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Original article

In vitro Anticancer, Antibacterial and Antifungal Activity Analysis of Natural Flavonoid Hesperidin

Berfu Çerçi Öngün[1,2], Ergül Mutlu Altundağ[3], Gülcem Altınoğlu[4], Mümtaz Güran[5], Gizem Şanlıtürk[5,6], Masoud Afshani[7], Deniz Balcı[8]

[1]Department of Anatomy, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [2]Department of Anatomy, Faculty of Medicine, Ankara University, Çankaya, Ankara, Turkey; [3]Department of Biochemistry, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [4]Department of Neuroscience, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [5]Department of Medical Microbiology, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [6]Department of Chemistry, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [7]Department of Histology and Embryology, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey; [8]Department of Biological Sciences, Faculty of Arts and Sciences, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey

Abstract. Hesperidin, a plant flavonoid mainly found in citrus fruits, has been shown to have important biological activities, while its effects on glioblastoma cells and against pathogen microorganisms are largely unknown. In this study, the cell viability effect of hesperidin on human glioblastoma cells and the antibacterial and antifungal properties against a comprehensive set of microorganisms in vitro were explored. To assess the anti-proliferative effect of hesperidin on human glioblastoma cells at different time points, glioblastoma cells were treated with hesperidin at different concentrations. Cell viability was evaluated by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and cell nuclear morphology of hesperidin treated glioblastoma cells was studied using 4, 6-diamidino-2-phenylindole staining. Hesperidin's antimicrobial activity was screened with standard broth microdilution assays for antibacterial and antifungal activity screening. Antibacterial activity was further tested by Antibacterial well diffusion assay. Hesperidin significantly inhibited the cell viability in a concentration and time-dependent manner, where the point of decrease in cell viability has been found at 150 and 200 μM of hesperidin treatment for 48 and 72 h. Staining showed the induction of apoptosis in glioblastoma cells following hesperidin treatment for 72 h at increased concentrations. The antimicrobial activity tests for hesperidin revealed no significant antimicrobial activity including antibacterial and antifungal assays. It can be concluded that hesperidin possesses anti-cancer properties in glioblastoma cells in vitro resulting in reduced cell viability, proliferation and increased apoptosis, and may be effective against brain gliomas, with no significant antimicrobial activity.

Key words: Hesperidin, Anticancer, Anti-apoptosis, Antimicrobial, Antifungal

Introduction

Flavonoids, a group of natural polyphenolic compounds usually found in many plants and fruits, are known to have various bioactive properties, including anti-inflammatory, anti-oxidant, anti-viral, anti-diabetic and anti-cancer properties (1). To this end, they have received great interest with many studies supporting their diverse bioactivity (2, 3, 4). It has been illustrated that regular consumption of flavonoid-rich diets is associated with a lower risk of chronic diseases, including cancer (5).

Hesperidin (3, 5, 7-trihydroxy-4– methoxy-avanone-7-rhamnoglucoside), a member of the flavonoid family mainly found in citrus fruits, has also been shown to possess roles against inflammation, oxidative stress, hyperglycemia and cancer (6, 7). The anticancer properties of hesperidin, and flavonoids in general, have mainly been accredited to their antioxidant nature as well as their ability to inhibit angiogenesis and modulation of cell proliferation (8, 9, 10). Additionally, it has been illustrated to have high permeability for the blood brain barrier (BBB) (11), however, little is known about how hesperidin affects human glioblastoma multiforme (GBM) cells, which is the most aggressive and commonly occurring glioma of the central nervous system (CNS), accounting for more than 60% of all brain tumors.

Hesperidin is also studied for its antimicrobial activities; however, the studies did not report any significant effect in terms of killing of some pathogenic microorganisms. Despite that, comprehensive antimicrobial analysis of hesperidin is not performed yet. Therefore, there is a need for a comprehensive testing of this flavonoid against standard microorganisms to clarify the antimicrobial effects of hesperidin. Furthermore, hesperidin`s killing activity against microorganisms other than bacteria is lacking.

In this study, we have explored the cell viability effect of hesperidin on human GBM cells and also we have performed antimicrobial and antifungal assays against a comprehensive set of microorganisms *in vitro*.

Materials and Methods

Reagents

Hesperidin (≥80%, CAS no: 520-26-3) was purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). The stock solution of Hesperidin (200 mM) was dissolved in dimethyl sulfoxide (DMSO) where the DMSO concentration was kept below 0.1%.

Cell Culture

Cell culture experiment was performed on human GBM cell line DBTRG-05MG (ATCC® CRL-2020™) obtained from ATCC. Cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640) (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco™ ThermoFisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco™ ThermoFisher Scientific, Waltham, MA, USA) at 37 °C in humidified air with 5% CO_2. Cells were then detached using 0.25% of Trypsin-EDTA solution (Gibco™ ThermoFisher Scientific, Waltham, MA, USA) after reaching confluence.

Cell Viability Assay

Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St Louis, MO, USA). The cells were seeded in 94-well culture plates at a density of 7 x 10^4 cells per well, and treated with hesperidin at different concentrations and time points. After incubation for 24, 48 or 72 h, 10 µL of MTT solution (0.5 mg/mL) was added to each well. Next, 100 mL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) was added into the culture medium, following a 4 h of incubation period at 37°C. It is also important to note that as a positive control, a major chemotherapeutic agent, 5-Fluorouracil (5-FU) (Sigma-Aldrich, St Louis, MO, USA) was used. The cell viability assay was then determined by measuring the spectrophotometrical absorbance at 595 nm with a Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). The percentage of viable cells was calculated by using the following equation;

$$\%\text{Viable cells} = (\text{Abss}_{\text{ample}} - \text{Abs}_{\text{blank}}) / (\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) * 100$$

To calculate the concentration of extract required for 50% inhibition of cell viability, the mean of triplicate experiments for each dose was employed.

DAPI Staining

Cell nuclear morphology of hesperidin treated GBM cells was studied using DAPI, or 4,6-diamidino2-phenylindole staining. The GBM cells treated with hesperidin at indicated concentrations were washed with

PBS (pH 7.4), fixed with ice cold 70% ethanol and resuspended in DAPI (Sigma-Aldrich, St Louis, MO, USA), followed by incubation at 37°C for 15 min and washing with phosphate-buffered saline solution (PBS). After staining, the cells were mounted on a glass slide and examined under fluorescence microscopy (x40).

Antibacterial Activity Screening

Broth microdilution assay for antibacterial Minimum Inhibitory Concentration (MIC) determination. Antimicrobial activity of hesperidin was tested by conducting Broth Micro dilution assay. The antibacterial activity of hesperidin which dissolved in 0.1%DMSO was tested quantitatively against six standard bacteria. A panel of selected standard bacterial strains including gram-positive (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 11706, *Escherichia coli* ATCC 25922) were utilized to evaluate the antibacterial activity of hesperidin.

Firstly, the agent was distributed to the wells by making serial dilutions in 96-well plates. Bacterial strains were normally kept at -80 °C and cultured in the blood agar two days before antimicrobial testing. After over-night incubation at 37 °C, the turbidity of each bacterial suspension was set at 0.5 MacFarland standard in the saline solution (corresponds to 1×10^7CFU/mL). Then, final bacterial concentration was adjusted to 1×10^5CFU/mL.

The samples were tested by making 2-fold serial dilutions separately. Subsequently, from each bacterial suspension 50μL was added into every well plate except for the control well. DMSO was tested as a negative control. Finally, the plates were incubated under aerobic conditions at 37 °C for 24 hours. The MICs were reported by determining the last invisible growth of the serial dilution in the 96-well plate.

Well Diffusion Assay. Antibacterial activity of hesperidin was also assessed using well diffusion assay on MHA. Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 were used ac bacterial strains to represent gram-positive and gram-negative bacteria. Preparing the bacterial suspension was done by adjusting the turbidity of the solution as 0.5 McFarland. After the inoculation of bacterial strains on MHA agar plates, 50 μl of the hesperidin solution which dissolved in 0.1%DMSO poured into the wells (diameter = 6mm) and incubated at 37°C. After 24 hours, the growth inhibition zones were determined in diameter. The zone of inhibition was given in millimeters (mm).

Antifungal Activity Screening

Broth microdilution assay for antifungal MIC determination. The MICs for *Candida albicans* ATCC 10231 were determined using the reference procedure of the Antifungal Susceptibility Testing of CLSI M27-A2 for the testing of yeasts as described in the relevant document (12). Quality control procedures were performed before the implementation of the testing protocols. Sterility and growth controls were used in the same testing line with hesperidin. Testing was performed in sterile 96-well microtiter plates with RPMI 1640 medium with L-glutamine, without sodium bicarbonate (NaHCO3, RPMI 1640; Gibco, Carlsbad, CA, USA) supplemented with 2% glucose, buffered to pH 7.0 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) medium. *C. albicans* strains were pre-cultured on RPMI 1640 Broth medium. Turbidity of fungi was prepared according to McFarland 0.5 scale to obtain a standard inoculum. Hesperidin was tested at dilutions starting from 5000 μg/mL concentrations. Dilutions were made with sterile distilled water. Amphotericin B was used as reference drug for *C. albicans* strain. Tests were performed under aseptic in vitro conditions. The lowest concentration that prevents the growth of fungi was determined as MIC.

Results

Hesperidin Reduced Cell Viability

Hesperidin is thought to act as an anti-cancer agent owing to its anti-proliferative actions in multiple cancer cell types (16). To assess the anti-proliferative effect of hesperidin on human GBM cells at 24 h, 48h and 72 h, GBM cells were treated with hesperidin at different concentrations (20, 40, 60, 80, 100, 150 and 200 μM). Chemotherapeutic agent, 5-FU (10 μM), was used as

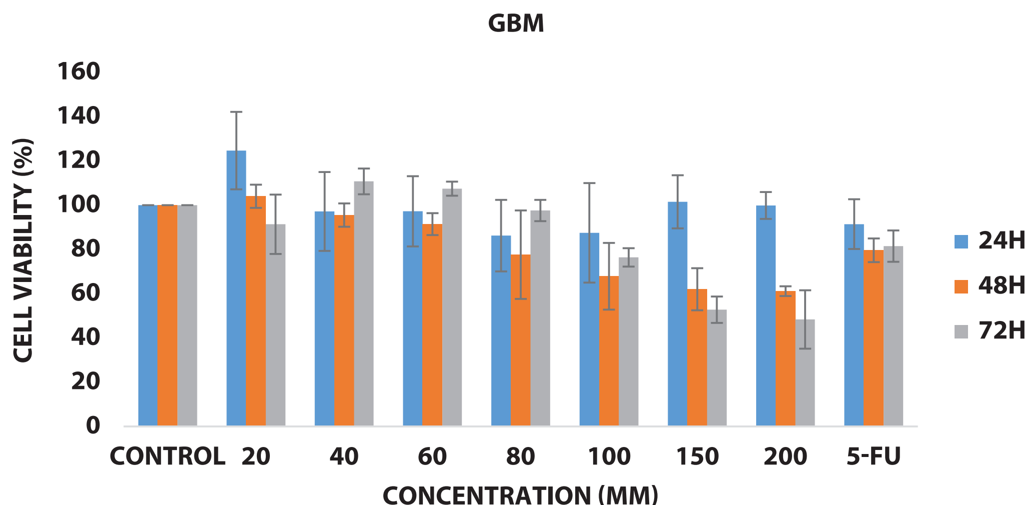

Figure 1. Hesperidin reduced GBM cell viability. GBM cells were treated with hesperidin at different concentrations (20, 40, 60, 80, 100, 150 and 200 µM) and were assessed at different time points (24, 48 and 72 h). Chemotherapeutic agent, 5-FU, was used as the positive control. Cell viability was measured by MTT assay. Cell viabilities were significantly reduced at 48 and 72 h with 150 and 200 µM hesperidin.

the positive control. The cell viability was significantly reduced in a concentration and time-dependent manner, as shown in Figure 1. Cell viability of hesperidin treatment has been found at 150 and 200 µM for 48 and 72 h. Percentage cell viability following 150 µM of hesperidin treatment has been found as 62% and 52%, while for 200 µM of hesperidin it was 61% and 48%, at 48h and 72 h, respectively. Hesperidin hasn't been found effective at the same concentrations at 24 h.

DAPI Staining

DAPI staining, used to study the cell nuclear morphology of hesperidin treated GBM cells, revealed the changes associated with apoptosis in GBM cells treated with hesperidin as shown in Figure 2. DAPI staining showed the induction of apoptosis in GBM cells at increased concentrations. Nucleus fragmentation and nuclear condensation occurred following hesperidin-treatment for 72 h at 100, 150 and 200 µM concentrations.

Antimicrobial Activity assays

Hesperidin`s antimicrobial activity was screened with antibacterial and antifungal assays. MICs for antibacterial activity after performing antibacterial broth microdilution assay were found to be above 5mg/ml for all tested bacterial strains meaning no significant antibacterial activity. Activity of ciprofloxacin which is a potent antibiotic molecule was found to be between 0.0038µg/ml-0.98µg/ml showing the control function for the assays. Furthermore, another antibacterial assay which is antibacterial well-diffusion assay approved the results for MIC testing as there were no growth inhibition zones around hesperidin-containing wells. For the ciprofloxacin controls 30mm and 35mm zones were measured.

Antifungal MICs were tested by antifungal broth micro dilution assay against the pathogen *C. albicans*. However, the tests resulted in no antifungal activity as similar to antibacterial assays. The MIC of antifungal control Amphotericin B was found to be 0.05mg/ml (Table 1).

Discussion

Natural products such as hesperidin have been shown to have a good safety profile in addition to their effectiveness in biological activities. Hesperidin has been shown to have several pharmacological activities such as anti-atherogenic, antihyperlipidemic, antidiabetic, anti-inflammatory, antihypertensive actions previously and now it is attributed to have a role in

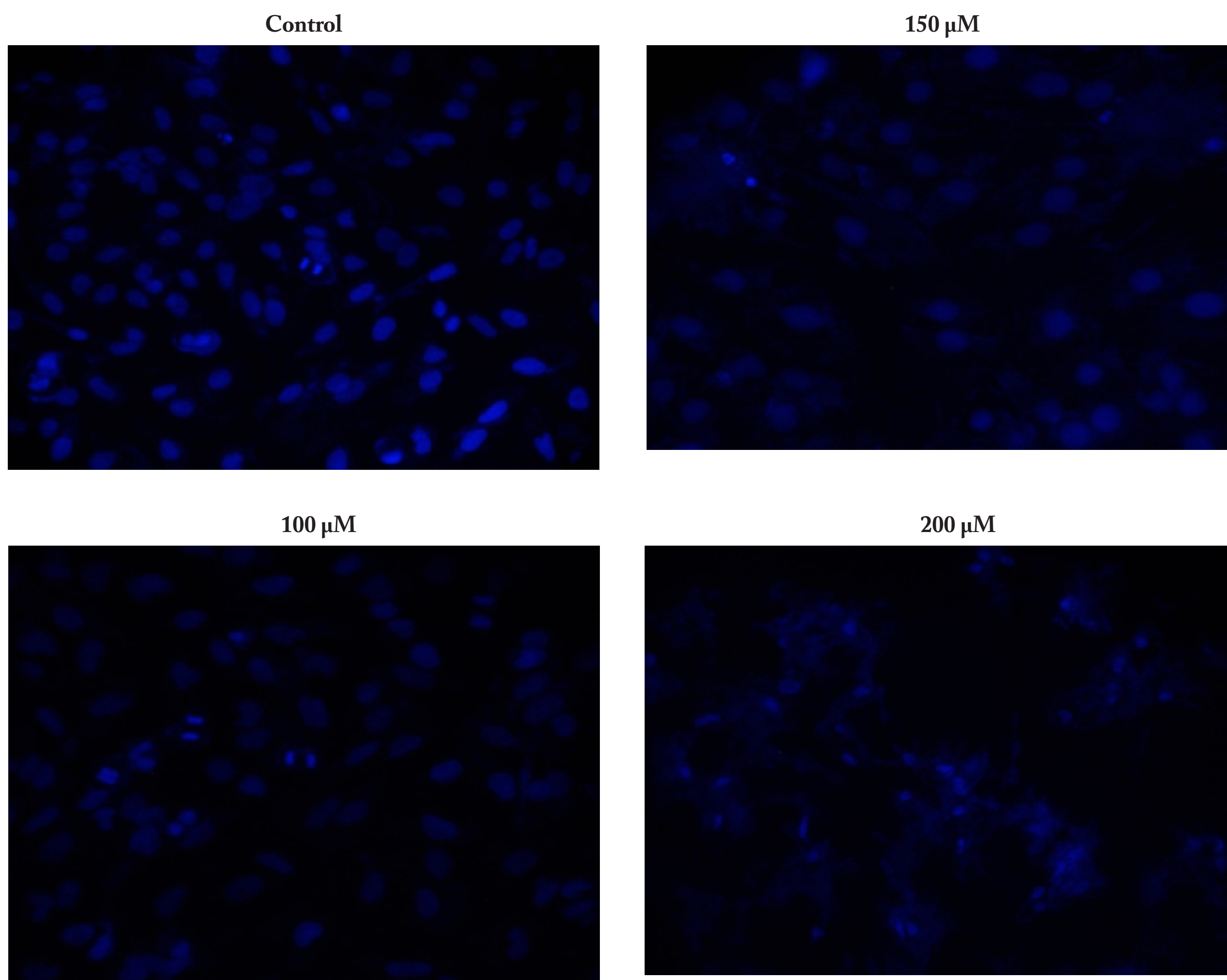

Figure 2. DAPI staining showing the induction of apoptosis in GBM cells at increased concentrations. Apoptotic cells were determined by DAPI staining and were imaged by fluorescence microscopy (x40).

Table 1. Results of antimicrobial activity assays

Name of the antimicrobial test	Name of the tested strain	MIC/Inhibition of growth zone	Control Group Ciprofloxacin	Control Group Amphotericin B
Antibacterial susceptibility – Broth micro dilution (MIC)	*S. aureus*	>5mg/ml	0.98μg/ml	-
	P. aeruginosa	>5mg/ml	0.03μg/ml	-
	E. coli	>5mg/ml	0.0038μg/ml	-
	K. pneumonia	>5mg/ml	0.0038μg/ml	-
	E. faecalis	>5mg/ml	0.0038μg/ml	-
Antibacterial well diffusion (zone of inhibition)	*S. aureus*	0	30mm	-
	E. coli	0	35mm	-
Antifungal susceptibility - Broth micro dilution (MIC)	*C. albicans*	>1.6mg/ml	-	0.05mg/ml

the treatment of Covid-19 as well (13). In the present study, we have analysed the cell viability effect of hesperidin on human GBM cells and also we have performed antimicrobial and antifungal assays against a comprehensive set of microorganisms *in vitro*.

Despite the regular treatment options including resection surgery, radiotherapy and chemotherapy, GBM often recurs (14). To this end, novel approaches are required to enhance the prognosis of GBM patients. Mounting research reveals that natural agents could open up a lot of possibilities for the development of new cancer treatment approaches (15, 16). Among these, hesperidin, a natural flavonoid mainly found in citrus fruits, has gained attention owing to its anti-cancerous effects. In a previous study hesperidin has been found to have an antitumor activity on brain glioblastoma cells (17). In the current study, MTT assay was performed to estimate the cell viability of cells. The result of the assay revealed that hesperidin treatment caused a significant inhibition of GBM cell viability in a time and dose-dependent manner, with greater decreases in cell viability when treated with 150 and 200 μM hesperidin on 48 and 72 h incubation period. This indicates the anti-proliferative effect of hesperidin on GBM cells and proved the inhibitory effect of hesperidin treatment on GBM cell viability.

Apoptosis is a physiological process of programmed cell death, and its evasion is considered as a pathological hallmark of cancer. Therefore, targeting apoptosis by re-establishing the defective apoptotic process is an effective approach in cancer treatment (18). To explore the effect of hesperidin treatment on the apoptotic process of GBM cells, DAPI staining assay was employed. DAPI is a fluorescent nucleic acid dye used to preferentially stain double-stranded DNA. It is used to study nuclear alterations and apoptotic body formation, both of which are involved with apoptosis (19). Nucleus fragmentation and nuclear condensation occurred following hesperidin treatment for 72 h at 100, 150 and 200 μM concentrations, validating hesperidin induced cell death via the apoptotic pathway in GBM cells, and its anti-proliferative actions.

Recent studies have explored the anti-cancer effects of hesperidin on glioma cells both *in vivo* and *in vitro*. Zhang and colleagues investigated the effect of hesperidin treatment on cerebrally implanted C6 glioma cells in rats and the proliferation of glioma cells was investigated. Their results showed prolonged survival of rats following hesperidin treatment, due to hesperidin decreasing cell proliferation, increasing apoptosis and causing cell cycle arrest. Investigation of tumor permeability also revealed a significant decrease in tumor permeability and edema (20). Additionally, another study that has explored the effects of hesperidin on GBM cells, along with the underlying signaling mechanisms *in vitro*, showed a marked inhibitory effect on cell viability upon hesperidin treatment and demonstrated the role of apoptotic death in the reduction of cell viability (21). Their findings indicated that hesperidin inhibited GBM cell proliferation by the induction of apoptosis via cell-cycle arrest, in accordance with many studies in the literature (22). Hesperidin has also previously been reported to induce cell growth arrest at G0/G1 or G2/M phase as an antiproliferative agent, where the transition from S phase into G2/M phase is considered as one of the cell cycle check-points (23, 24). In the previous study, hesperidin has been discovered to cause cell-cycle arrest in the G2/M phase in U-251 GBM cell line.

Hesperidin has not only shown effectiveness via apoptosis induction and cell cycle arrest in GBM cells, but also in other cell lines as well. In breast cancer cell line, MCF-7, it has been found to induce various apoptotic processes including DNA fragmentation, caspase-7 activation, and phosphatidyl-serine externalization, in addition to the accumulation of reactive oxygen species (ROS) and the activation of apoptosis signal-regulating kinase 1/ Jun N-terminal kinase (ASK1/JNK) pathway (25). Among these, the formation and accumulation of ROS are considered as the most important mechanism of hesperidin's apoptotic action. According to Zhang and colleagues, activation of mitochondrial pathway and high ROS levels together with calcium and adenosine triphosphate (ATP), are responsible for hesperidin- induced apoptosis in hepatocellular carcinoma cells (26). Evidence also showed that hesperidin caused caspase-dependent apoptosis in non-small cell lung cancer cell lines (A549 and NCI-H358) (27, 28).

In the present study, we have also analyzed the antimicrobial activities of hesperidin. Despite there are some studies available about antimicrobial activity of hesperidin, comprehensive antimicrobial analysis is lacking.

The antimicrobial assays of the current study resulted with no antimicrobial activity including antibacterial and antifungal assays. A previous study which investigated structure-activity relationship of polyphenols including hesperidin concluded that, hesperidin did not have antibacterial activity on gram-negative as well as gram-positive bacteria (29). This is explained by the chemical structure of hesperidin which does not contain hydroxyl group substituent on C7 position (29). Another polyphenol, quercetin for example, having a hydroxyl group at the mentioned position possesses a good antibacterial activity (30). Notably, Iranshahi et. al. reports data regarding significant antimicrobial action of hesperidin (31). In this respect, the contrary results are probably related to different methodologies used in mentioned studies and/or purity of tested compounds. Another study that focused on the antifungal activity of hesperidin and its derivatives did not report any antifungal activity similar to our study (32). Taken together, it can be concluded that, understanding the chemical structure and activity relationship of hesperidin may lead synthesis of hesperidin derivatives with antibacterial activity. This may provide multifunctional usage of hesperidin together with its antioxidant properties. Also, it was previously shown that some natural flavonoids may act synergistically or may improve each other`s biological activities when combined with other natural compounds and/or pharmacological agents which can be another alternative application to benefit from hesperidin (33, 34).

There are a few limitations to note for the current study as; (i) Hesperidin was low purity (≥80%), (ii) Lack of various clinical strains for the antimicrobial assays worth addressing for future research, and (iii) assays for other biological mechanisms such as toxicity or in-vivo experiments were not studied in the current study which limits further discussions. It is also important to note for hesperidin to exert its neuroprotective effects; it should be able to cross the blood brain barrier. However, the blood brain barrier is known to limit the passage of hesperidin into the central nervous system, leading to its poor bioavailability (35). Therefore, increasing hesperidin bioavailability with various methods, e.g. the use of nano-mediated delivery systems, is proposed.

Conclusion

In this study, we have analysed various biological activities of hesperidin. Our findings support the antitumor properties of hesperidin and its potential to function as a therapeutic agent, as hesperidin treatment not only resulted in reduced cell proliferation on GBM cells *in vitro*, but also increased apoptosis. The antimicrobial assays of the current study, on the other hand, revealed no antimicrobial activity of hesperidin. Therefore, our observations indicate the potential use of hesperidin as a possible candidate for the treatment of GBM.

Further in vivo research focusing on proven biological action of hesperidin such as anticancer, antioxidant properties may provide further evidence for the future beneficial therapeutic applications of hesperidin.

References

1. Wang TY, Li Q, Bi KS. Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. Asian J Pharm Sci 2018; 13(1): 12-23.
2. Krych J, Gebicka L. Catalase is inhibited by flavonoids. Int J Biol 2013; 58: 148-53.
3. Ragab FA, Yahya TAA, El-Naa MM, Arafa RK. Design, synthesis and structure–activity relationship of novel semi-synthetic flavonoids as antiproliferative agents. Eur J Med Chem 2014; 82: 506-20.
4. Tian SS, Jiand FS, Zhang K, et al. Flavonoids from the leaves of Carya cathayensis Sarg. inhibit vascular endothelial growth factor-induced angiogenesis. Fitoterapia 2014; 92: 34-40.
5. Willet WC. Balancing life-style and genomics research for disease prevention. Science 2002; 296: 695-8.
6. Mahmoud AM, Ashour MB, Abdel-Moneim A, Ahmed OM. Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proin ammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. J Diabetes Complications 2012; 26(6): 483-90.
7. SRKM Hewage, Piao MJ, Kang KA, et al. Hesperidin attenuates ultraviolet B-induced apoptosis by mitigating oxidative stress in human keratinocytes. BiomolTher (Seoul) 2016; 24(3): 312-9.
8. Yvonne O, Driss F, Dang PMC, et al. Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging

of hydrogen peroxide but not superoxide anion produced by human neutrophils. Biochem Pharmacol 2004; 68(10): 2003-8.
9. Afaq F, Saleem M, Krueger CG, Reed JD, Mukhtar H. Anthocyanin-and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-B pathways and inhibits skin tumorigenesis in CD-1 mice. Int J Cancer 2005; 113(3): 423-33.
10. Yang CS, Landau JM, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 2001; 21(1): 381-406.
11. Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C. Interaction between flavonoids and the blood-brain barrier: in vitro studies. J Neurochem 2003; 85(1): 180-92.
12. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard M27-A2. vol. 22, no. 15, 2nd ed., CLSI Document. Clinical and Laboratory Standards Institute, Villanova, PA, 2002.
13. Haggag YA, El-Ashmawy NE, Okasha KM. Is hesperidin essential for prophylaxis and treatment of COVID-19 Infection? Med Hypotheses 2020; 144.
14. Davis, ME. Glioblastoma: overview of disease and treatment. Clin J Oncol Nurs 2016; 20(5): 2-8.
15. Shu L, Cheung KL, Khor TO, Chen C, Kong AN. Phytochemicals: cancer chemoprevention and suppression of tumor onset and metastasis. Cancer Metastasis Rev 2010; 29(3): 483-502.
16. Nobili S, Lippi D, Witort E, et al. Natural compounds for cancer treatment and prevention. Pharmacol Res 2009; 59(6): 365-78.
17. Engin C, Iplik ES, Catmakas T, Cakmakoglu B. Hesperidin related apoptosis on brain glioblastoma. Ann. Med. Res. 2019; 26(10): 2255-8
18. Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 2005; 5(3): 231-7.
19. Hacker G. The morphology of apoptosis. Cell Tissue Res 2000; 301: 5-17.
20. Zhang XH, Zhang N, Meng X, Zhang Y, Qian Y, Xie YJ. Hesperetin inhibits the proliferation of cerebrally implanted C6 glioma and involves suppression of HIF1α/VEGF pathway in rats. Biomed Res 2017; 28(3).
21. Qiang L, Miao Z, Wang R, Yang J, Zhang D. Hesperetin Induces Apoptosis in Human Glioblastoma Cells via p38 MAPK Activation. Nutr Cancer 2019; 72(3): 1-8.
22. Aggarwal V, Tuli HS, Thakral F, et al. Molecular mechanisms of action of hesperidin in cancer: Recent trends and advancements. Exp Biol Med 2020; 245(5): 486-97.
23. Choi EJ. Hesperetin induced G1-phase cell cycle arrest in human breast cancer MCF-7 cells: involvement of CDK4 and p21. Nutr Cancer 2007; 59: 115–9.
24. Alshatwi AA, Ramesh E, Periasamy VS, Subash- Babu P. The apoptotic effect of hesperetin on human cervical cancer cells is mediated through cell cycle arrest, death receptor, and mitochondrial pathways. Fundam Clin Pharmacol 2013; 27: 581–92.
25. Palit S, Kar S, Sharma G, Das PK. Hesperetin induces apoptosis in breast carcinoma by triggering accumulation of ROS and activation of ASK1/JNK pathway. J Cell Physiol 2015; 230:1729–39
26. Zhang J, Song J, Wu D, Wang J, Dong W. Hesperetin Induces the Apoptosis of Hepatocellular Carcinoma Cells via Mitochondrial Pathway Mediated by the Increased Intracellular Reactive Oxygen Species, ATP AND Calcium. Med Oncol 2015; 32: 101
27. Xia R, Sheng X, Xu X, Yu C, Lu H. Hesperidin induces apoptosis and G0/G1 arrest in human non-small cell lung cancer A549 cells. Int J Mol Med 2018; 41: 464–72
28. Birsu Cincin Z, Unlu M, Kiran B, Sinem Bireller E, Baran Y, Cakmakoglu B. Anti-proliferative, apoptotic and signal transduction effects of hesperidin in non-small cell lung cancer cells. Cell Oncol 2015; 38: 195–204.
29. Xie , Chen J, Xiao A, Liu, L. Antibacterial Activity of Polyphenols: Structure-Activity Relationship and Influence of Hyperglycemic Condition. Molecules (Basel, Switzerland) 2017; *22*(11): 1913.
30. Yang D, Wang T, Long M, Li P. Quercetin: Its Main Pharmacological Activity and Potential Application in Clinical Medicine. Oxid Med Cell Longev 2020; 3: 1-13.
31. Iranshahi M, Rezaee R, Parhiz H, Roohbakhsh A, Soltani F. Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. Life Sci. 2015; 137: 125-32.
32. Bino A, Vicentini CB, Vertuani S, et al. Novel lipidized derivatives of the bioflavonoid hesperidin: Dermatological, cosmetic and chemopreventive applications. Cosmetics 2018; 5(4): 72.
33. Güran M, anlıtürk G, Kerküklü NR, Altundağ EM, Süha Yalçın A. Combined effects of quercetin and curcumin on anti-inflammatory and antimicrobial parameters in vitro. Eur J Pharmacol 2019; 859.
34. Altundağ EM, Toprak K, anlıtürk G, et al. Synergistic Combination of Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid and Natural Flavonoid Curcumin Exhibits Anticancer and Antibacterial Activity. Anticancer Agents Med Chem. 2021;21(10):1301-8.
35. Kumar PP, Kumar KTS, Nainita MK, et al. Cerebroprotective Potential of Hesperidin Nanoparticles Against Bilateral Common Carotid Artery Occlusion Reperfusion Injury in Rats and In silico Approaches. Neurotox Res. 2020; 37(2): 264-74.

Correspondence
Berfu Çerçi Öngün
Department of Anatomy, Faculty of Medicine, Eastern Mediterranean University, Famagusta, Northern Cyprus, Turkey.
E-mail: berfucerciongun@gmail.com