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Antioxidant and Apoptotic Effect of *Muscari muscarimi*, an Endemic Geophyte Species from Turkey

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Abstract

Muscari muscarimi Medik., which is an endangered and endemic geophyte species of Turkey, was studied for antioxidant, cytotoxic and apoptotic potentials. The antioxidant effect of the extracts (ethanol and methanol) provided from the flower and bulbs of *M. muscarimi* was tested through the antioxidant potency by phosphomolybdenum assay, the reducing power assay, metal chelating ability, nitric oxide scavenging and ABTS radical cation scavenging capability methods. Also, total flavonoid and saponin content of the extracts was specified. Cytotoxic activity of the extracts was assessed and screened against human MCF-7, HeLa and H1299 cancer cells. Terminal transferase dUTP nick end-labeling (TUNEL) assay was applied to cancer cells for the determination of the late apoptotic changes. Antioxidant potencies in bulb extracts were observed to be lower than the flower extracts. However, the cytotoxicity and TUNEL assay revealed that the bulb extracts exhibited more marked anticancer activity against H1299 cell line than the other cell lines. Based on the in vitro data, *M. muscarimi* warrants further studies to isolate novel compounds for chemotherapeutic use.

Keywords: Antioxidant activity, Apoptosis, Cancer cell lines, *Muscari muscarimi*, Turkey

1. Introduction

The use of plants as medicines has a history as old as mankind. Traditional medicinal plants have been used to cure several diseases for thousands of years in different parts of the World (Adebayo & Krettli, 2011). Turkey is one of the most prominent floristic regions on the earth due to its geologic, topographic and climatic features. Floristic studies have shown that Turkey houses about 12000 plant taxa on its soils and more than 3000 taxa among them are endemic (Hoekstra *et al*., 2005). Geophytes are a considerable component of this generous biodiversity and include a lot of significant endangered and endemic species such as *Muscari muscarimi* Medik. The genus *Muscari* Mill. was formerly classified in the family Liliaceae but recently has been reclassified in the family Asparagaceae (Mulholland *et al*., 2013). The total number of *Muscari* species recorded in Turkish flora is approximately 39, and 24 of them (61.5%) are endemic (Eker, 2019). *M. muscarimi* is a perennial bulbous plant known as 'misk sümbülü', growing in south-west Turkey. This endemic species is most fragrant species in the genus *Muscari* and has high ornamental potential. It bears fascinating dirty bluish-gray flowers that bloom between March and May each year. The indigenous populations of *M. muscarimi*, is critically affected by enhanced environmental impurity and urbanization (Ozel *et al*., 2015). It has been used in the folk medicine in Turkey. In addition to this, it has also been used as ornamental plants in gardens, dye and as food for animals and humans (Oztas *et al*., 2018). Some species of *Muscari* have been utilized in conventional medicine as hypoglycemic, diuretic, antirheumatic, antiverruca, antiallergic and expectorant (Kayıran & Özkan, 2017, Loizzo *et al*., 2010). Different investigations have also been notified to the antioxidant, antimicrobial and anticancer effects of *Muscari* (Mammadov *et al*., 2012, Mammadov *et al*., 2016, Eroğlu Özkan *et al*., 2018).

Antioxidants preserve the cell constituents towards oxidative loss caused by free radicals. Phenolic compounds, alkaloids, terpenoids and other secondary metabolites present in plants are superb in antioxidative effects. Researchers have found out that many of these antioxidant components have anticancer, antiinflammatory, antimutagenic, antimicrobial and antiviral potentials (Shahidi & Ambigaipalan, 2015). *Muscari* Mill. contains different phytochemicals with biological activities including homoisoflavonoids, glycosides and water-soluble polysaccharides (Urbancikova *et al*., 2002, Adinolfi *et al*., 1985). Lanosterol and tetranorlanosterol glycosides from *Muscari paradoxum* (bulbs) were assessed for their cytotoxic effect on HSC-2 cancer cells. Even though the tetranorlanostane glycosides did not display any cytotoxicity on HSC-2 cells, the lanostane glycosides exhibited high cytotoxic activity (Ori *et al*., 2003). It was reported that homoisoflavonoid compounds isolated from *M. neglectum* had antiinflammatory, estrogenic, antiestrogenic, anticancer and angioprotective bioactivities (Lim, 2014).

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Cancer is a fatal universal illness of these times and a crucial obstacle in the ever-increasing lifetime of affected inhabitants (Bray *et al*., 2018). Despite a general choice for cancer therapy, chemotherapy is restricted by drug resistance and noxious adverse effects (Hanahan & Weinberg, 2011). Natural products can inhibit cancer and restrain tumor growth via cell fate pathways containing apoptosis (Fulda, 2010). Cancer cells are invulnerable to apoptosis owing to the activation of anti-apoptotic proteins or suppression of pro-apoptotic proteins (Pandey *et al*., 2016). In recent times, a lot of plant extracts or their isolated compounds have been evaluated for apoptotic effect, and several mechanisms of actions have been proposed for their toxicity against cancer cells. Hence, in this study, *M. muscarimi*, which is an endemic and endangered species, was assessed for cytotoxicity and possible apoptotic ability taking into consideration that there has been no paper about such activities previously. The antioxidant activity of *M. muscarimi* was investigated through mainly five methods, and its total flavonoid and saponin content was also determined.

2. Materials and Methods

2.1. Plant material and extraction

Approximately, 250 g of *M. muscarimi* were picked up in May 2013 from Antalya (900 m), located in the southern part of Turkey and identified by (Voucher No: RM1001) Dr. Ramazan Mammadov, Muğla Sıtkı Koçman University, Turkey. The plant was divided into two parts as bulb and flowers and dried in shade at room temperature. The pulverised plant parts were separately subjected to solvent extraction in a shaker water bath with methanol and ethanol at 48-50°C for 6h. The extraction was repeated twice at the same condition. Then solvents were removed with a rotary evaporator (IKA RV, Germany), samples were lyophilised (Labconco FreeZone, USA). The crude samples were kept at -20°C until needed (Ozay & Mammadov, 2017).

2.2. Total flavonoid and saponin contents

The total amounts of flavonoid and saponin substances of the plant extracts were detected by using the aluminum chloride (Moreno *et al*., 2000) and vanillin-sulphuric acid (Hiai *et al*., 1976) colorimetric methods, respectively. These substances were expressed as quercetin (mg QEs/g) and quillaja (mg QAEs/g) equivalents, respectively.

2.3. Antioxidant activity assays

2.3.1. Total antioxidant capacity (TAC)

The phosphomolybdenum method was used to evaluate the TAC of the extracts. To keep it short, different extract solutions were mixed with the reagent solution and incubated at 95°C for 90 min. The absorbance values were determined at a wavelength of 695 nm (Prieto *et al*., 1999). TAC is denoted as ascorbic acid (mg AEs/g) equivalents.

2.3.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was applied as defined by Zengin *et al*., (2015). with small modifications. Extracts solutions were added to FRAP reagent which was mixed in advance. After measuring the absorbances at 593 nm, FRAP potential is denoted as Trolox (mg TEs/g extract) equivalents.

2.3.3. Metal chelating activity

Extracts solutions at different concentrations were added to $FeCl_2$. The reaction that started directly after adding ferrozine was read at 562 nm after being left for 10 min left at 25°C. Metal chelating effect is denoted as EDTA (mg EDTAEs/g extract) equivalents (Zengin *et al*., 2015).

2.3.4. ABTS radical scavenging activity

The scavenging activity towards ABTS (2,2 azino-bis (3-ethylbenzothiazloine-6-sulfonic acid)) radical was analyzed as described by Re *et al*., (1999) with slight modifications. Freshly prepared and diluted ABTS solution was joined in the extracts of *M. muscarimi* (20-1000 µg/mL), and the absorbances were read after 30 min at 734 nm. The outcomes were indicated as IC_{50}.

2.3.5. Nitric oxide (NO) scavenging activity

NO was produced from sodium nitroprusside (SNP) which read as defined by Balakrishnan *et al*., (2009) in the Griess reaction. SNP (5mM) in PBS was incubated with several concentrations (20-1000 µg/ml) of the extracts, and the tubes were kept waiting for 3 hours at 25°C. The absorbance value was determined at 546 nm wavelength. Ascorbic acid was employed as an antioxidant standard. The results were indicated as IC_{50}.

2.4. Cytotoxicity assay

HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma) and H1299 (non-small cell lung adenocarcinoma) human cancer cell lines were employed in this research. The cells were cultured in RPMI 1640 medium in a CO_2 incubator. 24 hours incubation after seeding into 96-well plates (2×10^3 cells/well), the medium was removed from the well leaving the adherent cells and cells were applied with extract for 72 hours in the range of 25-1000 µg/mL. After time was up, cell viability was determined by using CytotoxGlo kit (Promega, USA), in accordance with the manufacturer's instructions. The percentage of cell viability was calculated relative to control cells. A plot of cell viability (%) against concentration was created, and the concentration of the plant extract that decreased cell viability by 50% (IC_{50}) was calculated.

2.5. TUNEL assay

The apoptotic effects of *M. muscarimi* bulb extracts in HeLa, MCF-7 and H1299 cells were evaluated using the TUNEL assay. The cells were treated with IC_{50} values of each extract for 24 h at 37°C. To determine cell death, the *In Situ* Cell Death Detection Kit (Millipore, USA) was used in accordance with the manufacturer's instructions. The TUNEL-stained apoptotic cells were visualized by use of a microscope and then counted. The data were expressed as a percentage of the area of TUNEL-positive cells in 10 random fields.

2.6. Statistical analysis

Statistical analysis was performed using the software SPSS version 22.0 program. Statistical significance was determined using the one-way ANOVA. Multiple group comparisons were analyzed with Tukey's multiple comparison test. Data were expressed as mean ± standart error of three separate experiments. p value of < 0.05 was considered statistically significant.

3. Results

3.1. Antioxidant activity

Antioxidant activity of the bulb and flowers of *M. muscarimi* was assayed by using FRAP, ABTS, NO, phosphomolybdenum and metal chelating assays. Also, total amounts of flavonoid and saponin substances of the extracts were calculated by using the aluminum chloride and vanillin-sulphuric acid assays, respectively. The outcomes of all these assays are presented in Tables 1 and 2. According to the data that were obtained, the highest total flavonoid content was found for the methanolic flower extract as 27.22 mg QEs/g, while the highest total saponin content was detected for the methanolic bulb extract as 140.41 mg QAEs/g ($p < 0.05$) (Table 1). Flower methanolic extract had the highest ABTS (IC_{50} value=52.16 µg/ml) and NO (IC_{50} value=64.35 µg/ml) radical scavenging activities ($p < 0.05$) followed by the flower ethanolic extract (Table 2). Likewise, the highest results of the FRAP, phosphomolybdenum and metal chelating assays for *M. muscarimi* were determined in the flower methanolic extract as 77.53 mg TEs/g, 62.05 mg AEs/g and 24.10 mg EDTAEs/g extract, respectively.

Table 1. Total antioxidant capacity, total flavonoid and saponin contents of *M. muscarimi* extracts (mean±SE).

Extracts	TFC	TSC	TAC
BE	19.35±0.45^{b}	134.01±4.12^{a}	44.33±2.27^{b}
BM	15.33±0.63^{b}	140.41±4.35^{a}	47.24±2.41^{b}
FE	23.12±0.71^{a}	110.63±3.06^{c}	58.32±2.65^{a}
FM	27.22±0.75^{a}	128.17±3.52^{b}	62.05±2.73^{a}

TFC (Total flavonoid content): quercetin equivalents (mg QEs/g). TSC (Total saponin content): quillaja equivalents (mg QEs/g). TAC (Total antioxidant capacity): ascorbic acid equivalents (mg AEs/g). BM/FM: Bulb/Flower Methanol, BE/FE: Bulb/Flower Ethanol. Different letters in the same column indicate a significant difference ($p < 0.05$)

Table 2. Antioxidant activities of M. muscarimi extracts (mean ± SE).

Extracts	ABTS (IC_{50} µg/mL)	NO (IC_{50} µg/mL)	FRAP assay (mg TEs/g)	Metal chelating activity (mg EDTAEs/g)
BE	63.29±1.24^{b}	76.14±1.55^{b}	58.24±0.30^{c}	12.16±0.04^{b}
BM	68.70±1.38^{a}	81.42±1.67^{a}	62.48±0.33^{c}	14.03±0.07^{b}
FE	55.01±0.17^{c}	72.21±1.01^{b}	71.02±1.54^{b}	17.85±0.15^{b}
FM	52.16±0.12^{c}	64.35±0.56^{c}	77.53±1.63^{a}	24.10±0.26^{a}
Ascorbic acid	08.11±0.03^{d}	19.02±0.05^{d}	nt	nt

BM/FM: Bulb/Flower Methanol; BE/FE: Bulb/Flower Ethanol, TEs: Trolox equivalents, EDTAEs: EDTA equivalents, nt: no tested. Different letters in the same column indicate a significant difference ($p < 0.05$)

3.2. Cytotoxic activity and TUNEL assay

To evaluate the cytotoxic activity of the *M. muscarimi* bulb extracts at several concentrations (25-1000 µg/mL) towards MCF-7, HeLa and H1299 cancer cell lines for 72 h, CytotoxGlo assay was carried out. A decrease in viability in cancer cells was observed in a concentration-dependent manner ($p < 0.05$) (Fig. 1 and 2). The IC_{50} values (µg/mL) of ethanolic and methanolic bulb extracts in different cancer cell lines were tabulated (Table 3). Methanolic bulb extracts were found to have lower IC_{50} values in all cancer cells than ethanolic extracts. The IC_{50} values for the methanolic extract were 90.43, 140.13 and 58.20 µg/mL on MCF-7, HeLa and H1299 cells, respectively. The apoptosis-inducing potential of *M. muscarimi* bulb extract was evaluated in the cancer cell lines using the TUNEL assay after treatment with the extracts at their IC_{50} doses for 24 h. A remarkable rise in apoptotic cells was noticed in the H1299 cells treated with methanolic bulb (45.0 ± 2.38%) and ethanolic bulb (41.4 ± 2.35%) extracts, compared with the control ($p < 0.05$) (Fig. 3). Apoptotic cells in H1299 cells were shown in Fig. 4.

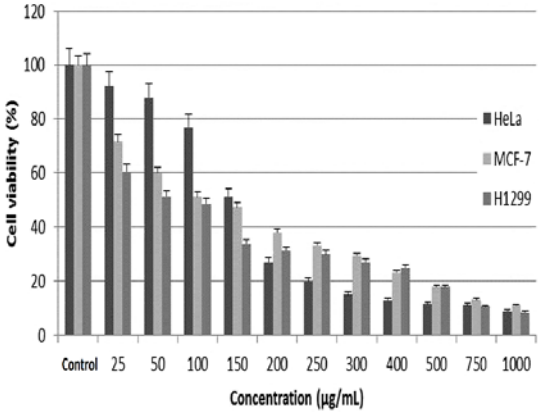

Figure 1. Cytotoxic activity of *M. muscarimi* ethanolic bulb extracts against different cancer cell lines. Data are presented as mean±SE.

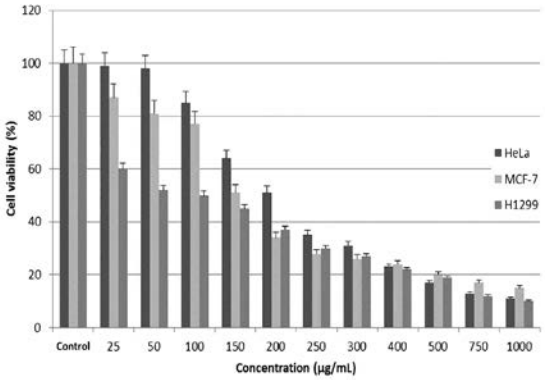

Figure 2. Cytotoxic activity of *M. muscarimi* methanolic bulb extracts against different cancer cell lines. Data are presented as mean±SE.

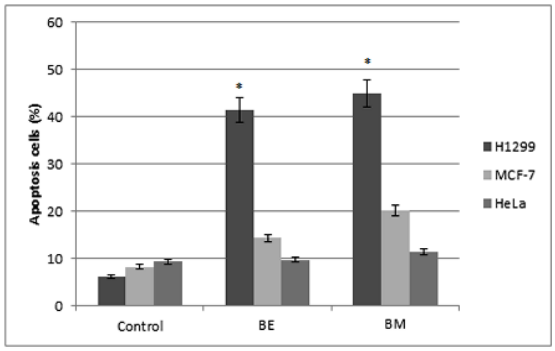

Figure 3. Apoptotic effects of *M. muscarimi* bulb extracts in different cancer cell lines. Data are presented as mean±SE.

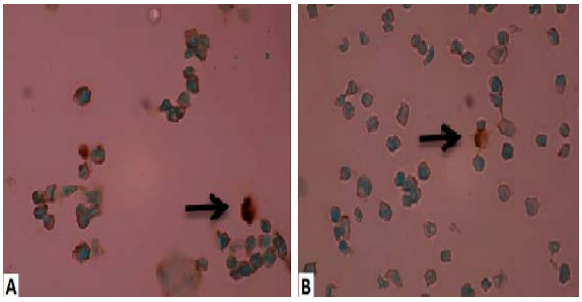

Figure 4. Apoptotic cells in H1299 cells treated by *M. muscarimi* bulb extracts (A: methanol, B: ethanol) using TUNEL assay. The arrows show the apoptotic cells.

Table 3. IC_{50} (µg/mL) values of *M. muscarimi* in different cancer cell lines

Extracts	HeLa	MCF-7	H1299
BE	205.01±1.46	135.08±1.28	62.05±1.24
BM	140.13±1.30	90.43±1.26	58.20±1.20

BE: Bulb Ethanol, BM: Bulb Methanol. Data are presented as mean±SE.

4. Discussion

The plants extensively include substances that high antioxidant activity owing to the being of phenolic complexes particularly flavonoids (Tungmunnithum *et al*., 2018). Antioxidants were called as bioactive substances that prevent the generation of free radicals or neutralize free radicals in living organisms. To prevent oxidative degradation of food, some antioxidants called synthetic (BHT, BHA, etc.) are extensively used in the food industry, but these synthetic antioxidants are suspected of being responsible for tumorigenesis (Lourenço *et al*., 2019). Therefore, the improvement and usage of more powerful antioxidants from natural resource are needed.

In studies evaluating the antioxidant activity of *M. muscarimi*, two free radicals (NO and ABTS) were used, and the studies determined the rate at which these radicals are scavenged by the extracts. Transition metals act as a catalyst for lipid peroxidation. Therefore, chelating these metals is considered as an important antioxidant mechanism (Zengin & Aktumsek, 2014). In this study using the phosphomolybdenum assay, FRAP assay and metal chelating activity, it was found that the flower extracts demonstrated higher antioxidant potency than the bulb extracts. We have previously published data representing the antioxidant activity of *M. muscarimi* using two assays namely, β-carotene/linoleic acid assay and DPPH assay and similarly, the flower extracts were detected to have higher antioxidant activity than the bulb extracts. Also, the highest total phenolic content was determined in the methanolic flower extract (38.13 mg GAEs/g) (Mammadov *et al*., 2016). In the current research, the highest total flavonoid content was determined in the methanolic flower extract as 27.22 mg QEs/g. Therefore, the higher antioxidant activity of methanolic flower extract may be depending on its total phenolic and flavonoid contents.

To assess the cytotoxic activity of the extracts of *M. muscarimi* on the growth of HeLa, MCF-7 and H1299 cell lines, the bulb extracts were examined. The low cytotoxicity of the flower extracts has directed us to this choice (data not shown). Also, in our previous report, the brine shrimp lethality test results of the *M. muscarimi* extracts showed that the flower extracts were less cytotoxic than the bulb extracts (Mammadov *et al*., 2016).

Many phytochemicals are biologically active, and they may interact to protect against cancer. Flavonoids are quite strong antioxidants, which scavenge free radicals, prevent the progression of cancer and protect towards oxidative stress related diseases (Abotaleb *et al*., 2019, Vrancheva *et al*., 2020). Flavonoids demonstrate powerful anticancer potencies against different cancer cells, mediated through coordinating of primary signaling pathways involved in the migration and invasion of cancer cells and metastatic spread, plus increase apoptosis (Ravishankar *et al*., 2013). It was reported that the chemical components of the genus *Muscari* are homo-isoflavanones, flavonoids, glycosides, alkaloids and terpenoids (Urbancikova *et al*., 2002; Lim, 2014). It has been shown that *M. racemosum* homoisoflavonoids have antimutagenic features and may be important for the prevention from cancer (Miadokova *et al*., 2002). In the current study, we found that the bulb extracts had higher saponin content than the flower extracts. Cytotoxicity of the bulb extracts containing a high amount of saponin was higher than the flower extracts. Studies in recent years have stated that saponins indicate remarkable anticancer activity, like anti-proliferation via mechanisms that contain induction of apoptosis (Man *et al*., 2010). Not surprisingly, the percentages of TUNEL-positive cells were escalated in all human cancer cell lines, especially in the H1299 cells, treated with the extracts comparison to their untreated controls, referring that the strong cytotoxic effects of the bulb extracts towards cancer cells are mediated by induction of apoptosis. Lanosterol glycosides, named scillasaponins E–G, were isolated from *M. paradoxum* bulb extract, and these isolated compounds exhibited cytotoxic activity against HSC-2 human oral squamous cell carcinoma cells with IC_{50} values ranging from 6.3 to 59 mg/mL when etoposide (positive control) had an IC_{50} value of 24 mg/mL (Ori *et al*., 2003). It can be concluded that the high amounts of saponins in the bulbs of *M. muscarimi* may result in cytotoxic activity.

5. Conclusion

In conclusion, this study is the first research that describes the potential antiproliferative and apoptotic efficacy on the cancer cells of *M. muscarimi*, which is an endemic and endangered geophyte species for Turkey. Further phytochemical and biological studies are needed to state the active constituents of *M. muscarimi*.

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