



## Research Article

# Didymin Exhibits Different Cytotoxicity Patterns on U-87 MG Human Glioblastoma Cells and Orchestrates the Release of Substance P (SP) and Interleukin-6 (IL-6) in a Time Depending Manner

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### Abstract

**Objectives:** This study aims to investigate the cytotoxic effects of didymin on the U-87 MG Human Glioblastoma cell line, besides, its effects on levels of Substance P and Interleukin 6 (IL-6) released from the cells.

**Methods:** The cytotoxic effects of didymin on glioblastoma were determined at the end of 24, 48, and 72 hours incubation periods. The effects on the levels of Substance P and Interleukin 6 (IL-6) determined via Elisa.

**Results:** Didymin in the range of 100 – 1,56  $\mu\text{M}/\text{ml}$  doses did not show cytotoxic effects at the end of the 24<sup>th</sup> and 48<sup>th</sup> hour. At the end of the 72-hours a cytotoxic effect was observed at a dose of 100  $\mu\text{M}/\text{ml}$ . SP increased at 100  $\mu\text{M}/\text{ml}$  and 50  $\mu\text{M}/\text{ml}$  doses at the end of the 24 and 48 hours incubation periods and decreased significantly at the end of the 72<sup>nd</sup> hour. IL-6 increased significantly at 100  $\mu\text{M}/\text{ml}$  at the end of the 24<sup>th</sup> hour. It caused a significant increase in each dose at the 48<sup>th</sup> hour, while a significant increase was observed only at the 50  $\mu\text{M}/\text{ml}$  dose at the 72<sup>nd</sup> hour.

**Conclusion:** The cytotoxic effect of didymin was observed in single dose. Didymin increased the level of inflammation marker IL-6, in a time and dose-dependent manner and decreased the level of SP at the end of the 72<sup>nd</sup> hour. This was the first study, which exhibits the effects of didymin on SP and IL-6 released from U-87.

**Keywords:** Cytotoxicity, didymin, glioblastoma, substance p, interleukin-6

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Tumors affecting the central nervous system currently represent 1.4% of newly diagnosed cancer types and 2.6% of total cancer deaths in 2015.<sup>[1]</sup> Brain tumors constitute 85-90% of all primary central nervous system tumors. Glioblastoma (GBM) accounts for approximately half of adult brain tumors and is the brain tumor associated with

the shortest survival time.<sup>[2]</sup> A multifaceted therapeutic intervention, including surgery, is preferred in the treatment of glioblastoma. Further work is needed to identify new compounds that can inhibit tumor growth and prevent the development of recurrent tumors to improve overall patient prognosis. In recent years, the use of natu-

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ral compounds in therapeutic intervention has increased. It is stated that almost one-third of the drugs approved for cancer by the United States Food and Drug Administration (USFDA) are natural products and their analogs.<sup>[3,4]</sup> Glioblastoma is a highly invasive and heterogeneous type of malignant brain tumor. Detailed molecular analyzes of glioblastoma; reveals cell growth, DNA repair, and dysregulation of signaling pathways such as receptor tyrosine kinase (RTK), phosphatidylinositol-3-kinase (PI3K), p53, and mitogen-activated protein kinase (MAPK).<sup>[5]</sup>

Flavonoids are secondary metabolites commonly found in various plants. Many citrus species accumulate large levels of flavonoids during development. Flavonoids have attracted the attention of researchers as they show therapeutic properties in the prevention and treatment of various diseases due to their anticancer, antioxidative, anti-inflammatory, and many more effects. Flavonoid glycosides are natural molecules in which flavonoids attach to sugar via a glycosidic bond and are a common component of many plants.<sup>[6]</sup>

Didymin is a dietary glycoside, found in citrus fruits, including mandarin orange, bergamot, orange, Origanum, and Vulgare Duanxueliu. Due to its citrus content and easy extraction, didymin has been accepted as an inexpensive, safe, and effective oral drug that does not cause toxicity in normal tissues.<sup>[7]</sup> It is emphasized that dietary flavonoids, which have a great place in nutrition, can play a role in the prevention of cancer safely and effectively. Studies show that didymin, a flavonoid glycoside, can reverse the negative effects on cancer. Although the molecular mechanisms have not been revealed today, it is known that didymin has therapeutic effects, antitumor, and anti-inflammatory potential on tumor cells. Cancer-related inflammation can accelerate tumor cell proliferation, survival, and migration.

Interleukin-6 (IL-6) is the primary cytokine that creates the inflammatory peri-tumoral environment. Its increased expression is observed in glioblastoma and directly affects patient survival.<sup>[8]</sup> Substance P (substance P (SP)), a neuropeptide, is an important mediator of neurogenic inflammation in the central and peripheral nervous systems. SP plays an important role in pain pathways and tumor cell proliferation, angiogenesis, tumor cell invasion, and metastasis, and SP has been shown to induce the expression of proinflammatory cytokines and chemokines such as IL-6 and IL-8.<sup>[9]</sup> Considering the literature studies, the cytotoxic, therapeutic, antitumor, and anti-inflammatory effects of various flavonoids on tumor cells increase the importance of these compounds. Didymin flavonoid is widely found in citrus fruits, its consumption is widespread and it is also important in terms of nutrition. Our aim in our study is to

investigate the cytotoxic and anti-inflammatory effects of this compound on the glioblastoma cell line.

## Methods

### Materials

Didymin was obtained AktinLab, China. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), trypsin – EDTA, MEM Non-essential amino acid, and sodium pyruvate were obtained from Gibco, USA. Genta antibiotic İbrahim Etem, Turkey, DMSO) was provided by Akdeniz University Tuberculosis Research Center (AKVUAM). WST-1 Proliferation Kit, Human Substance P Eliza kit, and Human Interleukin 6 (IL-6) Eliza Kit were obtained from Takara, Japan, and AFG Bioscience, USA respectively.

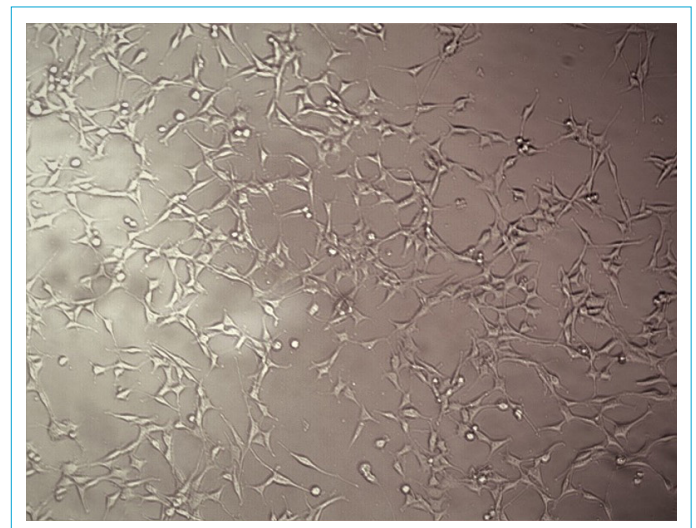
### Cell Culture

U-87 MG Human Glioblastoma cell line (ATCC) was a kindly gift from AKVUAM (Fig. 1).

U-87 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) containing 10% FBS (Gibco, USA), 2% glutamine, 1% non-essential amino acid (Gibco, USA) 0.5% sodium pyruvate (Gibco, USA) and 0.2% antibiotic (Genta Antibiotic, İbrahim Etem, Turkey). The cells were incubated at 37 °C and 5% CO<sub>2</sub> incubator.

### Preparation of Didymin Solution

10 mg of Didymin (AktinLab Cat No: APC-693) was dissolved in 1 ml of sterile DMSO, filtered, and stored in a -70°C until required. Didymin concentrations were prepared at a range of 100 µM/ml, 50 µM/ml, 25 µM/ml, 12,5 µM/ml, 6,25 µM/ml, 3,125 µM/ml and 1,56 µM/ml by diluting with a medium containing 1% FBS.



**Figure 1.** Characteristics of U-87 Cell Line.

### WST-1 Cell Proliferation Assay

To determine whether Didymin affected the growth of cancer cells the Takara WST-1 Cell Proliferation Assay Kit was used. Briefly, the cells were seeded at  $3 \times 10^3$  cells/well in 200  $\mu$ L complete medium on 96-well plates and allowed to attach for 24 hours. Then, media were removed and cells were treated with different concentrations (100-1,56  $\mu$ M/ml) of didymin. After the incubation period, the media were withdrawn, 90  $\mu$ L of serum-free medium and 10  $\mu$ L of WST-1 solution were added and incubated for 3 hours, then the absorbance values of the plates were measured at 450 nm wavelength in an Eliza Reader (Allsheng, China). All experiments were performed at least three times.

### Elisa Tests

To determine the possible effects of Didymin on the release of SP and IL-6, cells were divided into sterile 6-well plates at 105 cells/well. The levels of Substance P and IL-6 released from the cells to the medium were investigated using the Substance P ELISA Kit (AFG Cat No: EK711438) and the Human Interleukin 6 (IL-6) Elisa Kit (AFG Cat No: EK610267) respectively. Cells were treated with two concentrations (100  $\mu$ M/ml and 50  $\mu$ M/ml) of Didymin. At the end of the 24th, 48th and 72nd hours, the experiments were terminated and the media were collected and taken into Eppendorf tubes and studied according to manufacturers' protocol and the absorbance values were read in the Eliza kit reader at 450 nm wavelength.

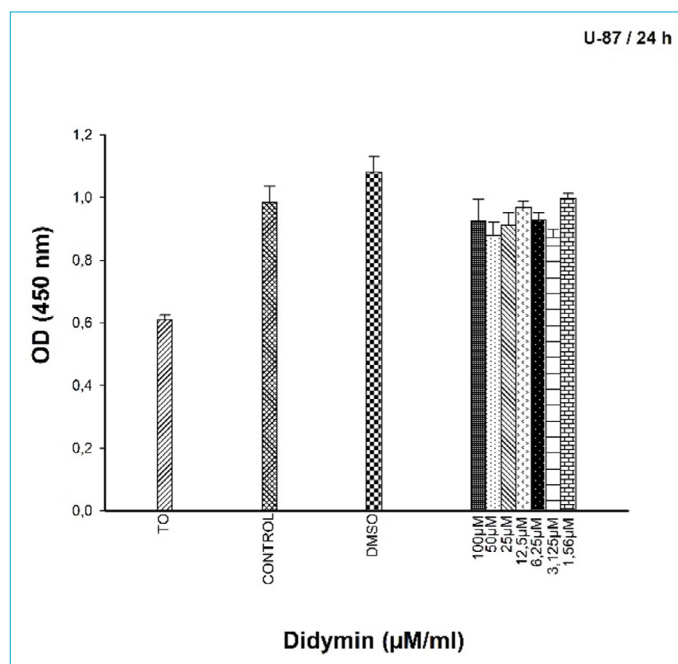
### Statistical Analysis

Statistical analyzes were evaluated using ANOVA and Dunnett Multiple Comparison Test in the GraphPad InStat program. The results of all analyzes performed with three replicates were given with their mean and standard deviations. The data were graphed using the Sigma Plot 10.0 program.

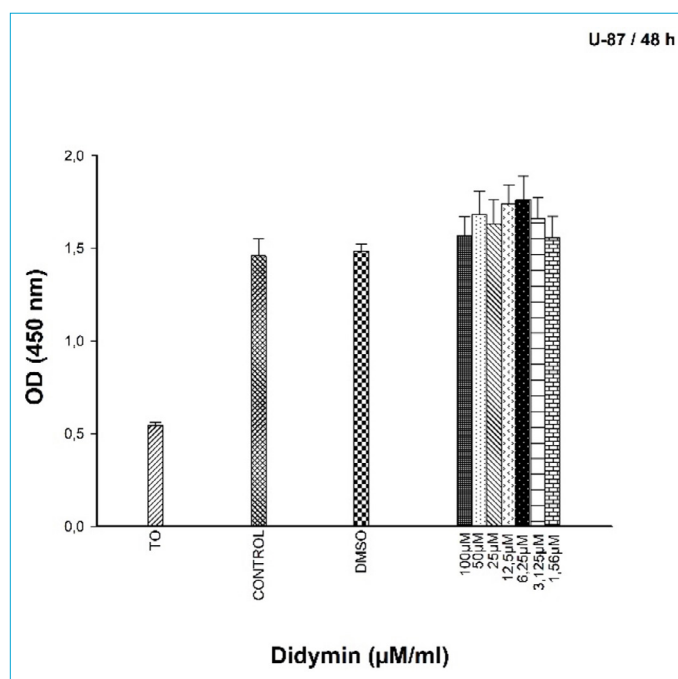
### Cytotoxic Effect of Didymin on the U-87 MG Glioblastoma Cell Line

The effect of didymin on cell proliferation was tested in U-87 MG glioblastoma cells. The doses tested in the range of 100 – 1,56  $\mu$ M/ml (Figs. 2 and 3) did not show cytotoxic effects on cells at the end of the 24<sup>th</sup> and 48<sup>th</sup> hours.

Didymin in the range of 100  $\mu$ M/ml showed a significant decrease in cell viability at the end of 72 hours as compared to the control group (80.7%, \*\*,  $p < 0.01$ ) in a dose-dependent manner at the end of the 72<sup>nd</sup> hour. There was no significant change in the other doses administered as compared to the control. The solvent control was applied as the concentration of the highest didymin concentration applied as the solvent control, and a significant decrease in cell viability was observed (83.1% \*\*,  $p < 0.01$ ) (Figs. 6, 7).



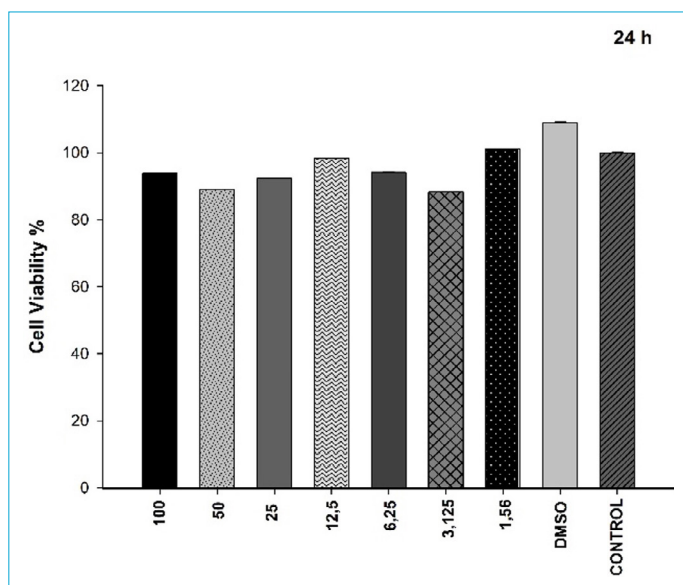
**Figure 2.** The effect of tested doses of didymin in the range of 100-1,56  $\mu$ M/ml on cell viability in the U-87 – MG cell line at the end of the 24-hour incubation period (TO: Time Zero, DMSO: Dimethyl sulfoxide).



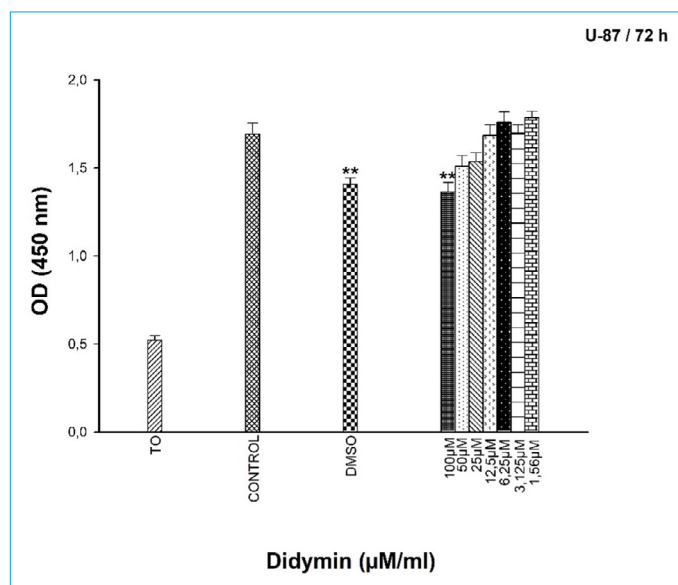
**Figure 3.** The effect of tested doses of didymin in the range of 100-1,56  $\mu$ M/ml on cell viability in the U-87 – MG cell line at the end of the 48 hour incubation period (TO: Time Zero, DMSO: Dimethyl sulfoxide).

### The Effect of Didymin on the Level of Substance P Released from Cells to the Medium

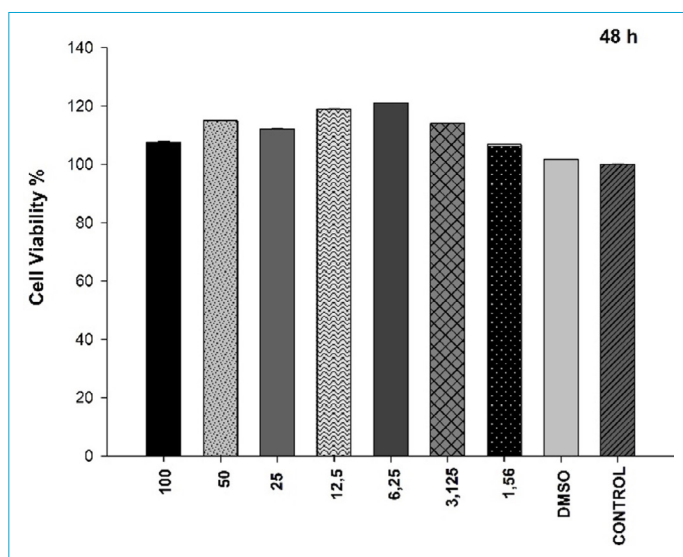
Didymin at doses of 100  $\mu$ M/ml and 50  $\mu$ M/ml, caused a significant increase in the level of SP released from the cells to the medium after the 24-hour and 48-hour incubation



**Figure 4.** % Cell viability of the tested doses of didymin in the range of 100-1,56 μM/ml on cells at the end of the 24-hour incubation period (T0: Time Zero, DMSO: Dimethyl sulfoxide).



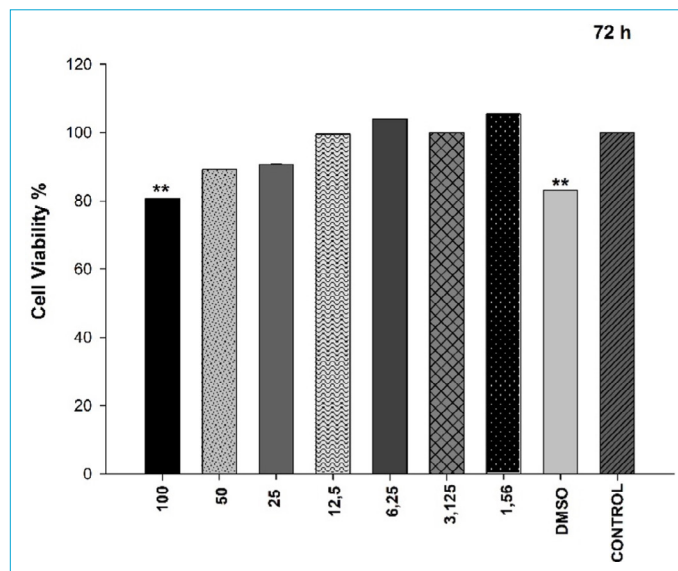
**Figure 6.** The effect of didymin on cell viability in the U-87 – MG cell line after 72 hours of incubation (\*\*,  $p < 0.01$ ) (T0: Time Zero, DMSO: Dimethyl sulfoxide).



**Figure 5.** % Cell viability of the tested doses of didymin in the range of 100-1,56 μM/ml on cell viability at the end of the 48 hour incubation period (T0: Time Zero, DMSO: Dimethyl sulfoxide).

periods as compared to the control (\*\*,  $p < 0.01$ , \*,  $p < 0.05$ ). Similarly, the solvent control DMSO caused an increase in the level of SP released into the medium at the end of the 24<sup>th</sup> hour compared to the control (\*,  $p < 0.05$ ) (Figs. 8, 9).

Didymin at doses of 100 μM/ml and 50 μM/ml, caused a statistically significant decrease in the level of SP released from the cells to the medium after 72 hours of incubation period as compared to the control (\*\*,  $p < 0.01$ ). Similarly, in the solvent control, DMSO decreased the level of SP as compared to the control (\*\*,  $p < 0.01$ ) (Fig. 10).

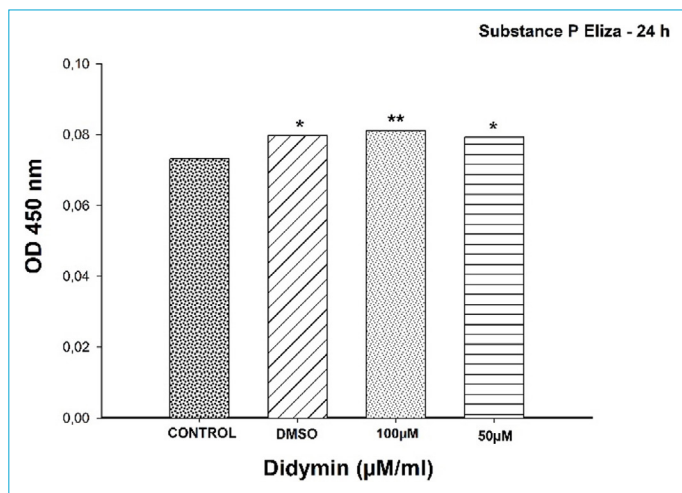


**Figure 7.** Percentage display of the effect of didymin doses in the range of 100-1,56 μM/ml on cell viability in the U-87 – MG cell line at the end of the 72 hour incubation period (\*\*,  $p < 0.01$ ) (T0: Onset time, DMSO: Dimethyl sulfoxide).

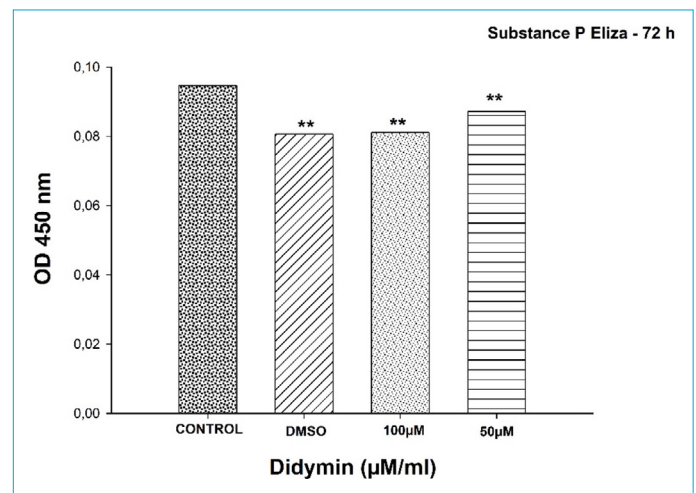
### The Effect of Didymin on the Level of Interleukin-6 Released from Cells to the Medium

Didymin significantly increased the level of IL-6 released at a dose of 100 μM/ml at the end of the 24<sup>th</sup> hour. It caused a significant increase in each dose at the 48<sup>th</sup> hour, while a significant increase was observed only at the 50 μM/ml dose at the 72<sup>nd</sup> hour.

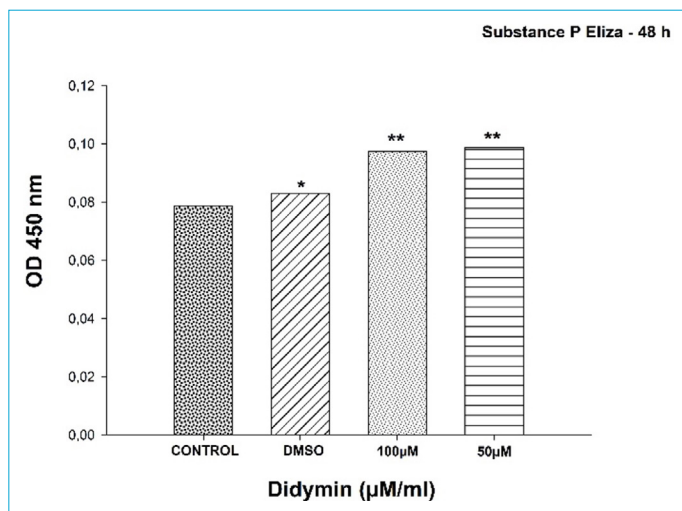




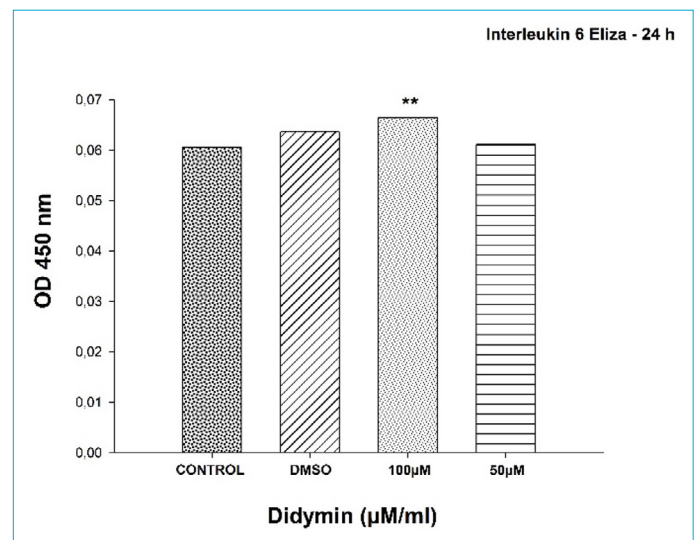
**Figure 8.** After the treatment of didymin at doses of 100 µM/ml and 50 µM/ml, the level of Substance P released from the cells to the medium at the end of the 24-hour incubation period was statically significant (\*\*,  $p < 0.01$ , \*,  $p < 0.05$ ) (T0: Time Zero, DMSO: Dimethyl sulfoxide).



**Figure 10.** After the administration of Didymin at doses of 100 µM/ml and 50 µM/ml, the level of Substance P released from the cells to the medium decreased at the end of the 72 hour incubation period (\*\*,  $p < 0.01$ ) (T0: Time zero, DMSO: Dimethyl sulfoxide).



**Figure 9.** After the treatment of didymin at doses of 100 µM/ml and 50 µM/ml, the level of Substance P released from the cells to the medium at the end of the 48-hour incubation was statically significant (\*\*,  $p < 0.01$ , \*,  $p < 0.05$ ) (T0: Time Zero, DMSO: Dimethyl sulfoxide).



**Figure 11.** The level of Interleukin 6 released from the cells (\*\*,  $p < 0.01$ ) (T0: Time zero, DMSO: Dimethyl sulfoxide) at the end of the 24-hour incubation period following the administration of Didymin at doses of 100 µM/ml caused a significant increase.

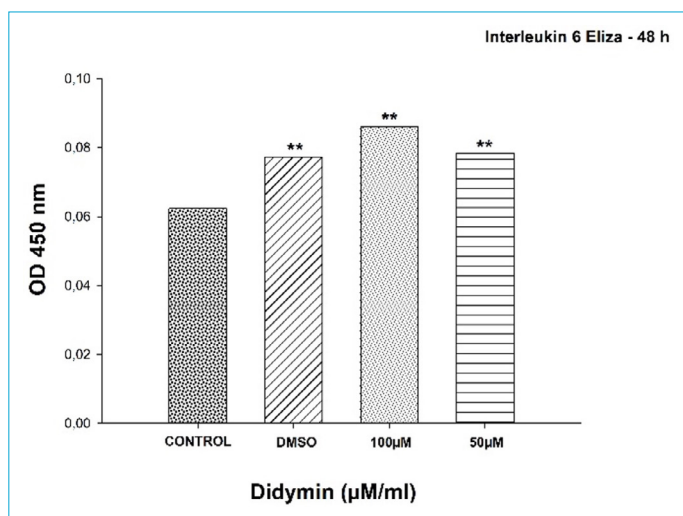
## Discussion

Cancer is an important public health problem that causes the most deaths in the world after heart diseases.<sup>[10]</sup> Detailed molecular analyzes of glioblastoma, a highly invasive and heterogeneous type of malignant brain tumor; reveals cell growth and dysregulation of signaling pathways such as DNA, RTK, PI3K, p53, and MAPK.<sup>[5]</sup>

Epidemiological studies have provided evidence that a high dietary intake of flavonoids with fruits and vegetables may be associated with a lower cancer prevalence in humans.<sup>[11]</sup> Especially naringenin, tangeretin, and hesperidin,

which are found in citrus fruits, have been the subject of much research and their effects on various types of cancer have been tried to be clarified. In a study conducted in 2019, it was discovered that hesperidin could be a natural therapeutic agent for GBM treatment and induce GBM cell apoptosis through p38 MAPK activation.<sup>[12]</sup>

Flavonoids taken naturally from the diet have been defined as more tolerable and less toxic than chemotherapeutic agents taken during the treatment of diseases. Studies have shown that co-administration of natural products with chemotherapeutics increases anti-tumor efficacy. The

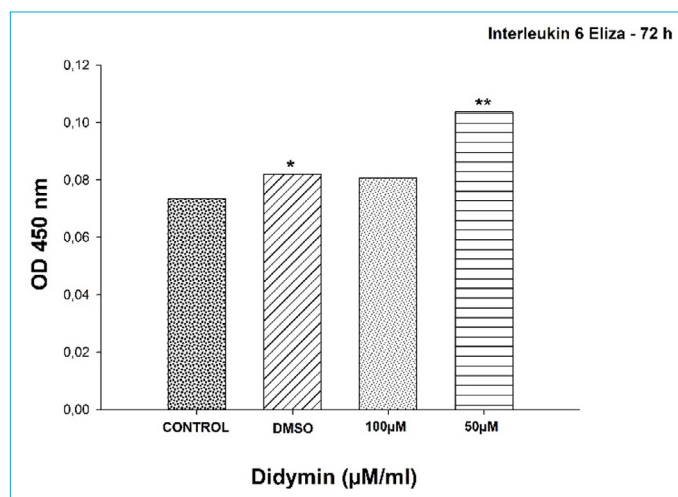


**Figure 12.** At the end of the 48 hour incubation period, Didymin and DMSO also caused a significant increase in the level of Interleukin 6 (\*\*,  $p < 0.01$ ) (T0: Time zero, DMSO: Dimethyl sulfoxide).

first study investigating the anticancer properties of didymin flavonoid was carried out by Hung et al. in 2010, and the mechanisms of action in human non-small cell lung cancer A549 and H460 cells were tried to be elucidated by in vitro and in vivo methods. The antiproliferative activity of didymin was determined in these cell lines, and the levels of p53, p21/WAF1, Fas/APO-1 receptor, and Fas ligand (FasL), which are strongly associated with signal transduction of apoptosis, were detected. It has been confirmed that Didymin dose-dependently inhibits the proliferation of cancer cells and the primary mechanism of apoptosis is apoptosis via the Fas/Fas ligand pathway. In the same study, Hung et al. showed in vivo that didymin significantly suppressed tumor growth in tumor-bearing mice without detectable side effects.<sup>[11]</sup>

In another study, Hsu et al. revealed that didymin can reverse phthalate ester-mediated breast cancer aggravation and suggested that it can be used as a safe and effective method against the negative effects of environmental toxins on cancer cells.<sup>[13]</sup>

Neuroblastoma (NB) is a solid pediatric tumor of the nervous system originating from neural progenitor cells.<sup>[14]</sup> As in many cancer and disease types, the activities of natural compounds have been investigated in the treatment of neuroblastoma. In a study investigating the effect of didymin on the survival and clonogenic potential of both p53 wild-type and p53 mutant neuroblastomas, the cytotoxic effects of three different flavonoids, including didymin, were examined in four different neuroblastoma cell lines, and it was revealed that it had the strongest effect compared to other flavonoids. Didymin had an effect of up to 75% in cell death of neuroblastomas compared to other



**Figure 13.** After the administration of didymin at doses of 100 µM/ml and 50 µM/ml, the level of Interleukin 6 released from the cells to the medium at the end of the 72 hour incubation period was increased (\*\*,  $p < 0.01$ , \*,  $p < 0.05$ ) (T0: Time zero, DMSO: Dimethyl sulfoxide).

flavonoids hesperidin and 2'-hydroxyflavanone (2HF), its effects on apoptosis were examined by the TUNEL method and it was shown that it induced increased cell death by fluorescent radiation. Didymin also potently inhibited the expression of proto-oncogenes N-Myc, as well as markers of proliferation (Ki67) and angiogenesis (CD31). In the in vivo part of the same study, mice were treated with didymin, with significant reductions in the number of tumors compared to the control group, while uncontrolled growth was observed in the control group. Histopathological examination of tumor xenograft sections revealed decreased expression of Ki67, CD31, and N-Myc in neuroblastomas; data supported the in vitro results of the study.<sup>[15]</sup>

In addition to its anti-cancer activities, the role of didymin in angiogenesis was tried to be clarified in 2018 by Shukla et al. Human umbilical vein endothelial cells (HUVEC) were induced and treated with didymin with vascular endothelial growth factor (VEGF) in in vitro and in vivo models, respectively. Didymin inhibited VEGF-induced cell proliferation and invasion in these cells, as well as ROS formation and activation of NF-κB. In addition, it showed antiangiogenic effects on the angiogenesis model in mice by preventing vessel formation and invasion of endothelial cells.<sup>[16]</sup>

In this study, the anticancer activity of the citrus flavonoid didymin, whose anticancer properties have not been investigated in glioblastoma cells before, was evaluated in U-87 Glioblastoma cells, and its effect on the level of IL-6 and SP was investigated. In our study, didymin flavonoid obtained from the Chinese company AktinLab was administered in the range of 100-1,56 µM/ml, and a significant decrease in cell viability was detected at a dose of 100 µM/ml at 72

hours incubation period as compared to the control. It was observed that the cell viability decreased at the end of the 72-hour depending on a dose depended manner. Similarly, the solvent was applied at the highest dose (100  $\mu\text{M}/\text{ml}$ ) of didymine which includes the highest concentration of DMSO, used as a control, and a cytotoxic effect was observed (\*\*,  $p < 0.01$ ). By the same, the cytotoxic effect observed in the experiments is due to the cytotoxic effects of DMSO itself. Hung et al., showed that the 20  $\mu\text{M}$  dose of didymine treatment in lung cancer A549 and H460 cells inhibited the proliferation of cells by 76.5% and 79.5%, while in another study, Singhal et al., showed that the 50 mmol/L doses of didymine in neuroblastoma cells reduced cell viability by 75%.<sup>[11,15]</sup>

In our study, while more cell death was expected at the doses we applied, 80.7 % cell viability was detected only at the highest dose. This struggle result might be related to the product itself. We speculate that the active substance does not show the expected effect due to a problem in the production process of the company. To clarify this situation, the experiments can be repeated in the same cell line with didymine could be obtained from different companies.

Substance P is the most important neuropeptide in cancer involved in neurogenic inflammation.<sup>[17]</sup> SP plays an important role in tumor cell proliferation, angiogenesis, tumor cell invasion, and metastasis.<sup>[9]</sup> Expression of SP is increased in GBM compared to normal cells.<sup>[18]</sup> In our study, the effect of didymine flavonoid on the level of SP released from glioblastoma cells into the medium was investigated, it increased by a 100  $\mu\text{M}/\text{ml}$  and 50  $\mu\text{M}/\text{ml}$  dose didymine treatments at the end of 24 and 48 hours of incubation periods and the levels were decreased significantly at the end of 72 hours. The solvent control DMSO reduced SP release at 72 hours. No study has been found in the literature on the effects of flavonoids on the level of SP released from cancer cells. It has been shown in studies that SP induces the expression of proinflammatory cytokines and chemokines such as IL-6 and IL-8 which contributed to our hypothesis.<sup>[19,20]</sup>

Increased expression of the IL-6 cytokine is observed in glioblastoma and directly affects the survival of the patient.<sup>[8]</sup> In the study of Kim et al., quercetin reduced IL-6 release and STAT3 phosphorylation in GBM cells.<sup>[21]</sup> Another study examined the effects of quercetin on the IL-6-induced STAT3 signaling pathway, resulting in a marked reduction in the proliferative and migration properties of glioblastoma cells.<sup>[22]</sup> In another study conducted in 2012, the effects of vetulin flavonoid obtained from açai fruit in reducing the production of lipopolysaccharide-induced proinflammatory cytokine tumor necrosis factor-alpha (TNF- $\alpha$ ) and IL-6

in peritoneal macrophages and mouse peritoneal macrophages were shown.<sup>[23]</sup>

In our study, at the end of the 24<sup>th</sup> hour, a significant IL-6 release was observed at a dose of 100  $\mu\text{M}/\text{ml}$  applied to cancer cells compared to the control, a significant release at each dose at the 48<sup>th</sup> hour, and a significant release at only 50  $\mu\text{M}/\text{ml}$  dose at the 72<sup>nd</sup> hour. Although this is the first study in the literature on Didymine and IL-6 release; In our study, it was observed that didymine significantly increased IL-6 releases with almost all doses at each incubation period. It was observed that the level of SP increased in the 24<sup>th</sup> and 48<sup>th</sup> hour incubation period and decreased at the end of the 72<sup>nd</sup> hour. The fact that didymine administration to glioblastoma cells causes an increase in two important cytokines in the inflammation and pain pathway reduces the possibility of this flavonoid being an effective therapeutic in the treatment of this type of cancer. Since there are not many studies in the literature investigating the effects of didymine flavonoid on SP and IL-6 levels, further studies should be conducted on this subject and the effect of didymine should be clarified.

## Conclusion

The data obtained in the study show that more studies are needed to be sure about the cytotoxic effect of didymine on glioblastoma cells. The cytotoxic effect of didymine was detected in only a single dose and the fact that the concentration of solvent contained in that dose was the primer reason for these cytotoxic results. Didymine increased the level of inflammation marker IL-6 in a time and dose-dependent manner and decreased the level of pain marker SP at the end of the 72 hours. This is the first study, which investigated the effects of didymine on SP and IL-6 released from U-87 MG cells. We suggest being careful about the usage of didymine on Glioblastoma.

## Disclosures

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**Ethics Committee Approval:** The study was approved by the Local Ethics Committee.

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

**Authorship Contributions:** Concept – N.B., E.Ş.; Design – N.B., E.Ş.; Supervision – N.B., E.Ş.; Materials – N.B., E.Ş.; Data collection &/or processing – N.B., E.Ş.; Analysis and/or interpretation – N.B., E.Ş.; Literature search – N.B., E.Ş.; Writing – N.B., E.Ş.; Critical review – N.B., E.Ş.

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