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Original scientific paper

NEW DATA ON THE DISTRIBUTION OF RARE PLANT SPECIES IN THE FLORA OF THE REPUBLIC OF N MACEDONIA

Vlado Matevski[1], Mitko Kostadinovski[2], Renata Ćusterevska[2], Olivera Matevska[3], Sara Cvetanoska[2], Cvetanka Cvetkoska[2], Angela Ivanova[2]

[1]Macedonian Academy of Sciences and Arts, 1000 Skopje, RN Macedonia
[2]Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University in Skopje, RN Macedonia
[3]Natural History Museum of the Republic of North Macedonia

*e-mail: vlado.matevski@manu.edu.mk

This article presents new data on the distribution of 15 taxa within the Flora of North Macedonia: *Anthemis auriculata* Boiss., *Astragalus hypoglottis* L. subsp. *gremlii* (Bur.) Greut. & Burdet, *Aubrieta* gracilis subsp. *scardica* (Wettst.) Phitos, *Bellardia trixago* (L.) All., *Coronilla coronata* L., *Fumana arabica* (L.) Spach, *Hippuris vulgaris* L., *Ilex aquifolium* L., *Lagoecia cuminoides* L., *Linum aroanum* Boiss. & Heldr., *Kitaibela vitifolia* Willd., *Odontites glutinosa* (M. Bieb.) Bentham, *Parietaria lusitanica* L., *Calamagrostis pseudo-phragmites* (Haller f.) Koeler, and *Romulea bulbocodium* (L.) Sebast. & Mauri. The article provides a list of new localities, contributing to the gradual completion of their distribution area within North Macedonia.

Kew words: flora; vascular plants; distribution; North Macedonia

INTRODUCTION

The results of floristic research conducted on the territory of Macedonia have been extensively documented in numerous scientific papers, spanning from the mid-19th century [1] until the present day. These findings have been comprehensively compiled in eight books of the Flora of the Republic of Macedonia (Flora of SR Macedonia, Flora of N Macedonia) [2–9]. Through continuous and ongoing floristic field research, new and previously unknown species have been discovered within the territory of North Macedonia, along with new localities for rare plant species that have been found to have a limited distribution within its borders.

The authors of this paper have conducted the latest field research in various regions of North Macedonia, leading to the identification of previously unknown localities for 15 vascular plant species. These species include *Anthemis auriculata* Boiss., *Astragalus hypoglottis* L. subsp. *gremlii* (Bur.) Greut. & Burdet, *Aubrieta gracilis* subsp. *scardica* (Wettst.) Phitos, *Bellardia trixago* (L.) All., *Coronilla coronata* L., *Fumana arabica* (L.) Spach, *Hippuris vulgaris* L., *Ilex aquifolium* L., *Lagoecia cuminoides* L., *Linum aroanium* Boiss. & Heldr., *Kitaibela vitifolia* Willd., *Odontites glutinosa* (M. Bieb.) Bentham, *Parietaria lusitanica* L., *Calamagrostis pseudophragmites* (Haller f.) Koeler, and *Romulea bulbocodium* (L.) Sebast. & Mauri.

MATERIAL AND METHODS

The new data presented in this study are derived from plant specimens collected during field research conducted by the authors in various regions of Macedonia. These herbarium specimens have been carefully processed and are currently stored at the Herbarium of the Institute of Biology, Faculty of Natural Sciences and Mathematics in Skopje

(MKNH). Detailed information regarding the geographical distribution, precise location, habitat, GPS coordinates, and collection dates are provided for each taxon. The comments section for each species includes references to relevant floristic literature. The authors have also supplemented the data with their personal observations on species habitats.

During the determination of the herbarium material, the authors consulted relevant literature, including "Prodromus Florae peninsulae Balcanicae, I-III" [10–12], "Flora Europaea, I-V" [13], "Flora of the Republic of Macedonia, 1(1-6)" [2–7], and other regional floras. They also referred to specific papers and databases focused on taxonomy, nomenclature, and chorology of the studied taxa. The nomenclature and taxonomy of the plants adhere to the Euro + Med (2006-) database [14]. Voucher specimens for all taxa have been carefully preserved within the Herbarium (MKNH).

RESULTS AND DISCUSION

Apiaceae

***Lagoecia cuminoides* L.** (Fig. 1 a, b)

Mk - Literature data: Demir Kapija, Bošava [15, 16], Veles-Ulanci; Dojran-Djopčeli, Star and Nov Dojran [7].

Mk - New locality: Negotino: Between Negotino and Krivolak, on clay, 200 m s.m., 2.06.2013 (coll. V. Matevski) (MKNH).

The Mediterranean plant species mentioned in the previous text is known to have a wide distribution within the narrow Mediterranean belt, stretching from Bulgaria to Portugal. Until now, it has been documented in only a few specific locations along the Vardar River, which is recognized as a phytogeographic corridor within the territory of North Macedonia. This corridor has facilitated the expansion of numerous Mediterranean plant species in a south-north direction. However, a new locality has recently been identified in the central part of North Macedonia, specifically in a steppe-like region around Negotino.

a) b)

Figure 1. a, b. *Lagoecia cuminoides* - Negotino-Krivolak

Aquifoliaceae

***Ilex aquifolium* L.** (Fig. 2).

Mk - Literature data: Nidže [17], Skopska Crna Gora [18], Javorlica, Garvan Klisura [16], Karadžica-Pepeljak; Nidže-Bela Reka [19], Demir Kapija-Došnica, Kožuf-Konjska Reka, Smrdliva Voda, Visoka Čuka, Keči Kaja, Belasica-v. Bansko [7].

Mk - New Locality: Jablanica-v. Vevčani, between v. Vevčani and Jankov Kamen, in the chesnut forest, 41.238288°N; 20.577483°E; 1168 m s.m., 10.07.2015 (coll. V. Matevski & O. Matevska) (MKNH)

To date, all recorded occurrences of this Mediterranean-Atlantic species within North Macedonia have been limited to the Vardar River and its tributaries, making them part of the Aegean catchment area. The vicinity of Vevčani (Jablanica Mountain) represents the sole known locality of this species in the western and southwestern regions of Macedonia, which falls within the Adriatic catchment area.

Figure 2. *Ilex aquifolium* - Jablanica Mt.: Vevčani

Asteraceae

***Anthemis auriculata* Boiss.** (Fig. 3)

Mk - Literature data: Dojran [20], Mariovo: Rasim Bej Most; Selečka Mt.- v. Kalen [21].

Mk - New locality: Kavadarci: v. Mrzen, dry grasslands, 10.06.2005 (coll. V. Matevski) (MKNH).

According to the Euro+Med Plant Base, Anthemis auriculata is known to have a restricted distribution in the Eastern Mediterranean and the southern regions of the Balkan Peninsula [AE (G) Bu Gr Mk Tu (A E)]. It is an intriguing and uncommon plant species with a limited range within the territory of North Macedonia. A new locality of this species has been identified in the central part of North Macedonia, specifically in Kavadarci - v. Mrzen. Recently, it has been the subject of diverse phytochemical research [22, 23].

Figure 3. *Anthemis auriculata* - Kavadarci: Mrzen

Cistaceae

***Fumana arabica* (L.) Spach** (Fig. 4)

Mk - Literature data: Dojran-Nikolič [24, 4].

Mk - New locality: Valandovo - Valandovsko Brdo, between Valandovo and v. Kosturino, in the belt of *Quercus coccifera*, 41.324581°N, 22.597107°E; 271 m s.m., 27.05.2005 (coll. V. Matevski) (MKNH)

Fumana arabica is naturally found in the southern part of Europe, ranging from Sardinia to Crimea. Previously, its presence in Macedonia was only documented in the vicinity of Dojran-v. Nikolic, as reported by Bornmuller [24] and Micevski [4]. However, the recent discovery of a locality near Valandovo confirms the continued existence of this taxon approximately 90 years later.

Figure 4. *Fumana arabica* - Valandovo - Valandovsko Brdo

Brassicaceae

***Aubrieta gracilis* subsp. *scardica* (Wettst.) Phitos** (Fig. 5)

Mk - Literature data: Šar Planina, Bistra, Korab, Dešat [4].

Mk - New locality: Galičica: Golem Kazan, screes, 40.936744°N, 20.825795°E, 2074 m s.m., 16.07.2010 (coll. R. Ćušterevska) (MKNH).

New locality for Galičica Mt.

Figure 5. *Aubrieta gracilis* subsp. *scardica* (Wettst.) Phitos - Galičica: Kazan

Fabaceae

***Astragalus hypoglottis* L. subsp. *gremlii* (Burnat) Greuter & Burdet** (Fig. 6)

Mk - Literature data: Krčin, Galičica [25, 6], Stogovo [26].

Mk - New localities: Jablanica Mt: Podgorečko lake, in mountain pastures and rocky, 1700–1900 m s.m., 7.9.2007 (coll. V. Matevski); Jablanica Mt - Vevčansko lake, Golina, mountain pastures and rocky, 41.244444°N, 20.536389°E; 1895 m s.m., 11.7.2015 (coll. V. Matevski & O. Matevska) (MKNH).

New locality is registered on Jablanica mountain (near Podgorečko and Vevčansko lake).

Figure 6. *Astragalus hypoglottis* subsp. *gremlii* - Jablanica: Vevčansko lake, Golina

***Coronilla coronata* L.**

Mk - Literature data: Skopje: Nova Breznica; Debar-Kosovrasti [6], Jakupica [27], Treska Gorge - Kapina, Poreče [28].

Mk - New locality: Galičica Mt: Krvov Kamen, pH 6,4; 1300 m s.m., SW, 34°, 17.6.2010 (coll. V. Matevski, A. Čarni & M. Kostadinovski) (MKNH)

New plant species for the Galičica mountain. It develops within the ass. *Querco pubescenis - Ostryetum carpinifoliae* Horv. 1938.

Linaceae

***Linum aroanium* Boiss. & Heldr.** (Fig. 7)

Mk - Locality from literature: Dešat-Krčin [7].

Mk - New locality: Galičica: 40,992891°N, 20,87048°E; 1488 m s.m., 08.07.2009 (coll. R. Ćušterevska) (MKNH)

Linum aroanum is a plant species native to the southern Balkans and Asia Minor, and it has a highly restricted distribution within North Macedonia. Previously, it was known to exist solely in subalpine pastures on Dešat Mountain-Krčin [7]. However, a newly discovered locality on Galičica Mountain has been found to thrive in similar habitats, at an elevation of approximately 1500 meters.

Figure 7. *Linum aroanium* Boiss. & Heldr. - Galičica: Tomoros

Malvaceae

***Kitaibela vitifolia* Willd.** (Fig. 8)

Mk - Literature data: Skopje: Vodno, Kitka, Skopska Crna Gora, Gorge of Treska, Pčinja; Demir Kapija; Crn Drmi-Lukovo; Gorna Belica [5].

Mk - New localities: Jablanica: Vevčani, between settlement Vevčani and Sveti Spas, in beech forest, 41.248997°N; 20.581352°E; 1289 m s.m., 8.09.2015 (coll. V. Matevski & O. Matevska) (MKNH)

Kitaibela vitifolia is tertiary plant species with Central Balkan and Asia Minor (Turkey) distribution. The primary occurrences of *Kitaibela vitifolia* are found in valleys and gorges, which are recognized as significant refuges for Tertiary plant species in the Balkan Peninsula. However, according to Stevanovic et al. [29], the habitats of this species are primarily anthropogenic in nature. These habitats include roadsides, vineyards, low scrublands, and the edges of degraded forests. This suggests that the occurrence of *K. vitifolia* is largely influenced by human activity. Consequently, *K. vitifolia* can be considered an element of the Tertiary flora that has successfully adapted to anthropogenic environments. Recently, similar habitats supporting this species have been discovered in Jablanica Mountain, near v. Vevčani, along forest roads within degraded beech and chestnut forests.

Figure 8. *Kitaibela vitifolia* - Jablanica: Vevčani

Scrophulariaceae

***Bellardia trixago* (L.) All.** (Fig. 9)

Mk - Literature data: Bitola-Crnovrška River (sub f. flaviflora Boiss.) [20].

Mk - New localities: Prilep: Debrešte-Debreška Krasta, dry grasslands, 41.484403°N, 21.339753°E; 760 m s.m., 23.06.2004 (coll. V. Matevski & M. Kostadinovski); Prilep: Mariovo-Sliva, wet places, 9.07.2013 (coll. V. Matevski, M. Kostadinovski & R. Ćuštere-vska); Prilep, v. Krivogaštani - Krajni Rid, hilly pastures, 41.345111°N; 21.317653°E, 674 m s.m., 17.06.2017 (coll. S. Cvetanovska) (MKNH); Bitola: Mariovo, between v. Rapeš and Staravina, 41.098092 ° N, 21.665271 ° E, 604 m, s.m., 18.06.2022 (coll. V. Matevski); Bitola: Rotino 41.0705955 °N; 21.197233 °E, 4.07.2023 (coll. M. Kostadinovski)

This plant species is exceptionally rare within the territory of North Macedonia and was previously known only in the vicinity of the city of Bitola [20]. However, recent discoveries have revealed its presence in three additional localities near Prilep, specifically in v. Debrešte, v. Krivogaštani, and Selečka Mountain.

***Odontites glutinosa* (M. Bieb.) Bentham** (Fig. 10)

Mk - Literature data: Baba, Luben [30]; Kozjak-Trojaci [31], Kapina-Oča [28], Vodno [32].

Mk - New locality: Prilep: v. Debrešte-Debreška Krasta, limestone, 780-850 m s.m., 17.06.2004 (coll. V. Matevski & M. Kostadinovski) (MKNH).

According to Bolliger [33], *Odontites glutinosa* (sub *Macrosyringion glutinosum*) is a plant species with a widespread but scattered distribution in the higher mountains of the Balkans, Anatolia, and the Caucasus. It can be found in the southern and southwestern parts of the Balkan Peninsula, including Serbia, Montenegro, Macedonia, Albania, Western and Southern Bulgaria, and Greece.

Within the territory of North Macedonia, *Odontites glutinosa* is considered a rare plant species. So far, it has only been documented in a few medium-high mountains, such as Luben, Kozjak-Pletvar, and Vodno. Typically, it is found in the belt of hilly pastures, similar to the newly discovered locality of the species on Debreška Krasta, near Prilep.

Figure 9. *Bellardia trixago* - Prilep: Debrešte-Debreška Krasta

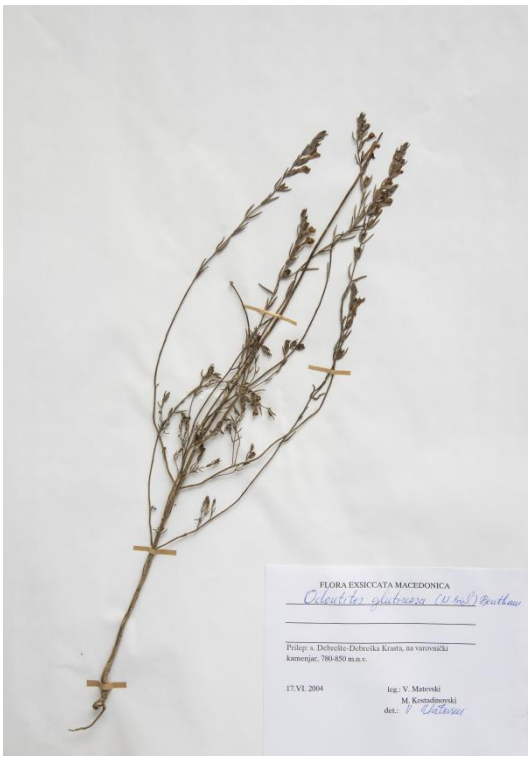

Figure 10. *Odontites glutinosa* - Prilep: Debrešte-Debreška Krasta

Hippuridaceae

***Hippuris vulgaris* L.**

Mk - Literature data: Ohrid and Struga Marsh [34, 6], Strumica [35].

Mk - New localities: Kratovo: v. Stracin-Suvo Ezero, peat bog, 42.185000°N; 22.002778°E; 918 m s.m., 19.06.2004 (coll. V. Matevski); Ohrid-Potpeš, 41.110636°N; 20.792288°E; 708 m s.m., 14.12.2020 (coll. V. Matevski & O. Matevska) (MKNH).

This amphibious plant species, widely distributed in the northern hemisphere, has a highly restricted range within the territory of North Macedonia. Previous data indicated that *Hippuris vulgaris* was solely known to exist in wetlands along Ohrid Lake and in the vicinity of Strumica [6]. However, recent investigations have confirmed its presence in Lake Ohrid and revealed another newly discovered locality near the village of Stracin, in the vicinity of Kratovo.

Urticaceae Juss.

***Parietaria lusitanica* L.** (Fig. 11)

Mk - Literature data: Dojran, Demir Kapija, Prilep:Kozjak, Kavadarci-Konopište [3].

Mk - New locality: Bitola - Mariovo: v. Grunište, under large granite rocks, 750 m s.m., 9.6.1996 (coll. V. Matevski & M. Kostadinovski) (MKNH)

In the Flora of Macedonia, the genus *Parietaria* is represented by three species: *Parietaria officinalis*, *P. diffusa*, and *P. lusitanica* [3]. The first two species are widespread throughout the territory, while *P. lusitanica* is an extremely rare plant, previously known from only a few localities. However, the distribution range of *P. lusitanica* in North Macedonia has expanded with the discovery of a new locality in the Mariovo area, near the village of Grunište. This species thrives beneath large granite rocks within the Crna Reka gorge, under the influence of a sub-Mediterranean climate.

Figure 11. *Parietaria lusitanica* - Bitola - Mariovo: v. Grunište

Figure 12. *Romulea bulbocodium* - Dojran: Debrešte-Nikolič

Iridaceae

***Romulea bulbocodium* (L.) Sebast. & Mauri (**Fig. 12)

Mk - Literature data: Dojran - Dojran Lake, Hasanli, Nikolič [36, 37, 38, 39, 40, 41], Valandovo - Anska Reka [36, 37, 39], Strumica - Bansko, Belasica, Novo Selo [36, 31, 35, 39], Bogdanci [36, 37], Gevgelija - Negorci [37], Radoviš, Plačkovica [42, 41].

Mk - New locality: Prilep: v. Krivogaštani - Krajni Rid, hilly pastures, 41.348892°N; 21.316575°E, 694 m s.m., 25.03.2017 (coll. S. Cvetanoska) (MKNH).

Romulea bulbocodium is a widely distributed plant species within the Mediterranean basin. It can be found along the Adriatic coast of the Balkan Peninsula and extends to the territories of Macedonia, Greece, Bulgaria, and Turkey [43, 44, 45, 46, 41]. In North Macedonia, it occurs primarily in the southern and southeastern parts, including the areas of Gevgelija, Bogdanci, Dojran, Valandovo, Strumica, and Radovis. It is typically found as a component of early spring therophytic non-nitrophilous vegetation, which develops under the influence of a sub-Mediterranean climate [41]. The populations of this species in all known Macedonian localities are predominantly located within the belt of *Quercus coccifera*. However, a newly discovered locality has been identified outside the *Quercus coccifera* area, specifically in hilly pastures in the central parts of North Macedonia, near Prilep.

Poaceae

***Calamagrostis pseudophragmites* (Haller f.) Koeler** (Fig. 13)

Mk - Literature data: Golešnica - Kadina Reka, 870 m s.m. (f. *persica* Boiss.); Skopska Crna Gora - Sv. Ilija, 1000 m s.m. (subvar. *exserta* Bornm.), Veles-Vardar [37, 38], Raduša [28].

Mk - New locality: Prilep: Mariovo-v. Bešište, gorge on the r. Satoka-Monastery of St. Petka, near the river, 800 m s.m., 20.07.1993 (coll. V. Matevski & M. Kostadinovski) (MKNH).

This plant species is typically found along water bodies, canals, and rivers throughout Europe. According to the online database "Plants of the World," its distribution range extends across various regions: Europe (central, southwestern, southeastern, and eastern), Asia-temperate (including Siberia, the Soviet Far East, Soviet Middle Asia, the Caucasus, western Asia, China, Mongolia, and eastern Asia), and Asia-tropical (India).

Within the territory of North Macedonia, this plant is considered very rare and has only been documented in the northern and central parts, specifically in the Skopje valley and the surroundings of Veles. However, a newly discovered locality has been identified in the Mariovo region, specifically in the Satoka River gorge.

Figure 13. *Calamagrostis pseudophragmites* - Prilep: Mariovo-v. Bešište

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НОВИ ПОДАТОЦИ ЗА РАСПРОСТРАНУВАЊЕТО НА РЕТКИ РАСТИТЕЛНИ ВИДОВИ ВО ФЛОРАТА НА РС МАКЕДОНИЈА

Владо Матевски[1], Митко Костадиновски[2], Рената Ќуштеревска[2], Оливера Матевска[3], Сара Цветаноска[2], Цветанка Цветкоска[2], Ангела Иванова[2]

[1] Македонска академија на науките и уметностите, 1000 Скопје, РС Македонија
[2] Институт за биологија, Природно-математички факултет,
Универзитет „Св. Кирил и Методиј“ во Скопје, РС Македонија
[3] Природонаучен музеј на Република Северна Македонија

Во овој труд се наведуваат нови податоци за распространувањето на 15 таксони од флората на РС Македонија: *Anthemis auriculata* Boiss., *Astragalus hypoglottis* L. subsp. *gremlii* (Bur.) Greut. & Burdet, *Aubrieta gracilis* subsp. *scardica* (Wettst.) Phitos, *Bellardia trixago* (L.) All., *Coronilla coronata* L., *Fumana arabica* (L.) Spach, *Hippuris vulgaris* L., *Ilex aquifolium* L., *Lagoecia cuminoides* L., *Linum aroanium* Boiss. & Heldr., *Kitaibela vitifolia* Willd., *Odontites glutinosa* (M. Bieb.) Bentham, *Parietaria lusitanica* L., *Calamagrostis pseudophragmites* (Haller f.) Koeler и *Romulea bulbocodium* (L.) Sebast. & Mauri. Со новите локалитети, постепено се заокружува нивниот ареал на територијата на РС Македонија.

Клучни зборови: флора; васкуларни растенија; дистрибуција; РС Македонија

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Original scientific paper

IRIS MEDITERRANEA spec. nov. - A NEW MEMBER OF NATIVE BALKAN FLORA AND VEGETATION

Milan Blažek[1], Vlado Matevski[2]

[1]Botanical garden in Průhonice, Botanical Institute of the Academy of Sciences of the Czech Republic, Czech Republic
[2]Macedonian Academy of Sciences and Arts, Research Center for Environment and Materials, 1000 Skopje, RN Macedonia

*e-mail: vlado.matevski@manu.edu.mk

Iris mediterranea species nova is a fertile natural species, similar to the vegetatively propagated, limited-fruiting cultural species *Iris germanica* L. (*Iris x germanica* auct. p.p.). The species have similar flowers, blue-purple, but with *Iris mediterranea* there do not occur the dark reddish-purple shades which are present in a significant number of clones of *Iris germanica*. In contrast with *Iris germanica,* except one clone found in culture, there are no known white-flowered plants of *Iris mediterranea* yet. In contrary, photos published in public discussions show samples of *Iris mediterranea* from distanced places in Greek mountains with bicolor flowers: standards are pure white, falls blue or even deep purple. Such color combination is not known in *Iris germanica*. Habitus is similar in both species, but *Iris mediterranea* has more abundant branching in many of its clones.

The majority of *Iris mediterranea* plants are similar in coloration to the known clones of *Iris trojana* Kerner ex Stapf, which correspond in flower size to selected large-flowered clones of *Iris mediterranea* known from culture. *Iris trojana* usually has also a larger number of flowers per stem.

In flowering time, *Iris mediterranea* is intermediate between *Iris germanica* and *Iris trojana*.

Key words: *Iris mediterranea*; new species; Balkan; flora

INTRODUCTION

In the Botanical Garden of the Botanical Institute of the Czechoslovak (later Czech) Academy in Průhonice, since the establishment of the garden and joining of Milan Blažek in this department (1963), a program of studying the relationships between the variability of wild and cultivated Irises has been carried out. The aim was to create study and exhibition collections of living plants with documentation leading to strictly systematic collections and to the conservation of endangered taxa [1]. The solution of the problem was associated with the corresponding gained experience and development of the necessary knowledge, based on gradually acquired representative study material.

Experimental hybridization was one of the basic working methods in the first decades, with the aim of explaining the relationships between species, together with the verification of breeding possibilities as interspecific hybridization has played an important role in the development of the genus *Iris*.

Particular attention was paid to the genetic resources of garden Irises and their botanical and genetic characteristics. This has resulted in one of the world's largest institutional collections, characterizing the complete historical development, and the largest collection of archaic cultivated Irises of the *Iris* section in relation to their natural resources. Maintaining this section of the collections is an ongoing priority for the garden.

There is a wealth of information in the literature on plants in this category - taxa on the boundary between wild and cultivated plants. Due to inconsistent interpretation of certain species names and their occasional misinterpretation, it has been and continues to be necessary to obtain as much rich, largely even unidentified living material as possible from all available sources. One of the key areas of interest was of course *Iris germanica* as a symbol of cultural Irises, the botanical nature of which was and is still inconsistently understood.

Iris germanica is the type species of the genus Iris. It is a name with a confusing interpretation since there are three biologically distinct groups to which the name *Iris germanica* is applied:

A - This view was supported by a series of cytological studies done in the previous century. If we here use the name *Iris germanica*, or *Iris germanica* sensu stricto, our interpretation of the name is that which we consider to be the closest to C. Linné's conception. This specification was supported by a series of cytological studies which confirmed its specifics. *Iris germanica* is a group of aneuploid sterile hybrids (2n = 44) [2, 3] that have been in culture throughout the world since ancient times. *Iris germanica* is one of the three names that C. Linné used for historical hybrid species of the genus Iris. The others are *I. sambucina* and *I. squalens*. These have 2n=24 and different ancestry [2, 4–11].

B - Many botanists use the name *Iris germanica* L. also for similar, but fertile tetraploids having 2n = 48. These were imported Irises from the East Mediterranean area, and awoke high interest in western Europe around 1900 and later. Many of them were collected as cultivated plants whose geographic origin is difficult to pinpoint. We treat them as *Iris germanica* sensu lato.

Views on the taxonomic value of them vary. Some authors treat these tetraploids as autonomous species, others refer to them as synonyms of *Iris germanica* L. This concept is mainly reflected in publications dealing with wild Irises [12–15]. In our work, we divide these tetraploid Irises into two groups, i.e., "germanicoids" and "aphylloids," according to their proximity to either *I. germanica* L. or *I. aphylla* L. [16].

C - Some practicing Iris growers use the name "*Iris germanica*" for all the tall bearded Irises, mostly with 2n = 48, and also for genetically distinct diploid (2n = 24) cultivars. By their origin, those diploids have little in common with *I. germanica* L., both *I. germanica* sensu stricto or sensu lato. They are close to *I. pallida* Lam. and *I. variegata* L.

Information on the origin of all known *Iris* cultivars is periodically compiled by the American Iris Society in the Alphabetical Check List. The first edition covers the history up to 1939, new registrations are now published annually.

Before 1900, diploid bearded Irises made up the majority of the tall bearded Irises in cultivation. Some of these cultivars were even the selected individuals of the original species, *I. pallida* Lam. or *I. variegata* L., not their hybrids.

Many other species were added to the hybridizing program later. Specialized gardeners call them *Iris x barbata* hort., and restrict using the name *Iris germanica* name to certain antique cultivated clones only. Naming them "*Iris germanica*" is improper. This is not in accordance with C. Linné's conception of the concerned species.

Most of present time tall bearded cultivars have in their pedigree *Iris germanica* sensu lato but extremely rarely *Iris germanica* sensu stricto.

NEW SPECIES

In the countryside of North Macedonia, Greece, and Albania, there is a native tall species of the genus *Iris*, which, due to its similarity to *Iris germanica* L. and known tetraploid species, is often identified as Linnaeus's *I. germanica* [14, 15].

The first encountered sample of it was from the University Botanical Garden in Zagreb, where it was then designated *Iris macedonica* Horvat. Unfortunately, the name *macedonica*, regardless of its factual rightness, could not be accepted for formal reasons.

These plants are not identical with any of the known species. This fact became the reason for the description under the new name - *Iris mediterranea* spec. nova. It is an endemic of the Prespa Tri-border.

MATERIALS AND METHODS

After the receipt of the first specimens from the Botanical Gardens of Zagreb (origin at Galičica, North Macedonia) and Sofia (origin at Ostravica, Albania) in the first years of the existence of the Průhonice Botanical Garden, this species, under the working name "*Iris macedonica*", was included among the open working problems of the working program.

The long processing time results from the necessity of obtaining sufficient samples of concerned plants in a region distanced from their place of occurrence, both in nature and in culture. With the long-

time limitation of possibilities to move outside our own national territory, maximum use was made of communication with botanical and horticultural specialists at the international level. In the course of time, we gradually obtained additional rich, representative collection of plants both from culture and nature.

Our successful sampling of these Irises *in situ* was only in the summer and autumn without the chance to see them in bloom and to select outstanding individuals. Nevertheless, our collected Irises, when they later bloomed, indicated that they came mostly from quite variable populations.

Samples of similar clones originating from Balkan countries were in the meantime obtained also from specialists of the American Iris Society and from European private collectors. The result was a collection that allowed long-term comparisons of plants of different origins at one workplace.

In the course of the research, significant monographic publications on the genus Iris, which refer to the territory of the Balkan Peninsula and Europe, were used [31–35].

This final, summarizing publication is realized in cooperation with Prof. Dr. Vlado Matevski, representative of the Institute of Biology, Faculty of Natural Sciences and Mathematics, Skopje, and Macedonian Academy of Sciences and Arts.

RESULTS AND DISCUSSION

The specific name *Iris mediterranea* was chosen in view of the geographically-sounding common names of a number of related species of the genus *Iris*, in order to emphasize their home area. This is the case of wild species, described according to their natural habitat, such as *Iris bohemica* Schmidt, *Iris bosniaca* Beck, *Iris croatica* Horvat, *Iris cypriana* Foster et Baker, *Iris hellenica* Mermygkas, *Iris hungarica* Waldst. et Kit., *Iris illyrica* Tommasini or *Iris kashmiriana* Baker, as well as of a species of clearly hybrid origin such as *Iris* x *germanica* L. In this case, however, Germany was the country of origin of the plants described, not the place of actual historical origin.

In contrast, *Iris mediterranea* is a species with a corresponding natural character, but it is commonly referred to in the botanical literature by the name of *Iris germanica*, a similar ancient hybrid with an unknown place of true origin. As a result, it has never been given enough attention, even though it can hardly be the Iris that has been described as *I. germanica* by C. Linné.

***Iris mediterranea* Blažek & Matevski spec. nova** (Figures 1–4)

Figure 1. The new species *Iris mediterranea* Blažek & Matevski on natural habitat in locality Matka near Skopje (N. Macedonia) – habitus (photo V. Matevski)

Description

Rhizome stout, horizontal. Stems 40–80 cm tall, stout, green, always branched, sometimes the lowest branch is below the middle of the stem and is further branched. *Leaf* length 30–60 cm, the dry leaf apices brown. *Inflorescences* with 4–8 flowers. *Bracts* at the apex of the inflorescences always with a dry, non-green terminal part; the mean length of the bracts at the top of the stem is about 30 mm, the width slightly less than half that. *Flowers* basically bicolored. Generally, *standards* are light blue or purplish blue, the *falls* are slightly or, more often, distinctly darker with a purple tinge (see Figures 7 and 8). *Standards* obovate 75 × 45 mm with a short haft (13 mm). *The beard* (a narrow band of about 3 mm long hairs) is darker toward the center of the flower, and usually pale yellow at the tip. Only occasionally the ends of the hairs are distinctly deep yellow. Sometimes faint blue tints are found in the bases of hairs in the beard. *Falls* slightly longer and narrower than standards, 80 × 45 mm wide. The beard reaches from the base to a third of the falls. *Stamens* 30–32 mm, filaments and anthers with equal length. *Ovary* 14–20 mm long. *Style* ca. 35 (38) mm long, with 8–9 mm long stigma lobes. *Fruits* oblong-oval 40–60 (75) mm long with a rounded, only slightly triangular cross-section and a pointed apex. *Seeds* usually 6–7 × 5 mm, bronze-brown to deep brown.

HOLOTYPE: North Macedonia, Skopje, Matka - gorge of the river Treska, on the left side of the artificial lake "Matka", on limestone rocks, 41.943199 N; 21.304656 E; 317 m.s.m. 07.05.2023 (Holotype: MKNH 00 – 90; Isotype: Průhonice) (Figure 4).

Distribution in North Macedonia: Skopje: Matka-Gorge of the Treska River; Struga: Gorge of Crni Drim River, between Struga and Debar; Prespa: Stenje village, near the stone beach.

Ecology of *Iris mediterranea*

From a geobotanical point of view, the typical population of the species *Iris mediterranea* in the nature develops on a carbonate substrate related to the communities of the dry grasslands which phytocoenologically belong to the newly described vegetation alliance *Diantho haematocalycis-Festucion hirtovaginatae* Matevski et al. 2018 [17].

At the same time, this species often occurs in crevices of calcareous rocks, which is part of the chazmophytic vegetation from the alliance *Ramondion nathaliae* Horvat ex Simon 1958, order *Potentilletalia speciosae* Quezel 1964 and the class *Asplenietea trichomanis* (Br.-Bl. In Meier et Br. -Bl. 1934) Oberd. 1977 (Fig. 1–3).

The population of this species on the territory of North Macedonia develops under the influence of the modified sub-Mediterranean climate, which penetrates to the Skopje Valley, through the famous phytogeographic corridor of the Vardar River, as well as in the Ohrid-Prespa region, where the sub-Mediterranean influence reaches through the Black Drim and Shkumba rivers. It is known that the valley of the Vardar river functioned as a phytogeographical corridor during the Pleistocene period, when the Demirkapija gorge was formed, with the descent of the Thessaloniki Lake, which connects to the Aegean Sea [18].

Figure 2. The new species *Iris mediterranea* Blažek & Matevski on natural habitat in Matka near Skopje N. Macedonia – flowers (photo V. Matevski)

Figure 3. The new species *Iris mediterranea* Blažek & Matevski on natural habitat in Matka (near Skopje, N Macedonia) – flower (photo V. Matevski)

Figure 4. *Iris mediterranea* Blažek & Matevski – Holotype (photo I. Blažev)

Characteristics of *Iris mediterranea*

Iris mediterranea spec. nova. belongs to the Eurasian species of the genus *Iris*, Section *Iris* (bearded Irises), with a branched stem whose height varies between 50 and 80 (up to 120) cm. The color of the flowers, as in similar species, is blue-purple. The shades vary from almost pure blue to deep purple flowers. Two-colored (bicolor) forms predominate, with the falls being darker and more purple in tone.

The yellow basic color is absent in this species. It is found only in hybrids with shorter local species. The occurrence of white-flowered individual of *Iris mediterranea* has so far been confirmed by only one record in culture. Occasionally, very rare individuals with white standards may be found.

The flowers of *Iris mediterranea* and similar species are generally similar in shape to each other in the proportions of the size of the individual parts. The visible and definable differences between them are mainly in the branching of the stems and the time of flowering. The bracts (spathe valves) of all these species are roughly two-thirds herbaceous at the time of peak flowering whilst the upper part is scarious, brownish.

Figure 5. *Iris mediterranea* from Vikos, blind collected, does not show any basic difference from the samples from Matka (photo M. Blažek in the home)

Figure 6. *Iris mediterranea* Blažek & Matevski – Botanical garden in Průhonice, Czech Republic (photo M. Blažek)

Stem height and leaf size vary in the given range, as in related species. Even differences in additional coloring of bracts give the impression of individual variation only. Some are slightly to strongly purple suffused, some not. Leaf coloration is uniform in *Iris mediterranea*. As in closely related Irises (*Iris germanica* or *Iris trojana*), a distinctive purple basal leaf color was not observed. It is, however, known among certain individuals of *Iris nyárádyana* [19], which is close to *Iris croatica* [20].

An important characteristic of each of these species is their position in nature. Among the taller tetraploid species, *Iris mediterranea* is the only species among the known plants of this category where the natural occurrence clearly prevails over the occurrence in culture.

In contrast, other species except *Iris croatica* predominate in culture. Thus, their names are based on descriptions of individuals from secondary habitats, with no subsequent possibility to study natural variability.

Basic features of *Iris mediterranea*

Flower color

The flowers are basically bicolored, where the standards have a light blue or purplish blue color, while the falls are slightly or, more often, distinctly darker with a purple tinge. The beard is usually pale yellow.

There is also variation in the veining on the sides of the beards, which varies from thin dark purple veins to heavy veining, sometimes accentuated by an obvious brown tinge on a yellowish ground.

Flower size

The standards are obovate with a short haft (13 mm), with a total length of 77 mm and a width of 48 mm. The falls are slightly longer and narrower, 82 mm long and 47 mm wide. The beard reaches from the base to a third of the falls.

Figure 7. *Iris mediterranea* Blažek & Matevski, dimensions of flower elements - Botanical garden in Průhonice, Czech Republic (photo M. Blažek)

Figure 8. *Iris mediterranea* Blažek & Matevski, flower variability - Botanical garden in Průhonice, Czech Republic (photo M. Blažek)

The ovary is 14–20 mm long, and the corresponding perianth tube is the same length.

The style arms are 36–38 mm long, followed by 8–9 mm long stigma lobes. The width of the arms is 14–16 mm.

Stamens 30–32 mm, filament and anther are of equal length.

Bracts

The bracts at the apex of the inflorescence always have a dry, non-green terminal part. As flowering progresses, its roughly one-quarter to one-third proportion increases at the expense of the herbaceous part. The latter is either green only or slightly to very strongly colored purple on a green ground. The mean length of the bracts at the top of the stem is about 30 mm, the width slightly less than half that.

Leaves

The leaves do not show any distinctive specific features in shape and size. Leaf length varies between 30 and 60 cm. The most common summer length is between 40–50 cm, and depending on the condition of the plant, there may be a difference of 15–20 cm between low and high fans within a clump. Winter leaves are short, but not completely lost.

The dry leaf apices are brown. Purple coloration of the leaf bases, as commonly encountered in Europe in, e.g., *Iris variegata* or *Iris aphylla*, has not been observed in this species.

Figure 9. *Iris mediterranea* Blažek & Matevski, branching - Botanical garden in Průhonice, Czech Republic (photo M. Blažek)

Stem

The stem, 40–80 cm tall, always branched and usually bears 4–8 flowers. Sometimes the lowest branch is below the middle of the stem and may be further branched. The number of flowers varies, of course, between individual stems of the same clone and often within a clump.

Fruits and seeds

The oblong-oval capsules with a rounded, only slightly triangular cross-section and a pointed apex are 40–60 (36–75) mm long and 20–28 mm wide. The ripe fruits open in the first half of August by opening the carpels from the top to the middle. The bronze-brown seeds begin to dry and darken to deep brown. An average number in a pod is about 50. Although the seeds are primarily ovoid in shape, the greater number of seeds growing in the capsule leads to being crowded and depressed. The ripe seeds are then mostly flattened on the sides and finally they develop in two rows in each of the thirds of the capsule. The dry seeds are usually 6–7 × 5 mm in size.

In culture, this species is not essentially different from related species. *Iris mediterranea* is evenly unproblematic, like *Iris germanica*, in contrast with *Iris albicans*, which is tender in colder climates.

Relation of *Iris mediterranea* to similar species - its position among similar Eurasian Irises

When describing *Iris mediterranea* as a species with its own name, it was necessary to define its position among other Irises, to analyze relationships, not only to *Iris germanica*, but also to other similar species, and also to compare it with known historical cultivars which occasionally give the impression of being native plants when they become naturalized.

In practice, by having to concentrate on the obvious, visible characters, the possibility of identification is quite limited. Conclusions depend on whether the species is judged by appearance of a chance sample or as a biological unit.

Reliable species identification requires a broader knowledge and cannot be made without comparisons. A simple, basic description of *Iris mediterranea* is sufficient for reliable identification only when considering a flowering natural population of the species within its native range. Identifying a single plant, removed from its natural habitat, is problematic for those who lack knowledge of related species. Here it is necessary to allow for the possibility of confusion with similar species, and to concentrate on their differences, which are essential but not readily apparent at first sight.

The flowering periods of *Iris mediterranea* and similar species do not completely overlap. *Iris mediterranea* fills the time space between the flowering of *Iris germanica* and *Iris trojana.*

These species are united not only by similar proportions but also by the color of their flowers: in a common sample of a medium-sized flower of *Iris mediterranea*, the flower size varies between the average size of *Iris germanica* or *Iris pallida* and that of *Iris trojana*. The shades of color are always just blue to purple in all of them, but the color shades vary within each species; they all contain only the basic colors of anthocyanin origin.

Generally, the standards tend to be blue and the falls purple shades. In this *Iris mediterranea* is close to *Iris germanica*, but even more so to *Iris trojana*, which some individuals of *Iris mediterranea* may strongly resemble.

All of these mutually similar species lack yellow flowers. White-flowered plants are commonly found only in *Iris germanica*, not in similar species.

Absence of yellow-flowered plants among all *Iris germanica* - like Irises is one of facts suggesting their distant genetic relationship to *Iris variegata*, although it is the only taller species occurring near natural localities of *Iris mediterranea* (in its northernmost areal).

The significant and clear, but not directly visible, unifying and separating element is in the *cytological sphere*. In all the taller species of the *Iris* section, the basic chromosome number is 12. With exception of *I. germanica* s.str. and *I. albicans* (2n = 44), other species are partly diploid, with 2n = 24, partly tetraploid, where 2n = 48 [11].

Both groups, diploids and tetraploids, are fully fertile, and the respective species interbreed readily.

Crossing occurs not only in culture but also in nature when two compatible species come into proximity with each other. The morphological character of interspecific hybrids and flowering time are usually intermediate.

However, it is not easy to interbreed diploids with tetraploids. In more distant hybrids, where species based on base numbers 8 and 12 are combined, fertility is limited [11, 21, 22]. Of the botanically described hybrids, *Iris germanica* L. (2n = 44) [2, 3] is such a basic distant hybrid.

Its close relative *Iris albicans* Lange, which is at the lower limit of the height of the Irises discussed here, has the same cytological problem.

Comparison of *Iris mediterranea* and *Iris germanica* L.

Characteristics of *Iris germanica* L.

Iris germanica L. is a small group of hybrids with limited fertility [3, 6, 11, 21, 23].

During 50 years of work on the completion of the Průhonice collection by collecting plants from various sources and obtaining them from all internationally active growers, after eliminating numerous duplicates, fewer than 30 clones could be found that can be unambiguously identified as *Iris germanica* L. Of the other Irises, described as species, is in the Mediterranean countries abundantly cultivated only *Iris albicans* Lange which is closest to it.

On the basis of published cytological analyses [11, 22], *Iris germanica* is the result of a cross between dwarf, early flowering west-Mediterranean *Iris lutescens* /2n = 40/ s. l., and a tall, later flowering *Iris* (2n = 24 or 2n = 48). Our verification cross of *Iris* ´Macrantha´ × *Iris lutescens* produced white seedlings that had all the basic parameters of the collected *Iris germanica* clones where white is also common.

Iris albicans seems to have originated from more southern parents. It freezes in winter in central European environments. It is shorter and earlier than *Iris germanica*, and is widely known in only two clones. Its white-flowered form is widespread in Mediterranean climates. The blue-flowered form, called the ´Madonna´ cultivar, is much more rare.

Figure 10. *Iris germanica* L. – Botanical garden in Průhonice, Czech Republic (photo M. Blažek)

Differences between *Iris germanica* L. and *Iris mediterranea*

The hue of the flower colors is little different. In particular, the color of the standards of *Iris mediterranea* is closer to pure blue, while *Iris germanica* is more often purple toned. Two-toned to bicolor flowers are found in both species. Monochromatic flowers, found occasionally in *Iris mediterranea*, may be so close to those of *Iris pallida*, that the flowers without green parts are confusable. Such cases are unknown in *Iris pallida* and *I. germanica*.

Stems and branching

The branching of *Iris germanica*, with one- or double-flowered apex and two to three single-flowered branches (4 flowers on the stem in total), is the minimum branching of *Iris mediterranea*. Branching is usually more abundant in *Iris mediterranea*. Similarly like with *I. germanica*, there is one flower between the bracts at the apex, or two. The two apical flowers are in *Iris mediterranea* sometimes slightly offset (1–2 cm), not consistently opposing each other between the supporting bracts.

Single flower at the top of the stem is in this species quite common.

A typical feature of the branching of *Iris mediterranea* is the secondary branching of the lower stem branches of the strongest individuals, so that the maximum branching on a single stem can be twice the minimum number of flowers compared to the four-flowered *Iris germanica* (up to 8). This brings *Iris mediterranea* close to *Iris trojana*.

Another difference is the somewhat *later flowering* of *Iris mediterranea*. It blooms in culture about a week after *Iris germanica*, thus following it and filling the time gap before *Iris trojana* blooms.

Fertility

The significant difference is in the cytology and the associated possibility of generative reproduction. Both species spontaneously produce fruits, but while in *Iris mediterranea* the ripe capsules are full of normally developed and germinating seeds after pollination by any compatible species, in *Iris germanica* a capsule full of seeds is not encountered. There are at most ten, and even these are often not viable. An exception is *Iris germanica* var. *Kharput*, where over 20 good seeds have been found in one fruit. This variety also has other specific features that distinguish it from other clones of *Iris germanica*.

The seeds of *Iris germanica* ripen in Central Europe in the first third of August, a few days earlier than those of *Iris mediterranea*. There is a small difference between concerned species.

Both have oval fruits but those of *Iris germanica* are more triangular in cross-section. The size of its capsules is 40-70 mm, the most common length is 50-60. Width varies proportionally from 14 to 24 mm, with 18 to 20 being common.

There is a considerable difference in the development of the seeds from the pollinated flowers of two species. In these experiments we have used widely the method received from P. Werckmeister. In *Iris germanica*, regardless of the pollinator, only a few of the large number of eggs (currently over 50 eggs in a single fruit) will produce viable seeds. Some of resulting, normal looking seeds have a properly formed embryo but a watery endosperm. When the embryo dries, it dies and the seed flattens to a thickness of just over 1-2 mm. Even in those seeds where a solid endosperm has formed, only a small number are able to germinate. Interestingly, some of the fresh endosperm-less seeds are visibly larger than those with a normal solid endosperm. They can be easily identified by the fact that they can be cut in half with a soft object. Well-developed seeds will not allow this.

The fruits open mostly by opening from the apex of the pod about halfway. There are different transitions between the different clones of *Iris germanica* to opening through the slits under the closed apex of the capsule, similar to the dwarf Irises of *Iris pumila* and *Iris lutescens* group. The basic shape of the seeds is ovoid, and because of their small number are uncrowded, so that this shape persists until the fruit is mature. Otherwise, the well-formed seeds do not differ from those of *Iris mediterranea*.

Table 1. Comparison of the differences between *Iris mediterranea* sp. nov. and *Iris germanica*

Feature	***Iris mediterranea***	***Iris germanica***
measurements	similar	similar
blooming time	a week later	a week earlier
prevailing purple shades of flowers	blue-purple	red-purple
flowers resembling *I. pallida*	occasionally	never
occurrence of albinos	extremely rare	almost 20 % of known clones
white standards, colored falls	extremely rare	never
count of flowers per stem	varying between 4 and 8	stable 4
more flowers on lower branches	occasionally	rarely
forming pods	abundantly	occasionally
quality of seeds	normal	mostly malformed
known obviously wild populations	frequent	not proved
geographic area in wild	limited to MK, AL, GR	unlimited
variability in wild populations	high	none
assumed territory of origin	South-Eastern Mediterranean	South-Western Mediterranean
assumed hybrid origin	not known	confirmed by cytological analyses
occurrence in culture	occasionally cultivated	exclusively cultivated (or escaped)
history of culture	till 1900 Mediterranean only	start not detectable, now world-wide

Hypothetical possibility of genetic relationships between *Iris germanica* and *Iris mediterranea*

Respecting *I. mediterranea* as a distinct species may facilitate the explanation of relationships of species whose phylogenetic development has been putatively subject of hybridization. From this point of view, it is possible to develop a new version of the explanation of the origin of the aneuploid hybrid *Iris germanica* sensu stricto (2n = 44): whether its tall parent from the x = 12 group was *Iris pallida* with 2n = 24 [3, 29] or one of 48-chromosome forms of "*Iris germanica*" *(= Iris germanica* sensu lato*)* [11, 22, 24].

In *Iris germanica*, when comparing various clones, there are indications that, as a hybrid with limited fertility, it did not evolve continuously from a single common base, but that it arose in multiple locations from different combinations of two types of parents: one from the group with 2n = 40 (*Iris lutescens* s. l.) by crossing with a tall diploid *Iris* with 2n = 24, or, more likely, with a tetraploid species with 2n = 48.

The character of the cultivated clones of this largely blind evolutionary branch suggests the likelihood that these Irises have repeatedly arisen from several shorter taxonomic units with some of the taller Irises. Only two native tall species are known in the wild of southern Europe: *Iris pallida* and *Iris mediterranea*.

The cytological barriers make the possibility of *Iris germanica* originating in *Iris pallida* less likely, but conversely the involvement of *Iris mediterranea* in the origin of hybrids with dwarf species is easily reproducible in practice at any time. At present, the ranges of these *Iris* species do not overlap, but this is an area with several thousand years of extensive human intervention in nature**.** If it were the case that the ancestor of *Iris germanica* was *Iris mediterranea*, it would be incorrect to refer to the original species (*Iris mediterranea*) by the name of its hybrid (*Iris germanica*).

Similarly, *Iris mediterranea* may also serve to studies concerning further species and hybrids occurring in Balkans.

The question arises as to why this simple hypothesis could not have been advanced by the authors of the cytological analyses of the middle of the last century explaining the origin of *Iris germanica*. The answer is simple: *Iris mediterranea* was not known at that time as a separate species.

Comparison of *Iris mediterranea* with *Iris trojana* Kerner ex Stapf

The difference in external similarity is slightly less than the difference between *Iris mediterranea* and *Iris germanica*, but there is considerable agreement in the characters linked to cytological conditions and underlying generative reproduction.

The practical problem is the lesser possibility of studying in detail the corresponding number and corresponding botanical reliability of compared plants. Obtaining an original individual of *Iris trojana* required an effort spanning several decades, yet resulted in only four clones that can be considered *Iris trojana*. One matches the description and portrait in the W. R. Dykes monograph, three were obtained under different names, one nameless [5, 23].

It has never been possible to verify the existence of an original natural population of *Iris trojana*, although a secondary occurrence is very likely in this species that has been cultivated for several centuries. In view of the full reproductive capacity of *Iris trojana*, the establishment of a generatively active natural population cannot be excluded.

Figure 11. *Iris trojana* Kerner ex Stapf – in Parc Floral in Paris, France (photo M. Blažek)

Therefore, an objective, full comparison with *Iris mediterranea* is not possible without further study. It can only be stated that *Iris trojana* known from gardens is, except for the height of the stem, slightly larger in size than the common wild plants of *Iris mediterranea*. Moreover, *Iris trojana* has more often double-flowered lateral branches and flowers a few days later. The classic clone of *Iris trojana* [8, 25] is also distinguished from most similar Irises by its unusually long bracts. These are reminiscent of the Asian species of the *Iris* section, the bracts of *Iris biliotti*, which is, however, poorly known as a natural species, although it is common in culture in the eastern Mediterranean.

Comparison of *Iris mediterranea* with *Iris pallida* Lam.

The relationship of *Iris mediterranea* to *Iris pallida* Lam., which is the only other native tall *Iris* species in the Balkans, is relatively easy to define, although the flowers of some exceptional individuals may at first sight strongly resemble each other: certain plants of *Iris mediterranea* have monochromatic blue flowers, sometimes with lavender tinge that cannot be distinguished from *Iris pallida* without seeing bracts. This situation is similar to the case in the past of *Iris sicula* Todaro, which was considered a form of *Iris pallida* by the flowers, not the other parts of the plant [6, 12].

However, introgression of *Iris pallida* does not seem very realistic due to the low ease of hybridization with *Iris mediterranea*. Pollination of *Iris pallida* with *Iris mediterranea* pollen (and vice versa) produces fruit filled with normal-looking seeds during opening of pods, but with a watery endosperm that dries out together with the embryo. These watery seeds tend to be slightly larger than normal, viable seeds. This is a common phenomenon when diploid and tetraploid tall Irises are crossed.

The size of the range of indisputably wild *Iris mediterranea* and that of indisputably wild *Iris pallida* is comparable, but geographic locations of wild populations of the two species do not overlap, which is a barrier to eventual contemporary natural hybridization. The two species meet only in culture at present, but we cannot speak about distanced past.

The clear difference from *Iris pallida* is the number of flowers per stem. The number of lateral branches is similar, but the difference is in the number of flowers in all bud sockets. In *Iris pallida* there are three and rarely four (or even five) flowers on the top of the stem and two or three on the side branches. Here, too, the lower branches may have secondary branching.

The dry leaf apices of *Iris mediterranea* are, as in most species, brown rather than silvery. This distinguishes them all from the grey-green leaves of *Iris pallida* with silvery leaf tips. And the most important characteristic that distinguishes *Iris pallida* from all other species of the genus (except related I. illyrica) is that the bracts (spathe-valves) are already dry before the first flower opens, without any chlorophyll-containing tissue.

Figure 12. *Iris mediterranea* (left) can be easily confused with *I. pallida* (right).
In order to identify the species, it is necessary to examine the vegetative parts (photo M. Blažek)

Figure 13. *Iris mediterranea* can be easily confused with *I. pallida*. In order to identify the species, it is necessary to examine the vegetative parts (photo M. Blažek)

Relationship of *Iris mediterranea* to *Iris croatica* Horvat

More similar to *Iris mediterranea* than *Iris pallida* is another tall Balkan species, *Iris croatica* [20].

These two species bloom at the same time, but the flowers of *Iris croatica* are slightly smaller and stalks are shorter than tallest individuals of *Iris mediterranea*. Some clones of *Iris croatica* can be confused with dark self-colored individuals of *Iris germanica*, but not with *Iris mediterranea*. *Iris croatica* has mostly darker, nearly self-colored flowers, never distinctly bicolored. Other differences are the very low or basal stem branching of *Iris croatica* and its bracts, which have a smaller proportion of non-green apical parts.

Figure 14. *Iris germanica* L., *Iris pallida* Lam. and *Iris croatica* Horvat – Botanical garden in Průhonice, Czech Republic Most individuals of *I. pallida* are not yet blooming (photo M. Blažek)

Relationship of *Iris mediterranea* to the *Iris sicula* group

Our new species, *Iris mediterranea*, is also related to a group of South-Eastern Mediterranean thermophilic Irises with different branching and generally taller growth (over 1 m) described from southernmost Europe and the Middle East: *Iris sicula* Tod. (1858), *Iris cypriana* Foster & Baker (1888), and *Iris mesopotamica* Dykes (1913).

No reliably identified living plants could be found for study and for objective comparison, nor sufficient convincing references of their original natural occurrence. Comparing the published differences between these three species indicates smaller differences than those which are evident in the high variation of wild *Iris mediterranea*. In flower color, *Iris sicula* with its relatives is close to monochromatic individuals of *Iris mediterranea*, and thus resembles *Iris pallida*. This in history has probably led to the association of *Iris sicula* with *Iris pallida: Iris pallida* subsp. *sicula* (Tod.) K. Richt. (1890), or supposing synonymity [2, 3, 6, 8, 10, 26].

Iris sicula Irises are highly important for the study of the phylogeny of tall species of section Iris with blue and purple flower color. While for a number of dwarf as well as taller Iris species it is possible to assume hybrid origin, for *Iris sicula*, just as for *Iris variegata*, we simply cannot find any eventual parent species for hybridization, leading to forming them as new species.

Comparison of *Iris mediterranea* and related species in frost hardiness and flowering time

Due to their ability to thrive in a relatively wide climatic range, neither *Iris mediterranea* nor related Irises, except *Iris albicans*, show any evidence of extremely southern origin in Europe. In Průhonice, Czech Republic, they bloom in mid-May and precede the mass flowering of tall Irises, included under the name *Iris barbata* of the Elatior group, by 1-2 weeks. Contrarily, they coincide with the Intermedia group, where the older purple cultivars cannot be distinguished from *Iris germanica.* The

original Intermedia group corresponds also by count of chromosomes (44) [1, 2, 4, 16, 23].

Flowering time alone, without comparison, is of relatively little help in species identification. Only the relative flowering time in one spot is of informative value. Since the difference in the onset of flowering of similar species is about a week backwards and forwards from *Iris mediterranea*, we are only able to compare them where they occur together in the same place. Only then is the species specificity visible.

After *Iris albicans, Iris germanica* is the first to flower among comparable species. It is followed by the tetraploid species. First is *Iris croatica*, then in close succession *Iris mediterranea* of wild origin and a few days later *Iris mediterranea* starts to flower. These are followed by *Iris trojana* and the latest is *Iris biliotti*, which is probably related to *Iris trojana* more than to European species. There is a similar order (except for the first and last species) in flower size. Of course, independently, besides the extreme individual variations, fluctuations depending on the condition of the plants are also observable.

Irises of the *Iris sicula* group bloom later than *Iris mediterranea.* They are not hardy enough in Central Europe, and probably not even in the territory where wild *Iris mediterranea* is at home. The latter peculiarity links them to *Iris albicans*, a close partner of *Iris germanica* (again a matter for study based in laboratory methods and experimental hybridization).

Study material of *Iris mediterranea* in institutions

In addition to the numerous populations in the three Balkan countries bordering Lake Prespa, there is a representative collection of wild-collected specimens and their seedlings in the Botanical Garden of the Botanical Institute of the Academy of Sciences of the Czech Republic. Individual plants of natural origin are maintained in other botanical gardens under the name *Iris macedonica* Horvat or *Iris germanica* s. l., or cultivar names ´Amas´ and ´Macrantha´, or eventually under local names according to their origin in Greece (Vikos etc.).

The oldest among all the nameless collections of garden specimens in the Průhonice collection is an Iris from the Romanian countryside and another, very similar clone, from the vicinity of Sofia.

A very similar clone reached the USA in the mid-20th century, where it was identified as *Iris varbosania.* In gardens it still spreads under the name *Iris varbossiana* [2, 13, 26]. However, this is a misidentification. *Iris varbosania* [27] is a diploid *Iris* described from Sarajevo. Unfortunately, the author K. Malý had no information about the cultivated Balkan *Iris* described earlier, which is identical and which the Danish botanist Jens Wilken Hornemann called *Iris neglecta. Iris neglecta* has its ancestry among hybrids of diploid species *Iris pallida* Lam. and *Iris variegata* L. [2, 5, 8].

There are other such cultivated clones of tetraploid Irises in historical human settlements in the Balkans. Their number is not large, but they are a significant element of traditional local garden culture. These Irises have long been the privilege of southern Europe. It was only as a result of increased introductory interest that new southern European tetraploid Irises began to be cultivated in other parts of Europe and the USA in the middle of the last century. These added to the small number of archaic tetraploid garden Irises introduced from the Middle East around 1900 and cultivated now worldwide.

The first of the new introductions (in 20th century) was the aforementioned "*Iris varbossiana*" - false *Iris varbosania* Malý. It has been spreading under this modified name ever since. Shortly afterwards, other clones of tetraploid - large-flowered archaic garden Irises, were coming not from breeders but from occasional collections in Greece. They were introduced as anonymous plants, named by original localities and entered the range of the world's Iris growers.

Of all the species described, they are next to *Iris trojana*, which is the closest to the newly described *Iris mediterranea*. Unlike the common specimens of natural *Iris mediterranea*, these historical garden clones show traces of selection. They probably originate from local natural populations or from selection among spontaneous seedlings in culture. At present it is no longer possible to assign them to wild plants, from which they differ qualitatively and quantitatively. They have often distinctive colors and, in particular, larger flowers than common specimens in nature. Their origin can no longer be deduced except by assuming that they are the result of non-professional selection in natural populations of *Iris mediterranea*.

In reality, surprisingly, they are less variable in color than wild forms. Mostly only two-toned clones are known, combining light blue standards with darker purple falls. The flowers are larger than the common specimens of *Iris mediterranea*, but otherwise show no signs of belonging to another species. In gardens they are commonly known as Vikos Irises or Skamnili (Skamneli) or Monodendri Irises [2, 9]. These probably in their history selected clones are not entirely identical to those with an obviously natural origin. On the other hand, wild spec-

imens from the rocks of Vikos Canyon do not show any specific difference from specimens originating from natural sites in Northern Macedonia or from Albanian territory.

Monochromatic – self-colored – Irises of these garden forms are almost not yet known, but in the Greek countryside in 2019 we found an attractive cultivated white-flowered specimen that resembles *Iris florentina*, the well-known albino *Iris germanica*, in its bluish hue and branching. But it has larger flowers of a slightly different shape, and testing pollination by different partners suggests that it is a tetraploid, not an aneuploid *Iris germanica* with limited fertility. When pollinated by any tetraploid *Iris*, it produces normal fruits, filled with good quality seeds.

Amas and Macrantha

Since the last century and before, Irises similar to *Iris germanica*, but with larger flowers, have become important in western Europe. These include the pair ´Amas´ (collected by M. Foster, named 1885) and ´Macrantha´ (named by van Tubergen 1907) [8, 10, 23, 25, 28, 30].

They were introduced into western Europe from Asia Minor, from secondary sites in a territory where there had been an interchange of inhabitants and cultures for several centuries. It is therefore not excluded that these Irises originated in the Balkans.

We acquired the first of them for the collection in the 1960s as a garden Iris in Zelenikovo (south of Skopje). The first identification led to the name ´Amas´, but after comparison with plants from other specialists, it turned out to be ´Macrantha´. The two clones are very similar to each other. Although they were described as two different plants long ago, they were not distinguished from each other in horticulture for many years.

In basic characteristics they are both slightly different from *Iris germanica*, as well as from known tetraploids of taller growth. They have the same kind of branching as *Iris germanica*, but larger flowers. Genetically, being fertile tetraploids, they are closer to *Iris mediterranea*.

CONCLUSIONS

The taxonomic evaluation of plants on the boundary between spontaneous wild plants and anthropogenic taxa tends often to be associated with inconsistent attitudes due to the different viewpoints of the authors.

Iris mediterranea sp. n. is not a new species in terms of biology. Its natural status is more convincing than that of the very similar species *Iris germanica* L. and *Iris trojana* Kern.

It belongs to the wild flora of all three countries bordering Lake Prespa and locally has reached a prominent position in Balkan in garden culture.

Only its name is new.

Current knowledge suggests moreover that *Iris mediterranea* most probably took an important position in the phylogeny of wild species and among natural as well as garden hybrids in the genus *Iris* of the section *Iris*, and thus becomes valuable working material for further study by modern laboratory methods.

We would be pleased if this work would also contribute to a more unified understanding of related species, valuable both botanically and culturally.

Acknowledgement. I am grateful to my colleagues from North Macedonia for their cooperation and help in closing this phase of work. I trust that the issue will be continued and developed further.

The collection of extensive plant material and discussions in search of answers to countless questions in the interest of understanding the group of closely related species necessarily required personal communication with many interested experts on international level. Without them, the work would not have been possible.

This work, apart from international partners, could hardly have also come into being without the long-term support and the considerable work of my parents, my wife Uljana, and the longstanding work of the gardeners and colleagues at the botanical garden in Průhonice.

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Holland: Horstink Yann

Hungary: Priszter Szaniszló

Russia: Rodionenko Georgi

Sweden: Höpfner Lars

USA: Edinger Phil, McDonald Nancy, Randolph Lowell Fitz, Warburton Beatrice

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IRIS MEDITERRANEA spec. nov. - НОВ ЧЛЕН НА ПРИРОДНАТА БАЛКАНСКА ФЛОРА И ВЕГЕТАЦИЈА

Милан Блажек[1] и Владо Матевски[2]

[1]Ботаничка Градина во Прихонице, Ботанички Институт при Академијата за науки на Чешката Република, Чешка Република
[2]Македонска академија на науките и уметностите, Истражувачки центар за животна средина и материјали, 1000 Скопје, РС Македонија

Iris mediterranea spec. nova е фертилен природен вид, сличен со ограничено фертилниот култивиран вид *Iris germanica* L. (*Iris* x *germanica* auct. p.p.). Двата вида имаат слични цветови, но кај *Iris mediterranea* не се појавуваат темноцрвени виолетови нијанси кои се присутни во значителен број на клонови од *Iris germanica*. За разлика од *Iris germanica*, освен еден клон пронајден во културата, кај *Iris mediterranea* сè уште не се познати растенија со бела боја на цветови. Хабитусот е сличен и кај двата вида, но *Iris mediterranea* има пообилно разгранување во многу од неговите клонови.

Поголемиот дел од растенијата на *Iris mediterranea* се слични во бојата на цветовите со познатите клонови на *Iris trojana* Kerner ex Stapf, кои одговараат во големината на цветот на избраните клонови со големи цветови на *Iris mediterranea* познати од културата. Но, *Iris trojana*, обично има и поголем број на цветови по стеблото.

Во периодот на цветање, *Iris mediterranea* е интермедиерна помеѓу *Iris germanica* и *Iris trojana*.

Помеѓу трите сродни видови - *Iris mediterranea, Iris germanica* и *Iris trojana*, новоопишниот вид *Iris mediterranea* е единствениот од трите наведени видови каде што постојат повеќекратни и променливи, генеративно репродуктивни природни популации, со јасно дефиниран географски ареал, кој вклучува и ненаселени оддалечени планински локации. Поради тоа, *I. mediterranea*, за разлика од сличните видови, не претставува таксон, кој во текот на неговата историска и сегашна дистрибуција, бил под антропогено влијание.

Клучни зборови: *Iris mediterranea*, нов вид за науката, *Iris germanica* L., Балкански Полуостров, флора

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Original scientific paper

2021 NATIONAL RED LIST OF FUNGI: FOCUSING ON CRITICALLY ENDANGERED SPECIES

Mitko Karadelev*, Slavica Tofilovska, Katerina Rusevska

Institute of Biology, Faculty of Natural Sciences and Mathematics,
Ss Cyril and Methodius University in Skopje, RN Macedonia

*e-mail: mitkok@pmf.ukim.mk

The National Red List plays a crucial role in the conservation of fungi, considering the significant threats they face, such as habitat loss and fragmentation, decline in symbiotic hosts, overexploitation, pollution, and climate change. The importance of protecting fungi is evident, and although unofficial red lists were created in the past (2000 and 2010), the new National Red List of Fungi of North Macedonia holds official status. This document includes a total of 64 species, each assigned to specific categories. Among them, six fungal species are classified as Critically Endangered (CR), nineteen as Endangered (EN), thirty-four as Vulnerable (VU), two as Near Threatened (NT), two as Least Concern (LC), and one species is categorized as Data Deficient. The majority of the listed taxa (fifty-three) belong to the phylum Basidiomycota, while ten taxa are associated with the phylum Ascomycota. In this article, we will focus on the Critically Endangered species, which are as follows: *Bovista paludosa* Lév, *Galerina sphagnorum* (Pers.) Kühner, *Galerina tibiicystis* (G.F. Atk.) Kühner, *Hyphoderma etruriae* Bernicchia, *Xeromphalina junipericola* G. Moreno & Heykoop, and *Zeus olympius* Minter & Diam.

Key words: IUCN Red List, fungi conservation, North Macedonia

INTRODUCTION

North Macedonia, known as a biodiversity hot-spot, exhibits a remarkable fungal diversity. The earliest publications on fungal diversity in North Macedonia primarily date back to the 1930s, but over the past three decades, there has been a notable increase in research focusing on fungal diversity and distribution. A significant milestone in the comprehensive and systematic study of fungal diversity across the entire country was the establishment of the Mycological Laboratory at the Faculty of Natural Sciences in Skopje. This endeavor resulted in the discovery of numerous new taxa for the national inventory. Although new species continue to be discovered in the country, the current number of macro fungi species exceeds 2,600 [1, 2]. Additionally, two species, *Astraeus macedonicus* Rusevska, Karadelev, Telleria & M.P. Martín [3] and *Clitopilus abprunulus* S.P. Jian, M. Karadelev & Zhu l. Yang [4], are newly described to science. The Macedonian Collection of Fungi (MCF) at the Faculty of Natural Sciences in Skopje houses approximately 25,000 dry specimens, representing around 4,000 different species. These specimens are digitized in the MACFUNGI database, which contains information on 37,000 collected specimens, representing 5,000 various species from Macedonia, the Balkans, and beyond. Distribution maps of critically endangered fungal species have also been published [5].

Regarding fungal conservation, an important initiative was undertaken by Karadelev [6], who prepared a Preliminary Red List of Fungi of Macedonia, comprising 67 species exclusively belonging to the class Basidiomycetes. The data from this list was utilized to generate an Official List of Strictly Protected and Protected Wild Species, which was published in the Official Gazette of the Republic of

Macedonia in 2011. However, in order to update the list, Karadelev and Rusevska [7] subsequently published an appendix to the red list of fungi, incorporating 213 species of Ascomycota and Basidiomycota. This updated list adhered to the rigorous criteria and categories of the IUCN Red List. Nevertheless, the latter publication lacked detailed descriptions of the conservation status for each species.

With the advancement of fungal research in North Macedonia, new insights have emerged regarding the distribution of macromycetes within the country. Consequently, it became imperative to conduct a revision of the Red List of Fungi of Macedonia. Drawing upon the newly acquired data on the distribution of macro fungi, adjustments have been made to the IUCN categories for several species, and new species have been assigned their respective categories. The entire list has undergone a comprehensive review, focusing particularly on the section pertaining to endangered species in the higher categories (Vulnerable, Endangered, and Critically Endangered). A total of 64 fungal species recorded in North Macedonia have now been assessed, aiming to determine their national conservation status in accordance with the Guidelines for Using the IUCN Red List Categories and Criteria [15].

METHODOLOGY

The development of the proposed National Red List of Fungi, which encompasses 64 taxa, relied on various sources of information. These sources included field research findings, published and unpublished species records, specimen collections, research notes, and contributions from other individual fungi collectors. All essential data were compiled to enable the final assessment of each species, taking into account the relevant IUCN criteria.

These criteria encompassed aspects such as distribution range, population trends, habitat and ecology, threats, and utilization and trade. The assessment process was carried out in three distinct stages, outlined as follows:

Pre-assessment

The research team conducted a thorough review of data from various literature sources, as well as data obtained from the Macedonian Collection of Fungi (MCF) and the MACFUNGI database, both housed at the Mycological Laboratory within the Faculty of Natural Sciences and Mathematics in Skopje. The unofficial Red List of Fungi for North Macedonia [7] was also carefully examined. In order to gather mycological specimens and facilitate laboratory analyses, field research was carried out, particularly for species with limited available data. The field research encompassed diverse geographical locations with varying climates, vegetation types, and substrates, including both deciduous and coniferous trees. Various habitats were explored, such as Greek juniper forests, beech forests, spruce forests, Macedonian pine forests, Bosnian pine forests, oak forests, as well as national parks, nature monuments, pristine forests, and other protected areas. Field activities took place during spring, summer, and especially autumn, which is the most conducive season for fungal growth. For species identification, standard methods were employed, including microscopy, application of reagents, and consultation of specialized identification books.

Assessment

The assessment process began with a desktop analysis of the species, following the IUCN criteria. The taxa were evaluated according to the IUCN Guidelines, specifically version 1.1 [15]. The members of the assessment team underwent prior training to utilize the Species Information Service (SIS), which is the IUCN's web application designed for conducting, managing, and storing species assessments for the IUCN Red List. SIS employs a standardized data format for assessments, ensuring consistency in classification systems and maintaining taxonomic integrity. In categorizing the species according to IUCN criteria, we also took into consideration the experiences of neighboring countries, certain European countries, and the fungi section of the IUCN Red List of Threatened Species [16].

All available data were meticulously analyzed, leading to the assignment of the appropriate category for each fungal species. The species taxonomy adheres to the latest nomenclature provided by Index Fungorum [17] and the MycoBank Database [18].

Review

The assessments of all fungal species underwent a rigorous review process by multiple independent international mycological experts. Their feedback, remarks, and recommendations were carefully considered and integrated into the assessments. Following this comprehensive review, the assessments were finalized. The red-listing data were then entered into the global Species Information Service (SIS) provided by the IUCN, accessible at https://www.iucnredlist.org/assessment/sis. Subse-

quently, the data was transferred to the Macedonian National Red List website, hosted under the Ministry of Environment and Physical Planning, available at http://redlist.moepp.gov.mk/. This website serves as the official platform for the Macedonian National Red List.

RESULTS AND DISCUSSION

The threat status of the sixty-four (64) assessed taxa is rendered in Table 1.

Table 1. The threat status of the assessed taxa of fungi

Alessioporus ichnusanus (Alessio, Galli & Littini) Gelardi, Vizzini & Simonini	EN – Endangered; B2ab(iii); D
Amanita caesarea (Scop.) Pers.	LC – Least Concern
Amanita curtipes E.-J. Gilbert	VU – Vulnerable, D1
Antrodia juniperina (Murrill) Niemelä & Ryvarden	VU – Vulnerable, B1ab(i,iii,iv); D1
Aspropaxillus giganteus (Sowerby) Kühner & Maire	NT – Near Threatened, D1
Baorangia emileorum (Barbier) Vizzini, Simonin i& Gelardi	VU – Vulnerable, B1ab(i, iii, iv)
Battarrea phalloides (Dicks.) Pers.	VU – Vulnerable, B1ab(i, iii, iv)
*Bovista paludosa*Lév	CR – Critically Endangered: C2a(i), D
Butyriboletu sregius (Krombh.) D. Arora & J.L. Frank	VU – Vulnerable, C2a(i)
Chlorophyllum agaricoides (Czern.) Vellinga	VU – Vulnerable, B2ab(iii,iv)
Cudonia circinans (Pers.) Fr.	VU – Vulnerable, D1
Daedaleopsis nitida (Durieu& Mont.) Zmitr. & Malysheva	VU – Vulnerable, B1ab (I,iii,iv); D1
Dentipellis fragilis (Pers.) Donk	VU – Vulnerable, D1
Disciseda bovista (Klotzsch) Henn	EN – Endangered, B1ab(iii) + 2ab(ii,iii,iv)
Galerina jaapii A.H. Sm. & Singer	EN – Endangered, B1ab(iii) + B2ab(i,ii,iv), D
Galerina sphagnorum (Pers.) Kühner	CR – Critically Endangered, B1ab (iii); C1
Galerina tibiicystis (G.F. Atk.) Kühner	CR – Critically Endangered, B1ab(iii), C1
Geastrum minimum Schwein.	VU – Vulnerable, B1ab(i, iii, iv, v)
Guepinia helvelloides (DC.) Fr.	EN – Endangered, B1ab(iii); B2ab(iii); D
Gyrodon lividus (Bull.) Sacc.	EN – Endangered, C2a(i)
Gyromitra gigas (Krombh.) Cooke	VU – Vulnerable, D1
Helvella atra J. König	VU – Vulnerable, B1ab(iii); D1
Hericium coralloides (Scop.) Pers.	EN – Endangered, C2a(i)
Hericium erinaceus (Bull.) Pers.	EN – Endangered, C2a(i)
Hortiboletus bubalinus (Oolbekk. & Duin) L. Albert & Dima	DD – Data Deficient
Hydnellum peckii Banker	EN – Endangered, B1ab(iii) + B2ab(i,ii,iv)
Hygrocybe cantharellus (Schwein.) Murrill	VU – Vulnerable, D1
Hygrocybe punicea (Fr.) P. Kumm.	EN – Endangered, C2a(i)
Hygrophorus marzuolus (Fr.) Bres.	VU – Vulnerable, D1
Hymenochaete cruenta (Pers.) Donk	EN – Endangered, D
Hyphoderma etruriae Bernicchia	CR – Critically Endangered, B2ab(iii); D
Inocutis tamaricis (Pat.) Fiasson & Niemelä	NT – Near Threatened, D1
Lactarius omphaliformis Romagn.	EN – Endangered, B1ab (i,iii,iv); B2ab (ii,iii,iv)
Langermannia gigantea (Batsch) Rostk.	LC - Least Concern
Lenzitopsis oxycedri Malençon & Bertault	EN – Endangered, D
Leucopaxillus compactus (P. Karst.) Neuhoff	VU – Vulnerable, D1
Microstoma protractum (Fr.) Kanouse	EN – Endangered, B1ab (i,iv); B2ab(ii,iv); D
Mitrula paludosa	EN – Endangered, B1ab(i,iii,iv); B2ab (ii,iii,iv); D
*Mycena juniperina*Aronsen	VU – Vulnerable, B1ab(i,iii,iv); D1
Mycenastrum corium (Guers.) Desv.	VU – Vulnerable, D1

Myriostoma coliforme (Dicks.) Corda	VU – Vulnerable, D1
Neolentinus cyathiformis (Schaeff.) Della Magg. & Trassin.	VU – Vulnerable, D1
Perenniporia medulla-panis (Jacq.) Donk	VU – Vulnerable, B1b(iii,iv,v)
Phylloporus pelletieri (Lév.) Quél.	VU – Vulnerable, B1ab(i,iii,iv)
Pilatotrama ljubarskyi (Pilát) Zmitrovich	VU– Vulnerable, D1
Plectania melastoma (Sowerby) Fucke	VU– Vulnerable, B1ab (i,iii,iv); D1
Poronia punctata (L.) Fr.	EN – Endangered, C2a; D
Psilocybe serbica M.M. Moser & E. Horak	VU – Vulnerable, D1
Pyrofomes demidoffii (Lév.) Kotl. & Pouzar	VU – Vulnerable, B1ab(i,iii); D1
Rubroboletus demonensis Vasquez, Simonini, Svetash., Mikšík &Vizzini	VU – Vulnerable, D1
Rubroboletus dupainii (Boud.) Kuan Zhao & Zhu L. Yang	VU – Vulnerable, D1
Rubroboletus rhodoxanthus (Krombh.) Kuan Zhao & Zhu L. Yang	VU – Vulnerable, C2a(i)
Rubroboletus satanas (Lenz) Kuan Zhao & Zhu L. Yang	VU – Vulnerable, C2a(i)
Sarcodon leucopus (Pers.) Maas Geest. & Nannf.	EN – Endangered, D
Sarcopeziza sicula (Inzenga) Agnello, Loizides & P. Alvarado	EN – Endangered, B2ab(iii); D
Sarcosphaera coronaria (Jacq.) J. Schröt.	VU – Vulnerable, D1
Skeletocutis odora (Sacc.) Ginns	EN – Endangered, D
Sparassis crispa (Wulfen) Fr.	EN – Endangered: B1ab(i,iii,iv); B2ab(ii,iii,iv)
Suillus americanus (Peck) Snell	VU – Vulnerable, B1ab(iii)+2ab(iii); D1
Tricholoma acerbum (Bull.) Quél.	VU – Vulnerable, C2a(i)
Tricholoma apium Jul. Schäff.	VU – Vulnerable, D1
Urnula craterium (Schwein.) Fr.	VU – Vulnerable, D1
Xeromphalina junipericola G. Moreno & Heykoop	CR – Critically Endangered, B2ab(iii); D
Zeus olympius Minter & Diam.	CR – Critically Endangered, D

The summary of the threat status, as shown in Table 2, is as follows: out of the identified fungus species, six species are classified as Critically Endangered (9.3 %), nineteen species are classified as Endangered (29.6 %), thirty-four species are classified as Vulnerable (53.1 %), two species are classified as Near Threatened (3.1 %), two species are classified as Least Concern (3.1 %), and one species has insufficient data to determine its status (1.5 %).

Table 2. Summary of numbers of N. Macedonia fungi per red list category

IUCN Red List Categories	Fungi
Extinct (EX)	0
Extinct in the Wild (EW)	0
Critically Endangered (CR)	6
Endangered (EN)	19
Vulnerable (VU)	34
Near Threatened (NT)	2
Least Concern (LC)	2
DD – Data Deficient	1
TOTAL	**64**

The majority of the taxa, consisting of fifty-three species (82.8 %), belong to the phylum Basidiomycota, while eleven species (17.1 %) are affiliated with the phylum Ascomycota.

The Critically Endangered species identified are as follows: *Bovista paludosa* Lév, *Galerina sphagnorum* (Pers.) Kühner, *Galerina tibiicystis* (G.F. Atk.) Kühner, *Hyphoderma etruriae* Bernicchia, *Xeromphalina junipericola* G. Moreno & Heykoop, and *Zeus olympius* Minter & Diam.

Bovista paludosa (Figure 1) is primarily found in wet habitats, particularly in high alpine peat bogs situated above 1,800 meters in altitude [8]. It is a species that thrives in association with mosses and is predominantly found in one of the most vulnerable and diminishing habitats – peat bogs and mires. These habitats face significant threats due to modifications in the hydrological system, pollution from surface water and air (eutrophication), natural succession and erosion, as well as forest management practices. In Macedonia, *Bovista paludosa* has been discovered in Sar Mountain (two sites) and Korab Mountain (a single site), specifically in wet peat bogs with *Sphagnum*. The population

size is small, and there is an expected decline in population trend.

The situation is similar for two *Galerina species*, *G. sphagnorum* (Figure 1) and *G. tibiicystis*, both of which have been found in wet habitats [5, 7]. Currently, only one site has been documented in the western part of the country, specifically in peat bogs with *Sphagnum* in Sar Mountain (Lukovo Pole) at an altitude of approximately 1,700 meters.

Considering the limited extent of peat bogs in North Macedonia, which serve as the primary habitats for these species, as well as the importance of peat bogs in the country and throughout Europe, it is crucial to protect these three species by conserving their habitat and preventing the degradation of sites where they currently occur or have the potential to occur.

The species *Hyphoderma etruriae* and *Xeromphalina junipericola* are found only in a single location, specifically in Greek juniper forests in the southeastern part of the country (Figure 2). *H. etruriae* is a wood-inhabiting fungus that thrives on old shrubs of juniper species, growing in hollows created by old wounds on trunks and large branches. It is endemic to southern Europe and has been recorded from only ten locations in two countries, Italy and North Macedonia [9, 10]. *X. junipericola* is an extremely rare species known only from North Macedonia, Spain, and Turkey [11, 12]. In North Macedonia, the population size is very small, with only two individuals observed.

The collection site is facing threats due to extensive exploitation of juniper trees by local inhabitants. The strong anthropogenic pressure on the habitat poses risks to habitat quality, area of occupancy, and the number of mature individuals. Consequently, the species is critically endangered due to the high risk of decline resulting from these factors.

Figure 1. *Bovista paludosa* (left) and *Galerina sphagnorum* (right), a critically endangered species from high alpine peat bogs.
Photo: Matthias Theiss

Figure 2. *Hyphoderma etruriae* (left) and *Xeromphalina junipericola* (right), a critically endangered species from Greek juniper forest in the south-eastern part of the country.
Photo: Mitko Karadelev

The species *Zeus olympius* (Figure 3) primarily grows on dead stems of young trees, twigs, and branches of Bosnian pine (*Pinus heldreichii*). It is known to occur in Bulgaria (Pirin Mountain, Slavyanka Mountain, Vitosa Mountain) and in Greece (Olympus Mountain, Pindus Mountain) [13, 14]. In North Macedonia, it has been observed at only one location, specifically in Galicica National Park, with a small population and a limited area of occupancy. The fungus appears to be restricted to the Bosnian pine and has never been found growing on any other pine species.

Figure 3. *Zeus olympius* from dead twigs and branches of Bosnian pine. Photo: Mitko Karadelev

CONCLUSION

The official National Red List of Fungi, consisting of 64 species, has been compiled and made accessible to the relevant conservation authorities and biodiversity programs. The list was created based on field research findings, published and unpublished species records, research notes, and information obtained from individual fungi collectors. All available data were thoroughly analysed, leading to the assignment of appropriate conservation categories for each fungal species. The red-listing data have been stored in the global Species Information Service (SIS) of the IUCN and subsequently transferred to the Macedonian National Red List website, overseen by the Ministry of Environment and Physical Planning. The summary of the threat status is as follows: six species are categorized as Critically Endangered (CR), nineteen as Endangered (EN), thirty-four as Vulnerable (VU), two as Near Threatened (NT), two as Least Concern (LC), and one species is classified as Data Deficient. The majority of the taxa (fifty-three) belong to the phylum Basidiomycota, while ten taxa are associated with the phylum Ascomycota. However, it is important to note that the Red List remains a dynamic document as new species may be discovered in future research, and there may be changes in the harvesting practices of important edible fungi by the local population.

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НАЦИОНАЛНА ЦРВЕНА ЛИСТА НА ГАБИ (2021), СО ПОСЕБЕН ОСВРТ НА КРИТИЧНО ЗАГРОЗЕНИТЕ ВИДОВИ

Митко Караделев, Катерина Русевска, Славица Тофиловска

Институт за биологија, Природно-математички факултет,
Универзитет „Св. Кирил и Методиј“ во Скопје, РС Македонија

Националната Црвена листа на габи претставува круцијален документ за заштита на габите во државата. При нејзината изработка се спроведоа бројни теренски истражувања, а беа инкорпорирани и податоци од публикувани и непубликувани наоди за различни видови габи, ексикати, како и информации од различни извори. Листата како официјален документ содржи податоци за 64 вида габи, од кои шест вида се категоризирани како критично загрозени (CR), деветнаесет вида се загрозени (EN), триесет и четири вида се ранливи (VU), два вида се речиси засегнати (NT), два се малку засегнати (LC) и еден вид е без доволно податоци (DD). Најголем дел од видовите (53) припаѓаат на типот Basidiomycota, додека десет вида се систематизирани во типот Ascomycota. Критично загрозените видови *Bovista paludosa*, *Galerina sphagnorum, Galerina tibiicystis*, *Hyphoderma etruriae*, *Xeromphalina junipericola* and *Zeus olympius* се посебно обработени и дискутирани во трудот.

Клучни зборови: IUCN Црвена листа; заштита на габите; Република Северна Македонија

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Original scientific paper

COMPARATIVE RESEARCH ON ANTIOXIDANT CONTENT IN THE FRUITS OF SELECT INDIGENOUS VARIETIES OF FRUITS, GRAPES AND VEGETABLES

Ana Selamovska, Viktor Gjamovski, Biljana Korunoska*, Milena Taseska-Gjorgjijevski, Klime Beleski, Duško Nedelkovski, Katerina Bandjo Orešković, Roze Djoljevska-Milenkovska, Viktor Rajčin

Institute of Agriculture, Ss. Cyril and Methodius University in Skopje, RN Macedonia

*e-mail: markovskabiljana@yahoo.com

In this research study, we presented the results of the chemical analysis conducted on fruits of indigenous varieties and populations of several fruit species, namely cherries, pomegranates and apples, as well as grapes and vegetables such as peppers and tomatoes. Among the samples analyzed, the pepper populations exhibited the highest concentration of vitamin C, measured at 51.25 mg/100g. Conversely, the lowest levels of vitamin C were observed in apples and grapes, approximately 9 mg/100g. When considering the overall antioxidant activity, tomatoes and peppers displayed the largest proportion of vitamin C, accounting for over 75 mg/100g. Furthermore, the highest quantity of anthocyanins was found in cherries, specifically 394.30 mg/kg FW (FW = Fresh Weight). Pomegranate varieties exhibited the greatest content of total phenols, measuring 5359.43 mg/kg FW, along with the highest fruit antioxidant activity, showing 81.58 % inhibition. As for apple varieties, they contained the highest amount of flavan-3-ols, reaching 517.98 mg/kg FW.

Our analysis revealed a positive correlation between total phenols and antioxidant activity, as well as between total phenols and flavan-3-ols. Additionally, a moderate negative correlation was identified between vitamin C and flavan-3-ols.

Key words: autochthonous; fruit species; grapes; vegetables; antioxidants

INTRODUCTION

During a period when human health is increasingly endangered by pollution from both non-living and living factors, the consumption of biologically active compounds, known as antioxidants, plays a crucial role in maintaining well-being. Including foods that are abundant in antioxidants and other vital chemicals in one's diet can significantly decrease the risk of specific diseases. Agricultural products serve as a valuable source of antioxidants, which actively combat cancer-causing free radicals and degenerative ailments. The level of antioxidants present in these products varies based on factors such as genetic makeup, environmental conditions, cultivation techniques, storage methods, fruit ripeness, and fruit processing Bassi *et al.* [2], Lakra *et al.* [19].

In agricultural products, the antioxidant activity is attributable to a range of chemical compounds, including carotenoids, chlorophyll, phenols, lycopene, vitamin C, anthocyanin's, organic acids, flavan-3-ols and others. Silva-Beltran *et al.* [39], George *et al.* [11].

Vitamins play a vital role in sustaining human life and health, as well as promoting growth and development of the body. Inadequate intake of certain vitamins can result in various diseases known as hypovitaminosis and avitaminosis. Vitamin C, known for its potent antioxidant properties, holds significant importance in several physiological functions. It regulates iron and calcium levels, controls blood sugar, reduces high blood pressure and „bad" cholesterol levels, enhances the immune system, and participates in cellular metabolism through oxidoreduction processes. Fruits and vegetables serve as sources of vitamin C, albeit in varying quantities.

High levels of vitamin C have been found in rose hips and actinidia (634.1-1008.3 mg/100g), hawthorn (500 mg/100g), black currant (300 mg/100g), wild strawberry and blueberry (80 mg/100g), and citrus fruits (50 mg/100g) Latocha *et al.* [21], Selamovska and Miskoska-Milevska, [38]. In terms of tomatoes, cultivated varieties contain 20.78 mg/100g of vitamin C, while wild varieties contain 26.22 mg/100g Kurina *et al.* [18].

Polyphenols are highly significant chemical compounds, categorized into two main groups: flavonoids (including anthocyanin's, flavan-3-ols, monomers and polymers, flavones, and dihydroflavones) and non-flavonoids (comprising hydroxybenzoic acid, hydroxycinnamic acid, their derivatives, stilbene compounds, and phenolic acids) Ribéreau-Gayon *et al.* [36]. These compounds exhibit antioxidant, anti-inflammatory and anticancer effects Block *et al.* [4], Goldner *et al.* [12], along with antimutagenic properties Sochor *et al.* [40]. They also possess antiallergenic qualities, reduce the risk of chronic diseases, cardiovascular and neurodegenerative disorders Vauzour *et al.* [43], provide protection against infections and UV radiation, lower blood pressure, decrease the risk of heart attacks and strokes by 20 %, reduce the risk of diabetes, and improve bone function. Moreover, they are crucial for the biosynthesis of vitamin C. Certain polyphenols contribute to the quality, color, and taste of fruits, Von Baer *et al.* [44], while others serve as protective agents for plants against biotic factors such as predators and pests, as well as abiotic factors like frost and drought, B. Korunoska [3]. Additionally, some polyphenols play specific physiological roles in plant development, Macheix *et al.* [23] and so on.

The polyphenol content in fruits is influenced by various factors such as the genotype, fruit maturity, soil-climatic conditions, and cultivation methods, Rodríguez-Delgado *et al.* [37], López-Roca *et al.* [22], Fernandez-Mar *et al.* [10]. Phenolic compounds, particularly anthocyanin's, are synthesized in higher amounts under low air temperatures and insufficient soil moisture Ratiu *et al.* [34].

Flavans-3-ols, a group of phenolic compounds, play a significant role in the astringency, bitterness, and structural properties of food products Ivanova and Dimovska, [14]. Catechins and their derivatives, including catechins, epicatechins, epigallocatechins, gallocatechins, and epicatechins-3-O-gallate, are prominent flavans-3-ols found in apples, blueberries, strawberries, and grapes. Flavans- 3-ols have been associated with reducing high blood pressure, body mass, and the risk of type 2 diabetes, as well as providing protection against vascular diseases Raman *et al.* [33], Osakabe [28].

Anthocyanin's are water-soluble pigments responsible for the coloration of leaves, flowers, and fruit skins. They are most commonly found in berry fruit species (e.g., black currant, blueberry, strawberry, raspberry), grapes, and certain tropical fruits Paz and Fredes [30], Panche *et al.* [29]. Pelargonidine, cyanidin, peonidine, delphinidine, petunidine, and malvidin are the major anthocyanin's present in fruits and grapes. Anthocyanin's exhibit antioxidant, anti-inflammatory, antitumor, anticancer, antibacterial, antimicrobial properties, and have a hepatoprotective effect Pešić *et al.* [31], Rauf *et al.* [35], Mattioli *et al.* [26]. They are also involved in the management of chronic diseases, particularly cardiovascular diseases, diabetes, and Alzheimer's disease Ćujić *et al.* [8].

Previous studies by Cevallos-Casals *et al.* [7], Marić *et al.* [24], Jovančević and Božović [15] have identified several anthocyanin's in the fruits of various Prunus species, including cyanidin-3-glycoside, cyanidin-3-rutinoside, cyanidin-3-amnoglycoside, cyanidin-3-gentiobioside, peonidine-3-glycoside and peonidine-3-rutinoside.

Given the increasing interest in functional foods and the significance of local varieties, our research aimed to analyse and quantify specific bioactive substances (antioxidants) in the fruits of different species and varieties.

MATERIAL AND METHODS

The study was conducted as part of the scientific project titled **"Antioxidant activity of fruits from indigenous varieties and populations of fruits, vegetables, and grapes".** The laboratory tests were carried out at the oenological laboratory located at the Institute of Agriculture in Skopje. Fruit samples were collected for analysis from various indigenous fruit varieties, including 9 varieties of pomegranate (Zumnarija, Bejnarija, Valandovska kisela, Valandovska kiselo-slatka, Hidjas, Kisela, Lifanka, Ropkavec, Karamustafa), 7 varieties of apple (Ubavocvetka, Shareno blago, Prespanka, Tetovka, Karapasha, Kozharka, Bela tetovka), 6 varieties of cherry (Ohridska brza, Ohridska rana, Ohridska crna, Dolga shishka, Dalbazlija, Ohridska bela), 4 varieties of grapes (Belo zimsko, Stanushina, Crven valandovski drenok, Crn valandovski drenok), 2 varieties of tomato (Skopski jabuchar, Volovsko srce), and 3 varieties of pepper (Vezen blag piper, Vezen lut piper, Kavardjik).

The content of vitamin C (mg/100g), total phenols (mg/kg FW), anthocyanin's (mg/kg FW), flavan-3-ols (mg/kg FW), and antioxidant activity of the fruits (% inhibition) were measured. The vitamin C content (mg/100g) was determined using the volumetric method, which involved titration of the filtrate with 2, 6-dichlorophenol indophenol, following Murray's method. The end point of the titration was to achieve a faint pink colour. Samples with a higher degree of red staining were treated with 50 mg of activated carbon before titration, until complete decolourization was achieved.

The content of total phenols, anthocyanin's, and flavan-3-ols was determined using a spectrophotometric method and expressed in mg/kg FW. The determination was carried out using an Agilent 8453 UV-VIS spectrophotometer. Prior to analysis, samples were prepared by taking approximately 5 g of homogenized material and transferring it to a laboratory flask. Then, 20 ml of a preprepared extraction solution (methanol: water: hydrochloric acid in a ratio of 70:30:0.1) was added to the flask. The mixture was subjected to ultrasonic treatment for 15 minutes followed by 30 minutes of stirring on a magnetic stirrer. The resulting clarified solution was transferred to a 25 ml laboratory flask and topped up to the mark with the same extraction solution. Total phenols were determined using the Folin-Ciocalteu method. A blank sample was prepared using distilled water instead of the tested sample, while the other reagents remained the same.

The determination of total anthocyanin's content was conducted using the Acid ethanol method, with ethanol chloride solution used as a blank test. The measurement of anthocyanin's content was performed on a spectrophotometer at a wavelength of 550 nm. P-dimethylaminocinnamaldehyde (p-DMACA) was employed to quantify the total flavan-3-ols in the tested samples, with methanol used as a control. The absorbance was measured at a wavelength of 640 nm.

The method for determining antioxidant activity involved assessing antiradical activity against the stable product DPPH (2,2-diphenyl-1-picrylhydrazil). Ascorbic acid was used as a standard to prepare a series of standard solutions. Spectrophotometric analysis was performed at a wavelength of 517 nm. The absorbance of the samples was measured individually and from the obtained results, the antioxidant activities were calculated as percentages of inhibition.

Correlation analysis was conducted between the examined parameters using the XL-Stat test (2014), yielding a coefficient of determination (R^2) and various standard parameters such as maximum and minimum values, average value and standard deviation for each parameter. The results of the examined parameters were presented as average values for each species' varieties.

RESULTS AND DISCUSSION

Table 1 presents the results of the analysis of vitamin C, total phenols, anthocyanin's, flavan-3-ols and antioxidant activity in fruits of various fruit species (pomegranate, apple, cherry), grapes and vegetables (tomato and pepper).

On average, peppers exhibited the highest vitamin C content (51.25 mg/100g). Apples and grape varieties had the lowest vitamin C content (approximately 9 mg/100g). According to our findings, tomatoes and peppers contributed the largest proportion of vitamin C to the total antioxidant activity, accounting for over 75 %. These fruits were rich in vitamin C, highlighting the importance of including them frequently in the diet to obtain this vitamin. Apples had the lowest contribution of vitamin C to the total antioxidant activity (15.52 %). A moderate negative correlation was observed between the content of vitamin C and flavan-3-ols (Table 3). In this case, the species with the lowest vitamin C content (apples and grapes) exhibited the highest content of flavan-3-ols (517.98 mg/kg FW in apples and 130.23 mg/kg FW in grapes). Pomegranates also had a high flavan-3-ol content (122.51 mg/kg FW). The data on vitamin C content in apples align with existing literature Boyer and Liu, [6], which reports an average vitamin C content of approximately 5.7 mg/100g in apple fruits, contributing less than 0.4 % to the total antioxidant activity. Although vitamin C is a potent antioxidant, these authors suggest that the antioxidant activity of apples is primarily driven by other antioxidant components, such as total phenols and flavan-3-ols, given that apples have the highest content of these substances compared to other crops. Kurina *et al.* [18], found higher vitamin C content in wild tomatoes (26.22 mg/100g) compared to cultivated tomatoes (20.78 mg/100g). George *et al.* [11] reported an average vitamin C content in tomatoes ranging from 2.50 to 26.50 mg/100g.

Table 1. Content of vitamin C, total phenols, anthocyanin's, flavan-3-ols and the antioxidant activity of fruits of several fruit species (pomegranate, apple, cherry), grapes and vegetables (tomato and pepper)

Species	Vitamin C (mg/100g)	Total phenols (mg/kg FW)	Anthocyanin's (mg/kg FW)	Flavan-3-ols (mg/kg FW)	Antioxidant activity (% inhibition)	% vitamin C of total anti-ox. activity
Pomegranate	23.67	5359.43	323.78	122.51	81.58	29.01
Apple	9.37	4383.06	7.11	517.98	60.37	15.52
Cherry	12.83	1386.25	394.30	69.15	43.36	29.59
Grape	9.00	1037.19	206.96	130.23	40.86	22.03
Tomato	38.25	685.79	63.74	48.04	48.32	79.16
Pepper	51.25	1846.73	46.09	30.73	65.45	78.30
Average	**24.06**	**2449.74**	**173.66**	**153.11**	**56.66**	**42.46**

Table 2. General statistical values for the comparative values of the examined parameters (vitamin C, total phenols, anthocyanin's, flavan-3-ols, antioxidant activity) of fruits (pomegranate, apple, cherry), grapes and vegetables (tomato, pepper

Variables	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Vitamin C	6	0	6	9.0000	51.2500	24.0617	17.3754
Total phenols	6	0	6	685.7900	5359.4300	2449.7417	1939.3541
Antocyans	6	0	6	7.1100	394.3000	173.6633	160.2637
Flavan-3-ols	6	0	6	30.7260	517.9800	153.1060	183.1157
Antiox. activity	6	0	6	40.8600	81.5800	56.6567	15.5443
% of vit. C from antiox. activity	6	0	6	15.5200	79.1600	42.2667	28.7098

Flavan-3-ols in grapes are primarily synthesized in the seeds (60 %) and stalks (20 %), with a smaller amount found in the fruit skin (approximately 15 %) Bourzeix *et al.* [5]. The typical flavan-3-ols present in grapes include (+) catechin, (-) epicatechin, (-) epicatechin-gallate and rarely (-) epicatechin-3-O-gallate Pineiro *et al.* [31]. The highest content of total phenols was observed in pomegranate (5359.43 mg/kg FW) and apple (4383.06 mg/kg FW), while tomato exhibited the lowest content (685.79 mg/kg FW). Pepper also showed a high content of phenols (1846.73 mg/kg FW). A moderate positive correlation was observed between total phenols and flavan-3-ols, while a very strong positive correlation was found between the content of total phenols and the antioxidant activity of the fruits (Table 4). Pomegranate, apple and pepper varieties displayed high levels of total phenols and exhibited the highest antioxidant activity (81.58 % inhibition in pomegranate, 65.45 % inhibition in pepper and 60.37 % inhibition in apple), Pešić *et al.* [32].

The high content of phenols contributes to sensory characteristics, enhances wine stability and exhibits antioxidant activity Landete [20], Alcalde *et al.* [1]. Krstić *et al.* [17] found a presence of 11.47 g/kg^{-1} total phenols in pepper and did not establish a statistically significant difference in the content of total phenols between hot and mild pepper populations. In our studies, cherries and pomegranate displayed the highest amount of anthocyanins (394.30 mg/kg FW and 323.78 mg/kg FW, respectively), while apples exhibited the lowest (7.11mg/kg FW). Grapes also showed a high content of anthocyanins (206.96 mg/kg FW).

The accumulation of anthocyanins is primarily influenced by genetic and external factors such as light and temperature. Previous studies have reported a high content of anthocyanins in pomegranate (15-270 mg/100g) by Ćujić *et al.* [8], Kaur and Kapoor [16] and Dumlu and Gürkan [9] (2,100–4,400 mg/l). Apples were found to contain 0-60 mg/100g of anthocyanins, sour cherries 2–450 mg/100g Cevallos-Casals *et al.* [7] and black grapes 192 mg/100g [16]. According to Honda *et al.* [13], during the synthesis of anthocyanins in apple fruits, five genes were ex-

pressed and the level of expression correlated with the concentration of anthocyanin's.

In the fruits of both wild and cultivated varieties, Tešović *et al.* [42] identified 17 anthocyanin's, including three in apples, four in plums, four in cherries, five in dogwoods, four in raspberries and five in blueberries. According to Mikulić - Petkovsek *et al.* [27], cyanidin-3-glycoside and cyanidin-3-rutinoside were the most common anthocyanin's found in Prunes species. In apple fruit skin, the main anthocyanin identified by Sun and Francis [41], was cyanidin-3-galactoside, followed by cyanidin-3-arabinoside and cyanidin-7-arabinoside. Various fruit species [42] contained similar anthocyanin's: apple and dogwood fruits contained cyanidin-3-arabinoside, plum and juniper fruits contained cyanidin-3-glycoside and peonidine-3-rutinoside. Kurina *et al.* [18] observed a higher anthocyanin's content in wild tomato varieties (125.30 mg/100g) compared to cultivated varieties (45.20 mg/100g). Markovski *et al.* [25] measured anthocyanin's content in pomegranate varieties ranging from 58.67 to 298.95 mg/l and phenol content from 1540.58 to 2614.59 mg/l.

Table 2 presents the general statistical values for the analyzed parameters (vitamin C, total phenols, anthocyanin's, flavan-3-ols, antioxidant activity) of fruits (pomegranate, apple, cherry), grapes and vegetables (tomato, pepper). Table 3 displays the correlation dependencies between the examined parameters. A moderate negative correlation was found between vitamin C and flavan-3-ols. A moderate positive correlation was observed between total phenols and flavan-3-ols. A very strong correlation was found between total phenols and antioxidant activity. In our case (Table 4), the statistical significance of the correlation between total phenols and antioxidant activity was determined ($p= 0.0395$). The highest coefficient of determination R^2 (Table 5) was found between the percentage of antioxidant activity attributed to vitamin C and the concentration of vitamin C, with approximately 89 % of the variation in the percentage of antioxidant activity explained by the concentration of vitamin C. Furthermore, 69 % of the variation in antioxidant activity was explained by the variations in the concentration of total phenols.

Table 3. Correlation dependencies between the examined parameters (vitamin C, total phenols, anthocyanin's, flavan-3-ols, antioxidant activity)

Variables	Vitamin C	Total phenol	Antocyans	Flavan 3 ols	Antiox. activity	% vitamin C of total anti-ox. activity
Vitamin C	**1**	-0.224	-0.4036	-0.558	0.3286	**0.9416**
Total phenols	-0.224	**1**	0.0669	0.561	**0.8330**	-0.4850
Antocyans	-0.403	0.066	**1**	-0.391	-0.0435	-0.4198
Flavan-3-ols	-0.558	0.561	-0.3918	**1**	0.1314	-0.6130
Antiox. activity	0.3286	**0.833**	-0.0435	0.131	**1**	0.0305
% of vit. C from antiox. activity	**0.9416**	-0.485	-0.4198	-0.613	0.0305	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4. The statistical significance of the above mentioned correlation between total phenols and the antioxidant activity

Variables	Vitamin C	Total phenol	Antocyans	Flavan 3 ols	Antiox. activity	% vitamin C of total anti-ox. activity
Vitamin C	**0**	0.6696	0.4275	0.2497	0.5249	**0.0050**
Total phenols	0.6696	**0**	0.8998	0.2462	**0.0395**	0.3296
Antocyans	0.4275	0.8998	**0**	0.4424	0.9348	0.4072
Flavan-3-ols	0.2497	0.2462	0.4424	**0**	0.8041	0.1957
Antiox. activity	0.5249	**0.0395**	0.9348	0.8041	**0**	0.9543
% of vit. C from antiox. activity	**0.0050**	0.3296	0.4072	0.1957	0.9543	**0**

Values in bold are different from 0 with a significance level alpha = 0.05

Table 5. Percentage of determination R^2between the examined parameters (vitamin C, total phenols, anthocyanin's, flavan-3-ols, antioxidant activity)

Variables	Vitamin C	Total phenol	Antocyans	Flavan-3-ols	Antiox. activity	% vitamin C of total anti-ox. activity
Vitamin C	**1**	0.0502	0.1629	0.3115	0.1080	0.8867
Total phenols	0.0502	**1**	0.0045	0.3153	0.6939	0.2352
Antocyans	0.1629	0.0045	**1**	0.1535	0.0019	0.1763
Flavan-3-ols	0.3115	0.3153	0.1535	**1**	0.0173	0.3757
Antiox. activity	0.1080	0.6939	0.0019	0.0173	**1**	0.0009
% of vit. C from antiox. activity	0.8867	0.2352	0.1763	0.3757	0.0009	**1**

CONCLUSIONS

Based on the analysis results of the chemical composition of fruits, including autochthonous varieties and populations of fruit species, grapes and vegetables, the following observations can be made: Autochthonous varieties of fruit species exhibited higher levels of total phenols, anthocyanin's, flavan-3-ols and displayed the highest antioxidant activity compared to autochthonous grape varieties, as well as tomato and pepper populations.

Among all fruit species, pomegranate showed the highest content of total phenols and the greatest fruit antioxidant activity. Apple varieties displayed the highest levels of flavan-3-ols. Cherries and pomegranates contained the highest amounts of anthocyanin's.

Grape varieties also demonstrated high levels of total phenols, anthocyanin's, and flavan-3-ols. In comparison to other fruit species and grapes, tomato and pepper fruits exhibited the highest vitamin C content.

Moreover, in tomato and pepper populations, vitamin C accounted for the largest proportion of the total fruit antioxidant activity (over 75 % inhibition).

Pepper populations contained higher amounts of vitamin C and total phenols and exhibited greater fruit antioxidant activity compared to tomato populations.

A very strong positive correlation was observed between the content of total phenols and the fruit antioxidant activity. Furthermore, a moderate positive correlation was found between total phenols and flavan-3-ols. Conversely, a moderate negative correlation existed between vitamin C and flavan-3-ols.

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КОМПАРАТИВНО ИСТРАЖУВАЊЕ НА СОДРЖИНАТА НА АНТИОКСИДАНТИТЕ ВО ПЛОДОВИТЕ ОД АВТОХТОНИ СОРТИ ОВОШЈЕ, ГРОЗЈЕ И ЗЕЛЕНЧУК

Ана Селамовска, Виктор Ѓамовски, Биљана Коруноска, Милена Тасеска-Ѓорѓијевски, Климе Белески, Душко Неделковски, Катерина Банџо Орешковиќ, Розе Џољевска-Миленковска, Виктор Рајчин

Земјоделски институт, Универзитет „Св. Кирил и Методиј“ во Скопје, РС Македонија

Во овој научен труд ги презентиравме резултатите од хемиската анализа направена на плодови од автохтони сорти и популации од повеќе овошни видови, имено, цреши, калинки и јаболка, како и на грозје и зеленчук (некои сорти пиперки и домати). Меѓу анализираните примероци, популациите на пиперка покажаа највисока концентрација на витамин Ц, и тоа 51,25 mg/100 g. Спротивно на тоа, најниско ниво на витамин Ц е забележано кај јаболката и грозјето, околу 9 mg/100 g. Кога ќе се земе предвид целокупната антиоксидантна активност, доматите и пиперките имаат најголем удел на витамин Ц, со над 75 mg/100 g. Понатаму, најголемо количество антоцијани е пронајдено во црешите, поточно 394,30 mg/kg FW (FW = свеж примерок). Сортите калинка покажаа најголема содржина на вкупни феноли, околу 5359,43 mg/kg FW и со тоа покажаа највисока антиоксидантна активност (81,58 % инхибиција). Што се однесува до сортите јаболка, тие покажаа најголема содржина на флаван-3-оли, достигнувајќи 517,98 mg/kg FW. Нашата анализа откри позитивна корелација помеѓу вкупните феноли и антиоксидативната активност, како и помеѓу вкупните феноли и флаван-3-олите. Дополнително, беше идентификувана умерена негативна корелација помеѓу витаминот Ц и флаван-3-олите.

Клучни зборови: автохтони; овошни видови; грозје; зеленчук; антиоксиданти

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Original scientific paper

THE INFLUENCE OF STIRRING RATE IN EMULSION SOLVENT EVAPORATION METHOD ON BIOPHARMACEUTICAL PROPERTIES OF MICROPARTICLES CONTAINING ACETAMINOPHEN

Tamara Georgievska[1*], Katerina Atkovska[2], Kiril Lisichkov[1]

[1]Ss. Cyril and Methodius University, Faculty of Technology and Metallurgy,
Institute for chemical and control engineering– Skopje, Republic of North Macedonia
[2]Ss. Cyril and Methodius University, Faculty of Technology and Metallurgy,
Institute for inorganic technology– Skopje, Republic of North Macedonia

*e-mail: tami.georgievska@gmail.com

Acetaminophen is widely used for managing musculoskeletal pain, particularly arthritis in elderly individuals, and for reducing fever associated with colds, flu, and infections. Cellulose-based microparticles were prepared using the acetone/liquid paraffin solvent system through the emulsion solvent evaporation technique. The objective of this study was to assess the influence of stirring rate on encapsulation efficiency, *in vitro* drug release, release kinetics, and microparticle morphology. Drug loading ranged from 13 % to 15 %, while encapsulation efficiency ranged from 67 % to 77 %. A directly proportional relationship between stirring speed and encapsulation efficiency was observed. Drug release followed the Higuchi model, with the release mechanism predominantly Fickian diffusion. In one trial, non-Fickian diffusion was observed. Microphotographs revealed the formation of both spherical and angular microparticles.

Key words: stirring rate; acetaminophen; microparticles; emulsion solvent evaporation technique; biopharmaceutical properties

INTRODUCTION

Interest in designing a microencapsulation process for active pharmaceutical ingredients (APIs) to achieve controlled drug release mechanism has grown significantly in recent decades. Sustained-release formulations are highly desirable in medicine and pharmacy as they reduce the frequency of administration, leading to improved patient compliance. Consequently, polymeric microparticles have been extensively investigated as a controlled drug delivery system.

For the encapsulation of hydrophilic drugs, the double emulsion method, followed by organic solvent evaporation or extraction, is most commonly employed [1, 2]. However, research studies using O_1/O_2 emulsions for microencapsulation have also been conducted [3]. Compared to spray-drying, the emulsion-solvent evaporation method (ESE) allows for the preparation of microparticles with controlled particle size [4]. However, careful selection of starting materials and preparation conditions is necessary to achieve higher encapsulation efficiency (EE), low residual solvent content, and acceptable extraction of the oil phase from the microparticle surface.

To obtain microparticles with desired properties using the ESE method, the following parameters are commonly varied: viscosity of the dispersed phase, volume ratio of dispersed phase to continuous phase, drug quantity in the dispersed phase, emulsifier content, stirring rate, temperature, and pressure [5]. The effects of solvent removal rate have been investigated in Poly(L-lactide) acid (PLLA) and poly(lactic-co-glycolic acid) (PLGA) microspheres [6, 7]. Additionally, the effect of ambient or reduced ambient pressure on solvent evaporation rate in the ESE method has been reported [8]. It has been observed that as the stirring rate increases, the average particle

size of microparticles decreases, as confirmed by relevant publications [9, 10]. However, no data correlating the influence of process parameter stirring time on particle shape, morphology, and surface topography was found at the time of literature review.

Due to the increasing prevalence of musculoskeletal pain (MSP), there is a growing demand for pharmacological treatments worldwide [11, 12]. Non-steroidal anti-inflammatory drugs (NSAIDs) with or without opioids are commonly administered for both acute and chronic MSP management [13]. However, in elderly populations, patients with comorbidities or contraindications may experience adverse effects, leading to the prescription or recommendation of acetaminophen as a therapy. Acetaminophen possesses analgesic and antipyretic properties with minimal anti-inflammatory activity, alongside minor gastrointestinal, renal, and vascular side effects. It has long been one of the most commonly administered drugs, both over-the-counter (OTC) and by prescription, for pain and fever [14].

Therefore, the aim of this study was to prepare, investigate, and characterize a hydroxypropyl cellulose (HPC)-based microsystem as a potential dosage form with controlled release of acetaminophen following oral administration.

MATERIALS AND METHODS

Materials

The active pharmaceutical ingredient (API) N-(4-hydroxyphenyl) acetamide (acetaminophen or paracetamol), hydroxypropyl cellulose as a polymer matrix, and Polyoxyethylene (80) sorbitan monooleate were obtained from Zhejiang Kangle Pharmaceutical Co., Ltd., Nippo Soda, and Sigma Aldrich, respectively. Liquid paraffin, n-hexane, and acetone were purchased from Alkaloid AD. All other chemicals used were of analytical grade.

Methods

Preparation of microparticles

The hydroxypropyl cellulose-based microparticles were prepared using the ESE technique. Briefly, the API was dissolved in acetone, and the polymer was dissolved in the drug solution using a magnetic stirrer until a viscous solution was formed. The resulting drug-polymer solution was extruded using a needle and syringe into an oil phase of liquid paraffin and surfactant (at a constant volume). The emulsion was stirred with a mechanical stirrer at three levels (low – 700 rpm, medium – 900 rpm, and high – 1100 rpm stirring rate) for four hours at ambient temperature to allow the evaporation of the organic solvent from the internal phase. Separation of the obtained microparticles was performed by decantation and filtration. Acetaminophen microparticles were left to stand still for 10–15 minutes to allow particle settlement under gravity. The microparticle residues were washed with n-hexane as a solvent to remove traces of oil. Drying of the microparticles was performed for 24 hours at room temperature.

Characterization of microparticles

Determination of yield of microparticles

The yield (%) of dried HPC microparticles was calculated as the ratio of dried microparticles to the total theoretical amount of starting ingredients.

Determination of encapsulation efficiency of acetaminophen

Microparticles were dissolved in methanol and water (45:55, *v*/*v* %) to achieve a final sample concentration of 0.02 mg/ml. The drug content was analyzed spectrophotometrically at 243 nm (UV spectrophotometer Pharo 600, Merck). The encapsulation efficiency was expressed as the percentage of entrapped acetaminophen in the polymer matrix with respect to the theoretical acetaminophen concentration.

In vitro drug release study

In vitro release from the microparticles was carried out using a magnetic stirrer for four hours at a stirring speed of 50 rpm and a temperature of 37±0.5°C in phosphate buffer pH 6.8, simulating the duodenal medium. The initial concentration of the microparticles was 0.2 mg/ml. Samples of the dissolution media (2 ml) were taken at predetermined time intervals and replaced by fresh buffer. The content of released drug was analyzed spectrophotometrically at 245 nm (UV spectrophotometer Pharo 600, Merck).

ATR-IR spectroscopy

ATR-IR spectra of pure polymer, API, and microparticles prepared at different operating conditions were measured using a Varian 660 FT-IR spectrometer, Varian Inc., equipped with an ATR module with a ZnSe crystal and a low-pressure clamp. The spectra were recorded in the region of 4000–550 cm^{-1}. All spectra were averaged from 16 scans per spectrum, and the resolution was set to 4 cm^{-1}.

Morphology of microparticles

The morphology of the microparticles was analyzed using a Zeiss Axioscope 5 microscope with a Zeiss Axiocam 208 color camera and a reflected light source, at a magnification range of 2.5x-50x.

RESULTS AND DISCUSSION

Methods

Preparation of microparticles

The stirring rate during the hardening phase of emulsified droplets in preparing HPC microparticles is considered an important factor affecting microparticle characteristics. Figure 1 illustrates the morphology of the acetaminophen-loaded microparticles obtained at different stirring rates. Spherical morphology of the HPC-loaded microparticles was observed when the highest investigated mixing speed was applied to remove solvent for forming microparticles. In contrast, angular and nonspherical morphology of the microparticles were observed when a low mixing speed was applied to form microparticles. The obtained microparticles were free-flowing granules, although some agglomeration can be observed from the micrographs (Figure 1). No sharp differences were noted in terms of surface porosity and smoothness.

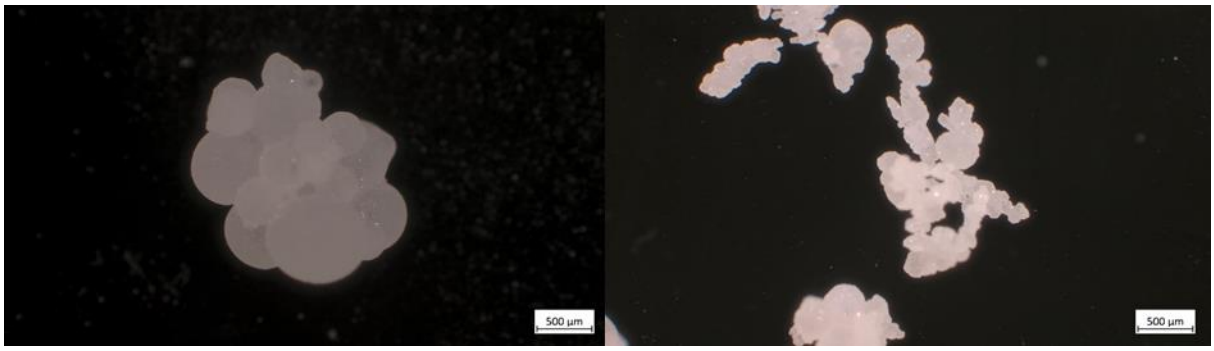

Figure 1. Micrographs of microparticles fabricated at different stirring rates F-1 (1100 rpm) and F-2 (700 rpm)

The physical state of the drug, polymer, and microparticles, as well as drug encapsulation in the polymer matrix, were investigated by ATR-IR spectroscopy. The FT-IR spectrum (Figure 2) showed the characteristic band of phenolic alcohol of acetaminophen at 3321 cm^{-1}, C=O belonging to an amide group at 1651 cm^{-1}, C=C (aromatic) at 1609 cm^{-1}, and N–H (amide) at 1561 cm^{-1} [15].

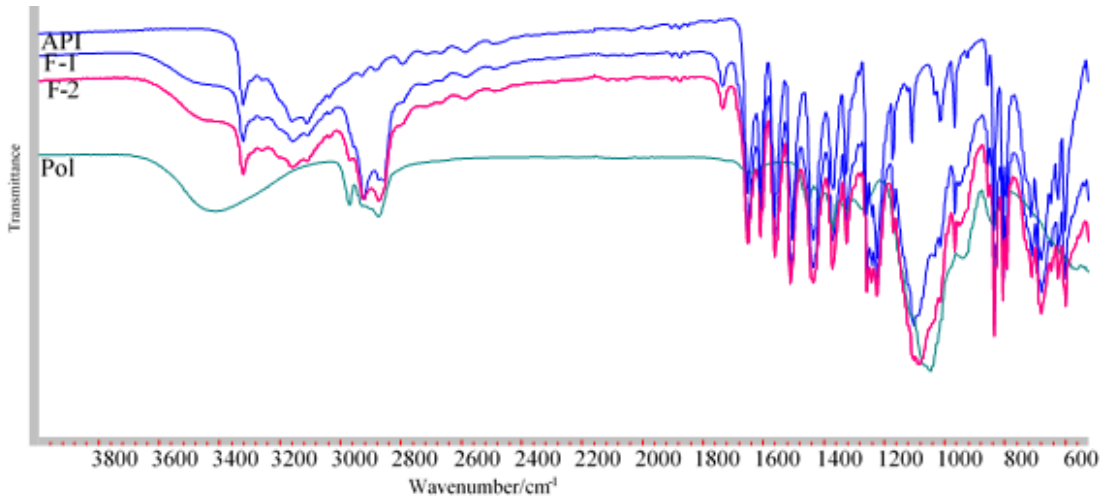

Fig. 2. FT-IR spectra of API, polymer (Pol) and microparticles F-1 and F-2

In the case of HPC, a broad band on the HPC spectrum was observed at 3411 cm^{-1}. The peaks in the region 2970–2874 cm^{-1} represent symmetric and asymmetric C-H stretching. The sharp peaks at

1373 cm^{-1} and 1069 cm^{-1} reflect C-H asymmetric deformations and C-O-C stretching, respectively. The peak at 1644 cm^{-1} is associated with the adsorbed water [16]. Absence of this peak is observed in the microparticles.

In the FTIR spectra of microparticles, there were no notable shifts in the characteristic bands for acetaminophen in the microparticles, indicating compatibility of the components. The new appearing band at 1735 cm^{-1} in the microparticles represents C=O stretching, arising from the residues of the used emulsifier. A slight increase in the intensity of the characteristic bands for acetaminophen in the trials (F-1 *vs*. F-2) at 3321 cm^{-1} and 1651 cm^{-1} can be observed, due to higher API content (14.7 % *vs*. 13.0 %) in the microparticulate delivery system. The FTIR data of HPC (control and microparticles) suggested that C-O-C stretching peaks were observed at a higher wavenumber (1069→1085 cm^{-1}) compared to control. The increase in wavenumber of the stretching peak might be attributed to a change in the bond strength [17].

The variation of stirring rate had a significant effect on the encapsulation of acetaminophen in HPC microparticles, as indicated by the FTIR spectra. The parameter encapsulation efficiency evaluates the successfulness of a drug delivery system (entrapped drug into the carrier). Based on the provided data in Table 1, we may recognize that the investigated drug carrier in our research exhibits satisfactory encapsulation efficiency ranging from 67.4 % to 76.6 %. A value for encapsulation efficiency approaching 65 % was considered suitable.

Table 1. Properties of acetaminophen microparticles

Formulation code	Stirring rate [rpm]	Yield [%]	Bulk density [gcm^{-1}]	Encapsulation efficiency [%]	Theoretical drug content [*w*/*w* %]	Amount of entrapped drug [*w*/*w* %]
F-1	1100	87.3	0.484	76.6	20.0	14.7
F-3	900	70.4	0.349	72.6	20.0	14.0
F-2	700	83.1	0.456	67.4	20.0	13.0

A proportional dependence is observed between stirring rate and encapsulation efficiency. These results agree with previous publication [9]. Yet, an inverse dependence has been observed in encapsulation of simvastatin in a polymeric blend of PLA and PCL [18]. No dependence between bulk density and stirring rate can be noticed.

Figure 3 exhibits the cumulative release profiles of acetaminophen from microparticles that have been produced at various stirring rates. As indicated by Figure 3 and Table 2, acetaminophen *in vitro* release from the microparticle formulations showed an initial burst effect that may be attributed to the presence of drug particles on the surface of the microparticles.

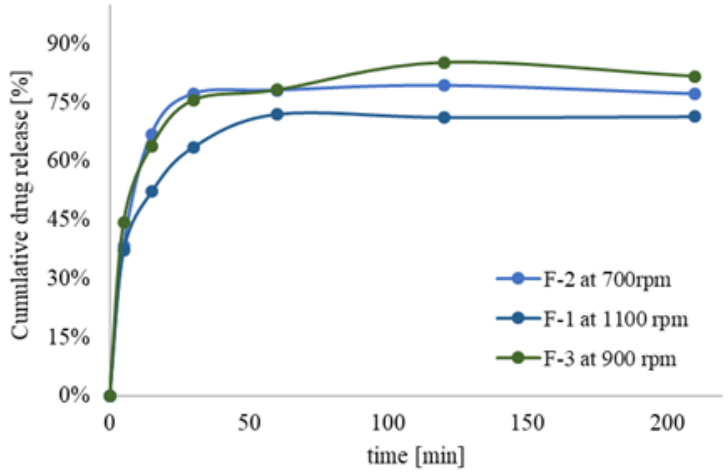

Figure 3. Cumulative percent release of acetaminophen from HPC-based-microparticles prepared at various stirring rates

The lowest initial burst effect was exhibited by F-1 formulation, whereas the highest initial burst effect was exhibited by F-2. Our microparticles were prepared by O_1/O_2 emulsion, in which the hy-

drophilic drug does not tend to migrate to the polar solvent, thus concentrating on the polymeric surface and leading to a burst effect. Nonetheless, the burst effect can be explained by a nonuniform encapsulation of the drug as a result of a nonstable emulsion during the preparation step "solvent removal". Such instability may cause migration of the encapsulated drug molecules toward the microparticle's surface, thus contributing to the initial burst effect [3]. Microparticles with the highest drug encapsulation efficiency (F-1) showed the lowest dissolution rate for all three specification points (Table 2). The specification points were selected based on USP monographs for Acetaminophen tablets and capsules with sustained release [19]. An inverse proportional dependence is observed between stirring rate and released drug at the 15th minute. The acetaminophen microparticles can be filled in gelatin capsules for peroral administration. According to the USP Pharmacopoeia, not less than 80 % in four hours should be released.

Table 2. Acetaminophen *in vitro* release from polymeric microparticles

Formulation code	**F-1 [1100 rpm]**	**F-3 [900 rpm]**	**F-2 [700 rpm]**
Time [minutes]		**Drug release [%]**	
15	54.16	67.76	71.05
60	76.77	84.07	83.76
210	76.12	88.08	82.75

By varying the stirring rate along with polymer content, the acetaminophen release rate can be controlled. The polymer content is expected to contribute to forming a thick polymeric wall that will slow the penetration of the dissolution medium into the polymeric microparticles, thus reducing the drug release and prolonging a lag time [3].

The *in vitro* release profiles were fitted with kinetic models to determine the mechanism of drug release [20, 21]. The fit parameters of zero order, first order, Higuchi model, and Korsemeyer-Peppas 60% are presented in Table 3. The n-value (graphically determined) of microparticles at different stirring rates was between 0.33 – 0.56, indicating that the drug release mechanism was Fickian diffusion (F-1 and F-3), whereas erosion and polymeric chain swelling occurred in F-2.

Table 3. Fitting parameters from kinetic assessment on drug release data

Formulation code	Zero order		First order		Higuchi		Korsemeyer-Peppas 60 %	
	D	R^2	K	R^2	K_H	R^2	n	R^2
F-1	0.0014	0.470	–6·106	0.470	0.028	0.664	0.33	0.999
F-3	0.0015	0.466	–7·106	0.467	0.030	0.659	0.36	1.0
F-2	0.001	0.289	–5·106	0.286	0.027	0.459	0.56	1.0

The assessment of release kinetics revealed that drug release from acetaminophen microparticles followed the Higuchi model. It was observed that, as the stirring rate increased, better compatibility with Q√t kinetics was displayed. Theoretically, it was expected that the prepared microparticles would be compatible with Q√t kinetics, being in a matrix structure [22]. This compatibility is an indicator that a matrix structure in the microparticulate form has been achieved rather than a solubility-controlled dosage form. Our findings are consistent with other investigations [3, 23–25].

CONCLUSION

HPC, which is a biodegradable polymer, was investigated in this study to produce acetaminophen-based microparticles with controlled release by a single ESE method. Microscopy analysis revealed spherical microparticles at the highest stirring rate. By UV-vis and FT-IR spectroscopy, the encapsulation of acetaminophen in HPC was confirmed. A direct dependency between encapsulation efficiency and stirring rate was observed. Yield

percentages may be improved by the scale-up process. An inverse proportional dependence is observed between stirring rate and released drug at the 15th minute. Drug release mainly follows Fickian diffusion, apart from one trial. By controlling the stirring rate, the solvent evaporates from the emulsion, so that the process of hardening and formation of HPC-based microparticles follows. Yet, further investigation into the influence of the following parameters: preparation temperature, reduction in ambient pressure, internal/external phase ratio, and emulsifier content has to be performed to attain a full screening-development study.

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ВЛИЈАНИЕТО НА БРЗИНАТА НА МЕШАЊЕТО НА РАСТВОРУВАЧОТ ВО МЕТОДОТ ЕМУЛЗИЈА-ИСПАРУВАЊЕ ВРЗ БИОФАРМАЦЕВТСКИТЕ СВОЈСТВА НА МИКРОЧЕСТИЧКИТЕ СО АЦЕТАМИНОФЕН

Тамара Георгиевска[1], Катерина Атковска[2], Кирил Лисичков[1]

[1]Универзитет „Св. Кирил и Методиј“ во Скопје, Технолошко-металуршки факултет, Институт за хемиско и контролно инженерство, Скопје, РС Македонија
[2]Универзитет „Св. Кирил и Методиј“ во Скопје, Технолошко-металуршки факултет, Институт за неорганска технологија, Скопје, РС Македонија

Ацефаминофенот е широко употребуван во третирањето на мускулноскелетните болки, главно за третирање на артритисот кај повозрасни луѓе и за намалување на температурата при настинки, грип и инфекции. Целулозните микрочестички се подготвуваат со техниката емулзија-испарување на растворувачот со примена на системот ацетон/течен парафин. Целта на ова истражување беше да се евалуира влијанието на брзина на мешањето врз: ефикасноста на инкапсулацијата, *in vitro* ослободување на лекот, кинетиката на ослободувањето и морфологијата на микрочестичките. Инкапсулираниот лек вариираше од 13 % до 15 %, додека ефикасноста на инкапсулацијата се движеше во рангот од 67 % до 77 %. Беше утврдена правопропорционална зависност помеѓу брзината на мешањето и ефикасноста на инкапсулацијата. Ослободувањето на лекот го следи Хигучиевиот модел, а механизмот на ослободувањето на лек е доминантно Фиковата дифузија. За една проба не беше согледана Фикова дифузија. Од микрофотографиите може да се дојде до заклучок дека се добиени микрочестички со сферна и неправилна морфологија.

Клучни зборови: брзина на мешање; ацетаминофен; микрочестички; техника на емулзија-испарување на растворувач; биофармацевтски својства

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