RESEARCH ARTICLE

Anthocyanins Profile Characterization of Common Grape Hyacinth (*Muscari neglectum* Guss. ex Ten.) Flowers Growing in Hungary by High-performance Liquid Chromatography Coupled to Diode Array Detector and Mass Spectrometry using the Electrospray Ionization Interface

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ABSTRACT

Objective: Anthocyanins are one of the biologically active substances group playing an important role in the state of physiological functions referring to human health. This research aimed to investigate the anthocyanins profiles in the grape hyacinth (*Muscari neglectum* Guss. ex Ten.).

Materials and methods: The identification of individual anthocyanins was carried using the method of high-performance liquid chromatography with diode array detection and mass spectrometry with electrospray ionization (DAD-ESI-MS) analysis. Chromatographic separation and tandem mass spectrometric analyses were performed on an Agilent 1100 HPLC system and Agilent 6410 triple quadrupole system equipped with an electrospray ion source (ESI) in positive ion mode.

Results: In the *M. neglectum* flowers (tepals), nine individual anthocyanins, containing delphinidin, petunidin, malvidine, pelargonidin aglycones were found. In this research, we report anthocyanin profiles for the *M. neglectum* flowers for the first time.

Conclusion: The obtained results concerning anthocyanins composition may be very useful for researchers in the field of the standardization and activity evaluation of extracts produced from *M. neglectum*.

Keywords: Anthocyanins, HPLC-DAD-ESI-MS, *Muscari neglectum.*

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INTRODUCTION

Muscari neglectum Guss. ex Ten. is a herbaceous perennial bulbous plant, related to genera known as "grape hyacinth" and particularly starch grape hyacinth[1] or common grape hyacinth[2] (Figure 1). *M. neglectum* has many synonyms found in scientific literature, the main ones are *Muscari racemosum* Lam. and DC., *Muscari racemosum* var. *neglectum* (Guss. ex Ten.) St.-Lag., *Hyacinthus neglectus* (Guss. ex Ten.) E.H.L. Krause. It is found in Ukraine, Moldova, Central, and Atlantic Europe, the Mediterranean, the Caucasus, Asia Minor, Iran, the south and southeast of the United States. In Russia, it grows absent-mindedly in the forest-steppe and steppe zones of the European part and the Ciscaucasia. Naturally, the species occurs in foothills, dry grassland, forests, and grassy

Figure 1: *Muscari neglectum* Guss.ex Ten.

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mountain slopes; the plant is ordinarily cultivated in gardens.[3] The flower buds, bulbs are commonly used in Mediterranean cuisine, especially in dishes from southern Italy. Flowers have a nutlike, sweet flavor and are utilized as a flavoring, while the bulbs taste like garlic and slightly bitter onion hybrid. *M. neglectum* flowers and buds may be pickled in vinegar; different parts including the leaves are edible in the grilled, boiled, raw or pickled state.[4,5]

Muscari species produce homoisoflavonoid compounds or homoisoflavonoids (HIFs) accumulated mainly in bulbs. A scillascillinoid homoisoflavanone was isolated and found out as 2′,5-dihydroxy-4′,7-dimethoxyspirol from the *M. neglectum* bulbs. Also,four previously discovered 3-benzyl-4-chromanones including 5,7-dimethoxy-3(4-methoxybenzyl)chroman-4-one, 4′-demethyl-3,9-dihydropunctatin) and scillascillin were isolated.[6] Two 3-benzylidene-4-chromanones, specifically, 7-hydroxy-5-methoxy-3-(3,4- dihydroxybenzylidene)chromane-4-one and 5,7-dihydroxy-6-methoxy-3-(3,4- duhydroxybenzylidene) chromane-4-one and as well as known homoisoflavanone were isolated from the bulbs.[7]

Succinic acid and uracil were detected in the aerial parts of *M. neglectum*. HIFs possess anti-inflammatory, antiestrogenic, estrogenic, angioprotective and anticancer biological activities.[8] *M. neglectum* HIFs were found to have an antioxidative effect.[9] The bulb extract containing 3-benzylidene-4-chromanones was found to have anticlastogenic and antimutagenic activities.[10] The HIFs obtained from the *M. neglectum* bulb ether extract were proved to have estrogenic activity in a dose-dependent manner by inducing MCF7 cell proliferation. The extract exhibited an antiestrogenic activity in the estradiol presence dose-dependently.[11] 32 bioactive compounds were identified in n-hexane extract from *M. neglectum* aerial parts in GC-MS analysis.[12] Alcoholic extract of *M. neglectum* has an antitrichomonal effect that was observed on *Trichomonas vaginalis* growth in vitro.[13] Different aqueous and ethanol extracts from *M. neglectum* herb and bulb showed antioxidant and cytotoxic activities.[14] Aerial parts and bulbs of *M. neglectum* contain essential oils that possess antimicrobial activity.[15] *M. neglectum* ethyl alcohol extracts from the bulb and leaves mixtures can be used as a wood preservative against fungi.[16]

Figure 2: Dried flowers of *M. neglectum.*

Ethanol extract of *M. neglectum* flowers was investigated by Iranian researchers.[17] It was characterized by total phenolic (18.2%), flavonoids (0.94%) and anthocyanin (0.11%) contents; extract demonstrated the anti-microbial antibacterial effect against *Candida albicans*, *Shigella flexneri*, *Aspergillus niger*, *Escherichia coli*. Based on the foregoing, this extract can be used as a natural coloring and preservative agent in foods and drugs.[17] To understand the chemical basis of the *M. neglectum* flowers' biological activity, the anthocyanin profile was determined using high-performance liquid chromatography and mass spectrometry.

EXPERIMENTAL

Materials and Methods

Chemicals and Reagents

HPLC gradient grade acetonitrile was acquired from Merck (Darmstadt, Germany). Acetic acid and methanol were purchased from Reanal-Ker (Budapest, Hungary).

Plant material

Flowers of *M. neglectum* were collected in Budapest, Hungary (47° 29' 19.20" N, 19° 2' 43.81" E) during the blooming phase in March-April 2019 and dried for one week at 25 °C (Figure 2). Plant samples were authenticated by Bokov D.O. in the Department of Pharmacognosy (Semmelweis University, Budapest). A voucher specimen was deposited at the Pharmaceutical Natural Sciences Department (Sechenov University, Moscow).

Sample Preparation

Dried and milled plant sample (0.3 g) was extracted with 20 mL of extraction solvent consisting of methanol-purified water-acetic acid (70:27:3). The ultrasound-assisted extraction was carried out in an ultrasonic bath at 30°C for 6 hours. The extract was filtered through filter paper and evaporated to dryness under reduced pressure with a rotary evaporator at 50°C. The dried extract was redissolved in HPLC grade methanol, and filtered through Phenex-RC 15 mm, 20 m syringe filters (Gen-Lab Ltd, Budapest, Hungary).

Determination of Anthocyanins

For chromatographic separation, an Agilent 1100 HPLC system (G1312A binary gradient pump, G1379A degasser, G1316A column thermostat, G1329A autosampler, and G1315C diode array detector) was used (Agilent Technologies, Waldbronn, Germany). Anthocyanins were separated on a Zorbax SB C18 column (150 mm × 3.0 mm, 3.5 µm; Agilent Technologies, Waldbronn, Germany) thermostated at 25 °C. Chromatograms were acquired at 520 nm, as the most selective wavelengths for the detection of anthocyanins. The UV spectra were recorded between 220 and 600 nm.

The following gradient elution program was applied at a mobile phase flow rate of 0.3 ml/min; where eluent A was

2.0% (v/v) formic acid in the water, eluent B was 2.0% (v/v) formic acid in acetonitrile: 0 min 10% B, 20 min 50% B, 25 min 100% B injection volume was 15 µL. Tandem mass spectrometric analyses were performed on an Agilent 6410 triple quadrupole system equipped with an electrospray ion source (ESI) in positive ion mode (Agilent Technologies, Palo Alto, CA, USA). Conditions were as follows: temperature: 350°C, nebuliser pressure: 45 psi (N_2); drying gas flow: 9 l/ min (N_2); capillary voltage: 4000V; fragmentor voltage: 100V. High purity nitrogen was used as collision gas, collision energy was changed between 10 and 50 eV according to differences in molecule structures. Fullscan mass spectra were recorded over the *m/z* range of 100–1600. The Masshunter B.01.03 software was used for data acquisition and qualitative analysis.

RESULTS AND DISCUSSION

The identification of individual anthocyanins was carried out based on the data of UV- spectra and mass spectrometry. UV-spectrum (1) and Mass spectrum (2) obtained in positive ion mode of compound No 5 (Muscarinin A) are shown in Figure 3.

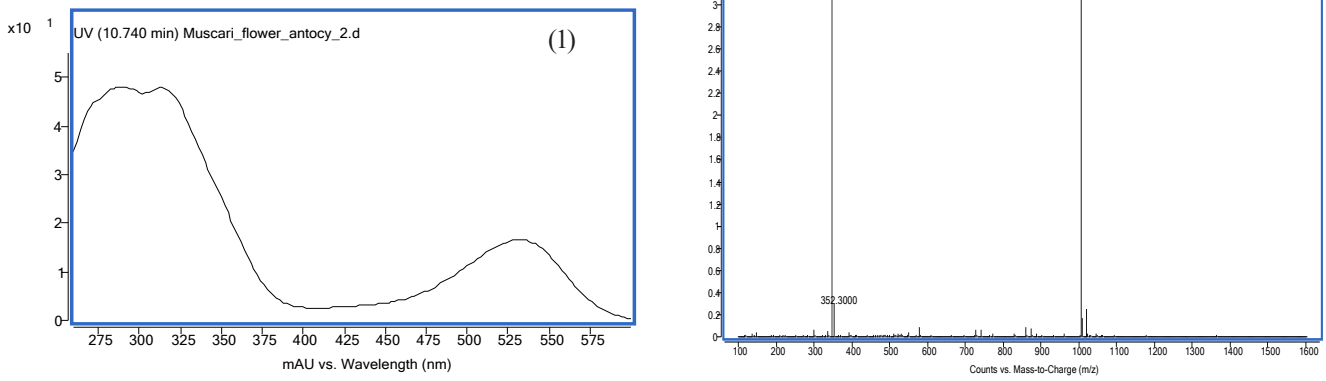


Figure 3: UV-spectrum (1) and Mass spectrum (2) obtained by scanning in positive ion mode of compound No 5 (Muscarinin A).

The UV spectra of compounds[1-10] showed characteristic absorption to anthocyanins structures. All of these compounds appeared as glycosides. The fragmentation behavior of anthocyanin-O-glycosides correlates with the glycosylation position. The HPLC chromatogram of the anthocyanin extract from the *M. neglectum* flowers is shown in Figure 4, mass spectra in Figure 5.

In total, 9 anthocyanins were identified in the tepals of *M. neglectum* (Figure 6, Table 1) for the first time. Peaks 1-3, 5-10, previously found in other grape hyacinths, were confirmed by the result of mass spectrometry.[18] Peak 4 had a molecular ion at a mass-to-charge ratio (*m/z*) 771 and three fragment ions, one at *m/z* 271 correspondings to pelargonidin aglycone, and two additional (*m/z* 609 and 447*)* produced by the neutral loss of *m/z* 162 Da, respectively, indicating the presence of two glucose units. The neutral loss of 176 Da (between fragment ions at *m/z* 447 and 271) pointed to a feruloyl group. Therefore, the compound was tentatively identified

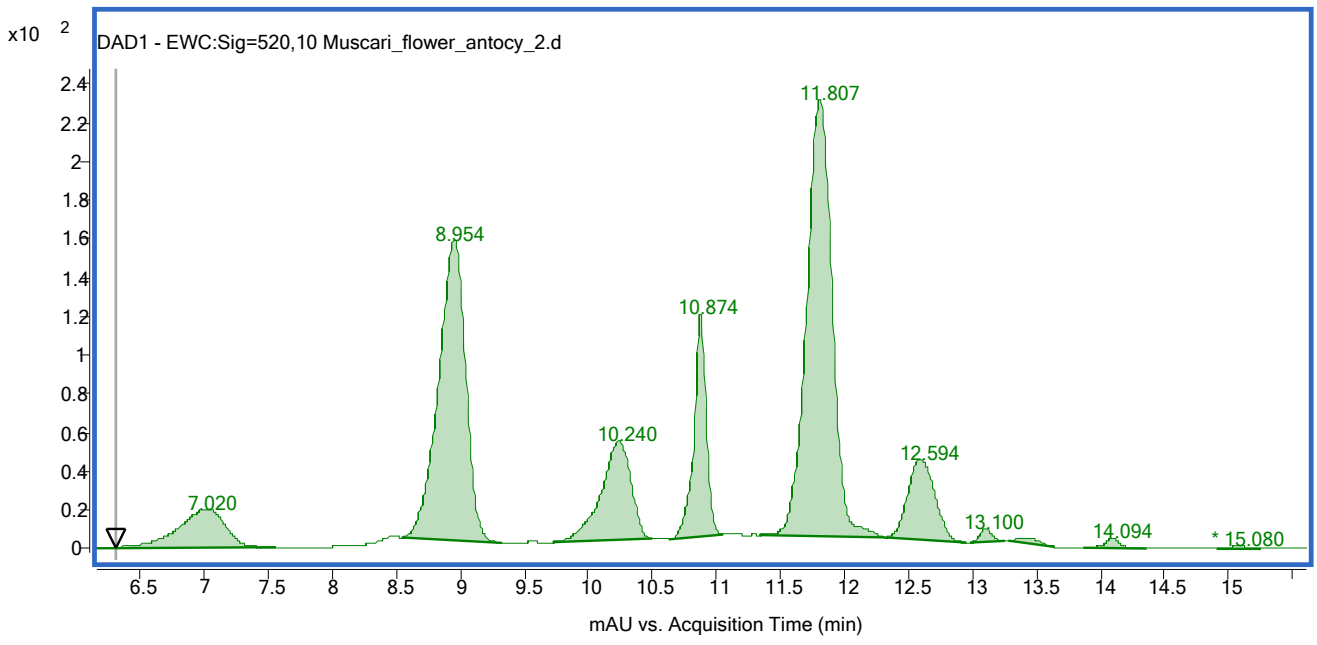


Figure 4: HPLC-UV chromatogram of *M. neglectum* anthocyanins.

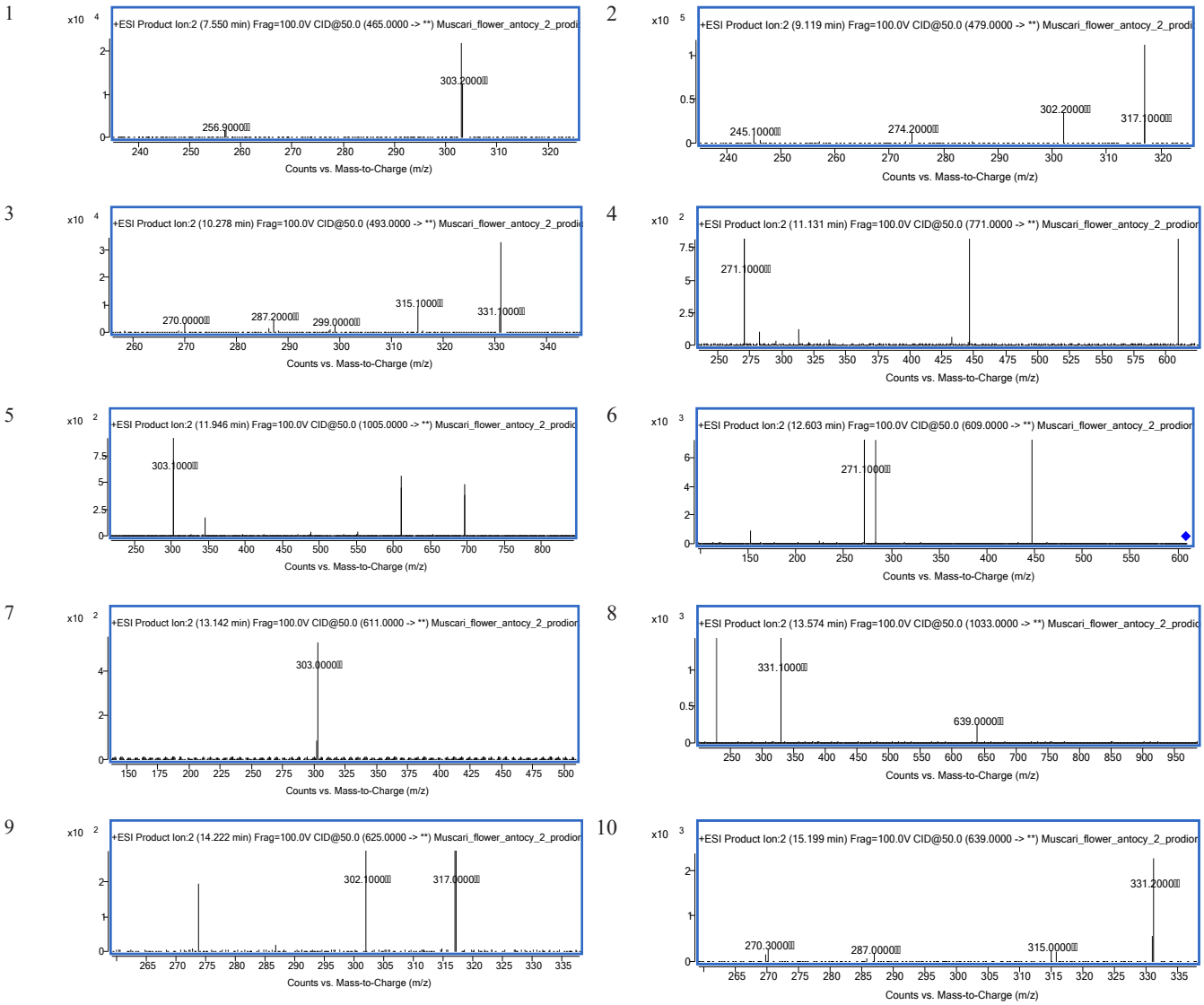
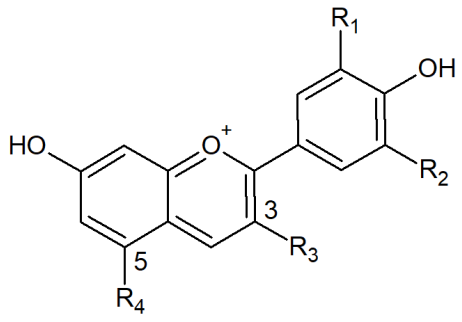


Figure 5: Mass spectra from the positive production mode analysis of *M. neglectum*.



1. R_1=OH, R_2=OH, R_3=OGlc, R_4=OH
2. R_1=OH, R_2= OCH_3, R_3= OGlc, R_4=OH
3. R_1=OCH_3, R_2= OCH_3, R_3= OGlc, R_4=OH
4. R_1=H, R_2= H, R_3=OferuloylGlc, R_4=OGlc; R_1=H, R_2= H, R_3=Oferuloyl(Glc)$_2$, R_4=OH
5. R_1=OH, R_2=OH, R_3= O-Glc-*p*-coumaroyl, R_4= OGlc(-Rha)-malonyl
6. R_1=H, R_2= H, R_3=OferuloylGlc, R_4=OH
7. R_1=OH, R_2=OH, R_3= OGlc-Rha, R_4=OH
8. R_1=OH, R_2=OH, R_3= O-Glc-*p*-coumaroyl, R_4= OGlc(-Rha)-malonyl + HCO
9. R_1=OH, R_2= OCH_3, R_3= OGlc-Rha, R_4= OH
10. R_1=OCH_3, R_2= OCH_3, R_3= OGlc-Rha, R_4=OH

Figure 6: Formulas of *M. neglectum* compounds No 1-10.

as pelargonidin-3-O-feruloyl glucoside-5-O-glucoside or pelargonidin- 3-O-feruloyl sophoroside.

The major peaks were No 2 (petunidin -3-O-glucoside) and No 5 (Muscarinin A). The structure of this major anthocyanins, as shown in Figure 6, represented about 65.58% of the total peak area.

Thus, in the *M. neglectum* flowers (tepals), nine individual anthocyanins were identified: delphinidin-3-O-glucoside

Table 1: Compounds detected in *M. neglectum*.

No	*Tr (min)*	*Precursor ion*	*Fragmentation*	*Molecular formula*	*Compound name*
1.	7.02	465	303, 229, 257	$C_{21}H_{21}O_{12}$	delphinidin-3-O-glucoside
2.	8.954	479	317, 302, 274, 245, 228, 217, 203	$C_{22}H_{23}O_{12}$	petunidin-3-O-glucoside
3.	10.24	493	331, 315, 299, 287, 270, 242, 179, 150	$C_{23}H_{25}O_{12}$	malvidine-3-O-glucoside
4.	10.874	771	609, 447, 271	$C_{37}H_{39}O_{18}$	pelargonidin-3-O-feruloyl glucoside-5-O-glucoside/ pelargonidin- 3-O-feruloyl sophoroside
5.	11.807	1005	697, 611, 303	$C_{45}H_{49}O_{26}$	muscarinin A
6.	12.594	609	447, 271	$C_{31}H_{29}O_{13}$	pelargonidin-3-O-feruloyl glucoside
7.	13.1	611	303	$C_{27}H_{31}O_{16}$	delphinidin-3-O-rutinoside
8.	13.407	1033	639, 331, 303	$C_{46}H_{49}O_{27}$	muscarinin A +formyl
9.	14.094	625	317, 302, 274	$C_{28}H_{33}O_{16}$	petunidin-3-O-rutinoside
10.	15.08	639	331, 315, 287, 270	$C_{29}H_{35}O_{16}$	malvidine-3-O-rutinoside

(1), petunidin-3-O-glucoside (2), malvidine-3-O-glucoside (3), pelargonidin-3-O- feruloyl glucoside-5-O-glucoside/ pelargonidin-3-O-feruloyl sophoroside (4), muscarinin A (5), pelargonidin-3-O-feruloyl glucoside (6), delphinidin-3-O-rutinoside (7), petunidin-3-O-rutinoside (8), and malvidine-3-O-rutinoside (9).

CONCLUSION

This research clearly shows that the applied HPLC-DAD-ESI-MS method is a powerful technique and utilized successfully for the characterization of anthocyanins in *M. neglectum* extract. Anthocyanins profile can serve asa chemotaxonomic indicator characterizing the biologically active compounds in *M. neglectum* herbal drugs and reported for the first time for this species. The obtained data can be used to enquire about biological activity, standardization of drugs, prepared from *M. neglectum*.

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