Total Antioxidant Potential, Total Phenolic Profile and Cytotoxic Activity Against Brain Cancer: Melocan and Galdirik§

Running head: Melocan and Galdirik as Potential Cytotoxic Agents

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SUMMARY

Research background. Brain cancer is known to be one of the most difficult types of cancer to be cure. It has serious impact on the lives of diagnosed people due to the insufficient treatment options and their side effects. Thus, the search for novel alternative treatments is ongoing. Melocan (Smilax excelsa L.) and Galdirik (Trachystemon orientalis) are significant contributors to both traditional culinary culture and traditional medicine around Black Sea; however, the evidence regarding their antioxidative and cytotoxic effects remains fairly limited.

Experimental approach. This study aimed to determine the antioxidant and cytotoxic activity of Smilax excelsa and Trachystemon orientalis, on the C6 glioblastoma cell line. Smilax excelsa and

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Trachystemon orientalis plants were dried and extracted; then, the total phenolic content (TPC) and their phenolic profiles were studied. In addition, their Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) were determined by using an assay kit. We also analyzed the total antioxidant activity (TAA) using the DPPH radical scavenging assay and the cytotoxic impact on the glioma cells via the 3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyltetrazolium (MTT) assay.

Results and conclusions. According to the results, the water extracts of both Smilax excelsa (1158.17 mg gallic acid eq./100 g DM) and Trachystemon orientalis (262 mg gallic acid eq./0 g DM) had higher TPC than the ethanol extracts. TAA were measured as 192.86 mg and 131.92 Trolox eq./100 g DM for the Smilax excelsa and Trachystemon orientalis, respectively. The MTT assay revealed that Trachystemon orientalis had a greater cytotoxic effect. In conclusion, the findings of the current study are promising.

Novelty and scientific contribution. This is the first study that aimed to evaluate the potential cytotoxic activity of two local Turkish plants, Smilax excelsa and Trachystemon orientalis, against C6 glioblastoma cells and the findings ensure that both plants may be used in the future as good therapeutic agents for treating cancer.

Keywords: C6 glioblastoma; Smilax excelsa L.; Trachystemon orientalis; brain cancer

INTRODUCTION

Cancer poses a significant challenge to human kind on a global scale. It is the second leading cause of death worldwide following coronary heart disorders, with an elevated mortality 10 million of deaths per year, and 19.3 million new cases are reported annually (1). Cancer is characterized by an inability to regulate or inhibit cell growth and multiplication, generating a tumor that may metastasize (2).

According to estimates from 2020, tumors that harm the brain and central nervous system were the cause of 251.329 fatalities in that year, making brain cancer the tenth deadliest disease for both sexes. Age, gender, family history (which increases the likelihood of developing brain cancer by 5 %), exposure to certain viruses and infections, head trauma, etc., can all have an impact on the incidence rates of brain cancer, as the case for other types of cancer. Additionally, tumors of the central nervous system (CNS) are found both in adults between the ages of 40 and 70 and in children. Brain cancer is a neoplasm that can grow in the brain and spinal cord (3). This tumor can be classified as benign or malignant based on its identification, origin, and rate of growth, and the tumors of the latter category may result in metastasis to other areas of the body. In addition, there are additional types of brain cancer that fall into those two main groups. One of these is glioma, a non-homogeneous group of
tumors that develop from the glial cells in the central nervous system. Glioma is also recognized as the most common brain cancer (4). It has a significant impact on people's quality of life because of the ineffectiveness of the existing treatments. Surgical methods, radiation therapy, and chemotherapeutic medications are frequently used to provide pain relief to patients with brain cancer and to extend their survival times. However, because of the deficiencies of these therapies, numerous inherent constraints are encountered (5).

Despite the improved understanding of the fundamental causes of brain tumor development, the number of survivors with various non-benign primary brain neoplasms has not increased to a notable degree. In fact, metastases make up the bulk of deadly brain tumors. Additionally, solid tumors are frequently encountered in children and are recognized as the primary cause of cancer-related mortality in children (6,7). All of these factors create a demand for better treatment methods and researchers have recently concentrated their efforts on finding a therapy that can treat cancer with fewer adverse effects than the previously mentioned treatment methods (8). Natural products are now the primary focus of treatment research, rather than pharmaceuticals. Plant secondary metabolites are frequently used in the medical industry. It is important to investigate these naturally occurring compounds as herbal or natural therapies in cancer treatment, as they have fewer side effects, are effective, are easy to access, and have the capacity to overcome resistance (9).

Many herbs used in medicine are derived from traditional healing plants and are used to cure a variety of illnesses, including cancer (10). One of the primary causes of cancer is the accumulation of reactive oxygen species in healthy cells. Thus, antioxidant molecules that significantly reduce the effects of oxidative stress can stop the transformation of healthy cells into cancerous ones. The antioxidant properties of plant-derived phytochemicals allow them to produce this result. Moreover, some of certain groups of polyphenolic compounds can exert other anticancer effects such as chemosensitization, metabolic modulation, metastatic inhibition, and apoptotic induction (11).

Melocan (Smilax excelsa L.) belongs to the Smilacaceae family, which is distinguished by its perennial members’ woody structures and spines and sizes of up to fifteen meters. This plant, which is extensively distributed throughout Northern Anatolia, is distinguished by having spines, bakka-type fruits, and roots that are narrow and cylindrical in shape. Spring marks the start of this plant's production of tiny shoots, which are consumed as vegetables. It is also used in cooking as playing a significant part in many different recipes. The roots are combined and sipped as tea (12).

Melocan is economically significant as it was historically used in Anatolia to cure stomach pain and bloating; recent studies have reported its role in breast cancer treatment (13). The rhizomes of this plant contain phenolic and saponin compounds that act against oxidation, fungi, and bacteria (14). This plant is a source of potent compounds that enable the species of this genus to combat cancer,
oxidation, mutation, inflammation, and bacteria (15). It has also been found that Melocan leaves have the capacity to protect kidneys from CCl4-induced nephrotoxicity by reducing the activity of antioxidant enzymes and preventing protein and lipid oxidation reactions, which simply support the integrity of the kidney's histological features and do not restore biological metrics. Furthermore, the plant shows a similar anti-amylase and anti-glucosidase activity to acarbose suggesting that it has the potential to treat diabetes mellitus (12).

Another widely distributed plant in East Bulgaria, the Caucasus, and Turkey, particularly along the Black Sea, is Galdirik (Trachystemon orientalis). This plant is considered as an edible plant since people in the Black Sea region typically include its flowers, rhizomes, leaves, and petioles in different recipes. According to recent reports, Galdirik contains anti-rheumatic, blood-purifying diuretic, antipyretic, and wound-healing properties (16). Additionally, in a number of studies large levels of phenolic compounds, including tannin, saponin and choline were reported in Galdirik. The effects of these compounds on antioxidants, antidiabetics, microbes, and fungi have also been researched (23).

According to Demir et al. (19), the enzyme superoxide dismutase, which is an essential component of the antioxidant defense mechanism developed by plants to avoid or lessen the harm caused by reactive oxygen species, is present in significant quantities in the plant's tissues. In addition to its antimutagenic activity (15), this plant's shoots were found to be also antimicrobial (27).

Although the different properties of these plants have been studied, no prior research has been conducted on their impact on glioma brain cancer cells. This study aimed to evaluate the antioxidant effect of the water and ethanol extracts of Melocan (Smilax excelsa L.) and Galdirik (Trachystemon orientalis) stems and leaves and their cytotoxicity potential against C6 glioblastoma cells.

MATERIALS AND METHODS

Materials

Fresh stems and leaves of S. excelsa L. and T. orientalis were gathered in September and October from the north Turkish village of Delikkaya near Ordu and Giresun. The settlement is 12 miles from the sea and is located at a height of roughly 300 m. The samples were first weeded and then washed three times to remove any remaining impurities. After being spread out thinly on a tray, they were dried in a convection oven (Electrolux Professional 260644 20 GN 2/1, Stockholm, Sweden) at 50 °C for 10 h. The samples were then stored at room temperature until they were used.

Reagents

Ethanol (≥99.8 %), gallic acid (GA) standard, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standards were from
Sigma Aldrich Co. (St. Louis, MO, USA); dimethyl sulfoxide (DMSO) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were sourced from Bio Basic Inc. (Ontario, Canada) and Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were procured from (Diagnovum, Ebsdorfergrund, Germany). Phosphate-buffered saline (PBS) (WISENT Inc., Quebec, Canada) and trypsin and sodium hydroxide were purchased from Merck KGaA (Darmstadt, Germany).

Proximate analyses
The total moisture content of the stems and leaves was assessed using the AOAC International technique 925.10 (the air oven method, gravimetric) (22) and the total ash content was determined using the 923.03 technique (the muffle furnace method, gravimetric) (23).

Extraction for the determination of the total phenolic content and antioxidant activity
Plant stems and leaves were used in combination for the analyses. A modified procedure using both water and ethanol solvents was utilized to prepare the plant extracts. A grinder (Sinbo, Scm 2934, Istanbul, Turkey) was used, for the initial grinding step. Water (25 mL) was added to 5 g of the ground sample. The extract was then subjected to a series of steps, including vortexing, 15 minutes of sonication, and 15 minutes of centrifugation at 8000×g (24) using a centrifuge (Heittich-Rotofix 32A, Tuttlingen, Germany). The same process was repeated to create a total amount of 100 mL of solvent for Galdirik and 75 mL for Melocan. The same procedure was used for the ethanol extraction, we added 25 mL of an ethanol:water (70:30 V/V) solution to about 5 g of the sample, vortexed the mixture, sonicated it for 15 minutes, and then centrifuged it at 8000×g for 15 minutes. These steps were repeated once more to produce a total solvent content of 50 mL for both samples (24).

Detection of the total phenolic content and major phenolic compounds
The total phenolic contents of the samples were assessed using the Folin-Ciocalteu colorimetric technique (24). First, 750 mL of a diluted Folin-Ciocalteu reagent solution with 100 mL of plant extract and 750 mL of 6% sodium carbonate solution were added after the Folin-Ciocalteu reagent solution had been diluted ten times with distilled water. After 1.5 h of incubation in the dark, the tubes were vortexed, and absorbance was measured at 750 nm using a blank solution made of water in place of the sample extract. This was undertaken using a Nanodrop spectrophotometer and plate reader Multiskan GO (Thermo Fischer Scientific, Germany). The amount of phenolic compounds was
determined using the gallic acid solution standard curve and is reported as mg of gallic acid equivalents/100g of DM. Triplicate analyses were carried out.

An HPLC (High Performance Liquid Chromatography) (Waters Turkey, W600 HPLC system with a PDA (photodiode array) detector, Istanbul, Turkey) was used to determine the major phenolic compounds in the plant samples. A Luna C18 column (150 x 4.60 mm pore size 100 Å, particle size 5 µm, Phenomenex, Torrance, CA, USA) was used as the stationary phase, while solvent A (Milli-Q water with 0.1 % (V/V) TFA) and solvent B (acetonitrile with 0.1 % (V/V) TFA) were used as the mobile phase (25). External standard calibration curves were used for quantification. All of the samples and calibration solutions were filtered through a 0.45-µm membrane filter and 2 mL of the filtered sample was placed into vials. The flow rate was 1 mL / min. Detections were conducted at 280, 312 and 360 nm wavelengths.

Total antioxidant activity
The total antioxidant activity of the Galdirik and Melocan extracts was assessed by their DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability (26). To achieve this, the extracts of this mixture were added to 2 mL of a freshly made DPPH solution that contained 1 mmol of DPPH reagent (freshly prepared). After 30 minutes in the dark, the test tubes were brought back into the light to measure the mixture’s absorbance at 517 nm. The results of this experiment are presented as mg Trolox equivalent (TE)/100 g dry mass (DM) of the plant material, with Trolox serving as the reference antioxidant.

Total antioxidant status / Total oxidant status (TAS/TOS)
The total antioxidant status of both extracts was evaluated also by using commercial TAS assay kits (Rel Assay Diagnostics, Gaziantep, Turkey). This test demonstrates how hydrogen peroxide oxidizes the free radical ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), which causes ABTS to lose its green or blue coloration and become stable. As the content of antioxidants increases, the color's intensity decreases. To achieve this, 18 µL of water and ethanol extract sample, standard, or H2O is combined with 300 µL of Reagent 1. After 30 seconds, the absorbance (A1) was measured spectrophotometrically at 660 nm. The mixture was then mixed with 45 µL of Reagent 2, and the absorbance (A2) was measured five minutes later at 37 °C and 660 nm. The total antioxidant status (TAS) was determined using the formula below, where \( \Delta A = A_2 - A_1 \):

\[
TAS = \left( \frac{\Delta A_{\text{H}_2\text{O}} - \Delta A_{\text{sample}}}{\Delta A_{\text{H}_2\text{O}} - \Delta A_{\text{standard}}} \right)
\]

Additionally, the oxidant status of both extracts was assessed using TOS commercial assay kit (Rel Assay Diagnostics, Gaziantep, Turkey). This assessment is based on the oxidation of Fe²⁺ to Fe³⁺. This ferric ion combines with xylene orange to form a complex, resulting in the creation of color that
can be observed spectrophotometrically. In this test, 45 µL of the sample or standard and 300 µL of Reagent 1 were combined, and after 30 seconds, the absorbance \( (A_1) \) was calculated spectrophotometrically at 530 nm. A volume of 15 µL of Reagent 2 was added to the mixture, and after incubation at 37 °C for 5 minutes, the absorbance \( (A_2) \) was measured. The overall oxidant status (TOS) was calculated using the formula below, where *: concentration of standard, \( \Delta A=A_2-A_1 \): 
\[
TOS=(\frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}})\cdot 10^* 
\]

**Cytotoxicity**

C6 glioblastoma culture

C6 glioblastoma cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in DMEM media with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin solution as supplements.

**Passaging protocol**

For the trypsinization of the cells, the cell culture medium was discarded and the cells were washed with PBS. Trypsin (1mL) was added to the cells and then the flask was placed in the incubator for 3 minutes (5 % CO\(_2\), 37 °C). The flask was then removed from the incubator, examined under a microscope for the presence of single cell suspensions, and tapped twice to ensure that every cell had detached completely from the flask surface. Then, 1 mL of DMEM was then added to terminate the trypsinization. The cells were resuspended by pipetting and transferred into a 15 mL centrifuge tube, where they were centrifuged for 2 minutes at 2000×g. Following the aspiration of the supernatant, 2 mL of DMEM was added to the pellet. This volume was split into two falcon tubes, to which 9 mL of DMEM was added bringing the total volume to 10 mL.

By using the MTT assay over a 24 h period, the cytotoxic impact of the Melocan and Galdirik water extracts on human glioma cancer cells were evaluated. The cells were incubated for 72 h at a density of 5x10\(^3\) cells/mL in a 96-well flat-bottomed cell culture plate. After removing the medium, different plant extract concentrations (ranging from 80 to 5 µg/mL) were applied to the cells for 48 h. The experiment was run in triplicate and three wells were utilized for each concentration. In order to dissolve the crystals, DMSO was added to each well after the addition of 10 µL MTT solution. Finally, absorbance \( (A) \) was measured at 570 nm. The \( A \) values were used to compute the percentage of cell viability using the following equation:

\[
\text{Cell viability}= \left( \frac{A_{\text{test sample}}}{A_{\text{control sample}}} \right) \cdot 100
\]
The logarithmic graph of the log concentration versus the percentage of cell viability was used to calculate the IC50. The IC50 value is the concentration (in µg/mL) that inhibits cell growth by 50 % (27).

Statistical analysis
All results were from at least two repetitions of experiments and the results are presented as mean value±standard deviation. The data were analyzed using the IBM SPSS Statistics software version 20, (IBM, New York, USA). A paired t-test was used to analyze the differences between the two extracts (water and 70 % ethanol:water) of each plant in order to determine whether they were statistically significant (p<0.05) (28). Analysis of variance (ANOVA) was used for the TAS/TOS and cytotoxic activity tests and the Duncan test was the post-hoc evaluation method used to determine the differences. Differences between samples were calculated at 95 % significance level. Pearson’s correlation matrix was used to detect the correlations between the antioxidant activity and the total phenolic contents of the samples (p<0.01 and p<0.05) were calculated using the same software.

RESULTS AND DISCUSSION

Quantitative determination of raw materials
The total moisture and ash contents of the Melocan and Galdirik samples were determined and shown in Table 1.

The moisture content of the dried leaves and stems of Melocan was determined to be 12.36 % due to the drying step, where that of the fresh edible parts of the plant was determined to be 82.6 % in a previous study (29). The ash content of Melocan (8.50 %) detected in this study was in line with previous findings reported in the literature where the ash content was found to be 6.77 % (30) and 7.10 % (31).

The results for Galdirik (29.02 %) were also slightly higher than the values reported in the literature; the ash content for the different genotypes of T.orientalis was between 9.2 and 17 % in a previous study (32). This might be related with the use of the whole plant in the study.

Total phenolic content and major phenolic compounds detected in plant extracts
The total phenolic content of the samples for the water and 70 % ethanol extracts were determined (as shown in Table 1), as the ability of plant phenolics to scavenge free radicals is among one of the most important anti-cancerous mechanisms.

The total phenolic contents of the stems and leaves of Melocan were determined to be 1158.17 mg and 293.89 mg gallic acid equivalent (GAE)/100 g DM, respectively, for the water and 70 % ethanol
extracts, indicating a significantly higher (p=0.003) concentration of phenolic compounds in the water extract. These findings are consistent with other findings in the literature, which show that the water extracts of these plants’ leaves contain higher concentrations of phenolic chemicals than the ethanol and infusion extracts (33). However, other researchers (12) have reported that the total phenolic content of the hexane extract of the leaves was 1930 mg GAE/100 g DM and the hexane extract of the stems was 710 mg GAE/g DM, revealing that the leaves of this plant contain a higher amount of phenolic compounds than the stems. However, in the present study, the combination of these two plant parts resulted in a favorable amount of phenolic compounds. Contradicting these findings, another group of researchers found that the phenolic content of the leaves of Melocan was 3060 and 3010 mg/100 g DM in water and ethanol, respectively, with higher amounts than were found in this study (33). The variations in the methodology and plant sections used may be the cause of these differences.

The total phenolic content of the Galdrik water and 70 % ethanol extracts were 262.42 and 235.67 mg GA/100 DM, respectively. The difference is statistically insignificant (p=0.165). In previous studies on Galdrik, the total phenolic content was found to be 9000 mg GA/100 g for water extracts and 2120 mg GA/100 g for ethanol extracts (34). The values found in the present study are lower than those established in the literature. Furthermore, they demonstrate that the Galdrik water extract has significantly higher levels of total phenolic content. These discrepancies can be attributed to the various extraction techniques used, differences in the initial concentrations, the differing effects of each plant portion used, and the various extraction solvents (16).

The major phenolic acids detected in Melocan were protocatechuic acid (5.35±0.04 mg/100 g), chlorogenic acid (5.91±0.02 mg/100 g) and chlorogenic acid derivatives (9.76±0.03 mg/100 g). The HPLC chromatogram for Melocan is given in Fig. 1. In the literature, Melocan has been reported to contain lanesol, violasterol A, transe-resveratrol, 5-O-caffeoylshikimic acid and 6-O-caffeoyl-β-d-fructofuranosyl-(2-1)-α-d-glucopyranoside (35). In another study, the researchers reported the phenolic components in Melocan shoot samples as caffeic acid (0.455 mg/100 g), ferulic acid (9.378 mg/100 g), rosmarinic acid (0.033 mg/100 g) and hydrocynamic acid (0.03 mg/100 g) by mentioning the impossibility to make any comparison with the literature (36). In Galdrik, no clear peaks were determined. This might be related to the extract properties or the HPLC conditions. Similarly to our findings, in the literature, only rutin and myricetin were detected infusions of Galdrik leaves and stems (37) or gallic acid, vanillic acid and rosmarinic acid abundantly (38).

Antioxidant potential of plant extracts
Assessing the radical scavenging activity of plants is a fundamental step for determining their anticancerous effects since oxidative stress is one of the major causes of cancer. The total antioxidant activity of the Melocan and Galdirik extracts was measured using the DPPH radical scavenging activity method, and the results are shown in Table 1. The DPPH radical scavenging approach determines the quantity of DPPH radicals reduced by antioxidants, which provide hydrogen or transfer electrons in order to create DPPH-H, a non-radical version of the DPPH radical.

In this study, the antioxidant content of Melocan was found to be higher in the water extract (192.85 mg/100g DM) than the 70 % ethanol extract (154.67 mg/100 g DM) (p>0.05).

According to previous studies, Melocan has a strong antioxidant activity of 62.36 mmol Fe^{+2}/kg. This result is comparable to that of another study, which used the β-carotene bleaching method and the linoleic acid system and concluded that this plant has significant antioxidant effect. In addition, it has been found via the phosphomolybdenum method that this plant has a high antioxidant content (almost 1200 µg α-tocopherol/g) (39).

Our findings suggest that the phenolic chemicals in Melocan contribute significantly to its antioxidant activity because there is a positive and linear association between the total phenolic content and the total antioxidant content.

In our study, the total antioxidant activity of the Galdirik extracts, were determined to be 131.92 and 73.81 mg TE/100 g DM for the water and 70 % ethanol extracts, respectively. As was the case in Melocan, the water extract of Galdirik had a higher antioxidant activity than the 70 % ethanol extract (p=0.165). The antioxidant activity of the Galdirik water and ethanol extracts was measured in a previous study using the ABTS free radical scavenging activity assay, and the results were 1725 and 240 mmoL TE/kg DM, respectively. In that study, the antioxidant content of this plant was comparable to that of broccoli and asparagus extracts, which had a value of 26.2 mmol TE/kg DM (34).

According to the results of the correlation assessment, for both of the water and 70% ethanol extracts, the total phenolic content and the total antioxidant activity correlated very well (0.998 for the 70% ethanol extracts and 0.993 for the water extracts (p<0.01).

**TAS/TOS of plant extracts**

The TAS value is an indicator that shows the activity of antioxidant compounds, while the TOS value shows the oxidant compounds produced by sample extracts. In the presence of the C6 glioblastoma cell line, the TAS and TOS were calculated for various amounts of Melocan and Galdirik water extracts, as shown in Fig. 2 and Fig. 3.

The TAS and TOS values of Melocan have never been the subject of a prior investigation. The water extract of a combination of stems and leaves was found to have an average TAS of 0.6 mmol Trolox
equivalent/L and a TOS of 3.64 μmol H₂O₂ equiv./L in the current investigation. In comparison to the control, the TAS value of the Melocan water extract was determined to be higher although not statistically significant (p=0.416). According to the results, Melocan does not exhibit significant oxidant activity, as the TOS value remains almost the same as that of the control sample. The antioxidants and oxidants that Melocan produces as a result of environmental influences or metabolic processes are represented by the TAS and TOS values, and no significant oxidant potential was detected.

For the TAS values of Galdirik, the average is 0.5 mmol TE/L, while the TOS is 2.3 μmol H₂O₂ equiv./L. The study findings demonstrate that the TAS value rises as the water extract concentration rises, and, at a 60 µg/mL concentration, it becomes comparable to the control (p>0.05). For the TOS results, the same Galdirik extract concentration (60 µg/mL) had a value of 2.4 μmol H₂O₂ equiv./L, which is slightly higher (p>0.05) than that of the control.

Cytotoxic activity of plant extracts determined by MTT assay

The cytotoxic activities of the water and 70 % ethanol extracts of the Melocan and Galdirik on the C6 glioblastoma cancer cells were examined using the MTT assay, and the results are shown in Fig. 4 and Fig. 5, respectively. MTT is reduced at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system. MTT is a colorimetric assay often used to determine the cell proliferation, viability and cytotoxicity. MTT test reveals cytotoxicity of test compound. Essentially, when a cell is alive, its metabolism causes the yellow tetrazolium dye to be reduced into purple formazan crystals, which are subsequently dissolved with the addition of DMSO (40). Thus, this method is used to quantify the color change as means of determining the number of cells that survived in the final stage.

According to the results of the current study, the Melocan extracts exerted a cytotoxic effect on the C6 glioblastoma cell line, with a higher effect produced by the water extract (IC50=7.73 μg/mL) than by the 70 % ethanol extract (IC50=10.05 μg/mL) (p>0.05) (Fig. 4). These findings are in line with the findings of the total phenolic and antioxidant activity, which also demonstrated that the water extract of this plant is more efficient than the 70 % ethanol extract.

However, in a previous study it was revealed that several chemicals extracted from Melocan have cytotoxic effects on the MCF-7 cell line (35). According to the same study, the chemicals violasterol A and solanesol, with IC50 values of 190.0 and 161.6 μM, respectively, exerted the greatest inhibitory effects on these types of breast cancer cells. Additionally, it was noted that the Smilax genus is unique due to its large amounts of steroidal saponins, chemicals that fall under the category of secondary metabolites and contribute to the biological effect exerted by many medicinal plants, and particularly their cytotoxic effects (41). Apoptosis and cytotoxicity on human osteosarcoma (U2OS) cells have
been proven to occur in *Smilax aspera*, one of the species in this genus (42). The same plant has been linked to cytotoxic action against lung cancer cells (43) as well as ovarian adenocarcinoma (OVCAR3), lung carcinoma (A549), and breast cancer (MDA-MB-231) cell lines (44).

The present results demonstrate that the water and 70 % ethanol extracts of Galdırık had the capacity to inhibit cell viability and the proliferation of C6 glioma cells in a dose-dependent manner (Fig. 5). For the water extract, the cell viability was 87.65 % at 1 µg/mL and decreased as the concentration increased reaching 11.33 % at 80 µg/mL. Similar to water, the 70 % ethanol extract also had a cytotoxic impact on the C6 cells, although it was less effective. In water, the cell viability peaked at 94.59 % at 1 µg/mL and dropped to 66.34 % at 80 µg/mL. Additionally, the IC50 for water was 4.47 µg/mL, meaning that it was more efficient than the ethanol extract as 50 % inhibition in cell viability is not achieved even at 80 µg/mL. The IC50 value reveals how much drug is needed to inhibit a biological process by half, and the lower IC50 value represents the higher potency. Therefore, the findings presented here, demonstrate that the water extract is more cytotoxic than the ethanol extract, supporting the earlier finding of this study, *i.e.*, that the water extract has more pronounced antioxidant activity due to its higher total phenolic content. Comparatively, it has been documented in the literature (45) that an ethanol extract (70 %) from a different plant, *Rhododendron brachycarpus*, has an anticancer effect on a number of human cancer cell lines, including MCF-7 breast cancer cell line, A549 lung cancer cell line, and Hep3B liver cancer cell line, among others. However, for the accurate determination of the cytotoxic activity and exact mechanism of action of these plants on glioblastoma, investigation of the blood-brain barrier crossing properties of their certain phenolic compounds (e.g. from flavonoids) is critically important (46). Therefore, more extensive studies are needed.

**CONCLUSIONS**

Overall, the results of this study revealed for the first time that both plants have the potential to be used in the treatment of glioblastoma cancer. Our results are in accordance with the literature data and highlight de novo the very important role of natural products in cancer research, in which both plants may be unique and good therapeutic agents for the therapy of cancer in medical care. Specifically, the Galdırık water extract has a lower IC50 value. This study may serve as a guide for future research that aims to identify the precise pathways controlled by this plant that have an impact on glioblastoma multiform. To confirm the *in vivo* effects, specifications of the molecular mechanism(s) of the plant cytotoxic activity and investigations of particular specialized components of the plants, are required.

**CONFLICTS OF INTEREST**
Authors state no conflict of interest for the study that competed with the objective, interpretation, and presentation of the results.

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AUTHORS’ CONTRIBUTION
D. Al Yassine and N. El Massri made the experiments of this study, formal analysis and writing the original draft. Z. Tacer Caba and G. Bulut made the conceptualization. Z. Tacer Caba and D. Akin made the supervision and methodology development. G. Demircan assisted in methodology development. Z. Tacer Caba, G. Bulut and D. Akin made the review and editing.

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REFERENCES


23. AOAC International. Method 923.03 Ash of Flour. In: AOAC International Official Methods of
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https://doi.org/10.1021/jf9801973

https://doi.org/10.1016/j.lwt.2013.11.040

https://doi.org/10.1016/j.foodres.2010.10.057

https://doi.org/10.5152/pcp.2021.20181


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Table 1. Proximate components, total phenolics and antioxidant activity of the samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>w (moisture)%</th>
<th>w (ash)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melocan</td>
<td>12.36 ± 0.05</td>
<td>8.50 ± 0.24</td>
</tr>
<tr>
<td>Melocan water extract</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melocan 70 % ethanol extract</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galdirkir</td>
<td>15.92±0.8</td>
<td>29.02±0.2</td>
</tr>
<tr>
<td>Galdirkir water extract</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galdirkir 70 % ethanol extract</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

w (total phenolics)/(mg GAE/100 g DM)  TAA/(mg TE/100 g DM)
(1158.17 ± 4.27)  (192.85 ± 5.53)  
(293.89 ± 0.01)  (154.67 ± 0.79) 
(262.57±1.59)  (132.60±0.14) 
(235.74±8.83)  (73.42±8.5) 

Values are presented as mean±standard deviation of duplicate analyses. Values of each sample in the same column with different letters (a-b) differ significantly (p<0.05). GAE=gallic acid equivalent, TAA=total antioxidant activity, TE=Trolox equivalent

Fig. 1. HPLC chromatogram of Melocan at 312 nm. Phenolic compounds in the sample are coded with numbers: (1) protocatechuic acid, 2) unidentified compound, 3) chlorogenic acid, and 4) chlorogenic acid derivatives
Fig. 2. Results of Melocan: a) total antioxidant status (TAS) and b) total oxidant status (TOS). The mean value±standard deviation of TAS and TOS results are given as mmol Trolox equivalent (TE)/L and µmol H₂O₂ equivalent (HE)/L, respectively. Values in the same column with different letters differ significantly (p<0.05)
Fig. 3. Results of Galdirik: a) total antioxidant status (TAS) and b) total oxidant status (TOS). The mean value±standard deviation of TAS and TOS results are given as mmol Trolox equivalent (TE)/L and μmol H₂O₂ equivalent (HE)/L, respectively. Values in the same column with different letters differ significantly (p<0.05)
Fig. 4. The cytotoxic activity of Melocan water and 70% ethanol extracts. Results are presented as mean value ± standard deviation of duplicate cytotoxic activity. Different small letters (a–c) among each concentration of an extract (e.g., water or 70% ethanol) differ significantly (p<0.05), and different capital letters (A–B) at each concentration of different extracts differ significantly (p<0.05).

Fig. 5. The cytotoxic activity of Galdirik water and 70% ethanol extracts. Results are presented as mean value ± standard deviation of duplicate cytotoxic activity. Different small letters (a–c) among each concentration of an extract (e.g., water or 70% ethanol) differ significantly (p<0.05), and different capital letters (A–B) at each concentration of different extracts differ significantly (p<0.05).