

In vitro cytotoxicity evaluation of *Marrubium vulgare* L. methanol extract

Mehmet Evren OKUR^{1*} , Nihal KARAKAŞ^{2,3} , Ayşe Esra KARADAĞ^{4,5} , Rabia YILMAZ³ , Fatih DEMİRCİ⁶ 

¹ University of Health Sciences, Faculty of Pharmacy, Department of Pharmacology, Üsküdar, İstanbul, Turkey

² İstanbul Medipol University, School of Medicine, Department of Medical Biology, Beykoz, İstanbul, Turkey

³ İstanbul Medipol University, Regenerative and Restorative Medicine Research Center, Beykoz, İstanbul, Turkey

⁴ İstanbul Medipol University, School of Pharmacy, Department of Pharmacognosy, Beykoz, İstanbul, Turkey

⁵ Anadolu University, Graduate School of Health Sciences, Tepebaşı, Eskişehir, Turkey

⁶ Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, Tepebaşı, Eskişehir, Turkey

* Corresponding Author. E-mail: evrenokurecz@gmail.com (M.E.O.); Tel. +90-216-418 96 16/2802.

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ABSTRACT: *Marrubium vulgare* L. (Lamiaceae) is a herbal drug used for centuries for many diseases. In this present study the plant material was acquired from commercial sources in pharma grade (PhEur 8.0) quality. The methanol extract of the aerial parts was evaluated for its *in vitro* cytotoxic activity by measuring the percentage of viable cells (U87 LN229 and T98G glioblastoma multiforme cell lines) using a luminescence system, and the antioxidant activities by ABTS and DPPH radical scavenging spectrophotometrically. As a result, the methanol extract of *M. vulgare* showed 48.97 ± 0.82 mg of GA/g corresponding to the total phenolic amounts, and moderate antioxidant activity (1.33 and 2.08) by ABTS[•] and DPPH[•] assays. To the best of knowledge, after evaluation of the cytotoxicity on *M. vulgare* treated U87 (IC₅₀:270.3 µM), LN229 (IC₅₀:343.9 µM) and T98G (336.6 µM) glioblastoma multiforme (GBM) cell lines, significant cytotoxic activities with 69.9% (*p*= 0.0081) and 71% cell viability (*p*= 0.0028) was observed in *M. vulgare* treated U87 and LN229 GBM cell lines, respectively. Overall, further *in vitro* and *in vivo* bioactivity studies based on phytochemistry on various *M. vulgare* preparations are worthwhile in order to discover bioactive secondary metabolites.

KEYWORDS: Cytotoxicity-1; glioblastoma multiforme cell lines-2; U87-3; LN229-4; T98G-5; *Marrubium vulgare*-6.

1. INTRODUCTION

Marrubium vulgare L. is a common plant of the Lamiaceae family and is known as “horehound”. It is native of North Africa, Western Asia, and Southern Europe. In 1910, *M. vulgare* was included in the European Pharmacopoeia. Flowered aerial parts and aqueous-methanol extracts are used in the treatment of stomach disorders and cough. It is also used as a sedative and anti-inflammatory agent in traditional medicine [1]. Also, there are several studies on the gastro-protective, anti-hypertensive, analgesic, hypoglycaemic, and antispasmodic effects [2-7]. More than 54 secondary metabolites were reported from *M. vulgare*. The major bioactive components are labdane diterpenes and flavonoids. Marrubiin is the main compound which is a diterpenoid isolated from *M. vulgare* aerial parts, however, the plant also contains vitexin, luteolin and apigenin [8]. Premarrubenol, premarrubiin and vulgarol also were characterized from *M. vulgare* among others. Other detailed phytochemical evaluations on various *M. vulgare* parts reported the presence of flavonoids, steroids, terpenoids, tannins, saponins, and essential oils [9-10].

Glioblastoma Multiforme (GBM) is one of the most aggressive and hard to treat brain cancer in adults. Highly invasive nature and heterogeneity of GBM lead to disabilities with current standard treatment methods. After diagnosis for GBM, surgical debulking of tumour with adjuvant radio- and chemotherapy are applied to the patients and these can only slightly increase survival rates [11-13]. Therefore, new therapeutic agents are needed. Thus cytotoxicity evaluation of plant extracts for their possible anti-cancer effects are common. As a novel approach, many studies revealed the feasibility of utilizing natural compounds in combination with radiotherapy and chemotherapy for the efficient treatment of cancers [14-16].

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In this present study, it was aimed to evaluate the *in vitro* antioxidant and the cytotoxic activity of the *M. vulgare* methanol extract. The initial plant material was acquired from commercial sources at PhEur 8.0 quality, which was evaluated for its percentage of viable cells (U87 and LN229 GBM cell lines) using a luminescence system, and the antioxidant activity by using the ABTS (=2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt and DPPH (=2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity by spectrophotometry. The total phenolic compounds (TPC) were also determined by using the Folin-Ciocalteu technique.

2. RESULTS AND DISCUSSION

2.1. Extraction

Pharmacopoeia quality plant material (marrubiin= 0.98%) was used for this study, which was confirmed by thin layer chromatography (TLC). An average 10% (w/w) extraction yield was achieved by MeOH maceration. Compared to the previous extraction results, the present obtained yield was above the average. The TLC was applied to confirm the quality of the plant extract prior the *in vitro* biological evaluations. The *in vitro* DPPH and ABTS radical scavenging antioxidant properties, also cytotoxicity against U87 and LN229 cancer cell lines were evaluated. The total phenolic content was also measured as reported below.

2.2. Antioxidant Activities

The MEOH extract ABTS• and DPPH• scavenging activity results are reported in detail in Table 1. The *M. vulgare* methanol extract showed relatively more antioxidant activity against DPPH (IC₅₀= 2.08 mg/mL) and ABTS (IC₅₀= 1.33 mg/mL) radicals compared to the standards Trolox and ascorbic acid, respectively.

Table 1. ABTS• and DPPH• scavenging activity results of *M. vulgare* MEOH extract.

	Extract	
	IC ₅₀ ± SD (mg/mL)	
ABTS•	1.33 ± 0.02	3.42 ± 0.04 (Trolox)
DPPH•	2.08 ± 0.02	4.54 ± 0.02 (Ascorbic acid)

The DPPH and ABTS radical scavenging assays are one of the most commonly used methods for evaluation of proton donating antioxidants, like phenolic compounds from plants. The DPPH• antioxidant analysis is based on the capacity of DPPH•, a stable free radical, to decolorize in the existence of antioxidants, thus a low value corresponds to a relatively general and good scavenging ability [17]. Previous reported *in vitro* antioxidant activity studies, it was observed that *M. vulgare* aerial parts showed different and varying results. It can be concluded that this may due to the differences of the locations and extraction methods of the plant material [18-21]. In addition the plant material of the previous studies were either grown in cell culture or collected from various sites without standardization concerns, which is one of the major differences of our plant material, resulting difficult comparisons in this aspect.

2.3. Total phenolic content of the extract

The total phenolic content of the *M. vulgare* MeOH extract was measured by using Folin-Ciocalteu technique [22]. The TPC of *M. vulgare* MeOH extract was calculated as gallic acid (GA) equivalent amount. The result suggests that TPC is present in a relative good amount in the extract. The TPC was found 48.97 mg GA/g. Phenolic substances display redox properties, which allow them to act as antioxidants. Based on the data obtained from performed experiments, a high correlation was found between the total phenolic contents and antioxidant activity for methanol extract of *M. vulgare*. It was observed that, when compared Brahmi and co-workers found similar results to the present study, and which is also comparable with other reports [18].

2.4. Cell viability on U87 and LN229 glioblastoma multiforme (GBM) cell lines

Cytotoxic effects of *M. vulgare* MeOH extract was tested on U87, LN229 and T98G GBM cell lines by measuring metabolically active cells. According to results obtained in the present study, cell viability was decreased at varying concentrations (0.1 mg/mL, 0.5 mg/mL and 1 mg/mL) in a concentration dependent manner. Cell viability of U87, LN229 and T98G cell lines upon 24 hours treatment with 0.1-1 mg/mL *M. vulgare* extract were given at Figure 1. Treatment with 1 mg/mL *M. vulgare* extract resulted in 69.9% viability of cells

on U87 cells ($p= 0.0081$), while LN229 cells displayed 71% of cell viability ($p= 0.0028$), respectively (Figure 1). As Temozolomide (TMZ) is the commonly used chemotherapeutic drug for standard therapy of GBM, in this study TMZ is also included in cell viability assays. Cell viability of 24 hours Temozolomide treated U87, LN229 and T98G GBM cell lines were given at Figure 2. TMZ treated GBM cell lines display drug resistancy at various concentrations and namely requires high doses for significant therapeutic responses, especially for LN229 and T98G cell lines. Conversely, U87 GBM cell line is semi-sensitive for 24 hours TMZ treatment as we recorded significant decrease when treated with 500 μM concentration.

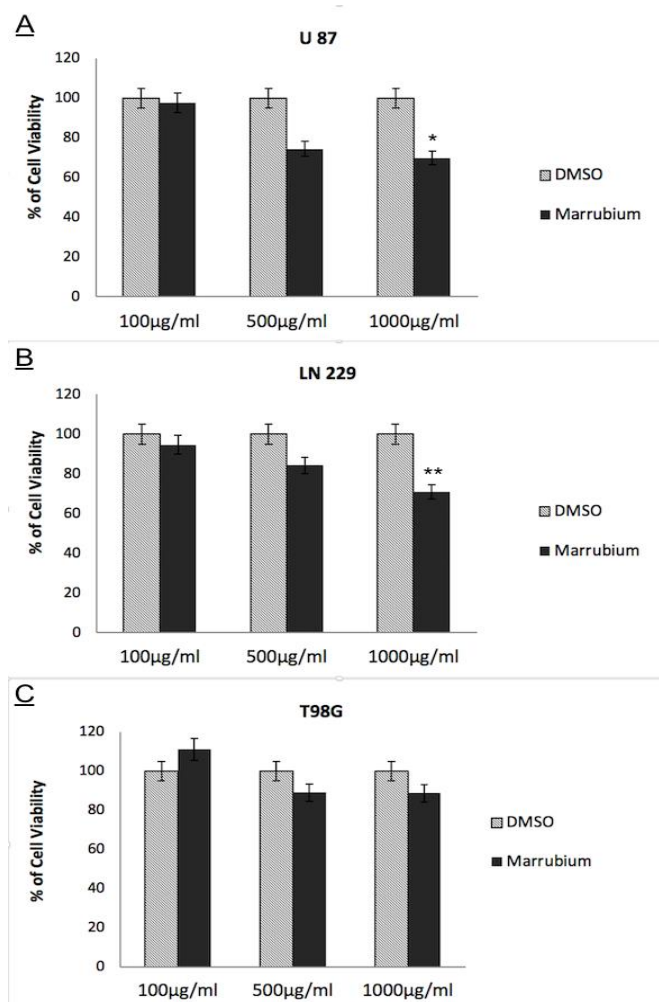


Figure 1. Cytotoxic effects of *M. vulgare* methanol extract on GBM Cell Lines. Cell viability of U87 (A), LN229 (B) and T98G (C) cell lines upon 24 hours treatment with 0.1-1 mg/mL *M. vulgare* extract significantly decreased. Data are expressed as \pm SEM ($0.003 < p^* \leq 0.01$ and $0.0005 < p^{**} \leq 0.003$).

Accordingly, the tested methanol extract showed 270.3 μM and 343.9 μM IC_{50} values for U87 and LN229 GBM cell lines, respectively (Figure 3). There was not significant decrease in cell viability on *M. vulgare* treated T98G cell line for tested concentrations, beside IC_{50} value was determined as 336.6 μM (Figure 3).

Cytotoxicity is a complicated process in animal and human systems, potentially involving direct cellular damage (e.g., with plant extracts, cytotoxic anticancer agent) and other systemic effects. One of the plant extract studies involves cytotoxic activities of *M. vulgare* on different cancers and very few studies reported anti-cancer effects of *M. vulgare*. In one of those studies, Zakari et al. [23] evaluated the *in vitro* cytotoxicity of *M. vulgare* essential oil was examined on cervical cancer line using a modified MTT assay. *M. vulgare* essential oil was inhibited the proliferation of HeLa cell lines. In another study, Hamedeyazdan et al. [24] evaluated *Marrubium persicum* methanolic extract antiproliferative activity with the MCF-7 breast cancer cell line using the MTT test for cell viability and cytotoxicity indices. They were found that the methanolic extract of *M. persicum* showed that growth of MCF-7 cells was inhibited by the extract in a dose and time dependent manner,

where a gradual increase of cytotoxicity effect has been achieved setting out on 200 $\mu\text{g}/\text{mL}$ concentration of the plant extract. Also they were evaluated antioxidant activity and determined total phenolic and flavonoids content of the extract. The antioxidant assay revealed that the extract was a strong scavenger of DPPH radicals. The total phenolic and flavonoids content of the plant extract was 409.3 mg gallic acid equivalent and 168.9 mg quercetin equivalent per 100g of dry plant material. In another study, authors were prepared *M. vulgare* leaves methanol extract by using rotary evaporator. They were evaluated in vitro cytotoxicity of this extract on Human colorectal cancer cells (HCT-116 cell line). Extract was significantly ($P < 0.05$) suppressed cell growth of HCT-116 at concentration of 250 $\mu\text{g}/\text{mL}$ [25, 26].

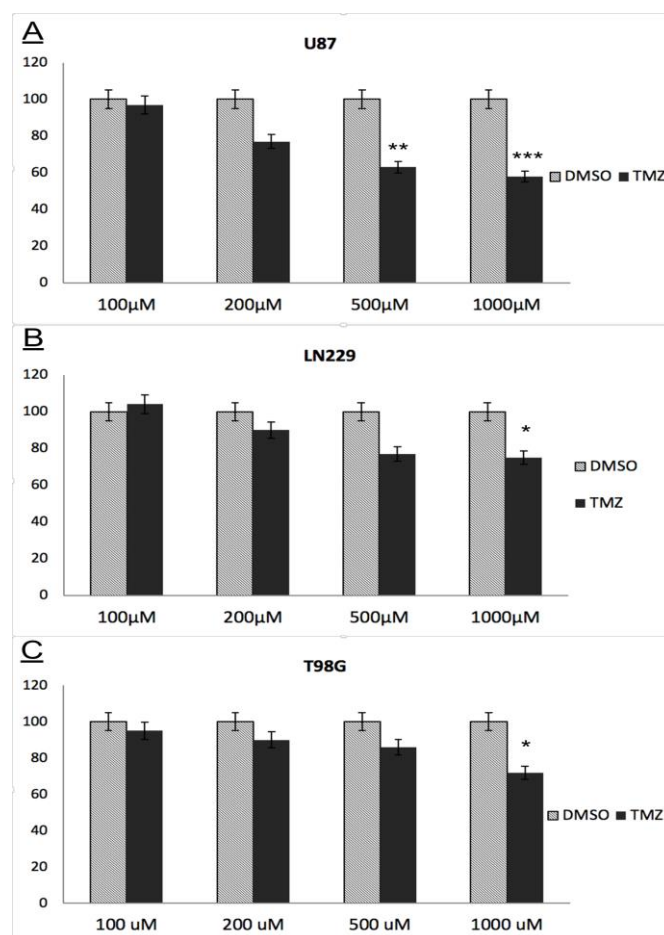


Figure 2. Cell viability of 24 hours Temozolomide (TMZ) treated GBM cell lines. U87 (A), LN 229 (B) and T98G (C) cell lines treated with increased doses of TMZ (100 μM -1000 μM). Data are expressed as \pm SEM. ($0.003 < p^* \leq 0.01$, $0.0005 < p^{**} \leq 0.003$ and $0.0005 \leq p^{***} \leq 0.0001$).

Eventhough, GBM is one of the mostly diagnosed and lethal cancers all around the world, relatively, there is no study investigating anti-cancer and anti-oxidant effects of *M. vulgare* on different GBM cell lines especially hard to treat with standard TMZ therapy. To the best of knowledge, this study is the first *in vitro* study on U87 and LN229 glioblastoma multiforme cell lines demonstrating the cytotoxic activity of *M. vulgare* MeOH extract. In a previous study, Paunovic et al. [27] prepared *M. vulgare* ethanolic extract by the modified pharmacopoeial percolation method. They were reported similar U251 glioma cell line results with the present study. They were concluded that *M. vulgare* ethanolic extract was dose-dependently reduced viability of melanoma (B16) and glioma (U251) cells. As in the study on brain tumors, this study demonstrates that *M. vulgare* extract may have a potential *in vitro* cytotoxic effect on brain cancers.

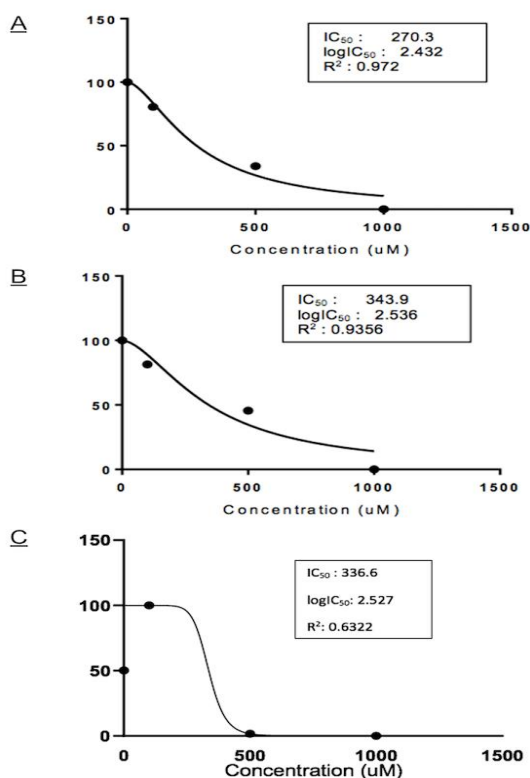


Figure 3. IC₅₀ value of *M. vulgare* methanol extract in A) U87, B) LN229, and C) T98G GBM cell lines.

3. CONCLUSION

The *in vitro* cytotoxicity against GBM brain cancer cell lines and correlated antioxidant activity of the *M. vulgare* extract showed notable results to study further. Especially, when considering that one of the major components is *Marrubium*, it is worth to validate the major compound effect. Also more in depth mechanical and selectivity studies are needed to prove the efficacy of *Marrubium* preparations.

4. MATERIALS AND METHODS

4.1. Materials

DPPH•, ABTS•, ascorbic acid, Temozolomide and Trolox were obtained from Sigma (Sternheim Germany). All chemicals used were highly pure - analytical grade if not otherwise stated.

4.2. Plant material and extraction

The Herba Marrubii conc. (PhEur 8.0) was purchased from Antonious Apotheke in Germany. For the extraction procedure, the plant materials were ground to the fine powder then, they were macerated initially with methanol for one day in a water bath shaker maintained at 25 ± 2°C. The extract was filtered using a 0.45 μm filter and then the filtrate was concentrated by a rotary evaporator (Heidolph, Schwabach-Germany). The prepared methanol extract was stored in an amber vial at 4°C for further experiments.

4.3. Antioxidant Activities

4.3.1. DPPH• Scavenging assay

The antioxidant capacity was determined in terms of hydrogen donating or radical scavenging ability using DPPH• by its capability to bleach the stable radical [28]. The reaction mix contained 100 μM DPPH• in methanol and several concentrations of the crude extract. After 30 minutes, absorbances were measured at 517 nm by using an UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 25 ± 2°C and the radical scavenging activity (RSA) was determined as the percentage of radical reduction as follows:

$$\text{DPPH}\cdot \text{ RSA \%} = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}}}{\text{Absorbance}_{\text{control}}} \right] \times 100 \quad (\text{Eq. 1})$$

4.3.2. ABTS• Scavenging assay

The antioxidant capacity of the extracts was evaluated by the ABTS• radical cation decolorization test according to Re *et al.* [29]. ABTS• solution was prepared by mixing aqueous ABTS• (7 mM) and potassium persulfate (2.45 mM). The mixture stored for 12-16 h in the dark at 25 ± 2°C. To regulate its absorbance at 734 nm, this final solution was diluted with ethanol. The test was carried out in triplicate. To determine absorbance of the extract, 990 µL ethanol was used instead of ABTS• in control. Firstly, the test was performed on Trolox as a standard [30]. The outcomes were signified as IC₅₀ as follows:

$$\text{ABTS}^\bullet \text{ RSA } \% = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}}}{\text{Absorbance}_{\text{control}}} \right] \times 100 \quad (\text{Eq. 2})$$

4.4. Total phenolic content of the extract

Folin-Ciocalteu method was used for determination of total phenolics content in the methanol extract. Folin-Ciocalteu's reagent (0.25 mL) and Na₂CO₃ (0.2 mL) were mixed to extract (5 mL). The mixtures were then incubated at 45°C for 15 minutes. The absorbance was determined at 765 nm by using an UV-Vis spectrophotometer at 25 ± 2°C. The total phenolic ingredient was measured from linear calibration curve (R² = 0.9811) [31] and the outcome was stated as mg gallic acid equivalent (GAE) /100 g extract [32].

4.5. Cytotoxicity Activity

4.5.1. Cell culture

U87-GBM (ATCC, #HTB-14), LN229-GBM (ATCC, #CRL-2611) and T98G-GBM (ATCC, #CRL-1690) cells were purchased from ATCC (U.S.). Then the cells were grown and expanded in DMEM (Gibco) medium with 10% fetal bovine serum (Gibco), 1% antibiotics (penicillin / streptomycin) at 37°C in 5% CO₂ incubator. The cells were then removed from the flask with Trypsin / EDTA 0.25% (Gibco) and seeded at a density of 5x10³ cells /well into 96 black well plates (Corning) for cell viability assay that measures metabolically active cells.

4.5.2. Cell viability assay

Extracts were dissolved in DMSO to prepare stock solutions, and serial dilutions were made using 1% DMSO as a final concentration to normalize measurements. Temozolomide was used a standard chemotherapeutic drug for GBM. After seeding into 96 well plates and cells were incubated at 37 °C in 5% CO₂ for a day. Then the culture medium was discarded and cells were treated with 100, 500 µg/mL, 1 mg/mL of *M. vulgare* extract as triplicates. After 24 hours of treatment, Cell Titer Glo reagent (Promega) added into each well and the percentage of viable cells was determined by reading luminescence (SpectraMax i3x Multi-Mode Detection Platform). Each of the viability experiments were performed as triplicates.

4.5.3. Statistical analysis

All statistical analyses were carried out by unpaired *Student's t-test* assuming equal variance. Differences were considered as statistically significant at 0.003 < p* ≤ 0.01, 0.0005 < p** ≤ 0.003 and 0.0005 ≤ p*** ≤ 0.0001. Data were expressed as the mean ± standard error of the mean (S.E.M.). Each of the experiments were performed as triplicates.

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Conflict of interest statement: The authors declared no conflict of interest.

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