0.1 mL activation solution to 5 mL XTT reagent. We then added 50 µL of the reaction solution to each well and the plate was incubated at 37°C in 5% CO_2 for 4 h. After incubation, the plate was swirled gently and the absorbance of the samples was measured using a spectrophotometer (ELISA reader PG Instruments) at a wavelength of 450 and 630 nm. DMEM (0.1% DMSO) was used as a negative control.

Statistical Analysis

We performed overall analyses using SPSS (Statistical package for social science for Windows, version 25.0, IBM SPSS Corp.; Armonk, NY). According to the results of ANOVA, we observed that the groups in which DXR, *P. cognatum* and both were administered in 48 hours were significantly different from the untreated controls ($p<0.05$).

RESULTS

Extraction of the plant material

P. cognatum was extracted with methanol, as illustrated above. Plant materials were dissolved in DMSO (Sigma-Aldrich). The final concentration of DMSO in cell lines was less than 0.1% to prevent any possible effect on the cytotoxicity levels.

Cytotoxiticity

Here, we investigated effect of *P. cognatum* extract on the cell viability of U87 cells. For this aim, we treated U87 cells with increasing concentrations (10–125 µg/mL) of *P. cognatum* extract for 24, 48, and 72 h. We evaluated the *in vitro* cytotoxic effect of *P.cognatum* methanolic extracts on U87 cell lines using the XTT assay. According to the assay, *P. cognatum* extract decreased the cell viability of U87 cells in a time and concentration-dependent manner, as compared to untreated controls. The most effective time for this was 48 hours.

As shown in Fig. 1A, decreases in the cell viability of U87 cells exposed to 10, 50, 100, and 125 µg/mL of the extract were 72.96%, 68.62%, 48.82% and 33.43% respectively, as compared to untreated controls at 48 h ($p<0.05$). The IC50 values was determined at 100 µg/mL for the methanolic extract of *P. cognatum* at 48 h for U87 cells.

Figure 1. a, b. Cell viability percentages of U87 cells after application of *P. cognatum* at different concentrations analyzed by XTT (a), cell viability percentages of U87 cells after application of DXR at different concentrations analyzed by XTT (b)

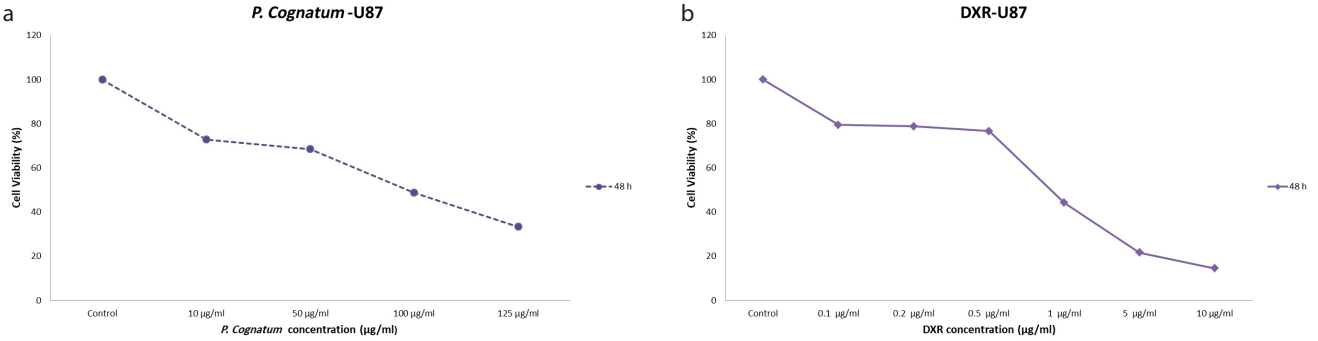
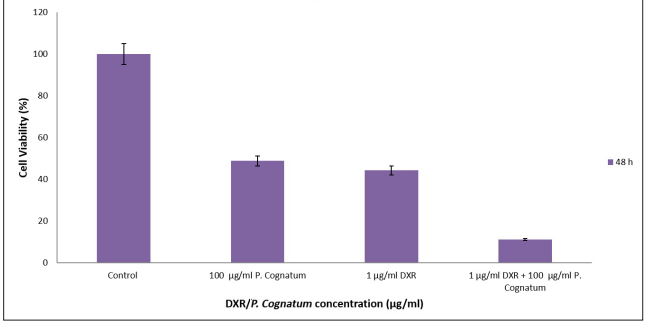


Figure 2. Percentage of cell viability of U87 cells induced by combination of DXR-*P. cognatum* IC50 concentrations, 48 h analyzed by XTT



Furthermore, we evaluated *in vitro* cytotoxicity activity of DXR on U87 cell line using the XTT assay and the results are given in Figure 1B. Results showed that DXR extract has a significant anti-cancer effect on glioblastoma cell in a concentration-dependent manner. DXR also exhibited a time-dependent inhibition of cell proliferation (Fig. 1B).

While U87 cells were exposed to 0.1, 0.2, 0.5, 1, 5, 10 µg/mL of extract, the cell viability for 48 h were 79.51%, 78.93%, 76.67%, 44.25%, 21.70% and 14.55% respectively, The IC50 values of DXR was 1 µg/mL.

While 100 µg/ml *P. cognatum* treatment for 48 h showed a 51.18 % cell death, 1 µg/mL DXR treatment showed a 55.75 % cell death. However, the combined treatment of 100 µg/mL *P. cognatum* and 1 µg/mL DXR for 48 h showed an 88.85% cell death (Fig. 2). This proves that a combined treatment of methanolic extract of *P. cognatum* and DXR inhibits U87 cell proliferation. Thus, *P. cognatum* increases the anti-cancer effect of DXR (Fig 2).

DISCUSSION

Heterogeneity, high motility of cell types, and ability to switch in proliferative/non-proliferative phases render the treatment of GBM very difficult (16). Radiotherapy and temozolomide (TMZ) are used in the treatment of GBM (17). However, TMZ resistance and unresponsiveness can be developed by some patients (18, 19). It was hypothesized that apoptosis-regulating genes and proteins such as *p53*, *p21*, *p16*, and *PTEN* are influenced by dysregulation (20). For these reasons, alternative treatments need to be developed.

One of the promising drugs in the treatment of GBM is DXR. DXR is known to inhibit biosynthesis through intercalation. It prevents the progression of the topoisomerase II enzyme, which enables the unfolding of DNA during transcription, stabilizes the topoisomerase-DNA complex during DNA duplication, thereby preventing the recombination of the DNA double helix (21). DXR cannot be applied systemically against GBM because it cannot reach therapeutic levels in the brain (8). Studies showed that local DXR application can prolong median survival and delay tumor growth, though complete remission cannot be achieved (22). TMZ and DXR are known to induce DNA damage by different mechanisms. A study hypothesized that TMZ-resistant GBM cells will remain sensitive to the cytotoxic effects of DXR. From other preliminary studies, DXR was shown to be an effective cytotoxic agent in TMZ-resistant GBM cells (23).

In recent studies, synergistic effects have been investigated with different plants in order to decrease the resistance of cancer cells to DXR (24). The combined use of different plants with DXR proves to increase its chemotherapeutic effects or reduce the resistance to DXR (25-27).

P. cognatum is used for adjuvant medicinal purposes in Turkey (28), reason why it is gaining grounds in research. An attempt was made to demonstrate its anti-cancer activity on different cell lines and direct them to define its content. There are studies showing that its extract has significant inhibitory effects on breast cancer. Eruygur et al. (29) showed that this extract prepared with ethanol is effective in MDA-MB-231 breast cancer cell line. Their results show that it has significant anti-cancer effects. Moreover, Sarac et al. (28) stated that *P. cognatum* decreased cell viability on the MCF-7 breast cancer cell line, with a little effect on the human umbilical vein endothelial cell line (HUVEC). Furthermore, Pekdemir et al. (30) stated that the methanol, acetone, and hexane extracts of the *P. cognatum Meissn* plant showed a significant decrease in cell viability of the MCF-7 cancer cell line compared to control, but not all doses of the ethanol extract

caused a significant decrease in cell viability. In addition, the plant is the most effective on MKN-45 gastric cancer cell line based on previous data and IC50 values.

This study supports the hypothesis that *P. cognatum* has anti-carcinogenic properties. Our study showed that the plant extract is effective in the U87 cell line and possibly potentiates the effectiveness of DXR in cancer treatment. Thus, we presume that it is beneficial to combine DXR with plants known to have anti-cancer effects in order to increase effectiveness against GBM. Previous reports also show that the combined application of the methanol extract of *P.cognatum* decrease the toxicity of DXR on GBM cells via decreasing IC50.

CONCLUSION

Methanolic extract of *P. cognatum* and DXR was tested for their potential anti-proliferative effect on the U87 cell line. The results showed that *P. cognatum* may have potential could be considered as alternative therapy in the future. In addition, it is presumed that *P. cognatum* increases the effect of DXR. Therefore, the results obtained in the present study form a basis for future clinical studies. The anti-cancer effect of the plant should be studied in detail as well as its mechanisms and signaling pathways.

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