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Antioxidant and Anticancer Effects of *Malva verticillata* Methanolic Extract

Duygu GENÇALP[1], Namık Refik KERKÜKLÜ[2], Özde BUDA[3], Göktürk BİNER[3], Emre TURGAL[3], Dilara POLAT[3], Fatma İRDEM[3], Nazife KASAPOĞLU[3], Ergül MUTLU ALTUNDAĞ[1*]

[1]Eastern Mediterranean University, Dr. Fazıl Küçük Faculty of Medicine, Department of Medical Biochemistry, Via Mersin 10, 99628, Turkey
[2]Eastern Mediterranean University, Faculty of Arts and Sciences, Department of Chemistry, Via Mersin 10, 99628, Turkey
[3]Eastern Mediterranean University, Dr. Fazıl Küçük Faculty of Medicine, Via Mersin 10, 99628, Turkey
*Corresponding author: ergul.altundag@emu.edu.tr

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Abstract

Objectives: *Malva verticillata* (*M. verticillata*), growing in North Cyprus, is an edible plant known as "mallow" in public. Current literature does not contain any data about the anticancer and antioxidant activities of the *M. verticillata* plant grows in Northern Cyprus.

Materials and Methods: In this study, *M. verticillata* methanolic extract was used to investigate the *in vitro* antioxidant and anticancer effects of the *M. verticillata* plant. The antioxidant potential of the Malva extract was determined using the α-diphenyl-p-picrylhydrazyl (DPPH) free radical scavenging method, the total phenolic content (TPC) test, and the total flavonoid content (TFC) test. Besides, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was used to investigate the anticancer potential of *M. verticillata* methanolic extract on MCF-7 cells.

Results: The highest antioxidant activity of *M. verticillata* methanolic extract was found to be 69.35 ± 3.3% at 70 mg/ml. TFC and TPC contents were calculated as 502 ± 1.8 µg/mg and 499 ± 7.5 µg/mg extract at 70 mg/ml, respectively. Evaluation of anticancer activity revealed that MCF-7 cell proliferation was significantly inhibited with increased extract concentrations of *M. verticillata*.

Conclusion: In this study, methanolic extract of *M. verticillata* plant has shown to possess significant antioxidant and anticancer activity.

Key Words: Antioxidant, Anticancer, Breast cancer (MCF-7), *Malva verticillata*

1. Introduction

Breast cancer is known to be the primary cause of cancer death in women worldwide. Although it is rare in men, 25.4 % of the total new cancer cases in women were diagnosed as breast cancer in 2018 (Bray et al., 2018). According to a study carried out by North Cyprus Cancer Registry, Ministry of Health, breast cancer was found to be the most common cancer type for female population in North Cyprus between 2007 and 2012 (Pervaiz et al., 2017). Breast cancer can be

treated in several ways including chemotherapy, radiation therapy, hormonal therapy and mastectomy (Maughan et al., 2010) (Radecka and Litwiniuk, 2016). However, all treatment strategies for breast cancer has some side effects and they have negative effects on patients quality of life (Taylor and Kirby, 2015) (Zurrida and Veronesi, 2015) (Klassen et al., 2017). Pain, lymphedema, hair loss, diarrhoea and peeling of skin are some of the side effects that decrease patients' quality of life. Therefore, the development of new treatment strategies with low side effects is needed to improve the survival rate of these patients. Nowadays, there is an increased interest in herbal medicine and natural products to treat diseases, and laboratory studies have gained momentum. Recent studies showed that different *Malva* species have anti-cancer, anti-inflammatory and antioxidant effects (Rayssan and Shawkat, 2019) (Khoury et al., 2020) (Mousavi et al., 2020) (DellaGreca et al., 2009).

M. verticillata, also known as "cluster mallow" or "Chinese mallow", is an edible plant which is a member of Malvaceae family. It grows in terrestrial habitats and it is mostly found in South East Asian countries and China (Ashok et al., 2020). Studies indicated that M. verticillata grows in China is a valuable source of natural antioxidants. Free radical scavenging activity of *M. verticillata* ethanol extract has been reported, and it is found to have a significant reducing power (Bao et al., 2018). In another study, water extract of *M. verticillata* seeds showed bone resorption suppression and osteoclastogenesis (Shim et al., 2016). Glycosyl glycerides isolated from the *M. verticillata* demonstrated cytotoxicity to A549, HCT-15, AGS, HepG2 cancer cells (Ko et al., 2018). However, there is no evidence about the antioxidant and anti-cancer potential of *M. verticillata* grows in North Cyprus. *M. verticillata* is a part of the natural flora of North Cyprus and is widely used in Cypriot cuisine. In the current study, we aimed to evaluate the potential antioxidant and in vitro anti-cancer properties on MCF-7 cells of *M. verticillata* methanolic extract.

2. Material and Methods

2.1. Chemicals: Methanol (CN: 24229), Gallic acid (CN: 398225), 1,1-diphenyl-2-picrylhydrazyl (DPPH, CN: D9132), Sodium carbonate (CN: 13418), Folin reagent (CN: F9252) and Quercetin (CN: Q4951)) were obtained from Sigma-Aldrich. Aluminum chloride (PC:10558030) was purchased from Thermo Fisher-Scientific.

2.2. Plant Material: Plant samples were collected from Taşkent, Kyrenia, Northern Cyprus (35.265204, 33.397465) in April 2019. Herbarium Botanists Prof. Dr. Neriman Özhatay from the Eastern Mediterranean University (EMU), Faculty of Pharmacy, identified the plant material as *M. Verticillata*. The herbarium specimen (Voucher No: DE 002) was pressed and deposited in the herbarium. Plant name was checked from http://www.theplantist.org.

2.3. Preparation of *M. verticillata* methanolic extract by Soxhlet extraction: Leaves of *M. verticillata* were separated carefully and washed with distilled water. Plant material was then dried at room temperature (25 °C) and crushed into powder. For Soxhlet extraction, 13 grams of *M. verticillata* powder and 300 ml of methanol was used. The process was completed in a total of 16 hours; two cycles at 70 °C each lasting for eight hours. Extracts were kept in the refrigerator at 4 °C until analysis.

2.4. Antioxidant Tests by α,α-diphenyl-β-picrylhydrazyl (DPPH) Assay: The antioxidant activities of *M. verticillata* methanol extract were determined using a DPPH reduction method with minor modifications (Alara et al., 2018a). *M. verticillata* extract was dissolved in distilled

water, and 5 µl from different concentrations (10, 20, 30, 40, and 70 mg/mL) was mixed with 195 µl DPPH solution in 96-well-plate. Then, the 96-well-plate was incubated at room temperature for 30 minutes in darkness. Gallic acid solution with varying concentrations (50, 100, 200, 499,600, 800 and 1000 µg/mL) was used as a standard. Following the incubation, the absorbance of the samples was measured at 517 nm with Varioskan Flash Multimode Microplate Reader (Thermo Scientific, USA). Following formula was used to calculate the inhibition percentages of the radical scavenging activity:

DPPH scavenging activity (% Inhibition) = [(Acontrol-Asample) /Acontrol] x 100

Acontrol =The absorbance of methanol mixed with DPPH solution
Asample =The absorbance of M. verticillata extract mixed with DPPH solution

2.5. TPC (Total phenolic content) Determination: To determine the TPC of *M. verticillata* extracts, Folin-Ciocalteu reagent method was used with modifications (Alara et al., 2018a) (Alara et al., 2018b). First, 50 µl from extract concentrations (10, 20, 30, 40 and 70 mg/ml) was added into 96-well plates. Then, 100 µl from folin reagent and 100 µl from Na2CO3 (sodium carbonate) solution were added to reaction mixtures. Gallic acid solution with varying concentrations (50, 100, 150, 200, 250, 1000 µg/ml) was used for the generation of the standard calibration curve *(y=0,0074x+0,2664; r2=0.978)*. Samples were incubated for 30 minutes at 25 °C prior to the absorbance measurement. Absorbance measurements were done at 765 nm using a Varioskan Flash Multimode Microplate Reader (Thermo Scientific, USA). The results are represented as µg gallic acid equivalent (GAE) per mg of *M. verticillata* extract (µg GAE/mg extract).

2.6. TFC (Total Flavonoid Content) Determination: AlCl3 (Aluminum chloride) colorimetric method was used to determine the TFC of the *M. verticillata* extracts with minor modifications(Kim et al., 2003). 50 µl from each extract solution (10, 20, 30, 40 and 70 mg/mL) was mixed with 50 µl of 2 % AlCl3 in 96-well plate. Then, the reaction mixtures were incubated at room temperature for 60 minutes. Different concentrations of quercetin (50, 100, 150, 200 and 250 µg/ml) were used for the generation of the standard calibration curve (y=0,0074x–0,0158; r2=0,9709). At the end of the incubation period, absorbance was measured at 420 nm via Varioskan Flash Multimode Microplate Reader (Thermo Scientific, USA). The results were expressed as µg quercetin equivalent (QE) per mg *M. verticillata* extract (µg QE/mg extract).

2.7. MCF-7 Cell Culture and Cell Viability Assay: Cell culture experiment was performed on MCF-7 human breast cancer cell line obtained from the American Type Culture Collection (ATCC, HTB-22). Dulbecco's modified Eagle's medium (DMEM- Merck, Germany) supplemented with 1 % L-glutamine, 1 % penicillin-streptomycin, 10 % fetal bovine serum (FBS; Hyclone Laboratories, USA) was used as cell culture media and cells were grown in a 5 % CO2 incubator at 37 °C. In order to determine the cell viability, MTT (Sigma, M2003) assay was performed (van der Heijden et al., 2004), and 5- Fluorouracil (5-FU) treated group was used as a positive control. Prior to the cell viability assay, 1x104 cells/well were seeded in 96 well plates in 100 µl of fresh culture medium and incubated for 24 hours at 37 °C. At the end of the incubation, cells were treated with *M. verticillata* extract at various concentrations (5,10, 20, 50, 100, 200 µg/mL) and 5 µM Fluorouracil (5-FU) for 24 hours. Then, 10 µl of 5 mg/ml MTT solution in PBS was added to each well, and left to incubate at 37 °C for four hours. At the end of incubation period,

the supernatant was removed, and DMSO (100 µl) was added to each well. The 96-well plates were placed into a microplate shaker for 5 minutes prior to the absorbance measurement. Absorbance was measured at 570 nm with a Varioskan Flash Multimode Microplate Reader (Thermo Scientific, USA). The following formula was used to calculate the percentage of cell viability:

% Viable cells= (Asample- Ablank) / (Acontrol-Ablank) x 100
Ablank= The absorbance of blank
Acontrol =The absorbance of control
Asample =The absorbance of M. verticillata extract

The experiment was performed in triplicate, and CalcuSyn Software program (Biosoft, Ferguson, USA) was used to calculate the concentration of extract required to inhibit 50 % of MCF-7 cell viability (IC50)

2.8. Statistical Analysis: Experiments were performed in triplicate and $P<0.05$ was considered to be statistically significant. Test results were calculated in Microsoft Excel 2015 software (Microsoft, Redmond, USA), and results were expressed as mean ± standard deviation (SD). GraphPad Prism Version 8 software was used to perform the statistical analyses. ANOVA/Dunnet's Multiple Comparison Test was used to determine the differences among groups.

3. Results and Discussion

Antioxidant activity of *M. verticillata* methanolic extract was determined by DPPH free radical scavenging assay, and reducing power of extract was determined by measuring its ability to act as a free radical scavenger. Gallic acid was used as standard and antioxidant activities of standard gallic acid concentrations at 50 and 800 mg/ml were determined as 6.13 % and 91.91 %, respectively (data not shown). *M. verticillata* methanolic extracts demonstrated simultaneous increase in DPPH radical scavenging activities in a dose-dependent manner. DPPH radical scavenging activity was calculated as 16,3 % ± 4,2 at 10 mg/mL of *M. verticillata* extract concentration. The highest DPPH scavenging activity of methanolic extract of *M. verticillata* was 69,35 ± 3,3 % at 70 mg/mL. The mean percentage of DPPH free-radical scavenging activity at different concentrations of extracts is shown in Table 1. As a basis, the total phenolic content of *M. verticillata* methanolic extract was determined using Folin-Ciocalteu reagent. The total phenolic content of the extract concentrations at 10, 20, 30, 40 and 70 mg/ml was determined as 455±14, 490±6.4, 492±2.4, 497±2.9 and 499±7.5 µg GAE /mg extract, respectively (Table 2). Total flavonoid content of *M. verticillata* methanolic extract was determined using aluminium chloride in a colorimetric method. The results were derived from the calibration curve (Data not shown) ($y = 0.0074x - 0.0158$, $R2 = 0.9709$) of quercetin (10–250 µg/mL) and expressed as µg Quercetin per mg *M. verticillata* extract (µg Quercetin / mg *M. verticillata* extract). Our results showed that flavonoid content of *M. verticillata* was 448±1.8 µg/mg quercetin equivalent at 10 mg/mL extract concentration. TFC of 20, 30, 40 and 70 mg/mL extracts was determined as 475±0.3, 481±0.2, 486±0.2 and 502±1.8 µg/mL, respectively (Table 3). To evaluate the cytotoxic effects, the methanolic extract of *M. verticillata* was subjected to MTT assay using breast cancer cell line MCF-7. MTT assay results showed concentration-dependent growth inhibition in MCF-7 breast cancer cell lines following *M. verticillata* methanolic extract (5-200 µg/ml) application. Results of the cytotoxicity evaluation against MCF-7 cells of the *M. verticillata* extract are shown in Figure 1. The lowest concentration (5 µg/mL) of the extract exhibited 22.92 % ($P=0.0008$) cell viability inhibition compared to control. In addition, increasing concentrations of the extract from 10, 20, 50, 100 to 200 µg/mL

resulted in an increase in cell viability inhibition on MCF-7 cells from 30.22, 38.39, 46.6, 49.74 % to 62.98 % compared to control (P<0.0001), respectively.

Table 1. DPPH radical scavenging activity of the different concentrations of *M. verticillata* methanolic extract. Results are expressed as % radical scavenging activity relative to 100 % radical scavenging activity of gallic acid as a reference. Values are expressed as mean ± standard deviation (n=3).

***M. verticillata* extract concentration (mg/mL)**	**DPPH radical scavenging activity (%)**
10 mg/mL	16,3 ± 4,2
20 mg/mL	29,67 ± 6,5
30 mg/mL	29,08 ± 2,2
40 mg/mL	37,84 ± 2,3
70 mg/mL	69,35 ± 3,3

Table 2. Table shows the total phenolic content of *Malva verticillata* extract expressed as µg gallic acid/mg of extract. Values are expressed as means ± standard deviation (n=3).

	***M. verticillata* extract concentration (mg/mL)**				
	10 mg/mL	**20 mg/mL**	**30 mg/mL**	**40 mg/mL**	**70 mg/mL**
GAE/mg of extract	455±14	490±6,4	492±2,4	497±2,9	499±7,5

Table 3: Table shows the total flavonoid content of *Malva verticillata* extract expressed as quercetin/mg of extract. Values are expressed as means ± standard deviation (n=3).

	***M. verticillata* extract concentration (mg/mL)**				
	10 mg/mL	**20 mg/mL**	**30 mg/mL**	**40 mg/mL**	**70 mg/mL**
µg quercetin/mg extract	448±1.8	475±0.3	481±0.2	486±0.2	502±1.8

Positive control 5µM 5-FU was found to decrease cell viability by 50.48 % (P<0.0001). Furthermore, methanolic extract of *M. verticillata* exhibited significant cell viability inhibition against the MCF-7 cells with an IC50 value of 71.39 µg/mL at 24 hours. Some medicinal plants have attracted attention as alternative cancer therapies because of their low toxicity and ease of affordability (Cassileth and Chapman, 1996). The discovery of novel potential products from bioactive plant

extracts for cancer treatment is the subject of various researches. The current study is focused on gathering information about the antioxidant activity of the *M. verticillata* and demonstrating the potential anticancer effect on MCF-7 breast cancer cell line.

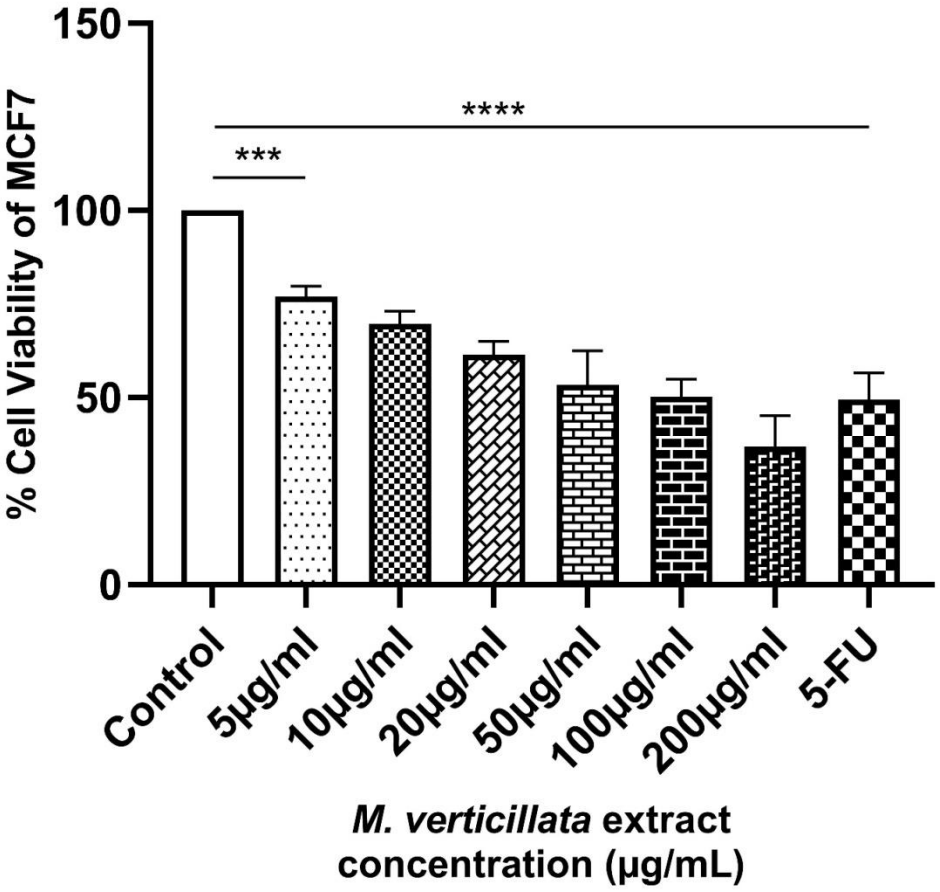

Figure 1: Effects of *M. verticillata* on MCF-7 cell viability. MCF-7 breast cancer cells were treated with different concentrations of *M. verticillata* methanol extract for 24 hours (p=0.0008***, p<0,0001****).

In this study, DPPH, TPC, and TFC tests were used to determine the antioxidant potential of *M. verticillata* methanolic extract. Previous studies have shown that other subtypes of the Malvaceae family have significant DPPH radical scavenging activity, high amount of total phenolic and total flavonoid contents (DellaGreca et al., 2009) (Güder and Korkmaz, 2012) (Choukri Beghdad et al., 2014). In one study, DPPH radical scavenging activity of *M. verticillata* leaves, seed and stem was reported (Bao et al., 2018). Based on our experimental results, *M. verticillata* methanolic leaf extract was found to have 29.67 % DPPH scavenging activity at 20 mg/mL. Total phenolic content evaluation also revealed phenolic content of the extract to be between 455±14 to 499±7.5 µg GAE /mg extract. The results suggest that *M. verticillata* methanolic extract has the potential antioxidant capability. Previous studies also reported that phenolic components have anticancer properties and capability to inhibit different types of cancer formation (Carocho and Ferreira, 2013) (Benetou et al., 2008) (Chahar et al., 2011). To understand the cytotoxicity effect of *M. verticillata* methanolic extract on breast cancer cells, MCF-7 cell line was selected to be

investigated throughout this study. Earlier findings support that M. sylvestris methanolic leaves extract inhibits cell viability of lymphoma cells by 68.65 % and melanoma cells by 76.53 % at 200 µg/mL concentration (Rayssan and Shawkat, 2019). Similarly, in this study, *M. verticillata* methanolic extract exhibited comparable effect with M. sylvestris and inhibited the MCF-7 cell viability by 62.98 % at 200 µg/mL extract concentration. As a result, we investigated the potential antioxidant and anticancer properties of *M. verticillata* which grows in North Cyprus, for the first time. The present findings provide preliminary data exposing *M. verticillata* grows in North Cyprus have potent cytotoxic activity against MCF-7 cells.

4. Conclusion

In summary, this study provides evidence for the significant antioxidant activity and anticancer effect of *M. verticillata* methanolic extract. *M. verticillata* has the potential antioxidant properties that could be beneficial to health either as potential therapeutic agent or as a dietary component. Further studies are required in order to reveal its mechanism, investigate the anticancer effects of *M. verticillata* on different cancer cell lines. al., 2010).

Acknowledgments

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Conflict of Interest

No conflict of interest was reported by the authors.

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