IDENTIFIKACIJA I BIOLOŠKA AKTIVNOST SLOBODNIH HLAPLJIVIH SPOJEVA RODA Veronica L. (PLANTAGINACEAE)

Nazlić, Marija

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

Marija Nazlić

IDENTIFIKACIJA I BIOLOŠKA AKTIVNOST SLOBODNIH HLAPLJIVIH SPOJEVA RODA *Veronica* L. (PLANTAGINACEAE)

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Marija Nazlić

IDENTIFICATION AND BIOLOGICAL ACTIVITY OF FREE VOLATILE COMPOUNDS OF THE GENUS *Veronica* L. (PLANTAGINACEAE)

DOCTORAL THESIS

Zagreb, 2023.

“Ovaj doktorski rad izrađen je na Odjelu za Biologiju, Prirodoslovno-matematičkog fakulteta, Sveučilišta u Splitu, pod vodstvom prof. dr. sc. Valerije Dunkić, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. “

“This doctoral thesis was made at the Department of Biology, Faculty of Science, University of Split, under the supervision of Valerija Dunkić, PhD, Full Professor, as a part of the Doctoral program of Biology at the University of Zagreb, Faculty of Science, Department of Biology. “

Izrada ovog doktorskog rada financirana je iz projekta HrZZ IP-2020-02-8425 pod nazivom „Hrvatske vrste roda *Veronica*: Fitotaksonomija i biološka aktivnost“, CROVeSPhyBA, kojeg vodi prof. dr. sc. Valerija Dunkić.

Mentorica: prof. dr. sc. Valerija Dunkić

Valerija Dunkić je redovita profesorica iz područja prirodnih znanosti, polje biologija, zaposlena na Prirodoslovno-matematičkom fakultetu Sveučilišta u Splitu. Od 2018. godine je i znanstveni suradnik iz područja biotehničkih znanosti polje prehrambena tehnologija. Doktorirala je 2006. godine na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu s temom rada: "Submikroskopske strukture i sekundarni metaboliti u žljezdanim stanicama nekih vrsta roda *Satureja* (Lamiaceae)".

Nositeljica je obaveznih kolegija Fiziologija bilja i Opća botanika, te izbornih kolegija Izolacija i primjena eteričnih ulja, Začinsko i aromatsko bilje i Osnove mediteranske prehrane na Preddiplomskom studiju Biologija i kemija i Biologija na Prirodoslovno-matematičkom fakultetu u Splitu, te na poslijediplomskom znanstvenom studiju „Istraživanje u edukaciji u području prirodnih i tehničkih znanosti" s izbornim kolegijima Biljne makromolekule i izolacija i Metabolizam kserofita. Na integriranom studiju Farmacije na Medicinskom fakultetu u Splitu nositeljica je kolegija Farmaceutska botanika, te na Sveučilišnom odjelu Mediteranska poljoprivreda u Splitu nositeljica je kolegija Opća botanika i Osnove fiziologije bilja.

U koautorstvu i autorstvu objavila je 54 znanstvena rada u časopisima citiranim u bazi Web of Science, četiri poglavlja u knjigama, te je sudjelovala na 42 znanstvena skupa. Bila je mentorica na 60 završnih i diplomskih radova.

Bila je suradnica na četiri projekta Ministarstvo znanosti i obrazovanja Republike Hrvatske od 1996. - 2014. godine. Od 2014. - 2018. godine bila je suradnica na projektu Hrvatske zaklade za znanost „Taksonomija, ekologija i uporaba rogača (*Ceratonia siliqua*) i lovora (*Laurus nobilis*) u Hrvatsko" (TEUCLIC) (HRZZ-IP-11-2013-3304). Od 2020. – 2022. godine bila je suradnica na projektu Europskog fonda za regionalni razvoj „Nove metode u suzbijanju štetnika masline primjenom biljnih hlapivih tvari".

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Recenzira znanstvene članke za brojne međunarodne časopise i bila je gost urednik tri specijalna izdanja časopisa Plants. Recenzirala je dva udžbenika iz Poljoprivredne botanike.

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Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

IDENTIFIKACIJA I BIOLOŠKA AKTIVNOST SLOBODNIH HLAPLJIVIH SPOJEVA RODA *Veronica* L. (PLANTAGINACEAE)

MARIJA NAZLIĆ

Doktorski rad izrađen je na Odjelu za Biologiju Prirodoslovno-matematičkog fakulteta u Splitu, Ruđera Boškovića 33

Sažetak:

Odabrane vrste roda *Veronica* (čestoslavice, porodica Plantaginaceae) - *V. saturejoides* ssp. *saturejoides, V. austriaca* ssp. *jacquinii* i *V. officinalis,* prikupljene su na prirodnim staništima u Republici Hrvatskoj. Za ove vrste istražen je fitokemijski sastav slobodnih hlapljivih spojeva eteričnih ulja i hidrosola plinskom kromatografijom-masenom spektrometrijom. Prevladavajući spojevi bili su heksahidrofarnezil aceton i heksadekanska kiselina. Iste vrste testirane su na antioksidativno, antiproliferativno i antifitovirusno djelovanje. Vrsta *V. saturejoides* ssp. *saturejoides* i *V. officinalis* pokazale su jača djelovanja od vrste *V. austriaca* ssp. *jacquinii* u većini testova. Mikromorfološkim istraživanjem u sve tri vrste identificirani su žljezdani trihomi u kojima se primarno događa sinteza istraživanih spojeva. U drugom dijelu doktorskog rada genetički su analizirane ITS regije i slobodni hlapljivi spojevi za 18 vrsta čestoslavica. Uz gore navedene vrste materijal je prikupljen i analiziran za: *V. longifolia, V. acinifolia, V. anagallis-aquatica, V. beccabunga, V. catenata, V. serpyllifolia, V. anagalloides, V. montana, V. arvensis, V. chamaedrys, V. dalmatica, V. persica, V. polita, V. cymbalaria* i *V. hederifolia*. Usporedbom klastera utvrđenim genetskim istraživanjima s fitokemijskom raspodjelom različitih hlapljivih tvari, donesen je zaključak o mogućnosti korištenja određenih hlapljivih komponenti kao kemofenetskih markera za rod *Veronica*.

Rad ima: 193 stranice, 219 literaturnih navoda, 42 slike, 32 tablice

Ključne riječi: čestoslavice, *Veronica,* slobodni hlapljivi spojevi, kemofenetski markeri, biološka aktivnost

Mentorica: Prof. dr. sc. Valerija Dunkić

Ocjenjivači:

1. Prof. dr. sc. Željka Vidaković-Cifrek, redovita profesorica, Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu, predsjednica povjerenstva

2. Prof. dr. sc. Zlatko Liber, redoviti profesor u trajnom zvanju, Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu, član

3. Izv. prof. dr. sc. Martina Grdiša, izvanredna profesorica, Agronomski fakultet, Sveučilište u Zagrebu, član

4. Doc. dr. sc. Ivica Šamanić, docent, Prirodoslovno-matematički fakultet, Sveučilište u Splitu, zamjenski član

University of Zagreb

Faculty of Science

Department of Biology

Doctoral thesis

IDENTIFICATION AND BIOLOGICAL ACTIVITY OF FREE VOLATILE COMPOUNDS OF THE GENUS *Veronica* L. (PLANTAGINACEAE)

MARIJA NAZLIĆ

Thesis performed at Department of Biology, Faculty of Science, University of Split, Ruđera Boškovića 33

Abstract:

Selected species of the genus *Veronica* (speedwells, family Plantaginaceae) - *V. saturejoides* ssp. *saturejoides*, *V. austriaca* ssp. *jacquinii* and *V. officinalis*, were collected in natural habitats in the Republic of Croatia. For these species, the phytochemical composition of free volatile compounds of essential oils and hydrosols was investigated by gas chromatography-mass spectrometry. The predominant compounds were hexahydrofarnesyl acetone and hexadecanoic acid. The same species were tested for antioxidant, antiproliferative and antiphytoviral activity. The species *V. saturejoides* ssp. *saturejoides* and *V. officinalis* showed stronger effects than *V. austriaca* ssp. *jacquinii* in most tests. Micromorphological research in all three species identified the glandular trichomes in which the synthesis of the investigated compounds primarily occurs. In the second part of the dissertation, the ITS regions and free volatile compounds of 18 species of speedwells were genetically analyzed. In addition to the above mentioned species, the material was collected and analyzed for: *V. longifolia, V. acinifolia, V. anagallis-aquatica, V. beccabunga, V. catenata, V. serpyllifolia, V. anagalloides, V. montana, V. arvensis, V. chamaedrys, V. dalmatica, V. persica, V. polita, V. cymbalaria* and *V. hederifolia*. By comparing the clusters obtained from genetic research with the phytochemical distribution of various volatile substances, a conclusion was reached about the possibility of using certain volatile components as chemophenetic markers for the genus *Veronica*.

Thesis has: 193 pages, 219 literature references, 42 figures, 32 tables

Key words: speedwells, *Veronica*, free volatile compounds, chemophenetic markers, biological activity

Supervisor: Valerija Dunkić, PhD, Full Professor

Reviewers:

1. Željka Vidaković-Cifrek, PhD, Full Professor, Faculty of Science, University of Zagreb, the president of the committee

2. Zlatko Liber, PhD, Full Professor- tenured position, Faculty of Science, University of Zagreb, member

3. Martina Grdiša, PhD, Associate Professor, Faculty of Agriculture, University of Zagreb, member

4. Ivica Šamanić, PhD, Assistant Professor, Faculty of Science, University of Split, substitute member

Sadržaj

1. UVOD

Proučavanje biokemijskih spojeva nastalih metaboličkim putevima u biljkama sakupljenim na prirodnim staništa iznimno je važno jer izolacijom i identifikacijom tih spojeva dobivamo uvid koji spojevi utječu ne samo na biljku u kojoj se nalaze, već neizravno i na druge biljke u blizini kao i na okoliš u cjelini [1]. Ovi spojevi su važni čimbenici u prilagodbi biljaka na abiotske stresne čimbenike. Štoviše, susjedne biljke detektiraju druge biljne hlapljive tvari kao „poruke“ o napadima biljojeda ili patogena, i posljedično prilagođavaju svoje metaboličke odgovore [2]. Prvi dio disertacije usmjeren je na identifikaciju slobodnih hlapljivih spojeva koji čine važan dio specijaliziranih biljnih metabolita, te biološku aktivnost kod vrsta *Veronica saturejoides* Vis. ssp. *saturejoides, Veronica officinalis* L. i *Veronica austriaca* L. ssp. *jacquinii* (Baumg.) Eb. Fisch. a objavljen je kroz prva tri rada (poglavlje 3.1., 3.2. i 3.3.). Drugi dio disertacije se bavi pitanjem slobodnih hlapljivih komponenti kao kemofenetskih markera za rod *Veronica* istraženih i objavljenih kroz dva znanstvena članka prikazanim u poglavljima 3.4. i 3.5.

1.1. Rod *Veronica* – čestoslavice

Vrste odabrane za istraživanje u okviru ove doktorske disertacije pripadaju rodu *Veronica* L. (čestoslavica) koji je najbrojniji rod unutar reda Lamiales (porodica Plantaginaceae) s oko 500 vrsta. Vrste ovog roda uglavnom rastu u umjerenim regijama sjeverne hemisfere, a značajan broj vrsta (približno 180) s *Hebe* kompleksom raste u regijama južne hemisfere (npr. u Australiji) [3], [4]. Ekstremna varijabilnost u morfologiji i vrlo dobra prilagodba različitim životnim uvjetima ovog roda omogućila je ovim biljnim vrstama rasprostranjenost na širokom rasponu staništa, od vodenih, močvarnih i šumskih staništa do stijena, pukotina, polja i ruderalnih staništa [3], [5]. Većina predstavnika raste u područjima s mediteranskom klimom [3]. Za Europu je opisano 67 vrsta roda *Veronica* [6] dok je u Hrvatskoj opisano 37 vrsta od kojih su tri endemične: *V. dalmatica* Padilla-García, Rojas-Andrés, López-González & M.M.Mart.Ort., *V. orbiculata* A. Kern. i vrsta koja je predmet ovog rada *V. saturejoides* Vis. ssp. *saturejoides* [7]. Čestoslavice su zeljaste trajnice ili jednogodišnje biljke s plavim, bijelim, ružičastim ili ljubičastim cvjetovima [8]. Stabljika je ponekad drvenasta, a može biti uspravna i polegnuta s nasuprotno smještenim listovima koji su ponekad skupljeni kao rozeta pri dnu stabljike [9]. Pojedinačni cvjetovi razvijaju se u pazušcima listova ili su raspoređeni u pazušne ili završne cvjetove. Čaška je podijeljena na četiri ili pet, često nejednakih, segmenata. Vjenčić se sastoji od 4 srasle latice. U cvjetovima su dva prašnika i jedan tučak koji se sastoji od dva srasla plodna lista. Plod je tobolac (čahura) [6]. Cvjetna

formula ovog roda je ↑K(4)C(4)A2G(2).

1.1.1. Vriskova čestoslavica – *Veronica saturejoides* ssp. *saturejoides*

Vriskova čestoslavica (Slika 1a i 1c) je endemična vrsta koja raste na stijenama Dinarskog gorja u Hrvatskoj, Bosni i Hercegovini i Crnoj Gori. Postoje još dvije podvrste ove biljke, jedna iz Albanije, *V. saturejoides* ssp. *munellensis*, i jedna iz Bugarske, *V. saturejoides* ssp. *kellereri* [10]. Vrsta *V. saturejoides* Vis. ssp. *saturejoides* je višegodišnja puzava biljka, koja naraste 10 – 30 cm u visinu i ima izduženo, donekle lignificirano korijenje. Ima dlakavu stabljiku koja je pri dnu drvenasta. Listovi su jednostavni, jedan nasuprot drugome, dugi 6 – 9 mm, integriranog ruba i nisu ili su malo dlakavi (Slika 1c). Pri dnu su skupljeni i formiraju rozetu. Cvjetovi su plavo-ljubičaste boje, dvospolni i nepravilni (zigomorfni). Po šest do dvanaest cvjetova formiraju duge terminalne grozdove duge svega 3 cm [11]. Cvate od kraja svibnja do lipnja (srpnja). Plod je tobolac srcolikog oblika, dlakav, dug 3,5 – 4 mm (Slika 1b i 1d).

(a)  (b) 

(c)  (d) 

Slika 1. *Veronica saturejoides* Vis. ssp. *saturejoides* – vriskova čestoslavica na prirodnom staništu na planini Dinari: (a) u cvatu (Autor: Milenko Milović, s dopuštenjem), (b) s plodovima (Autor: Marija Nazlić), (c) cvat – krupni plan (Autor: Milenko Milović, s dopuštenjem), (d) s plodovima – krupni plan (Autor: Marija Nazlić)

1.1.2. Ljekovita ili puzava čestoslavica – *Veronica officinalis* L.

Ljekovita čestoslavica je zeljasta trajnica s malim ljubičastim cvjetovima. Stabljika je polegnuta ili rjeđe pridignuta u donjim dijelovima, visine 10 – 50 cm, okrugla ili tupo četverobridna, obrasla stršećim dlakama (Slika 2). Ima listove dužine 1,5 – 5 cm, na kratkim peteljkama, koji su na rubu pilasti i dlakavi [7], [12]. Cvjetovi su dvospolni, zigomorfni s dva prašnika, jednim tučkom, skupljeni u grozdaste cvatove. Čaška je dlakava, duga 2,5 – 3 mm, a vjenčić koturastozvonast, promjera 8 mm, svijetloljubičast, blijedoplavičast ili rjeđe bijel. Cvate od svibnja do lipnja. Medonosna je biljka. Plod je tobolac veličine oko 4 mm, dlakav i naopako deltast do naopako srcast. Rasprostranjena je u Europi, na Azorskom otočju i u jugozapadnoj Aziji gdje raste u šumama, šumskim čistinama, od gorskog do pretplaninskog vegetacijskog pojasa, na umjereno suhim kiselim tlima. Prema dosadašnjem korištenju i znanstvenim istraživanjima, najznačajnija je ljekovita vrsta u rodu te se često koristi u pučkoj medicini za liječenje plućnih bolesti, jetre, slezene, bubrega, mokraćnog mjehura, kod kožnih bolesti, gihta i za zacjeljivanje rana [13], [14].

Slika 2. *Veronica officinalis* L. – ljekovita ili puzava čestoslavica na prirodnom staništu na planini Kamešnici (Autor: Marija Nazlić)

1.1.3. Tankolisna čestoslavica – *Veronica austriaca* L. ssp. *jacquinii*

Tankolisna ili žakenova čestoslavica (Slika 3) je višegodišnja zeljasta biljka koja raste na sunčanim, suhim mjestima, na kamenjarima i pašnjacima. Stabljika je visine (10) 25 – 50 (70) cm, blago puzajuća, češće uspravna. Stabljika je prekrivena trihomima duljine (0,6) 0,8 – 1,2 (1,5) mm, katkad manje ili više gola. Listovi su okruglasti ili široko lancetasti, rasperani, linearni ili linearno lancetasti, duž oboda više ili manje nazubljeni, pokriveni dlakama duljine (0,3) 0,4 – 1,2 (1,5) mm ili rjeđe goli. Listovi sterilnog dijela izdanka na vrhu stabljike su manje

razdijeljeni ili skoro cijeli. Grozdasti cvatovi nose 10 – 50 cvjetova na cvatnim stapkama duljine 2 – 10 cm. Stapke polaze iz pazušca gornjih listova stabljike. Čaška je četverodijelna, rjeđe podijeljena na pet linearno lancetastih, nejednakih režnjeva. Vjenčić je širine 7 – 10 mm, intenzivno plave boje, a latice nejednake i jajaste. Plod tobolac je spljoštena, okruglast, obrnuto srcast, na vrhu usječen, širok 4 – 5 mm, dug kao i čaška ili malo duži, na osnovici zaobljen, pokriven dlakama ili gol. Cvjeta od lipnja do srpnja [9], [12], [15], [16].

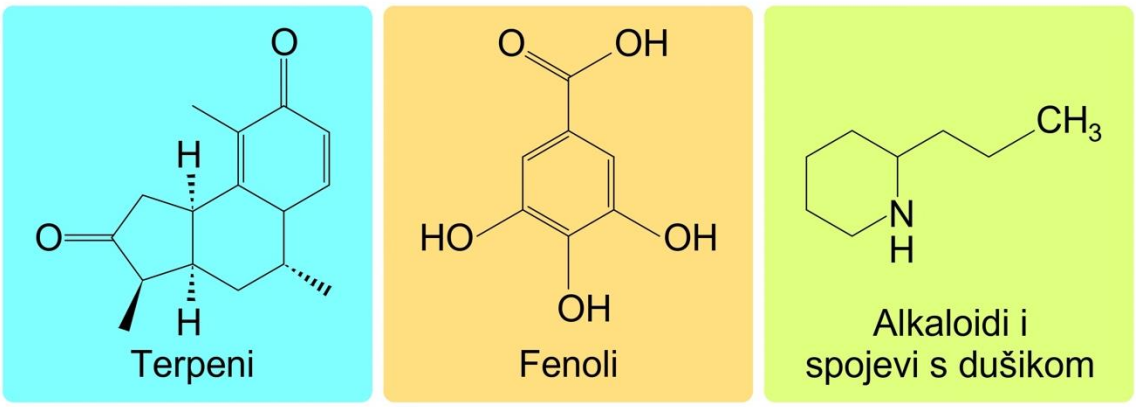
Slika 3. *Veronica austriaca* L. ssp. *jacquinii* (Baumg.) Eb. Fisch. – tankolisna čestoslavica (Autor: Marija Nazlić)

1.2. Specijalizirani metaboliti

Biljke su često izložene okolišnim stresnim uvjetima koji nepovoljno utječu na rast, razvoj ili urod. Stres može biti biotički, uzrokovan drugim organizmima, ili abiotički, koji proizlazi iz obilja ili manjka u fizičkim i kemijskim uvjetima okoliša. Okolišni uvjeti koji uzrokuju štetu biljkama uključuju poplave, suše, previsoke ili preniske temperature, visoku koncentraciju soli, neodgovarajuće mineralne hranjive tvari, te previše ili premalo svjetla. Stres izaziva širok raspon biljnih odgovora, od promjene u genskoj ekspresiji i staničnom metabolizmu do promjena u stopi rasta i prinosu usjeva [18].

Biljke rastu u svakom nastanjivom okolišu, a većina ih raste na kopnu. Suočene s brojnim navedenim stresnim uvjetima i izazovima, uz činjenicu da su sesilne, biljke su počele stvarati različite molekule za odbijanje napada životinja i ublažavanje okolišnih stresnih uvjeta.

Ove iste molekule daju biljci sposobnost otpuštanja mirisa, boja i toksičnosti [18], a pripadaju skupini specijaliziranih (sekundarnih) metabolita. Za razliku od primarnih metabolita, ovi metaboliti nemaju ulogu u glavnim metaboličkim procesima kao što su fotosinteza i disanje, ali zato su iznimno važni za adaptaciju biljke na različite okolišne uvjete. Specijalizirani metaboliti se na temelju načina biosinteze mogu podijeliti na – terpene, fenolne spojeve i spojeve koji sadrže dušik (Slika 4) [19]. Ovi metaboliti posjeduju različite biološke aktivnosti koje se mogu koristiti u medicini, farmaciji i prehrani (npr. konzerviranju hrane), budući da mikroorganizmi postaju sve otporniji na sintetske spojeve. Sintetski spojevi (npr. BHA – butilirani hidroksianizol) također mogu biti kancerogeni kada se koriste u konzerviranoj hrani [20], stoga se potraga za sigurnim prirodnim konzervansima (antioksidansa) za hranu nastavlja, kao i za prirodnim farmaceutskim pripravcima, te prirodnim pesticidima.



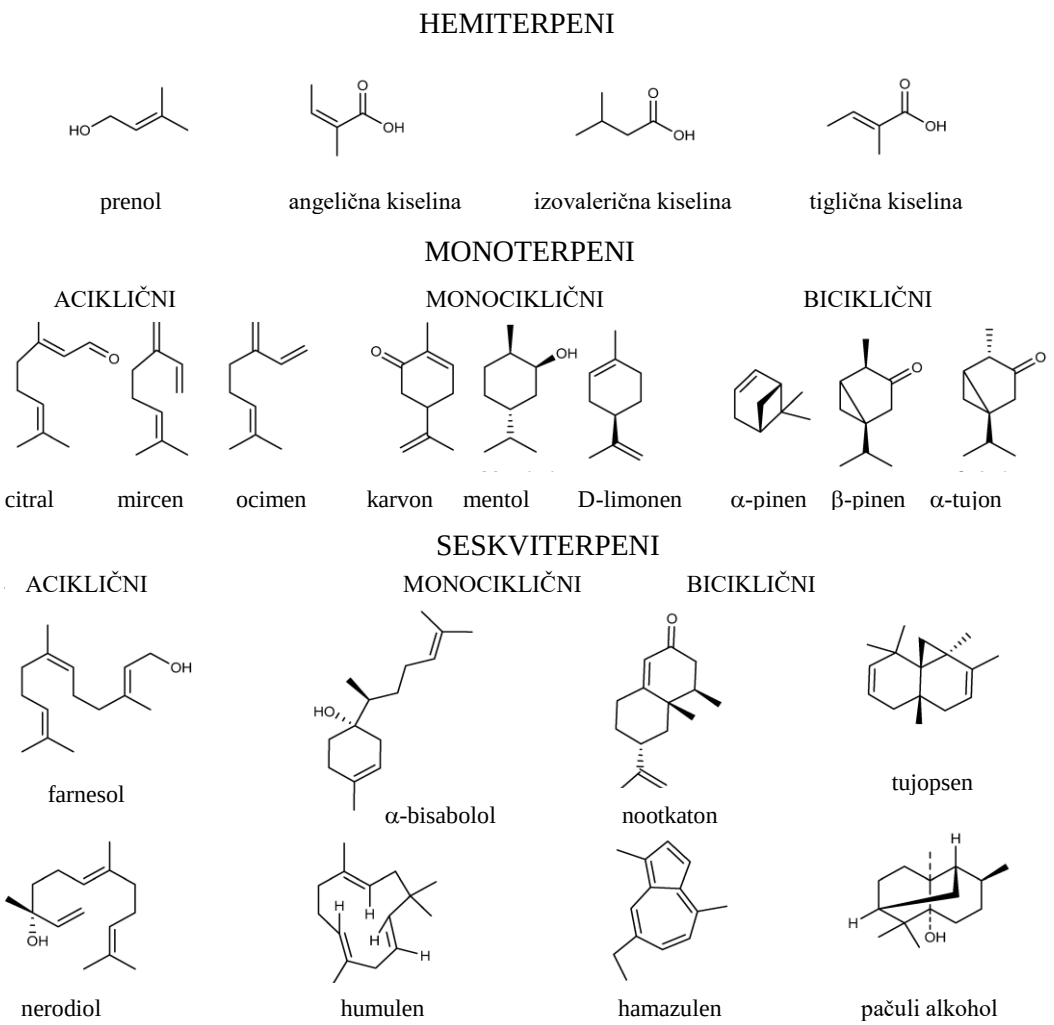
Slika 4. Glavne vrste specijaliziranih metabolita s obzirom na način biosinteze (strukture nacrtane u programu AutoCAD 2021)

Terpeni su najveća skupina sekundarnih metabolita, netopivi su u vodi, a sintetiziraju se putem mevalonske kiseline od izoprenskih jedinica. Fenolne tvari su derivati pentoza fosfatnog puta, puta šikiminske kiseline i fenilpropanoidnog puta. Spojevi s dušikom su organske tvari koje sadrže dušik u heterocikličkom prstenu [19].

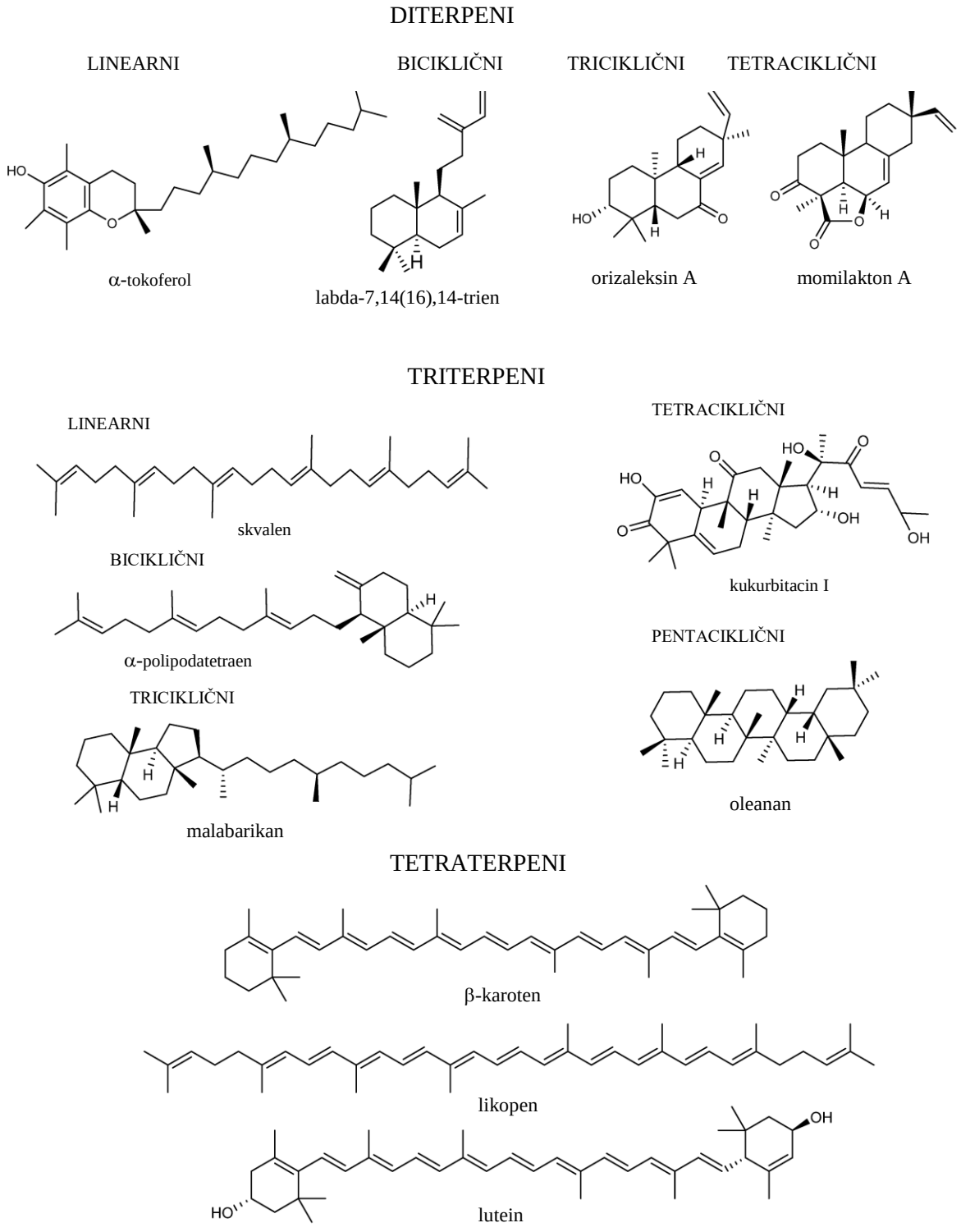
1.2.1. Slobodne hlapljive tvari – eterična ulja i hidrosoli (hidrolati)

Od davnina je poznato da cvjetni i vegetativni dijelovi mnogih vrsta biljaka otpuštaju tvari s određenim mirisom. Naše se znanje o distribuciji biljnih hlapljivih spojeva značajno proširilo posljednjih 30 godina zahvaljujući jednostavnoj metodi uzimanja uzoraka, takozvana „headspace“ metoda. Ti spojevi su izrazito lipofilni s molekularnom masom manjom od 300. Glavne kategorije spojeva koji ulaze u sastav slobodnih hlapljivih spojeva ili eteričnih ulja su: terpenoidi, derivati masnih kiselina, benzenoidi, fenilpropanoidi, C_5 razgranati spojevi i različiti

spojevi koji sadrže dušik ili sumpor. Veliko otkriće u zadnjih 20 godina bilo je da biljke otpuštaju širok raspon hlapljivih tvari prilikom napada herbivora [21]. Prilikom izolacije eteričnih ulja iz biljnog materijala, slobodne hlapljive tvari izolirane su u lipofilnoj i vodenoj frakciji. Vodene frakcije ili hidrosoli su kondenzirane vodene pare koje sadrže otopljene molekule eteričnog ulja i više u vodi topivih (polarnih) hlapljivih spojeva [22]. Zbog različite topljivosti hlapivih spojeva u vodi, ukupni sastav, a time i biološka aktivnost hidrosola razlikuje se od lipofilne frakcije ili eteričnog ulja. Hidrosoli se često odbacuju nakon ekstrakcije eteričnog ulja, ali studije pokazuju da su ti otpadni proizvodi bogati biološki aktivnim tvarima [22]–[24]. Zbog razlike u topljivosti hlapljivih spojeva u vodi, cjelokupni sastav, a time i biološka aktivnost hidrosola razlikuje se od eteričnih ulja. Hidrosoli iz raznih biljaka postaju sve važniji u prehrambenoj industriji, kozmetičkoj industriji, primjeni biljnih pesticida, tradicionalnoj farmaceutici, u aromaterapiji kao dio složenih formulacija i kao samostalni proizvodi; stoga bi njihova potencijalna uporaba trebala biti i dalje istraživana [25].



Slika 5. Strukture biljnih terpena – hemiterpeni, monoterpeni i seskviterpeni (preuzeto i prevedeno iz Ninkuu et al. [26])



Slika 6. Strukture biljnih terpena – diterpeni, triterpeni i tetraterpeni (preuzeto i prevedeno iz Ninkuu et al. [26])

Eterična ulja (EO) su vrlo složene mješavine spojeva, uglavnom monoterpena i seskviterpena. Osnovni put biosinteze hlapljivih spojeva se može prikazati u tri faze: (1) formacija C_5 izoprenskih jedinica, (2) kondenzacija dvije ili tri izoprenske jedinice da bi se na

kraju procesa formirali C_{10}, C_{15} i C_{20} prenil difosfata te na kraju (3) konverzija ovih spojeva u krajnje proizvode [21]. Trenutno se istražuje sastav više od 3000 eteričnih ulja, ali nisu sva pokazala značajnu biološku aktivnost, samo jedna desetina [27], pa je vrlo važno nastaviti s istraživanjem hlapljivih tvari biljaka. Neki od najčešćih spojeva koji se pojavljuju u sastavima eteričnih ulja su: pinen, mircen, limonen, kamfor, mentol, tujen, bisabolen, zingiberen, germakren, kariofilen, retinol, taksol, fitol, skvalen, karoten i likopen (Slike 5 i 6) [28].

1.2.2. Tehnike izolacije slobodnih hlapljivih spojeva

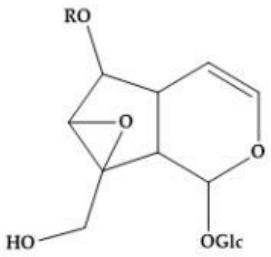
Izolacija slobodnih hlapljivih spojeva (FVC, *free volatile compounds*), koji su važni specijalizirani metaboliti biljaka, može se provesti klasičnom i „zelenom“ ekstrakcijom. Klasične tehnike ekstrakcije uključuju destilaciju vodenom parom, hidrodifuziju, hidrodestilaciju, destruktivnu destilaciju i hladno prešanje. Klasična metoda korištena u ovom radu je tehnika ekstrakcije Clevenger aparaturom u kojoj se biljni uzorak u vodi zagrijava da ispari hlapljive sastojke, koji se kondenziraju u hladilu. Ovom aparaturom na kraju ekstrakcije dobivaju se dva sloja, vodeni i uljni, koji se nakon završetka ekstrakcije odvajaju jedan od drugoga. Osim klasične hidrodestilacije u okviru istraživanja ove disertacije koristila se i zelena ekstrakcija mikrovalnom pećnicom. Obje metode pripadaju skupini hidrodestilacija u kojima se biljni uzorak nalazi u vodi ili je hidratiziran.

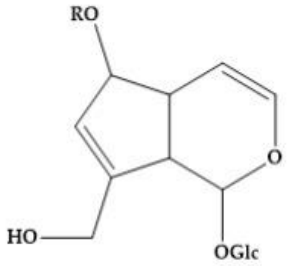
Općenito, tehnike „zelene“ ekstrakcije uključuju turbo destilaciju, ekstrakciju potpomognutu ultrazvukom, ekstrakciju potpomognutu mikrovalovima i tehnologiju trenutačnog kontroliranog pada tlaka. Ovisno o tehnici izolacije, sastav eteričnog ulja ekstrahiranog iz istog biljnog materijala može varirati. Na to utječu trajanje destilacije, temperatura i tlak. Zelena ekstrakcija zahtijeva manje vremena (manji utrošak električne energije) i manji utrošak vode nego tradicionalna ekstrakcija [29]. Postoje dvije vrste komercijalno dostupnih sustava mikrovalne ekstrakcije, a to su ekstrakcija u zatvorenim posudama pri kontroliranom tlaku i temperaturi, te u mikrovalnim pećnicama pri atmosferskom tlaku. Mikrovalna ekstrakcija se može koristiti za izdvajanje temperaturno osjetljivih spojeva kao što su eterična ulja iako je utvrđeno da je mikrovalna ekstrakcija neučinkovita ukoliko se provodi iz potpuno suhih ili svježih neosušenih materijala [30].

1.2.3. Glikozidi

Glikozidi su, uz fenole, od svih skupina specijaliziranih metabolita najznačajniji za čestoslavice, barem u dosadašnjim istraživanjima (Slika 7). Glikozidi su organski spojevi koji

sadrže šećernu komponentu povezanu glikozidnom vezom na neugljikohidratnu komponentu [19]. U brojnim istraživanjima posljednjih 30-ak godina glikozidi su dokazani kao dobri kemofenetski markeri (pojam objašnjen u zadnjem poglavlju uvoda, poglavlje 1.5.) na međurodnoj, a za neke rodove i međuvrsnoj razini. Ova uloga glikozida posebno za rod *Veronica* detaljnije je opisana u zadnjem poglavlju uvoda 1.5. (Kemofenetski markeri i molekularne analize).





R = H, katalpol
R = kafeoil
R = izoferuloil
R = protokatehuoil

R = benzoil
R= *p*-hidroksibenzoil
R = vaniloil

R = H, aukubin
R = cinamoil

Slika 7. Strukture derivata aukubina i katalpola izoliranih iz mnogih vrsta roda *Veronica* uključujući sekcije *Paederota, Pseudolysimachia, Veronicastrum, Omphalospora* i *Chamaedrys* (preuzeto i prevedeno iz Salehi i sur. [31])

1.2.4. Fenoli

Prijelaz ranih vaskularnih biljaka na kopnena staništa je bio uspješan u velikom dijelu zbog razvoja i usavršavanja raznolike skupine tvari općenito nazivane „fenoli“. Iako su većina fenolnih tvari komponente stanične stijenke, veliki broj tvari su toksini i tvari koje odbijaju herbivore, pigmenti cvjetova i plodova, arome biljnih organa (miris cvijeća i okus voća) i antioksidansi drva, kore i sjemenki. Sve ove funkcije biljnih fenolnih tvari su neophodne za opstanak vaskularnih biljaka [17]. Sintetiziraju se putem šikiminske kiseline i putem jabučne kiseline pomoću enzima fenilalanil amonijska lijaza [19]. Poznato je da imaju raznoliko biološko djelovanje uključujući antibakterijsko, antifungicidno, antivirusno, protuupalno, antiproliferativno i antioksidativno [32]. Glavne podvrste fenolnih tvari su: flavonoidi, antocijanidini, izoflavoni, halkoni, stilbeni, kumarini i furanokumarini, monolignoli te lignani nafta- i antra-kvinona kao i diarilheptanoidi [17]. Flavonoidi i fenolni spojevi također su opsežno proučavani, vjerojatno zbog njihove važnosti za biološku aktivnost biljaka. Poznato je da ovi spojevi imaju antialergijska, antivirusna, protuupalna, kardioprotektivna i

vazodilatatorska svojstva te, iznad svega, antioksidativni i potencijal hvatanja radikala [33]. Neki primjeri jednostavnih fenolnih kiselina su *trans*-cimetna kiselina, *p*-kumarinska kiselina, kavina i ferulinska kiselina [19].

1.2.5. Specijalizirani metaboliti roda *Veronica*

Najproučavaniji specijalizirani metaboliti roda *Veronica* su iridoidni glikozidi. Najznačajniji su aukubin i katalpol i njihovi derivati koji su izolirani u gotovo svim proučavanim vrstama [34]–[36]. Harput i sur. proučavali su iridoidne i feniletanoidne glikozide vrste *Veronica persica* [37]. Iz nadzemnih dijelova izolirani su novi feniletanoidni glikozid, persikozid i tri poznata feniletanoidna glikozida, akteozid, izoakteozid i lavandulifoliozid. Osim feniletanoidnih glikozida izolirali su i, heksitol, dulcitol i sedam poznatih iridoidnih glukozida, aukubin, veronikozid, amfikozid, 6-O-veratroil-katalpol, katalpozid, verprozid i verminozid [37]. Osim glikozida, druga značajna proučavana skupina specijaliziranih metabolita su fenolne tvari. Hong-Young je iz vrste *Veronica linariifolia* izolirao apigenin, luteolin, vaniličnu kiselinu, *p*-hidroksibenzojevu kiselinu, protokatehuinsku kiselinu, etil ester protokatehuinske kiseline izoeruličnu kiselinu, katehol i emodin [38]. Beara i sur. su istraživali fenole triju vrsta roda *Veronica*: *V. urticifolia*, *V. jacquinii* i *V. teucrium*. Od 30 identificiranih fenola najzastupljeniji su bili baikalin, hiperozid, izokvercetin, klorogenska kiselina i kininska kiselina. Genistein i baikalein otkriveni su prvi put u porodici Plantaginaceae i rodu *Veronica* [39]. Živković i sur. identificirali su fenolne i flavonoidne komponente vrste *Veronica urticifolia* [40]. Barreira i sur. proučavali su fenolne spojeve vrsta *V. montana*, *V. polita* i *V. spuria* te su pokazali da su flavoni prevladavali u sastavu (*V. montana*: sedam fenolnih kiselina, pet flavona, četiri feniletanoida i jedan izoflavon; kod vrste *V. polita*: 10 flavona, pet fenolnih kiselina, dva feniletanoida, jedan flavonol i jedan izoflavon i kod *V. spuria*: 10 fenolnih kiselina, pet flavona, dva flavonola, dva feniletanoida i jedan izoflavon). Vrsta *V. spuria* je imala najveći sadržaj svih skupina fenolnih spojeva, osim flavona [5].

Najmanje istraživani specijalizirani metaboliti čestoslavica su slobodni hlapljivi spojevi. Nekoliko istraživanja je identificirano pri pregledu literature. U prethodno istraživanoj vrsti *Veronica spicata* fitol je bio dominantan spoj u ukupnom sastavu eteričnog ulja [20]. Li [41] je identificirao komponente eteričnog ulja *V. linariifolia* i utvrdio da su glavne komponente cikloheksen, *β*-pinen, 1S-*α*-pinen, *β*-felandren, *β*-mircen i germakren D. Çelik i sur. istraživali su eterično ulje ekstrahirano iz *Veronica* sp. i utvrdili da su glavne komponente uglavnom linalol i karvakrol [42]. Istraživanje u okviru ove doktorske disertacije najviše novih saznanja

donosi upravo na području sastava slobodnih hlapljivih spojeva roda *Veronica* te njihove biološke aktivnosti.

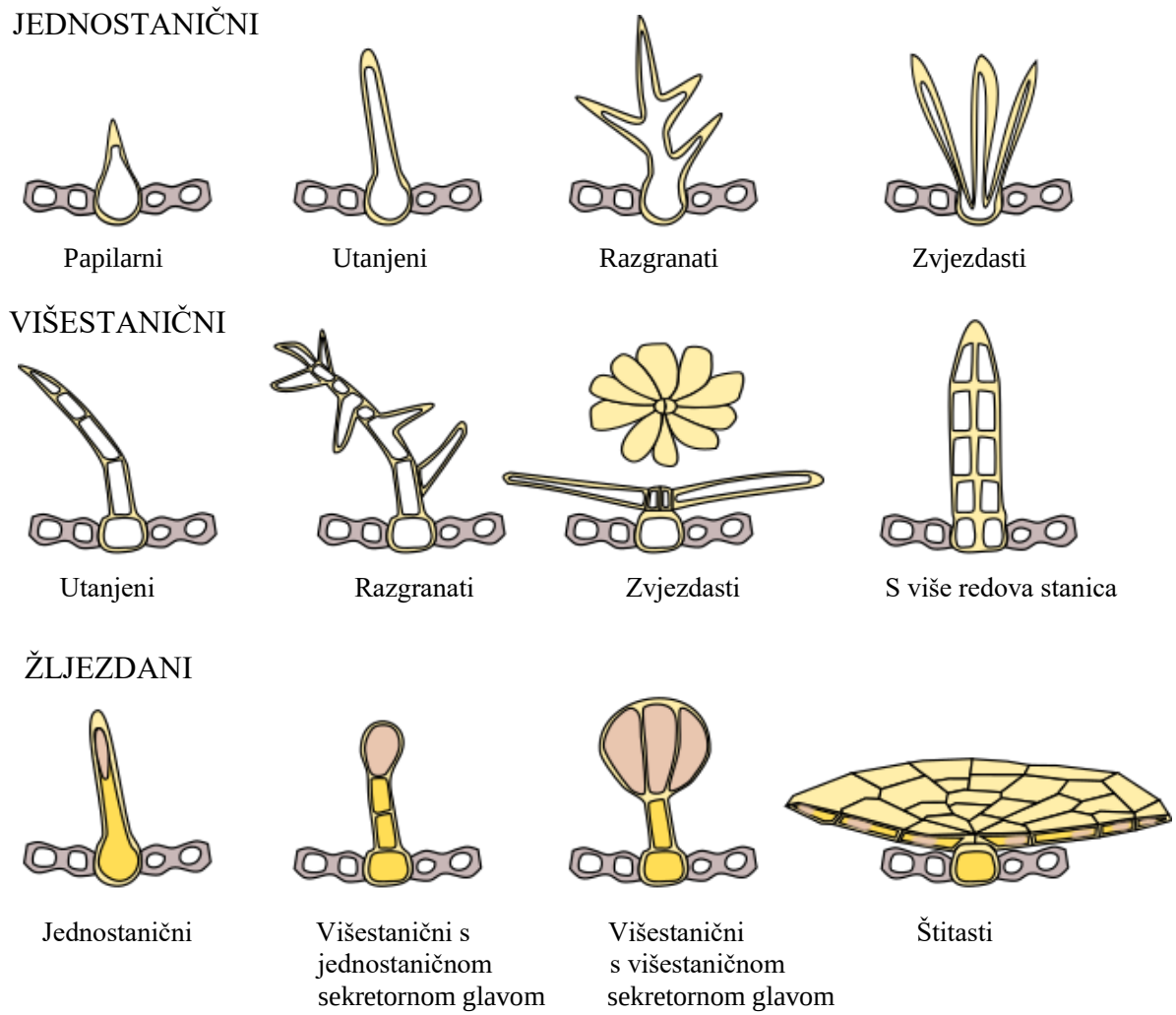
1.3. Trihomi – mjesta sinteze i prikupljanja slobodnih hlapljivih tvari

Akumulacija eteričnih ulja u biljkama općenito je ograničena na specijalizirane sekretorne strukture, naime, žljezdane trihome (dlake) koje su višestanične epidermalne žlijezde, koje se nalaze u nekim biljnim porodicama kao što su Lamiaceae, Asteraceae i Solanaceae, a koje luče terpene u subkutikularnu šupljinu na vrhu trihoma. Pohranjivanje terpenoida u tim strukturama također se može koristiti za ograničavanje rizika od toksičnosti za samu biljku. Morfologija ovih struktura mijenja se s fenologijom biljke ovisno o uvjetima navodnjavanja i uvjetima u okolišu te također prema toksičnosti intrakutikularnog sadržaja. Žljezdani trihomi aromatičnih biljaka dolaze u različitim oblicima i veličinama, kako bi osigurale određenu funkciju. Ova se funkcija uglavnom sastoji u zaštiti različitih biljnih organa i privlačenju oprašivača. Neki su znanstvenici klasificirali ove žlijezde u peltatne (štitaste) i glavičaste na temelju morfoloških kriterija; međutim, drugi su ih svrstali u kratkotrajne i dugotrajne žlijezde, na temelju načina lučenja. Kratkotrajne žlijezde su žlijezde koje brzo izlučuju hlapljive tvari kako bi zaštitile mlade organe. Dugotrajne žlijezde su žlijezde u kojima se sekretorna tvar postupno nakuplja u subkutikularnom prostoru i igraju ulogu u zaštiti zrelih organa kao što je cvijet, kao i u oprašivanju. Prema toj definiciji, zaključeno je da su glavičaste dlake kratkotrajne žlijezde, dok su štitaste (peltatne) dlake dugotrajne žlijezde. Razlika između ove dvije vrste žlijezda sastoji se od nekoliko aspekata poput strukture, načina lučenja i vremena lučenja [43].

1.3.1. Vrste i morfološka podjela trihoma

Trihomi su široko rasprostranjeni na površini različitih organa/tkiva u različitim biljkama te se općenito dijele na jednostanične ili višestanične, jednostavne i razgranate te na žljezdane i nežljezdane na temelju morfoloških značajki i funkcija [44]. Na Slici 8 prikazane su osnovne vrste biljnih trihoma: jednostanični i višestanični nežljezdani trihomi te žljezdani trihomi. Trihomi imaju različite oblike, kao što su npr. glavičasti i ljuskasti. Theobald i Barthlott dalje su podijelili trihome u tri kategorije na temelju distribucije listova: velike, male i žljezdane trihome. Veliki trihomi uglavnom su raspoređeni na abaksijalnoj (okrenuto suprotno od osnovne osi biljke) strani lista i rubovima te u vaskularnim snopovima; mali trihomi u

stomatalnom paracelularu; a žljezdani trihomi obično su pravilno raspoređeni u cijelom ili dijelu subepidermalnog tkiva na površini lista [44].

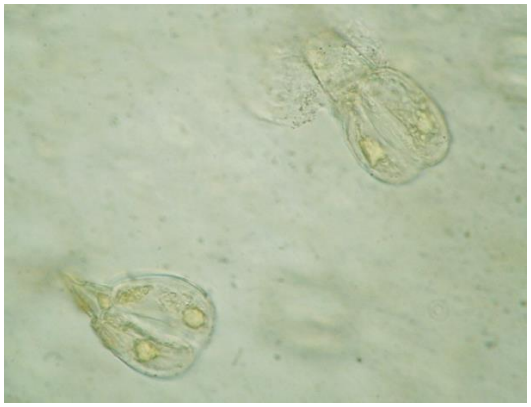
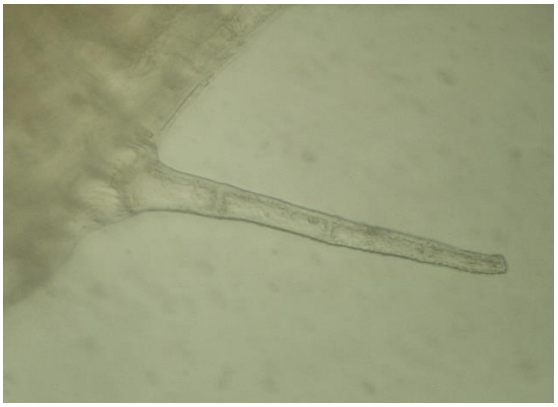


Slika 8. Morfološka podjela biljnih trihoma **(**preuzeto i prevedeno s https://www.bartleby.com [45]**)**

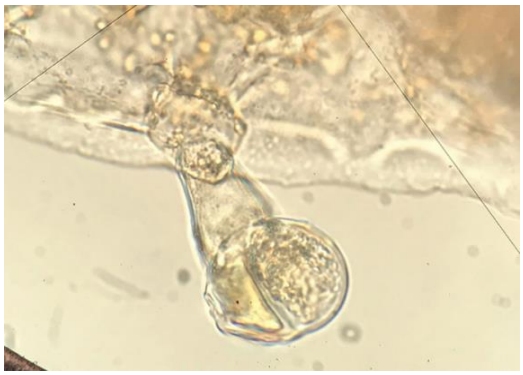
1.3.2. Trihomi roda *Veronica* i srodnih vrsta porodice Plantaginaceae

Trihomi čestoslavica su se počeli istraživati još u prošlom stoljeću. Kurer je prije 100 godina spomenuo postojanje nežljezdanih trihoma na dijelovima cvijeta neke vrste iz roda *Veronica* [46]. Također, Kraehmer i Baur opisali su te trihome u vrste *V. persica* Poir [47]. Ista vrsta nežljezdanih trihoma uobičajena je u mnogim drugim vrstama drugih porodica, npr. Lamiaceae [48]–[50]. Ti se trihomi sastoje od jedne stanice drške i dvije eliptično oblikovane stanice glave (Slike 9a, 10a i 11a). Nisu uspravne i mogu se opisati kao pripijene uz površinu. Isti tip glavičastih trihoma uočen je i u vrste *V. beccabunga* L. [51]. Isto tako, tip pognutog trihoma s dvostaničnom glavom zabilježen je kod vrste *Stachys recta* L. subsp. *recta* u

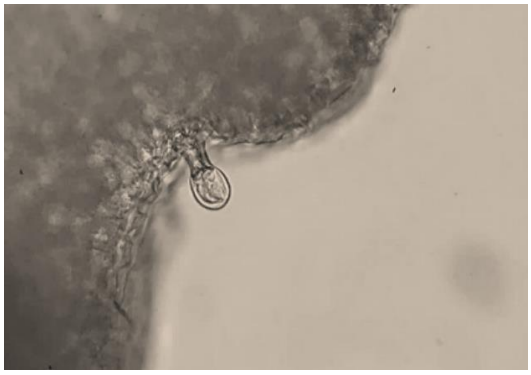
istraživanju Vundać i sur. [52]. Trihomi roda *Veronica* su istraživani i svjetlosnom mikroskopijom u okviru izrade diplomskih radova. Tako su uočeni nežljezdani (Slika 9b, 10b i 11b) jednostanični ili višestanični utanjeni trihomi te žljezdani glavičasti trihomi s dvostaničnom glavom (Slike 9a, 10a i 11a) [53].

(a)  (b) 

Slika 9. Trihomi vrste *Veronica spicata*: (a) Žljezdani trihomi*;* (b) Nežljezdani trihomi (Autor: Marija Nazlić)

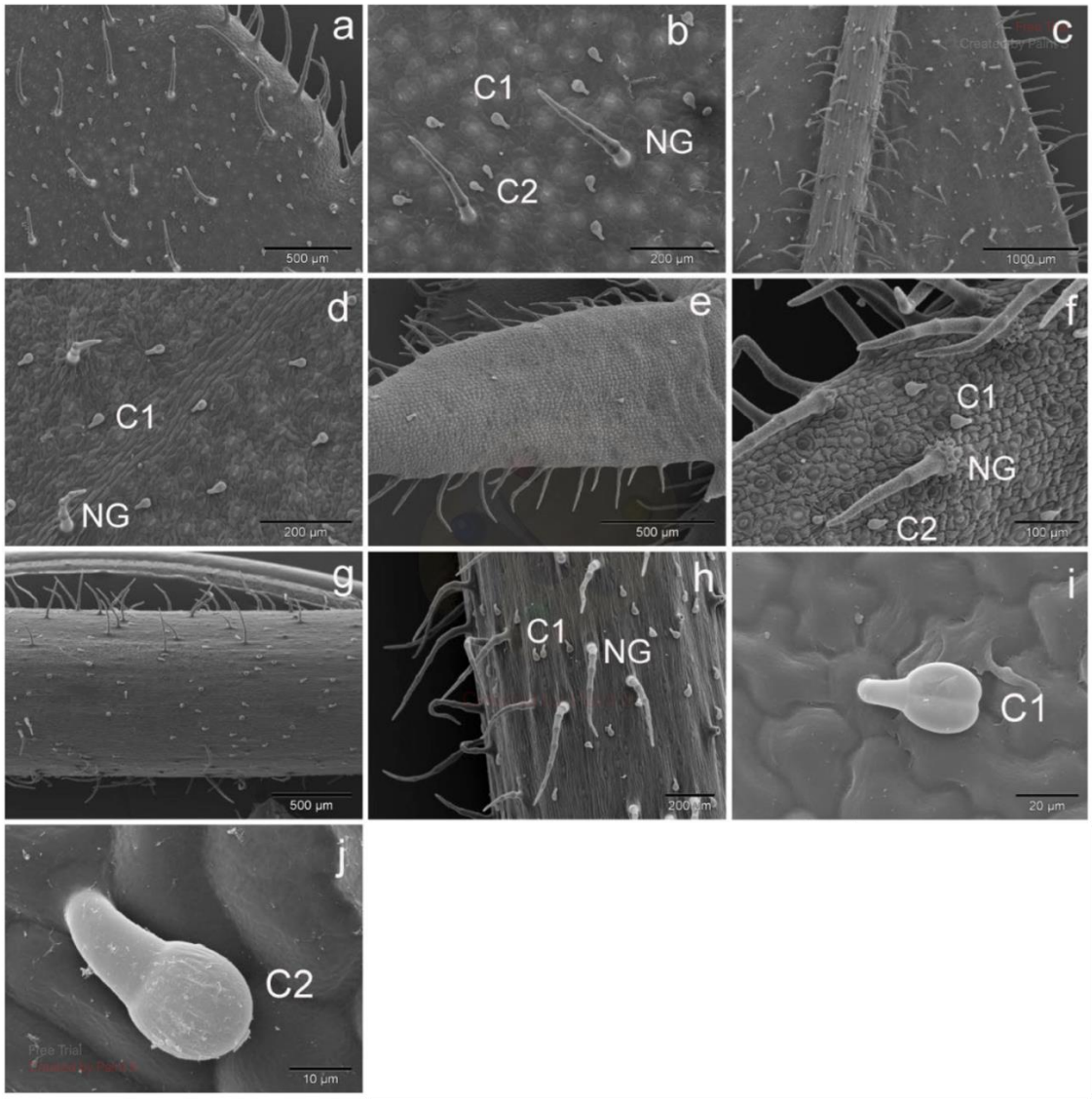
(a)  (b) 

Slika 10. Trihomi vrste *Veronica austriaca* ssp. *jacquinii*: (a) Žljezdani trihomi; (b) Nežljezdani trihomi (preuzeto s dopuštenjem iz diplomskog rada [53])

(a)  (b) 

Slika 11. Trihomi vrste *Veronica saturejoides* ssp. *saturejoides*: (a) Žljezdani trihomi; (b) Nežljezdani trihomi (preuzeto s dopuštenjem iz diplomskog rada [53])

Kremer i sur. su u novijem istraživanju mikromorfologije trihoma vrste *Veronica barrelieri* Schott ex Roem. et Schult [54] identificirali nežljezdane trihome vrlo slične onima do sada opisanima kroz već spomenute studije, utanjeni i višestanični, rijetko raspoređeni po različitim dijelovima biljke (Slika 12f i 12g, NG). Identificirana su i dva tipa glavičastih žljezdanih trihoma. Prvi tip trihoma je iste građe kao i u navedenim istraživanjima unutar diplomskog rada [53] s jednom stanicom stapke i dvije stanice glave žlijezde. Drugi tip trihoma prvi je put prijavljen kod roda *Veronica* [54], ali je karakterističan i identificiran unutar roda *Micromeria* [55], [56], a sastoji se od jedne stanice stapke i jedne stanice koja čini glavu žlijezde.



Slika 12. Žljezdani i nežljezdani trihomi vrste *Veronica barrelieri* Schott ex Roem. et Schult (a-j), na gornjoj ili adaksijalnoj (a,b) i donjoj ili abaksijalnoj (c,d) površini lista, čašci (e,f) i peteljci (g,h). Nežljezdani trihomi (NG - non-glandular), podtip 1 (C1 – capitate 1) i podtip 2 (C2 – capitate 2) žljezdanih glavičastih trihoma (preuzeto iz Kremer et al. [54])

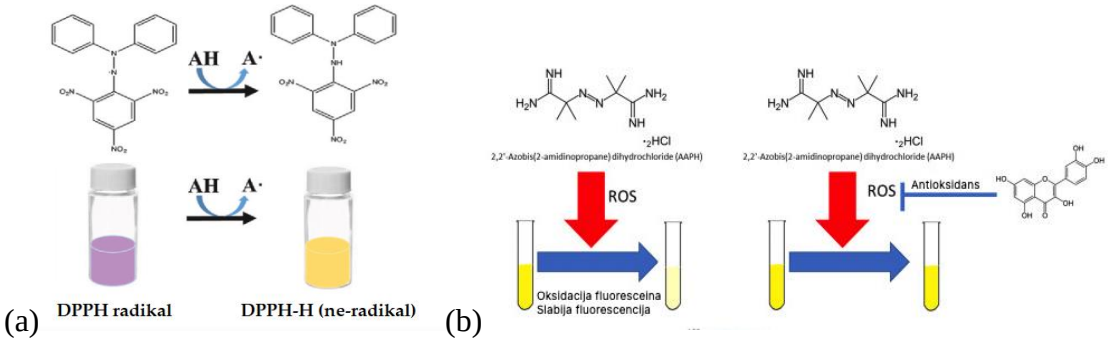
1.4. Biološka aktivnost specijaliziranih metabolita s naglaskom na eterična ulja

1.4.1. Antioksidativna aktivnost

Proces oksidativne fosforilacije u mitohondrijima je proces nužan i zajednički svim živim bićima jer proizvodi energiju potrebnu za normalno funkcioniranje. Ovaj proces kao nusprodukt proizvodi i slobodne radikale (ROS – *reactive oxygen species*, reaktivne kisikove čestice) koji mogu naštetiti stanicama [57], [58]. Antioksidansi su spojevi koji mogu usporiti ili zaustaviti oksidaciju tvari koje se mogu oksidirati, čak i kada se koriste u vrlo malim količinama u usporedbi s količinom tvari koju moraju zaštititi [59]. Ta svojstva daju mogućnost korištenja tih spojeva u očuvanju hrane od oksidacije te u reakcijama protuupalnog djelovanja. Postoji direktna veza između proizvodnje reaktivnih kisikovih čestica (ROS) i oksidativnih i upalnih stanja koji mogu voditi prema oboljenju od raka [60].

Antioksidativna aktivnost eteričnih ulja se lako može objasniti postojanjem fenolnih komponenti u njihovom sastavu, ali dokazano je da i druge nefenolne komponente mogu imati antioksidativnu aktivnost, iako nižu od fenola [61].

Najčešće korištene metode za utvrđivanje antioksidativne aktivnosti pojedine tvari ili ekstrakta su: DPPH (2,2-diphenyl-picrylhydrazyl), ORAC (*oxygen radical absorbance capacity*), TEAC (*Trolox equivalent antioxidant capacity*), ABTS (*2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid*) i test izbjeljivanja karotena. DPPH je karakteriziran kao stabilan slobodni radikal zahvaljujući delokalizaciji slobodnog elektrona preko cijele molekule, tako da se molekula ne dimerizira, kao što bi bio slučaj s većinom drugih slobodnih radikala. Delokalizacija elektrona također dovodi do tamnoljubičaste boje, koju karakterizira apsorpcijski spektar u otopini etanola sa maksimumom na oko 517 nm.



Slika 13. Ilustracija metoda za mjerenje antioksidativne aktivnosti: (a) DPPH (preuzeto i prilagođeno iz [62]), (b) ORAC (preuzeto i prilagođeno iz [63])

Kada se otopina DPPH pomiješa s onom supstrata (AH-antioksidans, Slika 13a) koji može donirati atom vodika, tada dolazi do reduciranog oblika molekule DPPH s gubitkom ljubičaste boje. ORAC je relativno nova analiza koja se može koristiti za testiranje antioksidativne aktivnosti hrane i drugih kemijskih tvari. Ciljna molekula koja se koristi za ovaj test je fluorescein (Slika 13b). Test se provodi korištenjem Troloxa (u vodi topljivog analoga vitamina E) kao standarda za određivanje Trolox ekvivalenta (TE). ORAC vrijednost se tada izračunava iz Trolox ekvivalenta i izražava kao ORAC jedinice ili vrijednost. Što je veća ORAC vrijednost, to je veća „antioksidativna moć". Ovaj se test temelji na stvaranju slobodnih radikala AAPH (2,2-azobis(2-amidopropan) dihidroklorid) i mjerenju smanjenja fluorescencije u prisutnosti hvatača slobodnih radikala [64]. Prior i sur. objavili su automatizirani ORAC test [65].

Različite tvari različito djeluju kao antioksidansi. Zato je u istraživanjima potrebno koristiti više metoda u svrhu izbjegavanja lažno pozitivnih ili negativnih rezultata. Metoda se bira i po procijenjenoj potencijalnoj primjeni ekstrakta koji se testira.

1.4.2. Antiproliferativna aktivnost

Rak se u svijetu smatra glavnim uzrokom smrti. Tumor ili rak je skupina bolesti u kojima dolazi do nekontroliranog rasta stanica i ima potencijal da se proširi na druge dijelove tijela. Rak je postao glavni uzrok smrtnosti u cijelom svijetu, bez obzira na ljudski razvoj. Rak pluća najrašireniji je karcinom (11,6 % od ukupnog broja slučajeva), za kojim slijede rak dojke kod žena (11,6 %), rak prostate (7,1 %) i kolorektalni karcinom (6,1 %). Prirodni proizvodi izolirani iz ljekovitog bilja korišteni su za liječenje raznih bolesti od davnina. Prva uporaba prirodnih proizvoda kao lijeka datira još 2600. godine prije Krista u Mezopotamiji. Zapisi "Ebers Papyrus" o više od 700 lijekova 1550. godine prije Krista također su dobro očuvani. Slično, tradicionalna kineska medicina dobro je dokumentirana tisućama godina, a indijski ajurvedski sustav prakticira se od 1. tisućljeća prije Krista [60]. Otkriće lijekova na bazi prirodnih proizvoda povezano je s nekim izazovima, kao što su dostupnost, identifikacija bioaktivnih spojeva, poteškoće u prikupljanju samoniklih vrsta i nekompatibilnost prirodnih proizvoda. Zbog ovih je poteškoća farmaceutska industrija preusmjerila svoj glavni fokus na sintetske spojeve radi otkrića novih lijekova. No, primjena sintetičkih lijekova uvedenih devedesetih godina 20. stoljeća nije ispunila očekivanja. Brojevi odobrenih lijekova od strane američke Uprave za hranu i lijekove (FDA) bili su niski te je iz tih razloga oživio interes za otkrivanje lijekova na bazi prirodnih proizvoda [60].

Iako su istraživanja o primjeni EO-a (eteričnih ulja, *essential oils*) kao antikancerogenih terapijskih agensa relativno nova, otprilike polovica konvencionalnih kemoterapijskih sredstava je biljnog podrijetla, s otprilike 25 % izravno dobivenim iz biljaka, a 25 % su kemijski modificirane verzije fitoprodukata. Jedna od takvih molekula je paklitaxel (čiji je najčešći naziv robne marke Taxol) koji je izvorno izveden iz kore drveta *Taxus brevifolia* [66]. Odobrenje Taxol®-a 1993. označilo je veliki ulazak terpenoida u područje borbe protiv raka, a ovaj lijek je i dalje vrlo važan u liječenju refraktornih karcinoma jajnika, dojke i drugih vrsta raka. Tijekom desetljeća drugi istaknuti prirodni terpenoidi postali su neophodni za suvremenu farmakoterapiju raka dojke. [67].

EO zbog svoje kemijske građe imaju sposobnost prolaženja kroz stanične membrane i djeluju na različite stanične mete uključene u različite putove. EO povećavaju unutarstanične razine ROS/RNS što rezultira apoptozom u stanicama raka. EO također moduliraju mehanizme popravljanja DNA djelujući kao inhibitori DNA polimeraze i dovode do cijepanja PARP-a (*poly(ADP ribose) polymerase*) što također dovodi do apoptoze u stanicama raka [68]. Antitumorsko djelovanje terpenoida povezano je s aktiviranjem fenomena stanične smrti (apoptoze) u stanicama raka bez utjecaja na normalne stanice [69].

Jedna od glavnih poteškoća u istraživanjima antiproliferativnih aktivnosti eteričnih ulja povezana je s racionalizacijom učinka: budući da su EO složene smjese stotina sastojaka, njihovo djelovanje na stanice raka zbroj je svake pojedinačne aktivnosti, modulirano svim potencijalnim sinergijama. Međutim, ovo ne bi trebalo obeshrabriti studije o upotrebi cjelovitog EO, osobito u kombinaciji s konvencionalnim kemoterapijama, jer se te smjese mogu smatrati obećavajućim izvorima novih antitumorskih sredstava. Stoga je istraživanje antitumorskih svojstava EO sada jako važno i trebalo bi imati isti interes kao i konvencionalniji kemoterapijski tretmani sintetskim sredstvima protiv raka [69]. Terapeutska aktivnost biljnih ekstrakata obično je posljedica sinergijskog i istodobnog djelovanja nekoliko kemikalija. S obzirom na složenu prirodu mnogih bolesti, uključujući rak i degenerativne bolesti, nije iznenađujuće da oslanjanje na otkriće lijekova na bazi jedne izolirane komponente nije uspjelo pružiti učinkovite lijekove [18].

U literaturi se sugerira da jedan test ne mora točno odražavati učinke istraživanih spojeva. Najčešći primjenjivani testovi za procjenu antiproliferativne aktivnosti prirodnih tvari uključuju klonogene testove (*in vitro* metode kojima se određuje preživljavanje stanica): MTT, MTS, LDH i SRB. Stupanj citotoksičnosti ili stanična održivost tvari mogu varirati i uvelike ovise o testovima koji su korišteni za procjenu te ukazuju na to da je odabir odgovarajuće *in vitro* analize citotoksičnosti ključan za sprječavanje lažno pozitivnih ili lažno negativnih

rezultata. *In vitro* istraživanje koje se bavi farmakološkim svojstvima može se smatrati preliminarnim korakom za prikupljanje prvih informacija o aktivnosti sirovih ekstrakata, njihovih frakcija ili izoliranih spojeva [67]. Test korišten u istraživanjima unutar ovog doktorskog rada je MTS (*3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium*) test koji se temelji na enzimskoj pretvorbi MTS-a s mitohondrijskom reduktazom. Protokol MTS testa temelji se na redukciji spoja MTS tetrazolija u živim stanicama sisavaca (i stanicama drugih vrsta) kako bi se stvorio obojeni spoj formazan koji je topiv u mediju stanične kulture. Ako ima više živih stanica boja otopine je ljubičasta, a ako ima više mrtvih stanica boja otopine je žuta. Metoda je kolorimetrijska [70], [71].

1.4.3. Antifitovirusna aktivnost

Ekološke ("zelene") strategije prevencije i prirodna sredstva kontrole biljnih virusa danas su posebno važni za podršku organskoj proizvodnji i zamjeni sintetičkih kemikalija biološkim antivirusnim lijekovima. Ovakav pristup modernoj poljoprivredi još uvijek je u razvoju, a jedan od ciljeva znanstvenika je pronaći bezopasne i ekološki prihvatljive antifitovirusne agense. Virus mozaika duhana (TMV) model je virusa u biljnoj virologiji i vrlo važan patogen za poljoprivredne usjeve, koji uzrokuje značajne gubitke prinosa. TMV pripada virusima s pozitivnim lancem RNA i kodira dva proteina koji funkcioniraju kao replikaze (molekularne težine 126 kDa i 183 kDa), protein za kretanje (30 kDa) koji olakšava kretanje virusa između stanica domaćina i protein omotača (17,5 kDa) koji ima važnu ulogu u stvaranju viriona [72]. Različiti biljni proizvodi kao što su eterična ulja, flavonoidi, polifenoli i organski, alkoholni i vodeni ekstrakti iz biljaka korišteni su protiv niza biljnih bolesti uzrokovanih virusima, fitopatogenim bakterijama, gljivicama, biljkama parazitskih nematoda te parazitskih i neparazitskih korova [73]–[76] s ciljem pronalaženja proizvoda na prirodnoj bazi korisnih za zaštitu bilja od patogena. Eterična ulja i hidrosoli roda *Veronica* do sada nisu istraživani na području djelovanja protiv fitovirusa te su rezultati dobiveni u okviru ove disertacije prvi prijavljeni za ove ekstrakte.

1.5. Biološka aktivnost specijaliziranih metabolita iz ekstrakata biljaka roda *Veronica*

1.5.1. Pregled najopsežnijih istraživanja biološke aktivnosti

Vrste roda *Veronica* koriste se u tradicionalnoj medicini za liječenje raznih bolesti, uključujući gripu, respiratorne bolesti i karcinom, te kao diuretici [20] zbog prirodno većeg

sadržaja fenolnih spojeva i iridoida. Posljednjih godina neka su istraživanja pokazala da se vrste iz ovog roda mogu koristiti i za liječenje mentalnih poremećaja [77] te nekih tipova dijabetesa [78]. Korištenje ovih biljaka u tradicionalnoj medicini u zemljama diljem svijeta potaknulo je interes za njihovim proučavanjem s obzirom na njihov kemijski sastav i biološku aktivnost. Mnoge biološke aktivnosti različitih ekstrakata zabilježene su u novijim studijama [31], [79]. Na primjer, metanolni i etil-acetatni ekstrakti vrste *V. spicata* testirani su na antimikrobno djelovanje, a vrijednosti MIC-a bile su između 1,25 i 5,00 mg/mL. Ovaj biljni ekstrakt također je pokazao značajno antioksidativno djelovanje, posebno metanolni ekstrakti cvijeća i lišća s IC50 i DPPH vrijednostima od 8,21 µg/mL odnosno 8,69 µg/mL [20]. Ertas i sur. istraživali su antimikrobno djelovanje fenolnih ekstrakata vrste *Veronica thymoides* subsp. *pseudocinerea,* a utvrđena vrijednost MIC-a bila je 31,25 mg/mL za metanolni ekstrakt protiv bakterije *Escherichia coli* [80].

Vrsta *Veronica officinalis,* koja je jedna od triju istraživanih vrsta u ovoj disertaciji, tradicionalno se koristi u medicini balkanskih naroda. Mocan i sur. istraživali su antioksidativnu aktivnost za etanolske ekstrakte fenolnih spojeva za vrstu *V. officinalis* te dobili rezultat od 157,99 mg Trolox ekvivalenta/g suhe tvari [81]. Valyova i sur. također su potvrdili antioksidativnu aktivnost fenolnih ekstrakata za *V. officinalis* u svojoj studiji [82]. Nadzemni dijelovi ljekovite čestoslavice koriste se za liječenje jetre, slezene, bolesti bubrega i mjehura, kao i za zacjeljivanje rana, kožnih lezija, ekcema i čireva [20], [80], [83].

Živković i sur. proučavali su fenolne spojeve, antioksidativno i antineurodegenerativno djelovanje vrste *V. austriaca* ssp. *jacquinii* i njihovi rezultati su pokazali da ova vrsta ima značajno antioksidativno i antineurodegenerativno djelovanje [77]. Mnoge druge vrste čestoslavica i biološka aktivnost njihovih specijaliziranih metabolita, posebno fenolnih spojeva, istražene su i pokusi su pokazali da imaju antioksidativnu [77], [80], [81], [84], antimikrobnu [20], [40], [81], [85], citotoksičnu i antitumorsku aktivnost [40].

Dosadašnja istraživanja antiproliferativne aktivnosti vrsta roda *Veronica* rađene su uglavnom na fenolnim ekstraktima. Može se reći da je vrlo malo vrsta čestoslavica proučavano na njihovu citotoksičnu aktivnost *in vitro* i *in vivo*. Uglavnom su metanolni i vodeni ekstrakti različitih vrsta roda *Veronica* testirani na staničnim linijama raka. Metanolni ekstrakt vrsta *V. cymbalaria, V. hederifolia, V. pectinata var. glandulosa, V. persica* i *V. polita* pokazale su značajno citotoksično djelovanje protiv epidermalnog karcinoma i stanica melanoma. Izolirana ekstrakt pokazao je citotoksičnost ovisnu o dozi [86]. Metanolni ekstrakt jestive vrste *V. americana* pokazao je citotoksično djelovanje protiv staničnih linija raka HF-6 (debelo crijevo) i PC-3 (prostata) [87]. U drugoj studiji istraživana je citotoksična aktivnost metanolnog

ekstrakta vrsta *V. cuneifolia* ssp. *cuneifolia* i *V. cymbalaria*. Rezultati su pokazali da bolju citotoksičnu aktivnost ima *V. cuneifolia* ssp. *cuneifolia*. Obje vrste su inhibirale diobu stanica raka Hep-2, RD i L-20B [88]. Flavonoidi izolirani iz vrste *V. sibirica* zaustavili su diobu stanica raka dojke MCF-7, vrijednost IC50 za enzimsku pretvorbu MTT-a bila je 42 µg/mL. Mehanizam inhibicije diobe stanica raka uključivao je apoptozu [79].

1.5.2. Ostala istraživanja biološke aktivnosti

Sva dosadašnja istraživanja biološke aktivnosti specijaliziranih metabolita roda *Veronica* provedena su s nekom vrstom fenolnog ekstrakta pripremljenog korištenjem različitih otapala (metanol, etanol, aceton ili voda) ili ekstraktom glikozida.

Osim gore navedenih aktivnosti ekstrakata čestoslavica, proučavano je i antibakterijsko, antifungalno i antiparazitsko djelovanje. Biljke roda *Veronica* odavno su poznate po svom korištenju u narodnoj medicini, a posebno vrsta *V. officinalis*, tj. ljekovita čestoslavica [20], [81]. Unatoč njihovoj širokoj upotrebi u narodnoj medicini, podaci o antimikrobnom djelovanju su još uvijek nedostatni. Antibakterijsko djelovanje protiv Gram-pozitivnih i Gram-negativnih vrsta ovisi o vrsti ekstrakta (korišteno otapalo, ekstrahirani dio, vrsta itd.). Kao primjer, ispitivana su antimikrobna svojstva ekstrakata nadzemnih dijelova biljke *V. spicata* s metodom difuzije i metodom mikrodilucije. Utvrđeno je da su bakterijski sojevi korišteni u istraživanju osjetljivi na metanolne i etil-acetatne ekstrakte, s vrijednostima minimalne inhibitorne koncentracije (MIC) između 1,25 i 5 mg/mL metodom mikrodilucije, dok su vodeni ekstrakti bili neaktivni [20]. U drugom radu, koristeći metodu mikrodilucije, Živković i sur. istraživali su antibakterijski učinak metanolnog ekstrakta biljke *V. urticifolia* na Gram-negativne bakterije *Escherichia coli, Enterococcus faecalis i Pseudomonas aeruginosa* te Gram-pozitivne bakterije *Staphylococcus aureus, Listeria monocytogenes i Bacillus cereus*. Nakon mjerenja minimalne baktericidne koncentracije (MBC) i MIC vrijednosti, utvrđeno je da je najosjetljiviji soj *Staphylococcus aureus*. Zaključeno je da antistafilokokni učinak ova biljka posjeduje zbog glavnog fenolnog spoja akteozida, koji bi mogao inhibirati ugradnju leucina i poremetiti sintezu proteina [40]. Drugi kemijski spojevi također mogu biti odgovorni za ovo antibakterijsko djelovanje, npr. *β*-sitosterol, kampesterol, stigmasterol, hispidulin i flavonoidi. Ovi su rezultati važni za ljudsko zdravlje jer je *S. aureus* patogena bakterija koju je teško liječiti s razvojem otpornosti na antibiotike. Iz tog su razloga korisne prirodne alternativne terapije za rješavanje ovog problema. Druge studije pokazale su antibakterijsko djelovanje metanolnih, etanolnih ili vodenih ekstrakata iz *V. urticifolia* Jacq., *V. orchidea* Crantz, *V. persica* i *V. montana* L. protiv Gram-pozitivnih i Gram-negativnih bakterija [31]. Nisu sve studije dokazale pozitivne

antibakterijske učinke. U jednoj studiji ekstrakti vrste *V. anagallis-aquatica* testirani su pomoću testa difuzije protiv pet sojeva bakterija i dva soja kvasca. Nijedan od ekstrakata ove čestoslavice nije pokazao značajnu inhibiciju u usporedbi s pozitivnom kontrolom (gentamicin) [89]. Čestoslavice su u istraživanjima pokazale i antifungalno, antivirusno i antiparazitsko djelovanje. Mocan i sur. su istraživali antifungalna svojstva vrste *V. persica* protiv *Aspergillus niger* i *Penicillium hirsutum* difuzimetrijskom metodom. Antifungalni učinak bio je veći za *A. niger* nego za *P. hirsutum* i to zbog fenolnih spojeva [81]. Koristeći istu metodu, antifungalni učinci ekstrakta vrste *V. persica* protiv *Candida albicans* i *A. niger* prikazani su u drugoj studiji [90]. Rezultati su pokazali da je najveći antifungalni učinak za oba gljivična patogena postignut pri koncentraciji od 300 μg/mL ekstrakta. Dunkić i sur. pokazali su u drugoj studiji antifungalni učinak metanolnog ekstrakta vrste *V. spicata* na MIC vrijednosti u rasponu od 1,25 mg/mL do 5 mg/mL [20]. Kao rezultat ovih pozitivnih podataka, čestoslavice se potencijalno mogu smatrati dobrim prirodnim terapeutskim alternativama za blage gljivične infekcije [31]. Novo biološko svojstvo, tj. antivirusno djelovanje (protiv herpes simplex virusa HSV1 i HSV2), za vrstu *V. persica* je dokazano u nedavnom istraživanju koje su proveli Sharifi-Rad i sur. [91]. U ovoj studiji, etanolni ekstrakt vrste *V. persica* testiran je na Vero stanicama zaraženim s obje vrste virusa. Jače antivirusno djelovanje pronađeno je u 80 % metanolnoj frakciji ekstrakta *V. persica* tijekom i nakon infekcije stanica virusima, što ukazuje na interferenciju ekstrakta s intracelularnim ulaskom virusa i moguću inhibiciju virusne unutarstanične replikacije endogenih herpetičkih virusa. HSV1 bio je puno osjetljiviji na djelovanje metanolne frakcije u usporedbi s HSV2. Ovo antivirusno djelovanje ukazuje na korisnost ekstrakta biljke *V. persica* u kombinaciji s antivirusnim lijekovima (kao što je aciklovir) za smanjenje ozbiljnosti simptomatskih epizoda oralne herpetičke infekcije, koja se vraća kada je imunološki sustav oslabljen [31].

Nisu istraživana samo biološka djelovanja ekstrakata čestoslavica. U jednom istraživanju Pinto-Zevallos i sur. istraživali su sadržaj hlapljivih spojeva klasaste čestoslavice (*V. spicata*) koji su se proizvodili u biljci prilikom napada herbivora. Zaključili su da biljka proizvodi različite hlapljive tvari prilikom napada herbivora u svrhu privlačenja predatora koji će se hraniti herbivorom koji je napao biljku [92].

1.6. Molekularne analize i kemofenetski markeri

Rod čestoslavica (*Veronica*) nekada je bio klasificiran u porodicu Scrophulariaceae uglavnom na temelju morfoloških karakteristika, ali je na temelju rezultata istraživanja sekvenci molekule DNA premješten i trenutno se nalazi u porodici Plantaginaceae [93].

U Hrvatskoj je prema bazi Flora Croatica Database zabilježeno 40 vrsta čestoslavica [7] koje su raspoređene u 7 podrodova: *Beccabunga, Chamaedrys, Veronica, Cochlidiosperma, Stenocarpon, Pocilla* i *Pentasepalae*.

Podrod *Beccabunga*
- *V. acinifolia* L.
- *V. anagallis-aquatica* L.
- *V. anagalloides* Guss.
- *V. beccabunga* L.
- *V. catenata* Pennell
- *V. scardica* Griseb.
- *V. serpyllifolia* L.
- *V. serpyllifolia* L. ssp. *humifusa*
- *V.a peregrina* L.

Podrod *Chamaedrys*
- *V. arvensis* L.
- *V. chamaedrys* L.
- *V. dillenii* Crantz
- *V. vindobonensis* M. A. Fisch.
- *V. verna* L.

Podrod *Veronica*
- *V. alpina* L.
- *V. aphylla* L.
- *V. montana* L.
- *V. officinalis* L.
- *V. scutellata* L.
- *V. urticifolia* Jacq.

Podrod *Cochlidiosperma*
- *V. cymbalaria* Bodard
- *V. hederifolia* L.
- *V. sublobata* M. A. Fisch.
- *V. triloba* (Opiz) Opiz

Podrod *Stenocarpon*
- *V. saturejoides* Vis.

Podrod *Pocilla*
- *V. agrestis* L.
- *V. opaca* Fr.
- *V. persica* Poir.
- *V. polita* Fr.
- *V. praecox* All.
- *V. triphyllos* L.

Podrod *Pentasepalae*
- *V. austriaca* L. ssp. *austriaca*
- *V. austriaca* L. ssp. *jacquinii*
- *V. dalmatica*
- *V. teucrium* L. ssp. *crinita*
- *V. teucrium* L. ssp. *pseudochamaedrys* (Jac.) Nyman
- *V. orbiculata* A. Kern.
- *V. orsiniana* Ten.
- *V. prostrata* L.

Albach i Taskova i njihovi suradnici istraživali su iridoidne glikozide roda *Veronica* i zaključili da se raspodjela tih tvari u različitim vrstama roda podudara s molekularnom filogenijom roda i tako pokazali da kemija roda može poslužiti kao dobar pokazatelj

međuvrsnih i međurodovskih veza [34]–[36], [94], [95]. Napredak u analitičkim metodama, posebice kromatografiji, zatim elektrokemijskim metodama detekcije (konduktometrijska, potenciometrijska, voltametrijska, amperometrijska i kulometrijska mjerenja), dovelo je do napretka u kemijskim studijama sve do metaboličkog profiliranja biljnih vrsta [1]. Kemotaksonomske studije temeljene isključivo na identifikaciji malih molekula postale su zastarjele kao alati za proučavanje filogenetskih odnosa viših biljaka zbog pojave mnogo moćnijih molekularnih tehnika i novih metoda analize genetskog materijala. Zato je predložen i u posljednje vrijeme se koristi novi termin za područje studija usmjerenih na identifikaciju karakterističnih specijaliziranih metabolita: kemofenetika biljaka. Kemofenetske studije su studije čiji je cilj opisivanje niza specijaliziranih sekundarnih metabolita u određenom rodu [96]. Dakle, kemofenetske studije pridonose fenetskom opisu roda ili vrste, slično anatomskim, morfološkim i kariološkim opisima, pa se tvari koje se identificiraju kao specijalizirani metaboliti karakteristični za pojedinu vrstu, rod ili porodicu, zovu kemofenetski markeri.

Iridoidni glikozidi korišteni su kao kemofenetski markeri na različitim taksonomskim razinama. U okviru istraživanja kemofenetskih markera za rod *Veronica*, Taskova i sur. izolirali su 16 iridoidnih glikozida i dali vezu između kemijskog sastava i osnovnog broja kromosoma [97]. Mehrvarz i sur. istraživali su kemijski sastav odabranih vrsta roda *Veronica*, iridoida i flavonoida, te proučavali njihovu važnost za sistematiku i filogenetsku asocijaciju ovih vrsta. Analiza 4 vrste roda *Veronica* (*V. persica, V. polita, V. francispetae, V. siaretensis*) pokazala je kvalitativno konstantan sastav uzoraka iridoida svih vrsta, neovisno o okolišnim uvjetima [98]. Crişan i sur. istraživali su metodom LC-MS 12 vrsta čestoslavica analizirajući sadržaj aukubina i katalpola koji pripadaju iridoidnim glikozidima. Prema njima, iridoidni glikozidi su se pokazali kao vrlo važni prirodni spojevi zbog svoje farmakološke aktivnosti i važnosti za kemofenetiku [99]. Općenito, najčešći iridoidi roda *Veronica* su aukubin i katalpol [37], [97], [99] za koje se pokazalo da djeluju antitoksično, protuupalno, antioksidativno, štite od osteoporoze i degeneracije živčanog sustava [99], [100]. Albach i sur. također su istraživali iridoidne glikozide aukubin i katalpol u rodu *Veronica* i biljnoj vrsti *Paederota lutea* te su na temelju sastava ovih spojeva došli do zaključka o povezanosti ova dva roda, *Veronica* i *Paederota* [101]. Ovo dokazuje da su iridoidi vrlo dobar marker za kemofenetiku biljnih vrsta, kao što su i Saracoglu i sur. potvrdili u svom istraživanju [102].

Za razliku od fitokemijskih istraživanja čestoslavica koja su uglavnom bila usmjerena na sadržaj glikozida, fenola i flavonoida, slobodni hlapljivi spojevi znatno su manje proučavani [41], [42], [80]. Molekularna filogenija i taksonomija roda *Veronica* dobro je istražena [3], [4], [10], [93], [95], [103]–[108]. Zbog paralelne morfološke evolucije uočene u tom rodu

(taksonomski različite vrste razvijaju slične morfološke karakteristike) [3], ne preporuča se oslanjati samo na morfologiju biljke pri određivanju i klasifikaciji vrsta roda. Stoga se za pouzdano i precizno određivanje vrste preporučuje analiza DNA barkodirane regije, kao što je ITS1-5.8SrDNA-ITS2, (nuklearni geni koji su naslijeđeni od oba roditelja) i trnL-trnF regije (geni iz kloroplasta koji se obično nasljeđuju od ženskog roditelja) [105].

Iako je regija ITS1-5.8SrDNA-ITS2 vjerojatno najpopularniji molekularni marker u biljkama za DNA identifikaciju biljnih vrsta, ova sekvenca može biti hipervarijabilna i stoga teška za analizu ako se sekvenciranje izvodi na tradicionalan način, Sangerovom metodom. Ovo posebno vrijedi za biljke hibridnog i/ili poliploidnog podrijetla. Kako je pojava poliploidije primijećena kod nekoliko vrsta čestoslavica [105], potencijalno puno preciznija identifikacija vrste može se dobiti novom metodom sekvenciranja sljedeće generacije (NGS, *next generation sequencing*). Ovom metodom moguće je sekvencirati mnogo više varijanti gena (markera) i dobiti mnogo kvalitetnije podatke, s većom rezolucijom, i time otkriti genetičku raznolikost kao što su polimorfizmi jednog nukleotida (SNP), umetanja/brisanja (indeli), homopolimerne regije i mikrosateliti, što sve zajedno omogućuje precizniju identifikaciju vrsta. NGS metoda već se primjenjuje u mnogim slučajevima: hvatanje varijacija alela ITS2 kod komaraca [109], raznolikost jedinica rDNA u rodu *Nicotiana* [110], autentičnost prehrambenih proizvoda biljnog podrijetla [111] i identifikacija ljekovitog bilja [112].

2. HIPOTEZE I CILJEVI ISTRAŽIVANJA

Cilj ovog doktorskog rada bio je iz odabranih vrsta roda *Veronica* izolirati i identificirati slobodne hlapljive spojeve iz eteričnih ulja i hidrosola, odrediti njihovu kemofenetsku (fitotaksonomsku) važnost te istražiti biološko djelovanje izoliranih specijaliziranih metabolita. Uz navedeno, jedan od ciljeva je također bio istražiti mikromorfologiju trihoma odabranih vrsta *Veronica* kao mjesta sinteze eteričnih ulja. U svrhu ostvarivanja navedenih ciljeva formirane su tri hipoteze.

Hipoteza 1: Odabrane vrste roda *Veronica* posjeduju žljezdane trihome u kojima se sintetiziraju raznovrsni slobodni hlapljivi spojevi.

Hipoteza 2: Pojedine identificirane komponente ili kemoskupine koje se nalaze u sastavu eteričnih ulja mogu biti kemofenetski markeri za rod *Veronica*.

Hipoteza 3: Slobodni hlapljivi spojevi koji se nalaze u sastavu eteričnog ulja i hidrosola posjeduju antioksidativnu i antiproliferativnu aktivnost te time doprinose biološkoj aktivnosti vrsta roda *Veronica* zajedno sa svim drugim specijaliziranim metabolitima.

3. RADOVI

3.1. Endemic *Veronica saturejoides* Vis. ssp. *saturejoides* – Chemical Composition and Antioxidant Activity of Free Volatile Compounds

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Endemic *Veronica saturejoides* Vis. ssp. *saturejoides*–Chemical Composition and Antioxidant Activity of Free Volatile Compounds

Marija Nazlić [1], Dario Kremer [2], Renata Jurišić Grubešić [2], Barbara Soldo [1], Elma Vuko [1], Edith Stabentheiner [3], Dalibor Ballian [4,5], Faruk Bogunić [4] and Valerija Dunkić [1,*]

1 Faculty of Science, University of Split, Ruđera Boškovića 33, HR-21000 Split, Croatia; marija.nazlic@pmfst.hr (M.N.); barbara@pmfst.hr (B.S.); elma@pmfst.hr (E.V.)
2 Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia; dkremer@pharma.hr (D.K.); rjurisic@pharma.hr (R.J.G.)
3 Institute of Biology, Karl-Franzens University, Schubertstrasse 51, A-8010 Graz, Austria; edith.stabentheiner@uni-graz.at
4 Faculty of Forestry, University of Sarajevo, Zagrebačka 20, BIH-71000 Sarajevo, Bosnia and Herzegovina; balliandalibor9@gmail.com (D.B.); faruk_bogunic@yahoo.com (F.B.)
5 Slovenian Forestry Institute, Večna pot 2, SI-1000 Ljubljana, Slovenia
* Correspondence: dunkic@pmfst.hr; Tel.: +38-521-619-296

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Abstract: Chemical profile and antioxidant activity of the species *Veronica saturejoides* Vis. ssp. *saturejoides* (Plantaginaceae)—which is endemic to Croatia, Bosnia and Herzegovina and Montenegro —were investigated. Volatile compounds produced by glandular trichomes (composed of one stalk cell and two elliptically formed head cells according to scanning electron microscope investigation) were isolated from the plants collected in two locations. Additionally, as a part of specialized metabolites, total polyphenols, total tannins, total flavonoids and total phenolic acids were determined spectrophotometrically. In the lipophilic volatile fractions-essential oils, the most abundant compounds identified were hexahydrofarnesyl acetone, caryophyllene oxide and hexadecanoic acid. In total, the class of oxygenated sesquiterpenes and the group of fatty aldehydes, acids and alcoholic compounds dominated in the essential oils. In the hydrophilic volatile fractions-hydrosols, the most abundant compounds identified were *trans-p*-mentha-1(7),8-dien-2-ol, *allo*-aromadendrene and *(E)*-caryophyllene. A group of oxygenated monoterpenes and the sesquiterpene hydrocarbons dominated in the hydrosols. Antioxidant activity of essential oils and hydrosols was tested with two methods: 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC). Essential oils showed higher antioxidant activity than hydrosols and showed similar antioxidant activity to *Rosmarinus officinalis* essential oil. Obtained results demonstrate that this genus is a potential source of volatiles with antioxidant activity.

Keywords: antioxidant activity; DPPH; GC; GC-MS; glandular trichomes; ORAC; polyphenols; *Veronica saturejoides*; volatile compounds

1. Introduction

The genus *Veronica* L. is the largest within the order Lamiales (family Plantaginaceae) with about 450 species. The extreme variability in morphology and the very good adaptation to the different living conditions of this genus has allowed it to be widely distributed on a wide range of habitats, from aquatic, swamp and forest habitats to rocks, cracks, fields and ruderal habitats [1]. Most representatives grow in areas with a Mediterranean climate [2]. Between 30 and 40 species of the genus *Veronica* have

been described in Croatia [3], but the species studied in this research is the only endemic species. *Veronica saturejoides* Vis. ssp. *saturejoides* grows on the rocks in the Dinaric Mountains in Croatia, Bosnia and Herzegovina and Montenegro. There are two other subspecies of this plant, one from Albania, *V. saturejoides* ssp. *munellensis*, and one from Bulgaria, *V. saturejoides* ssp. *kellereri* [4]. *V. saturejoides* Vis. ssp. *saturejoides* is a perennial crawling plant, which grows 10-30 cm long and has elongated, somewhat lignified roots. It has a hairy stem which is woody at the base. The leaves are simple, opposite each other, 6–9 mm long, have an integrated edge and are not or only slightly hairy on the leaves. The blue flowers are androgynous and zygomorphic [5].

Genus *Veronica* has been extensively phylogenetically investigated and the relationship between the distribution of iridoid glycosides and phylogeny has been reported [6,7]. Flavonoid and phenolic compounds have also been extensively studied, probably because of their importance for the biological activity of plants. These compounds are known to have anti-allergenic, antiviral, anti-inflammatory, cardioprotective and vasodilator properties and, above all, antioxidant and radical scavenging potential [8]. Species of the genus *Veronica* are used in traditional medicine for the treatment of various diseases, including influenza, respiratory diseases and cancer, and as diuretics [9] because of their natural richness in phenolic compounds and iridoids. In recent years, some research has shown that species from this genus could also be used to treat mental disorders [10] and some types of diabetes [11].

Free volatiles of the genus *Veronica* are not very well studied. To our knowledge, *V. saturejoides* ssp. *saturejoides* has not yet been studied, which encouraged our team to study free volatile components of this endemic species, especially in terms of comparing volatile components from essential oil (EO) and from water residues, and to test the antioxidant activity of these extracts, since this species grows under extreme environmental conditions and we assumed that it develops volatile components that could have antioxidant activity. In this research, volatile substances are extracted from glandular trichomes, which are later shown in the pictures. Free volatiles were isolated in lipophilic and water fraction. Water fractions or hydrosols are condensed water vapors containing dissolved molecules of EOs and more water-soluble (polar) volatile compounds [12]. Due to the different solubility of the volatile compounds in water, the overall composition and thus the biological activity of the hydrosol differs from lipophilic fraction or essential oil. Hydrosols are often discarded after the EO extraction process. However, research has shown that these waste products are rich in biologically active substances [12]. Hydrosols from various plants are becoming increasingly important in the food industry, the cosmetics industry, the application of plant pesticides, traditional pharmaceuticals, in aromatherapy as part of complex formulations and as independent products; therefore, their potential use should be further investigated [13].

We now know that plants are exposed to many stresses in their environment. Due to their sedentary lifestyles, they develop specialized metabolites in response to these extreme weather conditions [14] (for this plant, drought and high levels of sunlight due to growing on mountain peaks) and biological stress such as pathogen infection. Specialized plant metabolites have all kinds of biological activities that can be used in medicine, pharmacy and food preservation, as microorganisms become increasingly resistant to synthetic compounds. These synthetic compounds (e.g., BHA-butylated hydroxyanisole) can also be carcinogenic when used in canned foods [9], therefore the search for safe natural food preservatives goes on and this research contributes to the study of natural products as potential antioxidants in food preservations. What is more, it is basic research on the bioactive compounds of *Veronica* genus.

2. Results and Discussion

2.1. Gas Chromatography and Mass Spectrometry (GC-MS) Analysis of the Free Volatile Compounds from Essential Oils and Hydrosols

Two samples of EO and hydrosol obtained from *V. saturejoides* ssp. *saturejoides* were analyzed. The results are presented in Table 1. The yield of EO for two samples, Prenj (PS) and Kamešnica (KS), was 0.07% and 0.03% respectively. This endemic species is rich in volatile compounds that have been

studied in oils and hydrosols. The compounds are listed in the order of their elution from the column (Table 1). In total, the class of oxygenated sesquiterpenes (PS 54.25% and KS 36.46%) and the group of fatty aldehydes, acids and alcoholic compounds (PS 17.4% and KS 43.85%) dominate in the oils, if one considers the results obtained. Within the hydrosol, the oxygenated monoterpenes (PS 31.75% and KS 40%) and the sesquiterpene hydrocarbons (PS 36.46% and KS 34.53%) dominate (Table 1, Figure 1).

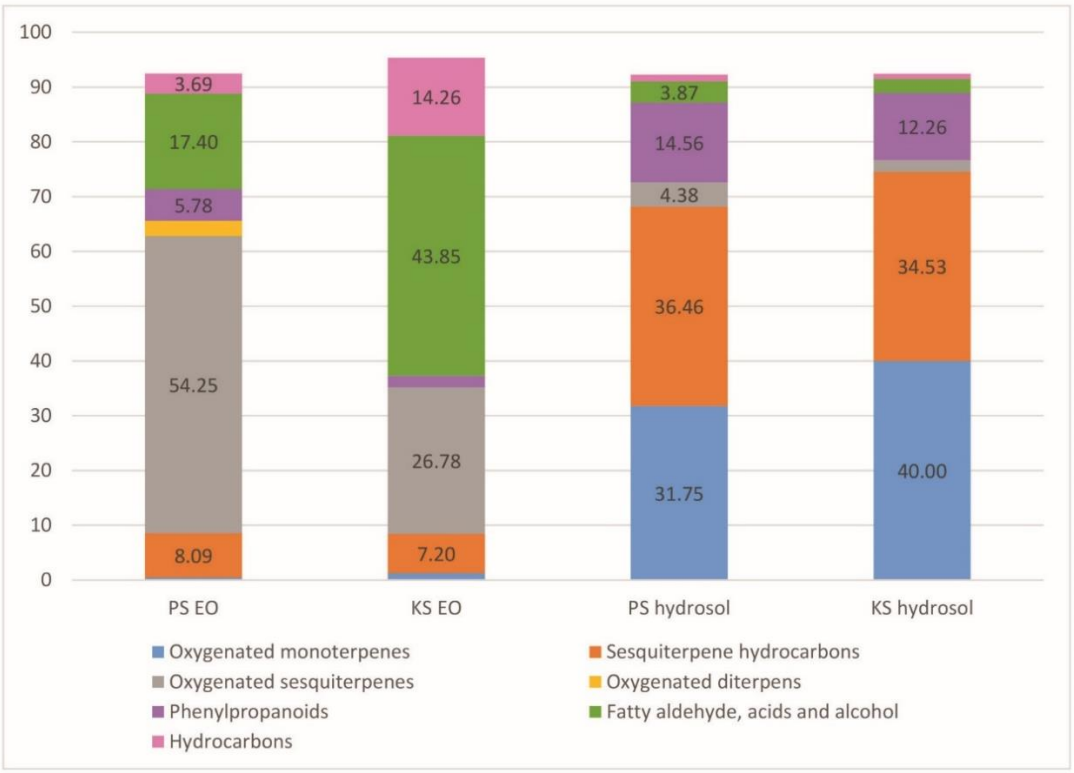

Figure 1. Distribution of volatile compounds by category in essential oils (%) and hydrosols (%); PS-Prenj sample, KS-Kamešnica sample.

In the PS essential oil, 25 compounds were identified, which account for 92.47% of the total oil. The most abundant compound was hexahydrofarnesyl acetone (30.13%). A total of 21 compounds were identified in KS essential oil, representing 95.38% of the total oil, with the most abundant compound being hexadecanoic acid (37.31%). Table 1 shows that the number of compounds detected and identified in hydrosol was lower than in EO. This is logical since non-polar compounds are less soluble in water. In the PS hydrosol, we identified 17 compounds, representing 92.29% of the total hydrosol; in the KS hydrosol, we identified 16 compounds, representing 92.43% of the total hydrosol. In the hydrosol samples, the most abundant compound was *trans-p*-mentha-1(7),8-dien-2-ol, which represented 31.75% and 36.63% of the total hydrosol in the PS and KS, respectively. These results are consistent with the fact that hydrosols contain dissolved molecules of EO, considering that more than half of the compounds that we identified in hydrosol can also be found in the essential oil [12].

Hexahydrofarnesyl acetone (phytone) and hexadecenoic acid (palmitic acid) are important components in both samples of the EOs analyzed in this study. According to the literature, phytone shows strong antimicrobial activity and broad-spectrum inhibition against various fungal strains [15]. Palmitic acid has antioxidant, nematidical, pesticide, antiandrogenic, hemolytic and 5-alpha reductase activities [16]. Palmitic acid was also found in the EO of the species *Veronica thymoides* P. H. Davis subsp. *pseudocinerea* M. A. Fischer, where it accounted for 5.4% of the total EO [17].

The presence of hydrocarbons is significant, especially in the oil PS where pentacosan dominates with 6.28%. Sesquiterpenes (E)-caryophyllene and caryophyllene oxide were identified in both

oil samples. Caryophyllene oxide is particularly present in the PS oil with 20.25%, while it is almost ten times less present in KS oil (2.34%). Sesquiterpenes are also significantly represented and dominate in the analyzed hydrosols. The proportion of identified (E)-caryophyllene in the hydrosol samples (24.52% and 12.35%) is significantly higher than in the oil samples, followed by allo-aromadendrene (8.13% and 11.53%) and germacrene D (2.56% and 4.67%), while caryophyllene oxide is least present in the hydrosols samples (Table 1). Volatile components rich in sesquiterpenes are known to have antifungal, antimicrobial, anticancer and antioxidant properties [18–21]. In addition to these sesquiterpenes and *trans-p*-mentha-1(7),8-dien-2-ol in the analyzed hydrosol samples, methyl eugenol was significantly present with 13.35% in the PS hydrosol and 11.92% in the KS hydrosol. Phenylpropanoids such as methyl eugenol and identified Z-methyl isoeugenol (1.25% and 4.16%) occur in plants under stress conditions, such as ultraviolet radiation and pathogen attack [22].

In the previously investigated *Veronica spicata* L., phytol was the dominant compound (21.13%) in the total EO [9]. In this research phytol was only present in the PS essential oil (Table 1) (2.82%). A further comparison of the compounds found in *V. spicata* L. and *V. saturejoides* shows that nine compounds were found in both plant species: (E)-caryophyllene, spathulenol, caryophyllene oxide, γ- eudesmol, phytol, docosane, tricosane, tetracosane and pentacosane. Li (2002) [23] identified essential oil components of *V. linariifolia* Pall. ex Link and found that the main components were cyclohexene (25.83%), β-pinene (11.61%), 1S-α-pinene (10.65%), β-phellandrene (10.49%), β-myrcene (10.42%), and germacrene D (4.99%). If we compare these results with ours, we can conclude that EO of *V. saturejoides* is richer in oxygenated sesquiterpenes and *V. linariifolia* in sesquiterpene hydrocarbons. Çelik et al. investigated EOs extracted from *Veronica* sp. and found that the main components were mainly linalool (4.18%) and carvacrol (7.28%) [24]. Research on free volatile compounds of the genus *Veronica* is very scarce, therefore it is important that our research is supported by the micromorphology of trichomes as a visible evidence of the place of synthesis of free volatile compounds.

Table 1. Chemical composition of the essential oil (%±SD) and hydrosols (%±SD) from the two samples from aerial parts of *V. saturejoides* Vis. ssp. *saturejoides*.

			Essential Oil Samples (%)		Hydrosol Samples (%)	
Component	**RI [a]**	**RI [b]**	**PS**	**KS**	**PS**	**KS**
Oxygenated monoterpenes			**0.44**	**1.23**	**31.75**	**40**
trans-p-Mentha-1(7),8-dien-2-ol	1187	1803	-	1.23 ± 0.01	31.75 ± 0.01 [b]	36.63 ± 0.01 [a]
Verbenone	1204	1705	-	-	-	0.76 ± 0.01
endo-Fenchyl acetate	1218	-	-	-	-	2.61 ± 0.01
Piperitone oxide	1365	-	0.44 ± 0.01	-	-	-
Sesquiterpene hydrocarbons			**8.09**	**7.2**	**36.46**	**34.53**
(*E*)-Caryophyllene *	1424	1585	0.94 ± 0.01 [b]	2.46 ± 0.01 [a]	24.52 ± 0.01 [a]	12.35 ± 0.01 [b]
Z-Methyl isoeugenol	1451	-	-	-	1.25 ± 0.01 [b]	4.16 ± 0.01 [a]
allo-Aromadendrene	1465	1662	2.35 ± 0.01 [a]	0.68 ± 0.01 [b]	8.13 ± 0.01 [b]	11.53 ± 0.01 [a]
β-Chamigrene	1476	1724	-	0.44 ± 0.01	-	1.17 ± 0.01
γ-Muurolene	1478	1685	0.39 ± 0.01 [b]	0.87 ± 0.01 [a]	-	0.65 ± 0.03
Germacrene D	1482	1692	2.78 ± 0.01 [a]	1.77 ± 0.01 [b]	2.56 ± 0.01 [b]	4.67 ± 0.01 [a]
δ-Cadinene	1517	1745	1.63 ± 0.01 [a]	0.98 ± 0.01 [b]	-	-
Oxygenated sesquiterpenes			**54.25**	**26.78**	**4.38**	**2.1**
Spathulenol	1577	2101	0.22 ± 0.01 [b]	0.87 ± 0.01 [a]	-	-
Caryophyllene oxide *	1581	1955	20.25 ± 0.01 [a]	2.34 ± 0.01 [b]	0.26 ± 0.01 [b]	0.84 ± 0.05 [a]
γ-Eudesmol	1632	2175	0.74 ± 0.01 [a]	0.33 ± 0.01 [b]	0.28 ± 0.01	-
α-Muurolol	1645	2181	2.91 ± 0.01	-	-	-
α-Bisabolol	1685	2210	-	-	0.32 ± 0.05	-
Hexahydrofarnesyl acetone	1839	2113	30.13 ± 0.01 [a]	23.24 ± 0.01[b]	3.52 ± 0.01 [b]	1.26 ± 0.01 [a]
Oxygenated diterpens			**2.82**	-	-	-
Phytol	1942	2610	2.82 ± 0.03	-	-	-

Table 1. *Cont.*

Component	RI [a]	RI [b]	Essential Oil Samples (%)		Hydrosol Samples (%)	
			PS	KS	PS	KS
Phenylpropanoids			**5.78**	**2.06**	**14.56**	**12.26**
Benzaldehyde	964	1513	-	-	0.75 ± 0.01 [a]	0.34 ± 0.01 [b]
2-Methoxy-4-vinylphenol	1294	2178	2.56 ± 0.01	-	0.46 ± 0.01	-
Methyl eugenol	1403	2005	1.28 ± 0.01 [a]	1.13 ± 0.01[b]	13.35 ± 0.01 [a]	11.92 ± 0.01 [b]
Benzyl benzoate	1754	-	1.94 ± 0.01 [a]	0.93 ± 0.01[b]	-	-
Fatty aldehyde, acids and alcohol			**17.4**	**43.85**	**3.87**	**2.59**
n-Nonanal	1100	1389	-	-	0.16 ± 0.01	-
Hexyl 2-methyl butanoate	1233	1425	-	-	1.13 ± 0.01 [b]	1.82 ± 0.01 [a]
Nonanoic acid	1267	2149	0.12 ± 0.01	-	-	-
Dodecanoic acid	1564	2480	0.59 ± 0.01 [b]	1.67 ± 0.01 [a]	-	-
1-Hexadecanol	1874	2371	7.73 ± 0.01 [a]	4.53 ± 0.01 [b]	1.32 ± 0.01	-
Hexadecanoic acid	1959	2912	7.88 ± 0.01 [b]	37.31 ± 0.01 [a]	1.26 ± 0.01 [a]	0.77 ± 0.01 [b]
Oleic acid	2133	-	1.08 ± 0.01 [a]	0.34 ± 0.03 [b]	-	-
Hydrocarbons			**3.69**	**14.26**	**1.27**	**0.95**
Heneicosane *	2100	2100	0.42 ± 0.02 [b]	0.68 ± 0.07 [a]	-	-
Docosane *	2200	2200	0.73 ± 0.01 [b]	3.27 ± 0.01 [a]	1.27 ± 0.01 [a]	0.95 ± 0.01 [b]
Tricosane *	2300	2300	1.27 ± 0.01 [b]	4.03 ± 0.01 [a]	-	-
Tetracosane *	2400	2400	0.59 ± 0.05	-	-	-
Pentacosane *	2500	2500	0.68 ± 0.01 [b]	6.28 ± 0.01 [a]	-	-
Total (%)			**92.47**	**95.38**	**92.29**	**92.43**

RI[a], retention indices on capillary column VF5-ms; RI[b], retention indices on capillary column CP-Wax 52; RI, identification by comparison to literature [25], and/or homemade library, comparison of mass spectra with those in mass spectral libraries NIST02 and Wiley 9; * co-injection with reference compounds; –, not identified; SD = standard deviation of triplicate analysis; significant differences were determined using multiple *t*-test. [a],[b]—Mean values with different superscript letters indicate a statistically significant difference between data from two locations ($p < 0.05$); PS—Prenj sample, KS—Kamešnica sample.

2.2. Micromorphological Traits

In general, micromorphological investigations of *Veronica* species are rare, so we have decided to conduct the micromorphological research on this plant to find site of essential oil production. On the stems, leaves, and the calyces of *V. saturejoides* non-glandular and glandular trichomes could be observed. According to scanning electron microscope (SEM) investigation, non-glandular trichomes (Figure 2a,b,g,h) were unbranched, bi-cellular to multicellular, uniseriate, and folded at different levels. They could be noted as attenuate trichomes [17]. The length of these trichomes varied from very short to long trichomes (Figure 2g,h). These trichomes protect the plant from water loss and maintain the positive microclimate. The surfaces of these trichomes showed a warty appearance due to the occurrence of cuticular micropapillae (Figure 2g,h). Leaves and flowers were sparsely covered by non-glandular trichomes (Figure 2a–f), while the stem was characterized by a relatively dense indumentum of these trichomes (Figure 2g,h). The existence of non-glandular trichomes on flower parts of *Veronica* species was mentioned 100 years ago by Kurer [26]. Additionally, Kraehmer and Baur described these trichomes in *V. persica* Poir [27]. The same type of non-glandular trichomes is common in many other Lamiaceae species [28–30].

Glandular capitate trichomes could be observed on stems, leaves, and the calyces of *V. saturejoides* ssp. *saturejoides*. These trichomes were composed of one stalk cell and two elliptically formed head cells (Figure 2e). They were not upright, and could be described as clinging to the surface. All investigated plant parts were sparsely covered by capitate trichomes. The same type of capitate trichomes was noticed in *V. beccabunga* L. [31]. Likewise, an inclined trichome type with a bicellular head was reported in *Stachys recta* L. subsp. *recta* by Vundac et al. [32]. Comparable capitate trichomes with only one elliptically formed head cell could be observed on SEM micrographs of *Marrubium vulgare* L. (Lamiaceae) in research by Haratym and Weryszko-Chmielewska [28]. Moreover, Hanlidou et al. described a similar trichome type ("short and ordinarily bent") in *Calamintha menthifolia* Host. [33].

Kremer et al. also found an inclined trichome type with one head cell in *Micromeria croatica* (Pers.) Schott [34]. Although the yield of EO was considerably higher in PS (0.07%) than in KS (0.03%), the micrographs did not show any significant difference between the samples for the number of capitate trichomes on calyces and leaves (Table 2, Figure 2c–f). A slightly higher number of capitate trichomes was found on stems from PS (Figure 2g). The obtained difference in EO yield between samples could be due to other reasons, such as climatic conditions or possible mechanical damage of the glandular trichomes.

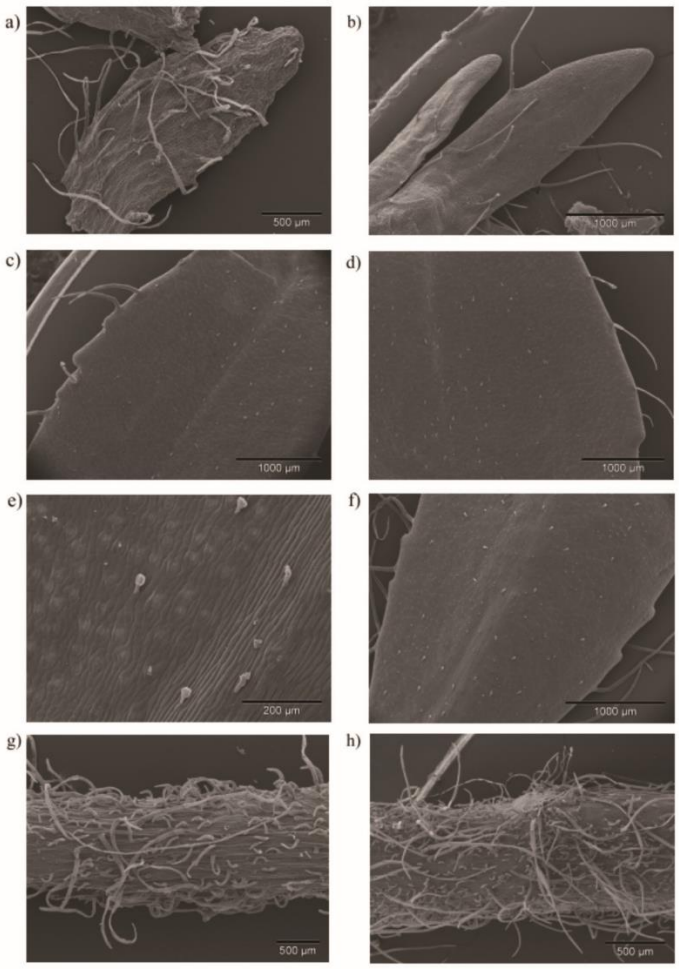

Figure 2. SEM micrographs of the different trichome types of *V. saturejoides* ssp. *saturejoides*. Rare (**a**,**b**) and dense (**g**,**h**) indumentum of non-glandular trichomes on the calyx (**a**,**b**) and stem (**g**,**h**); glandular trichomes on the adaxial (**c**,**d**) and abaxial (**e**,**f**) leaf surface; micrographs of Prenj (**a**,**c**,**e**,**f**,**g**) and Kamešnica (**b**,**d**,**h**) sample.

Table 2. Occurrence and frequency of trichomes on aerial parts of *Veronica saturejoides* ssp. *saturejoides*.

Sample	Trichome	Leaf		Calyx	Stem
	Type	Adaxial	Abaxial		
Prenj	Attenuate *	±	±	+	++
	capitate C1	±/+	±/+	−/±	±/+
Kamešnica	attenuate	±	±	+	+/++
	capitate C1	±/+	±/+	−/±	±

Note: * attenuate, non-glandular trichomes; trichomes: − absent, ± rare, + present, ++ abundant.

2.3. Polyphenol Analysis in Dry Plant Material

Plant polyphenols are natural biologically active compounds that can also be synthesized in the laboratory. They have been shown to be good antioxidants, anti-neurodegenerative and anticancer agents [35]. In our research, as is shown in Table 3, the highest content was found for total phenolic acids in *V. saturejoides* ssp. *saturejoides* (Kamešnica-KS; 1; A525 nm), while the yield of total flavonoids (TF) was found to be very low and the same for both investigated specimens of *V. saturejoides* ssp. *saturejoides* (Table 2). Harput et al. found that total phenolic content was 200.20 mg/g in *V. officinalis* L., 139.92 mg/g in *V. peduncularis* M. Bieb., 127.64 mg/g in *V. orientalis* Mill., and 83.15 mg/g in *V. baranetzkii* Bordz. [36]. Comparing these results with ours, it can be seen that *V. saturejoides* has a similar total phenolic content to *V. baranetzkii*. Ertas et al. found that the total phenolic content in methanolic extracts of *V. thymoides* subsp. *pseudocinerea* was 248.37 ± 3.68 mg/g and that total flavonoid content was 47.02 ± 0.21 mg/g [17].

Table 3. Contents of total polyphenols (TP), total tannins (T), total flavonoids (TF), and total phenolic acids (TPA) in *V. saturejoides*.

Species	TP (mg/g DW)	T (mg/g DW)	TF (mg/g DW)	TPA (505 nm) (mg/g DW)	TPA (525 nm) (mg/g DW)
V. saturejoides (KS)	86.9 ±1.4 [a]	2.3 ± 1.3	0.8 ± 0.00	33.1 ± 1.7 [a]	65.6 ± 0.2 [a]
V. saturejoides (PS)	70.9 ± 0.9 [b]	1.7 ± 0.5	0.8 ± 0.00	19.5 ± 0.2 [b]	45.4 ± 2.1 [b]

Note: DW, dry weight; SD = standard deviation of triplicate analysis; significant differences were determined using multiple *t*-test. [a],[b]—Mean values with different superscript letters indicate a statistically significant difference between data from two locations ($p < 0.05$); KS—Kamešnica sample, PS—Prenj sample.

The absorbances obtained at 505 nm refer to rosmarinic acid, and the results at 525 nm represent the chlorogenic acid content. According to the pharmacopoeial expressions ($A_{505nm} \times 2.5/m$; $A_{525nm} \times 5.3/m$), and taking into account specific absorbances of standard phenolic acids (rosmarinic acid $A^{1\%}_{1cm} = 400$; chlorogenic acid $A^{1\%}_{1cm} = 180$), chlorogenic acid predominates in the tested plant samples.

2.4. Phenolic Compounds in Hydrosols

Phenolic compounds in hydrosols were analyzed for better explanation of antioxidant activity of the hydrosols. Vanillin, cinnamic acid, and protocatechuic acid (3, 4-dihydroxybenzoic acid) were confirmed. All three compounds were found in hydrosol of plants collected in Kamešnica (KS). In hydrosols of PS (from Prenj), polyphenolic compounds were not detected (Table 4). In all analyzed samples from Kamešnica, protocatechuic acid was the most abundant compound, with an average concentration of 7.33 ± 0.35 mg L^{-1}. Phenolic compounds generally have a protective role in plants and in their acclimatization to environmental conditions, so that the difference between detected phenols in PS and KS hydrosols can be attributed to exposure to different stresses (i.e., water stress). Concentrations of vanillin and cinnamic acid ranged from 0.11 mg to 0.22 mg L^{-1}. The average vanillin concentration was 0.22 ± 0.01 mg L^{-1} and that of cinnamic acid was 0.12 ± 0.02 mg L^{-1}. Low amounts of polyphenols were expected due to their insolubility in water. In four types of *V. spicata* plant extracts (methanol, ethyl-acetate, water at 25 °C, and water at 45 °C), cinnamic acid and vanillin were not confirmed [9]. The concentration of protocatechuic acid was in the range from 0.008 ± 0.001% (in methanol) to 0.151 ± 0.012% (water at 45 °C). In ethyl-acetate extracts, protocatechuic acid was not found [9]. Beara et al. also found significant amounts of protocatechuic acid in 70% aqueous acetone extracts of *V. teucrium* and *V. jacquinii* [37]. Stojković et al. found that protocatechuic acid was the main compound in water extracts of *V. montana* L. [38].

Table 4. Polyphenolic analyses of hydrosols.

Samples	Vanillin	Cinnamic Acid	Protocatechuic Acid
V. saturejoides (KS)	0.22 ± 0.01	0.12 ± 0.02	7.33 ± 0.35
V. saturejoides (PS)	-	-	-

Results expressed in mg/L of hydrosol sample; KS—Kamešnica sample, PS—Prenj sample.

2.5. Antioxidant Activity

In Table 5, we can see that KS oil from the Kamešnica Mountains showed slightly higher oxygen radical absorbance capacity (ORAC) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) activity. In the polyphenolic analyses in the sample from Prenj Mountain, we could not detect any polyphenols, only in the sample from the Kamešnica Mountain, so we can conclude that this small difference in antioxidant activity could be due to this finding. This is the first report on the antioxidant activity of the free volatiles of *Veronica* species, so we cannot compare it with other results for *Veronica* species, but we can compare our results with other relevant EOs and hydrosols commonly used in food preservation, preservation, pharmacy and cosmetics. In comparison with reports on other plants, Aazza et al. [39] investigated the ORAC activity of hydrosols of several medicinal plants (*Lavandula officinalis, Origanum majorana, Rosmarinus officinalis, Salvia officinalis, Thymus vulgaris, Cinnamomum verum* and *Syzgium aromaticum*). The hydrosol KS tested in our research has higher ORAC activity than *Salvia officinalis* and similar activity to *Rosmarinus officinalis* [39]. Bentayeb et al. [40] reported antioxidant activity for 10 EOs of the plants often used as spices. ORAC antioxidant activity of *V. saturejoides* according to their reported values has similar activity as rosemary and basil EO. Viuda-Martos et al. [41] reported antioxidant activity for five plants used in Mediterranean and *V. saturejoides* showed similar DPPH activity to rosemary oil which is in accordance to other reported results [41]. Antioxidant activity for metabolites of the genus *Veronica* was mostly tested on different extracts of phenolic compounds and on iridoid glucosides. Harput et al. [42] tested the DPPH activity of methanolic extracts from fourteen different *Veronica* species and reported the highest antioxidant activity in *V. officinalis* with an IC50 value of 40.93 μg/mL [42]. Mocan et al. [43] confirmed the antioxidant activity for *V. officinalis* with TEAC method 157.99 ± 6.58 mg TE/g. Živković et al. [10] tested methanolic extracts of *V. teucrium* and *V. jacquinii* and they were stronger than previously reported by Harput et al. [42] for *V. officinalis*, with IC50 values of 28.49 ± 0.6 for *V. teucrium* and 37.63 ± 0.6 μg/mL for *V. jacquinii*, although both have lower activity than the standard (BHT-butylated hydroxytoluene and BHA-butylated hydroxyanisole) [10]. Kwak et al. reported antioxidant activity for iridoid glucosides in which the ethanolic extract showed higher activity than Trolox [44]. In our previous study, we tested the DPPH activity of various extracts of *V. spicata*, and the IC50 value showed the highest activity in methanolic extracts of flowers and leaves with values of 8.21 ± 0.06 μg/mL and 8.69 ± 0.06 μg/mL, i.e., higher than the values for standards BHT and BHA according to the values in the study by Živković et al. [10].

We have also tested the most abundant compound in EOs hexahydropharnesyl acetone with DPPH and ORAC methods, and it did not show any antioxidant activity, so we can assume that the antioxidant activity comes from another compound in EO, or is probably the result of synergistic work between different compounds. Synergistic activity has been demonstrated in some studies. Amorati et al. tested and compared the antioxidant activity for 5 different EOs, their hydrocarbon and oxygenated fractions and also for two compounds characteristic for these EOs, thymol and carvacrol. The results showed that in 4 out of 5 samples, the total oil has a higher antioxidant activity than its fractions, and in 3 out of 5 samples, the total oil has a higher activity than isolated single active compounds, so that we can conclude that the synergy between different compounds in the essential oil plays a crucial role in its activity [45]. From our results, we can also conclude that the activity does not originate from the most abundant compound, but from the interplay between all compounds from the EO. Our previous research showed similar results with phenolic extracts, since for *V. spicata*, it was shown that there is a negative correlation between the amount of phenolic compounds and antioxidant activity, so that it was concluded that other substances such as terpenoids and proteins

may have an influence on antioxidant activity [9]. We have shown that *Veronica* species have free volatile compounds (terpenoids) and that they have antioxidant activity. Other potential biological activity such as antimicrobial, antiviral and antiproliferative should be carried out to give better insight into the full potential of using these compounds in pharmacy or food preservation.

Table 5. Antioxidant potential of *V. saturejoides* essential oil and hydrosol determined by ORAC and DPPH method.

	Essential Oil		Hydrosols		Hexahydropharnesyl Acetone
Antioxidant Assay	**PS**	**KS**	**PS**	**KS**	
ORAC (Trolox eq)	255.1 ± 2.54	256.5 ± 5.73	0.559 ± 0.059	0.679 ± 0.036	-
DPPH (Trolox eq)	20.73 ± 0.21 ^{b}	44.32 ± 0.13 ^{a}	0.225 ± 0.062	0.323 ± 0.014	-
DPPH (% inhibition)	46.23 ± 4.37 ^{b}	66.99 ± 2.98 ^{a}	34.84 ± 0.89 ^{b}	49.26 ± 1.7 ^{a}	-
DPPH (IC 50)	10.88 ± 1.24 ^{b}	7.16 ± 0.12 ^{a}	-	-	-

ORAC, oxygen radical absorbance capacity, results for EOs expressed as μmol of Trolox equivalents (TE) per g of EO (10 mg/mL) and for hydrosols as μmol of Trolox equivalents (TE) per g of the total (undiluted) tested hydrosol sample; DPPH, results for EOs expressed as μmol of Trolox per g of EO (10 mg/mL) and for hydrosols as μmol of Trolox per g of absolute hydrosol, IC50 expressed in mg/mL for EOs; –showed no activity; SD = standard deviation of triplicate analysis; significant differences were determined using multiple t-test. ^{a},^{b}—Mean values with different superscript letters indicate a statistically significant difference between data from two locations ($p < 0.05$) KS—Kamešnica sample, PS—Prenj sample.

3. Materials and Methods

3.1. Herbal Material

Randomly selected samples of wild-growing plants of *V. saturejoides* Vis. were collected at the end of the blooming period in July 2018 from two locations, one in Croatia and one in Bosnia and Herzegovina: sample 1 (Prenj sample – PS) – Prenj Mountain (Bosnia and Herzegovina; 43°43′16″ N, 18°07′03″ E; 1525 m a.s.l.; Voucher No. HFK-HR 121/2018); sample 2 (Kamešnica sample – KS) –Kamešnica Mountain (Croatia; 43°43′33″ N, 16°51′57″ E; 1568 m a.s.l.; Voucher No. HFK-HR 122/2018). Voucher specimens of herbal material were deposited in the "Fran Kušan" herbarium (HFK-HR), Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

For gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC and GC-MS) analyses, samples were air dried for three weeks in a single layer in a well-ventilated room and protected from direct sunlight. Dried plant material was placed in double paper bags labeled with the sample number and stored in a dry place away from light until analysis.

For micromorphological studies of trichomes, samples from seven plants per locality were fixed in FAA (formalin/96% ethanol/acetic acid/water: 5/70/5/20). After three days, the samples were transferred to 70% ethanol (Kemika, Zagreb, Croatia) and stored in fridge until analysis.

3.2. GC and GC-MS Analyses

Dried aerial parts (50 g) for each location were hydrodistilled for three hours using Clevenger-type apparatus. For each sample, we collected lipophilic (extracted in the pentane part in the inner tube of the Clevenger apparatus) and hydrophilic volatile compounds (extracted in the water part in the inner tube of the Clevenger apparatus) fractions and stored in fridge until analysis. Both phases were analyzed with GC and GC-MS. GC was performed by gas chromatograph (model 3900, Varian Inc., Lake Forest, CA, USA) that is supplied with a flame ionization detector (FID), mass spectrometer (model 2100T; Varian Inc.), non-polar capillary column VF-5ms (30 m × 0.25 mm inside diameter, coating thickness 0.25 μm, Palo Alto, CA, USA) and polar capillary column CP-Wax 52 CB (30 m × 0.25 mm i.d., coating thickness 0.25 μm, Palo Alto, CA, USA). The chromatographic conditions for the analysis of lipophilic fraction (essential oils) were: FID detector temperature 300 °C, injector temperature 250 °C. The gas carrier was helium at 1 mL min^{-1}. The conditions for the VF-5ms column were: temperature

60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1}, and held isothermal for 25 min. Conditions for the CP Wax 52 column were: temperature 70 °C isothermal for 5 min, and then increased to 240 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. The injected volume was 2 µL and the split ratio was 1:20. The MS conditions were: ion source temperature 200 °C, ionization voltage 70 eV, mass scan range 40–350 mass units [9]. The individual peaks for all samples were identified by comparing their retention indices of n-alkanes with those of authentic samples and literature [25]. The chromatographic conditions for the analysis of hydrophilic fraction (hydrosols) were the same, however the injection was done with a headspace injection needle and there was no split ratio (splitless mode). The procedure for each hydrosol sample was as follows: 2 g of hydrosol was added in the glass bottle and closed with a metal cap with septum. The headspace needle was injected in the glass bottle closed with metal cap with septum. The glass bottle was first placed with the hydrosol sample in water at 40 °C and left there for 20 min without the needle to allow volatile compounds to evaporate from the water. The needle was then injected and left there for 20 min so that the volatile compounds could be adsorbed on the resin needle. The injection needle was then inserted into a GC inlet and left there for 20 min to ensure that all volatile compounds from the resin were resorbed into the injection liner. The MS conditions were the same as for volatile compounds from EO and the individual peaks for all samples were identified by comparing of their retention indices of n-alkanes with those of authentic samples and literature [25]. The results for all samples were measured in three independent analyses and expressed as percentage (%) of each compound in a total EO or hydrosol (Table 1). All values were calculated as the mean of three independent results with standard deviation.

3.3. Micromorphological Traits

For SEM investigation, stem, leaf, and calyx samples were transferred from 70% ethanol to 70% acetone and then further dehydrated (70%, 90% and 100% acetone) and subjected to critical point drying using CO_2 as drying medium (CPD030; Bal-tec, Balzers, Liechtenstein). The samples were then sputtered with gold (Sputter Coater, AGAR) and examined under an XL30 ESEM (FEI) SEM with an acceleration voltage of 20 kV in high vacuum mode [46]. Common terminology [47] was used to describe the trichomes.

3.4. Phenolic Compounds in Hydrosols

The phenolic compounds of the hydrosols were separated by high- performance liquid chromatography (HPLC) on a Series 200 Perkin Elmer HPLC system (Waltham, MA, USA), equipped with a thermostated autosampler, vacuum degasser, binary pump, thermostated column section, UV/VIS detector and the TotalChrom Workstation software package (version 6.2.1, Perkin Elmer, Waltham, MA, USA) used for the analyses. Phenolic compounds were separated with the C18 column (Ultra-Aqueous C-18, 250 × 4.6 mm, 5 Å) (Restek, Bellefonte, PA, USA) and by gradient chromatography. Chromatography conditions were set according to the methodology by Jukic Spika et al. [48].

The identification of phenolic compounds in the hydrosols was performed by comparing the retention time with that of the pure standard. The quantification of the phenolic compounds was performed using the calibration curve of the standard and the results were expressed in mg of each phenolic compound per L of the sample. The standard and solvents were of analytical grade, and were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water (Milli-Q) was used for the preparation of all solvents.

3.5. Polyphenol Analysis

3.5.1. Apparatus and Chemicals

A Soxhlet apparatus was used for drug extraction. The quantitative analysis of the polyphenolic substances was performed with an Agilent 8453 UV/Vis spectrophotometer (Agilent, Germany) equipped with PC -HP 845x UV—Visible System (Agilent, Germany) and 1 cm quartz cuvettes.

With the exception of the Folin–Ciocalteu phenol reagent (FCR), casein (Merck, Darmstadt, Germany) and quercetin (Roth, Karlsruhe, Germany) all chemicals and reagents for polyphenol analysis were of analytical quality grade and were supplied by Kemika (Zagreb, Croatia).

3.5.2. Total Polyphenol and Tannin Analysis (Folin–Ciocalteu Phenol Reagent (FCR) Procedure)

Total polyphenol (TP) and tannin (T) analysis was based on a reaction with FCR and spectrophotometric determination of TP and T at 720 nm-indirect analysis after precipitation T with casein [46,47]. The plant material (0.250 g; above ground parts) was previously extracted with methanol (30%, v/v), using water bath (70 °C) for 15 min [49,50]. The contents of TP and T in *V. saturejoides* extracts were evaluated in three independent analyses and were expressed as mg/g of dry weight of herbal material [48,49] according to equation:

$$A = 1.069cexp. - 0.0029 \quad (1)$$

where A is absorbance and cexp. is measured concentration (μg/mL).

Total polyphenol and tannin analysis (FCR procedure) were based on a reaction with FCR and spectrophotometric determination of TP and T (indirect analysis after precipitation with casein) at 720 nm. Tannin was used as the standard substance.

3.5.3. Total Flavonoid (TF) Analysis (TF Procedure)

TF included hydrolysis of glycosides (extraction of 0.20 g of powdered plant material with acetone, 25% HCl and 0.5% hexamethylenetetramine, using boiling water bath for 30 min), then extraction of total flavonoid aglycones with ethyl acetate, and complexation with $AlCl_3$ [51,52]. Absorbance of the yellow complex was measured at 425 nm and concentration was calculated as quercetin using the following equation:

$$\% = A \times 0.772/b; [A = \text{absorbance; } b = \text{mass of dry plant material (g)}] \quad (2)$$

where b is mass of the dry plant material (g) and 0.772 represents the conversion factor related to the specific absorbance of quercetin at 425 nm (i.e., 810). TF concentration was measured in three independent analyses and expressed as mg/g of dry weight of herbal material.

3.5.4. Determination of Total Phenolic Acids (TPA) (TPA Procedure)

TPA procedure was performed according to official pharmacopoeial method [52] for determination of hydroxycinnamic derivatives. TPA were determined spectrophotometrically (three independent analyses) in extracts of *V. saturejoides* samples (0.200 g of powdered drug; ethanol 50% v/v; boiled water bath under a reflux condenser; 30 min), using the nitrite-molybdate reagent of Arnow, in a sodium hydroxide medium.

TPA content, expressed as rosmarinic acid (λ = 505 nm), was calculated from the equation:

$$A \times 2.5/m; [A = \text{absorbance; } m = \text{mass of the substance to be examined (g)}] \quad (3)$$

where m is mass of the dry plant material (g) and 2.5 represents the conversion factor related to the specific absorbance of rosmarinic acid at 505 nm (i.e., 400).

TPA content, expressed as chlorogenic acid (λ = 525 nm), was calculated from the equation:

$$A \times 5.3/m; \text{ [A = absorbance; m = mass of the substance to be examined (g)]} \quad (4)$$

where 5.3 represents the conversion factor related to the specific absorbance of chlorogenic acid at 525 nm (i.e., 188).

Conversion factors refer to the preparation of plant samples for spectrophotometric determination of flavonoid and phenolic acid contents according to official pharmacopoeial methods, considering the specific absorbances of standards.

3.6. Antioxidant Activity of Essential Oils and Hydrosols

3.6.1. Oxygen Radical Absorbance Capacity Assay (ORAC)

The assay was performed in a Perkin–Elmer LS55 spectrofluorimeter, using 96-well white polystyrene microtiter plates (Porvair Sciences, Leatherhead, UK) according to a method described by Fredotovic et al. [53], with some adjustments due to different extracts. We had hydrophilic assay for hydrosols and lipophilic assay for EOs. For the hydrophilic assay, each reaction contained 180 µL of fluorescein (1 µM), 70 µL 2,2′-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH, Acros Organics) (300 mM), and 30 µL of plant extracts or reference standard Trolox (6.25–50 µM) (Sigma–Aldrich). For the antioxidant test for hydrosols, all experimental solutions and samples were prepared in a phosphate buffer (0.075 mM, pH 7.0). We used absolute hydrosol and dissolved it in phosphate buffer 20× and 40× for the experiments. For the lipophilic assay, EO were dissolved in acetone (10 mg in 1 mL acetone). The EO acetone dilutions were further dissolved 40× and 80× in the phosphate buffer for the experiments. The measurements were performed in triplicate by a method described in Fredotovic et al. [53]. The ORAC values of hydrosols are expressed as µmol of Trolox equivalents (TE) per g of the total (undiluted) tested hydrosol sample. The ORAC values of EOs were expressed as µmol of Trolox equivalents (TE) per g of essential oil. The results were obtained from three independent experiments.

3.6.2. Measurement of the DPPH Radical Scavenging Activity

The antioxidant capacity of the extracts was assessed by the DPPH method previously described by Mensor et al. and Payet et al. [54,55]. This method is based on the reduction of alcoholic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (Sigma–Aldrich) in the presence of a hydrogen-donating antioxidant using 96-well microtiter plates. Plant extracts as described in the ORAC method were used (acetone-dissolved essential oils and absolute hydrosols). We pipetted 100 µL methanol (Kemika, Zagreb, Croatia) and 200 µL standard and/or sample into each well. We prepared serial dilutions of standard and samples by pipetting 100 µL from the first row with a multichannel pipette into the wells in the second row and so on to the last row, where 100 µL of the solution is ejected after mixing. In the first column, in 96-well plates, a blank sample was always added. For EOs, the acetone and methanolic solution were used as blank and for hydrosols, water and methanolic solution were used as blank. The reaction starts by adding 100 µL of a methanolic solution of DPPH (200 µM) to each well. The initial absorbance at 517 nm was measured immediately, using MetOH as blank value. After 60 min incubation, the absorbance was measured again and the percentage of DPPH inhibition was calculated according to the following formula by Yen and Duh [56]:

$$\% \text{ inhibition} = ((AC(0) - AA(t))/AC(0)) \times 100,$$

where AC(0) is the absorbance of the control at t = 0 min, and AA(t) is the absorbance of the antioxidant at t = 1 h. All measurements were performed in triplicate. The standard curve was generated by plotting the percentage of inhibition of standard with corresponding µmol of Trolox. From the standard curve, results for EOs were expressed as µmol of Trolox per g of EO and for hydrosols as µmol of

Trolox per g of absolute hydrosol. Because of the data from other relevant literature we also expressed IC50 values for EOs expressed in mg/mL.

For both antioxidant methods, we also tested the activity of the most abundant compound in EOs using the same method as for total oils. We used pure standard of the hexahydropharnesyl acetone (BOC Sciences, Shirley, NY, USA), the concentration of the solution was 1 mg per g of acetone and was then further diluted in phosphate buffer up to the concentration of 100 µg/g.

3.7. Statistical Analysis

Statistical analysis was performed in GraphPad Prism Version 9. All data are expressed as mean ± SEM ($n \geq 3$). The statistical significance for free volatile compounds, total phenolic compounds and antioxidant activity was assessed by multiple *t*-test, $p < 0.05$. Statistical tests were performed separately for lipophilic (essential oils) and hydrophilic fractions (hydrosols).

4. Conclusions

Veronica (family Plantaginaceae) is a very large and versatile plant genus, rich in biologically active specialized metabolites. In this research, free volatile compounds and their antioxidant activity were studied for the first time in *V. saturejoides* ssp. *saturejoides* from the two localities. The main volatile compounds in essential oils were hexahydrofarnesyl acetone and hexadecanoic acid. The main volatile compound in hydrosols was trans-1(7),8-p-mentadien-2-ol. For the genus *Veronic*a, the most studied specialized metabolites are iridoid glycosides, because of their importance in phylogeny and phenolic compounds, which have a great antioxidant activity. Our research showed that essential oils exhibit stronger antioxidant activity than hydrosols. Comparing the results from phenolic compounds in dry material of other investigated *Veronica* species with the results of phenolic and volatile compounds of this *Veronica* species, we can conclude that essential oils and hydrosols are valuable sources of potentially biologically active compounds. Free volatile compounds of the genus *Veronica* are only just being studied and we believe that such compounds have great potential as antioxidants in pharmacy and food technology.

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References

1. Barreira, J.C.M.; Dias, M.I.; Zivkovic, J.; Stojkovic, D.; Sokovic, M.; Santos-Buelga, C.; Ferreira, I.C.F.R. Phenolic profiling of *Veronica* spp. grown in mountain, urban and sandy soil environments. *Food Chem.* **2014**, *163*, 275–283. [CrossRef]
2. Albach, D.C.; Martínez-Ortega, M.M.; Chase, M.W. *Veronica*: Parallel morphological evolution and phylogeography in the Mediterranean. *Plant Syst. Evol.* **2004**, *246*, 177–194. [CrossRef]
3. Flora Croatica Database. Available online: http://hirc.botanic.hr/fcd (accessed on 20 January 2019).
4. Albach, D.C.; Von Sternburg, M.; Scalone, R.; Bardy, K.E. Phylogenetic analysis and differentiation of *Veronica* subgenus *Stenocarpon* in the Balkan Peninsula. *Bot. J. Linn. Soc.* **2009**, *159*, 616–636. [CrossRef]
5. Silic, C. *Endemic Plants (Endemične Biljke)*, 1st ed.; "Svjetlost", OOUR Zavod za udžbenike i nastavna sredstva: Sarajevo, Bosnia and Herzegovina; Zavod za udžbenike i nastavna sredstva: Belgrade, Serbia, 1984.
6. Albach, D.C.; Grayer, R.J.; Kite, G.C.; Jensen, S.R. *Veronica*: Acylated flavone glycosides as chemosystematic markers. *Biochem. Syst. Ecol.* **2005**, *33*, 1167–1177. [CrossRef]

7. Albach, D.C.; Jensen, S.R.; Özgökce, F.; Grayer, R.J. *Veronica*: Chemical characters for the support of phylogenetic relationships based on nuclear ribosomal and plastid DNA sequence data. *Biochem. Syst. Ecol.* **2005**, *33*, 1087–1106. [CrossRef]
8. Ganzera, M.; Strum, S. Recent advances on HPLC/MS in medicinal plant analysis—An update covering 2011–2016. *J. Pharm. Biomed. Anal.* **2018**, *147*, 211–233. [CrossRef]
9. Dunkic, V.; Kosalec, I.; Kosir, I.J.; Potocnik, T.; Cerenak, A.; Zovko Koncic, M.; Vitali, D.; Dragojevic Muller, I.; Kopricanec, M.; Bezic, N.; et al. Antioxidant and antimicrobial properties of *Veronica spicata* L. (Plantaginaceae). *Curr. Drug Targets* **2015**, *16*, 1660–1670. [CrossRef]
10. Zivkovic, J.C.; Barreira, J.C.M.; Savikin, K.P.; Alimpic, A.Z.; Stojkovic, D.S.; Dias, M.I.; Santos-Buelga, C.; Duletic-Lausevic, S.N.; Ferreira, I.C.F.R. Chemical profiling and assessment of antineurodegenerative and antioxidant properties of *Veronica teucrium* L. and *Veronica jacquinii* Baumg. *Chem. Biodivers.* **2017**, *14*, e1700167. [CrossRef]
11. Sharifi-Rad, M.; Tayeboon, G.S.; Sharifi-Rad, J.; Iriti, M.; Varoni, E.M.; Razazi, S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts. *Cell. Mol. Biol.* **2016**, *62*, 80–85. [CrossRef]
12. Hamedi, A.; Pasdaran, A.; Zebarjad, Z.; Moein, M. A Survey on Chemical Constituents and Indications of Aromatic Waters Soft Drinks (Hydrosols) Used in Persian Nutrition Culture and Folk Medicine for Neurological Disorders and Mental Health. *J. Evid. -Based Complement Altern. Med.* **2017**, *22*, 744–752. [CrossRef]
13. Rajeswara Rao, B.R. Hydrosols and Water-Soluble Essential Oils: Their Medicinal and Biological Properties. In *Recent Progress in Medicinal Plants*, 1st ed.; Govil, J.N., Bhattacharya, S., Eds.; Studium Press LLC: New York, NY, USA, 2013; Volume 36, pp. 119–141.
14. Thomford, N.E.; Senthebane, D.A.; Rowe, A.; Munro, D.; Seele, P.; Maroyi, A.; Dzobo, K. Natural products for drug discovery in the 21st century: Innovations for novel drug discovery. *Int. J. Mol. Sci.* **2018**, *19*, 1578. [CrossRef] [PubMed]
15. Balogun, O.S.; Ajayi, O.S.; Adeleke, A.J. Hexahydrofarnesyl Acetone-Rich Extractives from *Hildegardia barteri*. *J. Herbs Spices Med. Plants* **2017**, *23*, 393–400. [CrossRef]
16. Silva, R.O.; Sousa, F.B.; Damasceno, S.R.; Carvalho, N.S.; Silva, V.G.; Oliveira, F.R.; Sousa, D.P.; Aragão, K.S.; Barbosa, A.L.; Freitas, R.M.; et al. Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. *Fundam. Clin. Pharmacol.* **2014**, *28*, 455–464. [CrossRef] [PubMed]
17. Ertas, A.; Boga, M.; Kizil, M.; Ceken, B.; Goren, A.C.; Hasimi, N.; Demirci, S.; Topcu, G.; Kolak, U. Chemical profile and biological activities of *Veronica thymoides* subsp. pseudocinerea. *Pharm. Biol.* **2015**, *53*, 334–339. [CrossRef]
18. Saikia, D.; Parihar, S.; Chanda, D.; Ojha, S.; Kumar, J.K.; Chanotiya, C.S.; Shanker, K.; Negi, A.S. Antitubercular potential of some semisynthetic analogues of phytol. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 508–512. [CrossRef]
19. Costa, J.P.; Ferreira, P.B.; De Sousa, D.P.; Jordan, J.; Freitas, R.M. Anticonvulsant effect of phytol in a pilocarpine model in mice. *Neurosci. Lett.* **2012**, *523*, 115–118. [CrossRef]
20. Pongprayoon, U.; Baeckström, P.; Jacobsson, U.; Lindström, M.; Bohlin, L. Antispasmodic Activity of β-Damascenone and E-Phytol Isolated from *Ipomoea pes-caprae*. *Planta Med.* **1992**, *58*, 19–21. [CrossRef]
21. Pejin, B.; Kojic, V.; Bogdanovic, G. An insight into the cytotoxic activity of phytol at in vitro conditions. *Nat. Prod. Res.* **2014**, *28*, 2053–2056. [CrossRef]
22. Tan, K.H.; Nishida, R. Methyl eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. *J. Insect Sci.* **2012**, *12*, 56. [CrossRef]
23. Li, F. Analysis of chemical constituents of essential oil in *Veronica linariifolia* by gas chromatography-mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 822–825.
24. Çelik, E.; Yuvali Çelik, G.; Meysun, A. Essential Oil Composition and Antibacterial Activity of Some Plant Species. *J. Appl. Biol. Sci.* **2010**, *1*, 45–48.
25. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4.1 ed.; Allured Publishing: Carol Stream, IL, USA, 2017.
26. Kurer, G.A. *Kutikularfalten und Protuberanzen an Haaren und Epidermen*; Druck, Gebr: Leemann, Germany, 1917.
27. Kraehmer, H.; Baur, P. *Weed Anatomy*; Wiley-Blackwell: Oxford, UK, 2013.
28. Haratym, W.; Weryszko-Chmielewska, E. Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare* L. (Lamiaceae). *Flora* **2017**, *231*, 11–20. [CrossRef]

29. Giuliani, C.; Pellegrino, R.; Tirillini, B.; Bini, L.M. Micromorphological and chemical characterisation of *Stachys recta* L. subsp. *serpentini* (Fiori) Arrigoni in comparison to *Stachys recta* L. subsp. *recta* (Lamiaceae). *Flora Morphol. Distrib. Funct. Ecol. Plants* **2008**, *203*, 376–385. [CrossRef]
30. Dunkic, V.; Kremer, D.; Grubesic, R.J.; Rodriguez, J.V.; Ballian, D.; Bogunic, F.; Stesevic, D.; Kosalec, I.; Bezic, N.; Stabentheiner, E. Micromorphological and phytochemical traits of four *Clinopodium* L. species (Lamiaceae). *S. Afr. J. Bot.* **2017**, *111*, 232–241. [CrossRef]
31. Kristen, U.; Lockhausen, J. The leaf glands of *Veronica beccabunga* L.: Ultrastructure and a possible pathway of secretion. *Israel J. Bot.* **1985**, *34*, 147–156. [CrossRef]
32. Bilusic Vundac, V.; Stabentheiner, E.; Brantner, A.; Plazibat, M. Morphology and distribution of trichomes on leaves in seven Croatian taxa of the genus *Stachys* (Lamiaceae). *Phyton* **2011**, *51*, 161–176.
33. Hanlidou, E.; Kokkini, S.; Bosabalidis, A.M.; Bessière, J.M. Glandular trichomes and essential oil constituents of *Calamintha menthifolia* (Lamiaceae). *Plant Syst. Evol.* **1991**, *177*, 17–26. [CrossRef]
34. Kremer, D.; Stabentheiner, E.; Dunkic, V.; Müller, I.D.; Vujic, L.; Kosalec, I.; Ballian, D.; Bogunic, F.; Bezic, N. Micromorphological and chemotaxonomical traits of *Micromeria croatica* (Pers.) Schott. *Chem. Biodivers.* **2012**, *9*, 755–768. [CrossRef]
35. Quideau, S.; Deffieux, D.; Douat-Casassus, C.; Pouységu, L. Plant polyphenols: Chemical properties, biological activities and synthesis. *Angew. Chem. -Int. Ed.* **2011**, *50*, 586–621. [CrossRef]
36. Harput, U.S.; Karadeniz, A.; Genç, Y.; Saraçoglu, I. Comparative Bioactivity Studies on Four *Veronica* species. *FABAD J. Pharm. Sci.* **2009**, *34*, 67–72.
37. Beara, I.; Zivkovic, J.; Lesjak, M.; Nadpal, J.; Savikin, K.; Maksimovic, Z.A.; Jankovic, T. Phenolic profile and anti-inflammatory activity of three *Veronica* species. *Ind. Crop. Prod.* **2015**, *63*, 276–280. [CrossRef]
38. Stojkovic, D.S.; Zivkovic, J.; Sokovic, M.; Glamoclija, J.; Ferreira, I.C.F.R.; Jankovic, T.; Maksimovic, Z. Antibacterial activity of *Veronica montana* L. extract and of protocatechuic acid incorporated in a food system. *Food Chem. Toxicol.* **2013**, *55*, 209–213. [CrossRef] [PubMed]
39. Aazza, S.; Lyoussi, B.; Miguel, M.G. Antioxidant activity of some Morrocan hydrosols. *J. Med. Plants Res.* **2011**, *5*, 6688–6696. [CrossRef]
40. Bentayeb, K.; Vera, P.; Rubio, K.; Nerín, K. The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils. *Food Chem.* **2014**, *148*, 204–208. [CrossRef]
41. Viuda-Martos, M.; Navajas, Y.R.; Zapata, E.S.; Fernández-López, J.; Perez-Alvarez, J.A. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour Fragr. J.* **2010**, *25*, 13–19. [CrossRef]
42. Harput, U.Ş.; Genç, Y.; Khan, N.; Saracoglu, I. Radical scavenging effects of different *Veronica* species. *Rec. Nat. Prod.* **2011**, *5*, 100–107.
43. Mocan, A.; Vodnar, D.C.; Vlase, L.; Crișan, O.; Gheldiu, A.M.; Crișan, G. Phytochemical characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from Romania and their antioxidant and antimicrobial properties. *Int. J. Mol. Sci.* **2015**, *16*, 21109–21127. [CrossRef]
44. Kwak, J.H.; Kim, H.J.; Lee, K.H.; Kang, S.C.; Zee, O.P. Antioxidative iridoid glycosides and phenolic compounds from *Veronica peregrina*. *Arch. Pharm. Res.* **2009**, *32*, 207–213. [CrossRef]
45. Amorati, R.; Foti, M.C.; Valgimigli, L. Antioxidant activity of essential oils. *J. Agric. Food Chem.* **2013**, *61*, 10835–10847. [CrossRef]
46. Kremer, D.; Dragojevic, I.; Dunkic, V.; Vitali Cepo, D.; Stabentheiner, E.; Oberländer, A.; Bezic, N.; Kosalec, I. Chemical traits and antimicrobial activity of endemic *Teucrium arduini* L. from Mt Biokovo (Croatia). *Cent. Eur. J. Biol.* **2012**, *7*, 941–947. [CrossRef]
47. Payne, W.W. A glossary of plant hair thermonology. *Brittonia* **1978**, *30*, 239–255. [CrossRef]
48. Jukic Spika, M.; Zanetić, M.; Kraljic, K.; Soldo, B.; Ljubenkov, I.; Politeo, O.; Skevin, D. Differentiation between Unfiltered and Filtered Oblica and Leccino cv. Virgin Olive Oils. *J. Food Sci.* **2019**, *84*, 877–885. [CrossRef] [PubMed]
49. Rodríguez, J.V.; Grubesic, R.J.; Kremer, D.; Kokot, V. Quality Assessment of Two Spectrophotometric Procedures for Polyphenol Determination and Application in *Moltkia petraea* Species. *J. Chin. Chem. Soc.* **2016**, *63*, 677–687. [CrossRef]
50. Schneider, G. To determine the tannin with casein. Zur Bestimmung der Gerbstroffe mit Casein. *Arch. Pharm.* **1976**, *309*, 38–44. [CrossRef] [PubMed]

51. Christ, B.; Mueller, K.H. On the serial determination of the content of flavonol derivatives in drugs. *Arch. Pharm. ber Dtsch. Pharm. Ges.* **1960**, *293*, 1033–1042. [CrossRef]
52. *European Pharmacopoeia*, 9th ed.; Council of Europe: Strasbourg, France, 2016; Volume 2.
53. Fredotovic, Z.; Sprung, M.; Soldo, B.; Ljubenkov, I.; Budic-Leto, I.; Bilusic, T.; Cikes-Culic, V.; Puizina, J. Chemical composition and biological activity of *Allium cepa* L. and *Allium* × *cornutum* (Clementi ex Visiani 1842) methanolic extracts. *Molecules* **2017**, *22*, 448. [CrossRef]
54. Payet, B.; Sing, A.S.C.; Smadja, J. Assessment of antioxidant activity of cane brown sugars by ABTS and DPPH radical scavenging assays: Determination of their polyphenolic and volatile constituents. *J. Agric. Food Chem.* **2005**, *53*, 10074–10079. [CrossRef]
55. Mensor, L.L.; Menezes, F.S.; Leitão, G.G.; Reis, A.S.; dos Santos, T.C.; Coube, C.S.; Leitão, S.G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother. Res.* **2001**, *15*, 127–130. [CrossRef]
56. Yen, G.C.; Duh, P.D. Scavenging Effect of Methanolic Extracts of Peanuts Hulls on Free-Radical and Active-Oxygen Species. *J. Agric. Food Chem.* **1994**, *42*, 629–632. [CrossRef]

3.2. Wild Species *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides* — Biological Potential of Free Volatiles

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Wild Species *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides*—Biological Potential of Free Volatiles

Marija Nazlić [1], Željana Fredotović [1], Elma Vuko [1], Lea Fabijanić [2], Dario Kremer [3], Edith Stabentheiner [4], Mirko Ruščić [1] and Valerija Dunkić [1,*]

[1] Faculty of Science, University of Split, Ruđera Boškovića 33, HR-21000 Split, Croatia; mnazlic@pmfst.hr (M.N.); zfredotov@pmfst.hr (Ž.F.); elma@pmfst.hr (E.V.); mrus@pmfst.hr (M.R.)
[2] Bartul Kašić High School, Ante Starčevića 9, HR-23250 Pag, Croatia; lea.fabijanic2@skole.hr
[3] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia; dkremer@pharma.hr
[4] Institute of Biology, Karl-Franzens University, Schubertstrasse 51, A-8010 Graz, Austria; edith.stabentheiner@uni-graz.at
* Correspondence: dunkic@pmfst.hr; Tel.: +385-21-619-296

Abstract: Extracts from plants of the genus *Veronica* have been and continue to be used in traditional medicine to treat various diseases throughout the world. Although often considered a weed, many scientific reports demonstrate that these plants are a source of valuable biologically active compounds and their potential for horticulture should be investigated and considered. In this study, free volatile compounds of essential oils (EO) and hydrosols were extracted from two species: *Veronica officinalis*, which is most commonly used in traditional medicine, and *Veronica saturejoides*, an endemic plant that could be obtained by cultivation in horticulture. Volatiles were analyzed by gas chromatography coupled with mass spectrometry (GC, GC-MS). The most abundant compounds identified in the EOs were hexadecanoic acid in *V. officinalis* EO and caryophyllene oxide in *V. saturejoides* EO. The hydrosols were characterized by a high abundance of caryophyllene oxide in *V. saturejoides* hydrosol and of *p*-vinyl guaiacol for *V. officinalis* hydrosol. The sites where the volatile compounds are synthesized and stored were analyzed using SEM (Scanning Electron Microscopy); glandular and non-glandular trichomes were detected on stems, leaves and the calyx. Further, to investigate the activity of the free volatile compounds against pathogens, isolated volatile compounds were tested on the antiphytoviral activity against tobacco mosaic virus (TMV) infection. The hydrosols of both investigated species and EO of *V. officinalis* showed significant antiphytoviral activity. To further investigate the biological potential of these extracts they were also tested for their antiproliferative and antioxidant activities. The results indicate that these compounds are a valuable source of potential anticancerogenic agents that should be investigated in future studies. The presented results are the first report of hydrosol and EO activity against TMV infection, suggesting that these extracts from *Veronica* species may be useful as natural-based antiphytoviral agents.

Keywords: antioxidant activity; antiphytoviral activity; antiproliferative activity; essential oil; free volatile compounds; GC-MS; hydrosol; speedwell

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1. Introduction

The genus *Veronica* of the family Plantaginaceae grows predominantly in temperate Northern Hemisphere regions, with a smaller number of species growing in Southern Hemisphere regions and in Australia [1,2]. The many species of this plant family, about 450, show the great ecological adaptability of the genus *Veronica*. Species of this genus grow in wet and dry habitats, as well as in the marine belt and mountains [3].

The studied species *Veronica officinalis* L. (Figure 1b) (common speedwell) and *Veronica saturejoides Vis.* ssp. *saturejoids* (Figure 1a) (savory leafed speedwell) grow on Dinaric Massif (Republic of Croatia). Both species are perennial herbaceous plants with small

attractive violet flowers. *Veronica officinalis* is slightly taller (10–50 cm) and has longer leaves (1.5–5 cm) than *V. saturejoides* ssp. *saturejoides* (stem length 10–30 cm, leaf length 1–3 cm). The latter species is a plant endemic to Croatia [4].

(**a**) (**b**)

Figure 1. Investigated plants in their natural habitat: (**a**) *Veronica saturejoides* ssp. *saturejoides* on Dinara Mountain; (**b**) *Veronica officinalis* L. on Kamešnica Mountain.

In general, the study of chemical compounds produced by wild plants is extremely important because these compounds ultimately affect not only the plant in which they are found, but also indirectly other plants in the vicinity as well as the environment as a whole [5]. These compounds are important factors in plant adaptation to abiotic stresses. Moreover, neighboring plants detect other plant volatiles as 'messages' about herbivore or pathogen attacks, and consequently adapt their metabolism responses [6]. Plants of the genus *Veronica* are used in traditional medicines in countries around the world, which sparked interest in the studying these plants in terms of their chemical composition and biological activity. Many different biological activities of the various extracts have been reported in recent studies [7,8]. For example, methanolic and ethyl-acetate extracts of *V. spicata* were tested for antimicrobial activity and MIC values were between 1.25 and 5.00 mg/mL. This plant extract has also shown substantial antioxidant activity, especially the methanol extracts of flowers and leaves with IC_{50} and DPPH values of 8.21 µg/mL and 8.69 µg/mL, respectively [9]. Ertas et al. reported antimicrobial activity for phenolic extracts of *Veronica thymoides* subsp. *pseudocinerea* and the MIC value determined was 31.25 mg/mL for methanol extract against *Escherichia coli* [10].

Veronica officinalis, which is the subject of this study, is traditionally used in the medicine of Balkan peoples. Mocan et al. reported antioxidant activity for ethanol extracts of phenolic compounds for *V. officinalis* to be 157.99 ± 6.58 mg Trolox equivalents/g d.w [11]. Valyova et al. also confirmed antioxidant activity of phenolic extracts for the *V. officinalis* in their study [12]. The aerial parts of speedwells are used to treat liver, spleen, kidney, and bladder diseases, as well as snakebites wound healing, skin lesions, eczema and ulcers [9,10,13].

Green prevention strategies and means of virus control of natural origin are particularly important today to support organic production and the replacement of synthetic chemicals with biologically based antivirals. This approach to modern agriculture is still

under development, and one of our goals is to find harmless and environmentally friendly antiphytoviral agents. Tobacco mosaic virus (TMV) is a model virus in plant virology and a very important pathogen for agricultural crops, causing significant yield losses. TMV belongs to the positive-strand RNA viruses and encodes two proteins that function as replicases (molecular weights 126 kDa and 183 kDa), a movement protein (30 kDa) that facilitates virus movement between host cells, and a coat protein (17.5 kDa) that plays an important role in virion formation [14]. Various plant products such as essential oils, flavonoids, polyphenols and organic, alcoholic and aqueous extracts from plants and natural compounds from other organisms such as fungal metabolites, have been used against a number of plant diseases caused by viruses, phytopathogenic bacteria, fungi, plant parasitic nematodes and parasitic and non-parasitic weeds [15–19] with the aim of finding natural-based products useful for plant protection against pathogens. Some of our previous studies and studies by other authors describe the activity of plant volatiles as natural antiphytoviral compounds [19–27]. Since this activity of Plantaginaceae family volatiles has not been tested so far, we investigated the antiphytoviral activity of both essential oil and hydrosol of *V. officinalis* and *V. saturejoides*, with the aim of increasing the knowledge about the antiphytoviral activity of essential oils and especially hydrosols, which have been very little studied in this regard. To further investigate the biological potential, the antiproliferative and antioxidant activity of these extracts was tested.

Thus, the aim of this study was to investigate the composition of the volatile compounds of these two species from the EOs and the water residues (hydrosols) and to discuss differences and similarities in composition. In addition, the biological potential of these extracts for possible use in horticulture were evaluated either to cultivate and thus preserve the endemic species *V. saturejoides* or to promote the cultivation of the wild plant *V. officinalis*. To our knowledge, this is the first report on the EO and hydrosol composition of *V. officinalis* and *V. saturejoides* from this site, and the first report on their biological activity, as well as on the micromorphology of the trichomes of *V. officinalis*.

2. Materials and Methods

2.1. Plant Material

Plant material for *V. officinalis* and *V. saturejoides* was collected from the sites at Dinara Massif (Table 1, Figure 1). The voucher specimens were deposited in the Faculty of Science Herbarium (PMFST-HR), University of Split, Croatia. For GC and GC-MS analyses, the samples were air dried in a single layer and protected from direct sunlight for three weeks. The dried plant material was kept in the dark in double paper bags labeled with the sample number and stored in a dry place.

Table 1. Locations of the plant material collection (for volatile compounds extraction).

	Locality	Coordinates	Altitude a.s.l. (m)	Date of Collection
Veronica officinalis L.	Kamešnica Mountain	43°43′03.1″ N 16°50′34.1″ E	1445 m	July 2021
Veronica saturejoides Vis. ssp. *saturejoides*	Dinara Mountain	44°3′2.6″ N; 16°22′52.9″ E	1650 m	July 2021

Above ground plant parts (stems, leaves, and flowers) of seven plants of *V. officinalis* were fixed in solution made from formalin, 96% ethanol, acetic acid and water in a volume ratio of 5:70:5:20. After three days, the samples were transferred to 70% ethanol and stored in the refrigerator. The study of the trichomes for *V. saturejoides* was carried out in the previous work [28].

2.2. GC and GC-MS Analyses

Dried above ground parts (50 g) for each sample were hydrodistilled for 3 h in a Clevenger-type apparatus. Three separate extractions were performed for each species and volatile compounds were collected from the lipophilic layer (pentane/diethyl ether) and water residues (hydrosols). Both phases were analyzed by gas chromatography (GC), and gas chromatography and mass spectrometry (GC-MS). GC was performed by gas chromatograph (model 3900, Varian Inc., Lake Forest, CA, USA) that is supplied with a flame ionization detector (FID), mass spectrometer (model 2100T; Varian Inc., Palo Alto, CA, USA), non-polar capillary column VF-5ms (30 m × 0.25 mm inside diameter, coating thickness 0.25 μm, Palo Alto, CA, USA) and polar capillary column CP-Wax 52 CB (30 m × 0.25 mm i.d., coating thickness 0.25 μm, Palo Alto, CA, USA). The chromatographic conditions for the analysis of lipophilic fraction (essential oils) were: FID detector temperature 300 °C, injector temperature 250 °C. The gas carrier was helium at 1 mL min^{-1}. The conditions for the VF-5ms column were: temperature 60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1}, and held isothermal for 25 min. Conditions for the CP Wax 52 column were: temperature 70 °C isothermal for 5 min, and then increased to 240 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. The injected volume was 2 μL and the split ratio was 1:20. The MS conditions were: ion source temperature 200 °C, ionization voltage 70 eV, mass scan range 40–350 mass units [9,28]. The individual peaks for all samples were identified by comparison of their retention indices of *n*-alkanes to those of authentic samples and literature [29,30], comparing it to our libraries from previous work [9,28] and to other previously published material for *Veronica* species [10,12,31,32]. The results are given as the mean of three extractions with standard deviation.

2.3. Micromorphological Traits

For micromorphological investigations internodes of stem, central parts of leaves (left or right from the main axis), and the whole calyx were used. Before the scanning electron microscopic (SEM) analysis, the samples were transferred from 70% (*v*/*v*) ethanol to 70% (*v*/*v*) acetone. Thereafter, the samples were subjected to a dehydration procedure, i.e., they were transferred from 70% (*v*/*v*) to 90% (*v*/*v*), and 100% (*v*/*v*) acetone. Each transfer from one to another concentration of acetone was repeated twice and lasted 15 min each at room temperature. With the use of CO_2 as the drying medium, the dehydrated samples were subjected to a process of critical point drying in CPD030 device (Bal-tec, Balzers, Liechtenstein). The samples thus prepared were sputter coated with gold in Sputter Coater device (Agar Scientific Ltd., Essex, UK) and researched under the scanning electron microscope XL30 ESEM (FEI Company, Eindhoven, Netherlands) with an acceleration voltage of 20 kV in high vacuum mode [33]. Common terminology [34] was used to describe the trichomes.

2.4. Antiphytoviral Activity

2.4.1. Virus and Plant Hosts

Leaves of *Nicotiana tabacum* L. cv. Samsun systemically infected with tobacco mosaic virus were used to prepare the virus inoculum as described by Vuko et al. [25]. Leaves of the native host *Datura stramonium* L. were pollinated with carborundum (Sigma-Aldrich, St. Louis, MO, USA) before virus inoculation, and the inoculum was diluted with inoculation buffer to obtain 5–30 lesions per inoculated leaf. Experiments were carried out when the plants had reached the 5–6 leaf stage. Care was taken to ensure that the experimental plants were as uniform in size as possible.

2.4.2. Antiphytoviral Activity Assay

Essential oil (final concentration adjusted to 500 ppm) or hydrosol (undiluted) were applied as a spray solution to the leaves of local host plants for three consecutive days prior to virus inoculation. Plants were then rubbed with the virus inoculum and treated once with the same spray solutions immediately after inoculation. The antiviral activity of

the essential oil and hydrosol was evaluated by the percentage inhibition of the number of local lesions on the leaves of the treated and control plants as described by Vuko et al. [25].

2.5. Cell Culture

Three cancer cell lines, Cervical Cancer Cell Line (HeLa), Human Colon Cancer Cell Line (HCT116) and Human Osteosarcoma Cell Line (U2OS), were grown in an incubator under humidified conditions with 5% CO_2 and 37 °C, in a Dulbecco's modified Eagle's medium (DMEM, EuroClone, Milan, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), and 1% antibiotics (penicillin and streptomycin, EuroClone).

2.6. Cell Proliferation Assay

The antiproliferative capacity of EO of *V. officinalis* L. and *V. saturejoides* Vis. ssp. *saturejoides* was determined on HeLa, HCT116 and U2OS cancer cells using the MTS-based CellTiter 96® Aqueous Assay (Promega). Cells were grown in an incubator at 37 °C and 5% CO_2 until they reached 80% confluence. They were counted using a handheld automated cell counter (Sceptre, Merck), and 5000 cells/well were seeded in 96-well plates with a serial dilution of EOs and hydrosols. The cells were then cultured for an additional 48 h. Thereafter, 20 µL MTS tetrazolium reagent (Promega) was added to each well and left in the incubator for an additional 3 h. Then, absorbance was measured at 490 nm using a microplate reader (Bio-Tek, EL808). IC_{50} values were calculated from three independent experiments using GraFit 6 data analysis software (Erithacus, East Grinstead, UK).

2.7. Antioxidant Activity of Essential Oils and Hydrosols

2.7.1. ORAC

The assay was performed in a Perkin–Elmer LS55 spectrofluorimeter, using 96-well white polystyrene microtiter plates (Porvair Sciences, Leatherhead, UK) following a method described by Fredotović et al. [35], with some adjustments based on different extracts. Hydrophilic assay was performed for hydrosols and lipophilic assay for EOs. Adjustments were made for the EO antioxidant assay. EOs were dissolved in acetone (10 mg in 1 mL acetone). The EO acetone dilutions were further dissolved 40× and 80× in the phosphate buffer for the experiments. For the hydrosol assays total undiluted hydrosols were used. All measurements were performed in triplicate according to the method described in Nazlić et al. [28].

2.7.2. DPPH

The antioxidant capacity of the extracts was determined using the DPPH method already described by Mensor et al. and Payet et al. [36,37] and adapted to tested plant extracts. Plant extracts as described in the ORAC method were used (acetone-dissolved essential oils and absolute hydrosols) for the assay. An amount of 100 µL of methanol (Kemika, Zagreb, Croatia) and 200 µL of sample was pipetted into each well. Serial dilutions of samples were prepared by pipetting 100 µL from the first row with a multichannel pipette into the wells in the second row and so on to the last row, where 100 µL of the solution was ejected after mixing. In the first column, in 96-well plates, a blank sample was always added. For EOs, the acetone and methanolic solution were used as blank and for hydrosols, water and methanolic solution were used as blank. The calculation and presentation of the results were performed according to the method described in the previous research by Nazlić et al. [28].

2.8. Statistical Analyses

Statistical analysis was performed in GraphPad Prism Version 9. All data are presented as mean ± SD ($n \geq 3$). Statistical significance for free volatile compounds and antioxidant activity was assessed by multiple *t*-test, $p < 0.05$. Statistical tests were performed separately for lipophilic (essential oils) and hydrophilic fractions (hydrosols).

3. Results and Discussion

3.1. Composition of Free Volatile Components

The chemical composition of the free volatile compounds of *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides* is shown in Table 2. The free volatile compounds were analyzed from the lipophilic-essential oil and aqueous-hydrosol fractions.

Table 2. Chemical composition of the essential oil (EO) and hydrosol (H) from the aerial parts of *Veronica officinalis* and *Veronica saturejoides* Vis. ssp. *saturejoides* (Plantaginaceae).

			V. officinalis V. saturejoides		***V. officinalis V. saturejoides***	
			Essential Oils		**Hydrosols**	
Component	**RI[1]**	**RI[2]**	**Mean ± SD (%)**		**Mean ± SD (%)**	
Monoterpene hydrocarbons			NI	NI	0.15	0.89
α-Thujene	924	1012	NI	NI	NI	0.23 ± 0.01
α-Pinene *	935	1017	NI	NI	NI	0.66 ± 0.03
β -Phellandrene	1002	1195	NI	NI	0.15 ± 0.03	-
Oxygenated monoterpenes			1.36	12.39	13.48	15.93
1,8-Cineole	1026	1210	NI	NI	0.76 ± 0.01	NI
γ-Terpinene	1057	1225	NI	NI	2.61 ± 0.01	NI
Linalool	1095	1506	0.52 ± 0.01 [b]	0.89 ± 0.05 [a]	4.72 ± 0.01 [a]	1.39 ± 0.01 [b]
allo-Ocimene	1128	1390	0.22 ± 0.15	NI	NI	NI
Camphor	1151	1499	NI	NI	0.72 ± 0.01	NI
Borneol	1176	1719	NI	NI	1.59 ± 0.01	NI
α-Terpineol	1184	1660	NI	0.88 ± 0.01	3.08 ± 0.03 [a]	2.79 ± 0.01 [b]
trans-1(7),8-p-Mentadien-2-ol	1187	1803	0.62 ± 0.01	10.62 ± 0.02	NI	11.75 ± 0.01
Sesquiterpene hydrocarbons			13.32	12.93	7.76	7.6
α-Copaene	1377	1484	0.78 ± 0.01	NI	NI	NI
E-Caryophyllene *	1424	1585	6.78 ± 0.04 [b]	7.63 ± 0.01 [a]	0.56 ± 0.01 [b]	1.39 ± 0.01 [a]
allo-Aromadendrene	1465	1662	1.32 ± 0.01 [a]	0.87 ± 0.01 [b]	2.59 ± 0.01 [b]	3.87 ± 0.01 [a]
β-Chamigrene	1478	1724	NI	NI	0.27 ± 0.12	NI
Germacrene D	1481	1692	NI	2.61 ± 0.01	NI	2.34 ± 0.01
δ-Selinene	1492	1756	3.32 ± 0.01	NI	4.34 ± 0.01	NI
δ-Cadinene	1517	1745	1.12 ± 0.01 [b]	1.82 ± 0.01 [a]	NI	NI
Oxygenated sesquiterpenes			6.54	34.04	15.74	25.82
Spathulenol	1577	2101	NI	1.8 ± 0.01	5.25 ± 0.01	NI
Caryophyllene oxide *	1581	1955	1.42 ± 0.01 [b]	23.65 ± 0.01 [a]	7.52 ± 0.01 [b]	21.28 ± 0.01 [a]
Viridiflorol	1592	2099	NI	NI	NI	1.53 ± 0.01
γ-Eudesmol	1632	2175	1.82 ± 0.01 [a]	0.2 ± 0.03 [b]	NI	0.23 ± 0.01
α-Muurolol	1645	2181	3.30 ± 0.01 [b]	7.86 ± 0.01 [a]	2.38 ± 0.01	2.37 ± 0.01
α-Cadinol	1655	2208	NI	NI	NI	0.41 ± 0.01
α-Bisabolol	1685	2210	NI	NI	0.59 ± 0.03	NI
α-Bisabolol oxide	1748	2511	NI	0.53 ± 0.01	NI	NI
Phenolic compounds			0.27	10.23	11.59	23.31
p-Vinyl guaiacol	1313	2156	0.27 ± 0.03	NI	11.59 ± 0.01	NI
Methyl eugenol	1403	2005	NI	10.23 ± 0.01	NI	23.31 ± 0.01
Phenylpropanoids			6.02	1.41	1.75	2.61
Z-Methyl isoeugenol	1451	2045	1.46 ± 0.03 [a]	1.41 ± 0.01 [b]	1.75 ± 0.01 [b]	2.16 ± 0.03 [a]
Benzyl benzoate	1760	2613	4.56 ± 0.01	NI	NI	NI

Table 2. *Cont.*

			V. officinalis V. saturejoides		*V. officinalis V. saturejoides*	
			Essential Oils		**Hydrosols**	
Component	**RI[1]**	**RI[2]**	**Mean ± SD (%)**		**Mean ± SD (%)**	
Fatty aldehyde, acids, alcohol, esters and ketones			56.56	22.33	36.84	14.58
Isopentyl acetate	863	1127	NI	6.24 ± 0.01	0.24 ± 0.09 [b]	0.59 ± 0.01 [a]
Benzaldehyde	952	1508	0.98 ± 0.01 [b]	4.29 ± 0.01 [a]	9.25 ± 0.01 [a]	8.87 ± 0.01 [b]
Benzene acetaldehyde	1036	1633	0.48 ± 0.01	NI	4.75 ± 0.01 [a]	3.68 ± 0.01 [b]
n-Nonanal	1100	1389	0.89 ± 0.02	NI	1.68 ± 0.01 [a]	0.28 ± 0.01 [b]
Hexyl 2-methyl butanoate	1233	1425	NI	NI	0.21 ± 0.01 [a]	0.15 ± 0.03 [b]
Menthyl acetate	1294	1550	NI	NI	1.58 ± 0.02	NI
(E)-β-Damascone	1384	1819	NI	NI	6.69 ± 0.01	NI
β-Ionone	1487	1935	17.88 ± 0.01	NI	10.74 ± 0.01	NI
Hexahydrofarnesyl acetone	1839	2113	13.92 ± 0.01 [a]	6.18 ± 0.01 [b]	0.25 ± 0.03 [b]	0.39 ± 0.03 [a]
1-Hexadecanol	1874	2371	1.79 ± 0.01 [a]	1.51 ± 0.03 [b]	NI	NI
Hexadecanoic acid	1959	2912	20.62 ± 0.01 [a]	4.11 ± 0.01 [b]	1.45 ± 0.01 [a]	0.62 ± 0.02 [b]
Hydrocarbons			10.86	1.12	1.78	0.49
Eicosane *	2000	2000	4.21 ± 0.01 [a]	1.12 ± 0.01 [b]	1.51 ± 0.04	NI
Heneicosane *	2100	2100	0.98 ± 0.17	NI	0.27 ± 0.01	NI
Docosane *	2200	2200	2.13 ± 0.01	NI	NI	NI
Tricosane *	2300	2300	NI	NI	NI	NI
Tetracosane *	2400	2400	0.83 ± 0.01	NI	NI	0.49 ± 0.01
Pentacosane *	2500	2500	2.71 ± 0.04	NI	NI	NI
Total identification (%)			**94.93**	**95.45**	**89.09**	**91.23**

Retention indices (RIs) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) and CPWax 52 (RI^2). Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [29] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; * co-injection with reference compounds; NI, not identified; SD, standard deviation of triplicate analysis. Significant differences for every volatile compound present in both species were determined using multiple *t*-test. [a,b] Mean values in the same row with different superscript letters indicate a statistically significant difference between data from two species ($p < 0.05$); [a] for the higher abundance of the compound, [b] for the lesser abundance of the compound.

The major compounds in the lipophilic fraction of *V. officinalis* are: hexadecanoic acid (20.62%), β-ionone (17.88%), hexahydrofarnesyl acetone (13.92%) and *E*-caryophyllene (6.78%). These compounds were also identified in the hydrosol fraction of *V. officinalis* but with much lower proportions, except for β-ionone, which has a high proportion of 10.74%. Moreover, the phenolic compound *p*-vinyl guaiacol (11.59%) is the most represented compound in the hydrosol fraction of *V. officinalis*, followed by benzaldehyde (9.25%), caryophyllene oxide (7.52%) and *(E)*-β-damascone (6.69%).

The oxygenated sesquiterpenes are the major group in both fractions of *V. saturejoides* ssp. *saturejoids* (Table 1, Figure 2) with a predominant caryophyllene oxide compound (23.65% in EO and 21.28% in H). Moreover, among the phenolic compounds, only methyl eugenol was identified as the most abundant compound, in the EO-fraction with 20.23% and in the hydrosol fraction it with 23.31%. Among the oxygenated monoterpenes in *Veronica saturejoides* ssp. *saturejoids* in both fractions, *trans*-1(7),8-p-mentadien-2-ol was the most abundant compound (10.62% in EO and 11.75% in H).

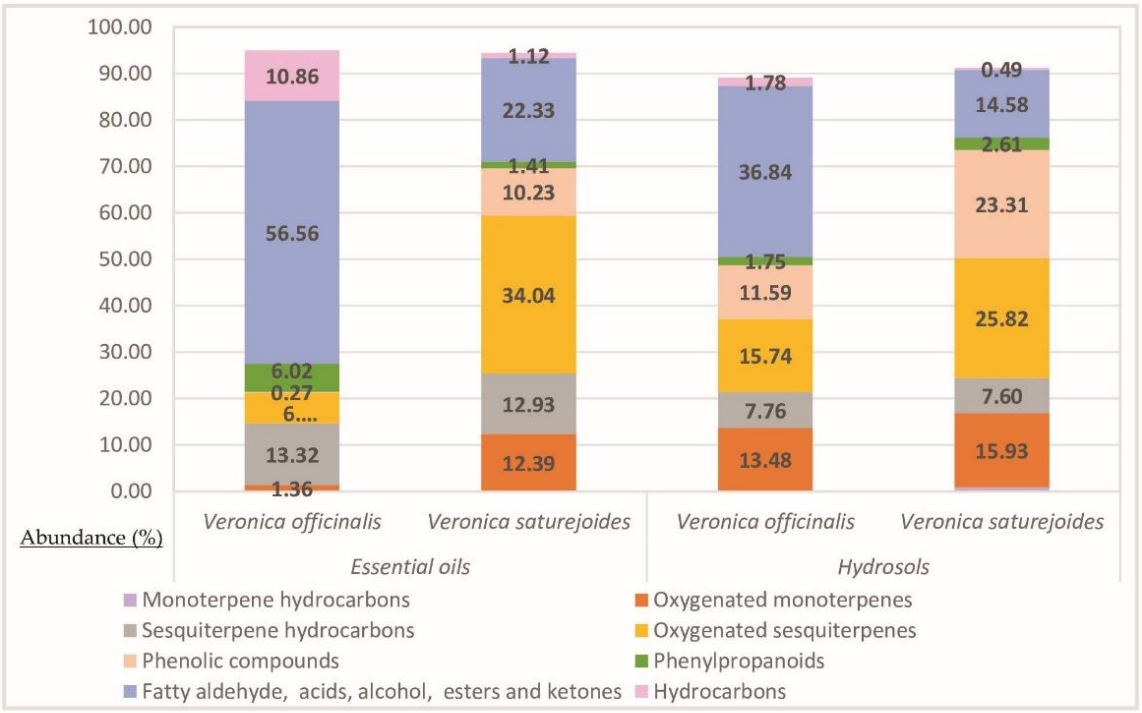

Figure 2. Volatile compounds distribution by categories for all samples of essential oils and hydrosols for the investigated species *V. officinalis* and *V. saturejoides* ssp. *saturejoides*.

In the previous studies, composition of EO and hydrosol for the species *V. saturejoides* ssp. *saturejoides* was analyzed but from two different sites (Kamešnica and Prenj Mountain) [28]. In hydrosols from these two locations *trans*-1(7),8-p-mentadien-2-ol was the most abundant compound, with a percentage of 31.75% for Prenj Sample and 36.63% for the Kamešnica sample. Caryophyllene oxide was also present in both fractions of *V. saturejoides* (essential oil and hydrosol) which is consistent with the results of this current research. The composition of the EO of *V. spicata* was also previously analyzed and the study found that the most abundant compound was phytol (21.13%) [9]. Not many studies have been carried out on the composition of the volatile compounds of other *Veronica* species. Valyova et al. studied extracts of the Bulgarian species *V. officinalis* by GC-MS and identified in ethanol extracts terpinen-4-ol, neophytadiene, hexahydrofarnesyl acetone, vitamin E, phytol and squalene for the first time in the genus *Veronica* [12]. Other research on the EOs of the genus *Veronica* were carried on *V. thymoides* subsp. *pseudocinerea* [10], *V. linariifolia* [31] and *Veronica* sp. [32]. Ertas et al. found that the most abundant constituent of the essential oil from *Veronica thymoides* subsp. *pseudocinerea* was hexatriacontene (21%) which belongs to the hydrocarbon compound group [10]. In another research of the essential oil composition of *Veronica linariifolia* Pall. ex Link the major constituents were cyclohexene (25.83%), β-pinene (11.61%), 1S-α-pinene (10.65%), β-phellandrene (10.49%), β-myrcene (10.42%), and germacrene D (4.99%) (monoterpene and sesquiterpene hydrocarbons) [31]. Çelik et al. studied the essential oils extracted from *Veronica* sp. and found that the main components were mainly linalool (4.18%) and carvacrol (7.28%) [32].

3.2. Glandular and Non-Glandular Trichomes

Glandular trichomes are 'bio-factories' of volatile compounds that are part of EOs. They play an important role in protecting plants from herbivores and pathogens and in attracting pollinators [38]. Two types of glandular trichomes can be seen on the surface of the studied plants of *V. officinalis*: peltate and capitate (Figure 3). Peltate glandular trichomes are composed of a basal cell, a very short stalk cell and a multicellular head with a fairly large subcuticular space (Figure 3c). These trichomes are very rare and they were noticed only on the calyx of *V. officinalis*. Nazlić et al. [28] did not describe peltate trichomes in *Veronica saturejoides* ssp. *saturejoides*. On the other hand, peltate trichomes are common in some other plant species, especially in Lamiaceae [39,40]. Capitate glandular trichomes could be further divided in two subtypes. Subtype 1 (C1) capitate trichomes consist of a stalk cell and two elliptically shaped head cells with a subcuticular space (Figure 3g). They are present on the adaxial and abaxial sides of the leaf, on the calyx and on the stem. Subtype 2 (C2) capitate trichomes are uniseriate, unbranched, multicellular, long and folded at different levels (Figure 3e,h). They consist of several (most often four to five) stalk cells and a head cell with a subcuticular space. They are present on the stem and calyx of *V. officinalis*. Only C1 trichomes are present on the leaf surface and they appear to be denser than on the stem and calyx. C1 trichomes were also observed on the surface of stem, leaves and calyx of *V. saturejoides* ssp. *saturejoides*. On the other hand, the C2 trichomes were not found in *V. saturejoides* ssp. *saturejoides* [28]. According to available data, C1 capitate trichomes were previously observed in *Veronica beccabunga* L. [41]. Comparable, but more or less upright capitate trichomes with a short stalk and two-celled head were observed in *Marrubium vulgare* L. [39,40] and in endemic *Salvia smyrnea* L. from Turkey [39,40].

Non-glandular (NG) trichomes also have a protective function in plants. They can protect plants from herbivores and prevent greater water loss through transpiration [38]. NG trichomes were observed on the calyxes, leaves and stems of *V. officinalis*. According to SEM investigation the NG trichomes are unbranched and uniseriate. It can also be observed that they are short (two-celled) or longer (multicellular) trichomes (Figure 3b,f). These trichomes are folded at different levels. The NG trichomes are denser on the stem surface than on leaves and calyxes (Figure 3). The presence of the same type of NG trichomes was noted before on aerial parts of *V. saturejoides* [28], on the flower parts of *Veronica* sp. [42] and for *V. persica* Poir. [43].

3.3. Antiphytoviral Activity

The numerous biological activities of essential oils known to date and their role in the interaction of plants with their biotic and abiotic environment suggest that these specialized plant metabolites are much more than just plant fragrances. Hydrosols are a by-product of essential oil distillation, making the usability of all products of this process ecologically and biologically desirable. Therefore, the antiphytoviral activity is imposed as a continuation of the study of the biological activities of this genus, especially since this activity of the volatiles of the genus *Veronica* has never been tested before.

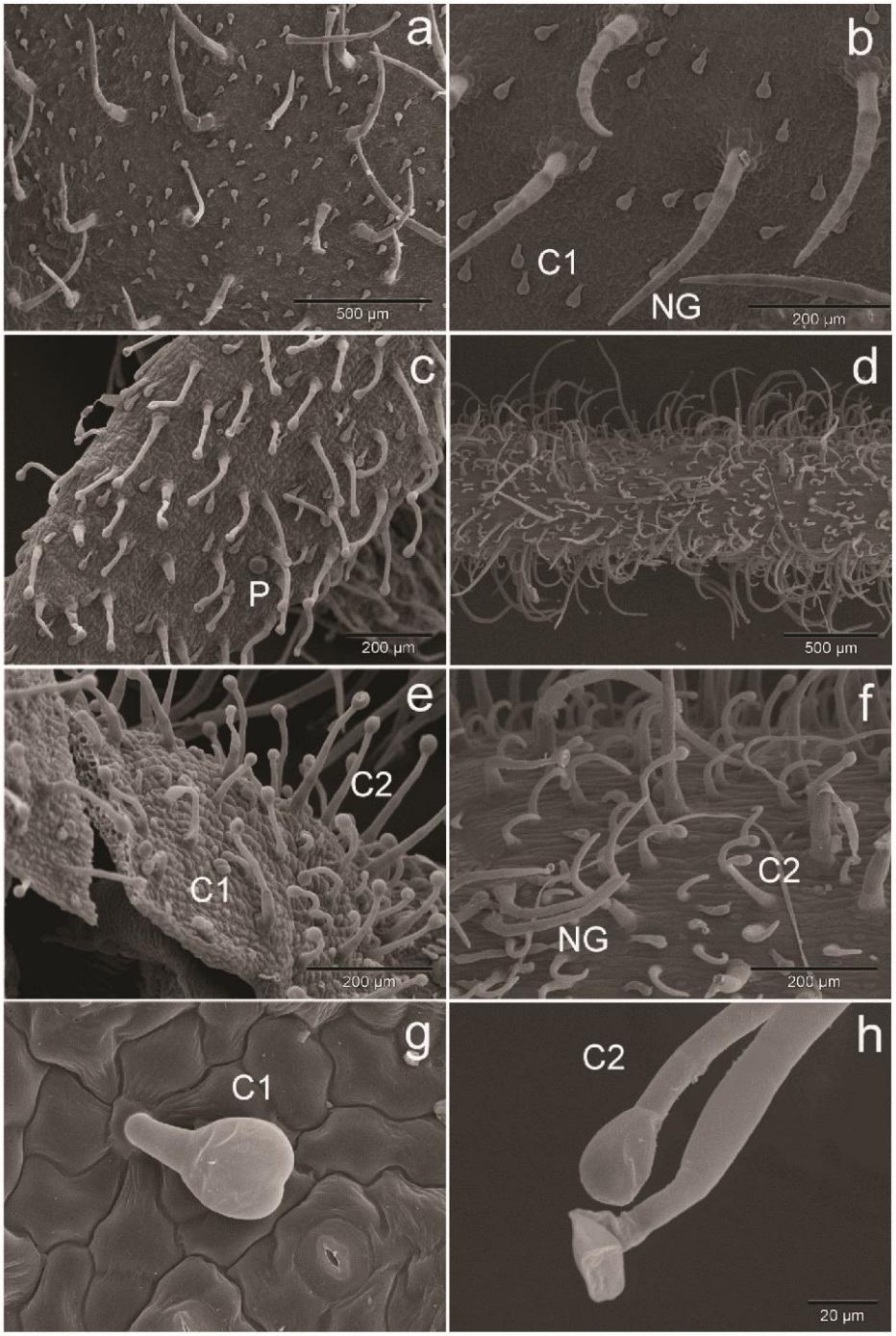

Figure 3. *V. officinalis* SEM micrographs showing different types of trichomes on adaxial (**a**,**g**) and abaxial (**b**) leaf surface, on the sepal (**c**,**e**) and stem (**d**,**f**,**h**). Non-glandular trichomes (NG), peltate trichomes (P), subtype 1 (C1) and subtype 2 (C2) of capitate trichomes.

The results show that plants treated with hydrosol (H) of *V. officinalis* and *V. saturejoides* prior to TMV infection significantly reduced the number of local lesions compared to control plants. In the essential oil (EO) treated plants, the number of local lesions decreased only on the leaves of the plants treated with *V. officinalis* EO (Table 3), while the plants treated with *V. saturejoides* EO in the preliminary experiment developed similar or even more severe infection than the control plants. On the third day post inoculation, the inhibition of lesions on the leaves of the plants treated with *V. officinalis* H and *V. saturejoides* H was 84.69% and 74.11%, respectively, while it was 59.43% on the leaves of the plants treated with *V. officinalis* EO (Figure 4). Until the seventh day after inoculation, the inhibition of local lesions was still pronounced in all treated groups, with 79.09% and 68.38% on the leaves of plants treated with *V. officinalis* H and *V. saturejoides* H, respectively, and 62.70% on the leaves of plants treated with *V. officinalis* EO. We compared these results with the antiphytoviral activity of plant volatiles reported in some of our previous studies and studies by other authors [19–27]. Thus, essential oils isolated from the aromatic species *Satureja montana* ssp. *variegata* and *Teucrium arduini* inhibited TMV infection by 29.2% and 25.7%, and cucumber mosaic virus (CMV) infection by 24.1% and 21.9%, respectively [20]. The essential oils of species of the genus *Micromeria* (*M. graeca*, *M. fruticulosa* and *M. croatica*) showed antiphytoviral activity on plants infected with satellite RNA associated cucumber mosaic virus (satCMV) with an activity of 59.3%, 43.6% and 34.5%, respectively [19,24,44]. The essential oils extracted from four species of the genus *Teucrium* (*T. polium*, *T. flavum*, *T. montanum* and *T. chamaedrys*) were shown to reduce the number of lesions in the host plants infected with CMV, with the essential oil of *T. polium* having the strongest effect with an activity of 41.4% [23]. The essential oils of *Hypericum perforatum* ssp. *veronense*, *Eryngium alpinum* and *E. amethystinum* showed promising antiviral activity rates of 88%, 77.8% and 80.5%, respectively [22,25]. In addition, among the essential oils extracted from 29 indigenous Chinese aromatic plants, the oils of ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass caused more than 50% inhibition of TMV at the concentrations tested [26,27]. In addition to plant extracts, inhibitors of plant viruses derived from metabolites of microbes are also considered as potential alternatives to chemical pesticides [45]. Ningnanmycin isolated from the fermentation broth of *Streptomyces noursei* var. *Xichangensis* exhibits comprehensive antiphytoviral activity and is characterized by increased resistance, excellent efficiency and low toxicity in host plants by acting through expression of pathogenesis-related proteins, increasing salicylic acid biosynthesis and inducing systemic resistance in host plants [46–48].

Table 3. Number of local lesions (LLN) on leaves of treated *Datura stramonium* plants inoculated with tobacco mosaic virus and on leaves of control plants (C) on the third, fifth, and seventh day post inoculation (dpi). Treated plants were sprayed with hydrosol (H) and essential oil (EO) of *V. officinalis* (V.off) or *V. saturejoides* (V.sat) for three consecutive days prior to and once immediately after inoculation.

		LLN		
dpi	**Mean ± SD**		**Mean ± SD**	
3rd	C	5.86 ± 2.39	C	6.55 ± 1.24
	V. off H	0.83 ± 0.31 *	V.off EO	2.57 ± 0.18 *
	V. sat H	1.50 ± 0.62 *	V.sat EO	n.a.
5th	C	8.95 ± 2.95	C	8.39 ± 2.20
	V. off H	1.36 ± 0.58 *	V.off EO	2.75 ± 0.58 *
	V. sat H	2.24 ± 0.63 *	V.sat EO	n.a.
7th	C	10.55 ± 3.62	C	9.67 ± 2.24
	V. off H	2.10 ± 0.87 *	V.off EO	3.39 ± 0.50 *
	V. satH	3.02 ± 0.89 *	V.sat EO	n.a.

SD, standard deviation of triplicate analysis; n.a., no activity; significant differences were determined by *t*-test; * statistically significant differences between control and EO/H treatment data ($p < 0.05$).

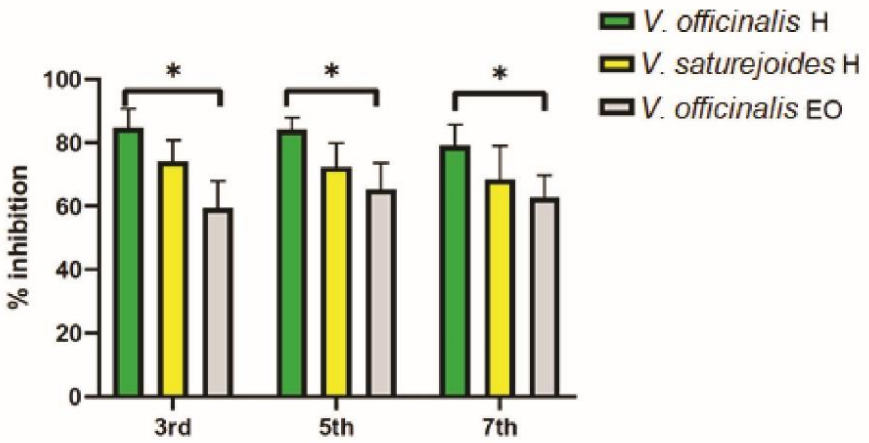

Figure 4. Percentage of inhibition of lesions on leaves of treated *Datura stramonium* plants inoculated with tobacco mosaic virus compared to control plants on the third, fifth and seventh day post inoculation. Treated plants were sprayed with hydrosol (H) and essential oil (EO) of *V. officinalis* or *V. saturejoides* for three consecutive days prior and once immediately after inoculation. Error bars show standard deviation of triplicate analyses; significant differences were determined by *t*- test and marked with * ($p < 0.05$).

This and previous studies dealing with essential oils and hydrosols of aromatic plant species [20–25] show that volatile plant compounds can increase the resistance of plants to viral pathogens. The activity of hydrosol of both *Veronica* species is even more promising than that of essential oils, and the presented results suggest the need for further studies on the antiviral activity of volatiles of *Veronica* species. The activity of the tested extracts is probably related to their chemical composition, where a synergistic effect of the volatiles could activate plant signaling pathways and lead to increased resistance to viral infections. Among the major constituents of the essential oil (Table 2), β-ionone and hexadecanoic acid are abundant in *V. officinalis* EO (17.88% and 20.62%, respectively), in contrast to *V. saturejoides* EO, where these constituents are less abundant (hexadecanoic acid) or absent (β-ionone). In addition, δ-selinene (3.32%), benzyl benzoate (4.56%), docosane (2.13%) and pentacosane (2.71%) are noteworthy, which are also detected only in *V. officinalis* EO, but not in *V. saturejoides* EO. Comparing the essential oil composition with that of hydrosol, α-terpineol, *allo*-aromadendrene, benzene acetaldehyde, *n*-nonanal and hexyl 2-methylbutanoate are listed in similar abundance in both *V. saturejoides* H and *V. officinalis* H, while they are less abundant or absent in *V. saturejoides* EO. The above similarities and differences in the composition of the tested extracts may be useful for future predictions of their efficacy in plant protection.

The reported antiphytoviral activity of *Veronica* species should be analyzed in more detail in the future, including further studies to evaluate their efficacy against viral diseases under field conditions.

3.4. Antiproliferative Activity

In the present study antiproliferative activity of the essential oil (EO) and hydrosol of two *Veronica* species was analyzed. This was of particular interest to us because there are no data in the literature on the antiproliferative activity of *V. saturejoides* and *V. officinalis*. The results of this study showed significant antiproliferative activity of the oil and hydrosol of these species. Essential oil and hydrosol of *V. saturejoides* collected from two different locations (Kamešnica sample (KS*) and Dinara sample (DS)) and *V. officinalis* (Kamešnica sample (KS)) were tested on three cancer cell lines: HeLa (human cervical cancer cell line), HCT116 (colon cancer cell line) and U2OS (osteosarcoma cell line). The best antiprolifera-

tive activity on all three cell lines tested was shown by the essential oil of *V. saturejoides* KS (Figure 5a). A particularly significant difference in the antiproliferative power of *V. saturejoides* KS compared to *V. saturejoides* DS and *V. officinalis* KS was observed in the HeLa and U2OS cell lines. Interestingly, the hydrosol showed slightly different results in contrast to the essential oil (Figure 5b). The hydrosol of *V. saturejoides* KS and DS showed similar antiproliferative effect on all the tested cancer cells. The hydrosol of *V. officinalis* DS showed the strongest antiproliferative activity on HeLa and HCT116 cell lines with IC_{50} values of 64.93% and 45.93%, respectively. *V. saturejoides* KS hydrosol had the best growth inhibition on the U2OS cell line with IC_{50} value of 48.23%.

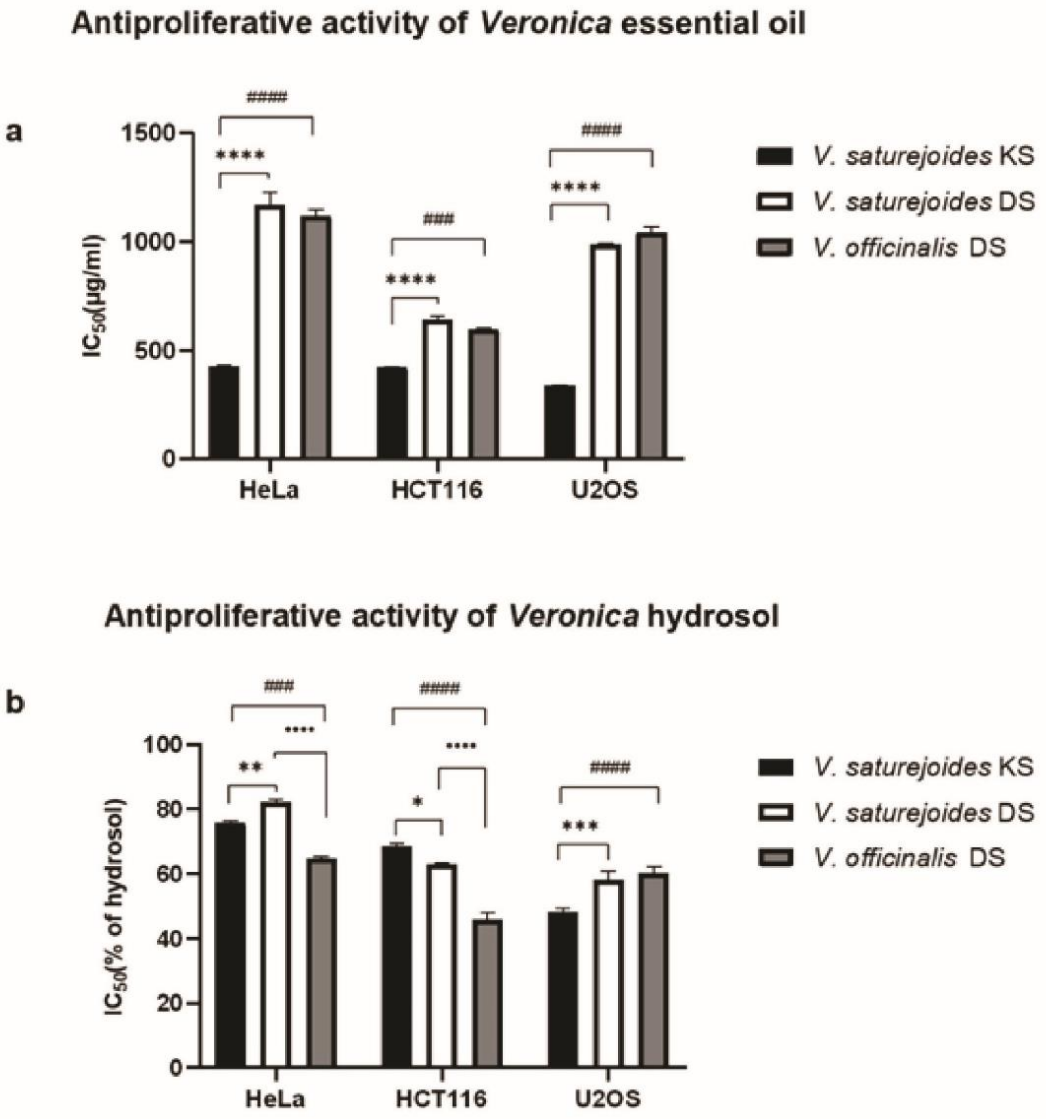

Figure 5. Antiproliferative activity of essential oil (**a**) and hydrosol (**b**) of *V. saturejoides* KS, DS and *V. officinalis* determined by MTS-based cell proliferation test. Results are expressed as the mean of three independent experiments ± SD (presented as error bars). Statistical analyzes were performed using the two-way ANOVA method followed by the Tukey's multiple comparisons test, and significant differences were labeled as * ($^{\#}$ or $^{\bullet}$) $p < 0.05$; ** ($^{\#\#}$ or $^{\bullet\bullet}$) $p < 0.01$; *** ($^{\#\#\#}$ or $^{\bullet\bullet\bullet}$) $p < 0.001$; and **** ($^{\#\#\#\#}$ or $^{\bullet\bullet\bullet\bullet}$) $p < 0.0001$.

Species of the genus *Veronica* have long been used in traditional medicine to treat a number of diseases including cancer, influenza, hernia, cough, respiratory diseases and many others [49–51]. Studies have shown that these plants are a source of secondary metabolites that are largely responsible for their excellent biological activity, such as antimicrobial, antioxidant and anti-inflammatory properties. Although various *Veronica* extracts have been used in folk medicine for the treatment of cancer, very few species have been studied for their cytotoxic and anticancer activity [7]. Previous research on various *Veronica* species has mainly led to the isolation of biologically active compounds such as iridoid glucosides, which have been found to be excellent natural anticancer agents [52–57]. Moreover, methanolic and aqueous extracts of several *Veronica* species have been tested on different cancer cell lines. The cytotoxic activity of methanolic extracts of five *Veronica* species (*V. cymbalaria, V. hederifolia, V. pectinata* var. *glandulosa, V. persica* and *V. polita*) was tested against KB (human epidermoid carcinoma) and B16 (mouse melanoma) cells. All species showed similar dose-dependent effects against tested cells [54]. Methanolic extract of *V. americana* managed to stop the division of the two cancer cells of HF-6 (colon) and PC -3 (prostate) cancer cell lines [58]. Feng et al. showed that flavonoids isolated from *V. sibirica* (Vtfs) induced dose-dependent apoptosis in breast cancer cells MCF-7 with IC_{50} of 42 µg/mL [59]. Aqueous extracts of *V. cuneifolia* subsp. *cuneifolia* and *V. cymbalaria* showed moderate cytotoxic activity against Hep-2 (human epidermoid carcinoma), RD (human rhabdomyosarcoma) and L-20B (transgenic murine L-cells) [60]. Water fractions of the methanolic extracts of *V. persica* and *V. crista-galli* effectively inhibited proliferation of HeLa and MCF-7 cells but showed no toxicity against normal fibroblast cell line [61]. These water fractions contained a high concentration of flavonoids and phenols, which is why they exhibited significant antioxidant activity in addition to their cytotoxic activity.

The results of this study show for the first time that both the essential oil and hydrosol of the species *V. saturejoides* KS, *V. saturejoides* DS and *V. officinalis* KS have significant antiproliferative activity and thus possible chemotherapeutic properties, which is why they deserve further investigation. It is necessary to conduct further studies with the essential oil and hydrosol of *V. saturejoides* and *V. officinalis* and their major constituents on different cancer cell lines to determine the mechanism of antiproliferative action and to evaluate the possibility of their use in medicine and pharmacology.

*Note: Chemical composition of the EO and hydrosol of *V. saturejoides* ssp. *saturejoides* from the Kamešnica location (KS) and plant material information was reported in the previous work by Nazlić et al. [28].

3.5. Antioxidant Activity

The antioxidant activity of the extracted volatile compound was analyzed by two methods, ORAC and DPPH, and the results are presented in the Table 4. The essential oil of *V. saturejoides* ssp. *saturejoides* showed higher antioxidant activity than the EO of *V. officinalis* by both methods. This could be due to a much higher content of oxygenated sesquiterpenes, especially the compound caryophyllene oxide. The hydrosols showed slightly different results. In the DPPH method, hydrosol of *V. saturejoides* showed higher activity, and in the ORAC method, hydrosol of *V. officinalis* showed higher activity (Table 4). Hydrosols of *V. saturejoides* also have higher content of oxygenated sesquiterpenes. Sesquiterpenes have been previously reported to possess numerous biological activities [62,63]. Comparing these results with those previously reported for *V. saturejoides* ssp. *saturejoides* from two other locations (Kamešnica and Prenj Mountain), it can be seen that the antioxidant activity of EO of *V. saturejoides* (Dinara sample) showed similar ORAC activity to the other two previously reported EOs, but the DPPH activity seems to be lower than the EOs of *V. saturejoides* from Kamešnica and Prenj Mountain [28]. The sample from Kamešnica seems to have the highest antioxidant activity, as was also shown for antiproliferative activity. *V. officinalis* EO has lower antioxidant activity than the *V. saturejoides* EO sample from this research report (Dinara sample) and also lower than the two previously reported EOs. Therefore, the conclusion can be drawn that *V. officinalis* EO has lower antioxidant activity

than *V. saturejoides* EO. Comparing the results for the hydrosols, it is evident from Table 4 and previous results for *V. saturejoides* [28] that both the hydrosols of *V. officinalis* and *V. saturejoides* have lower antioxidant activity than the previously reported hydrosols from the two locations of *V. saturejoides*. Comparing these results with reports on other plants, it appears that *V. officinalis* hydrosol has higher antioxidant ORAC activity than *Salvia officinalis* hydrosol [64]. Reports on the antioxidant activity of other extracts (phenolic compounds and iridoid glycosides) from *Veronica* species indicate that these plants have significant antioxidant potential. Valyova et al. studied phenolic extracts of *V. officinalis* and found that ethyl acetate extracts exhibited excellent antioxidant activity based on DPPH and ABTS assays [11]. Harput et al. investigated antioxidant activity for four *Veronica* species (*V. orientalis*, *V. baranetzkii*, *V. officinalis* and *V. peduncularis*) and reported the strongest antioxidant activity for aqueous extracts of *V. officinalis* (IC_{50} 54.19 µg/mL). This could be due to the highest reported total phenolic content being for this species (200.20 mg/g) [65]. In another study, Harput et al. reported antioxidant activity against SO for *V. chamaedrys* (IC_{50}) to be higher than the standards (BHA and quercetin) and the highest IC_{50} value for *V. officinalis* against DPPH radical to be 40.93 µg/mL [66]. Živković et al. investigated antioxidant activity of three *Veronica* species and reported highest antioxidant activity for *V. teucrium* 70% aqueous acetone extracts (IC_{50} 12.58 µg/mL) [67]. Dunkić et al. reported even higher antioxidant activity for methanol extracts of flowers of *V. spicata* with IC_{50} 8.21 µg/mL [9]. Sharifi-Rad et al. reported DPPH antioxidant activity for methanol extract of aboveground parts of *V. persica* to be IC_{50} 30 µg/mL. [68]. All these results show that speedwells should be further researched for their in vivo antioxidant activities or for potential usage in food preservations.

Table 4. Antioxidant potential of *V. officinalis* and *V. saturejoides* ssp. *saturejoides* of the essential oil and hydrosol determined by ORAC and DPPH method.

	Essential Oils (Mean ± SD)	
Antioxidant Assay	***V. officinalis***	***V. saturejoides* ssp. *saturejoides***
ORAC (Trolox eq)	58.75 ± 3.42 [b]	263.29 ± 4.89 [a]
DPPH (% inhibition)	15.51 ± 1.67	19.11 ± 4.09
DPPH (IC 50)	31.34 ± 2.91 [b]	15.99 ± 4.17 [a]
	Hydrosols (mean ± SD)	
Antioxidant Assay	*V. officinalis*	*V. saturejoides* ssp. *saturejoides*
ORAC (Trolox eq)	0.307 ± 0.011	0.258 ± 0.013
DPPH (% inhibition)	27.74 ± 0.77 [b]	38.39 ± 5.83 [a]

ORAC (oxygen radical absorbance capacity) results for EOs expressed as µmol of Trolox equivalents (TE) per g of EO (10 mg/mL) and for hydrosols as µmol of Trolox equivalents (TE) per g of the total (undiluted) tested hydrosol sample; DPPH, results are expressed in percentage of inhibition for the EO concentration of 10 mg per mL of acetone and for the hydrosols in percentage of inhibition for the absolute (undiluted) hydrosol; for the EOs, results are also expressed in IC50 value in mg/mL; SD = standard deviation of triplicate analysis; significant differences were determined using multiple *t*-test. [a,b] Mean values in the same row with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$), [a] for the higher activity, [b] for the lower activity.

4. Conclusions

In this study, the free volatile compounds and their biological activities of two speedwells, *V. officinalis* and *V. saturejoides* ssp. *saturejoides*, from the Dinaric Massif, were analyzed. The most abundant compound in the extracts was caryophyllene oxide for EO and hydrosol of *V. saturejoides*. Hexadecanoic acid and *p*-vinyl guaiacol were the most abundant compounds in the EO and hydrosols of *V. officinalis*, respectively. The volatile compounds extracted from the essential oils and hydrosols showed significant antiphytoviral activity, antiproliferative and antioxidant activity. The results for antiphytoviral activity show that

hydrosol of *V. officinalis* and *V. saturejoides* reduced the number of local lesions on the plants infected with the TMV virus compared to control plants. Essential oil of *V. officinalis* also showed results against TMV infection, while EO of *V. saturejoides* showed no antiphytoviral activity. The best antiproliferative activity on all three cell lines tested was shown by the essential oil of *V. saturejoides* from the Kamešnica location. However, for the hydrosols the results showed that *V. officinalis* had the strongest antiproliferative activity against HeLA and HCT116 cell lines. Antioxidant activity of the essential oil of *V. saturejoides* ssp. *saturejoides* appeared to be higher than the activity of the *V. officinalis* EO by both tested methods. Hydrosols gave different results, as hydrosols from the *V. officinalis* showed higher activity by the ORAC method and hydrosols from *V. saturejoides* showed higher activity by the DPPH method. These results show that plants of the genus *Veronica* are more than just a weed, as they are often considered, but a valuable source of biologically active compounds for human use and for neighboring plants if used in horticulture. Further research could be focused on in vivo studies. Furthermore, it would be valuable to grow these plants from seed in a controlled environment and compare chemical composition and biological activity to the species growing in the wild.

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References

1. Albach, D.C.; Martinez-Ortega, M.M.; Chase, M.W. *Veronica*: Parallel morphological evolution and phylogeography in the Mediterranean. *Plant Syst. Evol.* **2004**, *246*, 177–194. [CrossRef]
2. Albach, D.C.; Martínez-Ortega, M.M.; Fischer, M.A.; Chase, M.W. A new classification of the tribe Veroniceae-problems and a possible solution. *Taxon* **2004**, *53*, 429–452. [CrossRef]
3. Sharifi-Rad, M.; Tayeboon, G.S.; Sharifi-Rad, J.; Iriti, M.; Varoni, E.M.; Razazi, S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts. *Cell. Mol. Biol.* **2016**, *62*, 80–85. [CrossRef]
4. Nikolić, T. Flora Croatica Database. Available online: http://hirc.botanic.hr/fcd (accessed on 22 May 2021).
5. Reynolds, T. The evolution of chemosystematics. *Phytochemistry* **2007**, *68*, 2887–2895. [CrossRef] [PubMed]
6. Loreto, F.; Dicke, M.; Schnitzler, J.P.; Turlings, T.C.J. Plant volatiles and the environment. *Plant Cell Environ.* **2014**, *37*, 1905–1908. [CrossRef]
7. Salehi, B.; Shetty, M.S.; Anil Kumar, N.V.; Živković, J.; Calina, D.; Docea, A.O.; Emamzadeh-Yazdi, S.; Kılıç, C.S.; Goloshvili, T.; Nicola, S.; et al. *Veronica* plants—Drifting from farm to traditional healing, food application, and phytopharmacology. *Molecules* **2019**, *24*, 2454. [CrossRef]
8. Xue, H.; Chen, K.X.; Zhang, L.Q.; Li, Y.M. Review of the Ethnopharmacology, Phytochemistry, and Pharmacology of the Genus *Veronica*. *Am. J. Chin. Med.* **2019**, *47*, 1193–1221. [CrossRef]
9. Dunkić, V.; Kosalec, I.; Kosir, I.; Potočnik, T.; Cerenak, A.; Koncic, M.; Vitali, D.; Muller, I.; Kopricanec, M.; Bezić, N.; et al. Antioxidant and antimicrobial properties of *Veronica spicata* L. (Plantaginaceae). *Curr. Drug Targets* **2015**, *16*, 1660–1670. [CrossRef]

10. Ertas, A.; Boga, M.; Kizil, M.; Ceken, B.; Goren, A.C.; Hasimi, N.; Demirci, S.; Topcu, G.; Kolak, U. Chemical profile and biological activities of *Veronica thymoides* subsp. *pseudocinerea*. *Pharm. Biol.* **2015**, *53*, 334–339. [CrossRef]
11. Mocan, A.; Vodnar, D.C.; Vlase, L.; Crișan, O.; Gheldiu, A.M.; Crișan, G. Phytochemical characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from Romania and their antioxidant and antimicrobial properties. *Int. J. Mol. Sci.* **2015**, *16*, 21109–21127. [CrossRef]
12. Valyova, M.; Hadjimitova, V.; Stoyanov, S.; Ganeva, Y.; Petkov, I. Free radical scavenging activity of extracts from Bulgarian *Veronica officinalis* L. and GC-MS analysis of ethanol extract. *Internet J. Aesthetic Antiaging Med.* **2008**, *2*, 2–6. [CrossRef]
13. Kwak, J.H.; Kim, H.J.; Lee, K.H.; Kang, S.C.; Zee, O.P. Antioxidative iridoid glycosides and phenolic compounds from *Veronica peregrina*. *Arch. Pharm. Res.* **2009**, *32*, 207–213. [CrossRef] [PubMed]
14. Ishibashi, K.; Ishikawa, M. Replication of Tobamovirus RNA. *Annu. Rev. Phytopathol.* **2016**, *54*, 55–78. [CrossRef]
15. Othman, B.A.; Shoman, S.A. Antiphytoviral Activity of the *Plectranthus tenuiflorus* on Some Important Viruses. *Int. J. Agric. Biol.* **2004**, *6*, 844–849.
16. Rusak, G.; Krajačić, M.; Krsnik-Rasol, M.; Gutzeit, H.O. Quercetin influences response in *Nicotiana megalosiphon* infected by satellite-associated cucumber mosaic virus/Quercetin beeinf lusst die Reaktion von *Nikotiana magalosiphon* gegenüber Satelliten-assoziierten Gurkenmosaikvirus-Infektionen. *J. Plant Dis. Prot.* **2007**, *114*, 145–150. [CrossRef]
17. Krcatović, E.; Rusak, G.; Bezić, N.; Krajačić, M. Inhibition of tobacco mosaic virus infection by quercetin and vitexin. *Acta Virol.* **2008**, *52*, 119–124.
18. Moeini, A.; van Reenen, A.; Van Otterlo, W.; Cimmino, A.; Masi, M.; Lavermicocca, P.; Valerio, F.; Immirzi, B.; Santagata, G.; Malinconico, M.; et al. α-costic acid, a plant sesquiterpenoid from *Dittrichia viscosa*, as modifier of Poly (lactic acid) properties: A novel exploitation of the autochthone biomass metabolite for a wholly biodegradable system. *Ind. Crops Prod.* **2020**, *146*, 112134. [CrossRef]
19. Vuko, E.; Dunkić, V.; Bezić, N.; Ruščić, M.; Kremer, D. Chemical Composition and Antiphytoviral Activity of Essential Oil of *Micromeria graeca*. *Nat. Prod. Commun.* **2012**, *7*, 1227–1230. [CrossRef]
20. Dunkić, V.; Bezić, N.; Vuko, E.; Cukrov, D. Antiphytoviral activity of *Satureja montana* L. ssp. variegata (host) P. W. Ball essential oil and phenol compounds on CMV and TMV. *Molecules* **2010**, *15*, 6713–6721. [CrossRef]
21. Dunkić, V.; Bezić, N.; Vuko, E. Antiphytoviral activity of essential oil from endemic species *Teucrium arduini*. *Nat. Prod. Commun.* **2011**, *6*, 1385–1388. [CrossRef]
22. Dunkić, V.; Vuko, E.; Bezić, N.; Kremer, D.; Ruščić, M. Composition and antiviral activity of the essential oils of *Eryngium alpinum* and *E. amethystinum*. *Chem. Biodivers.* **2013**, *10*, 1894–1902. [CrossRef] [PubMed]
23. Bezić, N.; Vuko, E.; Dunkić, V.; Ruščić, M.; Blažević, I.; Burčul, F. Antiphytoviral Activity of Sesquiterpene-Rich Essential Oils from Four Croatian *Teucrium* species. *Molecules* **2011**, *16*, 8119–8129. [CrossRef] [PubMed]
24. Vuko, E.; Rusak, G.; Dunkić, V.; Kremer, D.; Kosalec, I.; Rada, B.; Bezić, N. Inhibition of satellite RNA associated cucumber mosaic virus infection by essential oil of *Micromeria croatica* (Pers.) Schott. *Molecules* **2019**, *24*, 1342. [CrossRef]
25. Vuko, E.; Dunkić, V.; Ruščić, M.; Nazlić, M.; Mandić, N.; Soldo, B.; Šprung, M.; Fredotović, Ž. Chemical Composition and New Biological Activities of Essential Oil and Hydrosol of *Hypericum perforatum* L. ssp. *veronense* (Schrank) H. Lindb. *Plants* **2021**, *10*, 1014. [CrossRef]
26. Lu, M.; Han, Z.; Xu, Y.; Yao, L. In Vitro and In Vivo Anti-Tobacco Mosaic Virus Activities of Essential Oils and Individual Compounds. *J. Microbiol. Biotechnol.* **2013**, *23*, 771–778. [CrossRef]
27. Bishop, C.D. Antiviral Activity of the Essential Oil of *Melaleuca alternifolia* (Maiden amp; Betche) Cheel (Tea Tree) Against Tobacco Mosaic Virus. *J. Essent. Oil Res.* **1995**, *7*, 641–644. [CrossRef]
28. Nazlić, M.; Kremer, D.; Jurišić-Grubešić, R.; Soldo, B.; Vuko, E.; Stabentheiner, E.; Ballian, D.; Bogunić, F.; Dunkić, V. Endemic *Veronica saturejoides* Vis. ssp. *saturejoides*–Chemical Composition and Antioxidant Activity of Free Volatile Compounds. *Plants* **2020**, *9*, 1646. [CrossRef]
29. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing: Carol Stream, IL, USA, 2017; ISBN 978-1-932633-21-4.
30. Babushok, V.I.; Linstrom, P.J.; Zenkevich, I.G. Retention Indices for Frequently Reported Compounds of Plant Essential Oils. *J. Phys. Chem. Ref. Data* **2011**, *40*, 1–47. [CrossRef]
31. Li, F. Analysis of chemical constituents of essential oil in *Veronica linariifolia* by gas chromatography-mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 822–825.
32. Çelik, E.; Çelik, G.Y.; Meysun, A. Essential Oil Composition and Antibacterial Activity of Some Plant Species. *J. Appl. Biol. Sci.* **2010**, *1*, 45–48. [CrossRef]
33. Kremer, D.; Müller, I.D.; Dunkić, V.; Vitali, D.; Stabentheiner, E.; Oberländer, A.; Bezić, N.; Kosalec, I. Chemical traits and antimicrobial activity of endemic *Teucrium arduini* L. from Mt Biokovo (Croatia). *Cent. Eur. J. Biol.* **2012**, *7*, 941–947. [CrossRef]
34. Payne, W.W. A glossary of plant hair thermonology. *Brittonia* **1978**, *30*, 239–255. [CrossRef]
35. Fredotović, Ž.; Šprung, M.; Soldo, B.; Ljubenkov, I.; Budić-Leto, I.; Bilušić, T.; Cikeš-Čulić, V.; Puizina, J. Chemical composition and biological activity of *Allium cepa* L. and *Allium* × *cornutum* (Clementi ex Visiani 1842) methanolic extracts. *Molecules* **2017**, *22*, 448. [CrossRef]
36. Mensor, L.L.; Menezes, F.S.; Leitão, G.G.; Reis, A.S.; dos Santos, T.C.; Coube, C.S.; Leitão, S.G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phyther. Res.* **2001**, *15*, 127–130. [CrossRef]

37. Payet, B.; Sing, A.S.C.; Smadja, J. Assessment of antioxidant activity of cane brown sugars by ABTS and DPPH radical scavenging assays: Determination of their polyphenolic and volatile constituents. *J. Agric. Food Chem.* **2005**, *53*, 10074–10079. [CrossRef]
38. Hazzoumi, Z.; Moustakime, Y.; Joutei, K.A. Essential Oil and Glandular Hairs: Diversity and Roles. In *Essential Oils—Oils of Nature*; El-Shemy, H.A., Ed.; IntechOpen: London, UK, 2019; pp. 1–16.
39. Haratym, W.; Weryszko-Chmielewska, E. Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare* L. (Lamiaceae). *Flora Morphol. Distrib. Funct. Ecol. Plants* **2017**, *231*, 11–20. [CrossRef]
40. Baran, P.; Aktaş, K.; Özdemir, C. Structural investigation of the glandular trichomes of endemic *Salvia smyrnea* L. *S. Afr. J. Bot.* **2010**, *76*, 572–578. [CrossRef]
41. Kristen, U.; Lockhausen, J. The leaf glands of *Veronica beccabunga* L.: Ultrastructure and a possible pathway of secretion. *Isr. J. Plant Sci.* **2015**, *34*, 147–156. [CrossRef]
42. Kurer, G.A. *Kutikularfalten und Protuberanzen an Haaren und Epidermen*; Druck, Gebr. Leeman: Dresden, Germany, 1917.
43. Kraehmer, H.; Baur, P. *Weed Anatomy*; Wiley-Blackwell: Hoboken, NJ, USA, 2013; ISBN 978-0-470-65986-1.
44. Ruščić, M.; Vuko, E.; Bezić, N.; Kremer, D.; Dunkić, V. Croatian *Micromeria fruticulosa*-essential oil composition and antiphytoviral activity. *Nat. Prod. Commun.* **2017**, *12*, 1133–1135. [CrossRef]
45. An, M.; Zhou, T.; Guo, Y.; Zhao, X.; Wu, Y. Molecular Regulation of Host Defense Responses Mediated by Biological Anti-TMV Agent Ningnanmycin. *Viruses* **2019**, *11*, 815. [CrossRef] [PubMed]
46. Han, Y.; Luo, Y.; Qin, S.; Xi, L.; Wan, B.; Du, L. Induction of systemic resistance against tobacco mosaic virus by Ningnanmycin in tobacco. *Pestic. Biochem. Physiol.* **2014**, *111*, 14–18. [CrossRef] [PubMed]
47. Fan, H.; Song, B.; Bhadury, P.S.; Jin, L.; Hu, D.; Yang, S. Antiviral activity and mechanism of action of novel thiourea containing chiral phosphonate on tobacco mosaic virus. *Int. J. Mol. Sci.* **2011**, *12*, 4522–4535. [CrossRef] [PubMed]
48. Gao, D.; Wang, D.; Chen, K.; Huang, M.; Xie, X.; Li, X. Activation of biochemical factors in CMV-infected tobacco by ningnanmycin. *Pestic. Biochem. Physiol.* **2019**, *156*, 116–122. [CrossRef] [PubMed]
49. Tomassini, L.; Brkic, D.; Serafini, M.; Nicoletti, M. Constituents of *Veronica hederifolia* and *Veronica polita*. *Fitoterapia* **1995**, *66*, 382.
50. Su, B.N.; Zhu, Q.X.; Jia, Z.J. Aquaticol, a novel bis-sesquiterpene from *Veronica anagallis-aquatica*. *Tetrahedron Lett.* **1999**, *40*, 357–358. [CrossRef]
51. Graham, J.G.; Quinn, M.L.; Fabricant, D.S.; Farnsworth, N.R. Plants used against cancer—An extension of the work of Jonathan Hartwell. *J. Ethnopharmacol.* **2000**, *73*, 347–377. [CrossRef]
52. Chari, V.M.; Grayer-Barkmeijer, R.J.; Harborne, J.B.; Osterdahl, B.-G. An acylated allose-containing 8-hydroxyflavone glycoside from *Veronica filiformis*. *Phytochemistry* **1981**, *20*, 1977–1979. [CrossRef]
53. Taskova, R.; Handjieva, N.; Peev, D.; Popov, S. Iridoid glucosides from three *Veronica* species. *Phytochemistry* **1998**, *49*, 1323–1327. [CrossRef]
54. Harput, U.S.; Saracoglu, I.; Inoue, M.; Ogihara, Y. Anti-inflammatory and cytotoxic activities of five *Veronica* species. *Biol. Pharm. Bull.* **2002**, *25*, 483–486. [CrossRef]
55. Saracoglu, I.; Harput, U.S.; Inoue, M.; Ogihara, Y. New phenylethanoid glycosides from *Veronica pectinata* var *glandulosa* and their free radical scavenging activities. *Chem. Pharm. Bull.* **2002**, *50*, 665–668. [CrossRef]
56. Saracoğlu, I.; Harput-Hudaverdi, U.Ş.; Nagatsu, A.; Varel, M. Cytotoxic Activity and Structural Aspects of Iridoids Isolated from *Veronica thymoides* subsp. *pseudocinerea*. In Proceedings of the Natural Products with Pharmaceutical, Nutraceutical, Cosmetic, and Agrochemical Interest, 7th Joint Meeting of AFERP, ASP, GA, and PSE (49th Annual Meeting of the American Society of Pharmacognosy), Athens, Greece, 8 July 2008; p. 1046.
57. Saracoğlu, I.; Harput-Hudaverdi, U.Ş. In vitro Anticancer Activity and Structure-Activity Relationships of Iridoid Glucosides. In Proceedings of the 9th International Symposium on Pharmaceutical Sciences, Ankara, Turkey, 23–26 June 2009.
58. Moreno-Escobar, J.A.; Bazalda, S.; Villarreal, M.L.; Bonilla-Barbosa, J.R.; Mendoza, S.; Rodríguez-López, V. Cytotoxic and antioxidant activities of selected Lamiales species from Mexico. *Pharm. Biol.* **2011**, *49*, 1243–1248. [CrossRef] [PubMed]
59. Feng, K.; Jiang, R.; Sun, L.W. Studies on the anti-tumor activity in vitro of the flavonoid extract in round leaf speedwell. In Proceedings of the First International Conference on Cellular, Molecular Biology, Biophysics and Bioengineering, Lushan, China, 17–18 September 2010.
60. Saracoglu, I.; Oztunca, F.H.; Nagatsu, A.; Harput, U.S. Iridoid content and biological activities of *Veronica cuneifolia* subsp. *cuneifolia* and *V. cymbalaria*. *Pharm. Biol.* **2011**, *49*, 1150–1157. [CrossRef] [PubMed]
61. Mohadjerani, M.; Asadollahi, S. *Veronica crista-galli* Steven and *Veronica persica* Poir. as anticancer and antioxidant plants in-vitro. *Trends Phytochem. Res.* **2019**, *3*, 61–66. [CrossRef]
62. Assaeed, A.; Elshamy, A.; El Gendy, A.E.N.; Dar, B.; Al-Rowaily, S.; Abd-ElGawad, A. Sesquiterpenes-rich essential oil from above ground parts of *Pulicaria somalensis* exhibited antioxidant activity and allelopathic effect on weeds. *Agronomy* **2020**, *10*, 399. [CrossRef]
63. Chappell, J.; Coates, R.M. Sesquiterpenes. *Compr. Nat. Prod. II Chem. Biol.* **2010**, *1*, 609–641. [CrossRef]
64. Aazza, S.; Lyoussi, B.; Miguel, M. Antioxidant activity of some Morrocan hydrosols. *J. Med. Plants Res.* **2011**, *5*, 6688–6696. [CrossRef]
65. Harput, U.S.; Saracoglu, I.; Genc, Y. Comparative Bioactivity Studies on Four *Veronica* species. *Fabad J. Pharm. Sci.* **2009**, *34*, 67–72.
66. Harput, U.Ş.; Genç, Y.; Khan, N.; Saracoglu, I. Radical scavenging effects of different *Veronica* species. *Rec. Nat. Prod.* **2011**, *5*, 100–107.

67. Živković, J.; Ćebović, T.; Maksimović, Z. In vivo and in vitro antioxidant effects of three *Veronica* species. *Cent. Eur. J. Biol.* **2012**, *7*, 559–568. [CrossRef]
68. Sharifi-Rad, J.; Sharifi-Rad, M.; Salehi, B.; Iriti, M.; Roointan, A.; Mnayer, D.; Soltani-Nejad, A.; Afshari, A. In vitro and in vivo assessment of free radical scavenging and antioxidant activities of *Veronica persic*a Poir. *Cell. Mol. Biol.* **2018**, *64*, 57–64. [CrossRef]

3.3. Free Volatile Compounds of *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. and Their Biological Activity

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Free Volatile Compounds of *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. and Their Biological Activity

Marija Nazlić [1], Željana Fredotović [1], Elma Vuko [1], Nenad Vuletić [1], Ivica Ljubenkov [1], Dario Kremer [2], Renata Jurišić Grubešić [3], Edith Stabentheiner [4], Marko Randić [5] and Valerija Dunkić [1,*]

[1] Faculty of Science, University of Split, Ruđera Boškovića 33, HR-21000 Split, Croatia; mnazlic@pmfst.hr (M.N.); zfredotov@pmfst.hr (Ž.F.); elma@pmfst.hr (E.V.); nenov@pmfst.hr (N.V.); iljubenk@pmfst.hr (I.L.)
[2] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia; dkremer@pharma.hr
[3] Faculty of Medicine, University of Rijeka, Braće Branchetta 20, HR-51000 Rijeka, Croatia; renatajg@medri.uniri.hr
[4] Institute of Biology, Karl-Franzens University, Schubertstrasse 51, A-8010 Graz, Austria; edith.stabentheiner@uni-graz.at
[5] Biology Public Institution "Priroda", Grivica 4, HR-51000 Rijeka, Croatia; marko.randic@ju-priroda.hr
* Correspondence: dunkic@pmfst.hr; Tel.: +385-21-619-296

Abstract: The composition of free volatile compounds of essential oils (EO) and hydrosols (Hy) from four different localities of the species *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. were analyzed by gas chromatography coupled with mass spectrometry. In the EOs, the most abundant compounds identified were hexahydrofarnesyl acetone (23.34–52.56%), hexadecanoic acid (palmitic acid, 26.71–58.91%) and octadecanol acetate (0–6.24%). The hydrosols were characterized by high abundance of methyl eugenol (23.35–57.93%), *trans-p*-mentha-1(7),8-dien-2-ol (5.24–7.69%) and thymol (3.48–9.45%). Glandular trichomes were analyzed using SEM (Scanning Electron Microscopy), as they are the sites of synthesis of free volatile compounds. We have detected glandular trichomes, consisting of a one stalk cell and two elliptically shaped head cells, and non-glandular (unbranched, bi-cellular to multicellular) trichomes on stems, leaves and the sepals. Data for volatile compounds from EOs and hydrosols were analyzed using Principal Component Analyses (PCA) to demonstrate variations in the composition of the volatile compounds identified. Isolated samples of EO and hydrosols were analyzed for their antioxidant activity using two methods, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ORAC (Oxygen Radical Absorbance Capacity). The essential oils showed higher antioxidant activity than the hydrosols in ORAC method, but lower activity by the DPPH method. The isolates were also tested for their antiproliferative activity on different types of cancer cells and also on two lines of healthy cells, and the results showed that the extracts were not toxic to the cell lines tested. Total polyphenols, total tannins, total flavonoids and total phenolic acids were also analyzed and determined spectrophotometrically. The free volatile compounds of *Veronica austriaca* ssp. *jacquinii* can be considered as a safe natural product.

Keywords: *Veronica*; volatile compounds; GC-MS; antioxidant activity; DPPH; ORAC; essential oil; hydrosol; antiproliferative activity; PCA

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1. Introduction

Genus *Veronica* L. is divided into 13 subgenera according to the latest classifications and belongs to the Plantaginaceae family (formerly belonged to Scrophulariaceae) [1]. The large number of species (about 500) is an indication of the high ecological adaptability of this genus, in which species grow in wet and dry habitats, by the sea and in the mountains [2,3], but they mostly grow in regions with a Mediterranean precipitation regime [4]. In Croatia there are about 40 species of *Veronica* [5].

Species *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. (Figure 1) is a perennial herbaceous plant with an oblique, cylindrical rhizome. There are numerous stems, or only one, upright, tall (10) 30–70 cm, covered with gray hairs. Leaves are round or broadly lanceolate, pinnately lobed, pinnately or double-pinnately divided. The lobes are narrow, linear or linearly lanceolate, entire or serrated, more or less in-rolled, hairy or rarely glabrous. The leaves of the sterile part of the shoot at the top of the stem are almost whole. The flowers are arranged in 2–4 lateral, opposite racemes. The seeds are flattened, almost round [6].

Figure 1. *Veronica austriaca* ssp. *jacquinii* in its natural habitat (Lika, Croatia).

The literature search revealed that the most studied specialized metabolites of *V. austriaca* ssp. *jacquinii* and the genus *Veronica* in general include iridoid glucosides, phenylethanoids and flavonoid glycosides [7], so this is the first study on the chemical composition of essential oils (EO) and hydrosols from this species. Živković et al. studied phenolic compounds, antioxidative and antineurodegenerative effect of *V. austriaca* ssp. *jacquinii* and their results showed that this species has significant antioxidant and antineurodegenerative activity [8]. Many other *Veronica* species and the biological activity of their specialized metabolites, especially phenolic compounds, were investigated and the experiments showed that they have antioxidant [8–12], antimicrobial [7,11,13,14], cytotoxic and antitumor activities [13]. All these studies were performed with some kind of phenolic extract prepared using different solvents (methanol, ethanol, acetone or water). Hydrosols, that are the subject to this research, are condensed water vapors containing dissolved molecules of essential oils and more water-soluble (polar) volatile compounds [15]. Due to the difference in solubility of volatile compounds in water, the entire composition and hence biological activity of hydrosols differ from that of essential oils. Hydrosols are often discarded after essential oil extraction, but studies show that these waste products are rich in biologically active substances [15–17] so their potential use should be further researched. Essential oils are a very complex mixture of compounds, mainly monoterpenes and sesquiterpenes, and currently more than 3000 essential oils are investigated for their composition but not all of them have shown significant biological activity, only one tenth [18], so it is very important to continue investigating plant volatiles. Due to the natural richness in phenolic compounds and iridoids, species from the genus *Veronica* are widely used in traditional medicine in treating various diseases, influenza, respiratory diseases, cancer and as diuretic [14,19] and this is probably the reason why the interest in

the chemistry and biological activity of the specialized metabolites of this genus began at the beginning of the last century. In the traditional medicine of the Balkan people, the aboveground parts of the species *V. officinalis* are used to treat liver, spleen, kidney, and bladder diseases, for the treatment of snake bites, for wound healing, skin damage, eczema, and ulcers [9,14,20]. Since the biological activity of the volatile compounds of the genus *Veronica* (apart from our earlier research on *V. saturejoides* ssp. *saturejoides*) has not been investigated to date, our team decided to investigate the biological activity of the extracted volatile compounds as well. Therefore, the aim of this study was to investigate the volatile compounds of this species especially with regard to comparison of the volatile components of essential oil and of water residues (hydrosols), as well as discussing differences and similarities in the composition, taking into account the different locations where the plant material was collected. To our knowledge, this is the first report on the composition of the essential oil and hydrosols and their biological activity, as well as on the micromorphology of the trichomes of *V. austriaca* L. ssp. *jacquinii*.

2. Results and Discussion

2.1. Volatile Compounds from Essential Oils and Hydrosols

The isolation and identification of the four samples of essential oils and four related hydrosols were determined by GC and GC-MS; the results are calculated as relative amounts of the compounds expressed in percentage and are reported in the Tables 1 and 2. The compounds are listed in the order of their elution from the column. The yields of EOs from four locations were 0.38%, 0.47%, 0.51% and 0.64%, respectively. The objective was to determine the similarities and differences in the volatile components depending on the population. In all EO samples, more than 90% of the total oil was identified, with hexahydrofarnesyl acetone (23.34–52.56%) and hexadecanoic acid (26.71–58.91%) being the most abundant (Figure S1). These components were also identified in all hydrosol samples, but in much lower percentage ranging from 0.48% to 7.70% when both components are observed. Apart from these two components, *E*-caryophyllene and (*Z*)-methyl isoeugenol are present in all four EO samples and in all four hydrosol samples. The major components in the hydrosols are methyl eugenol (23.35–57.93%), *trans*-*p*-mentha-1(7),8-dien-2-ol (5.24–7.69%) and thymol (3.48–9.45%) (Tables 1 and 2).

In the Figure 2a composition is presented based on the Tables 1 and 2 (according to %—relative peak area). It can be seen from the Figure 2a. that the composition of all the EO samples is characterized by high percentage of "Oxygenated sesquiterpenes" and "Acids, alcohols and esters categories". This is in agreement with our previous results for the EOs of *V. saturejoides* [12]. According to a literature review, GC-MS studies have been performed on only a few *Veronica* species. Ertas et al. found that the most abundant constituent of the essential oil from *Veronica thymoides* subsp. *pseudocinerea* is hexatriacontene (21%) which belongs to the hydrocarbon compound group [9]. In our previous research in the oil of *V. spicata* L., the most abundant compound was the diterpene phytol [14]. In another research of the essential oil composition of *Veronica linariifolia* Pall. ex Link the major constituents were cyclohexene (25.83%), β-pinene (11.61%), 1S-α-pinene (10.65%), β-phellandrene (10.49%), β-myrcene (10.42%), and germacrene D (4.99%) (monoterpene and sesquiterpene hydrocarbons) [21]. Çelik et al. studied the essential oils extracted from *Veronica* sp., and found that the main components were mainly linalool (4.18%) and carvacrol (7.28%) [22]. Valyova et al. studied extracts of the Bulgarian species *Veronica officinalis* by GC-MS and found the following composition in was found in the ethanol extract of the aboveground parts: terpenes, saturated and unsaturated fatty acids and esters, steroids, p-hydroxyphenylethyl alcohol, maltol and loliolide. In terms of content, β-sitosterol was the most abundant. In their study, they also identified terpinen-4-ol, neophytadiene, hexahydrofarnesyl acetone, vitamin E, phytol and squalene for the first time in the genus *Veronica* [23]. As in the above-mentioned research, we have also found hexahydrofarnesyl acetone in both *V. saturejoides* and *V. jacquinii*, and it was in both species the main EO compound. In addition to this compound, hexadecanoic (palmitic) acid was

also found in high percentage in all the EO samples examined. All four samples of the hydrosols of *V. austriaca* ssp. *jacquinii* have similar composition with methyl eugenol as the most abundant compound (Table 2, Figure S2). This compound could be responsible for the higher antioxidant activity of the hydrosol compared to the EO activity (measured with the DPPH method). The main difference between the composition of EO and hydrosol (Figure 2) is that non-polar compounds such as fatty acids and oxygenated sesquiterpenes are the main compound categories in EOs, whereas more polar compounds such as phenolic acids and oxygenated monoterpenes are the most abundant in hydrosols. Other specialized metabolites have also been studied for this species. Živković et al. discovered flavonoids derived from flavones—luteolin and isoscutellarein and found that acteoside is the most dominant compound in *V. austriaca* ssp. *jacquinii*. They also detected quercetin derivates in this species [8].

Table 1. Chemical composition of the essential oil from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii*.

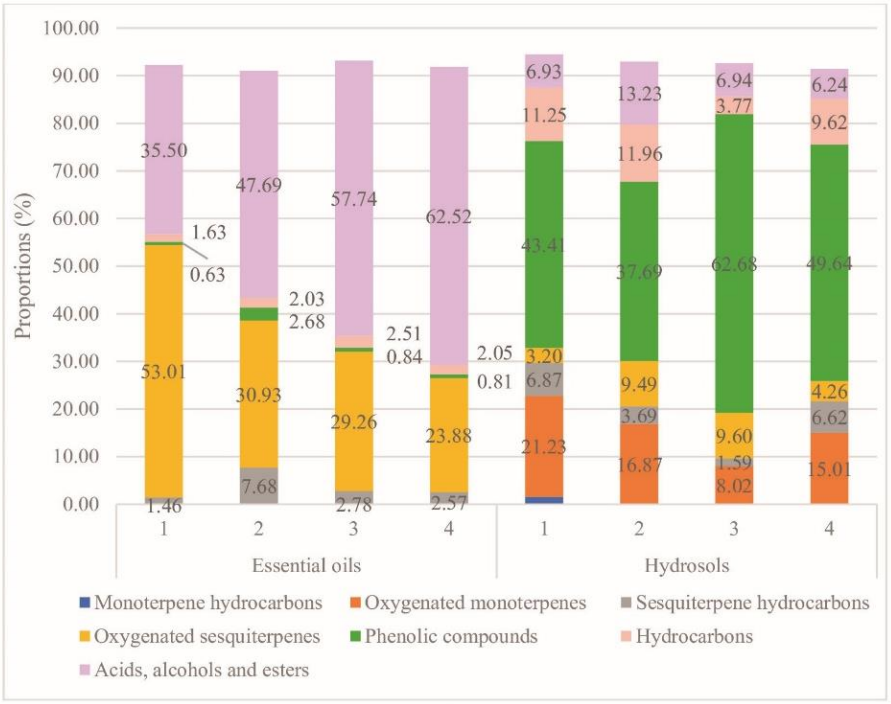
			Mr	St	Br	GJ
Component	**RI[1]**	**RI[2]**	**EO ± SD (%)**	**EO ± SD (%)**	**EO ± SD (%)**	**EO ± SD (%)**
Sesquiterpene hydrocarbons			1.46	7.68	2.78	2.57
E-Caryophyllene *	1424	1585	0.31 ± 0.01 ^{d}	2.35 ± 0.01 ^{a}	1.52 ± 0.01 ^{c}	2.15 ± 0.01 ^{b}
δ-Cadinene	1517	1745	1.15 ± 0.01 ^{b}	1.84 ± 0.01 ^{a}	0.99 ± 0.02 ^{c}	0.42 ± 0.01 ^{d}
allo-Aromadendrene	1465	1662	-	0.88 ± 0.01	-	-
β-Chamigrene	1476	1724	-	-	0.27 ± 0.02	-
Germacrene D	1482	1692	-	2.61 ± 0.05	-	-
Oxygenated sesquiterpenes			53.01	30.93	29.26	23.88
Spathulenol	1577	2101	-	1.84 ± 0.01 ^{a}	0.45 ± 0.01 ^{c}	0.54 ± 0.01 ^{b}
β-Caryophyllene oxide *	1581	1955	0.45 ± 0.01 ^{b}	0.62 ± 0.01 ^{a}	0.48 ± 0.01 ^{b}	-
γ-Eudesmol	1632	2175	-	0.25 ± 0.03	-	-
α-Bisabolol oxide	1748	2511	-	0.37 ± 0.01	-	-
Hexahydrofarnesyl acetone	1839	2113	52.56 ± 0.01 ^{a}	27.85 ± 0.01 ^{c}	28.33 ± 0.01 ^{b}	23.34 ± 0.01 ^{d}
Phenolic compounds			0.63	2.68	0.84	0.81
Methyl eugenol	1403	2005	-	1.26 ± 0.01	-	-
(Z)-Methyl isoeugenol	1451	2070	0.63 ± 0.03 ^{c}	1.42 ± 0.01 ^{a}	0.84 ± 0.01 ^{b}	0.81 ± 0.01 ^{b}
Acids, alcohols and esters			35.5	47.69	57.74	62.52
1-Hexadecanol	1874	2371	-	0.57 ± 0.03	-	-
Hexadecanoic acid	1959	2912	26.71 ± 0.02 ^{d}	47.12 ± 0.01 ^{c}	54.53 ± 0.05 ^{b}	58.91 ± 0.03 ^{a}
Oleic acid	2133	2998	2.35 ± 0.01 ^{a}	-	0.51 ± 0.03 ^{b}	-
Octadecanol acetate	2209	2211	6.24 ± 0.01 ^{a}	-	2.26 ± 0.01 ^{c}	3.61 ± 0.01 ^{b}
1-Heptatriacotanol	2309	2309	-	-	0.44 ± 0.01	-
Hydrocarbons			1.63	2.03	2.51	2.05
Eicosane *	2000	2000	-	1.16 ± 0.01 ^{b}	0.45 ± 0.01 ^{c}	1.24 ±0.04 ^{a}
Heneicosane *	2100	2100	0.53 ± 0.02 ^{a}	0.35 ± 0.01 ^{b}	-	0.53 ± 0.01 ^{a}
Docosane *	2200	2200	0.38 ± 0.01 ^{c}	0.29 ± 0.01 ^{c}	0.81 ± 0.01 ^{a}	-
Tricosane *	2300	2300	-	-	0.63 ± 0.01	-
Tetracosane *	2400	2400	-	0.23 ± 0.01 ^{c}	0.62 ± 0.01 ^{a}	0.28 ± 0.01 ^{b}
Pentacosane *	2500	2500	0.72 ± 0.04	-	-	-
Total identification (%)			92.03	91.01	93.13	91.83

Retention indices (RI) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) [24] and CPWax 52 (RI^2) [25]; Identification method: RI, comparison of RIs with those listed in a homemade library, reported in the literature [24], and/or authentic samples; comparison of mass spectra with those in mass spectral libraries NIST02 and Wiley 9; * co-injection with reference compounds; %—relative peak area; SD, standard deviation. Significant differences for every volatile compound present in more than one location were determined 2way ANOVA followed by Šídák's multiple comparisons test. ^{a,b,c,d}—Mean values in the same row with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$).

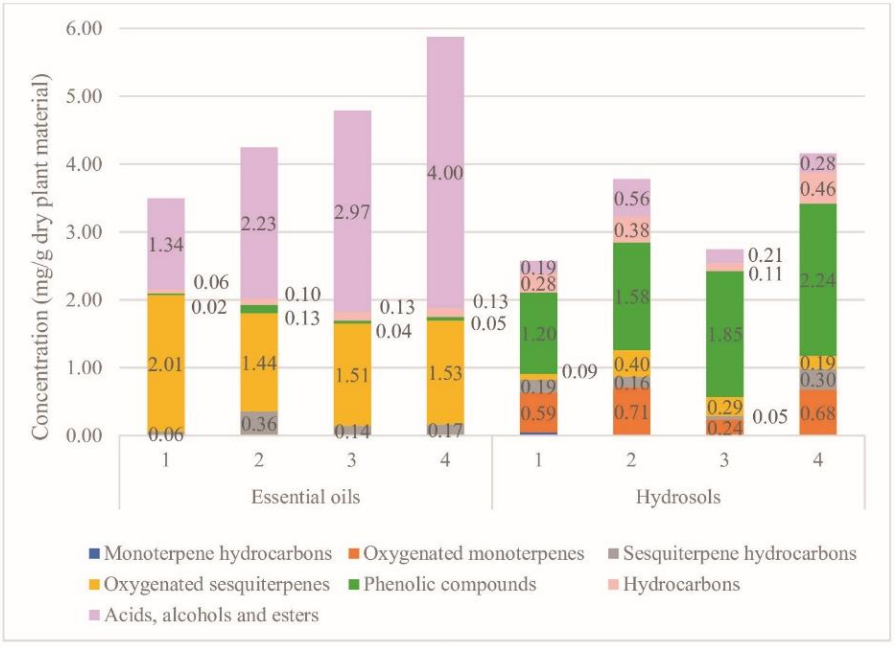
Table 2. Chemical composition of the hydrosols from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii*.

			Mr	St	Br	GJ
Component	**RI[1]**	**RI[2]**	**Hy ± SD (%)**	**Hy ± SD (%)**	**Hy ± SD (%)**	**Hy ± SD (%)**
Monoterpene hydrocarbons			1.56	-	-	-
α-Thujene	924	1032	0.68 ± 0.01	-	-	-
β-Phellandrene	1002	1194	0.88 ± 0.03	-	-	-
Oxygenated monoterpenes			21.23	16.87	8.02	15.01
trans-Linalool oxide *	1088	1434	0.36 ± 0.04	-	-	-
n-Nonanal	1100	1389	4.35 ± 0.01 ^{a}	2.82 ± 0.01 ^{b}	-	-
Borneol	1176	1719	1.56 ± 0.01 ^{a}	0.51 ± 0.01 ^{b}	-	-
Camphor	1151	1499	2.18 ± 0.01 ^{b}	0.92 ± 0.01 ^{c}	-	3.53 ± 0.01 ^{a}
Pinocarvone	1160	1565	2.00 ± 0.01	-	-	-
trans-p-Mentha-1(7),8-dien-2-ol	1187	1803	7.69 ± 0.01 ^{a}	5.24 ± 0.01 ^{d}	7.44 ± 0.01 ^{b}	6.37 ± 0.02 ^{c}
Hexyl 2-methyl butanoate	1233	1425	1.26 ± 0.01 ^{c}	3.12 ± 0.03 ^{b}	-	4.36 ± 0.01 ^{a}
Menthyl acetate	1294	1550	1.83 ± 0.03 ^{b}	4.26 ± 0.01 ^{a}	0.58 ± 0.01 ^{d}	0.75 ± 0.06 ^{c}
Sesquiterpene hydrocarbons			6.87	3.69	1.59	6.62
E-Caryophyllene *	1424	1585	2.65 ± 0.01 ^{a}	1.33 ± 0.01 ^{b}	0.66 ± 0.02 ^{d}	0.73 ± 0.01 ^{c}
δ-Cadinene	1517	1745	2.36 ± 0.01 ^{a}	-	0.93 ± 0.06 ^{b}	2.38 ± 0.08 ^{a}
allo-Aromadendrene	1465	1662	1.52 ± 0.01 ^{a}	-	-	1.24 ± 0.01 ^{b}
β-Chamigrene	1478	1724	0.34 ± 0.01	-	-	-
Germacrene D	1482	1692	-	2.36 ± 0.01 ^{a}	-	2.27 ± 0.01 ^{b}
Oxygenated sesquiterpenes			3.20	9.49	9.60	4.26
Spathulenol	1577	2101	-	-	-	1.23 ± 0.01
β-Caryophyllene oxide *	1581	1955	2.18 ± 0.01 ^{a}	1.27 ± 0.01 ^{b}	1.10 ± 0.01 ^{c}	0.50 ± 0.01 ^{d}
γ-Eudesmol	1632	2175	-	-	-	-
α-Muurolol	1645	2181	-	1.23 ± 0.01	-	-
α-Cadinol	1655	2208	-	2.45 ± 0.01	-	-
α-Bisabolol	1685	2210	0.54 ± 0.03 ^{b}	-	0.50 ± 0.01 ^{b}	1.32 ± 0.01 ^{a}
α-Bisabolol oxide	1748	2511	-	-	0.30 ± 0.01 ^{b}	0.51 ± 0.01 ^{a}
Hexahydrofarnesyl acetone	1839	2113	0.48 ± 0.01 ^{d}	4.54 ± 0.04 ^{b}	7.70 ± 0.02 ^{a}	0.70 ± 0.01 ^{c}
Phenolic compounds			43.41	37.69	62.68	49.64
Thymol *	1289	2154	8.35 ± 0.05 ^{b}	9.45 ± 0.02 ^{a}	3.48 ± 0.01 ^{d}	4.18 ± 0.01 ^{c}
Thymol acetate	1349	-	3.66 ± 0.01 ^{a}	2.27 ± 0.01 ^{c}	-	2.43 ± 0.03 ^{b}
Methyl eugenol	1403	2005	30.23 ± 0.02 ^{c}	23.35 ± 0.01 ^{d}	57.93 ± 0.01 ^{a}	41.85 ± 0.01 ^{b}
(Z)-Methyl isoeugenol	1451	2070	1.17 ± 0.01 ^{c}	2.62 ± 0.06 ^{a}	1.27 ± 0.01 ^{b}	1.18 ± 0.01 ^{c}
Acids, alcohols and esters			6.93	13.23	6.94	6.24
1-Hexadecanol	1874	2371	-	-	2.44 ± 0.01	-
Hexadecanoic acid	1959	2912	4.57 ± 0.01 ^{b}	6.28 ± 0.02 ^{a}	2.25 ± 0.01 ^{c}	1.89 ± 0.01 ^{d}
Oleic acid	2133	2998	0.28 ± 0.01 ^{d}	4.85 ± 0.01 ^{a}	0.46 ± 0.01 ^{c}	3.79 ± 0.01 ^{b}
Octadecanol acetate	2209	-	1.54 ± 0.01 ^{a}	1.18 ± 0.01 ^{b}	0.57 ± 0.02 ^{c}	0.56 ± 0.01 ^{c}
1-Heptatriacotanol	2309	2309	0.54 ± 0.01 ^{c}	0.92 ± 0.01 ^{b}	1.22 ± 0.01 ^{a}	-
Hydrocarbons			11.25	11.96	3.77	9.62
Eicosane *	2000	2000	1.52 ± 0.04 ^{a}	-	0.43 ± 0.01 ^{c}	1.37 ± 0.01 ^{b}
Heneicosane *	2100	2100	0.71 ± 0.01 ^{a}	-	0.29 ± 0.01 ^{c}	0.56 ± 0.06 ^{b}
Docosane *	2200	2200	1.15 ± 0.01 ^{a}	-	0.36 ± 0.01 ^{b}	1.19 ± 0.01 ^{a}
Tricosane *	2300	2300	0.63 ± 0.01 ^{b}	0.85 ± 0.02 ^{a}	-	-
Tetracosane *	2400	2400	-	0.48 ± 0.01 ^{c}	0.87 ± 0.01 ^{a}	0.68 ± 0.01 ^{b}
Pentacosane *	2500	2500	0.67 ± 0.01 ^{a}	0.25 ± 0.01 ^{b}	-	-
Hexacosane *	2600	2600	2.54 ± 0.01 ^{b}	3.08 ± 0.01 ^{a}	0.97 ± 0.02 ^{c}	0.83 ± 0.03 ^{d}
Heptacosane *	2700	2700	3.14 ± 0.01 ^{b}	3.22 ± 0.01 ^{a}	0.29 ± 0.01 ^{d}	1.03 ± 0.01 ^{c}
Octacosane *	2800	2800	0.89 ± 0.01 ^{c}	4.08 ± 0.01 ^{a}	0.56 ± 0.02 ^{d}	3.96 ± 0.01 ^{b}
Total identification (%)			94.45	92.93	92.6	91.39

Retention indices (RI) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) [24] and CPWax 52 (RI^2) [25]; Identification method: RI, comparison of RIs with those listed in a homemade library, reported in the literature [24], and/or authentic samples; comparison of mass spectra with those in mass spectral libraries NIST02 and Wiley 9; * co-injection with reference compounds; %—relative peak area; SD, standard deviation. Significant differences for every volatile compound present in more than one location were determined using 2way ANOVA followed by Šídák's multiple comparisons test. ^{a,b,c,d}—Mean values in the same row with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$).



(a)



(b)

Figure 2. Volatile compounds distribution by categories for all samples of essential oils and hydrosols from four locations; (**a**) based on the relative peak area identification (data from Tables 1 and 2); (**b**) based on the concentration of volatile components in dry plant material (data from Tables S1 and S2).

In the Supplementary Materials in the Tables S1 and S2 we have recalculated compositions of volatile compounds from EOs and hydrosols according to the yields from dry plant material. The main categories are presented in the Figure 2b. If we compare data from Figure 2a,b, the main difference is that in the Figure 2b it is well presented that concentration of volatiles in hydrosols is lower than in the EOs but the distribution of the main categories remains the same as they are distributed in the Figure 2a. It can be seen that in the EOs compounds belonging to the "Oxygenated sesquiterpenes" and "Acids, alcohols and esters" are the most abundant (1.44–2.01 and 1.34–4.00 mg/g, respectively) and compounds belonging to the "Phenolic compounds" are the most abundant in the hydrosols (1.20–2.24 mg/g).

2.2. Principal Component Analyses

PCA analyses were performed for volatile compounds from EOs and hydrosols with the amount greater than 2% (Figure 3). In addition to volatiles from the *V. austriaca* ssp. *jacquinii* from this research we also included volatile compounds of *V. saturejoides* from our previous research that is presented in Table 1 in Nazlić et al. [12] to see whether the two species will differentiate from each other and if volatile compounds could be a distinguishing feature for this genus (Figure 3a). PC1 and PC2 for volatile compounds from EOs and hydrosols explained 50.04% of the variance and distinguished *V. saturejoides* from *V.austriaca* ssp. *jacquinii*. In addition, hydrosols were distinguished from the EOs. This was to be expected, as mentioned earlier, the composition of volatile compounds in the EOs is somewhat different than in the hydrosols. The components differentiating EOs from hydrosols are located in the negative region of PC1 and PC2 for both species. The major components that differentiate *V. austriaca* ssp. *jacquinii* from *V. saturejoides* for hydrosols are *trans-p*-1(7),8-mentha-dien-2-ol, *allo*-aromadendrene, *Z*-methyl isoeugenol, germacrene D and *E*-caryophyllene (Figure 3b). The main components that differentiate *V. austriaca* ssp. *jacquinii* from *V. saturejoides* for the EOs are 1-hexadecanol, α-muurolol, tricosane, pentacosane, 2-methoxy-4-vinylphenol, octadecanol acetate, *β*-caryophyllene oxide and hexadecanoic acid (Figure 3b). From the Figure 3, it can be seen that the two species are best distinguished on the basis of the volatile components of the hydrosols. To our knowledge, this is the first PCA analysis of volatile compounds for the genus *Veronica*. However, numerous other experiments have been conducted for other species, suggesting that volatile compounds can be used as a discriminating factor between species/cultivars [26–28].

2.3. Micromorphology of the Trichomes

Glandular trichomes are part of plant organism where compounds that make essential oils are synthesized. Their role in protecting plants from herbivores and pathogens is very important and as pollinator attractors as well [29]. Volatile compounds from EOSs are generally less investigated than some other specialized metabolites so this is also the reason why we chose to investigate the cells in which these compounds are produced. Both non-glandular and one type of glandular trichomes were observed on calyxes, leaves and the stems of *V. austriaca* ssp. *jacquinii*. According to SEM-investigation non-glandular trichomes vary from bi-celled to multicelled (Figure 4a,f,h). They are unbranched, uniseriate and folded at different levels, while the length varied from short to long hairs. Their surface showed a warty appearance due to the presence of cuticular micropapillae (Figure 4a). These type of trichomes could be noted as attenuate hairs [30]. Calyxes, stems and adaxial leaves side were moderately dense covered by non-glandular trichomes (Figure 4a,e,h), while on the abaxial leaves side these trichomes were mainly distributed along the main vein and leaf edge (Figure 4c). This type of non-glandular hairs was described in our previous research on the *V. saturejoides* ssp. *saturejoides* [12]. Existence of non-glandular trichomes on flower parts of *Veronica* species was mentioned by Kurer [31]. Kraehmer and Baur found non-glandular trichomes in *V. persica* Poir. [32]. Attenuate, non-glandular trichomes are commonly known from other plant species [33–35].

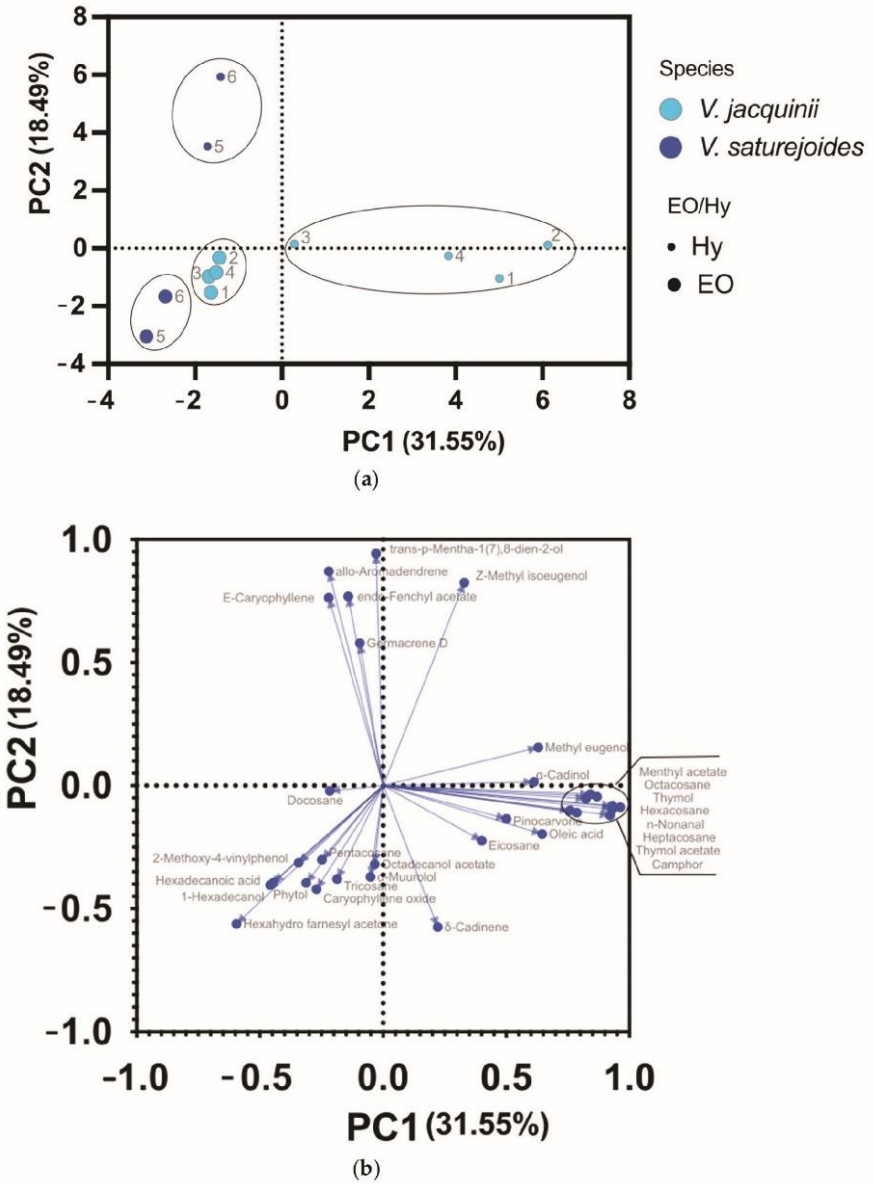


Figure 3. PCA analyses of volatile compound in the amount larger than 2% from essential oils (EO) and hydrosols (Hy) of *V. austriaca* ssp. *jacquinii* from four locations. (**a**) PCA score plot allocating different species into clusters; (**b**) PCA loading plots of volatiles from the first and second principal component.

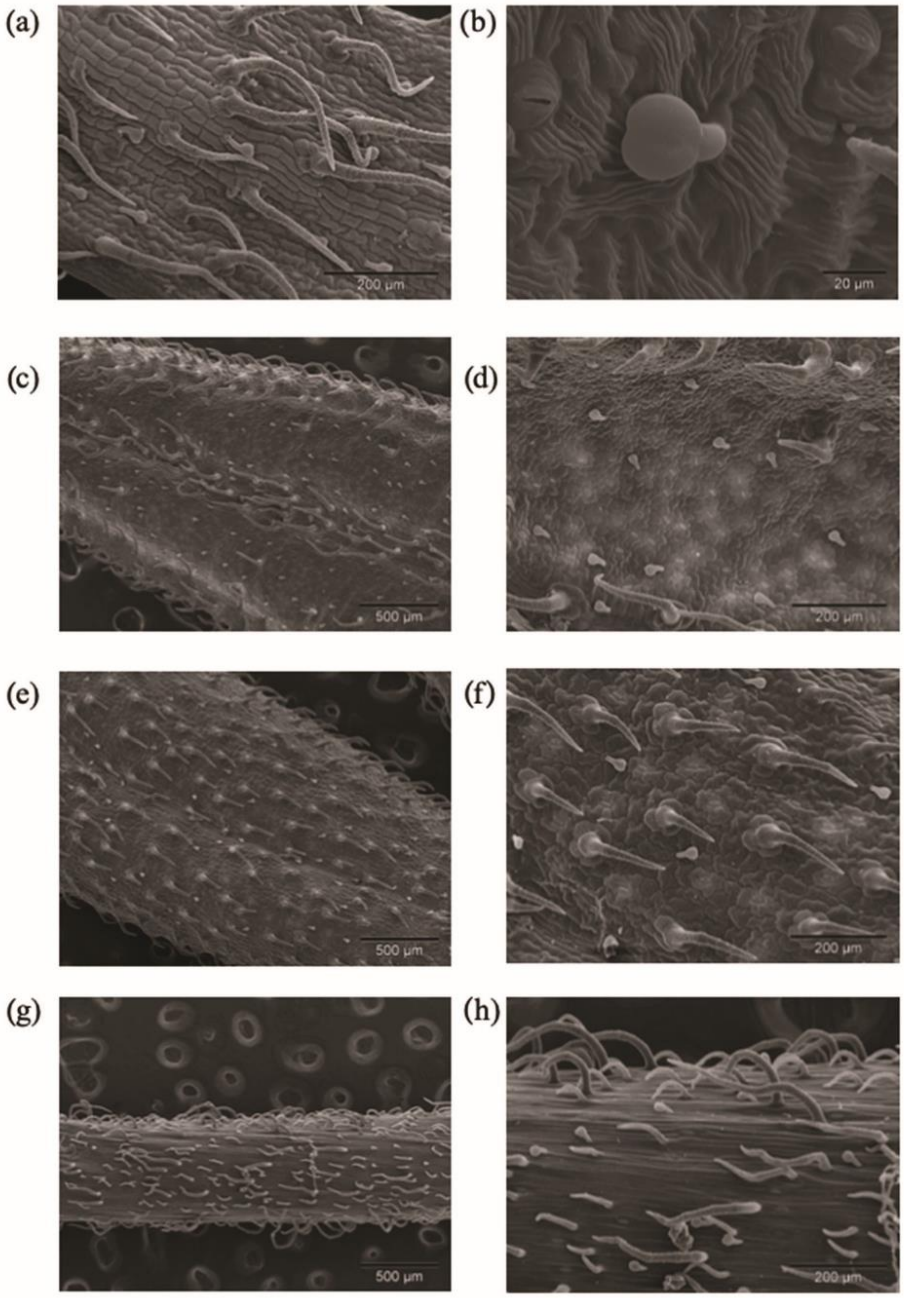


Figure 4. *V. austriaca* ssp. *jacquinii* SEM micrographs showing differences in the distribution of the trichomes on sepal (**a**,**b**), abaxial (**c**,**d**) and adaxial (**e**,**f**) leaf surface, and stem (**g**,**h**).

The glandular trichomes of *V. austriaca* ssp. *jacquinii* belong to capitate type of trichomes and consist of one stalk cell and two elliptically shaped head cells (Figure 4b). These trichomes were not upright but could be described as clinging to the surface. They were observed on calyxes, leaves and the stems. All investigated plant parts were moderately dense covered by capitate trichomes. This type of glandular trichomes was also noted by our team in *V. saturejoides* ssp. *saturejoides* [12]. Kristen and Lockhausen (1985) found

the same type of capitate trichomes in *Veronica beccabunga* L. [36]. A glandular, capitate, inclined trichome type with a bicellular head is also known from *Stachys recta* L. ssp. *recta* [37]. Haratym and Weryszko-Chmielewska found the same type of capitate trichomes with two-celled head on the stem and leaves of *Marrubium vulgare* L. (Lamiaceae) [38]. Comparable glandular trichomes, but with only one elliptically shaped head cell, were found on *Satureja thymbra* L., *Thymus capitatus* (L.) Hoffmanns, *Majorana syriaca* (L.) Rafin. [33], *Calamintha menthifolia* Host. [39], *Geranium macrorrhizum* L. and *G. dalmaticum* (Beck) Rech. f. [40]. In general comparison with the previously investigated *V. saturejoides* [12], we can say that the trichomes of *V. austriaca* ssp. *jacquinii* are denser on all parts of the above ground parts (stem, leaves and calyxes).

2.4. Phenolic Compounds in Hydrosols

The phenolic compounds in the hydrosols were also investigated by the HPLC method to compare the results with our previous studies on *V. saturejoides*. We did not find any phenolic compounds in any of the samples examined from four sites of *V. austriaca* ssp. *jacquinii*. In our previous research, in one sample of *V. saturejoides* we detected vanillin, cinnamic acid and protocatechuic acid. The hydrosol sample from this location had higher antioxidant activity than the plant sample of the same species, but from different location, which did not contain any phenolic compounds [12]. When compared with the results for *V. austriaca* ssp. *jacquinii*, the hydrosol sample of *V. saturejoides* in which phenolic compounds were detected showed higher antioxidant activity than all four samples from *V. jacquinii*. These results are consistent with many previous studies that have shown the antioxidant activity of phenolic compounds. Although phenolic compounds were not detected by HPLC in our hydrosol samples, these compounds have been reported in hydrosols of other plant species such as *Rosa damascena*, where Ulusoy et al. detected tocopherol and carotene [41]. Vlachou et al. studied several plant species to possibly use them as agricultural by-products, and they discovered some valuable compounds in hydrosol extracts of the barks of *Pinus* and *Eucalyptus* species, such as catechin, epicatechin, taxifolin and phenolic acids [42]. From these and many other studies, it can be concluded that hydrosols are valuable by-products of essential oil production and have many potentials in food preservation and industry in the future (e.g., prevention of biofilm formation on utensils and surfaces, natural antimicrobial agents in food industry) [17].

2.5. Antioxidant Activity

The antioxidant activity of the specialized metabolites has been only partially studied for this genus, but all previous studies showed good antioxidant properties, especially for iridoids and phenols. Harput et al. compared the bioactivity of chemical compounds in four species of the genus *Veronica*. Their study showed that a plant with higher phenolic content (*V. officinalis*) also had better antioxidant properties against DPPH [10] while in another study they found that *V. chamaedrys* had significant antioxidant activity against superoxide and *V. officinalis* against DPPH and nitric oxide [43]. In their another study DPPH activity was detected for water extracts of *V. cymbalaria*, *V. hederifolia*, *V. pectinata*, *V. persica* and *V. polita* species. The highest activity was detected for *V. polita* [44]. From Table 3, it can be seen that the results of antioxidant activity for EOs and hydrosols differ when comparing two methods (ORAC and DPPH). If we look at the results for ORAC method, highest activity showed St sample (6.6 $\pm$ 0.47 μmol/g of EO) which is not the case for results obtained with DPPH method. In this method Mr sample showed the highest activity (IC_{50} 246.55 $\pm$ 14.19 mg/mL). Results for hydrosols showed that GJ sample had the highest activity when using ORAC method (1.41 $\pm$ 0.149 μmol/g of hydrosol). The highest activity using the DPPH method showed Br sample of hydrosol (7.263 $\pm$ 0.593 mg/mL). When EOs and hydrosols are compared, it can be seen that EOs show higher activity in ORAC method while hydrosols show higher activity in DPPH method. Compared to our previous research on *V. saturejoides* ssp. *saturejoides* EOs of *V. jacquinii* have lower antioxidant activity in both methods [12]. Considering the study of Aazza et al. [45] who investigated the ORAC

activity of hydrosols of several medicinal plants (*Lavandula officinalis*, *Origanum majorana*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thymus vulgaris*, *Cinnamomum verum* and *Syzygium aromaticum*), the investigated *Veronica* hydrosols in the concentration of the volatiles of 10 mg/mL from all four locations have higher ORAC antioxidant activity than *S. officinalis*, *L. officinalis*, *R. officinalis* and *C. verum* hydrosol. A review of antioxidant research on other specialized metabolites from the genus *Veronica* (phenolic compounds and iridoid glycosides) shows that many of them have significant antioxidant activity [12].

Table 3. Antioxidant potential of *V. austriaca* ssp. *jacquinii* of the essential oil and hydrosol determined by ORAC and DPPH method.

Essential Oils				
Antioxidant Assay	**1 (Mr)**	**2 (St)**	**3 (Br)**	**4 (GJ)**
ORAC (Trolox eq)	4.25 ± 0.42^{a}	6.6 ± 0.47^{a}	4.87 ± 0.49^{a}	4.92 ± 0.38^{a}
DPPH (Trolox eq)	0.135 ± 0.02^{a}	0.06 ± 0.002^{a}	0.09 ± 0.008^{a}	0.02 ± 0.002^{a}
DPPH (% inhibition)	2.22 ± 0.17^{a}	1.16 ± 0.05^{a}	1.51 ± 0.15^{a}	0.48 ± 0.06^{a}
DPPH (IC 50)	246.55 ± 14.19^{a}	528.47 ± 17.57^{c}	428.96 ± 21.88^{b}	1138.4 ± 39.03^{d}
Hydrosols				
Antioxidant Assay	**1 (Mr)**	**2 (St)**	**3 (Br)**	**4 (GJ)**
ORAC (Trolox eq)	1.24 ± 0.089^{a}	0.884 ± 0.041^{a}	1.33 ± 0.069^{a}	1.41 ± 0.149^{a}
DPPH (Trolox eq)	0.355 ± 0.019^{a}	0.209 ± 0.017^{a}	0.342 ± 0.026^{a}	0.085 ± 0.008^{a}
DPPH (% inhibition)	64.66 ± 3.61^{b}	47.092 ± 3.67^{a}	68.841 ± 5.623^{b}	35.528 ± 3.532^{c}
DPPH (IC 50)	7.73 ± 0.431^{a}	10.617 ± 0.827^{ab}	7.263 ± 0.593^{a}	14.073 ± 1.4^{b}

ORAC, oxygen radical absorbance capacity, results for EOs expressed as µmol of Trolox equivalents (TE) per g of EO (10 mg/mL) and for hydrosols as µmol of Trolox equivalents (TE) per g of the total (undiluted) tested hydrosol sample (10 mg volatiles/mL of hydrosol); DPPH, results for EOs expressed as µmol of Trolox per g of EO (10 mg/mL) and for hydrosols as µmol of Trolox per g of absolute hydrosol, IC50 expressed in mg/mL for EOs; SD = standard deviation of triplicate analysis; significant differences were determined using 2Way ANOVA followed by Tukey's multiple comparison test. ^{a,b,c,d}—Mean values in the same row with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$).

In our previous research, we tested the antioxidant activity of the most abundant compound in the EOs, hexahydrofarnesyl acetone. This compound did not show antioxidant activity, so it was concluded that probably the antioxidant activity comes from another compound in EO or is the result of a synergistic effect between different compounds [12]. The results of the current research are consistent with these findings, as it can be seen that there are large differences in the activity of the EOs from the *V. austriaca* ssp. *jacquinii* and *V. saturejoides*, although the same major components are present in both oils. On the other hand, it can be inferred from this and previous research, that one compound which is more abundant in the hydrosols than in EOs may have an impact on the antioxidant activity of these extracts. The compound trans-p-mentha-1(7),8-dien-2-ol is present in all four samples of *V. jacquinii* hydrosols. This compound is not present in the EOs of *V. jacquinii* so this could be the reason for higher activity of the hydrosols in DPPH method. This compound is also present in the EO sample of *V. saturejoides*, which showed higher antioxidant activity than all other samples tested (from this study and from the other location of the same species from the previous study). Looking at the chemical structure of trans-p-mentha-1(7),8-dien-2-ol, it is an isomer of carveol which according to Kaur et. al. has high antioxidant activity potential [46]. They tested the antioxidant activity of carveol by four methods and concluded that the high antioxidant activity of carveol probably comes from an unsaturated hydroxyl group [46]. Looking again at the chemical structure of trans-p-mentha-1(7),8-dien-2-ol [47], we can see that this compound also has this hydroxyl

group. Therefore, in future research, this compound should be tested for its antioxidant activity to see if this hypothesis is true. The other compound that could be the reason for the antioxidant activity of hydrosols is methyl eugenol which is major compound for all four samples of the tested hydrosols, especially in Br sample that showed the highest antioxidant activity in DPPH method and in GJ sample that shoed the highest activity in ORAC method.

The final activity is probably result of synergistic activity of the compounds present in EOs and hydrosols. Synergistic activity has been experimentally demonstrated in some studies. Amorati et al. found that the total oil tested on antioxidant activity had a higher activity than isolated single active compounds [48]. In their experiments, Huang et al. confirmed the synergistic effect of the minor components of cinnamon EO against *S. pullorum* [49]. In our previous studies on *V. spicata*, it was shown that there can also be a negative correlation between the amount of phenolic compounds and antioxidant activity [14]. This finding favors the possibility that other compounds (terpenoids and proteins) have an impact on the antioxidant activity. The results also show the importance of using different methods when evaluating antioxidant activity of plant extracts as different methods showed the different extract with the highest activity. This is expected as different compounds have different reactions with the compounds and radicals used in antioxidant methods [45].

2.6. Antiproliferative Activity of Essential Oils and Hydrosols

Cytotoxic effect of the essential oil and hydrosol of *V. austriaca* ssp. *jacquinii* was tested on three cancer cell lines (HeLa, U2OS and HCT116) and two healthy cell lines (HaCaT and RPE1). The results showed that neither the EO nor the hydrosol exhibited toxicity to both cancer cells and healthy human cell lines (Figure 5a,b). The IC_{50} values were extremely high, over 1.5 mg/mL for the oil and over 3 mg/mL for the hydrosol. HeLa cells exhibited the highest resistance, requiring 3.47 mg/mL of the EO and 4.55 mg/mL of the hydrosol to inhibit cell growth by 50%. The colon (HCT116) and osteosarcoma (U2OS) cells showed slightly lower but still significant resistance to the effects of EO and hydrosol, with IC_{50} values of 1.9 mg/mL and 2.7 mg/mL for the EO and 3.65 and 4.54 mg/mL for the hydrosol, respectively. The EO and hydrosol also did not prevent the division of the healthy cells. The RPE1 cells, representing the epithelial cell model, were not sensitive to the effect of the oil and hydrosol with IC_{50} values of 2.82 and 4.61 mg/mL, respectively. In addition, the oil and hydrosol showed no toxicity to human keratinocytes (HaCaT) cells (IC_{50} values were 1.93 mg/mL for the EO and 3.72 mg/mL for the hydrosol). Two major compounds of the EO, hexahydrofarnesyl acetone and palmitic acid, were also tested on the antiproliferative activity. As expected, given the results on the cytotoxic activity of the EO, none of the compounds tested showed cytotoxic activity against cancer and healthy cell lines. The IC_{50} values for hexahydrofarnesyl acetone were above 4 mg/mL and for palmitic acid much lower, but still high enough not to inhibit proliferation of both cancer and healthy cells (IC_{50} > 1 mg/mL) (Figure 5c).

Various extracts from the aerial parts of *Veronica* species are used in folk medicine worldwide to treat many types of cancer [4]. Nevertheless, very few *Veronica* species have been studied for their cytotoxic activity in vitro and in vivo. Mainly methanolic and aqueous extracts of different *Veronica* species have been tested on cancer cell lines. The methanol extract of *V. cymbalaria*, *V. hederifolia*, *V. pectinata* var. *glandulosa*, *V. persica* and *V. polita* showed significant cytotoxic activity against KB epidermal carcinoma and melanoma cells. The isolated chloroform fraction showed dose dependent cytotoxicity [44]. The methanolic extract of the edible species *V. americana* showed cytotoxic activity against HF-6 (colon) and PC-3 (prostate) cancer cell lines [50]. The aqueous extract of *V. cuneifolia* subsp. *cuneifolia* and *V. cymbalaria* inhibited the division of Hep-2RD and L-20B cancer cells more than the healthy cell line, Vero cell line [51]. Flavonoids (Vtfs) isolated from *V. sibirica* stopped the division of MCF-7 (breast) cancer cells, the IC_{50} value was 42 ug/mL. The mecha-

nism of inhibition of cancer cell division involved apoptosis, which was concentration dependent [52].

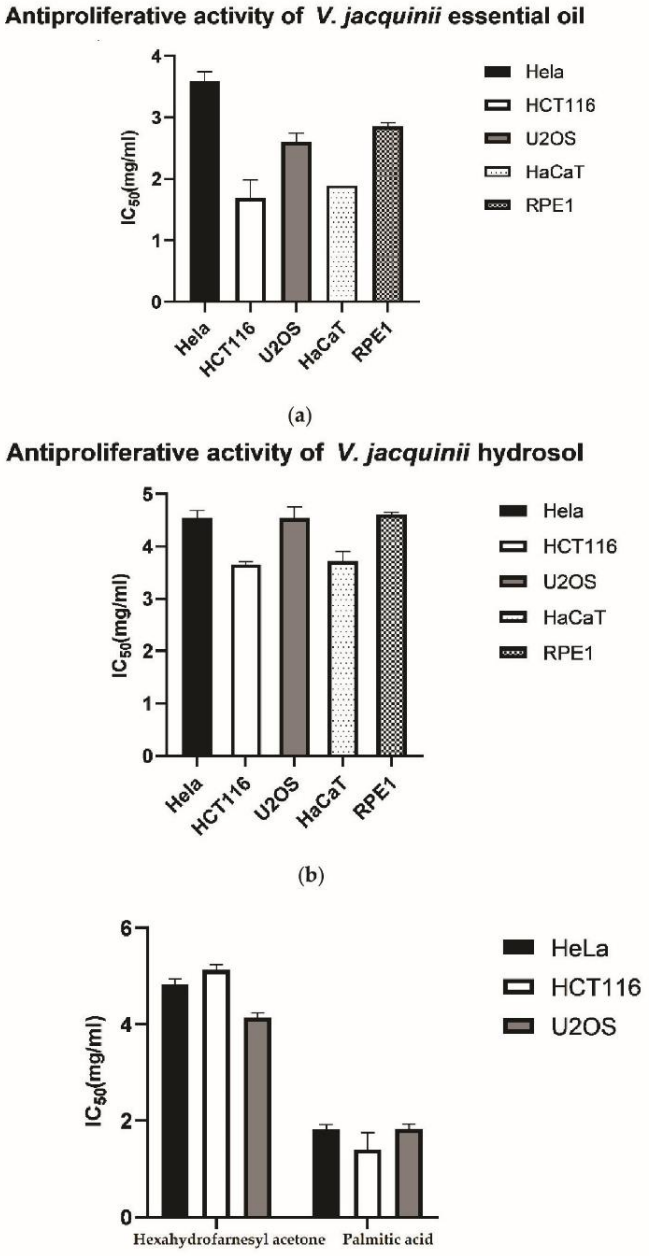


Figure 5. Antiproliferative activity of: (**a**) *V. austriaca* ssp. *jacquinii* EO; (**b**) *V. austriaca* ssp. *jacquinii* hydrosol; (**c**) Standards of the most abundant compounds.

Considering the results obtained in the present study, the essential oil and hydrosol of *V. austriaca* ssp. *jacquinii* can be considered as a safe natural product.

2.7. Polyphenol Analysis in Dry Plant Material

Polyphenols are natural compounds that help the plant to protect itself from the ultraviolet radiation or aggression by pathogens [53]. Due to their good antioxidant, anti-neurodegenerative and anticancer properties, they are commonly consumed in the diet [54]. Table 4 shows the results for the polyphenol analyses *V. austriaca* ssp. *jacquinii* from four locations in Croatia. The highest content was found for total phenolic acids (TPA) in *V. austriaca* ssp. *jacquinii* from the Location Br while the yield of total flavonoids (TF) was found to be very low and similar for all investigated specimens of the studied species. Harput et al. conducted a study on a few *Veronica* species and found that total phenolic content (TP) was 200.20 mg/g in *V. officinalis* L., 139.92 mg/g in *V. peduncularis* M. Bieb., 127.64 mg/g in *V. orientalis* Mill., and 83.15 mg/g in *V. baranetzkii* Bordz. [10]. In the previous research of our team, species *V. spicata* had higher quantities of TP from methanol extracts than *V. austriaca* ssp. *jaquinii* (129.43 mg/g from dry weight of flowers and 141.28 mg/g from dry weight of leaves) [14]. In another study on the species *V. saturejoides* ssp. *saturejoides*, the TP content from methanol extracts was slightly higher in the Kamešnica sample (86.9 mg/g from dry weight of plant material) and similar in the Prenj sample (70.9 mg/g dry weight of plant material) compared to *V. austriaca* ssp. *jacquinii* (Table 4) [12].

Table 4. Contents of total polyphenols (TP), total tannins (T), total flavonoids (TF), and total phenolic acids (TPA) in *V. austriaca* ssp. *jacquinii*.

Sample	TP (mg/g DW)	T (mg/g DW)	TF (mg/g DW)	TPA1 (505 nm) (mg/g DW)	TPA2 (525 nm) (mg/g DW)
1 (Mr)	55.98 ± 0.30^{c}	8.29 ± 0.70^{a}	1.29 ± 0.00^{a}	15.68 ± 2.10^{b}	19.91 ± 2.00^{a}
2 (St)	70.60 ± 7.60^{b}	9.06 ± 6.40^{a}	2.10 ± 0.00^{a}	20.07 ± 1.20^{ab}	17.83 ± 1.20^{a}
3 (Br)	78.79 ± 1.30^{a}	8.98 ± 1.40^{a}	2.05 ± 0.00^{a}	26.58 ± 2.00^{a}	24.16 ± 1.80^{a}
4 (GJ)	66.55 ± 0.50^{b}	8.58 ± 0.80^{a}	1.47 ± 0.00^{a}	19.47 ± 0.60^{b}	17.38 ± 0.50^{a}

Note: DW, dry weight; SD = standard deviation of triplicate analysis; significant differences were determined using Tukey's multiple comparison test. ^{a,b,c}—Mean values in each column with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$).

3. Materials and Methods

3.1. Plant Material

Plant material for *V. austriaca* ssp. *jacquinii* was collected from four locations in Croatia (Table 5, Figure 6). Voucher specimens were deposited in the "Fran Kušan" hebarium (HFK-HR), Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. For GC-MS analyses samples were air dried in a single layer and protected from direct sunlight for three weeks. Dried plant material was placed in double paper bags labeled with the sample number and stored in a dry place protected from light until analysis.

Table 5. Locations of the plant material collection (for volatile compounds extraction).

	Locality	Coordinates	Altitude a.s.l. (m)	Date of Collection	Abbrev.
1.	Mrkopalj	45°18′59″ N; 14°50′43″ E	820	June 2019	Mr
2.	Stupačinovo	44°32″21″ N; 15°09′51″ E	971	June 2019	St
3.	Lika, Brezovac	44 47′42″ N; 15°34′28″ E	798	June 2020	Br
4.	Gornje Jelenje	45°21′50″ N; 14°37′32″ E	880	May 2020	GJ

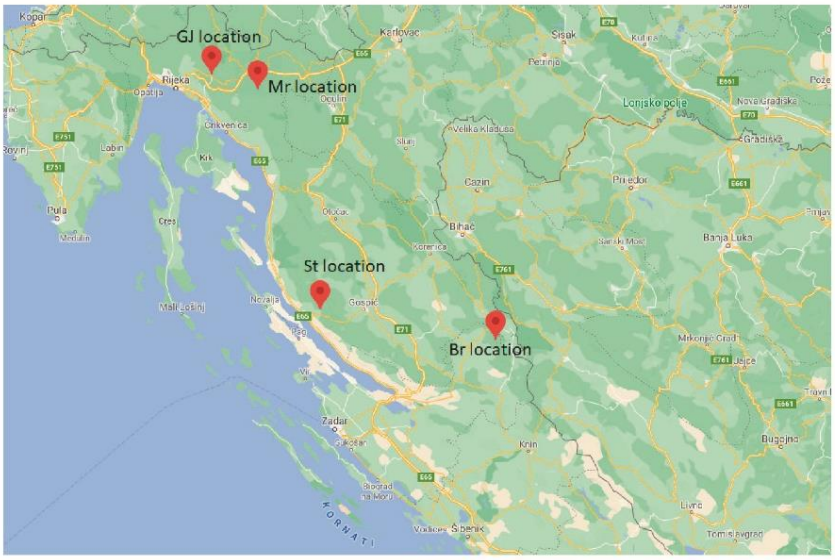


Figure 6. Map with the places of collection of the plant material.

For micromorphological investigations of trichomes samples of seven plants were fixed in FAA (formalin/96% ethanol/acetic acid/water—5/70/5/20). After three days, samples were transferred to 70% ethanol.

3.2. Extraction of Volatiles from Hydrosols

After the extraction of EOs and hydrosols with Clevenger type apparatus, in order to determine antioxidant activity of hydrosols and compare it with EO antioxidant activity, volatiles from the hydrosols have been extracted with pentane/diethyl ether mixture. 5 mL of each hydrosol was extracted in a separation funnel with the mixture of 3 mL of pentane and 3 mL of diethyl ether. The organic layer was separated and dried over the anhydrous sodium sulphate. To calculate the exact concentration of hydrosol the organic solvent was evaporated. The volatile compounds that remained were weighed and the concentration for each hydrosol sample was calculated and expressed in mg/mL of hydrosol for the antioxidant activity. In the Table 6 masses of dry plant material and calculated yields of EOs and hydrosols are presented.

Table 6. Yield of obtained volatile compounds from EOs and hydrosols.

Location	Mass of Dry Plant Material Used for Isolations of Volatiles (g)	Mass of EO (mg)	Yield of EO (%)	Mass of Volatiles from Hy (mg)	Yield of Volatiles from Hy (%)
1 (Mr)	50	190	0.38	138	0.28
2 (St)	30	140	0.47	126	0.42
3 (Br)	35	180	0.51	104	0.30
4 (GJ)	25	160	0.64	113	0.45

3.3. GC and GC-MS Analyses

Dried above ground parts (25—50 g) for each sample were hydrodistilled for 3 h in Clevenger type apparatus. For each sample (three separate extractions for each) volatile compounds and water residues (hydrosols) were collected. Both phases were analyzed with gas chromatography and mass spectrometry (GC-MS) according to a method de-

scribed in our recent research [12]. GC was performed by gas chromatograph (model 3900, Varian Inc., Lake Forest, CA, USA) that is supplied with a flame ionization detector (FID), mass spectrometer (model 2100T; Varian Inc.), non-polar capillary column VF-5ms (30 m × 0.25 mm inside diameter, coating thickness 0.25 μm, Palo Alto, CA, USA) and polar capillary column CP-Wax 52 CB (30 m × 0.25 mm i.d., coating thickness 0.25 μm, Palo Alto, CA, USA). The chromatographic conditions for the analysis of lipophilic fraction (essential oils) were FID detector temperature 300 °C, injector temperature 250 °C. The gas carrier was helium at 1 mL min^{-1}. The conditions for the VF-5ms column were temperature 60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1}, and held isothermal for 25 min. Conditions for the CP Wax 52 column were: temperature 70 °C isothermal for 5 min, and then increased to 240 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. The injected volume was 2 μL and the split ratio was 1:20. The MS conditions were: ion source temperature 200 °C, ionization voltage 70 eV, mass scan range 40–350 mass units [14]. The individual peaks for all samples were identified by comparison of their retention indices of *n*-alkanes to those of authentic samples and literature [24,55], comparing it to our libraries from previous work [12,14] and to other previously published material for *Veronica* species [9,21–23]. The results are expressed as the mean value of three analyses with standard deviation.

3.4. Micromorphological Traits

For SEM-investigation, stem, leaf and calyx samples were transferred from 70% ethanol to 70% acetone, then further dehydrated (70%, 90% and 100% acetone) and subjected to critical point drying using CO_2 as the drying medium (CPD030; Baltec). After that, samples were sputter coated with gold (Sputter Coater, AGAR) and examined under the scanning electron microscope XL30 ESEM (FEI) with 20 kV acceleration voltages in high vacuum mode [56]. Common terminology [30] was used in the description of trichomes.

3.5. Phenolic Compounds in Hydrosols

The phenolic compounds of the hydrosols were separated by high-performance liquid chromatography (HPLC) on a Ultra Aqueous C18 column (250 × 4.6 mm, 5 mm; Restek; Bellefonte, PA, USA) according to a method described in Nazlić et al. [12]. Gradient chromatography conditions were set according to the methodology by Jukić Špika et al. [57]. Solvents used for mobile phase were 0.2% phosphoric acid (A), methanol (B) and acetonitrile (C). At 0 min, the gradient was 96% A, 2% B, and 2% C. During the first 40 min, ratios were changed from the initial value to 50% A, 25% B, and 25% C and were subsequently changed to 40% A, 30% B, and 30% C from 40 to 45 min. The gradient was changed to 50% B and 50% C from 45 to 60 min, and kept at these values until 70 min. The obtained solvent ratio was retained for the last 10 min to achieve the stability of the column to the initial conditions.

The standard and solvents were of analytical grade and were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). In the preparation of all solvents deionized water (Milli-Q) was used.

3.6. Polyphenol Analysis

3.6.1. Apparatus and Chemicals

Apparatus

Water-bath, reflux condenser, UV/Vis spectrophotometer Agilent 8453 (Agilent, Karlsruhe, Germany) with PC-HP 845x UV-Visible System (Agilent, Karlsruhe, Germany) and 1 cm quartz cells were used for all absorbance measurements. Filtration of prepared sample solutions was performed by using 0.20 μm Minisart-plus membrane filter (Sartorius AG, Goettingen, Germany).

Reagents and solutions (TP/T)

Pro analysi chemicals, as well as double distilled water were used throughout the work. The solution of 30% methanol (Kemika, Zagreb, Croatia) was used for plant material extraction. The solution of 33% sodium carbonate decahydrate (Kemika, Zagreb, Croatia) was used for sample preparation.

Except for the Folin–Ciocalteu phenol reagent (FCR), casein (Merck, Darmstadt, Germany) and quercetin (Roth, Karlsruhe, Germany), all chemicals and reagents for polyphenol analysis were of analytical quality grade and were supplied by Kemika (Zagreb, Croatia).

3.6.2. Total Polyphenol and Tannin Analysis (Folin–Ciocalteu Phenol Reagent (FCR) Procedure)

Total polyphenols (TP) and tannins (T) in aboveground parts of *V. austriaca* ssp. *jacquinii* were determined by using the prevalidated *FCR procedure* for polyphenols analysis according to Jurišić Grubešić et al. [58].

The finely ground above-ground plant portions (0.25 g) were extracted with 80 mL of 30% methanol in a reflux flask, heating on a boiling water bath for about 15 min. After cooling, the extract was filtered into a 100.0 mL volumetric flask and made up to the mark with 30% (*v/v*) methanol. 2.0 mL of the filtrate was mixed with 8.0 mL of distilled water and 10.0 mL of sodium acetate solution (1.92 g of sodium acetate trihydrate and 0.34 mL of acetic acid were mixed and made up to 100.0 mL with distilled water). The buffer solution maintains a constant pH value of the medium (pH = 5), which is optimal for the precipitation of tannins. The solution obtained as described was labeled as solution 1 (S1). 10.0 mL of S1 was shaken with 50 mg of casein on a shaker for 45 min. The solution was filtered, and the resulting filtrate was solution 2 (S2).

1.0 mL of S1 and S2 were mixed separately in 10.0 mL volumetric flasks with 0.5 mL of Folin-Ciocalteu's phenolic reagent and made up to the mark with 33% (*m/v*) sodium carbonate decahydrate solution. The absorbances of the obtained blue solutions were measured at 720 nm, with distilled water as a blank. The value given by S1 corresponds to the content of TP, while the difference between the values obtained for S1 and S2 represents the content of T bound to casein.

A calibration curve was developed using tannin as a standard substance. For this purpose, 10 mg of tannin was dried at 80 °C and dissolved in 100.0 mL of distilled water (standard stock solution). The working standard was prepared by mixing 5.0 mL of standard stock solution and 5.0 mL of buffer solution. The concentration range, obtained by diluting the volume from 0.2 to 1.2 mL of working standard to 10.0 mL with buffer solution (corresponding to a tanning concentration of 0.001 to 0.006 mg/mL), gives a linear increase in absorbance.

For the measured absorbance values of S1 and S2, the corresponding concentrations from the calibration diagram are obtained. The difference between the TP content (obtained by measuring S1), and the content determined for S2, represents the content of tannins precipitated with casein [59].

The contents of TP and T in *V. austriaca* ssp. *jacquinii* extracts were evaluated in three independent analyses and were expressed as mg/g of dry weight of herbal material according to following equation:

$$\text{TP (mg/g)} = A_{S1}/0.0025$$

$$\text{T (mg/g)} = (A_{S1}/0.0025) - (A_{S2}/0.0025)$$

A_{S1}, A_{S2} = measured absorbance of S1 and S2, respectively.

3.6.3. Total Flavonoid (TF) Analysis—TF Procedure

TF Procedure was performed as follows: 0.200 g of powdered herbal drug is extracted individually for 30 min with 20 mL of acetone, 2 mL of 25% hydrochloric acid and 1 mL of 0.5% hexamethylenetetramine solution, heating to reflux in a water bath. The hydrolysate

was passed through a cotton ball, and the drug residue on the cotton wool was re-extracted with 20 mL of acetone, heating to boiling for 10 min. This solution was also passed through a cotton ball, and the acetone extraction described above was repeated a total of 3 times. The combined filtrates were diluted with acetone to 100.0 mL.

20.0 mL of the hydrolysate was mixed with 20 mL of water and then extracted first with 15 mL and three times with 10 mL each of ethyl acetate. The combined ethyl acetate phases were washed twice with 40 mL each of water, passed through cotton wool and diluted with ethyl acetate to 50.0 mL. Two portion of 10.0 mL of this solution were transferred to two 25.0 mL volumetric flasks. To each flask was added 0.5 mL of 0.5% aqueous sodium citrate solution. An additional 2 mL of aluminum chloride solution was added to one flask (2 g of aluminum chloride hexahydrate was dissolved in 100.0 mL of 5% methanolic acetic acid solution). Both flasks were then made up to 25.0 mL with 5% methanolic acetic acid solution. After 45 min, the absorbances of the solutions with aluminum chloride were measured at 425 nm, in a 1 cm thick layer. The compensating solution was a previously prepared solution without aluminum chloride.

The mass concentration of flavonoids is calculated as quercetin, according to the expression:

$$TF\ (\%) = A \times 0.772/b\ (A = \text{absorbance; } b = \text{drug weight expressed in g}).$$

3.6.4. Determination of Total Phenolic Acids (TPA) (TPA Procedure)

TPA Procedure in selected plant samples was performed by spectrophotometric method prescribed by the European Pharmacopoeia [60].

In 0.200 g of powdered herbal drug was added 80 mL of 50% ethanol and heated in a reflux flask on a boiling water bath for 30 min. After cooling and filtration, the filtrate was made up to 100.0 mL with 50% ethanol in a volumetric flask (stock solution). The test solution was prepared by mixing 1.0 mL of stock solution with 2.0 mL of 0.5 M hydrochloric acid, 2.0 mL of solution obtained by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100.0 mL of water and 2.0 mL 8.5% sodium hydroxide solution. The flask was made up to 10.0 mL with distilled water. The compensating solution was obtained by diluting 1.0 mL of stock solution with water to 10.0 mL. The absorbance of the resulting solutions was immediately measured at 505 nm (expressed as rosmarinic acid) and 525 nm (expressed as chlorogenic acid).

The mass fraction of phenolic acids calculated and expressed as rosmarinic acid (TPA1):

$$TPA1\ (\%) = A \times 2.5/m$$

where A is the measured absorbance at 505 nm, taking the specific absorbance of rosmarinic acid at 505 nm to be 400; m—mass of the drug expressed in grams.

The mass fraction of phenolic acids calculated and expressed as chlorogenic acid (TPA2):

$$TPA2\ (\%) = A \times 5.3/m$$

where A is the measured absorbance at 525 nm, taking the specific absorbance of chlorogenic acid at 525 nm to be 188; m—mass of the drug expressed in grams.

3.7. Antioxidant Activity of Essential Oils and Hydrosols

3.7.1. ORAC

The assay was performed in a Perkin–Elmer LS55 spectrofluorimeter, using 96-well white polystyrene microtiter plates (Porvair Sciences, Leatherhead, UK) according to a method described by Fredotović et al. [61], with some adjustments due to different extracts described in our previous research [12]. All measurements were performed in triplicate by a method described in Nazlić et. al. [12].

3.7.2. DPPH

The antioxidant capacity of the extracts was assessed by the DPPH method previously described by Mensor et al. and Payet et al. [62,63] and adjusted to our plant extracts as described in the method from our previous research [12]. Calculation and expressing the results were carried out according to the method described in our previous research in Nazlić et al. [12].

3.8. Cell Culture

We received three cancer cell lines: Cervical Cancer Cell Line (HeLa), Human Colon Cancer Cell Line (HCT116) and Human Osteosarcoma Cell Line (U2OS), and two healthy cell lines: Human Epidermal Keratinocyte Line (HaCaT) and Retinal Pigmented Epithelial Cells (RPE1) as a gift from Professor Janoš Terzić from the School of Medicine, University of Split. Cells were grown in an incubator under humidified conditions with 5% CO_2 and 37 °C, in a Dulbecco's modified Eagle's medium (DMEM Euroclone, Milano, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), and 1% antibiotics (penicillin and streptomycin, EuroClone).

3.9. Cell Proliferation Assay

The antiproliferative capacity of the EO of *V. austriaca* ssp. *jacquinii* was determined on HeLa, HCT116 and U2OS cancer cells and in healthy cells HaCaT and RPE1 using the MTS-based CellTiter 96® Aqueous Assay (Promega, Madison, WI, USA). Cells were grown in an incubator at 37 °C and 5% CO_2 until they reached 80% confluence. They were counted using a handheld automated cell counter (Scepter, Merck), and 5000 cells/well were seeded in 96-well plates with a serial dilution of the EOs and hydrosols. The cells were then cultured for an additional 48 h. Thereafter, 20 µL MTS tetrazolium reagent (Promega, Madison, WI, USA) was added to each well and left in the incubator for a further 3 h. Then, absorbance was measured at 490 nm using a microplate reader (Bio-Tek, EL808). IC_{50} values were calculated from three independent experiments using GraFit 6 data analysis software (Erithacus, East Grinstead, UK).

3.10. Statistical Analyses

Statistical analysis was performed in GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± SD ($n \geq 3$). Data included in the PCA analyses was data obtained from the GC-MS analyses. The statistical significance for free volatile compounds, total phenolic compounds and antioxidant activity was assessed by 2way ANOVA followed by Šídák's multiple comparisons test and Tukey's multiple comparison test, $p < 0.05$. Statistical tests were performed separately for lipophilic (essential oils) and hydrophilic fractions (hydrosols).

4. Conclusions

In this study, the free volatile compounds of the species *V. austriaca* ssp. *jacquinii* and their biological activity were reported. The volatile compounds extracted in essential oils and hydrosols from four locations were compared. Hexahydrofarnesyl acetone and hexadecanoic acid were the major components of the essential oils from all four locations. The major components of the hydrosols were methyl eugenol and thymol. Our research has shown that hydrosols can exhibit stronger antioxidant activity than essential oils, even though they contain fewer dissolved molecules in them. As they showed no toxicity towards healthy cells they could be used as a safe product in cosmetics or for food preservation. Essential oils and hydrosols from the genus *Veronica* are just beginning to be studied in more detail as other compounds were more investigated for this genus, such as iridoids and phenolic compounds. We believe that the free volatile compounds from this genus are also valuable resources and should be further investigated in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10112529/s1. Table S1. Chemical composition of the essential oil from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii* calculated based on the masses of EOs extracted from dry plant material; Table S2. Chemical composition of the hydrosols from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii* calculated based on the masses of volatile compounds extracted from hydrosols from dry plant material; Figure S1. RIC chromatograms of the four samples of EOs of *V. jacquinii* with marked major compounds; Figure S2. RIC chromatograms of the four samples of hydrosols of *V. jacquinii* with marked major compounds.

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References

1. Albach, D.C.; Martínez-Ortega, M.M.; Fischer, M.A.; Chase, M.W. A new classification of the tribe Veroniceae-problems and a possible solution. *TAXON* **2004**, *53*, 429–452. [CrossRef]
2. Sharifi-Rad, M.; Tayeboon, G.S.; Sharifi-Rad, J.; Iriti, M.; Varoni, E.M.; Razazi, S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of Veronica persica phenolic-rich extracts. *Cell. Mol. Boil.* **2016**, *62*, 80–85. [CrossRef]
3. Barreira, J.C.; Dias, M.I.; Živković, J.; Stojković, D.; Soković, M.; Santos-Buelga, C.; Ferreira, I.C. Phenolic profiling of Veronica spp. grown in mountain, urban and sandy soil environments. *Food Chem.* **2014**, *163*, 275–283. [CrossRef]
4. Salehi, B.; Shetty, M.S.; Kumar, N.V.A.; Živković, J.; Calina, D.; Docea, A.O.; Emamzadeh-Yazdi, S.; Kılıç, C.S.; Goloshvili, T.; Nicola, S.; et al. Veronica Plants—Drifting from Farm to Traditional Healing, Food Application, and Phytopharmacology. *Molecules* **2019**, *24*, 2454. [CrossRef] [PubMed]
5. Nikolić, T. Flora Croatica Database. Available online: http://hirc.botanic.hr/fcd (accessed on 15 September 2021).
6. Živković, J.Č. *Morphological, Chemical and Pharmacological Characterization of Selected Veronica L. Species (Plantaginaceae)*; University of Belgrade: Belgrade, Serbia, 2014.
7. Stojkovic, D.; Živković, J.; Sokovic, M.; Glamočlija, J.; Ferreira, I.C.; Janković, T.; Maksimović, Z. Antibacterial activity of Veronica montana L. extract and of protocatechuic acid incorporated in a food system. *Food Chem. Toxicol.* **2013**, *55*, 209–213. [CrossRef]
8. Živković, J.; Barreira, J.C.M.; Šavikin, K.P.; Alimpić, A.Z.; Stojkovic, D.; Dias, M.I.; Santos-Buelga, C.; Duletic-Lausevic, S.; Ferreira, I.C.F.R. Chemical Profiling and Assessment of Antineurodegenerative and Antioxidant Properties ofVeronica teucriumL. andVeronica jacquiniiBaumg. *Chem. Biodivers.* **2017**, *14*, e1700167. [CrossRef]
9. Ertaş, A.; Boğa, M.; Kizil, M.; Çeken, B.; Gören, A.C.; Haşimi, N.; Demirci, S.; Topçu, G.; Kolak, U. Chemical profile and biological activities of *Veronica thymoides* subsp. *pseudocinerea*. *Pharm. Biol.* **2014**, *53*, 334–339. [CrossRef]
10. Harput, U.S.; Saracoglu, I.; Genc, Y. Comparative Bioactivity Studies on Four Veronica species. *FABAD J. Pharm. Sci.* **2009**, *34*, 67–72.
11. Mocan, A.; Vodnar, D.C.; Vlase, L.; Crişan, O.; Gheldiu, A.-M.; Crişan, G. Phytochemical Characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from Romania and Their Antioxidant and Antimicrobial Properties. *Int. J. Mol. Sci.* **2015**, *16*, 21109–21127. [CrossRef]
12. Nazlić, M.; Kremer, D.; Jurišić-Grubešić, R.; Soldo, B.; Vuko, E.; Stabentheiner, E.; Ballian, D.; Bogunić, F.; Dunkić, V. Endemic Veronica saturejoides Vis. ssp. saturejoides–Chemical Composition and Antioxidant Activity of Free Volatile Compounds. *Plants* **2020**, *9*, 1646. [CrossRef]
13. Živković, J.; Barreira, J.C.; Stojković, D.; Ćebović, T.; Santos-Buelga, C.; Maksimović, Z.; Ferreira, I.C. Phenolic profile, antibacterial, antimutagenic and antitumour evaluation of Veronica urticifolia Jacq. *J. Funct. Foods* **2014**, *9*, 192–201. [CrossRef]
14. Dunkić, V.; Kosalec, I.; Košir, I.; Potočnik, T.; Čerenak, A.; Končič, M.; Vitali, D.; Muller, I.; Kopricanec, M.; Bezić, N.; et al. Antioxidant and antimicrobial properties of *Veronica spicata* L. (Plantaginaceae). *Curr. Drug Targets* **2015**, *16*, 1660–1670. [CrossRef]

15. Hamedi, A.; Pasdaran, A.; Zebarjad, Z.; Moein, M. A Survey on Chemical Constituents and Indications of Aromatic Waters Soft Drinks (Hydrosols) Used in Persian Nutrition Culture and Folk Medicine for Neurological Disorders and Mental Health. *J. Evid.-Based Integr. Med.* **2017**, *22*, 744–752. [CrossRef]
16. Cid-Pérez, T.S.; Ávila-Sosa, R.; Ochoa-Velasco, C.E.; Rivera-Chavira, B.E.; Nevárez-Moorillón, G.V. Antioxidant and Antimicrobial Activity of Mexican Oregano (*Poliomintha longiflora*) Essential Oil, Hydrosol and Extracts from Waste Solid Residues. *Plants* **2019**, *8*, 22. [CrossRef]
17. D'Amato, S.; Serio, A.; López, C.C.; Paparella, A. Hydrosols: Biological activity and potential as antimicrobials for food applications. *Food Control.* **2018**, *86*, 126–137. [CrossRef]
18. Sharifi-Rad, J.; Sureda, A.; Tenore, G.C.; Daglia, M.; Sharifi-Rad, M.; Valussi, M.; Tundis, R.; Sharifi-Rad, M.; Loizzo, M.R.; Oluwaseun Ademiluyi, A.; et al. Biological activities of essential oils: From plant chemoecology to traditional healing systems. *Molecules* **2017**, *22*, 70. [CrossRef]
19. Beara, I.; Živković, J.; Lesjak, M.; Ristić, J.; Šavikin, K.; Maksimović, Z.; Janković, T. Phenolic profile and anti-inflammatory activity of three Veronica species. *Ind. Crop. Prod.* **2015**, *63*, 276–280. [CrossRef]
20. Kwak, J.H.; Kim, H.J.; Lee, K.H.; Kang, S.C.; Zee, O.P. Antioxidative iridoid glycosides and phenolic compounds from Veronica peregrina. *Arch. Pharm. Res.* **2009**, *32*, 207–213. [CrossRef]
21. Li, F. Analysis of chemical constituents of essential oil in Veronica linariifolia by gas chromatography-mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 822–825.
22. Béjaoui, A.; Chaabane, H.; Jemli, M.; Boulila, A.; Boussaid, M. Essential Oil Composition and Antibacterial Activity of Origanum vulgare subsp. glandulosum Desf. at Different Phenological Stages. *J. Med. Food* **2013**, *16*, 1115–1120. [CrossRef] [PubMed]
23. Valyova, M.; Hadjimitova, V.; Stoyanov, S.; Ganeva, Y.; Petrov, T.; Petrov, I. Free radical scavenging activity of extracts from Bulgarian *Veronica officinalis* L. and GC-MS analysis of ethanol extract. *Internet J. Aesthetic Antiaging Med.* **2009**, *2*. [CrossRef]
24. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing Corporation: Carol Stream, IL, USA, 2007; ISBN 978-1-932633-21-4.
25. NIST Chemistry WebBook. Available online: https://webbook.nist.gov/ (accessed on 12 March 2021).
26. Popović, M.; Špika, M.J.; Bratinčević, M.V.; Ninčević, T.; Mateško vić, A.; Mandušić, M.; Rošin, J.; Nazlić, M.; Dunkić, V.; Vitanović, E. Essential Oil Volatile Fingerprint Differentiates Croatian cv. Oblica from Other *Olea europaea* L. Cultivars. *Molecules* **2021**, *26*, 3533. [CrossRef]
27. Dosoky, N.S.; Satyal, P.; Barata, L.M.; Da Silva, J.K.R.; Setzer, W.N.; Silva, D. Volatiles of Black Pepper Fruits (*Piper nigrum* L.). *Molecules* **2019**, *24*, 4244. [CrossRef]
28. Lei, G.; Li, J.; Zheng, T.; Yao, J.; Chen, J.; Duan, L. Comparative Chemical Profiles of Essential Oils and Hydrolate Extracts from Fresh Flowers of Eight Paeonia suffruticosa Andr. Cultivars from Central China. *Molecules* **2018**, *23*, 3268. [CrossRef]
29. Hazzoumi, Z.; Moustakime, Y.; Joutei, K.A. Essential Oil and Glandular Hairs: Diversity and Roles. In *Essential Oils-Oils of Nature*; El-Shemy, H.A., Ed.; IntechOpen: London, UK, 2019; pp. 1–16. [CrossRef]
30. Payne, W.W. A glossary of plant hair thermonology. *Brittonia* **1978**, *30*, 239–255. [CrossRef]
31. Kurer, G.A. *Kutikularfalten Und Protuberanzen an Haaren Und Epidermen*; Druck, Gebr. Leeman: Dresden, Germany, 1917.
32. Kraehmer, H.; Baur, P. *Weed Anatomy*; Wiley-Blackwell: Oxford, UK, 2013; ISBN 978-0-470-65986-1.
33. Werker, E.; Ravid, U.; Putievsky, E. Structure of glandular hairs and identification of the main components of their secreted material in some species of the labiatae. *Isr. J. Bot.* **1985**, *34*, 31–45. [CrossRef]
34. Xiang, C.; Dong, Z.-H.; Peng, H.; Liu, Z.-W. Trichome micromorphology of the East Asiatic genus Chelonopsis (Lamiaceae) and its systematic implications. *Flora Morphol. Distrib. Funct. Ecol. Plants* **2010**, *205*, 434–441. [CrossRef]
35. Seyedi, Z.; Salmaki, Y. Trichome morphology and its significance in the systematics of Phlomoides (Lamiaceae; Lamioideae; Phlomideae). *Flora Morphol. Distrib. Funct. Ecol. Plants* **2015**, *213*, 40–48. [CrossRef]
36. Kristen, U.; Lockhausen, J. The leaf glands of Veronica Beccabunga L.: Ultrastructure and a possible pathway of secretion. *Isr. J. Plant Sci.* **2015**, *34*, 147–156. [CrossRef]
37. Bilušić Vundać, V.; Stabentheiner, E.; Brantner, A.; Plazibat, M. Morphology and distribution of trichomes on leaves in seven Croatian taxa of the genus Stachys (Lamiaceae). *Phyton* **2011**, *51*, 161–176.
38. Haratym, W.; Weryszko-Chmielewska, E. Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare* L. (Lamiaceae). *Flora Morphol. Distrib. Funct. Ecol. Plants* **2017**, *231*, 11–20. [CrossRef]
39. Hanlidou, E.; Kokkini, S.; Bosabalidis, A.M. Glandular trichomes and essential oil constituents of Calamintha menthifolia (Lamiaceae). *Plant Syst. Evol.* **1991**, *177*, 17–26. [CrossRef]
40. Kremer, D.; Vitali Čepo, D.; Dunkić, V.; Dragojević, M.; Ivna Kosalec, I.; Bezić, N.; Stabentheiner, E. Phytochemical and Micromorphological Traits of Geranium dalmaticum and G. macrorrhizum (Geraniaceae). *Nat. Prod. Commun.* **2013**, *8*, 645–650. [CrossRef]
41. Ulusoy, S.; Boşgelmez-Tınaz, G.; Seçilmiş-Canbay, H. Tocopherol, Carotene, Phenolic Contents and Antibacterial Properties of Rose Essential Oil, Hydrosol and Absolute. *Curr. Microbiol.* **2009**, *59*, 554–558. [CrossRef] [PubMed]
42. Vlachou, P.; Stathopoulou, K.; Georgousaki, K.; Lemonakis, N.; Aligiannis, N.; Skaltsounis, A.; Fokialakis, N. Exploitation of agricultural by-products for the recovery of bioactive compounds with applications in cosmetic industry. *Planta Med.* **2015**, *81*, PW_147. [CrossRef]

43. Harput, U.Ş.; Genç, Y.; Khan, N.; Saracoglu, I. Radical scavenging effects of different Veronica species. *Rec. Nat. Prod.* **2011**, *5*, 100–107.
44. Harput, U.S.; Saracoglu, I.; Inoue, M.; Ogihara, Y. Anti-inflammatory and Cytotoxic Activities of Five Veronica Species. *Biol. Pharm. Bull.* **2002**, *25*, 483–486. [CrossRef]
45. Aazza, S. Antioxidant activity of some Morrocan hydrosols. *J. Med. Plants Res.* **2011**, *5*. [CrossRef]
46. Kaur, N.; Chahal, K.K.; Kumar, A.; Singh, R.; Bhardwaj, U. Antioxidant activity of Anethum graveolens L. essential oil constituents and their chemical analogues. *J. Food Biochem.* **2019**, *43*, e12782. [CrossRef] [PubMed]
47. National Center for Biotechnology Information PubChem Compound Summary for CID 6428442, trans-1(7), 8-p-Menthadien-2-ol. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/trans-1_7_8-p-Menthadien-2-ol (accessed on 30 July 2021).
48. Amorati, R.; Foti, M.C.; Valgimigli, L. *Antioxidant Activity of Essential Oils. Journal of Agricultural and Food Chemistry*; American Chemical Society: Washington, DC, USA, 2018; Volume 61. [CrossRef]
49. Huang, Z.; Pang, D.; Liao, S.; Zou, Y.; Zhou, P.; Li, E.; Wang, W. Synergistic effects of cinnamaldehyde and cinnamic acid in cinnamon essential oil against *S. pullorum*. *Ind. Crop. Prod.* **2021**, *162*, 113296. [CrossRef]
50. Moreno-Escobar, J.A.; Bazaldúa, S.; Villarreal, M.L.; Bonilla-Barbosa, J.R.; Mendoza, S.; López, V.R. Cytotoxic and antioxidant activities of selected Lamiales species from Mexico. *Pharm. Biol.* **2011**, *49*, 1243–1248. [CrossRef]
51. Saracoglu, I.; Oztunca, F.H.; Nagatsu, A.; Harput, U.S. Iridoid content and biological activities ofVeronica cuneifolia-subsp.cuneifoliaandV. cymbalaria. *Pharm. Biol.* **2011**, *49*, 1150–1157. [CrossRef]
52. Xue, H.; Chen, K.X.; Zhang, L.Q.; Li, Y.M. Review of the Ethnopharmacology, Phytochemistry, and Pharmacology of the Genus Veronica. *Am. J. Chin. Med.* **2019**, *47*, 1193–1221. [CrossRef] [PubMed]
53. Pandey, K.B.; Rizvi, S.I. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
54. Quideau, S.; Deffieux, D.; Douat, C.; Pouységu, L. Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis. *Angew. Chem. Int. Ed.* **2011**, *50*, 586–621. [CrossRef] [PubMed]
55. Babushok, V.I.; Linstrom, P.J.; Zenkevich, I.G. Retention Indices for Frequently Reported Compounds of Plant Essential Oils. *J. Phys. Chem. Ref. Data* **2011**, *40*, 043101. [CrossRef]
56. Kremer, D.; Muller, I.; Dunkic, V.; Vitali, D.; Stabentheiner, E.; Oberländer, A.; Bezić, N.; Kosalec, I. Chemical traits and antimicrobial activity of endemic Teucrium arduini L. from Mt Biokovo (Croatia). *Open Life Sci.* **2012**, *7*, 941–947. [CrossRef]
57. Špika, M.J.; Žanetić, M.; Kraljić, K.; Soldo, B.; Ljubenkov, I.; Politeo, O.; Škevin, D. Differentiation Between Unfiltered and Filtered Oblica and Leccino cv. Virgin Olive Oils. *J. Food Sci.* **2019**, *84*, 877–885. [CrossRef]
58. Grubešić, R.J.; Vuković, J.; Kremer, D.; Vladimir-Knežević, S. Spectrophotometric method for polyphenols analysis: Prevalidation and application on Plantago L. species. *J. Pharm. Biomed. Anal.* **2005**, *39*, 837–842. [CrossRef]
59. Rodríguez, J.V.; Grubešić, R.J.; Kremer, D.; Kokot, V. Quality Assessment of Two Spectrophotometric Procedures for Polyphenol Determination and Application inMoltkia petraeaSpecies. *J. Chin. Chem. Soc.* **2016**, *63*, 677–687. [CrossRef]
60. *Council of Europe European Pharmacopoeia*, 4th ed.; European Directorate for the Quality of Medicines and HealthCare (EDQM): Strasbourg, France, 2014.
61. Fredotović, Ž.; Šprung, M.; Soldo, B.; Ljubenkov, I.; Budić-Leto, I.; Bilušić, T.; Čikeš-Čulić, V.; Puizina, J. Chemical Composition and Biological Activity of Allium cepa L. and Allium × cornutum (Clementi ex Visiani 1842) Methanolic Extracts. *Molecules* **2017**, *22*, 448. [CrossRef] [PubMed]
62. Mensor, L.L.; Menezes, F.S.; Leitão, G.G.; Reis, A.; Dos Santos, T.C.; Coube, C.S.; Leitão, S.G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother. Res.* **2001**, *15*, 127–130. [CrossRef] [PubMed]
63. Payet, B.; Sing, A.S.C.; Smadja, J. Assessment of Antioxidant Activity of Cane Brown Sugars by ABTS and DPPH Radical Scavenging Assays: Determination of Their Polyphenolic and Volatile Constituents. *J. Agric. Food Chem.* **2005**, *53*, 10074–10079. [CrossRef] [PubMed]

Table S1. Chemical composition of the essential oil from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii* calculated based on the masses of EOs extracted from dry plant material.

			Mr	St	Br	GJ
Component	**RI[1]**	**RI[2]**	**EO ± SD (mg/g dry plant material)**	**EO ± SD (mg/g dry plant material)**	**EO ± SD (mg/g dry plant material)**	**EO ± SD (mg/g dry plant material)**
Sesquiterpene hydrocarbons			**0.056**	**0.358**	**0.143**	**0.165**
E-Caryophyllene*	1424	1585	0.012 ± 0.00038	0.109 ± 0.00047	0.078 ± 0.00051	0.138 ± 0.00064
δ-Cadinene	1517	1745	0.044 ± 0.00038	0.086 ± 0.00047	0.051 ± 0.00103	0.027 ± 0.00064
allo-Aromadendrene	1465	1662	-	0.041 ± 0.00047	-	-
β-Chamigrene	1476	1724	-	-	0.014 ± 0.00103	-
Germacrene D	1482	1692	-	0.122 ± 0.00233	-	-
Oxygenated sesquiterpenes			**2.014**	**1.443**	**1.505**	**1.529**
Spathulenol	1577	2101	-	0.086 ± 0.00047	0.023 ± 0.00051	0.035 ± 0.00064
β-Caryophyllene oxide*	1581	1955	0.017 ± 0.00038	0.029 ± 0.00047	0.025 ± 0.00051	-
γ-Eudesmol	1632	2175	-	0.012 ± 0.0014	-	-
α-Bisabolol oxide	1748	2511	-	0.017 ± 0.00047	-	-
Hexahydrofarnesyl acetone	1839	2113	1.997 ± 0.00038	1.299 ± 0.00047	1.457 ± 0.00051	1.494 ± 0.00064
Phenolic compounds			**0.024**	**0.125**	**0.043**	**0.052**
Methyl eugenol	1403	2005	-	0.0588 ± 0.00047	-	-
(Z)-Methyl isoeugenol	1451	2070	0.024 ± 0.00114	0.066 ± 0.00047	0.043 ± 0.00051	0.052 ± 0.00064
Acids, alcohols and esters			**1.341**	**2.226**	**2.969**	**4.001**
1-Hexadecanol	1874	2371	-	0.027 ± 0.0014	-	-
Hexadecanoic acid	1959	2912	1.015 ± 0.00076	2.199 ± 0.000467	2.804 ± 0.00257	3.770 ± 0.00192
Oleic acid	2133	2998	0.089 ± 0.00038	-	0.026 ± 0.00154	-
Octadecanol acetate	2209	2211	0.237 ± 0.00038	-	0.116 ± 0.00051	0.231 ± 0.00064
1-Heptatriacotanol	2309	2309	-	-	0.023 ± 0.00051	-
Hydrocarbons			**0.06114**	**0.095**	**0.129**	**0.131**
Eicosane*	2000	2000	-	0.054 ± 0.00047	0.023 ± 0.00051	0.079 ± 0.00256
Heneicosane*	2100	2100	0.02014 ± 0.00076	0.016 ± 0.00047	-	0.034 ± 0.00064
Docosane*	2200	2200	0.014 ± 0.00038	0.014 ± 0.00047	0.042 ± 0.00051	-
Tricosane*	2300	2300	-	-	0.032 ± 0.00051	-
Tetracosane*	2400	2400	-	0.011 ± 0.00047	0.032 ± 0.00051	0.018 ± 0.00064
Pentacosane*	2500	2500	0.027 ± 0.00152	-	-	-
Total identification (mg/g)			**3.496**	**4.247**	**4.789**	**5.878**

Retention indices (RI) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) [24] and CPWax 52 (RI^2) [25]; Identification method: RI, comparison of RIs with those listed in a homemade library, reported in the literature [24], and/or authentic samples; comparison of mass spectra with those in mass spectral libraries NIST02 and Wiley 9; * co-injection with reference compounds; SD, standard deviation.

Table S2. Chemical composition of the hydrosols from four locations from aerial parts of *Veronica austriaca* ssp. *jacquinii* calculated based on the masses of volatile compounds extracted from hydrosols from dry plant material

			Mr	St	Br	GJ
Component	**RI[1]**	**RI[2]**	**Hy ± SD (mg/g dry plant material)**	**Hy ± SD (mg/g dry plant material)**	**Hy ± SD (mg/g dry plant material)**	**Hy ± SD (mg/g dry plant material)**
Monoterpene hydrocarbons			**0.047**	**-**	**-**	**-**
α-Thujene	924	1032	0.023 ± 0.00028	-	-	-
β -Phellandrene	1002	1194	0.024 ± 0.00083	-	-	-
Oxygenated monoterpenes			**0.585**	**0.708**	**0.238**	**0.679**
trans -Linalool oxide*	1088	1434	0.010 ± 0.00110	-	-	-
n-Nonanal	1100	1389	0.120 ± 0.00028	0.118 ± 0.00042	-	-
Borneol	1176	1719	0.043 ± 0.00028	0.021 ± 0.00042	-	-
Camphor	1151	1499	0.060 ± 0.00028	0.039 ± 0.00042	-	0.160 ± 0.00045
Pinocarvone	1160	1565	0.055 ± 0.00028	-	-	-
trans-p-Mentha-1(7),8-dien-2-ol	1187	1803	0.212 ± 0.00028	0.220 ± 0.00042	0.220 ± 0.00030	0.288 ± 0.00090
Hexyl 2-methyl butanoate	1233	1425	0.035 ± 0.00028	0.131 ± 0.00126	-	0.197 ± 0.00045
Menthyl acetate	1294	1550	0.050 ± 0.00083	0.179 ± 0.00042	0.018 ± 0.00030	0.034 ± 0.00271
Sesquiterpene hydrocarbons			**0.189**	**0.155**	**0.047**	**0.300**
E-Caryophyllene*	1424	1585	0.073 ± 0.00028	0.056 ± 0.00042	0.019 ± 0.00059	0.033 ± 0.00045
δ-Cadinene	1517	1745	0.065 ± 0.00028	-	0.028 ± 0.00177	0.108 ± 0.00362
allo-Aromadendrene	1465	1662	0.042 ± 0.00028	-	-	0.056 ± 0.00045
β-Chamigrene	1478	1724	0.009 ± 0.00028	-	-	-
Germacrene D	1482	1692	-	0.099 ± 0.00042	-	0.103 ± 0.00045
Oxygenated sesquiterpenes			**0.088**	**0.398**	**0.285**	**0.194**
Spathulenol	1577	2101	-	-	-	0.056 ± 0.00045
β-Caryophyllene oxide*	1581	1955	0.060 ± 0.00028	0.053 ± 0.00042	0.033 ± 0.00030	0.023 ± 0.00045
γ-Eudesmol	1632	2175	-	-	-	-
α-Muurolol	1645	2181	-	0.052 ± 0.00042	-	-
α-Cadinol	1655	2208	-	0.103 ± 0.00042	-	-
α-Bisabolol	1685	2210	0.015 ± 0.00083	-	0.015 ± 0.00030	0.060 ± 0.00045
α-Bisabolol oxide	1748	2511	-	-	0.009 ± 0.00030	0.023 ± 0.00045
Hexahydrofarnesyl acetone	1839	2113	0.013 ± 0.00028	0.190 ± 0.00168	0.228 ± 0.00059	0.032 ± 0.00045
Phenolic compounds			**1.197**	**1.582**	**1.854**	**2.243**
Thymol*	1289	2154	0.230 ± 0.00138	0.397 ± 0.00084	0.103 ± 0.00030	0.189 ± 0.00045
Thymol acetate	1349	-	0.101 ± 0.00028	0.095 ± 0.00042	-	0.110 ± 0.00136
Methyl eugenol	1403	2005	0.834 ± 0.00055	0.980 ± 0.00042	1.713 ± 0.00030	1.891 ± 0.00045
(Z)-Methyl isoeugenol	1451	2070	0.032 ± 0.00028	0.110 ± 0.00252	0.038 ± 0.00030	0.053 ± 0.00045
Acids, alcohols and esters			**0.191**	**0.557**	**0.206**	**0.281**
1-Hexadecanol	1874	2371	-	-	0.072 ± 0.00030	-
Hexadecanoic acid	1959	2912	0.126 ± 0.00028	0.264 ± 0.00084	0.067 ± 0.00030	0.085 ± 0.00045
Oleic acid	2133	2998	0.008 ± 0.00028	0.204 ± 0.00042	0.014 ± 0.00030	0.171 ± 0.00045
Octadecanol acetate	2209	-	0.042 ± 0.00028	0.050 ± 0.00042	0.017 ± 0.00059	0.025 ± 0.00045
1-Heptatriacotanol	2309	2309	0.015 ± 0.00028	0.039 ± 0.00042	0.036 ± 0.00030	-
Hydrocarbons			**0.279**	**0.380**	**0.114**	**0.461**
Eicosane*	2000	2000	0.042 ± 0.00110	-	0.013 ± 0.00030	0.062 ± 0.00045

Heneicosane*	2100	2100	0.020 ± 0.00028	-	0.009 ± 0.00030	0.025 ± 0.00271
Docosane*	2200	2200	0.032 ± 0.00028	-	0.011 ± 0.00030	0.054 ± 0.00045
Tricosane*	2300	2300	0.017 ± 0.00028	0.036 ± 0.00084	-	-
Tetracosane*	2400	2400	-	0.020 ± 0.00042	0.026 ± 0.00030	0.031 ± 0.00045
Pentacosane*	2500	2500	0.018 ± 0.00028	0.010 ± 0.00042	-	-
Hexacosane*	2600	2600	0.070 ± 0.00028	0.129 ± 0.00042	0.029 ± 0.00059	0.038 ± 0.00136
Heptacosane*	2700	2700	0.087 ± 0.00028	0.135 ± 0.00042	0.009 ± 0.00030	0.047 ± 0.00045
Octacosane*	2800	2800	0.025 ± 0.00028	0.171 ± 0.00042	0.017 ± 0.00059	0.179 ± 0.00045
Total identification (mg/g)			**2.576**	**3.670**	**2.744**	**4.158**

Retention indices (RI) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) [24] and CPWax 52 (RI^2) [25]; Identification method: RI, comparison of RIs with those listed in a homemade library, reported in the literature [24], and/or authentic samples; comparison of mass spectra with those in mass spectral libraries NIST02 and Wiley 9; * co-injection with reference compounds; SD, standard deviation.

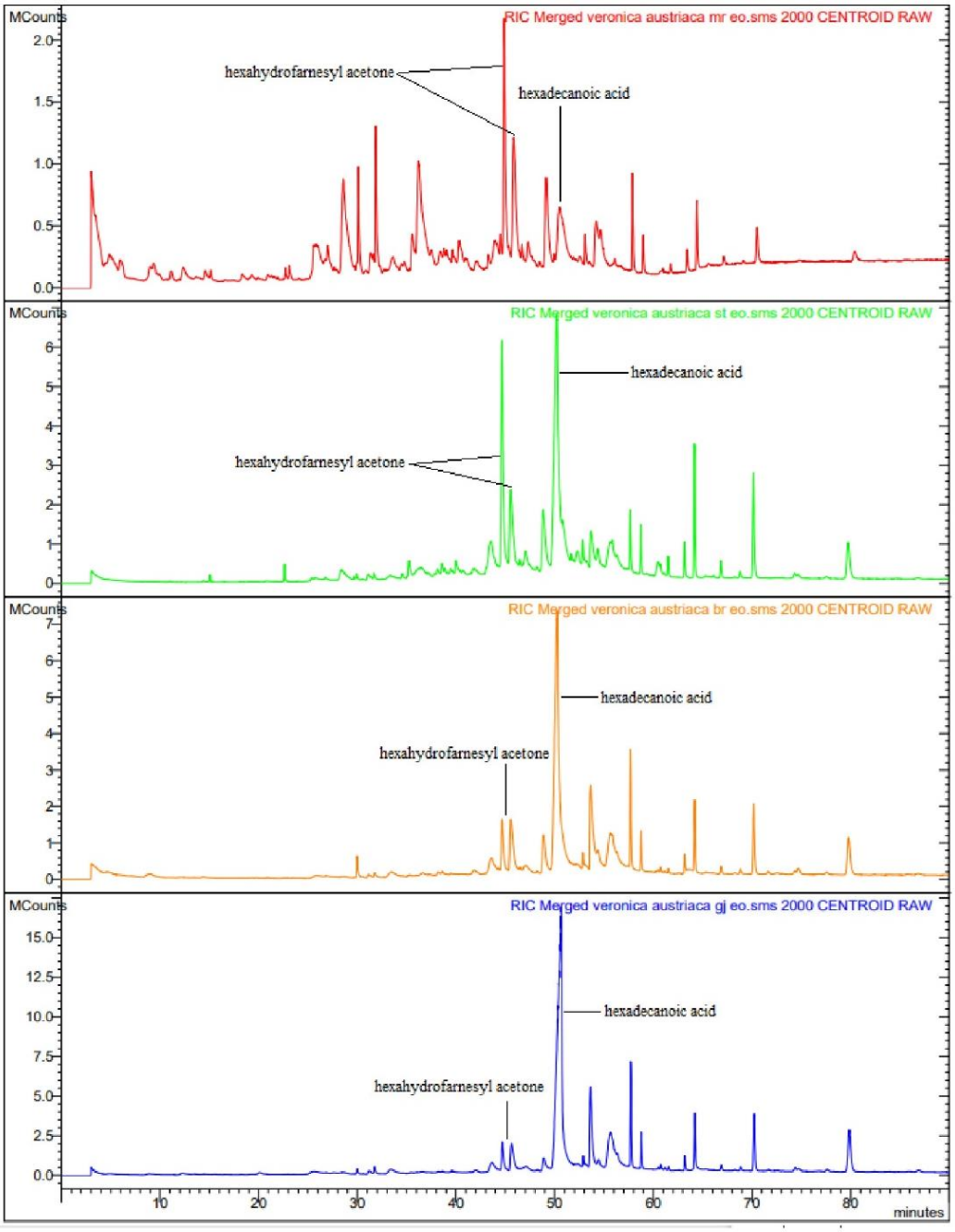

Figure S1. RIC chromatograms of the four samples of EOs of *V. jacquinii* with marked major compounds

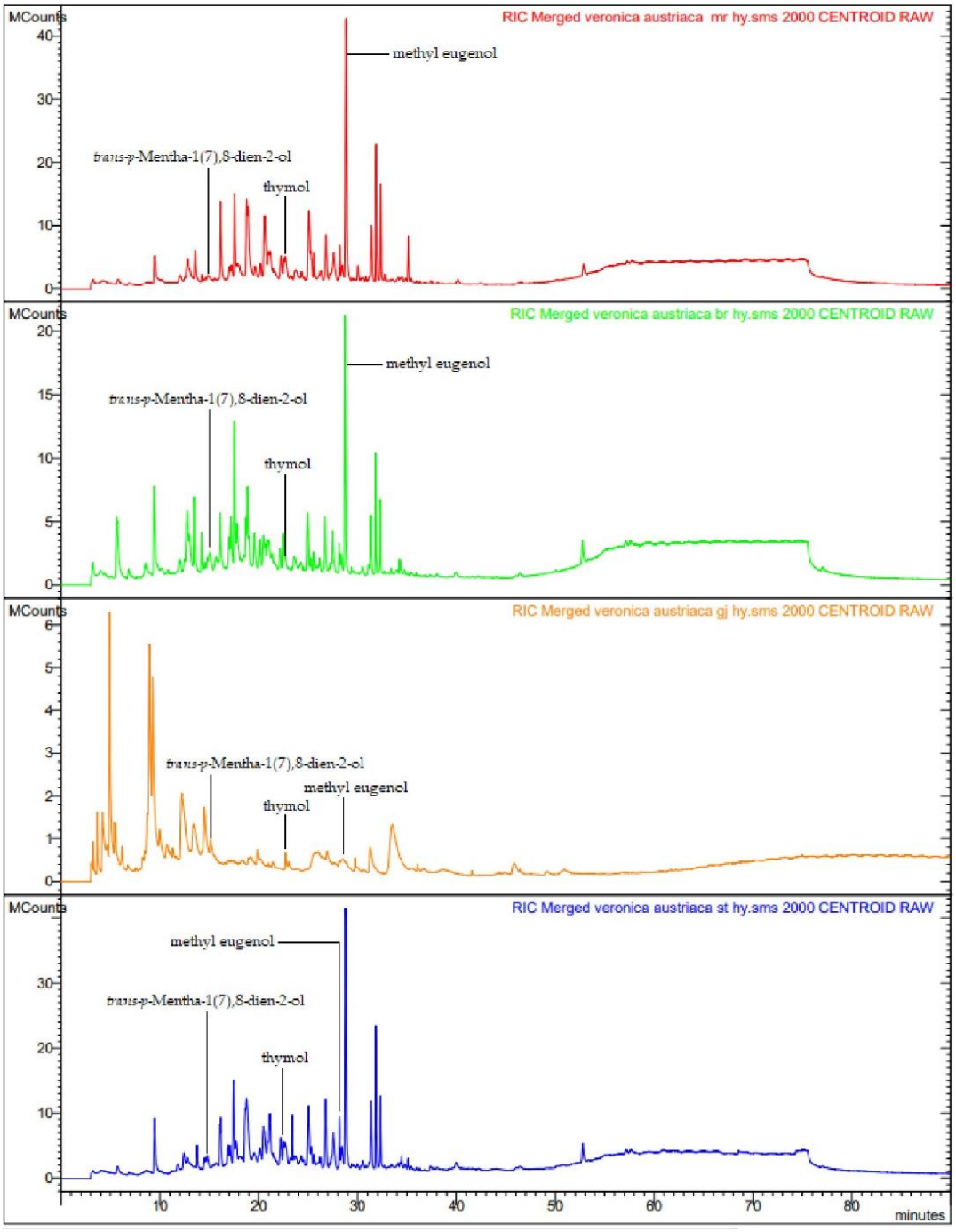

Figure S2. RIC chromatograms of the four samples of hydrosols of *V. jacquinii* with marked major compounds

3.4. Hydrodistillation and Microwave Extraction of Volatile Compounds: Comparing Data for Twenty-One *Veronica* Species from Different Habitats

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Hydrodistillation and Microwave Extraction of Volatile Compounds: Comparing Data for Twenty-One *Veronica* Species from Different Habitats

Valerija Dunkić [1], Marija Nazlić [1], Mirko Ruščić [1], Elma Vuko [1], Karla Akrap [1], Snježana Topić [1], Milenko Milović [2], Nenad Vuletić [1], Jasna Puizina [1], Renata Jurišić Grubešić [3], Siniša Srečec [4] and Dario Kremer [5,*]

[1] Faculty of Science, University of Split, Rudera Boškovića 33, 21000 Split, Croatia; dunkic@pmfst.hr (V.D.); mnazlic@pmfst.hr (M.N.); mrus@pmfst.hr (M.R.); elma@pmfst.hr (E.V.); kakrap@pmfst.hr (K.A.); topic@pmfst.hr (S.T.); nenov@pmfst.hr (N.V.); puizina@pmfst.hr (J.P.)
[2] Antun Vrančić Grammar School, Put Gimnazije 64, 22000 Šibenik, Croatia; milenko.milovic@skole.hr
[3] Faculty of Medicine, University of Rijeka, Braće Branchetta 20, 51000 Rijeka, Croatia; renatajg@medri.uniri.hr
[4] Križevci College of Agriculture, Milislava Demerca 1, 48260 Križevci, Croatia; ssrecec@vguk.hr
[5] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia
* Correspondence: dkremer@pharma.hr; Tel.: +38-514-619-422

Abstract: Free volatile compounds were isolated from 21 Croatian *Veronica* species studied by hydrodistillation (HD) and microwave extraction (ME) and analyzed by gas chromatography coupled with mass spectrometry. Principal Component Analysis (PCA) distinguished some clusters based on the relative proportion of major compounds, such as hexadecanoic acid, hexahydrofarnesyl acetone, phytol, *E*-caryophyllene, and caryophyllene oxide, which were identified in all species studied by both isolation methods. In addition to these compounds, germacrene D, δ-selinene, and eicosane were also identified in five samples from dry habitats isolated using ME. *Allo*-aromadendrene and β-ionone are particularly abundant in five species from wet habitats isolated by both methods. The peculiarities of *Veronica* species from moderate habitats isolated with HD are benzene acetaldehyde, *n*-nonanal, and the identification of significant compounds from the hydrocarbon class, while the peculiarity of ME is (*E*)-β-damascenone. In this article, we present new results on the phytochemical characterization of *Veronica* species from different habitats. The biological potential of these compounds should be further investigated for a better understanding and utilization of the specialized plant metabolites.

Keywords: *Veronica*; volatile compounds; microwave extraction; GC-MS; hexahydrofarnesyl acetone; hexadecanoic acid; phytol

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1. Introduction

The genus *Veronica* L. from the family Plantaginaceae (formerly Scrophulariaceae) (order Lamiales) includes about 500 species that are distributed slightly more over the Northern Hemisphere [1]. A significant number of species (approximately 180) with the *Hebe* complex are spread throughout the Southern Hemisphere, i.e., New Zealand, Australia, and New Guinea [2]. Sixty-two *Veronica* species have been described for Europe [3] and thirty-seven for Croatia [4]. *Veronica* species are characterized by extreme variability in morphology, life forms, and habitats [2]. The ability to adapt to different living conditions has allowed these species to inhabit a variety of habitats, from aquatic and wetland habitats to rocky and dry habitats [2,5,6]. Most representatives of the genus *Veronica* grow in areas with a Mediterranean climate and from the sea level to high alpine regions [2]. *Veronica* species are herbs with a sometimes-woody stock and opposite low leaves. The floral leaves usually alternate. Solitary flowers develop in the leaf-axils or are arranged in axillary or terminal racemes. The calyx is divided into four or five, often unequal, segments. The

corolla is rotated to the campanulate and different in color. There are two exerted stamens and one pistil in the flowers. The fruit is a capsule [3].

It is well known that plants are exposed to many environmental stresses, such as droughts, extreme temperatures, nutrient deficiencies, fires, flooding, salinity, excessive amounts of heavy metals, insect attacks, and various pathogenic microorganisms. These stresses affect their growth, development, and reproduction. As a response to environment stresses, weather conditions, and pathogen attacks, they produce specialized metabolites [7]. Tan and Nishida [8] found that phenylpropanoids, such as methyl eugenol and Z-methyl isoeugenol, occur in plants under the influence of pathogen attacks and ultraviolet radiation.

Phytochemical studies on species of the genus *Veronica* have mostly been focused on the content of glycosides, phenols, and flavonoids [9–17]. The free volatile compounds (VCs) of the *Veronica* species, on the other hand, have been much less studied [6,14,18–21]. One of the reasons for this is that research on VCs has mainly focused on plant families that are commonly known to be rich sources of these compounds, such as Lamiaceae, Geraniaceae, Asteraceae, Rutaceae, Lauraceae, and Myrtaceae.

The isolation of free VCs, which are important specialized metabolites of plants, can be done by classical and green extraction. Classical extraction techniques include steam distillation, hydro-diffusion, hydrodistillation, destructive distillation, and cold pressing. Green extraction techniques include turbo distillation, ultrasonic-assisted extraction, microwave-assisted extraction, and instant controlled pressure drop technology. Depending on the isolation technique, the composition of the essential oil extracted from the same plant material may vary. This is influenced by the duration of the distillation, the temperature, the pressure, and the quality of the plant material. Green extraction requires less time and less water than traditional extraction [22].

In this paper, we describe the phytochemical characterization of free VCs obtained by classical hydrodistillation (HD) and green microwave extraction (ME) from twenty-one *Veronica* species distributed in Croatia. The studied species of the genus *Veronica* were grouped by habitat so that the results could be easily summarized. The aim of this work was to obtain new data on the characterization of VCs as specialized metabolites. Qualitative and quantitative differences in the composition of these compounds can be identified using different isolation techniques, and the identification of these differences is very important for further biological research. For most of the studied *Veronica* species, data on the composition of volatile compounds are presented for the first time.

2. Results

2.1. Investigation of Veronica Species

Twenty-one species of the genus *Veronica* collected through field research were classified into three groups of habitats based on the general humidity of the habitat (dry, wet, and moderate). VCs isolated by classical HD and green ME were analyzed by gas chromatography (GC) coupled with mass spectrometry (MS) and the results are shown in Supplementary Tables S1–S6. In Tables S1–S6, the identified compounds are arranged according to the time of occurrence on the nonpolar capillary column (VF5-ms) and according to the eight corresponding classes (monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, oxygenated diterpenes, phenolic compounds, hydrocarbons, and a common group of acids, alcohols, and esters).

2.2. Volatile Compounds of Veronica Species from Dry Habitats

The five *Veronica* species (*V. austriaca* L. ssp. *austriaca*, *V. austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch., *V. cymbalaria* Bodard, *V. dalmatica* Padilla-García, Rojas-Andrés, López-González et M. M. Mart. Ort., and *V. saturejoides* Vis. ssp. *saturejoides*) from dry habitats were studied (Figure 1). The term 'dry habitat' encompasses *Veronica* species that grow in open, rocky, sunny, or dry areas. The compound classes shown in Figure 2 for these studied species are listed according to Supplementary Tables S1 and S2, which compare the two isolation methods. The main classes with a percentage of identification greater than 50% are

oxygenated sesquiterpenes with 60.16% in *V. cymbalaria* obtained by HD and sesquiterpene hydrocarbons with 57.8% in *V. dalmatica* obtained by ME (Figure 2).

Figure 1. Photographs of *Veronica* taxa collected from dry habitats: *V. austriaca* ssp. *austriaca* (**a**), *V. austriaca* ssp. *jacquinii* (**b**), *V. cymbalaria* (**c**), *V. dalmatica* (**d**), and *V. saturejoides* ssp. *saturejoides* (**e**).

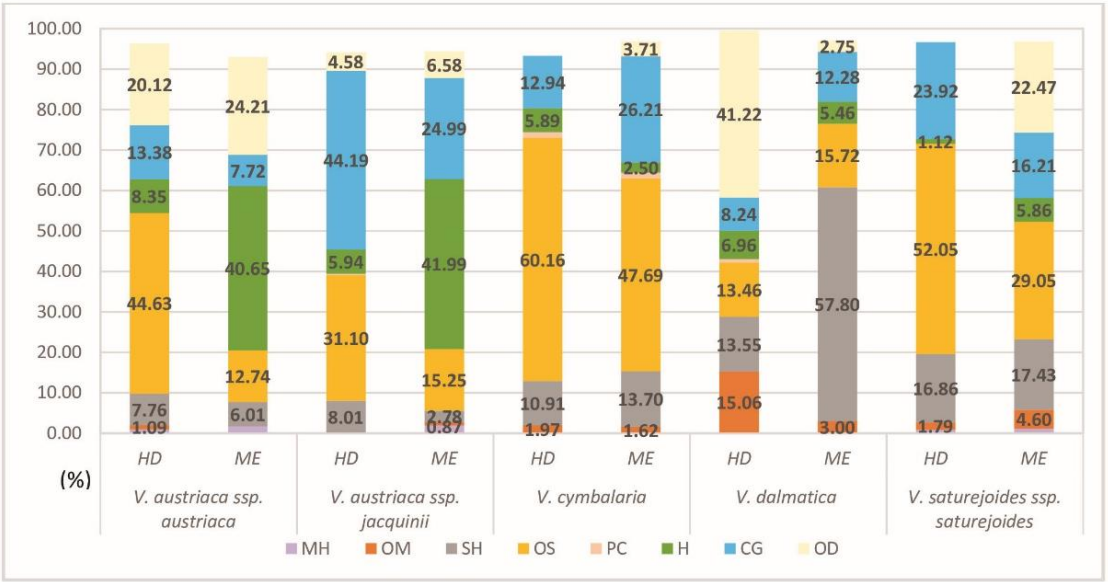

Figure 2. Relative content of volatile compounds in *Veronica* taxa collected from dry habitats. HD, hydrodistillation; ME, microwave extraction; MH, monoterpene hydrocarbons; OM, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; OS, oxygenated sesquiterpenes; PC, phenolic compounds; H, hydrocarbons; CG, common group of acids, alcohols, and esters; OD, oxygenated diterpenes.

The main constituents in the composition of VCs obtained by HD (Supplementary Table S1) are the following: The oxygenated sesquiterpene hexahydrofarnesyl acetone is the most abundant compound identified in *V. austriaca* ssp. *austriaca* (39.77%) and *V. cymbalaria* (36.33%). Hexadecanoic acid is the most abundant compound in *V. austriaca* ssp. *jacquinii* (32.17%). In the composition of the endemic species *V. dalmatica*, the oxygenated diterpene phytol is the most abundant with a percentage of identification of 41.22% (Figure 2), while in the composition of *V. saturejoides* ssp. *saturejoides* caryophyllene oxide is the most abundant (34.53%).

In the composition of VCs obtained via ME, phytol is the most abundant compound identified in *V. austriaca* ssp. *austriaca* (24.21%) and in *V. saturejoides* ssp. *saturejoides* (22.47%), while hexadecanoic acid is the most abundant compound identified in *V. austriaca* ssp. *jacquinii* (22.12%) (Figure 2; Supplementary Table S2). Caryophyllene oxide is the major compound in *V. cymbalaria* (32.72%), while *E*-caryophyllene is the most abundant compound in *V. dalmatica* (39.53%).

2.3. Volatile Compounds of Veronica Species from Wet Habitats

Five *Veronica* species (*V. anagallis-aquatica* L., *V. anagalloides* Guss., *V. beccabunga* L., *V. catenata* Pennell, and *V. longifolia* L.) were also collected from wet habitats (Figure 3). The term 'wet habitat' encompasses species that grow in lake or stream water. The compound classes for these studied species are shown in Figure 4, which compares the two isolation methods. Hexahydrofarnesyl acetone is the major compound in *V. anagallis-aqua*tica isolated by HD and ME (27.17% and 25.97%, respectively) (Supplementary Tables S3 and S4). It is also the predominant compound in *V. anagalloides* identified by both extraction methods (14.33% for HD and 19.12% for ME). In the same species, hexadecanoic acid was identified with a significant percentage (13.67%) by HD. The oxygenated diterpene phytol is the dominant compound in *V. beccabunga* (27.31% for HD and 34.54% for ME), *V. catenata* (29.92% for HD and 42.26% for ME), and *V. longifolia* (13.63% for HD and 37.18% for ME) isolated by both methods. *E*-caryophyllene, caryophyllene oxide, hexahydrofarnesyl acetone, phytol, hexadecanoic acid, and β-ionone were identified in all five *Veronica* species collected from wetland habitats. Monoterpene hydrocarbons were not isolated during green extraction in *Veronica* species from wet habitats (Supplementary Table S4).

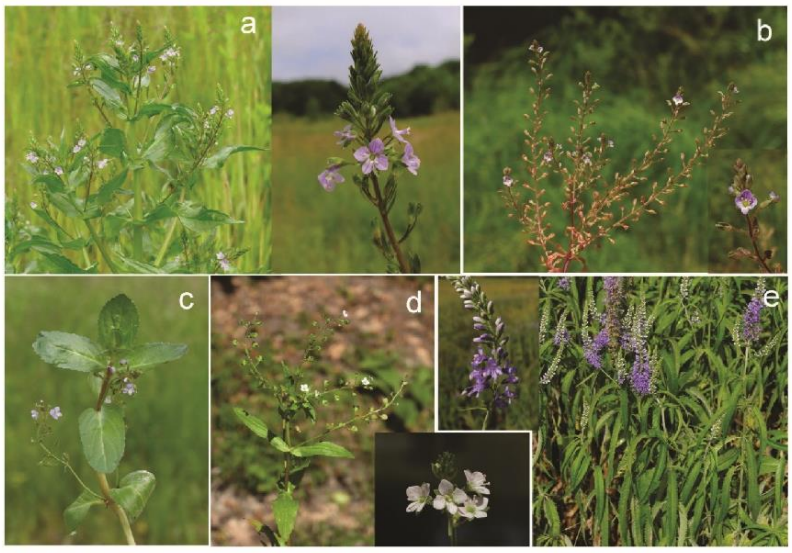

Figure 3. Photographs of *Veronica* taxa collected from wet habitats: *V. anagallis-aquatica* ssp. *anagallis-aquatica* (**a**), *V. anagalloides* (**b**), *V. beccabunga* (**c**), *V. catenata* (**d**), and *V. longifolia* (**e**).

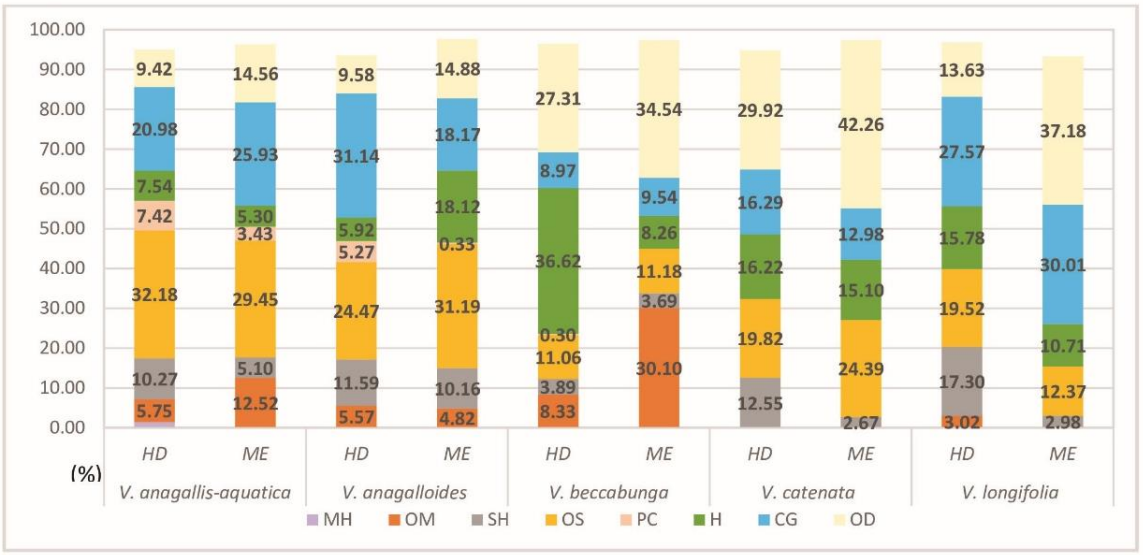

Figure 4. Relative content of volatile compounds in *Veronica* taxa collected from wet habitats. MH, monoterpene hydrocarbons; OM, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; OS, oxygenated sesquiterpenes; PC, phenolic compounds; H, hydrocarbons; CG, common group of acids, alcohols, and esters; OD, oxygenated diterpenes.

2.4. Volatile Compounds of Veronica Species from Moderate Habitats

Eleven species of the genus *Veronica* (*V. acinifolia* L., *V. arvensis* L., *V. chamaedrys* L., *V. hederifolia* L., *V. montana* L., *V. officinalis* L., *V. opaca* Fr., *V. persica* Poir., *V. polita* Fr., *V. serpyllifolia* L., and *V. urticifolia* Jacq.) were collected from moderate habitats (Figure 5). The term 'moderate habitat' encompasses Veronica species that grow in vineyards (*V. acinifolia*), orchards (*V. chamaedrys*), arable land (*V. arvensis*, *V. hederifolia*, *V. opaca*, *V. persica*, *V. polita*, and *V. serpyllifolia*), and mesophilic beech forests (*V. montana*, *V. officinalis*, and *V. urticifolia*). The isolation of the VCs was also carried out by both methods. The compound classes for the studied species are shown in Figure 6, which compares the two isolation methods. *E*-caryophyllene, caryophyllene oxide, hexahydrofarnesyl acetone, phytol, β-ionone, hexadecanoic acid, and docosane were identified in all eleven isolates obtained by HD (Supplementary Table S5). *V. acinifolia* is rich in β-ionone (17.01%), hexahydrofarnesyl acetone (15.37%), and caryophyllene oxide (7.71%). Hexahydrofarnesyl acetone (28.85%) is the most abundant compound in the composition of VCs in *V. hederifolia*. Additionally, the monoterpene hydrocarbon α-thujene (4.86%) was identified only in *V. hederifolia*. A peculiarity is the identification of γ-eudesmol in a high percentage (19.98%) only in *V. arvensis*. *V. chamaedrys*, *V. montana*, *V. persica*, *V. polita*, *V. serpyllifolia*, and *V. urticifolia* are rich in phytol (particularly *V. urticifolia* with an identification percentage of 47.55%). These species are also rich in heptacosane, as is *V. officinalis* with an identification percentage of 20.67%. Heptacosane, *E*-caryophyllene, and caryophyllene oxide were also the most abundant compounds in the composition of VCs obtained using HD from *V. opaca* with percentages greater than 11% (Supplementary Table S5).

Figure 5. Photographs of Veronica species collected from moderate habitats: *V. acinifolia* (**a**), *V. arvensis* (**b**), *V. chamaedrys* (**c**), *V. hederifolia* (**d**), *V. montana* (**e**), *V. officinalis* (**f**), *V. opaca* (**g**), *V. persica* (**h**), *V. polita* (**i**), *V. serpyllifolia* (**j**), and *V. urticifolia* (**k**).

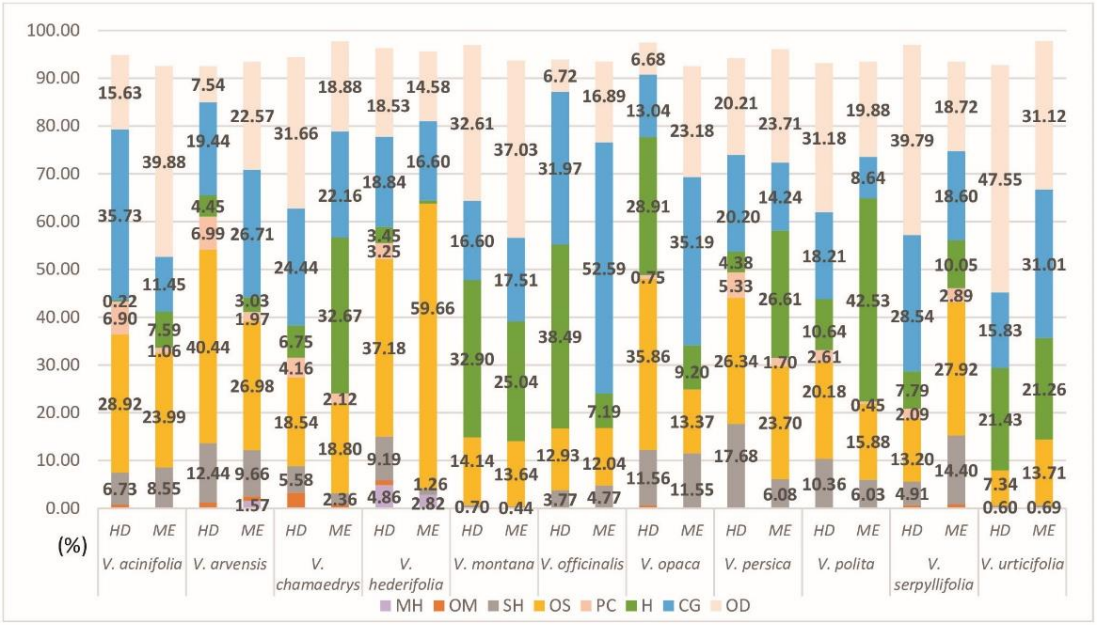

Figure 6. Relative content of volatile compounds in *Veronica* species collected from moderate habitats. MH, monoterpene hydrocarbons; OM, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; OS, oxygenated sesquiterpenes; PC, phenolic compounds; H, hydrocarbons; CG, common group of acids, alcohols, and esters; OD, oxygenated diterpenes.

The same major components identified in the isolates using HD are also predominant in the composition of the VCs isolated by ME in the 11 *Veronica* species collected from moderate habitats (Supplementary Table S6). The distinctive features are the high percentage of the hydrocarbon pentacosane (14.9%) identified in *V. montana* and the high percentage of heptacosane identified in *V. persica* and *V. polita* (14.28% and 15.13%, respectively). Another feature is that the isolate obtained by green extraction from *V. polita* is rich in octacosane (16.95%). The compound 3-hexen-*1-ol* was identified (22.04%) only in *V. officinalis* from isolates obtained by ME, and this component was not identified in other *Veronica* species studied either by classical or green extraction.

2.5. Principal Component Analysis Analyses of Volatile Compounds

Principal Component Analysis (PCA) analyses were performed for VCs with amounts greater than 2%. Separate analyses were performed for the classical (Figure 7) and microwave (Figure 8) extraction methods. Principal Component (PC)1 and PC2 for VCs from the Clevenger extraction explained 62.19% of the variance and distinguished four clusters. The first cluster consists of *V. austriaca* ssp. *austriaca*, *V. cymbalaria*, *V. hederifolia*, and *V. anagallis-aquatica* (Figure 7a). All of these species are characterized by a high relative content of hexahydrofarnesyl acetone (27.17–39.77%). This component is located in the negative region of PC1 and the positive region of PC2 (Figure 7b). The second cluster consists of *V. anagalloides*, *V. austriaca* ssp. *jacquinii*, *V. acinifolia*, *V. persica*, *V. longifolia*, *V. opaca*, and *V. arvensis*. These species are located around the center of the PCA score plot. They are all characterized by a moderate relative amount of hexadecanoic acid, hexahydrofarnesyl acetone, and phytol. The third cluster consists of *V. catenata*, *V. dalmatica*, *V. chamaedrys*, *V. polita*, *V. serpyllifolia*, *V. urticifolia*, *V. montana*, and *V. beccabunga*. The component that differentiates this cluster is phytol, which is located in the positive region of PC1 and PC2 (Figure 7b). The fourth cluster consists of two species: *V. saturejoides* ssp. *saturejoides* and *V. officinalis*. *V. saturejoides* ssp. *saturejoides* is characterized by a high relative concentration of caryophyllene oxide (34.53%). On the other hand, *V. officinalis* is characterized by a higher relative abundance of β-ionone, hexadecanoic acid, and heptacosane.

PC1 and PC2 for volatile compounds obtained by microwave extraction explained 51.23% of the variance. PCA analyses for volatile compounds obtained by microwave extraction gave no distinguishing features; however, we can point out some similarities among species (Figure 8). Species that are in the negative region of PC1 and PC2 include *V. catenata*, *V. urticifolia*, *V. montana*, *V. beccabunga*, *V. acinifolia*, and *V. longifolia* (Figure 8a). All of these species are characterized by a high relative concentration of phytol (24.21–47.55%). This component is located in the negative region of PC1 and PC2 (Figure 8b). Species located around the center of the PCA score plot include *V. anagalloides*, *V. polita*, *V. persica*, *V. arvensis*, *V. saturejoides* ssp. *saturejoides*, *V. chamaedrys*, *V. austriaca* ssp. *austriaca*, *V. serpyllifolia*, *V. officinalis*, *V. opaca*, and *V. austriaca* ssp. *jacquinii*. These species are all characterized by a moderate relative concentration of hexadecanoic acid, hexahydrofarnesyl acetone, and phytol. *V. hederifolia* was allocated out of all the species because of its very high relative percentage of hexahydrofarnesyl acetone (59.15%). *V. cymbalaria* has a high concentration of caryophyllene oxide and *V. dalmatica* has a high concentration of *E*-caryophyllene, so they were allocated out of all the other clusters.

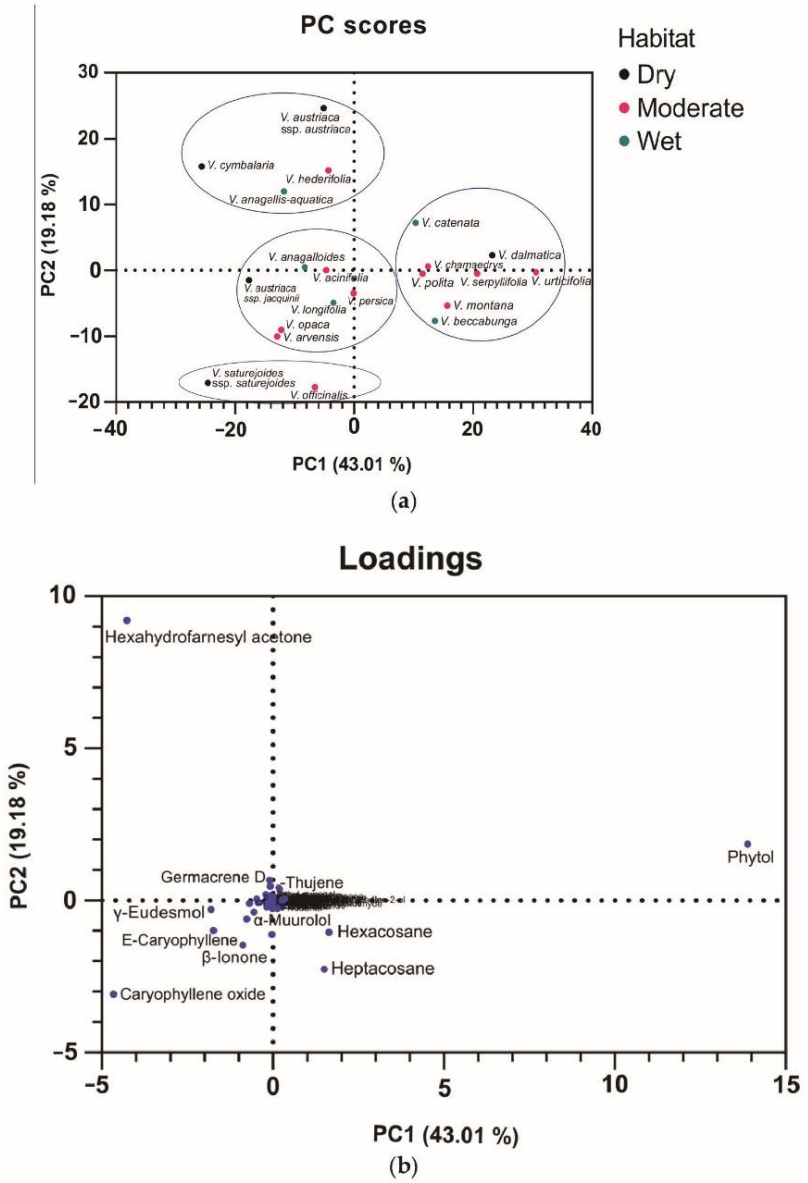

Figure 7. PCA analyses of volatile compounds of 21 *Veronica* species obtained by hydrodistillation. The PCA score plot allocating different species to clusters (**a**); PCA loading plots of volatiles from the first and second principal components (**b**).

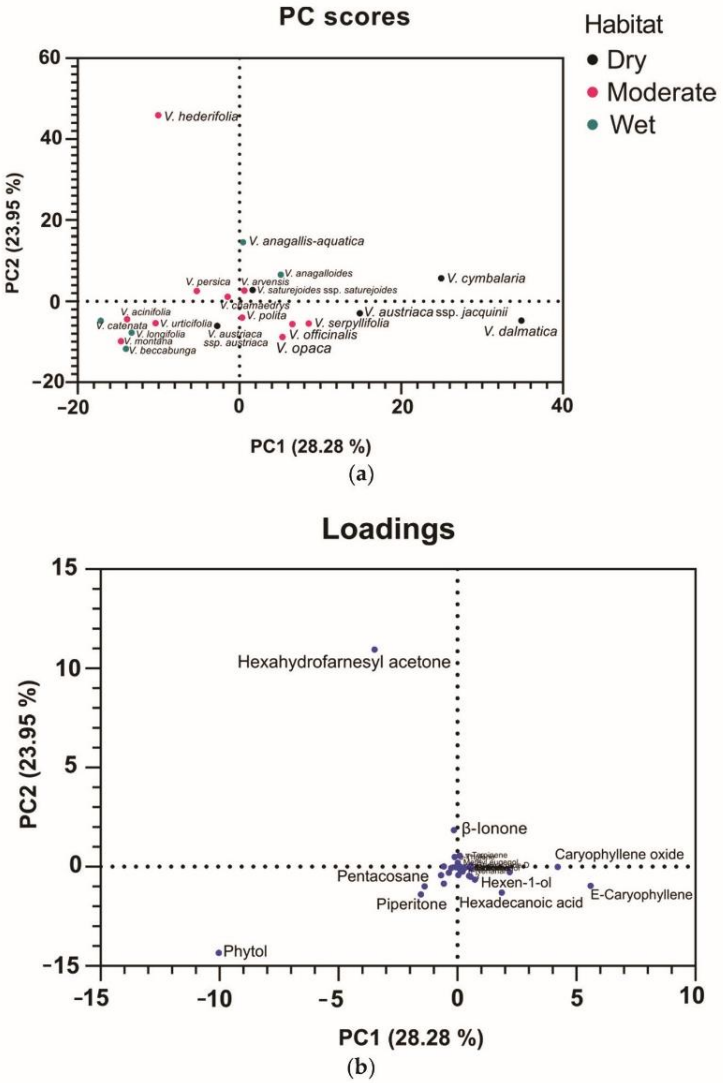

Figure 8. PCA analyses of volatile compounds of 21 *Veronica* species obtained by microwave extraction. The PCA score plot allocating different species to clusters (**a**); PCA loading plots of volatiles from the first and second principal components (**b**).

3. Discussion

The genus *Veronica* has undergone major changes in taxonomy over the last 20 years. It used to belong to the Scrophulariaceae family and then was transferred to the Plantaginaceae family following new genetic studies [1,2,23]. Albach and Taskova and their associates studied the iridoid glycosides of the genus *Veronica* and concluded that the distribution of these substances in the different species of the genus is consistent with the molecular phylogeny of the genus, thus showing that the chemistry of the genus can serve as a good indicator of interspecies and intergenus connections [10,11,24,25]. Chemosystematics (chemophenetics [26]) has been used throughout history to identify plants and other organisms and divide them into those that are suitable for use as food and those that should be avoided. One of the first researchers in this field was Greshoff (1909), who concluded

that researchers in chemistry and botany should work together to study the plant world [27]. Taskova et al. isolated 16 iridoid glycosides from the genus *Veronica* and established a link between the chemical composition and the basic chromosome number [28]. The analysis of four *Veronica* species (*V. persica*, *V. polita*, *V. francispetae* M. A. Fisch., and *V. siaretensis* E. Lehm.) showed a qualitatively constant composition of iridoid samples in all species, regardless of environmental conditions [29]. This is consistent with the results of this study, because some major constituents were found to be present in all species regardless of the habitat in which they live. These VCs are hexahydrofarnesyl acetone, phytol, and hexadecanoic acid. The most abundant iridoids in the genus *Veronica* are generally aucubin and catalpol [28,30,31]. Albach et al. investigated the iridoid glycosides aucubine and catalpol in the genus *Veronica* and the plant species of *Paederota lutea* Scop. and concluded that the genera *Veronica* and *Paederota* are related based on the composition of these compounds [32]. This proves that iridoids are a very good marker of the chemophenetics of plant species. In our study, the VCs in *Veronica* species were studied to determine whether some of the compounds could be used as chemophenetic markers for future research and to determine whether different habitats affect the composition of these compounds.

Dunkić et al. [33] investigated the composition of the essential oil of *Veronica spicata* L. and identified the predominant hydrocarbons (heptacosane and pentacosane). The other predominant compounds were the diterpenes phytol and isophytol, the oxygenated monoterpenes piperitone and piperitone oxide, and aliphatic ketones [33]. In the Bulgarian species *Veronica officinalis*, a GC-MS study on ethanolic extracts of the aerial parts gave following composition: terpenes (hexahydrofarnesyl acetone), saturated and unsaturated fatty acids and esters, steroids, *p*-hydroxyphenylethyl alcohol, maltol, and loliolid [34]. Ertas et al. investigated the phytochemical composition of *Veronica thymoides* P. H. Davis subsp. *pseudocinerea* M. A. Fischer, concluded that the major component of the essential oil of this species is hexatriacontene, and found that the most abundant fatty acids in this plant were linoleic acid and hexadecanoic acid [14]. Feng Li investigated the composition of the essential oil of *Veronica linariifolia* Pall. ex Link and found that the major constituents were cyclohexene (25.83%), β-pinene (11.61%), 1S-α-pinene (10.65%), β-phellandrene (10.49%), β-myrcene (10.42%), and germacrene D (4.99%) (monoterpene and sesquiterpene hydrocarbons) [18]. If we compare all these results, then we can see that some constituents, such as hexadecanoic acid, hexahydrofarnesyl acetone, phytol, and different hydrocarbons, were found very frequently in all studies. These results may suggest that these VCs could be used as chemophenetic markers for the genus *Veronica*. Looking at the clusters for both Clevenger and microwave-assisted extraction, we can see that humidity-based habitats do not affect the composition of volatile compounds, as all habitats are represented in most clusters. Numerous other experiments have shown that VCs can be used to discriminate between species and cultivars [35–38].

Regarding the relative content of volatile compounds in *Veronica* taxa collected from dry habitats, oxygenated sesquiterpenes form the main class of classically isolated compounds. The exception is the endemic species *V. dalmatica*, in which the proportion of oxygenated sesquiterpenes was similar for both isolation methods. In the species *V. dalmatica*, oxygenated diterpenes were the most abundant in HD and sesquiterpene hydrocarbons were dominant in green extraction (Figure 2). In the group of plants collected from wet habitats, the percentages of compound classes extracted by both methods were the same. The greatest variation was found in the identification percentage of oxygenated monoterpenes in *V. beccabunga* (Figure 4). The compounds in the composition of monoterpene hydrocarbons were generally the least identified and the most isolated in the species of moderate habitats *V. hederifolia* by both methods of isolation (Figure 6). The composition of *V. officinalis* was found to be dominated by a group of compounds consisting of acids, alcohols, and esters. One of these compounds was the alcohol 3-hexen-1-ol, which was isolated by green extraction. This component is known to be one of the most important in the composition of VCs [39]. This species is often used in herbal tea, so the presence of 3-hexen-1-ol was expected as this compound is widely found in fresh tea leaves [39]. With

this study on free VCs, we have increased our knowledge of the specialized metabolites that form the basis for further biological research.

Comparing the results from the extraction of VCs using classical hydrodistillation and microwave distillation, it can be seen that same main components were isolated by both methods but in different relative percentages. Some compounds were only isolated with either hydrodistillation or microwave distillation. This is logical as it is known that the process of hydrodistillation can negatively affect some of the compounds that are being decomposed due to high temperatures and long extraction times. On the other hand, microwave distillation can sometimes result in the isolation of fewer components as is stated in the study by Wu et al. [40]. They concluded in their research that hydrodistillation remains a better option for free VC extraction as it extracts the highest number of VCs. Looking at the results for the isolated VCs for the genus *Veronica* and the fact that all main compounds were extracted with both methods, microwave extraction should be considered when extracting VCs from a smaller amount (laboratory extraction) of sample because it is a greener choice that uses less water and energy and will not overheat the sample. In future analyses of *Veronica* species, it would be useful to investigate the composition of free VCs in water extracts (hydrosols) and compare the results of VC clustering with clustering based on genetic studies to identify potential chemophenetic (phytotaxonomic) markers among VCs.

4. Materials and Methods

4.1. Plant Material Collection and Preparation

Plant material was collected from March to July 2021 at different locations in Croatia (Table 1; Figure 9). All plant species were in the flowering stage. Voucher specimens were deposited at the Laboratory of Botany herbarium (HPMF-HR), Faculty of Science, University of Split, Croatia. All samples were air dried in a single layer and protected from direct sunlight for ten days.

Table 1. Details of the data collection and origin of the investigated *Veronica* taxa.

Taxa	Habitat	Locality	Latitude	Longitude	Altitude a.s.l. (m)	Voucher No.
V. austriaca ssp. *austriaca*	dry	Dinara Mt	44°02′20.1″ N	16°23′22.5″ E	1550	CROVeS-01-2021
V. austriaca ssp. *jacquinii*	dry	Brač Island	43°19′07.3″ N	16°36′08.5″ E	564	CROVeS-02-2021
V. cymbalaria	dry	Murter Island	43°48′36.6″ N	15°35′07.4″ E	37	CROVeS-03-2021
V. dalmatica	dry	Dubrovnik	42°39′19.1″ N	18°04′56.9″ E	58	CROVeS-04-2021
V. saturejoides ssp. *saturejoides*	dry	Dinara Mt	44°03′11.3″ N	16°23′29.7″ E	1697	CROVeS-05-2021
V. anagallis-aquatica ssp. *anagallis-aquatica*	wet	Split	43°31′43.5″ N	16°28′45.2″ E	22	CROVeS-06-2021
V. anagalloides	wet	Čikola River	43°49′36.2″ N	16°01′19.4″ E	45	CROVeS-07-2021
V. beccabunga	wet	Baške Oštarije	44°31′32.1″ N	15°10′34.2″ E	908	CROVeS-08-2021
V. catenata	wet	Trakoščan	46°15′30.3″ N	15°56′25.2″ E	240	CROVeS-09-2021
V. longifolia	wet	Oštarije	45°13′36.1″ N	15°16′18.2″ E	311	CROVeS-10-2021
V. acinifolia	moderate	Donji Karin	44°07′18.1″ N	15°36′13.7″ E	119	CROVeS-11-2021
V. arvensis	moderate	Hvar Island	43°10′42.3″ N	16°36′43.6″ E	38	CROVeS-12-2021
V. chamaedrys	moderate	Radoboj	46°09′49.4″ N	15°55′36.1″ E	260	CROVeS-13-2021
V. hederifolia	moderate	Zagreb	45°49′40.4″ N	15°58′59.6″ E	192	CROVeS-14-2021
V. montana	moderate	Papuk Mt	45°30′38.1″ N	17°39′57.2″ E	761	CROVeS-15-2021
V. officinalis	moderate	Kamešnica Mt	43°42′38.7″ N	16°50′47.9″ E	1225	CROVeS-16-2021
V. opaca	moderate	Split	43°30′32.3″ N	16°27′54.5″ E	67	CROVeS-17-2021
V. persica	moderate	Samoborsko gorje	45°49′41.6″ N	15°40′32.9″ E	301	CROVeS-18-2021
V. polita	moderate	Kaštel Žegarski	44°09′26.1″ N	15°51′56.0″ E	53	CROVeS-19-2021
V. serpyllifolia	moderate	Zagreb	45°49′40.3″ N	15°58′59.5″ E	192	CROVeS-20-2021
V. urticifolia	moderate	Plešivica Mt	45°45′05.7″ N	15°42′28.3″ E	350	CROVeS-21-2021

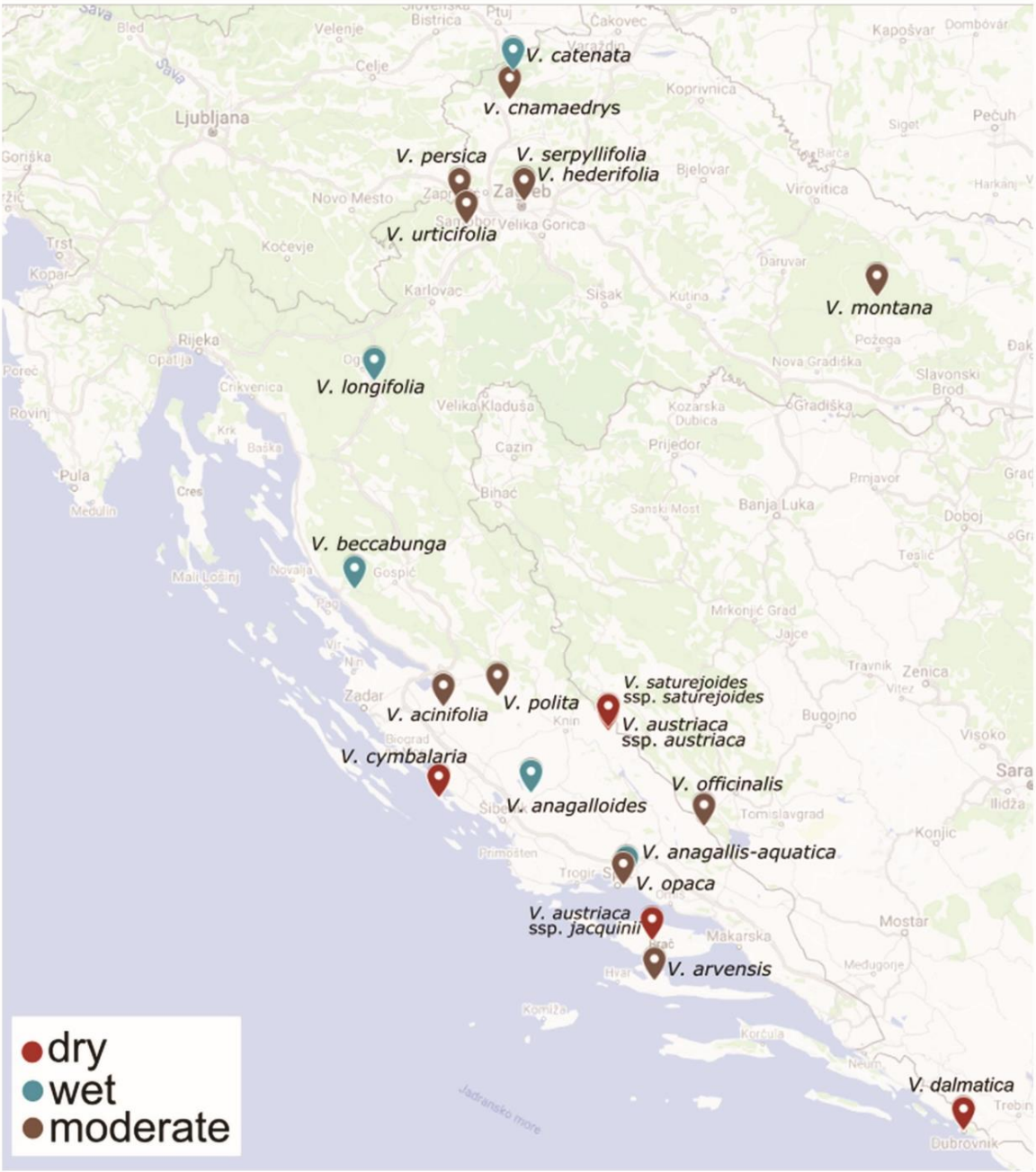

Figure 9. Map of the sites at which the studied *Veronica* taxa were collected.

Dried aboveground parts of the plant leaves, stems, and flowers (30–50 g) for each plant species were subjected to two different extraction methods: hydrodistillation and microwave-assisted extraction. The extracts, which were collected in pentane and diethyl ether (VWR, Radnor, PA, USA) from both extraction methods for all plant species, were dried over anhydrous sodium sulphate and stored at −20 °C until analysis.

4.2. Isolation of Volatile Compounds

4.2.1. Classical Isolation

The VCs were isolated by hydrodistillation in a Clevenger-type apparatus (Šurlan, Medulin, Croatia) for 2.5 h using 30–50 g of dried plant material. In the inner tube of the Clevenger apparatus, VCs of the investigated species were collected in a mixed solution of pentane and diethyl ether (2:1).

4.2.2. Green Isolation

Dried plant material (30–50 g for each plant species) was hydrated for 1 h before the isolation process. A Milestone 'ETHOS X' microwave laboratory oven (1900 W maximum) was used for microwave-assisted isolation. This oven is a 2.45 GHz multimode microwave reactor.

Regarding microwave-assisted distillation, a typical experiment was conducted at atmospheric pressure for 40 min at 500 W (98 °C). The distillation process started after 10 min. The distillate was collected in a side-tube using a pentane/diethyl ether trap, dried over anhydrous sodium sulphate, and stored at −20 °C until analysis.

4.3. GC and GC-MS Analyses

The above-described extracts were analyzed with a mass spectrometer (model 2100T; Varian Inc.) and a VF-5-ms non-polar capillary column (30 m with gas chromatography and mass spectrometry (GC-MS)) according to the method described in [6,19,21]. GC was performed by a gas chromatograph (model 3900, Varian Inc., Lake Forest, CA, USA) that was equipped with a flame ionization detector (FID), a mass spectrometer (model 2100T; Varian Inc.), a VF-5ms non-polar capillary column (inside diameter, 30 m × 0.25 mm; coating thickness, 0.25 µm; Palo Alto, CA, USA), and a CP-Wax 52 CB polar capillary column (i.d., 30 m × 0.25 mm; coating thickness, 0.25 µm; Palo Alto, CA, USA). The chromatographic conditions for the analysis of VCs were an FID detector temperature of 300 °C and an injector temperature of 250 °C. The gas carrier was helium at 1 mL min^{-1}. The conditions for the VF-5-ms column were a temperature of 60 °C (isothermal) for 3 min, which was then increased to 246 °C at a rate of 3 °C min^{-1} and held (isothermal) for 25 min. The conditions for the CP Wax 52 column were a temperature of 70 °C (isothermal) for 5 min, which was then increased to 240 °C at a rate of 3 °C min^{-1} and held (isothermal) for 25 min. The injected volume was 2 µL and the split ratio was 1:20. The MS conditions were: ion source temperature, 200 °C; ionization voltage, 70 eV; mass scan range, 40–350 mass units [33]. The individual peaks for all samples were identified by a comparison of their retention indices of *n*-alkanes to those of authentic samples and the studies [41,42], a comparison to our libraries from previous work, and a comparison to other previously published material for *Veronica* species [14,18,34]. The results are expressed as the mean value of three analyses with the standard deviation.

4.4. PCA Analyses

Statistical analysis was performed in GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA). All data in the tables are expressed as the mean ± SD ($n = 3$). Data included in the PCA analyses were obtained from the GC-MS analyses. PCA analyses were performed for VCs with amounts greater than 2%.

5. Conclusions

The results of this study show that hexahydrofarnesyl acetone, hexadecanoic acid, phytol, *E*-caryophyllene, and caryophyllene oxide are the major components identified by the classical (hydrodistillation) and green (microwave) methods of extraction regardless of the habitat of the 21 Croatian *Veronica* species studied. As these compounds were isolated in all species, they could be considered chemophenetic markers. Future research comparing clusters based on VCs and clusters resulting from genetic investigations might confirm this hypothesis. Looking at the results for the isolated VCs for the genus *Veronica* and the

fact that all main compounds were extracted with both methods, microwave extraction should be considered when extracting VCs because it is a greener choice that uses less water and energy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11070902/s1, Table S1. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of Veronica taxa collected on dry habitats, Table S2. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of Veronica taxa collected on dry habitats, Table S3. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of Veronica taxa collected on wet habitats, Table S4. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of Veronica taxa collected on wet habitats, Table S5. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of Veronica taxa collected on moderate habitats, Table S6. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of Veronica taxa collected on moderate habitats.

Author Contributions: Conceptualization, V.D. and M.N.; methodology, V.D. and M.N.; software, M.N.; validation, V.D., D.K. and M.N.; formal analysis, M.N., V.D., D.K. and E.V.; investigation, V.D., M.N., K.A., D.K., M.R., E.V., N.V., S.T., J.P., M.M., R.J.G. and S.S.; data curation, M.N. and V.D.; writing—original draft preparation, M.N., V.D., D.K. and E.V.; writing—review and editing, M.N., V.D., D.K. and E.V.; visualization, V.D.; M.N. and D.K.; supervision, V.D.; funding acquisition, V.D. All authors have read and agreed to the published version of the manuscript.

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References

1. Albach, D.C.; Martínez-Ortega, M.M.; Fischer, M.A.; Chase, M.W. A new classification of the tribe *Veroniceae*—Problems and a possible solution. *Taxon* **2004**, *53*, 429–452. [CrossRef]
2. Albach, D.C.; Martinez-Ortega, M.M.; Chase, M.W. *Veronica*: Parallel morphological evolution and phylogeography in the Mediterranean. *Plant Syst. Evol.* **2004**, *246*, 177–194. [CrossRef]
3. Walters, S.M.; Webb, D.A. *Veronica* L. In *Flora Europaea*; Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A., Eds.; Cambridge University Press: Cambridge, UK, 1972; Volume 3, pp. 242–251.
4. Nikolić, T. Flora Croatica Database. Available online: http://hirc.botanic.hr/fcd (accessed on 15 December 2021).
5. Barreira, J.C.M.; Dias, M.I.; Živković, J.; Stojković, D.; Soković, M.; Santos-Buelga, C.; Ferreira, I.C.F.R. Phenolic profiling of *Veronica* spp. grown in mountain, urban and sandy soil environments. *Food Chem.* **2014**, *163*, 275–283. [CrossRef] [PubMed]
6. Nazlić, M.; Kremer, D.; Grubešić, R.J.; Soldo, B.; Vuko, E.; Stabentheiner, E.; Ballian, D.; Bogunić, F.; Dunkić, V. Endemic *Veronica saturejoides* Vis. ssp. *saturejoides*—Chemical composition and antioxidant activity of free volatile Compounds. *Plants* **2020**, *9*, 1646. [CrossRef]
7. Thomford, N.E.; Senthebane, D.A.; Rowe, A.; Munro, D.; Seele, P.; Maroyi, A.; Dzobo, K. Natural products for drug discovery in the 21st century: Innovations for novel drug discovery. *Int. J. Mol. Sci.* **2018**, *19*, 1578. [CrossRef]
8. Tan, K.H.; Nishida, R. Methyl Eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. *J. Insect Sci.* **2012**, *12*, 56. [CrossRef] [PubMed]
9. Albach, D.C.; Grayer, R.J.; Jensen, S.R.; Özgökce, F.; Veitch, N.C. Acylated flavone glycosides from *Veronica*. *Phytochemistry* **2003**, *64*, 1295–1301. [CrossRef]
10. Albach, D.C.; Grayer, R.J.; Kite, G.C.; Jensen, S.R. *Veronica*: Acylated flavone glycosides as chemosystematic markers. *Biochem. Syst. Ecol.* **2005**, *33*, 1167–1177. [CrossRef]
11. Albach, D.C.; Jensen, S.R.; Özgökce, F.; Grayer, R.J. *Veronica*: Chemical characters for the support of phylogenetic relationships based on nuclear ribosomal and plastid DNA sequence data. *Biochem. Syst. Ecol.* **2005**, *33*, 1087–1106. [CrossRef]
12. Kwak, J.H.; Kim, H.J.; Lee, K.H.; Kang, S.C.; Zee, O.P. Antioxidative iridoid glycosides and phenolic compounds from *Veronica Peregrina*. *Arch. Pharm. Res.* **2009**, *32*, 207–213. [CrossRef]
13. Beara, I.; Živković, J.; Lesjak, M.; Ristić, J.; Šavikin, K.; Maksimović, Z.; Janković, T. Phenolic profile and anti-inflammatory activity of three *Veronica* species. *Ind. Crops Prod.* **2015**, *63*, 276–280. [CrossRef]

14. Ertas, A.; Boga, M.; Kizil, M.; Ceken, B.; Goren, A.C.; Hasimi, N.; Demirci, S.; Topcu, G.; Kolak, U. Chemical profile and biological activities of *Veronica thymoides* subsp. *pseudocinerea*. *Pharm. Biol.* **2015**, *53*, 334–339. [CrossRef] [PubMed]
15. Mocan, A.; Vodnar, D.C.; Vlase, L.; Crișan, O.; Gheldiu, A.M.; Crișan, G. Phytochemical characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from Romania and their antioxidant and antimicrobial properties. *Int. J. Mol. Sci.* **2015**, *16*, 21109–21127. [CrossRef]
16. Sharifi-Rad, M.; Tayeboon, G.S.; Sharifi-Rad, J.; Iriti, M.; Varoni, E.M.; Razazi, S. Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts. *Cell. Mol. Biol.* **2016**, *62*, 80–85. [CrossRef]
17. Živković, J.Č.; Barreira, J.C.M.; Šavikina, K.P.; Alimpić, A.Z.; Stojković, D.S.; Dias, M.I.; Santos-Buelga, C.; Duletić-Laušević, S.N.; Ferreira, I.C.F.R. Chemical profiling and assessment of antineurodegenerative and antioxidant properties of *Veronica teucrium* L. and *Veronica jacquinii* Baumg. *Chem. Biodivers.* **2017**, *14*, e1700167. [CrossRef] [PubMed]
18. Li, F. Analysis of chemical constituents of essential oil in *Veronica linariifolia* by gas chromatography-mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 822–825.
19. Nazlić, M.; Fredotović, Ž.; Vuko, E.; Fabijanić, L.; Kremer, D.; Stabentheiner, E.; Ruščić, M.; Dunkić, V. Wild Species *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides—Biological potential of free volatiles*. *Horticulturae* **2021**, *7*, 295. [CrossRef]
20. Tikvic, I.; Ugarkovic, D.; Peles, I.; KnežIc, I.; Medunic-Orlic, G.; Marinic, S.; Butorac, L.; Cmrlec, A.; Koharevic, R.; Nazlic, M.; et al. Assessment of forest ecosystem services and non-market forest functions in Park forest Marjan Split. *Sumar. List* **2017**, *141*, 277–285. [CrossRef]
21. Nazlić, M.; Fredotović, Ž.; Vuko, E.; Vuletić, N.; Ljubenkov, I.; Kremer, D.; Grubešić, R.J.; Stabentheiner, E.; Randić, M.; Dunkić, V. Free volatile compounds of *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. and their biological activity. *Plants* **2021**, *10*, 2529. [CrossRef]
22. Li, Y.; Fabiano-Tixier, A.-S.; Chemat, F. Essential oils: From conventional to green extraction. In *Essential Oils as Reagents in Green Chemistry*, 1st ed.; Sharma, S.K., Ed.; Springer Briefs in Molecular Science; Springer: Cham/Heidelberg, Germany; New York, NY, USA; Dordrecht, The Netherlands; London, UK, 2014; pp. 1–71. [CrossRef]
23. Albach, D.C.; Meudt, H.M.; Oxelman, B. Piecing together the "new" Plantaginaceae. *Am. J. Bot.* **2005**, *92*, 297–315. [CrossRef]
24. Taskova, R.M.; Albach, D.C.; Grayer, R.J. Phylogeny of *Veronica*—A combination of molecular and chemical evidence. *Plant Biol.* **2004**, *6*, 673–682. [CrossRef] [PubMed]
25. Jensen, S.R.; Albach, D.C.; Ohno, T.; Grayer, R.J. *Veronica*: Iridoids and cornoside as chemosystematic markers. *Biochem. Syst. Ecol.* **2005**, *33*, 1031–1047. [CrossRef]
26. Zidorn, C. Plant chemophenetics—A new term for plant chemosystematics/plant chemotaxonomy in the macro-molecular era. *Phytochemistry* **2019**, *163*, 147–148. [CrossRef] [PubMed]
27. Reynolds, T. The evolution of chemosystematics. *Phytochemistry* **2007**, *68*, 2887–2895. [CrossRef]
28. Taskova, R.; Peev, D.; Handjieva, N. Iridoid glucosides of the genus *Veronica* s.l. and their systematic significance. *Plant Syst. Evol.* **2002**, *231*, 1–17. [CrossRef]
29. Saeidi Mehrvarz, S.; Mahmoodi, N.O.; Asadian, R.; Bakhshi Khaniki, G. Iridoid and flavonoid patterns of the genus *Veronica* sect. *Alsinebe* subsect. *Agrestis* (Benth.) Stroh (Lamiales) and their systematic significance. *Aust. J. Crop Sci.* **2008**, *1*, 1–5.
30. Crişan, G.; Vlase, L.; Balica, G.; Muntean, D.; Ştefănescu, C.; Păltinean, R.; Tămaş, M.; Leucuţa, S. LC/MS analysis of aucubin and catalpol of some *Veronica* species. *Farmacia* **2010**, *58*, 237–242.
31. Harput, U.S.; Saracoglu, I.; Nagatsu, A.; Ogihara, Y. Iridoid glucosides from *Veronica hederifolia*. *Chem. Pharm. Bull.* **2002**, *50*, 1106–1108. [CrossRef]
32. Albach, D.C.; Held Gotfredsen, C.; Jensen, S.R. Iridoid glucosides of *Paederota lutea* and the relationships between *Paederota* and *Veronica*. *Phytochemistry* **2004**, *65*, 2129–2134. [CrossRef]
33. Dunkić, V.; Kosalec, I.; Kosir, I.; Potočnik, T.; Cerenak, A.; Koncic, M.; Vitali, D.; Muller, I.; Kopricanec, M.; Bezić, N.; et al. Antioxidant and antimicrobial properties of *Veronica spicata* L. (Plantaginaceae). *Curr. Drug Targets* **2015**, *16*, 1660–1670. [CrossRef]
34. Valyova, M.; Hadjimitova, V.; Stoyanov, S.; Ganeva, Y.; Petkov, I. Free radical scavenging activity of extracts from Bulgarian *Veronica officinalis* L. and GC-MS analysis of ethanol extract. *Internet J. Aesthet. Antiaging Med.* **2008**, *2*, 2–6. [CrossRef]
35. Popović, M.; Jukić Špika, M.; Veršić Bratinčević, M.; Ninčević, T.; Matešković, A.; Mandušić, M.; Rošin, J.; Nazlić, M.; Dunkić, V.; Vitanović, E. Essential oil volatile fingerprint differentiates Croatian cv. Oblica from other *Olea europaea* L. cultivars. *Molecules* **2021**, *26*, 3533. [CrossRef]
36. Craft, J.D.; Satyal, P.; Setzer, W.N. The Chemotaxonomy of common sage (*Salvia officinalis*) based on the volatile constituents. *Medicines* **2017**, *4*, 47. [CrossRef] [PubMed]
37. Wang, Y.; Li, X.; Jiang, Q.; Sun, H.; Jiang, J.; Chen, S.; Guan, Z.; Fang, W.; Chen, F. GC-MS analysis of the volatile constituents in the leaves of 14 Compositae plants. *Molecules* **2018**, *23*, 166. [CrossRef] [PubMed]
38. Giuliani, C.; Lazzaro, L.; Calamassi, R.; Calamai, L.; Romoli, R.; Fico, G.; Foggi, B.; Mariotti Lippi, M. A volatolomic approach for studying plant variability: The case of selected *Helichrysum* species (Asteraceae). *Phytochemistry* **2016**, *130*, 128–143. [CrossRef] [PubMed]
39. Nie, C.; Gao, Y.; Du, X.; Bian, J.; Li, H.; Zhang, X.; Wang, C.; Li, S. Characterization of the effect of *cis*-3-hexen-1-ol on green tea aroma. *Sci. Rep.* **2020**, *10*, 15506. [CrossRef]

40. Wu, C.; Wang, F.; Liu, J.; Zou, Y.; Chen, X. A comparison of volatile fractions obtained from *Lonicera macranthoides* via different extraction processes: Ultrasound, microwave, Soxhlet extraction, hydrodistillation, and cold maceration. *Integr. Med. Res.* **2015**, *4*, 171–177. [CrossRef]
41. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*; Allured Publishing: Carol Stream, IL, USA, 2017.
42. NIST Chemistry WebBook. Available online: https://webbook.nist.gov/ (accessed on 12 March 2021).

Table S1. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of *Veronica* taxa collected on dry habitats.

			V. austriaca ssp. *austriaca*	*V. austriaca* ssp. *jacquinii*	*V. cymbalaria*	*V. dalmatica.*	*V. saturejoides* ssp. *saturejoides*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Monoterpene hydrocarbons			**0.94**	**-**	**-**	**0.15**	**0.88**
α-Thujene	924	1012	0.94±0.01				
α-Pinene[c]	935	1017	-	-	-	-	0.88±0.01
β -Phellandrene	1002	1195	-	-	-	0.15±0.03	-
Oxygenated monoterpenes			**1.09**	**-**	**1.97**	**15.06**	**1.79**
1,8-Cineole	1026	1210	0.83±0.01	-	-	0.76±0.01	-
γ-Terpinene	1057	1225	-	-	-	2.61±0.01	-
Linalool	1095	1506	0.26±0.01	-	0.26±0.01	4.72±0.01	0.89±0.05
allo-Ocimene	1128	1390	-	-	0.22±0.15	-	-
Camphor	1151	1499	-	-	-	0.72±0.01	-
Terpinen-4-ol	1174	1686	-	-	0.22±0.01	-	-
Borneol	1176	1719	-	-	-	1.59±0.01	-
α-Terpineol	1184	1660	-	-	-	3.08±0.03	0.28±0.01
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	-	-	0.62±0.01	-	0.62±0.02
Menthyl acetate	1294	1550	-	-	0.65±0.01	1.58±0.02	-
Sesquiterpene hydrocarbons			**7.76**	**8.01**	**10.91**	**13.55**	**16.86**
α-Copaene	1377	1484	-	-	0.78±0.01	-	1.53±0.01
E-Caryophyllene'	1424	1585	1.23±0.01	8.01±0.01	3.95 ±0.04	3.48±0.01	9.43±0.01
allo-Aromadendrene	1465	1662	-	-	1.32±0.01	2.59±0.01	1.47±0.01

β-Chamigrene	1478	1724	-	-	-	0.27±0.12	-
Germacrene D	1481	1692	5.28±0.01	-	1.42±0.01	3.87±0.02	2.61±0.01
δ-Selinene	1492	1756	1.25±0.01	-	2.32±0.01	3.34±0.01	-
δ-Cadinene	1517	1745	-	-	1.12±0.01	-	1.82±0.01
Oxygenated sesquiterpenes			**44.63**	**31.1**	**60.16**	**13.46**	**52.05**
Spathulenol	1577	2101	-	-	-	0.25±0.01	1.8±0.01
Caryophyllene oxide*	1581	1955	4.86±0.01	13.98±0.01	10.92±0.01	0.52±0.01	34.53±0.01
Viridiflorol	1592	2099	-	-	-	-	1.05±0.01
γ-Eudesmol	1632	2175	-	-	9.61±0.01	-	0.2±0.03
α-Muurolol	1645	2181	-	-	3.30±0.01	2.38±0.01	7.06±0.01
α-Bisabolol	1685	2210	-	-	-	2.59±0.03	-
α-Bisabolol oxide	1748	2511	-	-	-	-	0.53±0.01
Hexahydrofarnesyl acetone*	1839	2113	39.77±0.01	17.12±0.01	36.33±0.01	7.72±0.13	6.88±0.01
Oxygenated diterpene			**20.12**	**4.58**	-	**41.22**	-
Phytol*	1942	2610	20.12±0.01	4.58±0.01	-	41.22±0.01	-
Phenolic compounds			**-**	**0.33**	**1.38**	**0.85**	**-**
Thymol*	1289	2154	**-**	**-**	0.82±0.01	**-**	**-**
p-Vinyl guaicol	1313	2156	-	-	0.27±0.03	-	-
Thymol acetate	1349	-	-	-	-	0.85±0.01	-
Methyl eugenol	1403	2005	-	0.33±0.01	**-**	**-**	**-**
(Z)-Methyl isoeugenol	1451	2070	**-**	**-**	0.29±0.01	**-**	**-**
Acids, alcohols and esters			**13.38**	**44.19**	**12.94**	**8.24**	**23.92**
Isopentyl acetate	863	1127	-	-	-	0.24±0.09	0.28±0.01

Benzaldehyde	952	1508	-	-	0.98±0.01	1.25±0.01	3.98±0.01
Benzene acetaldehyde	1036	1633	-	-	0.48±0.01	-	1.12±0.01
n-Nonanal	1100	1389	-	-	0.89±0.02	-	1.68±0.01
Hexyl 2-methyl butanoate	1233	1425	-	-	-	0.21±0.01	-
n-Decanol	1266	1711	-	-	0.11±0.05	-	0.98±0.01
2,4-Decadienal	1304	1764	2.23±0.01	-	-	-	4.25±0.01
(E)-*β*-Damascenone	1384	1819	-	-	-	0.69±0.01	-
β-Ionone	1487	1935	-	-	7.28±0.01	3.27±0.01	3.98±0.01
Benzyl benzoate	1760	2613	-	-	1.56±0.01	-	-
1-Hexadecanol	1874	2371	-	-	0.89±0.01	1.45±0.01	1.51±0.03
Hexadecanoic acid*	1959	2912	11.15±0.01	32.17±0.01	0.75±0.01	1.13±0.01	6.14±0.01
Oleic acid	2133	2998	-	12.02±0.01	-	-	-
Octadecanol acetate	2209	-	-	0.98±0.01	-	-	-
Hydrocarbons			**8.35**	**5.94**	**5.89**	**6.96**	**1.12**
Eicosane*	2000	2000	-	1.39±0.01	1.24±0.01	-	1.12±0.01
Heneicosane*	2100	2100	-	0.83±0.01	0.98±0.17	0.87±0.01	-
Docosane*	2200	2200	-	2.69±0.01	2.13±0.01	2.11±0.01	-
Tricosane*	2300	2300	-	-	-	1.96±0.01	-
Tetracosane*	2400	2400	-	-	0.83±0.01	1.77±0.01	-
Pentacosane*	2500	2500	3.92±0.01	1.03±0.01	0.71±0.04	0.25±0.03	-
Heptacosane*	2700	2700	3.74±0.01	-	-	-	-
Octacosane*	2800	2800	0.69±0.01	-	-	-	-
Total identification (%)			**96.27**	**94.15**	**93.25**	**92.53**	**96.62**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

Table S2. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of *Veronica* taxa collected on dry habitats.

			V. austriaca ssp. *austriaca*	*V. austriaca* ssp. *jacquinii*	*V. cymbalaria*	*V. dalmatica*	*V. saturejoides* ssp. *saturejoides*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Monoterpene hydrocarbons			**1.69**	**1.93**	**-**	**-**	**1.18**
α-Thujene	924	1012	1.69±0.01	-	-	-	-
α-Pinene*	935	1017	-	1.93±0.01	-	-	1.18±0.01
Oxygenated monoterpenes			-	**0.87**	**1.62**	**3**	**4.6**
Linalool	1095	1506	-	0.87±0.01	0.96±0.15	1.22±0.01	1.78±0.02
Camphor	1151	1499	-	-	-	-	0.52±0.01
α-Terpineol	1184	1660	-	-	-	1.78±0.13	0.88±0.01
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	-	-	-	-	1.42±0.03
Menthyl acetate	1294	1550	-	-	0.66±0.07	-	-
Sesquiterpene hydrocarbons			**6.01**	**2.78**	**13.7**	**57.8**	**17.43**
α-Copaene	1377	1484	0.57±0.03	-	-	-	0.89±0.03
E-Caryophyllene*	1424	1585	0.66±0.08	1.79±0.01	6.13 ±0.01	39.53±0.01	8.49±0.01
allo-Aromadendrene	1465	1662	-	-	2.28±0.01	15.43±0.01	-
Germacrene D	1481	1692	1.01±0.01	0.68±0.01	2.34±0.03	1.89±0.01	5.11±0.01
δ-Selinene	1492	1756	3.77±0.01	0.31±0.1	1.52±0.01	0.95±0.01	1.73±0.01
δ-Cadinene	1517	1745	-	-	1.43±0.01	-	1.21±0.01
Oxygenated sesquiterpenes			**12.74**	**15.25**	**47.69**	**15.72**	**29.05**
Spathulenol	1577	2101	-	-	-	4.76±0.01	2.25±0.04
Caryophyllene oxide*	1581	1955	2.97±0.01	6.64±0.01	32.72±0.01	7.52±0.15	8.43±0.01

Viridiflorol	1592	2099	-	-	-	-	0.65±0.02
γ-Eudesmol	1632	2175	-	-	0.61±0.07	-	-
α-Bisabolol	1685	2210	-	-	1.01±0.01	-	-
Hexahydrofarnesyl acetone*	1839	2113	9.77±0.01	8.61±0.01	13.35±0.01	3.44±0.02	17.72±0.01
Oxygenated diterpene			**24.21**	**6.58**	**3.71**	**2.75**	**22.47**
Phytol*	1942	2610	24.21±0.01	6.58±0.01	3.71±0.01	2.75±0.02	22.47±0.01
Phenolic compounds			**-**	**-**	**1.44**	**-**	**-**
Thymol*	1289	2154	-	-	0.45±0.01	-	-
(Z)-Methyl isoeugenol	1451	2070	-	-	0.99±0.16	-	-
Acids, alcohols and esters			**7.72**	**24.99**	**26.21**	**12.28**	**16.21**
Isopentyl acetate	863	1127	-	-	-	-	0.56±0.09
Benzaldehyde	952	1508	2.37±0.01	-	1.24±0.01	-	1.48±0.01
Benzene acetaldehyde	1036	1633	-	-	0.42±0.01	0.75±0.01	1.02±0.01
n-Nonanal	1100	1389	0.83±0.02	-	-	1.68±0.01	1.14±0.18
Hexyl 2-methyl butanoate	1233	1425	-	-	-	0.91±0.01	-
n-Decanol	1266	1711	-	1.52±0.05	-	-	1.88±0.01
2,4-Decadienal	1304	1764	-	-	-	-	0.55±0.01
(E)-β-Damascenone	1384	1819	-	-	-	0.29±0.06	-
β-Ionone	1487	1935	-	1.01±0.03	7.88±0.01	6.00±0.01	1.84±0.03
Benzyl benzoate	1760	2613	-	-	-	-	-
1-Hexadecanol	1874	2371	-	-	0.64±0.13	-	1.1±0.01
Hexadecanoic acid*	1959	2912	4.15±0.01	22.17±0.01	15.72±0.01	2.65±0.01	6.64±0.01
Oleic acid	2133	2998	0.37±0.01	0.29±0.01	0.31±0.01	-	-

Hydrocarbons			**40.65**	**41.99**	**2.5**	**5.46**	**5.86**
Eicosane*	2000	2000	1.84±0.01	1.12±0.01	0.24±0.01	1.51±0.04	0.12±0.01
Heneicosane*	2100	2100	0.77±0.04	0.44±0.02	0.88±0.01	0.87±0.01	2.89±0.01
Docosane*	2200	2200	1.17±0.05	1.63±0.1	-	0.48±0.06	1.59±0.01
Tricosane*	2300	2300	13.61±0.01	17.32±0.01	-	1.13±0.01	0.43±0.03
Tetracosane*	2400	2400	3.65±0.01	1.61±0.05	0.89±0.01	1.12±0.01	0.35±0.07
Pentacosane*	2500	2500	1.37±0.02	5.91±0.01	0.49±0.05	0.35±0.23	0.48±0.01
Hexacosane*	2600	2600	9.29±0.01	8.97±0.01	-	-	-
Heptacosane*	2700	2700	8.95±0.02	4.11±0.15	-	-	-
Octacosane*	2800	2800	-	0.88±0.01	-	-	-
Total identification (%)			**93.02**	**94.39**	**96.87**	**97.01**	**96.8**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

Table S3. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of *Veronica* taxa collected on wet habitats.

			*V. anagallis-aqua*tica	*V. anagalloides*	*V. beccabunga*	*V. catenata*	*V. longifolia*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Monoterpene hydrocarbons			**1.42**	**-**	**-**	**-**	**-**
β -Phellandrene	1002	1195	1.42±0.01	-	-	-	-
Oxygenated monoterpenes			**5.75**	**5.57**	**8.33**	**-**	**3.02**
1,8-Cineole	1026	1210	1.83±0.01	-	-	-	-
γ-Terpinene	1057	1225	0.49±0.05	2.53±0.01	-	-	-
Linalool	1095	1506	1.21±0.01	1.71±0.01	-	-	0.65±0.01
Terpinen-4-ol	1174	1686	-	0.68±0.1	-	-	-
α-Terpineol	1184	1660	-	-	-	-	0.82±0.07
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	2.22±0.01	0.65±0.04	1.85±0.01	-	1.55±0.01
Piperitone	1250	1719	-	-	5.79±0.01		
Menthyl acetate	1294	1550	-	-	0.69±0.01	-	-
Sesquiterpene hydrocarbons			**10.27**	**11.59**	**3.89**	**12.55**	**17.3**
α-Copaene	1377	1484	-	-	-	-	-
E-Caryophyllene[c]	1424	1585	5.49±0.01	4.07±0.01	2.75 ±0.01	4.11±0.02	4.13±0.01
allo-Aromadendrene	1465	1662	1.10±0.03	2.00±0.01	0.72±0.01	1.23±0.01	2.17±0.01
β-Chamigrene	1478	1724	-	-	-	-	0.29±0.01
Germacrene D	1481	1692	1.28±0.01	2.22±0.15	0.42±0.01	3.87±0.02	4.61±0.01
δ-Selinene	1492	1756	0.65±0.01	1.15±0.01	-	3.34±0.01	3.75±0.01
δ-Cadinene	1517	1745	1.75±0.01	2.15±0.1	-	-	2.35±0.01
Oxygenated sesquiterpenes			**32.18**	**24.47**	**11.06**	**19.82**	**19.52**

Spathulenol	1577	2101	0.65±0.01	2.85±0.01	-	0.55±0.01	2.56±0.01
Caryophyllene oxide[c]	1581	1955	4.36±0.01	4.91±0.01	4.22±0.01	1.55±0.01	5.58±0.01
Viridiflorol	1592	2099	-	-	0.60±0.16	-	1.75±0.01
γ-Eudesmol	1632	2175	-	-	0.11±0.01	-	-
α-Muurolol	1645	2181	-	-	-	-	0.55±0.01
α-Bisabolol	1685	2210	-	1.66±0.01	-	-	-
α-Bisabolol oxide	1748	2511	-	0.72±0.09	-	-	-
Hexahydrofarnesyl acetone[c]	1839	2113	27.17±0.01	14.33±0.01	6.13±0.01	17.75±0.02	9.08±0.01
Oxygenated diterpene			**9.42**	**9.58**	**27.31**	**29.92**	**13.63**
Phytol[c]	1942	2610	9.42±0.01	9.58±0.01	27.31±0.01	29.92±0.01	13.63±0.01
Phenolic compounds			**7.42**	**5.27**	**0.30**	**-**	**-**
Thymol*	1289	2154	**-**	**-**	-	**-**	**-**
p-Vinyl guaicol	1313	2156	2.46±0.01	-	-	-	-
Methyl eugenol	1403	2005	4.96±0.01	2.66±0.04	0.30±0.01	-	-
(Z)-Methyl isoeugenol	1451	2070	**-**	2.61±0.01	-	**-**	**-**
Acids, alcohols and esters			**20.98**	**31.14**	**8.97**	**16.29**	**27.57**
Isopentyl acetate	863	1127	-	-	-	-	-
Benzaldehyde	952	1508	2.78±0.01	-	-	0.25±0.01	0.91±0.01
Benzene acetaldehyde	1036	1633	4.27±0.01	6.62±0.01	1.58±0.01	-	1.19±0.01
n-Nonanal	1100	1389	-	3.08±0.01	2.85±0.02	1.65±0.04	8.18±0.01
Hexyl 2-methyl butanoate	1233	1425	-	2.03±0.03	-	-	-
n-Decanol	1266	1711	0.68±0.01	-	-	-	0.45±0.01
2,4-Decadienal	1304	1764	2.23±0.01	-	-	-	-

(E)-β-Damascenone	1384	1819	1.96±0.01	3.78±0.01	-	-	-
β-Ionone	1487	1935	4.41±0.01	1.62±0.01	1.28±0.01	4.37±0.01	7.10±0.01
Benzyl benzoate	1760	2613	-	-	0.56±0.15	-	-
Hexadecanoic acid*	1959	2912	4.65±0.01	13.67±0.01	2.72±0.01	10.02±0.01	9.74±0.01
Oleic acid	2133	2998	-	0.34±0.03	-	-	-
Hydrocarbons			**7.54**	**5.92**	**36.62**	**16.22**	**15.78**
Eicosane*	2000	2000	-	-	1.64±0.01	-	-
Heneicosane*	2100	2100	-	-	0.98±0.17	.	-
Docosane*	2200	2200	3.72±0.01	3.91±0.01	0.13±0.01	3.11±0.03	4.32±0.07
Tricosane*	2300	2300	1.69±0.04	-	1.18±0.02	4.16±0.01	1.28±0.15
Tetracosane*	2400	2400	0.35±0.02	-	0.83±0.01	3.79±0.01	1.29±0.01
Pentacosane*	2500	2500	-	2.01±0.01	0.51±0.04	0.28±0.1	6.81±0.01
Hexacosane*	2600	2600	-	-	16.21±0.01	-	2.08±0.17
Heptacosane*	2700	2700	0178±0.02	-	14.89±0.01	4.88±0.04	-
Octacosane*	2800	2800	-	-	0.25±0.02	-	-
Total identification (%)			**94.98**	**93.54**	**96.78**	**94.8**	**96.82**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

Table S4. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of *Veronica* taxa collected on wet habitats.

			V. anagallis-aquatica	*V. anagalloides*	*V. beccabunga*	*V. catenata*	*V. longifolia*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Oxygenated monoterpenes			**12.52**	**4.82**	**30.1**	**-**	**-**
1,8-Cineole	1026	1210	0.46±0.01	-	-	-	-
γ-Terpinene	1057	1225	7.58±0.01	3..81±0.01	-	-	-
Linalool	1095	1506	0.71±0.01	-	-	-	-
Camphor	1151	1499	0.91±0.01	-	-	-	-
Terpinen-4-ol	1174	1686	-	1.01±0.04	-	-	-
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	1.78±0.01	-	0.82±0.01	-	-
Piperitone	1250	1719	-	-	29.28±0.01	-	-
Menthyl acetate	1294	1550	1.08±0.01	-	-	-	-
Sesquiterpene hydrocarbons			**5.1**	**10.16**	**3.69**	**2.67**	**2.98**
E-Caryophyllene[c]	1424	1585	3.29±0.01	4.01±0.01	2.95 ±0.04	2.48±0.01	1.43±0.01
allo-Aromadendrene	1465	1662	-	2.11±0.01	0.32±0.02	0.19±0.01	1.55±0.01
Germacrene D	1481	1692	0.88±0.01	3.07±0.01	0.42±0.01	-	-
δ-Selinene	1492	1756	0.22±0.04	0.97±0.01	-	-	-
δ-Cadinene	1517	1745	0.71±0.01	-	-	-	-
Oxygenated sesquiterpenes			**29.45**	**31.19**	**11.18**	**24.39**	**12.37**
Spathulenol	1577	2101	0.65±0.01	-	-	0.65±0.1	0.81±0.01
Caryophyllene oxide[c]	1581	1955	2.55±0.01	8.58±0.01	1.62±0.01	6.52±0.01	1.53±0.01
Viridiflorol	1592	2099	-	0.81±0.01	-	-	0.75±0.01
α-Bisabolol	1685	2210	0.28±0.01	1.91±0.01	-	-	-

α-Bisabolol oxide	1748	2511	-	0.77±0.02	-	-	-
Hexahydrofarnesyl acetone*	1839	2113	25.97±0.01	19.12±0.01	9.56±0.01	17.22±0.01	9.28±0.01
Oxygenated diterpene			**14.56**	**14.88**	**34.54**	**42.26**	**37.18**
Phytol*	1942	2610	14.56±0.01	14.88±0.01	34.54±0.01	42.26±0.01	37.18±0.01
Phenolic compounds			**3.43**	**0.33**	**-**	**-**	**-**
Methyl eugenol	1403	2005	3.43±0.01	0.33±0.07	-	-	-
Acids, alcohols and esters			**25.93**	**18.17**	**9.54**	**12.98**	**30.01**
Isopentyl acetate	863	1127	-	-	-	-	0.22±0.03
Benzaldehyde	952	1508	1.63±0.01	-	-	-	-
Benzene acetaldehyde	1036	1633	9.67±0.01	-	2.48±0.01	-	4.17±0.01
n-Nonanal	1100	1389	1.82±0.01	2.12±0.01	0.89±0.02	-	2.68±0.01
Hexyl 2-methyl butanoate	1233	1425	-	0.44±0.01	-	-	-
n-Decanol	1266	1711	1.28±0.01	-	-	-	0.58±0.01
2,4-Decadienal	1304	1764	0.28±0.05	-	-	-	-
(E)-β-Damascenone	1384	1819	0.12±0.16	0.52±0.07	-	0.32±0.09	-
β-Ionone	1487	1935	6.36±0.01	4.22±0.01	1.43±0.01	0.27±0.01	16.22±0.01
Hexadecanoic acid*	1959	2912	4.77±0.01	9.17±0.01	4.74±0.01	5.81±0.01	6.14±0.01
Oleic acid	2133	2998	-	1.02±0.01	-	2.25±0.01	-
Octadecanol acetate	2209	-	-	0.68±0.03	-	4.33±0.01	-
Hydrocarbons			**5.3**	**18.12**	**8.26**	**15.1**	**10.71**
Eicosane*	2000	2000	0.72±0.01	1.12±0.01	-	-	-
Heneicosane*	2100	2100	0.18±0.01	0.33±0.04	-	-	-
Docosane*	2200	2200	2.22±0.01	5.62±0.01	4.15±0.01	-	2.82±0.01

Tricosane*	2300	2300	0.75±0.01	-	-	3.03±0.01	-
Tetracosane*	2400	2400	1.15±0.01	-	-	12.07±0.01	-
Pentacosane*	2500	2500	-	5.43±0.01	1.13±0.01	-	4.71±0.01
Hexacosane*	2600	2600	-	3.90±0.01	0.27±0.09	-	1.48±0.01
Heptacosane*	2700	2700	0.28±0.1	0.95±0.01	1.45±0.01	-	1.70±0.01
Octacosane*	2800	2800	-	0.77±0.15	1.26±0.01	-	-
Total identification (%)			**97.29**	**96.79**	**97.31**	**97.4**	**93.25**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

Table S5. Chemical composition of the VCs obtained by hydro-distillation from the aerial parts of *Veronica* taxa collected on moderate habitats.

			V. acinifolia	*V. arvensis*	*V. chamaedrys*	*V. hederifolia*	*V. montana*	*V. officinalis*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Monoterpene hydrocarbons			**-**	**-**	**-**	**4.86**	**-**	**-**
α-Thujene	924	1012	-	-	-	4.86±0.01	-	-
Oxygenated monoterpenes			**0.75**	**1.2**	**3.26**	**0.98**	**-**	**-**
Terpinen-4-ol	1174	1686	0.75±0.1	-	1.49±0.01	-	-	-
α-Terpineol	1184	1660	-	-	0.92±0.01	0.98±0.15	-	-
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	-	0.52±0.01	0.85±0.01	-	-	-
Hexyl 2-methyl butanoate	1233	1425	-	0.68±0.05	-	-	-	-
Sesquiterpene hydrocarbons			**6.73**	**12.44**	**5.58**	**9.19**	**0.7**	**3.77**
E-Caryophyllene[c]	1424	1585	4.46±0.01	6.21±0.01	2.43 ±0.01	4.11±0.01	0.13±0.01	3.12±0.01
allo-Aromadendrene	1465	1662	0.89±0.05	0.85±0.01	0.82±0.01	2.26±0.01	0.57±0.01	0.65±0.01
β-Chamigrene	1478	1724	-	0.77±0.03	-	-	-	-
Germacrene D	1481	1692	0.43±0.01	1.25±0.03	1.02±0.01	1.47±0.03	-	-
δ-Selinene	1492	1756	0.95±0.01	2.01±0.01	1.31±0.15	1.35±0.04	-	-
δ-Cadinene	1517	1745	-	1.35±0.01	-	-	-	-
Oxygenated sesquiterpenes			**28.92**	**40.44**	**18.54**	**37.18**	**14.14**	**12.93**
Spathulenol	1577	2101	0.95±0.01	-	-	0.58±0.01	-	
Caryophyllene oxide[c]	1581	1955	7.71±0.01	14.11±0.01	6.25±0.01	4.59±0.01	7.28±0.01	4.65±0.01
Viridiflorol	1592	2099	-	-	0.91±0.03	-	-	
γ-Eudesmol	1632	2175	-	19.98±0.01	0.56±0.01	3.16±0.01	-	0.88±0.01
α-Muurolol	1645	2181	-	-	-	-	-	2.38±0.01

α-Cadinol	1655	2208	-	-	-	-	-	0.72±0.01
α-Bisabolol	1685	2210	2.12±0.01	-	-	-	-	0.38±0.01
α-Bisabolol oxide	1748	2511	2.77±0.01	-	-	-	-	0.69±0.01
Hexahydrofarnesyl acetone[c]	1839	2113	15.37±0.01	6.35±0.01	10.82±0.01	28.85±0.01	6.86±0.01	3.25±0.02
Oxygenated diterpene			**15.63**	**7.54**	**31.66**	**18.53**	**32.61**	**6.72**
Phytol[c]	1942	2610	15.63±0.01	7.54±0.01	31.66±0.01	18.53±0.01	32.61±0.01	6.72±0.01
Phenolic compounds			**6.9**	**6.99**	**4.16**	**3.25**	**-**	**-**
Thymol[a]	1289	2154	-	-	-	0.56±0.1	-	-
p-Vinyl guaicol	1313	2156	2.41±0.01	3.11±0.01	2.64±0.01	2.22±0.01	-	-
Thymol acetate	1349	-	2.38±0.01	-	-	-	-	-
Methyl eugenol	1403	2005	2.11±0.01	2.16±0.02	1.52±0.01	0.47±0.1	-	-
(Z)-Methyl isoeugenol	1451	2070	-	1.72±0.01	-	-	-	-
Acids, alcohols and esters			**35.73**	**19.44**	**24.44**	**18.84**	**16.6**	**31.97**
Isopentyl acetate	863	1127	-	-	0.56±0.01	-	-	-
Benzaldehyde	952	1508	0.32±0.13	-	1.32±0.01	3.24±0.01	1.84±0.01	-
2-Pentyl furan	984	1230	-	4.52±0.01	-	-	-	-
Benzene acetaldehyde	1036	1633	3.97±0.01	2.69±0.01	7.28±0.01	-	3.34±0.01	-
n-Nonanal	1100	1389	2.18±0.01	2.91±0.01	1.81±0.1	2.71±0.01	-	-
Hexyl 2-methyl butanoate	1233	1425	2.45±0.01	-	-	-	-	-
n-Decanol	1266	1711	1.88±0.01	0.85±0.01	-	-	-	-
2,4-Decadienal	1304	1764	1.45±0.01	-	-	-	-	-
(E)-β-Damascenone	1384	1819	3.12±0.01	2.61±0.01	1.22±0.07	2.57±0.01	-	0.42±0.03
β-Ionone	1487	1935	17.01±0.01	2.69±0.01	4.41±0.01	3.07±0.01	2.18±0.01	18.11±0.01

Benzyl benzoate	1760	2613	-	-	2.11±0.01	-	-	-
Hexadecanoic acid	1959	2912	3.35±0.01	3.17±0.01	5.73±0.01	7.25±0.01	9.24±0.01	13.21±0.01
Oleic acid	2133	2998	-	-	-	-	-	0.23±0.07
Octadecanol acetate	2209	-	-	-	-	-	-	1.11±0.01
Hydrocarbons			**0.22**	**4.45**	**6.75**	**3.45**	**32.9**	**38.49**
Eicosane	2000	2000	-	-	-	-	0.31±0.01	-
Heneicosane	2100	2100	-	0.88±0.01	0.21±0.1	.	0.42±0.07	0.34±0.05
Docosane	2200	2200	0.22±0.01	1.32±0.01	3.14±0.01	0.81±0.02	3.34±0.02	2.15±0.01
Tricosane	2300	2300	-	0.65±0.01	0.32±0.03	0.46±0.01	0.53±0.01	0.65±0.07
Tetracosane	2400	2400	-	0.89±0.01	2.83±0.01	0.77±0.01	0.45±0.01	0.92±0.01
Pentacosane	2500	2500	-	0.71±0.01	0.56±0.01	0.21±0.03	10.47±0.01	11.89±0.01
Hexacosane	2600	2600	-	-	0.22±0.05	0.88±0.15	12.45±0.01	1.52±0.01
Heptacosane	2700	2700	-	-	2.30±0.01	0.32±0.05	3.82±0.01	20.67±0.01
Octacosane	2800	2800	-	-	-	-	1.11±0.01	0.35±0.04
Total identification (%)			**94.88**	**92.5**	**94.39**	**96.28**	**96.95**	**93.88**

Table 5. Continue

			V. opaca	*V. persica*	*V. polita*	*V. serpyllifolia*	*V. urticifolia*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Oxygenated monoterpenes			**0.63**	**-**	**-**	**0.68**	**-**
Linalool	1095	1506	0.42±0.01	-	-	-	-
Terpinen-4-ol	1174	1686	0.21±0.04	-	-	-	-
α-Terpineol	1184	1660	-	-	-	0.68±0.01	-

Sesquiterpene hydrocarbons			**11.56**	**17.68**	**10.36**	**4.91**	**0.6**
α-Copaene	1377	1484	-	-	-	-	-
E-Caryophyllene*	1424	1585	11.23±0.01	9.29±0.01	6.57 ±0.01	2.11±0.01	0.43±0.01
allo-Aromadendrene	1465	1662	-	2.81±0.01	0.42±0.01	0.28±0.01	0.17±0.04
β-Chamigrene	1478	1724	-	2.02±0.01	-	-	-
Germacrene D	1481	1692	0.33±0.01	0.75±0.01	1.06±0.03	0.67±0.01	-
δ-Selinene	1492	1756	-	2.81±0.01	2.31±0.04	1.85±0.02	-
Oxygenated sesquiterpenes			**35.86**	**26.34**	**20.18**	**13.2**	**7.34**
Spathulenol	1577	2101	-	-	-	0.88±0.01	-
Caryophyllene oxide*	1581	1955	11.75±0.01	10.11±0.01	7.55±0.01	4.19±0.01	0.38±0.01
Viridiflorol	1592	2099	-	-	0.87±0.08	-	-
γ-Eudesmol	1632	2175	4.62±0.01	0.72±0.01	0.29±0.01	0.21±0.01	-
α-Muurolol	1645	2181	1.73±0.01	1.71±0.01	-	-	-
α-Cadinol	1655	2208	1.06±0.01	-	-	-	-
α-Bisabolol	1685	2210	4.02±0.01	1.23±0.01	0.71±0.01	-	-
α-Bisabolol oxide	1748	2511	1.21±0.01	2.26±0.01	0.48±0.01	-	-
Hexahydrofarnesyl acetone*	1839	2113	11.47±0.01	10.31±0.01	10.28±0.01	7.92±0.01	6.96±0.01
Oxygenated diterpene			**6.68**	**20.21**	**31.18**	**39.79**	**47.55**
Phytol*	1942	2610	6.68±0.01	20.21±0.01	31.18±0.01	39.79±0.01	47.55±0.01
Phenolic compounds			**0.75**	**5.33**	**2.61**	**2.09**	-
p-Vinyl guaicol	1313	2156	0.75±0.01	3.14±0.01	2.26±0.01	0.52±0.01	-
Methyl eugenol	1403	2005	-	1.42±0.01	0.35±0.01	1.57±0.04	-
(Z)-Methyl isoeugenol	1451	2070	-	0.77±0.01	-	-	-

Acids, alcohols and esters			**13.04**	**20.2**	**18.21**	**28.54**	**15.83**
Benzaldehyde	952	1508	0.29±0.02	0.75±0.01	5.47±0.01	0.29±0.01	-
Benzene acetaldehyde	1036	1633	0.45±0.01	1.65±0.01	1.45±0.01	3.92±0.01	0.74±0.01
n-Nonanal	1100	1389	0.21±0.01	0.76±0.01	0.51±0.1	2.78±0.01	1.15±0.01
n-Decanol	1266	1711	-	1.08±0.01	0.43±0.01	0.96±0.01	-
2,4-Decadienal	1304	1764	-	0.44±0.07	-	-	-
(E)-*β*-Damascenone	1384	1819	-	2.57±0.01	0.32±0.04	0.77±0.01	2.14±0.01
β-Ionone	1487	1935	7.51±0.01	5.60±0.01	3.28±0.01	7.54±0.01	0.56±0.01
1-Hexadecanol	1874	2371	-	-	-	-	-
Hexadecanoic acid*	1959	2912	4.58±0.01	7.35±0.01	6.75±0.01	12.28±0.01	11.24±0.01
Hydrocarbons			**28.91**	**4.38**	**10.64**	**7.79**	**21.43**
Eicosane*	2000	2000	0.66±0.05	-	0.42±0.05	-	0.39±0.08
Heneicosane*	2100	2100	0.79±0.01	0.37±0.01	0.21±0.03	.	0.49±0.01
Docosane*	2200	2200	7.45±0.01	3.10±0.01	3.87±0.01	2.01±0.01	3.33±0.01
Tricosane*	2300	2300	1.71±0.01	0.25±0.01	0.37±0.02	0.48±0.04	1.21±0.01
Tetracosane*	2400	2400	2.11±0.01	-	0.81±0.01	0.77±0.01	0.25±0.01
Pentacosane*	2500	2500	4.14±0.01	-	0.36±0.01	0.98±0.01	0.48±0.01
Hexacosane*	2600	2600	0.34±0.01	-	4.27±0.05	3.13±0.01	0.32±0.05
Heptacosane*	2700	2700	11.71±0.01	0.21±0.01	0.33±0.01	0.42±0.03	13.82±0.01
Octacosane*	2800	2800	-	0.45±0.01	-	-	2.14±0.01
Total identification (%)			**97.43**	**94.14**	**93.18**	**97**	**93.15**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

Table S6. Chemical composition of the VCs obtained by microwave extraction from the aerial parts of *Veronica* taxa collected on moderate habitats.

			V. acinifolia	*V. arvensis*	*V. chamaedrys*	*V. hederifolia*	*V. montana*	*V. officinalis*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Monoterpene hydrocarbons			**-**	**1.57**	**-**	**2.82**	**-**	**-**
α-Thujene	924	1012	-	-	-	2.82±0.01	-	-
β -Phellandrene	1002	1195	-	1.57±0.01	-	-	-	-
Oxygenated monoterpenes			**-**	**0.93**	**0.76**	**-**	**-**	**-**
Terpinen-4-ol	1174	1686	-	-	0.76±0.01	-	-	-
trans-p-Mentha-*1(7),8*-dien-2-ol	1187	1803	-	0.93±0.01	-	-	-	-
Sesquiterpene hydrocarbons			**8.55**	**9.66**	**2.36**	**1.26**	**0.44**	**4.77**
E-Caryophyllene'	1424	1585	6.51±0.01	3.25±0.01	1.05 ±0.01	1.10±0.01	0.44±0.01	3.12±0.01
allo-Aromadendrene	1465	1662	0.21±0.07	1.81±0.01	0.82±0.01	0.16±0.01	-	1.65±0.01
Germacrene D	1481	1692	1.83±0.01	2.45±0.01	0.12±0.01	-	-	-
δ-Selinene	1492	1756	-	1.22±0.01	0.37±0.01	-	-	-
δ-Cadinene	1517	1745	-	0.93±0.01	-	-	-	-
Oxygenated sesquiterpenes			**23.99**	**26.98**	**18.8**	**59.66**	**13.64**	**12.04**
Spathulenol	1577	2101	0.25±0.01	-	-	-	-	
Caryophyllene oxide'	1581	1955	5.52±0.01	7.11±0.01	1.22±0.01	0.51±0.06	2.61±0.01	4.15±0.01
Viridiflorol	1592	2099	-	-	0.12±0.03	-	-	
γ-Eudesmol	1632	2175	-	2.32±0.01	0.77±0.01	-	-	-
α-Cadinol	1655	2208	-	-	-	-	0.69±0.01	-
α-Bisabolol	1685	2210	1.78±0.01	-	-	-	0.72±0.01	0.22±0.01
α-Bisabolol oxide	1748	2511	0.27±0.01	-	-	-	0.45±0.01	0.85±0.01

Hexahydrofarnesyl acetone*	1839	2113	16.17±0.01	17.55±0.01	16.69±0.01	59.15±0.01	9.17±0.01	6.82±0.01
Oxygenated diterpene			**39.88**	**22.57**	**18.88**	**14.58**	**37.03**	**16.89**
Phytol*	1942	2610	39.88±0.01	22.57±0.01	18.88±0.01	14.58±0.01	37.03±0.01	16.89±0.01
Phenolic compounds			**1.06**	**1.97**	**2.12**	**-**	**-**	**-**
p-Vinyl guaicol	1313	2156	0.58±0.01	1.51±0.01	0.84±0.01	-	-	-
Methyl eugenol	1403	2005	0.48±0.01	0.46±0.06	0.57±0.01	-	-	-
(Z)-Methyl isoeugenol	1451	2070	-	-	0.71±0.01	-	-	-
Acids, alcohols and esters			**11.45**	**26.71**	**22.16**	**16.6**	**17.51**	**52.59**
Isopentyl acetate	863	1127	-	-	-	-	-	1.04±0.01
3-Hexen-1-ol	873	1383		-	-	-	-	22.04±0.01
Benzaldehyde	952	1508	-	3.12±0.01	-	-	0.91±0.04	-
2-Pentyl furan	984	1230	-	0.51±0.01	-	-	-	-
Benzene acetaldehyde	1036	1633	-	1.24±0.01	3.22±0.01	-	2.31±0.01	-
n-Nonanal	1100	1389	1.46±0.01	2.31±0.01	0.85±0.1	-	-	6.72±0.01
Hexyl 2-methyl butanoate	1233	1425	0.24±0.01	-	0.75±0.1	-	-	1.61±0.01
n-Decanol	1266	1711	0.45±0.01	-	0.32±0.1	-	-	-
2,4-Decadienal	1304	1764	0.53±0.01	-	-	-	-	-
(E)-β-Damascenone	1384	1819	0.16±0.01	0.82±0.01	0.42±0.01	-	0.31±0.01	0.99±0.03
β-Ionone	1487	1935	4.09±0.01	7.29±0.01	0.77±0.01	15.03±0.01	7.68±0.01	7.79±0.01
Hexadecanoic acid*	1959	2912	4.52±0.01	17.42±0.01	15.83±0.01	1.57±0.01	5.81±0.01	12.40±0.01
Hydrocarbons			**7.59**	**3.03**	**32.67**	**0.68**	**25.04**	**7.19**
Heneicosane*	2100	2100	-	0.58±0.07	-	.	1.48±0.07	-
Docosane*	2200	2200	1.42±0.01	1.68±0.01	12.13±0.01	0.68±0.01	3.13±0.02	0.92±0.01

Tricosane*	2300	2300	0.42±0.01	0.77±0.01	0.15±0.07	-	0.88±0.01	-
Tetracosane*	2400	2400	-	-	5.55±0.01	-	0.95±0.01	-
Pentacosane*	2500	2500	5.75±0.01	-	8.36±0.01	-	14.90±0.01	0.15±0.03
Hexacosane*	2600	2600	-	-	0.13±0.02	-	-	0.54±0.01
Heptacosane*	2700	2700	-	-	5.22±0.01	-	3.70±0.01	5.52±0.01
Octacosane*	2800	2800	-	-	1.13±0.01	-	-	0.06±0.01
Total identification (%)			**92.52**	**93.42**	**97.75**	**95.6**	**93.66**	**93.48**

Table 6. Continue

			V. opaca	*V. persica*	*V. polita*	*V. serpyllifolia*	*V. urticifolia*
Component	RI[a]	RI[b]	VC±SD	VC±SD	VC±SD	VC±SD	VC±SD
Oxygenated monoterpenes			**-**	**-**	**-**	**0.86**	**-**
Linalool	1095	1506	-	-	-	0.86±0.01	-
Sesquiterpene hydrocarbons			**11.55**	**6.08**	**6.03**	**14.4**	**0.69**
α-Copaene	1377	1484	-	-	-	1.61±0.01	-
β-Elemene	1389	1593	-	1.29±0.01	1.16±0.01	2.29±0.01	-
E-Caryophyllene*	1424	1585	8.62±0.01	2.62±0.01	4.17 ±0.01	6.83±0.01	0.14±0.01
allo-Aromadendrene	1465	1662	0.85±0.01	0.82±0.05	0.52±0.02	-	-
β-Chamigrene	1478	1724	-	0.17±0.01	-	-	-
Germacrene D	1481	1692	0.53±0.01	0.35±0.01	0.07±0.01	3.24±0.01	0.55±0.01
δ-Selinene	1492	1756	1.55±0.01	0.83±0.01	0.11±0.01	0.43±0.01	-
Oxygenated sesquiterpenes			**13.37**	**23.7**	**15.88**	**27.92**	**13.71**

Spathulenol	1577	2101	-	-	-	1.25±0.01	-
Caryophyllene oxide[c]	1581	1955	6.75±0.01	3.14±0.01	1.48±0.01	14.74±0.01	0.49±0.04
Viridiflorol	1592	2099	-	-	0.44±0.01	-	-
γ-Eudesmol	1632	2175	2.62±0.01	1.22±0.01	-	2.52±0.01	-
α-Muurolol	1645	2181	0.75±0.01	-	-	-	-
α-Cadinol	1655	2208	0.42±0.01	-	-	0.82±0.01	-
α-Bisabolol	1685	2210	2.02±0.01	0.63±0.01	1.41±0.01	1.21±0.01	-
α-Bisabolol oxide	1748	2511	0.81±0.03	0.24±0.07	1.73±0.01	0.84±0.01	-
Hexahydrofarnesyl acetone[b]	1839	2113	5.87±0.01	18.47±0.01	10.82±0.01	6.54±0.01	13.22±0.01
Oxygenated diterpene			**23.18**	**23.71**	**19.88**	**18.72**	**31.12**
Phytol[b]	1942	2610	23.18±0.01	23.71±0.01	19.88±0.01	18.72±0.01	31.12±0.01
Phenolic compounds			**-**	**1.7**	**0.45**	**2.89**	**-**
p-Vinyl guaicol	1313	2156	-	0.74±0.01	0.45±0.01	1.64±0.01	-
Methyl eugenol	1403	2005	-	0.95±0.01	-	1.25±0.01	-
Acids, alcohols and esters			**35.19**	**14.24**	**8.64**	**18.6**	**31.01**
Benzaldehyde	952	1508	1.16±0.01	0.39±0.01	-	0.85±0.01	0.49±0.01
1-Octen-3-ol	974	1433	-	-	-	-	1.67±0.01
Benzene acetaldehyde	1036	1633	2.09±0.01	2.05±0.01	0.46±0.01	1.25±0.01	7.65±0.03
n-Nonanal	1100	1389	1.64±0.01	1.06±0.02	0.51±0.01	1.28±0.01	0.42±0.05
2,4-Decadienal	1304	1764	-	0.34±0.03	-	-	-
(E)-β-Damascenone	1384	1819	2.51±0.01	0.55±0.01	0.65±0.01	1.56±0.01	0.16±0.01
β-Ionone	1487	1935	7.52±0.01	4.54±0.01	1.33±0.01	5.74±0.01	4.51±0.01
Benzyl benzoate	1760	2613	0.59±0.01	-	-	-	-

Hexadecanoic acid*	1959	2912	19.68±0.01	5.31±0.01	5.69±0.01	7.71±0.01	16.11±0.01
Oleic acid	2133	2998	-	-	-	0.21±0.01	-
Hydrocarbons			**9.2**	**26.61**	**42.53**	**10.05**	**21.26**
Eicosane*	2000	2000	0.29±0.15	1.51±0.01	0.30±0.01	-	-
Heneicosane*	2100	2100	0.59±0.02	0.32±0.05	0.13±0.15	-	.
Docosane*	2200	2200	3.48±0.01	2.85±0.01	2.45±0.01	4.20±0.01	2.76±0.01
Tricosane*	2300	2300	0.76±0.01	0.14±0.01	0.37±0.02	0.30±0.03	0.18±0.01
Tetracosane*	2400	2400	-	0.84±0.02	0.89±0.01	2.59±0.01	2.71±0.01
Pentacosane*	2500	2500	-	5.27±0.01	1.76±0.01	0.18±0.07	8.91±0.01
Hexacosane*	2600	2600	0.44±0.05	0.17±0.15	4.55±0.01	2.74±0.01	0.53±0.04
Heptacosane*	2700	2700	0.75±0.03	14.28±0.01	15.13±0.01	-	5.75±0.01
Octacosane*	2800	2800	2.89±0.01	1.23±0.01	16.95±0.02	-	0.42±0.05
Total identification (%)			**92.49**	**96.04**	**93.41**	**93.44**	**97.79**

Retention indices (RIs) were determined relative to a series of n-alkanes (C8–C40) on capillary columns VF5-ms (RI^a) and CPWax 52 (RI^b); Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [41] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; *co-injection with reference compounds; -, not identified; SD, standard deviation of triplicate analysis.

3.5. Free Volatile Compounds as Chemophenetic Markers—Comparison with ITS2 and ITS1-5.8S-ITS2 Sequence Data for 18 Species of the Genus *Veronica*

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Free Volatile Compounds as Chemophenetic Markers—Comparison with ITS2 and ITS1-5.8S-ITS2 Sequence Data for 18 Species of the Genus *Veronica*

Marija Nazlić [1], Dario Kremer [2], Željana Fredotović [1], Željka Trumbić [3], Valerija Dunkić [1] and Jasna Puizina [1,*]

[1] Faculty of Science, University of Split, Ruđera Boškovića 33, 21000 Split, Croatia; mnazlic@pmfst.hr (M.N.); zfredotov@pmfst.hr (Ž.F.); dunkic@pmfst.hr (V.D.)
[2] Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia; dkremer@pharma.hr
[3] Department of Marine Studies, University of Split, Ruđera Boškovića 37, 21000 Split, Croatia; zeljka.trumbic@unist.hr
* Correspondence: puizina@pmfst.hr; Tel.: +385-21-619-200

Abstract: (1) Background: The purpose of this study was to compare the free volatile compounds of 18 *Veronica* species (Plantaginaceae), as previously analyzed by gas chromatography coupled with mass spectrometry, with their DNA sequences for internal transcribed spacers ITS2 and ITS1-5.8S-ITS2 of the nuclear ribosomal DNA. (2) Methods: Two sets of DNA sequence data were generated and used for phylogenetic analysis: ITS2 sequences (~360 bp) obtained by next-generation sequencing and ITS1-5.8S-ITS2 sequences (~580 bp) sequenced by the Sanger sequencing method. Clustering from previously analyzed free volatile compounds was performed by Ward's method. (3) Results: Both sets of DNA sequence data showed that the 18 analyzed *Veronica* species were grouped into eight main groups corresponding to the following subgenera: *Pentasepalae*, *Pocilla*, *Chamaedrys*, *Veronica*, *Beccabunga*, *Cochlidiosperma*, *Stenocarpon* and *Pseudolysimachium*. Results of the clustering analysis of free volatile compounds showed better clustering when using microwave-extracted volatiles. Three clusters were detected with the following main compounds: hexahydrofarnesyl acetone, hexadecanoic acid, phytol, caryophyllene oxide and *(E)*-caryophyllene. (4) Conclusion: The phylogenetic analysis of ITS2 data obtained by NGS technology and ITS1-5.8S-ITS2 data obtained by Sanger sequencing resulted in the grouping of 18 *Veronica* species into eight subgenera, which is in accordance with the existing classification. Statistical testing showed that there was no correlation between such clustering of *Veronica* species and clustering that was based on free volatile compounds. The achieved results can be viewed in the light of parallel evolution among some of the species of the *Veronica* genus as well as the fact that volatile compound composition can be influenced by environmental factors or epigenetic modifications.

Keywords: *Veronica*; volatile compounds; chemophenetic markers; molecular phylogeny; ITS1; ITS2; NGS; Sanger

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1. Introduction

The genus *Veronica* of the family Plantaginaceae consists of 450 species, growing mainly in the temperate regions of the Northern Hemisphere, while a smaller number of species grow in the mountains of the tropical and temperate regions of the Southern Hemisphere regions [1,2]. Many species testify to the great ecological adaptability of the genus *Veronica*, in which species grow in wet and dry habitats, by the sea and in the mountains [3].

One of the first phytochemical studies of the genus *Veronica* was related to iridoid glycosides, which proved to be taxonomically important [4,5]. This genus was once placed in the family Scrophulariaceae based mainly on morphological characters, but based on

the results of DNA sequence studies, it was transferred and is currently in the family Plantaginaceae [6]. Albach et al., Jensen et al. and Taskova et al. studied the iridoid glycosides of the genus *Veronica* and concluded that the distribution of these compounds in the different species of the genus is consistent with the molecular phylogeny of the genus, thus showing that the genus chemistry can serve as a good indicator of links between species and within the genus [7–10]. Advances in analytical methods, especially chromatography, followed by electronic detection methods, accelerated chemical studies to the point of metabolic profiling of plant species. In general, research on chemical compounds produced by plants is extremely important because these compounds ultimately affect not only the plant in which they are found but indirectly other plants in the environment as well as the environment as a whole [11]. Iridoid glycosides have been used as chemophenetic markers at different taxonomic levels [12–16]. As a part of research on chemophenetic markers for the genus *Veronica*, Taskova et al. isolated 16 iridoid glycosides and established a link between chemical composition and basic chromosome number [16]. Mehrvarz et al. studied the chemical composition of selected species of the genus *Veronica*, iridoids and flavonoids and investigated their significance for the systematic and phylogenetic assignment of these species. The analysis of four species of the genus *Veronica* (*V. persica*, *V. polita*, *V. francispetae*, *V. siaretensis*) showed a qualitatively constant composition of iridoids of all species, regardless of environmental conditions [17]. Crişan et al. studied 12 species of the genus *Veronica* with LC-MS, analyzing the content of aucubin and catalpol belonging to iridoid glycosides, and also confirmed their importance in chemophenetics [15]. Albach et al. studied the iridoid glycosides aucubin and catalpol in the genus *Veronica* species and the species *Paederota lutea* and, based on the composition of these compounds, concluded that these two genera *Veronica* and *Paederota* are related [18]. Molecular analyses showed that *Paederota* is a sister group next to *Veronica*, and the composition of iridoid glycosides supported this as the same compounds were detected in *Paederota* species [18]. This proves that iridoids are a very good marker for the chemophenetics of plant species, as confirmed by Saracoglu et al. in their studies [19].

The free volatile compounds, as specialized metabolites of the genus *Veronica*, have been much less studied than other metabolites such as glycosides, phenols and flavonoids. Only a few research studies could be found before our research [20–23]. Our recent studies on the composition of volatile compounds in 21 Croatian *Veronica* species indicate the diversity and richness of isolated metabolites, and for most of the *Veronica* species studied, these data were presented for the first time [24].

The molecular phylogeny and taxonomy of the genus *Veronica* are well studied [2,25–29]. Due to the parallel morphological evolution observed in this genus (a taxonomically distinct species develops similar morphological characters) [2], it is not recommended to rely solely on plant morphology for the identification and classification of species in the genus *Veronica*. Therefore, DNA analyses are recommended for reliable and accurate species identification. DNA barcoding regions that have been shown to be useful for the phylogenetics of *Veronica* are ITS1-5.8SrDNA-ITS2 (nuclear genes which are inherited from both parents) and trnL-trnF regions (genes from the chloroplast which are usually inherited from the female parent) [25].

Although the ITS1-5.8SrDNA-ITS2 region is probably the most popular molecular marker in plants for the DNA identification of plant species (DNA barcoding) this sequence can be hypervariable and therefore difficult to analyze when sequenced in a conventional way, using the Sanger method. This is particularly important for plants of hybrid and/or polyploid origin. Since the occurrence of polyploidy has been observed in several species of the genus *Veronica* [25], we hoped that with the new method of next-generation sequencing (NGS, next-generation sequencing), much more accurate identification of species would be possible. With this method, it is possible to sequence many more gene variants (markers) and obtain data of much better quality and higher resolution, revealing genetic diversity such as single nucleotide polymorphisms (SNPs), insertions/deletions (indels), homopolymeric regions and microsatellites, which in aggregate will allow accurate species

identification. The NGS method is already used in many cases: the detection of ITS2 allelic variation in mosquitoes [30], diversity of rDNA unit in *Nicotiana* [31], authenticity of plant foods [32] and identification of medicinal plants [33].

This study provides an overview of the phytochemical composition of free volatile compounds of selected species of the genus *Veronica* from Croatia, which have been published previously; however, in this work, cluster classification based on free volatile compounds (FVCs) is performed. Moreover, this clustering is compared with molecular classification based on the ITS (internal transcribed spacer) regions of ribosomal genes. One of the main objectives of this study is to investigate whether free volatiles can also be a good chemophenetic marker for classification between species and between genera. Several recent studies indicated the diversity and richness of isolated metabolites from the genus *Veronica*, and some of them proved to be reliable taxonomic markers. The combination of such studies with molecular phylogenetic analyses provides a good basis for exploring the potential relationship between the distribution of free volatile compounds and phylogeny.

2. Materials and Methods

2.1. ITS2 and ITS1-5.8S-ITS2 Sequencing

2.1.1. DNA Extractions

Genomic DNAs of 18 *Veronica* species listed in Table 1 (locations shown in Figure 1) were extracted from young silica-dried leaves using NucleoSpin Plant II, Mini kit for DNA from plants (Macherey-Nagel™; Cat. No. 740770.5, 52355 Düren, Germany) according to the manufacturer's instructions. For each species studied, DNA was separately isolated from three individuals. Voucher specimens were deposited at the Laboratory of Botany herbarium (HPMF-HR), Faculty of Science, University of Split, Croatia.

Table 1. List of 18 *Veronica* species with location collection information used for this research (the same plant material was used for the previously published research [24]).

Taxa	Locality	Latitude	Longitude	Altitude a.s.l. (m)	Voucher no.
V. austriaca L. ssp. *jacquini* i	Brač Island	43°19′07.3″ N	16°36′08.5″ E	564	CROVeS-02-2021
V. cymbalaria	Murter Island	43°48′36.6″ N	15°35′07.4″ E	37	CROVeS-03-2021
V. dalmatica	Dubrovnik	42°39′19.1″ N	18°04′56.9″ E	58	CROVeS-04-2021
V. saturejoides ssp. *saturejoides*	Dinara Mt	44°03′11.3″ N	16°23′29.7″ E	1697	CROVeS-05-2021
V. anagallis-aquatica	Split	43°31′43.5″ N	16°28′45.2″ E	22	CROVeS-06-2021
V. anagalloides	Čikola River	43°49′36.2″ N	16°01′19.4″ E	45	CROVeS-07-2021
V. beccabunga	Baške Oštarije	44°31′32.1″ N	15°10′34.2″ E	908	CROVeS-08-2021
V. catenata	Trakošćan	45°15′30.3″ N	15°56′25.2″ E	240	CROVeS-09-2021
V. longifolia	Oštarije	45°13′36.1″ N	15°16′18.2″ E	311	CROVeS-10-2021
V. acinifolia	Donji Karin	44°07′18.1″ N	15°36′13.7″ E	119	CROVeS-11-2021
V. arvensis	Hvar Island	43°10′42.3″ N	16°36′43.6″ E	38	CROVeS-12-2021
V. chamaedrys	Radoboj	46°09′49.4″ N	15°55′36.1″ E	260	CROVeS-13-2021
V. hederifolia	Zagreb	45°49′40.4″ N	15°58′59.6″ E	192	CROVeS-14-2021
V. montana	Papuk Mt	45°30′38.1″ N	17°39′57.2″ E	761	CROVeS-15-2021
V. officinalis	Kamešnica Mt	43°42′38.7″ N	16°50′47.9″ E	1225	CROVeS-16-2021
V. persica	Samoborsko gorje	45°49′41.6″ N	15°40′32.9″ E	301	CROVeS-18-2021
V. polita	Kaštel Žegarski	44°09′26.1″ N	15°51′56.0″ E	53	CROVeS-19-2021
V. serpyllifolia	Zagreb	45°49′40.3″ N	15°58′59.5″ E	192	CROVeS-20-2021

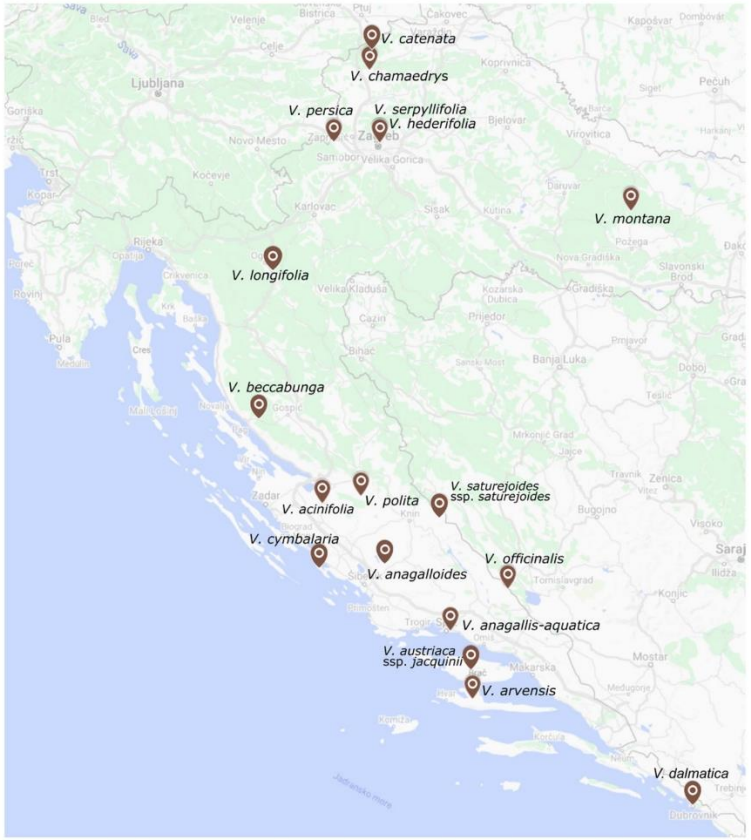

Figure 1. Map of locations of material collection.

2.1.2. PCR Amplification and Sequencing by Sanger

The ITS1-5.8S-ITS2 region of 35S rDNA was amplified by PCR using the primers ITS-1 (5′GTTTCCGTAGGTGAACCTGC3′) described by Bezić et al. [34] and ITS-4 (5′TCCTCCGCTTATTGATATGC3′) described by White et al. [35]. The amplified products were visualized and confirmed by 1% agarose gel electrophoresis, extracted from the gel and isolated using a Plasmid Mini Kit (Qiagen, Hilden, Germany). Purified DNA was sent to Macrogen (Amsterdam, The Netherlands) for sequencing. The ITS1-5.8S-ITS2 sequences were deposited in GenBank under the following accession numbers: OQ564378-OQ564387 (Table S1).

2.1.3. Sequence Analysis

The DNA sequences were assembled and aligned in ClustalW implemented in MEGA11 [36], and the alignment was manually refined. Alignments in fasta format are available as Datas S1 and S2. To infer phylogenetic relationships from the newly obtained *Veronica* ITS sequences and other closely related *Veronica* species, the sequences were subjected to similarity search against the non-redundant nucleotide sequence database using the NCBI (National Centre for Biotechnology Information) BLASTN network service. Four species from the Plantaginaceae family were taken into analysis as outgroup species, and details of their GenBank accession numbers and publications are shown in Table S2 [8,10,25,28,37–40]. The final dataset of ITS2 sequences contained 57 sequences, and there were a total of 327 positions. The evolutionary history was inferred by using the Maximum Parsimony method implemented in MEGA 11 under default parameters. Tree

#1 out of 5 most parsimonious trees (length = 323) is shown in Figure 2. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option).

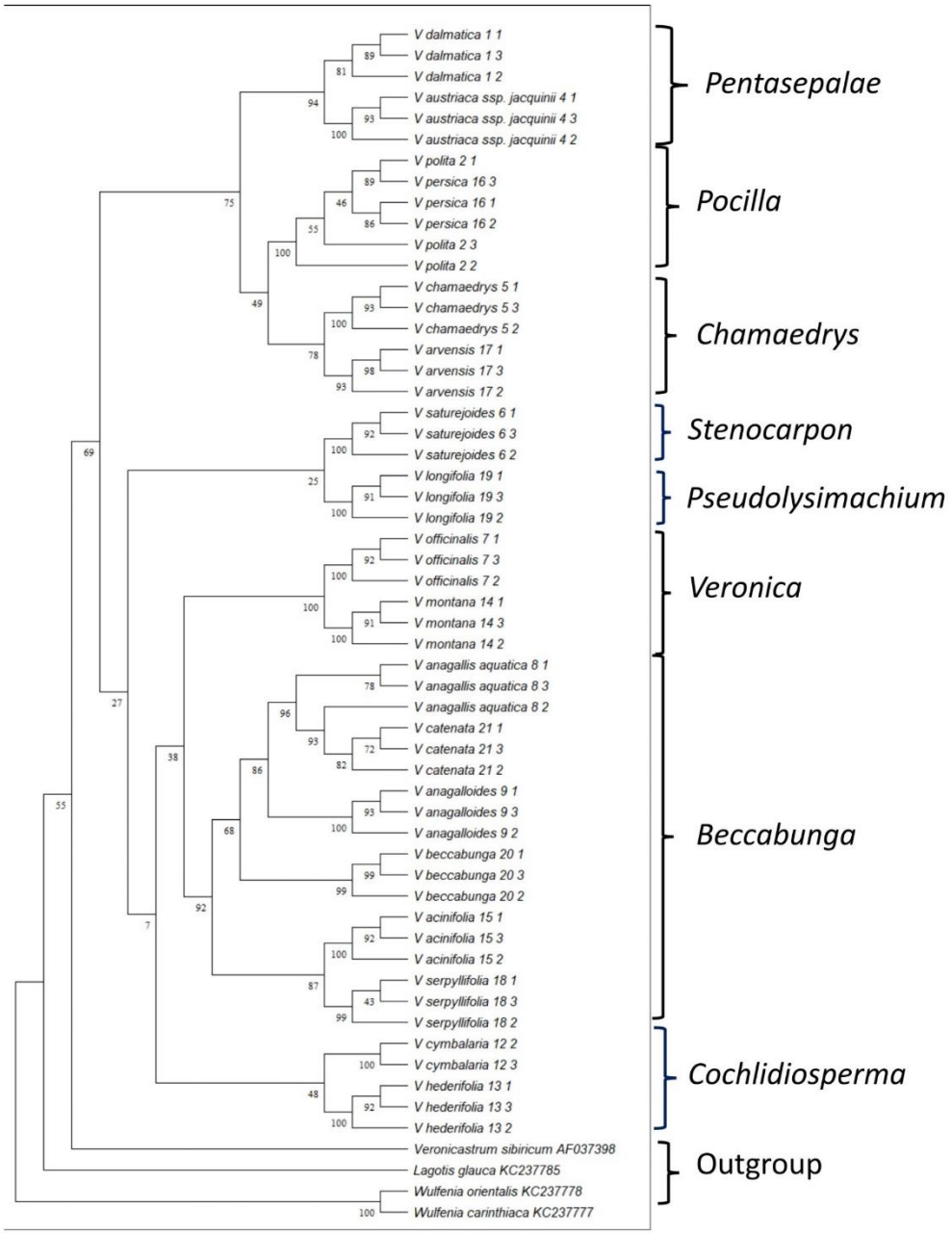

Figure 2. Evolutionary relationships of the studied *Veronica* species inferred from ITS2 sequence analysis by the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Subgeneric affiliation is shown.

The final dataset of ITS1-5.8S-ITS2 sequences contained 28 sequences, and there were a total of 528 positions in the final dataset. The evolutionary history was inferred by using the maximum likelihood method and general time-reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4567)). All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). The bootstrap consensus tree was inferred from 500 replicates. Evolutionary analyses were conducted in MEGA11 [36].

2.1.4. NGS (Illumina Sequencing) of ITS2 Region and Data Analyses

Preparation of Illumina libraries, PCR and sequencing was outsourced to Novogene (Cambridge, UK). Target ITS2 region was amplified from genomic DNA extractions using ITS3 (5′ GCATCGATGAAGAACGCAGC 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) universal primers described by White et al. [35]. The same amount of PCR products from each sample was pooled, end-repaired, A-tailed and ligated with Illumina adapters. NovaSeq PE250 Sequencing System was used to sequence the libraries. Paired-end reads were assigned to samples based on their unique barcode, after which barcode and primer sequences were removed. Quality filtering of the raw reads was performed with Novogene proprietary pipeline and included discarding reads shorter than 60 bp, reads with >10% of uncertain nucleotides (N) and reads with >50% of low-quality nucleotides (Qscore $\leq$ 5). The quality of paired-end reads at every step was inspected with FastQC v0.11.8 [41]. Clean reads were merged using FLASH v1.2.7 [42], and chimeras were removed by comparing them to the Gold database using UCHIME algorithm v.7.0.1001 [43]. The resulting effective clean tags were further screened for contamination by comparison with the UNITE database [44] of the eukaryotic nuclear ribosomal ITS regions using SortMeRNA v2.1 [45]. Tags that did not match any of the *Veronica* plant species in the database were discarded from further analyses. Contigs (ribotypes) of the ITS2 region for each sample were assembled de novo as fragments using MIRA v5rc1 [46] in draft mode. Assembly in fragment mode is recommended for single gene projects or small plasmids. Since the tag number was high (mean 90,774, min. 32,731–max. 116,647), lossless digital normalization was enabled, and other assembly parameters were kept as default. Contigs that contained more than 20% of reads and displayed highest coverage were selected as representative ribotypes, and the longest of them were selected for phylogenetic analyses (Table S4). Purification of effective tags, quality control and assembly of contigs were performed using the resources of the Isabella computing cluster hosted by the University Computing Centre, University of Zagreb (SRCE), Croatia.

2.2. Review Protocol

In order to obtain data for the cluster analyses of volatile compounds, the data for the free volatile compounds (FVCs) for 18 selected *Veronica* species used for this paper were obtained from one previously published paper by our team [24]. The volatile compounds were isolated by two methods: hydrodistillation in a Clevenger-type apparatus (Šurlan, Medulin, Croatia) and microwave-assisted extraction (Milestone 'ETHOS X' microwave laboratory oven, 1900 W maximum, Sorisole, Italy) for 2.5 h using 30–50 g of dried plant material. The distillate consists of two layers: a lipophilic layer collected in a side tube using a pentane/diethyl ether trap and a water layer (hydrosol). For this study, we only used volatile compounds from lipophilic layer, because it is standard procedure in most papers when obtaining data for cluster analyses of volatile compounds [47–51]. For this paper, we presented percentages of FVCs from both extractions in Table 2. Percentages for FVCs were obtained with gas chromatography–mass spectrometry. Method for FVC analyses is presented in detail in paper by Dunkić et al. [24].

Table 2. Free volatile compounds most abundant in all investigated *Veronica* species—Clevenger hydrodistillation (HD) and microwave extraction (MW) (relative percentage, %).

Subgenus/Species	Hexahydrofarnesyl Acetone		Hexadecanoic Acid		Caryophyllene Oxide		*(E)*-Caryophyllene		Phytol		Pentacosane		Germacrene D	
	HD	MW	HD	MW	HD	MW	HD	MW	HD	MW	HD	MW	HD	MW
Pentasepalae														
V. austriaca ssp. *jacquinii*	17.12	8.61	32.17	22.17	13.98	6.64	8.01	1.79	4.58	6.58	1.03	5.91	-	0.68
V. dalmatica	7.72	3.44	1.13	2.65	0.52	7.52	3.48	39.53	41.22	2.75	0.25	0.35	3.87	1.89
Pseudolysimachium														
V. longifolia	9.08	9.28	9.74	6.14	5.58	1.53	4.13	1.43	13.63	37.18	6.81	4.71	3.87	-
Beccabunga														
V. acinifolia	15.37	16.17	3.35	4.52	7.71	5.52	4.46	6.51	15.63	39.88	-	5.75	0.43	1.83
V. anagallis-aquatica	27.17	25.97	4.65	4.77	4.36	2.55	5.49	3.29	9.42	14.56	-	-	1.28	0.88
V. beccabunga	6.13	9.56	2.72	4.74	4.22	1.62	2.75	2.95	27.31	34.54	0.51	1.13	0.42	0.42
V. catenata	17.75	17.22	10.02	5.81	1.55	6.52	4.11	2.48	29.92	42.26	0.28	-	-	-
V. serpyllifolia	7.92	6.54	12.28	7.71	4.19	14.74	2.11	6.83	39.79	18.72	0.98	0.18	0.67	3.24
V. anagalloides	14.33	19.12	13.67	9.17	4.91	8.58	4.07	4.01	9.58	14.88	2.01	5.43	2.22	3.07
Veronica														
V. montana	6.86	9.17	9.24	5.81	7.28	2.61	0.13	0.44	18.53	37.03	10.47	14.90	-	-
V. officinalis	3.25	6.82	13.21	12.40	4.65	4.15	3.12	3.12	32.61	16.89	11.89	0.15	-	-
Stenocarpon														
V. saturejoides	6.88	17.72	6.14	6.64	34.53	8.43	9.43	8.49	-	22.47	-	0.48	2.61	5.11
Chamaedrys														
V. arvensis	6.35	17.55	3.17	17.42	14.11	7.11	6.21	3.25	7.54	22.57	0.71	-	1.25	2.45
V. chamaedrys	10.82	16.69	5.73	15.83	6.25	1.22	2.43	1.05	31.66	18.88	0.56	8.36	1.02	0.12
Pocilla														
V. persica	10.31	18.47	7.35	5.31	10.11	3.14	9.29	2.62	20.21	23.71	-	5.27	0.75	0.35
V. polita	10.28	10.82	6.75	5.69	7.55	1.48	6.57	4.17	31.18	19.88	0.36	1.76	1.06	0.07
Cochlidiosperma														
V. cymbalaria	36.33	13.35	0.75	15.72	10.92	32.72	3.95	6.13	-	3.71	0.71	0.49	1.42	2.34
V. hederifolia	28.85	59.15	7.25	1.57	4.59	0.51	4.11	1.10	18.53	14.58	0.21	-	1.47	-

2.3. Statistical Analyses

Cluster Analyses Based on Free Volatile Compounds

Cluster analysis (CA) was based on chemical constituents obtained by classical extraction—hydrodistillation with Clevenger apparatus and microwave-assisted water extractions in an amount of at least 2%. Dendrograms of Euclidean distances were prepared according to Ward's method to verify the affinities determined in the molecular analysis. CA was performed using the Statistica 7 software (StatSoft Inc., Tulsa, OK, USA).

The relationship between matrices of Euclidean distances describing chemical components of species and genetic distances obtained by Sanger and NGS methods was assessed using Mantel test as implemented in vegan package for R v4.2.2 [52]. Spearman rank correlation was chosen as a measure of association, and significance was calculated through 9999 permutations. Genetic distances for the Mantel test were calculated in MEGA11 [34] using Kimura 2-parameter model with 1000 bootstrap iterations [53].

3. Results

3.1. ITS2 and ITS1_5.8S-ITS2 Data Analyses

With the aim of evaluating the usefulness of next-generation sequencing (NGS) for the authentication of species from the genus *Veronica*, we performed NGS sequencing of the ITS2 region of 18 *Veronica* species (data available as BioProject PRJNA944611). Each species was represented by three plants. On average, 99086 paired-end reads were produced per sample (Table S3). After quality control, filtering and chimera removal, an average of 99.4% of reads remained and were used to generate effective clean tags. The proportion of contaminant non-*Veronica* species tags was generally low (4.8% on average), except for sample E (*V. chamaedrys*) where 30.9% of tags were removed (Table S3).

Out of 18 *Veronica* species, the identity of 11 species was successfully confirmed by blasting randomly chosen 5000 sequences for the ITS2 region, as these data were consistent with the results of anatomical-morphological identification. For the remaining seven species, identification based on the ITS2 region was doubtful and did not agree with their morphological identification. For these samples, an additional analysis of the complete ITS1-5.8S-ITS2 region was performed by the PCR amplification of this region and its classical Sanger sequencing (Table S1).

The number of reads/tags after the clean and merge procedures is shown in Table S2. Clean effective tags were assembled into anywhere between 21 to 237 contigs per sample, with one largest contig/ribotype recreated with the highest coverage and a length of approximately 360 nucleotides for most samples. Information on the assembled contigs supported by at least 20% of the reads for each sample is listed in Table S3, and their nucleotide sequences are given in fasta format in Data S1. These contigs/ribotypes that had the highest coverage were selected as representative ITS2 ribotypes, deposited in GenBank under unique GenBank accession numbers (Table S1), and the longest of those were chosen for phylogenetic analyses (Figure 2).

Both sets of DNA sequence data (ITS2 and ITS1-5.8S-ITS2) showed that the 18 analyzed *Veronica* species were divided into eight major groups corresponding to the following subgenera: *Pentasepalae*, *Pocilla*, *Chamaedrys*, *Veronica*, *Beccabunga*, *Cochlidiosperma*, *Stenocarpon* and *Pseudolysimachium* (Figures 2 and 3). The most numerous subgenus was *Beccabunga*, which contained six species: *V. beccabunga*, *V. catenata*, *V. acinifolia*, *V. serpyllifolia*, *V. anagaloides* and *V. anagalis-aquatica*. This subgenus is much better supported in the phylogenetic tree with ITS1-5.8S-ITS2 sequences (bootstrap support = 99) (Figure 3) than in the tree containing only ITS 2 sequences (bootstrap support = 92) (Figure 2). Monophyletic group *Cochlidiosperma*, with two species *V. cymbalaria* and *V. hederifolia*, was the least supported group in both the ITS2 and the ITS1-5.8S-ITS2 phylogenetic trees (Figures 2 and 3). The subgenera *Stenocarpon* and *Pseudolysimachium* are represented by only a single species *V. saturejoides* and *V. longifolia*, respectively.

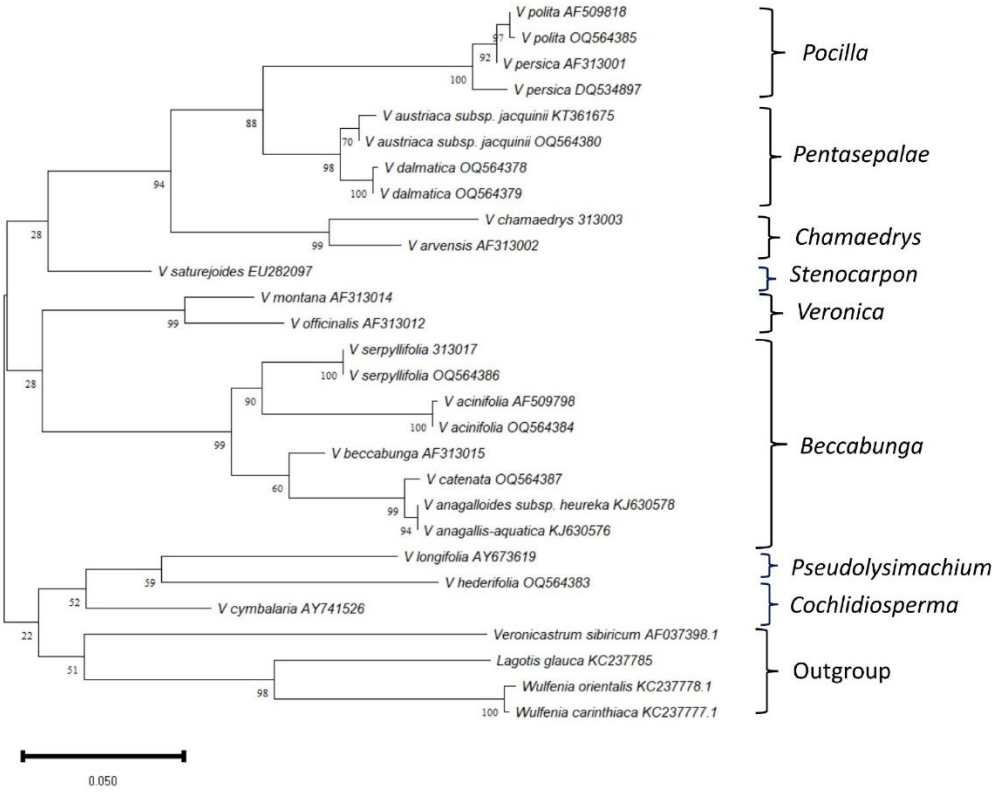

Figure 3. Evolutionary relationships of the studied *Veronica* species inferred from ITS1-5.8S-ITS2 sequence analysis by the maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Subgeneric affiliation is shown.

A comparison of the two phylogenetic analyses and their results—the two phylogenetic trees—suggests that the analysis of the longer ITS1-5.8S-ITS2 sequences yielded better subgenus classification than the analysis of the ITS2 sequences. This is likely due to the fact that longer Sanger-generated sequences (~580 bp) produce more variable and informative sites (positions) than shorter NGS sequences (~360 bp). Thus, our results indicate that ITS2 data are of rather limited value for *Veronica* species identification and phylogenetic analysis.

3.2. Statistical Analyses

3.2.1. Cluster Analyses Based on Free Volatile Compounds

The complete table of volatile compounds of these *Veronica* species was previously published in a paper by Dunkić et al. [24]. Free volatile compounds were extracted with two methods of hydrodistillation, with the Clevenger apparatus and microwave hydrodistillation. Table 2 lists the compounds with the highest relative percentages for all species selected for genetic research. Cluster analyses were performed for this study, and the results are shown in Figures 4 and 5. A total of 40 volatiles were used for this analysis. Figure 4 shows the clustering for the volatiles extracted with the Clevenger apparatus. Two main groups can be observed, which we described with five major compounds: phytol, hexahydrofarnesyl acetone, caryophyllene oxide, *(E)*-caryophyllene and hexadecanoic acid. The first group includes *V. persica*, *V. acinifolia*, *V. chamaedrys*, *V. arvensis*, *V. cymbalaria*, *V. anagalloides*, *V. longifolia*, *V. austriaca* ssp. *jacquinii*, *V. polita*, *V. montana*

and *V. anagallis-aquatica*. This group cannot be described with only one chemotype, but we can observe three subgroups. The subgroup *V. persica*/*V. acinifolia*/*V. chamaedrys* can be described with compounds in the following order: phytol > hexahydrofarnesyl acetone > caryophyllene oxide > (*E*)-caryophyllene > hexadecanoic acid. The subgroup *V. arvesis*/V. cymbalaria/V. anagalloides/V. longifolia/V. austriaca ssp. jacquinii is defined with compounds in the following order: hexahydrofarnesyl acetone ≈ hexadecanoic acid ≈ caryophyllene oxide > (*E*)-caryophyllene ≈ phytol. The third subgroup *V. polita*/*V. montana*/*V. anagallis-aquatica* can be described with compounds in the following order: phytol ≈ hexahydrofarnesyl acetone > hexadecanoic acid ≈ caryophyllene oxide > (*E*)-caryophyllene. The second cluster consists of *V. serpyllifolia*, *V. hederifolia*, *V. dalmatica*, *V. saturejoides*, *V. catenata*, *V. officinalis* and *V. beccabunga*. This cluster is specified by the main compounds in the following order: phytol ≈ caryophyllene oxide ≈ hexahydrofarnesyl acetone > hexadecanoic acid > (*E*)-caryophyllene.

In Figure 5, which shows the clusters of volatiles extracted with a microwave apparatus, three clusters can be observed. The first cluster includes *V. persica*, *V. polita*, *V. serpyllifolia*, *V. arvensis*, *V. chamaedrys*, *V. saturejoides*, *V. anagalloides* and *V. anagallis-aquatica*. This cluster is specified by the major compounds in the following order: hexahydrofarnesyl acetone > phytol > hexadecanoic acid ≈ caryophyllene oxide > *(E)*-caryophyllene. The second cluster consists of *V. officinalis*, *V. austriaca* ssp. *jacquinii*, *V. cymbalaria*, *V. dalmatica* and *V. hederifolia*. This cluster is specified by major compounds in the following order: phytol ≈ caryophyllene oxide ≈ hexahydrofarnesyl acetone > hexadecanoic acid > (*E*)-caryophyllene. It can be seen that this type of percentage ratio of volatile compounds is observed in the second cluster for Clevenger isolation (species compared in both clusters are *V. officinalis*, *V. dalmatica* and *V. hederifolia*). The third cluster includes *V. acinifolia*, *V. montana*, *V. longifolia*, *V. catenata* and *V. beccabunga*. For all these species, the prevailing compound is phytol, and other compounds are evenly distributed in relative percentage.

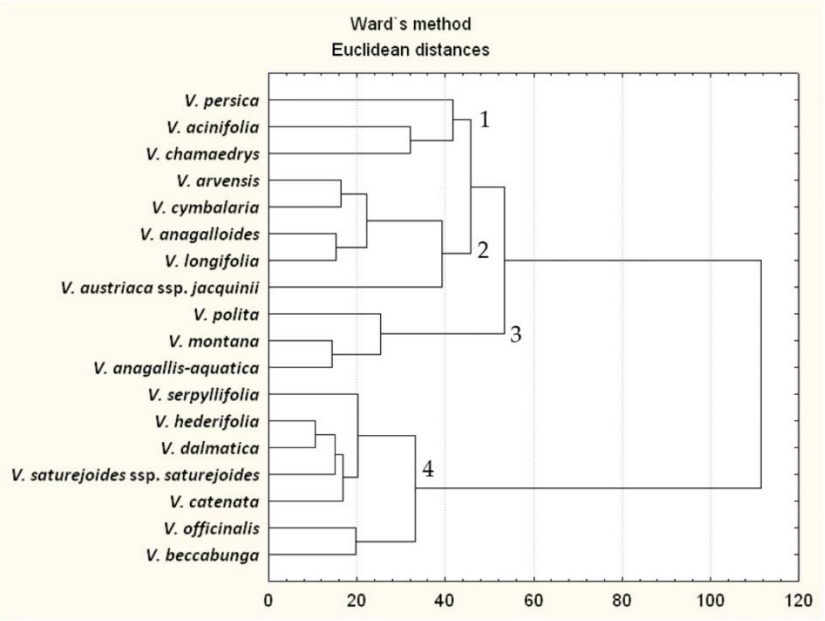

Figure 4. Dendrograms of Euclidean distances using Ward's method based on chemical constituents obtained by classical extraction—hydrodistillation with Clevenger apparatus in an amount equal to or greater than 2%. 1-phytol; 2-hexahydrofarnesyl acetone; 3- phytol/hexahydrofarnesyl acetone; 4-phytol/caryophyllene oxide/hexahydrofarnesyl acetone.

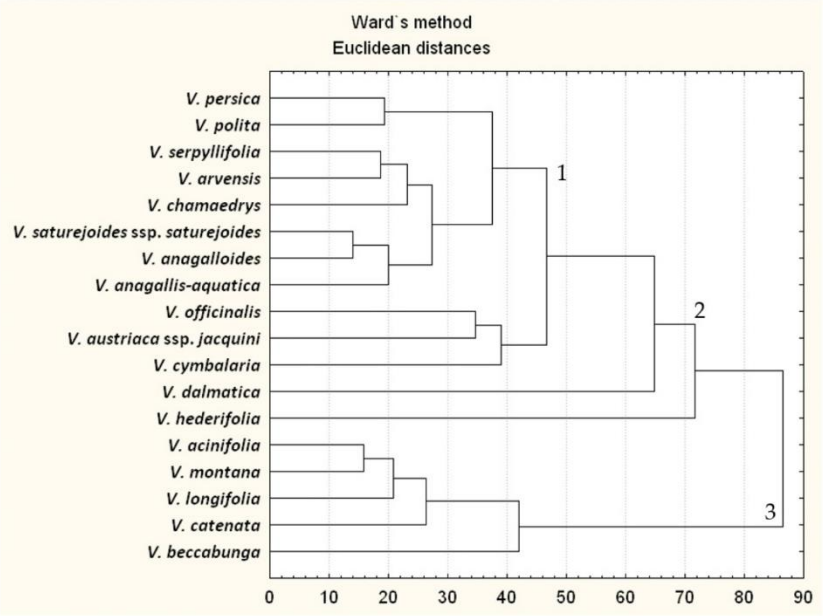

Figure 5. Dendrograms of Euclidean distances using Ward's method based on chemical constituents obtained by microwave-assisted water extractions in an amount equal to or greater than 2%. 1-hexahydrofarnesyl acetone; 2-phytol/caryophyllene oxide/hexahydrofarnesyl aceton; 3-phytol.

3.2.2. Mantel Test

In order to compare the grouping of the 18 studied *Veronica* species based on the ITS sequences with the grouping based on volatile components, a Mantel test was performed to compare the Euclidean distances of volatile components and Kimura genetic distances from DNA sequence data (Data S3). However, the result showed that there was no correlation between these two sets of data, and it was not statistically significant.

4. Discussion

Although it was possible to identify most of the analyzed *Veronica* species studied from their ITS2 sequence data generated by NGS sequencing, more accurate identification was achieved by using the longer ITS1-5.8S-ITS2 sequences obtained by classical Sanger sequencing. In addition, better results of the phylogenetic analysis and clustering of *Veronica* species to eight subgenera were obtained based on ITS1-5.8S-ITS2 sequences than with data based on ITS2 sequences. This is likely due to the fact that longer sequences generated by the Sanger sequencing method (~580 bp) produced more variable and informative positions than shorter NGS sequences (~360 bp). However, the abundance of ITS2 sequences obtained with NGS technology, along with ITS1 sequences also sequenced with NGS for 18 *Veronica* species, which are not shown in this paper because they are less informative than the ITS2 sequences, will be used for the detailed analysis of the diversity of contigs/ribotypes within each species and their molecular evolution and genome structure, and such analyses are underway.

The results of the two phylogenetic analyses (based on ITS2 and ITS1-5.8S-ITS2) are largely consistent with previous publications. According to our and previously published data on molecular-phylogenetic analyses of the *Veronica* species [1,2,6,8,10,28,54,55], the species from Croatia selected for this research belong to eight subgenera: *Pseudolysimachium* (*V. longifolia*), *Beccabunga* (*V. acinifolia*, *V. anagallis-aquatica*, *V. beccabunga*, *V. catenata*, *V. serpyllifolia*, *V. anagalloides*), *Veronica* (*V. montana*, *V. officinalis*), *Chamaedrys*

(*V. arvensis*, *V. chamaedrys*), *Pentasepalae* (*V. austriaca*, *V. dalmatica*), *Stenocarpon* (*V. saturejoides*), *Pocilla* (*V. persica*, *V. polita*) and *Cochlidiosperma* (*V. cymbalaria*, *V. hederifolia*).

Comparing the two clusters created based on the free volatile compounds (Figures 4 and 5) and based on the molecular data for ITS regions (Figure 2), it can be said that clustering based on the volatile compounds from microwave extraction resulted in clusters that better fit the molecular clusters. The first cluster (Figure 5) consists of species belonging to *Chamaedrys*, *Pocilla*, *Stenocarpon* and *Beccabunga* subgenera. *Chamaedrys* and *Pocilla* are closely related in the molecular ITS clusters. The second cluster includes species from *Cochlidiosperma*, *Veronica* and *Pentasepalae* subgenera. The third cluster consists of species from *Veronica* and *Beccabunga* subgenera. In Figure 2, these two subgenera, *Veronica* and *Beccabunga*, are closely related. Compounds in these species that were present in all species regardless of the habitat and conditions under which they grew include hexahydrofarnesyl acetone, hexadecanoic acid, caryophyllene oxide and *(E)*-caryophyllene. Other compounds also present in almost all species samples are phytol, germacrene D and pentacosane. The different relative percentages and their mutual ratio are probably the result of the ecological conditions in which they live. Since no correlation was found between the ITS cluster and the volatile cluster, it can be concluded that these compounds are not good interspecies (belonging to the same genus) chemophenetic markers, but some of them can be defined as chemophenetic markers for the whole genus *Veronica*. To back up this result, we reviewed volatile compounds studied in other genera belonging to the family Plantaginaceae and found that compositions of volatiles differed in major compounds but had some of the same compounds as *Veronica* species. Hammami et al. studied essential oil of *Plantago afra* and found that the major constituents were thymol (14.3%), 3-[4-(t-Butyl) phenyl] furan-2,5-dione (12.7%), hexadecanoic acid (8.9%) and eudesmane [56]. Al-Mazroa et al. studied essential oils of *Plantago amplexicaulis* and *Plantago boissieri*, and the results showed the main composition for *P. amplexicaulis* was hexadecanoic acid and 3 -methyl undecane. *P. boissieri* major compounds were found to be bicyclo-2,2, 1-heptane,2-(2-propenyl and l-dodecane-3-ol [57]. Essential oil major constituents of *Conobea scoparioides* were found to be thymol methyl ether (62%), thymol (16%) and α-phellandrene (14%) in a study by de Lima et al. [58]. In another study by Brandao et al., *Dizygostemon riparius* essential oil major constituents were found to be endo-fenchyl acetate and endo-fenchol, followed by *(E)*-caryophyllene and caryophyllene oxide in smaller relative percentages [59]. Bajer et al. studied the essential oil composition of *Plantago lanceolata* leaves and found the following major compounds: hexadecanoic acid, linalool and pentyl vinyl ketone. They also identified hexahydrofarnesyl acetone but in small relative percentages (1.81–2.99%) [60]. In another study on the essential oils of *Plantago lanceolata* and *Plantago major*, the major compounds identified were different from those in Bajer et al.'s research: metaraminol, bifemelane, metossamina and pterin-6-carboxylic acid in *P. lanceolata* and 2-dodecen-1-yl (-) succinic anhydride, benzenemethanol, α-(1-aminoethyl)-2,5- dimethoxy, dl-phenylephrine and nortriptyline in *P. major* [61]. Fons et al. also studied *Plantago lanceolata* essential oil and found the major compounds of leaf essential oil to be oct-1-en-3-ol and *(E)*-4(3-oxo-2.6.6-trimethylcyclohex-2-en-1-yl)-3-buten-2-ol [62]. They found hexahydrofarnesyl acetone as a major compound but only in the fruits of this plant [62]. One more study on the Plantaginaceae family reported hexahydrofarnesyl acetone as a detected compound in the FVC composition. Roudbaraki et al. studied *Digitalis nervosa* leaf volatile compounds and found that the major compounds were *trans*-pinocamphone and hexadecanoic acid, followed by caryophyllene oxide and phytol [63]. Comparing all these results to the identified volatiles of *Veronica* species and their relative percentage, it can be concluded that the identified volatile compounds of the genus *Veronica* were not found outside the genus in this combination, at least not until this moment. Some major compounds mentioned earlier in these identified percentage ratios can be defined as chemophenetic markers for the genus *Veronica*. Hexahydrofarnesyl acetone is particularly noteworthy, which appears in high percentages in all *Veronica* species studied and appears in other Plantaginaceae genera in much smaller relative percentages than in *Veronica* species. Other reported identi-

fications of this compound are studied in seeds or fruits, so they are not comparable to our study [62,64]. A similar situation is found with caryophyllene oxide, *(E)*-caryophyllene, phytol, germacrene D and pentacosane. After reviewing all these Plantaginaceae volatiles, it can be concluded that hexadecanoic acid is not a good chemophenetic marker as it appears in many other species outside the *Veronica* genus. Our study is not the first that found compounds that are chemophenetic markers for the genus but not for the subgenus level. Mehrvarz et al. found similar results in their study, in which they detected constant and characteristic iridoid and flavonoid profiles in selected *Veronica* species, which is useful in analyzing taxonomic problems at a specific level (intergenus level) [17] but not for distinguishing species belonging to the same genus. Reviewing the glycosides that were detected in the species of genus *Veronica*, Taskova et al. found that aucubin and catalpol were found in all the species they investigated (many of them were part of this study by our team: *V. polita*, *V. persica*, *V. chamaedrys*, *V. cymbalaria*, *V. montana*, *V. officinalis*, *V. longifolia*, *V. anagallis-aquatica*, *V. peregrina*, *V. beccabunga*, *V. serpyllifolia*, *V. acinifolia*) [10]. In their research, they also found no correlation between clade membership and phytochemical components due to intraclade variability. This is also the case with free volatile compounds from our research. Taskova et al. also concluded in their research of the New Zealand snow hebe that chemical profiles can provide valuable data for taxonomic problems at the subsection rank [65]. Further research on the free volatile compounds as chemophenetic markers could include some sections of the genus *Veronica* that grow in the Southern Hemisphere, such as *Hebe*, *Parahebe*, *Heliohebe*, *Detzneria* and *Derwentia*, as they were once considered to be different genera.

The Mantel test for the comparison between the Euclidean distances of volatile components and Kimura genetic distances from DNA sequence data showed that there was no correlation between the chemical and genetic data groups. Genetic distances are typically based on molecular markers that evolve slowly over time and are subject to random mutations. Moreover, the observed cases of parallel evolution and in the genus *Veronica* [2,37] can additionally complicate the correlation of genetic data with the chemical composition of volatile components. Volatile components as well as other plant metabolites may be influenced by more rapid changes such as changes in gene expression, environmental factors or epigenetic modifications. Therefore, it is important to consider multiple lines of evidence when studying the evolutionary relationships between organisms, including genetic, morphological and biochemical data. Further studies will shed more light on this interesting question.

5. Conclusions

This is the first report of the comparison between the free volatile compounds and DNA sequence data in Croatian *Veronica* species and a useful contribution to the better understanding of interspecies relationships in this genus. We did not find any correlations between the *Veronica* subgenera membership and the composition of free volatile compounds. This could be explained by the fact that volatile components as well as other plant metabolites may be influenced by more rapid changes such as changes in gene expression, environmental factors or epigenetic modifications. Major components identified by the classical (hydrodistillation) and green (microwave) methods of extraction regardless of the habitat isolated in all 18 species selected for this study were hexahydrofarnesyl acetone, hexadecanoic acid, phytol, *(E)*-caryophyllene and caryophyllene oxide. Since these compounds were not identified as major compounds in these relative percentage ratios in any other genera from the Plantaginaceae family, they could be considered as chemophenetic markers for the genus.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9050524/s1eo. Table S1: List of taxa, GenBank accession numbers for the ITS2 and ITS1-5.8S-ITS2 sequences of *Veronica* species obtained in this study, Table S2: List of taxa, GenBank accession numbers for the ITS1-5.8S-ITS2 sequences and references for the previously published sequences used in this study, Table S3: Number of raw reads, effective tags and contigs/ribotypes obtained after cleaning, merging, chimera removal and assembly of ITS2 region amplicons of *Veronica* plants with NovaSeq Illumina platform, Table S4: Statistics of assembled contigs/ribotypes of ITS2 region of *Veronica* plants that incorporated > 20% of clean tags, Data S1: Sequences of assembled contigs/ribotypes of ITS2 region of *Veronica* plants that incorporated > 20% of clean tags, Data S2: Sequences of ITS1-5.8S-ITS2 region of *Veronica* species obtained in this study and combined with earlier published sequences from GenBank, Data S3: Results of Mantel test.

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References

1. Albach, D.C.; Martínez-Ortega, M.M.; Fischer, M.A.; Chase, M.W. A New Classification of the Tribe Veroniceae-Problems and a Possible Solution. *Taxon* **2004**, *53*, 429–452. [CrossRef]
2. Albach, D.C.; Martínez-Ortega, M.M.; Chase, M.W. *Veronica*: Parallel Morphological Evolution and Phylogeography in the Mediterranean. *Plant Syst. Evol.* **2004**, *246*, 177–194. [CrossRef]
3. Sharifi-Rad, M.; Tayeboon, G.S.; Sharifi-Rad, J.; Iriti, M.; Varoni, E.M.; Razazi, S. Inhibitory Activity on Type 2 Diabetes and Hypertension Key-Enzymes, and Antioxidant Capacity of *Veronica* Persica Phenolic-Rich Extracts. *Cell Mol. Biol.* **2016**, *62*, 80–85. [CrossRef] [PubMed]
4. Grayer-Barkmeijer, R.J. A Chemosystematic Study of *Veronica*: Iridoid Glucosides. *Biochem. Syst. Ecol.* **1973**, *1*, 101–110. [CrossRef]
5. Grayer, R.J.; Chase, M.W.; Simmonds, M.S.J. A Comparison between Chemical and Molecular Characters for the Determination of Phylogenetic Relationships among Plant Families: An Appreciation of Hegnauer's "Chemotaxonomie Der Pflanzen". *Biochem. Syst. Ecol.* **1999**, *27*, 369–393. [CrossRef]
6. Albach, D.C.; Meudt, H.M.; Oxelman, B. Piecing Together the "New" Plantaginaceae. *Am. J. Bot.* **2005**, *92*, 297–315. [CrossRef]
7. Albach, D.C.; Grayer, R.J.; Jensen, S.R.; Özgökce, F.; Veitch, N.C. Acylated Flavone Glycosides from *Veronica*. *Phytochemistry* **2003**, *64*, 1295–1301. [CrossRef]
8. Albach, D.C.; Jensen, S.R.; Özgökce, F.; Grayer, R.J. *Veronica*: Chemical Characters for the Support of Phylogenetic Relationships Based on Nuclear Ribosomal and Plastid DNA Sequence Data. *Biochem. Syst. Ecol.* **2005**, *33*, 1087–1106. [CrossRef]
9. Jensen, S.R.; Albach, D.C.; Ohno, T.; Grayer, R.J. *Veronica*: Iridoids and Cornoside as Chemosystematic Markers. *Biochem. Syst. Ecol.* **2005**, *33*, 1031–1047. [CrossRef]
10. Taskova, R.M.; Albach, D.C.; Grayer, R.J. Phylogeny of *Veronica*—A Combination of Molecular and Chemical Evidence. *Plant Biol.* **2004**, *6*, 673–682. [CrossRef]
11. Reynolds, T. The Evolution of Chemosystematics. *Phytochemistry* **2007**, *68*, 2887–2895. [CrossRef] [PubMed]

12. Tundis, R.; Loizzo, M.; Menichini, F.; Statti, G.; Menichini, F. Biological and Pharmacological Activities of Iridoids: Recent Developments. *Mini-Rev. Med. Chem.* **2008**, *8*, 399–420. [CrossRef] [PubMed]
13. Harput, U.S.; Saracoglu, I.; Inoue, M.; Ogihara, Y. Phenylethanoid and Iridoid Glycosides from *Veronica persica*. *Chem. Pharm. Bull.* **2002**, *50*, 869–871. [CrossRef] [PubMed]
14. Harput, U.S.; Saracoglu, I.; Nagatsu, A.; Ogihara, Y. Iridoid Glucosides from *Veronica hederifolia*. *Chem. Pharm. Bull.* **2002**, *50*, 1106–1108. [CrossRef]
15. Crişan, G.; Vlase, L.; Balica, G.; Muntean, D.; Ştefănescu, C.; Păltinean, R.; Tămaş, M.; Leucuţa, S. LC /MS Analysis of Aucubin and Catalpol of Some *Veronica* Species. *Farmacia* **2010**, *58*, 237–242.
16. Taskova, R.; Peev, D.; Handjieva, N. Iridoid Glucosides of the Genus *Veronica* s.l. and Their Systematic Significance. *Plant Syst. Evol.* **2002**, *231*, 1–17. [CrossRef]
17. Saeidi Mehrvarz, S.; Mahmoodi, N.O.; Asadian, R.; Bakhshi Khaniki, G. Iridoid and Flavonoid Patterns of the Genus *Veronica* Sect. Alsinebe Subsect. Agrestis (Benth.) Stroh (Lamiales) and Their Systematic Significance. *Aust. J. Crop. Sci.* **2008**, *1*, 1–5. [CrossRef]
18. Albach, D.C.; Held Gotfredsen, C.; Jensen, S.R. Iridoid Glucosides of Paederota Lutea and the Relationships between *Paederota* and *Veronica*. *Phytochemistry* **2004**, *65*, 2129–2134. [CrossRef]
19. Saracoğlu, I.; Harput, Ü.Ş.; Ogihara, Y. Acylated Flavone Glycosides from *Veronica pectinata* var. *glandulosa* and *V. persica*. *Turk. J. Chem.* **2004**, *28*, 751–759.
20. Li, F. Analysis of Chemical Constituents of Essential Oil in *Veronica linariifolia* by Gas Chromatography-Mass Spectrometry. *Chin. J. Anal. Chem.* **2002**, 822–825.
21. Kwak, J.H.; Kim, H.J.; Lee, K.H.; Kang, S.C.; Zee, O.P. Antioxidative Iridoid Glycosides and Phenolic Compounds from *Veronica peregrina*. *Arch. Pharm. Res.* **2009**, *32*, 207–213. [CrossRef] [PubMed]
22. Çelik, E.; Yuvali Çelik, G.; Meysun, A. Essential Oil Composition and Antibacterial Activity of Some Plant Species. *J. Appl. Biol. Sci.* **2010**, *1*, 45–48. [CrossRef]
23. Ertas, A.; Boga, M.; Kizil, M.; Ceken, B.; Goren, A.C.; Hasimi, N.; Demirci, S.; Topcu, G.; Kolak, U. Chemical Profile and Biological Activities of *Veronica thymoides* Subsp. *pseudocinerea*. *Pharm. Biol.* **2015**, *53*, 334–339. [CrossRef] [PubMed]
24. Dunkić, V.; Nazlić, M.; Ruščić, M.; Vuko, E.; Akrap, K.; Topić, S.; Milović, M.; Vuletić, N.; Puizina, J.; Jurišić Grubešić, R.; et al. Hydrodistillation and Microwave Extraction of Volatile Compounds: Comparing Data for Twenty-One *Veronica* Species from Different Habitats. *Plants* **2022**, *11*, 902. [CrossRef] [PubMed]
25. Rojas-Andrés, B.M.; Low, E.; Albach, D.C.; Prebble, J.M.; Meudt, H.M.; Garnock-Jones, P.J. Is Genome Downsizing Associated with Diversification in Polyploid Lineages of *Veronica*? *Bot. J. Linn. Soc.* **2015**, *178*, 243–266. [CrossRef]
26. Albach, D.C.; Meudt, H.M. Phylogeny of *Veronica* in the Southern and Northern Hemispheres Based on Plastid, Nuclear Ribosomal and Nuclear Low-Copy DNA. *Mol. Phylogenet. Evol.* **2010**, *54*, 457–471. [CrossRef]
27. Bardy, K.E.; Albach, D.C.; Schneeweiss, G.M.; Fischer, M.A.; Schönswetter, P. Disentangling Phylogeography, Polyploid Evolution and Taxonomy of a Woodland Herb (*Veronica chamaedrys* Group, Plantaginaceae s.l.) in Southeastern Europe. *Mol. Phylogenet. Evol.* **2010**, *57*, 771–786. [CrossRef]
28. Albach, D.C.; Von Sternburg, M.; Scalone, R.; Bardy, K.E. Phylogenetic Analysis and Differentiation of *Veronica* Subgenus Stenocarpon in the Balkan Peninsula. *Bot. J. Linn. Soc.* **2009**, *159*, 616–636. [CrossRef]
29. Bardy, K.E.; Schönswetter, P.; Schneeweiss, G.M.; Fischer, M.A.; Albach, D.C. Extensive Gene Flow Blurs Species Boundaries among *Veronica barrelieri*, *V. orchidea* and *V. spicata* (Plantaginaceae) in Southeastern Europe. *Taxon* **2011**, *60*, 108–121. [CrossRef]
30. Batovska, J.; Cogan, N.O.I.; Lynch, S.E.; Blacket, M.J. Using Next-Generation Sequencing for DNA Barcoding: Capturing Allelic Variation in ITS2. *G3 Genes Genomes Genet.* **2017**, *7*, 19–29. [CrossRef]
31. Matyášek, R.; Renny-Byfield, S.; Fulneček, J.; Macas, J.; Grandbastien, M.A.; Nichols, R.; Leitch, A.; Kovařík, A. Next Generation Sequencing Analysis Reveals a Relationship between RDNA Unit Diversity and Locus Number in Nicotiana Diploids. *BMC Genom.* **2012**, *13*, 1–12. [CrossRef] [PubMed]
32. Delgado-Tejedor, A.; Leekitcharoenphon, P.; Aarestrup, F.M.; Otani, S. Evaluating the Usefulness of Next-Generation Sequencing for Herb Authentication. *Food Chem. Mol. Sci.* **2021**, *3*, 100044. [CrossRef] [PubMed]
33. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE* **2010**, *5*, e8432. [CrossRef] [PubMed]
34. Bezić, N.; Šamanić, I.; Dunkić, V.; Besendorfer, V.; Puizina, J. Essential Oil Composition and Internal Transcribed Spacer (ITS) Sequence Variability of Four South-Croatian *Satureja* Species (Lamiaceae). *Molecules* **2009**, *14*, 925–938. [CrossRef]
35. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press Inc.: New York, NY, USA, 1990; pp. 315–322.
36. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
37. Albach, D.C.; Chase, M.W. Paraphyly of Veronica (Veroniceae; Scrophulariaceae): Evidence from the Internal Transcribed Spacer (ITS) Sequences of Nuclear Ribosomal DNA. *J. Plant Res.* **2001**, *114*, 9–18. [CrossRef]
38. Muñoz-Centeno, L.M.; Albach, D.C.; Sánchez-Agudo, J.A.; Martínez-Ortega, M.M. Systematic Significance of Seed Morphology in *Veronica* (Plantaginaceae): A Phylogenetic Perspective. *Ann. Bot.* **2006**, *98*, 335–350. [CrossRef]

39. Wagstaff, S.J.; Garnock-Jones, P.J. Evolution and Biogeography of the *Hebe* Complex (Scrophulariaceae) Inferred from ITS Sequences. *N. Z. J. Bot.* **1998**, *36*, 425–437. [CrossRef]
40. Surina, B.; Pfanzelt, S.; Einzmann, H.J.R.; Albach, D.C. Bridging the Alps and the Middle East: Evolution, Phylogeny and Systematics of the Genus *Wulfenia* (Plantaginaceae). *Taxon* **2014**, *63*, 843–858. [CrossRef]
41. Andrews, S. *FastQC: A Quality Control Tool for High Throughput Sequence Data*; Babraham Institute: Cambridge, UK, 2018.
42. Magoč, T.; Salzberg, S.L. FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies. *Bioinformatics* **2011**, *27*, 2957–2963. [CrossRef]
43. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME Improves Sensitivity and Speed of Chimera Detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
44. Abarenkov, K.; Zirk, A.; Piirmann, T.; Pöhönen, R.; Ivanov, F.; Nilsson, R.H.; Kõljalg, U. *UNITE General FASTA Release for Eukaryotes 2*; Version 16.10.2022; UNITE Community; Available online: https://doi.org/10.15156/BIO/2483914 (accessed on 14 March 2023).
45. Kopylova, E.; Noé, L.; Touzet, H. SortMeRNA: Fast and Accurate Filtering of Ribosomal RNAs in Metatranscriptomic Data. *Bioinformatics* **2012**, *28*, 3211–3217. [CrossRef] [PubMed]
46. Chevreux, B.; Pfisterer, T.; Drescher, B.; Driesel, A.J.; Müller, W.E.G.; Wetter, T.; Suhai, S. Using the MiraEST Assembler for Reliable and Automated MRNA Transcript Assembly and SNP Detection in Sequenced ESTs. *Genome Res.* **2004**, *14*, 1147–1159. [CrossRef] [PubMed]
47. Craft, J.D.; Satyal, P.; Setzer, W.N. The Chemotaxonomy of Common Sage (*Salvia officinalis*) Based on the Volatile Constituents. *Medicines* **2017**, *4*, 12. [CrossRef] [PubMed]
48. Lim Ah Tock, M.J.; Kamatou, G.P.P.; Combrinck, S.; Sandasi, M.; Viljoen, A.M. A Chemometric Assessment of Essential Oil Variation of Three *Salvia* Species Indigenous to South Africa. *Phytochemistry* **2020**, *172*, 112249. [CrossRef] [PubMed]
49. Nikolić, J.S.; Zlatković, B.K.; Jovanović, S.Č.; Stojanović, G.S.; Marin, P.D.; Mitić, Z.S. Needle Volatiles as Chemophenetic Markers in Differentiation of Natural Populations of *Abies alba*, *A.* x Borisii-Regis, and *A. cephalonica*. *Phytochemistry* **2021**, *183*, 112612. [CrossRef]
50. Kremer, D.; Dunkić, V.; Radosavljević, I.; Bogunić, F.; Ivanova, D.; Ballian, D.; Stešević, D.; Matevski, V.; Ranđelović, V.; Eleftheriadou, E.; et al. Phytochemicals and Their Correlation with Molecular Data in *Micromeria* and *Clinopodium* (Lamiaceae) Taxa. *Plants* **2022**, *11*, 3407. [CrossRef]
51. Niederbacher, B.; Winkler, J.B.; Schnitzler, J.P. Volatile Organic Compounds as Non-Invasive Markers for Plant Phenotyping. *J. Exp. Bot.* **2015**, *66*, 5403–5416. [CrossRef]
52. Oksanen, J.; Simpson, G.; Blanchet, F.; Kindt, R.; Legendre, P.; Minchin, P.; O'Hara, R.; Solymos, P.; Stevens, M.; Szoecs, E.; et al. Vegan: Community Ecology Package. R Package 2022. Available online: https://CRAN.R-project.org/package=vegan (accessed on 14 March 2023).
53. Kimura, M. A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef]
54. Doostmohammadi, M.; Bordbar, F.; Albach, D.C.; Mirtadzadini, M. Phylogeny and Historical Biogeography of *Veronica* Subgenus *Pentasepalae* (Plantaginaceae): Evidence for Its Origin and Subsequent Dispersal. *Biol.* **2022**, *11*, 639. [CrossRef]
55. Albach, D.C.; Martınez-Ortega, M.M.; Fischer, M.A.; Chase, M.W. Evolution of Veroniceae: A Phylogenetic Perspective. *Ann. Mo. Bot. Gard.* **2004**, *91*, 275–302.
56. Hammami, S.; Debbabi, H.; Jlassi, I.; Joshi, R.K.; Mokni, R. El Chemical Composition and Antimicrobial Activity of Essential Oil from the Aerial Parts of *Plantago afra* L. (Plantaginaceae) Growing Wild in Tunisia. *South Afr. J. Bot.* **2020**, *132*, 410–414. [CrossRef]
57. Al-Mazroa, S.A.; Al-Wahaibi, L.H.; Mousa, A.A.; Al-Khathlan, H.Z. Essential Oil of Some Seasonal Flowering Plants Grown in Saudi Arabia. *Arab. J. Chem.* **2015**, *8*, 212–217. [CrossRef]
58. De Lima, E.J.; Fontes, S.S.; Nogueira, M.L.; Silva, V.R.; Santos, L.D.S.; D'Elia, G.M.; Dias, R.B.; Sales, C.B.S.; Rocha, C.A.G.R.; Bezerra, D.P.; et al. Essential Oil from Leaves of *Conobea scoparioides* (Cham. & Schltdl.) Benth. (Plantaginaceae) Causes Cell Death in HepG2 Cells and Inhibits Tumor Development in a Xenograft Model. *Biomed. Pharmacother.* **2020**, *129*, 110402. [CrossRef]
59. Brandão, C.M.; Cavalcante, K.S.B.; de M. Teles, R.; Georgiana, G.E.; Monteiro, O.S.; Andrade, E.H.A.; Maia, J.G.S. Composition and Larvicidal Activity of the Oil of *Dizygostemon riparius* (Plantaginaceae), a New Aromatic Species Occurring in Maranhão, Brazil. *Chem. Biodivers.* **2020**, *17*, e2000462. [CrossRef]
60. Bajer, T.; Janda, V.; Bajerová, P.; Kremr, D.; Eisner, A.; Ventura, K. Chemical Composition of Essential Oils from *Plantago lanceolata* L. Leaves Extracted by Hydrodistillation. *J. Food Sci. Technol.* **2016**, *53*, 1576–1584. [CrossRef]
61. Haghighi, S.R.; Yazdinezhad, A.; Bagheri, K.; Sharafi, A.; Haghighi, S.R.; Sharafi, A. Original Article Volatile Constituents and Toxicity of Essential Oils Extracted from Aerial Parts of *Plantago lanceolata* and *Plantago major* Growing in Iran. *Pharm. Biomed. Res.* **2022**, *8*, 205–224.
62. Fons, F.; Rapior, S.; Gargadennec, A.; Andary, C.; Bessière, J.M. Volatile Components of *Plantago lanceolata* (Plantaginaceae). *Acta Bot. Gall.* **1998**, *145*, 265–269. [CrossRef]
63. Roudbaraki, S.J.; Nori-Shargh, D. The Volatile Constituent Analysis of *Digitalis nervosa* Steud. & Hochst. Ex Benth. from Iran. *Russ. Chem. Bull.* **2016**, *65*, 1148–1150. [CrossRef]

64. Seifi, H.; Masoum, S.; Seifi, S.; Ebrahimabadi, E.H. Chemometric Resolution Approaches in Characterisation of Volatile Constituents in *Plantago ovata* Seeds Using Gas Chromatography-Mass Spectrometry: Methodology and Performance Assessment. *Phytochem. Anal.* **2014**, *25*, 273–281. [CrossRef]
65. Taskova, R.M.; Kokubun, T.; Ryan, K.G.; Garnock-Jones, P.J.; Jensen, S.R. Phenylethanoid and Iridoid Glycosides in the New Zealand Snow Hebes (*Veronica*, Plantaginaceae). *Chem. Pharm. Bull.* **2010**, *58*, 703–711. [CrossRef] [PubMed]

Data 3

Sanger

	Mantel statistic r	Signifance
Clevenger 2%	0.1643	0.0739
Microwave 2%	0	0.5052

NGS contigs

	Mantel statistic r	Signifance
Clevenger 2%	0.01682	0.4504
Microwave 2%	0.05229	0.3644

Table S1: GenBank accession numbers for the ITS2 and ITS1-5.8S-ITS2 sequences of *Veronica* species obtained in this study

Species	ITS2 GenBank number	ITS1-5.8S-ITS2 GenBank number
V. dalmatica_1	OQ594828	
V. dalmatica_2	OQ594829	OQ564378
V. dalmatica_3	OQ594830	OQ564379
V. polita_1	OQ594831	
V. polita_2	OQ594832	
V. polita_3	OQ594833	OQ564385
V. austriaca subsp. *jacquinii_1*	OQ594834	
V. austriaca subsp. *jacquinii_2*	OQ594835	OQ564380
V. austriaca subsp. *jacquinii_3*	OQ594836	
V. chamaedrys_1	OQ594837	
V. chamaedrys_2	OQ594838	
V. chamaedrys_3	OQ594839	
V. saturejoides_1	OQ594840	
V. saturejoides_2	OQ594841	
V. saturejoides_3	OQ594842	
V. officinalis_1	OQ594843	
V. officinalis_2	OQ594844	
V. officinalis_3	OQ594845	
Veronica anagallis-aquatica_1	OQ594846	
Veronica anagallis-aquatica_2	OQ594847	
Veronica anagallis-aquatica_3	OQ594848	
Veronica anagalloides_1	OQ594849	
Veronica anagalloides_2	OQ594850	
Veronica anagalloides_3	OQ594851	
Veronica cymbalaria_1	OQ594852	OQ564383
Veronica cymbalaria_2	OQ594853	
Veronica hederifolia_1	OQ594854	
Veronica hederifolia_2	OQ594855	
Veronica hederifolia_3	OQ594856	
Veronica montana_1	OQ594857	
Veronica montana_2	OQ594858	
Veronica montana_3	OQ594859	
Veronica acinifolia_1	OQ594860	
Veronica acinifolia_2	OQ594861	OQ564384
Veronica acinifolia_3	OQ594862	
Veronica persica_1	OQ594863	
Veronica persica_2	OQ594864	
Veronica persica_3	OQ594865	
Veronica arvensis_1	OQ594866	
Veronica arvensis_2	OQ594867	
Veronica arvensis_3	OQ594868	
Veronica serpyllifolia_1	OQ594869	
Veronica serpyllifolia_2	OQ594870	
Veronica serpyllifolia_3	OQ594871	OQ564386
Veronica longifolia_1	OQ594872	
Veronica longifolia_2	OQ594873	
Veronica longifolia_3	OQ594874	
Veronica beccabunga_1	OQ594875	
Veronica beccabunga_2	OQ594876	
Veronica beccabunga_3	OQ594877	
Veronica catenata_1	OQ594878	

Table S2. List of taxa, GenBank accession numbers, and references for the previously published **ITS1-5.8S-ITS2** sequences used in this study.

Species	GenBank accession number	References
V. austriaca subsp. *jacquinii*	KT361675	Rojas-Andres et al. 2015
V. persica	DQ534897	Kaplan and Hasanoglu, unpublished
V. persica	AF313001	Albach and Chase 2001
V. polita	AF509818	Albach et al. 2004.
V. chamaedrys	AF313003	Albach and Chase 2001
V. arvensis	AF313002	Albach and Chase 2001
V. hederaefolia	AJ548981	Ronald et al 2003
V. cymbalaria	AY741526	Munoz-Centeno et al. 2006
V. longifolia	AY673619	Albach et al. 2005
V. saturejoides	EU282097	Albach et al. 2009
V. montana	AF313014	Albach and Chase 2001
V. officinalis	AF313012	Albach and Chase 2001
V. serpyllifolia	AF313017	Albach and Chase 2001
V. acinifolia	AF509798	Albach et al. 2004.
V. beccabunga	AF313015	Albach and Chase 2001
V. anagallis-aquatica	KJ630576	Meudt et al 2014, unpublished
V. anagalloides ssp. *heureka*	KJ630578	Meudt et al 2014, unpublished
Satureja montana	EU823287	Bezić et al 2009
Satureja subspicata	EU823288	Bezić et al. 2009
Clinopodium macrostemun	JQ668083	Drew and Sytsma 2012

TABLE S3

SAMPLE_NAME	BIOSAMPLE_Accession	SAMPLE_LIBRARY	N_RAW_READS	N_CLEAN_READS	N_combined_TAGs	N_NoChimeras_TAGs	%_EFFECTIVEE_TAGS	N_VERONICA_TAGS	% VERONICA TAGS	N_MIRA_CONTIGS
V_dalmatica_1_1	SAMN33750235	A_1_1_ITS2	99170	99030	96561	94567	97.93	85523	90.44	75
V_dalmatica_1_2	SAMN33750236	A_1_2_ITS2	91956	91780	88927	87823	98.76	82013	93.38	77
V_dalmatica_1_3	SAMN33750237	A_1_3_ITS2	100371	100322	96622	95647	98.99	91423	95.58	75
V_polita_2_1	SAMN33750238	B_2_1_ITS2	117622	117563	107339	107207	99.88	105367	98.28	95
V_polita_2_2	SAMN33750239	B_2_2_ITS2	96097	95968	88392	88290	99.88	87269	98.84	54
V_polita_2_3	SAMN33750240	B_2_3_ITS2	89965	89920	87326	87293	99.96	86856	99.50	58
V_austriaca_ssp._jacquinii_4_1	SAMN33750241	D_4_1_ITS2	119907	119808	116616	116036	99.50	114254	98.46	74
V_austriaca_ssp._jacquinii_4_2	SAMN33750242	D_4_2_ITS2	100021	99938	97073	96182	99.08	93677	97.40	81
V_austriaca_ssp._jacquinii_4_3	SAMN33750243	D_4_3_ITS2	103492	103367	99851	99458	99.61	98139	98.67	65
V_chamaedrys_5_1	SAMN33750244	E_5_1_ITS2	111564	111420	45652	44678	97.87	32719	73.23	144
V_chamaedrys_5_2	SAMN33750245	E_5_2_ITS2	81824	81788	32767	32731	99.89	19732	60.29	81
V_chamaedrys_5_3	SAMN33750246	E_5_3_ITS2	98652	98583	37127	37047	99.78	27326	73.76	113
V_saturejoides_6_1	SAMN33750247	F_6_1_ITS2	110118	109960	107707	107272	99.60	102952	95.97	46
V_saturejoides_6_2	SAMN33750248	F_6_2_ITS2	79468	79386	77540	76454	98.60	72475	94.80	33
V_saturejoides_6_3	SAMN33750249	F_6_3_ITS2	119515	119188	116407	115514	99.23	111913	96.88	52
V_officinalis_7_1	SAMN33750250	G_7_1_ITS2	108578	108508	106087	104684	98.68	98242	93.85	55
V_officinalis_7_2	SAMN33750251	G_7_2_ITS2	101570	101506	98811	98166	99.35	95953	97.75	70
V_officinalis_7_3	SAMN33750252	G_7_3_ITS2	119158	119123	117819	114705	97.36	91303	79.60	45
V_anagallis_aquatica_8_1	SAMN33750253	H_8_1_ITS2	92198	92127	89345	89064	99.69	88129	98.95	74
V_anagallis_aquatica_8_2	SAMN33750254	H_8_2_ITS2	114194	114102	104546	102997	98.52	96382	93.58	62
V_anagallis_aquatica_8_3	SAMN33750255	H_8_3_ITS2	105189	105142	103654	103529	99.88	102623	99.12	48
V_anagalloides_9_1	SAMN33750256	I_9_1_ITS2	101446	101210	98526	97505	98.96	93298	95.69	33
V_anagalloides_9_2	SAMN33750257	I_9_2_ITS2	97201	97115	94545	94099	99.53	90993	96.70	28
V_anagalloides_9_3	SAMN33750258	I_9_3_ITS2	90001	89960	88627	85694	96.69	66812	77.97	26
V_cymbalaria_12_2	SAMN33750259	L_12_2_ITS2	98852	98741	95049	94695	99.63	93394	98.63	48
V_cymbalaria_12_3	SAMN33750260	L_12_3_ITS2	110751	110717	108966	108819	99.87	107680	98.95	44
V_hederifolia_13_1	SAMN33750261	M_13_1_ITS2	97262	97207	95256	95095	99.83	93862	98.70	62
V_hederifolia_13_2	SAMN33750262	M_13_2_ITS2	91395	91319	89062	88982	99.91	88157	99.07	43
V_hederifolia_13_3	SAMN33750263	M_13_3_ITS2	81516	81485	80624	80606	99.98	80402	99.75	34
V_montana_14_1	SAMN33750264	N_14_1_ITS2	104059	103867	96735	95990	99.23	92367	96.23	53
V_montana_14_2	SAMN33750265	N_14_2_ITS2	87383	87199	84829	84701	99.85	84051	99.23	41
V_montana_14_3	SAMN33750266	N_14_3_ITS2	101471	101315	99917	99806	99.89	99041	99.23	21
V_acinifolia_15_1	SAMN33750267	O_15_1_ITS2	106781	106711	103739	103639	99.90	102995	99.38	49
V_acinifolia_15_2	SAMN33750268	O_15_2_ITS2	95376	95338	92162	92118	99.95	91773	99.63	45
V_acinifolia_15_3	SAMN33750269	O_15_3_ITS2	94945	94910	93305	92985	99.66	91134	98.01	35
V_persica_16_1	SAMN33750270	P_16_1_ITS2	93769	93704	91205	90885	99.65	89513	98.49	51
V_persica_16_2	SAMN33750271	P_16_2_ITS2	89534	89488	87091	86942	99.83	82700	95.12	39
V_persica_16_3	SAMN33750272	P_16_3_ITS2	95497	95459	94157	94093	99.93	93249	99.10	27
V_arvensis_17_1	SAMN33750273	R_17_1_ITS2	111527	111405	72609	72504	99.86	72027	99.34	236
V_arvensis_17_2	SAMN33750274	R_17_2_ITS2	97022	96924	61615	61370	99.60	60258	98.19	227
V_arvensis_17_3	SAMN33750275	R_17_3_ITS2	96080	96029	76481	76439	99.95	76164	99.64	237
V_serpyllifolia_18_1	SAMN33750276	S_18_1_ITS2	86830	86801	84476	84409	99.92	83936	99.44	62
V_serpyllifolia_18_2	SAMN33750277	S_18_2_ITS2	99773	99713	96855	96804	99.95	96240	99.42	60
V_serpyllifolia_18_3	SAMN33750278	S_18_3_ITS2	93019	92971	91713	91692	99.98	91233	99.50	26
V_longifolia_19_1	SAMN33750279	T_19_1_ITS2	90015	89946	87610	85949	98.10	76678	89.21	57
V_longifolia_19_2	SAMN33750280	T_19_2_ITS2	82787	82743	80186	79466	99.10	76895	96.76	58
V_longifolia_19_3	SAMN33750281	T_19_3_ITS2	116597	116556	115184	114357	99.28	110041	96.23	25
V_beccabunga_20_1	SAMN33750282	U_20_1_ITS2	80357	80293	78372	78312	99.92	77905	99.48	54
V_beccabunga_20_2	SAMN33750283	U_20_2_ITS2	90182	90129	84239	84134	99.88	83557	99.31	73
V_beccabunga_20_3	SAMN33750284	U_20_3_ITS2	106037	106004	104700	104669	99.97	104463	99.80	45
V_catenata_21_1	SAMN33750285	V_21_1_ITS2	101760	101351	98857	97295	98.42	90562	93.08	45
V_catenata_21_2	SAMN33750286	V_21_2_ITS2	91688	91648	89271	89241	99.97	88905	99.62	49
V_catenata_21_3	SAMN33750287	V_21_3_ITS2	110001	109957	108624	108392	99.79	107146	98.85	45

TABLE S4

name	length	av.qual	N_reads	mx.cov.	av.cov	GC%	CnIUPAC	CnFunny	CnN	CnX	CnGap	CnNoCov
A_1_1_fragments_draft_norm_c1	364	40	74413	54883	20541.6	60.99	0	0	0	0	7	0
A_1_2_fragments_draft_norm_c1	364	40	66848	44872	22889.88	61.26	0	0	0	0	4	0
A_1_3_fragments_draft_norm_c1	364	40	78606	60337	22854.45	60.99	0	0	0	0	6	0
B_2_1_fragments_draft_norm_c1	385	43	98136	91478	70962.01	59.74	0	0	0	0	5	0
B_2_2_fragments_draft_norm_c1	359	40	81575	68078	53572.79	60.45	0	0	0	0	2	0
B_2_3_fragments_draft_norm_c1	359	40	75317	64012	55723.26	60.45	0	0	0	0	2	0
D_4_1_fragments_draft_norm_c1	364	40	92710	65389	21275.46	59.89	0	0	0	0	8	0
D_4_2_fragments_draft_norm_c1	364	40	79897	58079	18122.81	59.89	0	0	0	0	7	0
D_4_3_fragments_draft_norm_c1	364	40	88291	64837	20472.3	59.89	0	0	0	0	11	0
E_5_1_fragments_draft_norm_c1	361	40	16365	8549	5113.48	61.5	0	0	0	0	8	0
E_5_1_fragments_draft_norm_c3	181	39	7508	7111	3517.57	55.25	0	0	0	0	0	0
E_5_2_fragments_draft_norm_c1	258	40	5753	4507	2044.95	62.02	0	0	0	0	3	0
E_5_2_fragments_draft_norm_c2	361	40	7367	3962	1880.48	61.5	0	0	0	0	4	0
E_5_3_fragments_draft_norm_c1	361	40	12525	6098	3478.36	61.5	0	0	0	0	5	0
E_5_3_fragments_draft_norm_c2	253	40	6813	6245	2436.61	61.66	0	0	0	0	0	0
F_6_1_fragments_draft_norm_c1	362	40	95275	68569	52935.61	57.46	0	0	0	0	3	0
F_6_2_fragments_draft_norm_c1	362	40	67442	54304	44425.19	57.46	0	0	0	0	1	0
F_6_3_fragments_draft_norm_c1	362	40	102938	83366	66775.94	57.46	0	0	0	0	0	0
G_7_1_fragments_draft_norm_c1	361	40	83141	73332	41233.9	57.34	0	0	0	0	1	0
G_7_2_fragments_draft_norm_c1	361	40	80449	63805	39355.12	57.34	0	0	0	0	4	0
G_7_3_fragments_draft_norm_c1	361	40	83923	72714	50097.7	57.34	0	0	0	0	3	0
H_8_1_fragments_draft_norm_c1	362	40	70161	44935	20000.14	55.52	0	0	0	0	2	0
H_8_2_fragments_draft_norm_c1	362	40	86513	67719	41147.08	56.91	0	0	0	0	1	0
H_8_3_fragments_draft_norm_c1	362	40	96706	66630	29633.76	55.8	0	0	0	0	2	0
I_9_1_fragments_draft_norm_c1	362	40	91287	78247	58931.15	56.35	0	0	0	0	0	0
I_9_2_fragments_draft_norm_c1	362	40	88811	76829	56861.77	56.35	0	0	0	0	2	0
I_9_3_fragments_draft_norm_c1	362	40	65571	53448	49088.37	56.35	0	0	0	0	1	0
L_12_2_fragments_draft_norm_c1	361	40	81768	64062	38377.32	58.45	0	0	0	0	7	0
L_12_3_fragments_draft_norm_c1	361	40	102135	87116	52025.31	58.45	0	0	0	0	0	0
M_13_1_fragments_draft_norm_c1	358	40	82108	60341	38213.61	61.73	0	0	0	0	1	0
M_13_2_fragments_draft_norm_c1	358	40	75424	56424	35632.27	61.73	0	0	0	0	2	0
M_13_3_fragments_draft_norm_c1	358	40	76471	60224	32913.36	61.73	0	0	0	0	2	0
N_14_1_fragments_draft_norm_c1	361	40	89955	76463	59144.41	55.4	0	0	0	0	2	0
N_14_2_fragments_draft_norm_c1	361	40	81518	71228	53577.57	55.4	0	0	0	0	0	0
N_14_3_fragments_draft_norm_c1	396	40	96678	83670	67148.31	55.3	0	0	0	0	0	0
O_15_1_fragments_draft_norm_c1	359	40	94898	80235	66854.81	55.99	0	0	0	0	2	0
O_15_2_fragments_draft_norm_c1	359	40	85512	71060	64437.41	55.99	0	0	0	0	2	0

4. RASPRAVA

4.1. Kemijski sastav specijaliziranih metabolita

Slobodne hlapljive tvari: eterična ulja i hidrosoli

Slobodne hlapljive tvari iz roda *Veronica* do sada nisu šire istraživane. Za istraživanje kemijskog sastava (s posebnim naglaskom na slobodne hlapljive tvari) i biološku aktivnost tih ekstrakata u okviru ove disertacije odabrane su vrste: *Veronica saturejoides* Vis. ssp. *saturejoides*, *Veronica officinalis* L. i *Veronica austriaca* L. ssp. *jacquinii* (Baumg.) Eb. Fisch. Prema pregledu literature, kemijski sastav FVC-ova (*free volatile compounds*, slobodne hlapljive tvari) i njihova biološka aktivnost za ove vrste još nije istražena, pa je ovo prvi pregled slobodnih hlapljivih komponenti za ove vrste, posebno u smislu usporedbe hlapljivih komponenti iz eteričnog ulja (EO, *essential oil*) i iz vodenih ostataka (HY, hidrosola, *hydrosols*), ispitivanja antioksidativnog i antiproliferativnog djelovanja ovih ekstrakata, te mikromorfološke karakterizacije trihoma. Budući da ove vrste rastu i u ekstremnim uvjetima okoliša, pretpostavljeno je da razvijaju hlapljive komponente koje bi mogle imati antioksidativno djelovanje, kao i neka druga biološka djelovanja.

Za vriskovu čestoslavicu (*V. saturejoides* ssp. *saturejoides*) za biološku aktivnost i identifikaciju FVC-ova istražene su tri lokacije: s planine Prenj (PS) (rad u sklopu poglavlja 3.1.), s planine Kamešnica (KS) (rad u sklopu poglavlja 3.1.) i s planine Dinara (DS) (rad u sklopu poglavlja 3.2.). U eteričnom ulju PS (Prenj sample, uzorak s planine Prenj) identificirano je 25 spojeva koji čine 92,47 % ukupnog ulja. Najzastupljeniji spoj bio je heksahidrofarnezil aceton (30,13 %). U eteričnom ulju KS (Kamešnica sample, uzorak s planine Kamešnica) identificiran je ukupno 21 spoj, što predstavlja 95,38 % ukupnog ulja, a najzastupljenija je heksadekanska kiselina (37,31 %). Tablica 1 iz poglavlja 3.1. pokazuje da je broj spojeva otkrivenih i identificiranih u hidrosolu manji nego u EO. To je i logično jer su nepolarni spojevi manje topljivi u vodi. U PS hidrosolu identificirano je 17 spojeva, što predstavlja 92,29 % ukupnog hidrosola; u hidrosolu KS identificirano je 16 spojeva, što predstavlja 92,43 % ukupnog hidrosola. U uzorcima hidrosola najzastupljeniji spoj bio je *trans*-*p*-menta-1(7),8-dien-2-ol, koji je predstavljao 31,75 % odnosno 36,63 % ukupnog hidrosola u PS i KS. Ovi rezultati su u skladu s činjenicom da hidrosoli sadrže otopljene molekule EO, s obzirom da se više od polovice spojeva koji su identificirani u hidrosolu nalaze i u eteričnom ulju [22].

Heksahidrofarnezil aceton (fiton) i heksadekanska kiselina (palmitinska kiselina) važne su komponente u oba uzorka analiziranih EO. Prema literaturi, fiton pokazuje snažno

antimikrobno djelovanje i inhibiciju širokog spektra protiv različitih sojeva gljivica [113]. Palmitinska kiselina ima antioksidacijsku, nematidnu, pesticidnu, antiandrogenu, hemolitičku i 5-alfa reduktaznu aktivnost [114]. Palmitinska kiselina je također pronađena u EO vrste *Veronica thymoides* P. H. Davis subsp. *pseudocinerea* M. A. Fischer, gdje je činila 5,4 % ukupnog EO [80].

Prisutnost ugljikovodika je značajna, posebno u EO PS uzorka gdje dominira pentakosan sa 6,28 %. Seskviterpeni *(E)*-kariofilen i kariofilen oksid identificirani su u oba uzorka ulja. Kariofilen oksid je posebno prisutan u PS ulju sa 20,25 %, dok ga je u KS ulju gotovo deset puta manje (2,34 %). Seskviterpeni su također značajno zastupljeni i dominiraju u analiziranim hidrosolima. Udio identificiranog *(E)*-kariofilena u uzorcima hidrosola (24,52 % i 12,35 %) značajno je veći nego u uzorcima ulja, a zatim slijede *allo*-aromadendren (8,13 % i 11,53%) i germakren D (2,56 % i 4,67 %), dok je kariofilen oksid najmanje prisutan u uzorcima hidrosola (Tablica 1, poglavlje 3.1). Poznato je da hlapljive komponente bogate seskviterpenima imaju antifungalna, antimikrobna, antikancerogena i antioksidativna svojstva [115]–[118]. Uz ove seskviterpene i *trans*-*p*-menta-1(7),8-dien-2-ol u analiziranim uzorcima hidrosola značajno je prisutan metil eugenol i to 13,35 % u PS hidrosolu i 11,92 % u KS hidrosolu.

Glavni FVC-ovi *V. officinalis* su: heksadekanska kiselina (20,62 %), *β*-ionon (17,88 %), heksahidrofarnezil aceton (13,92 %) i *(E)*-kariofilen (6,78 %). Ovi spojevi također su identificirani u frakciji hidrosola *V. officinalis*, ali s mnogo manjim udjelima, osim *β*-ionona, koji ima visok udio od 10,74 %. Štoviše, fenolni spoj *p*-vinil gvajakol (11,59 %) najzastupljeniji je spoj u frakciji hidrosola *V. officinalis*, a slijede ga benzaldehid (9,25 %), kariofilen oksid (7,52 %) i *(E)*-*β*-damaskon (6,69 %). Oksigenirani seskviterpeni glavna su skupina u obje frakcije *V. saturejoides* ssp. *saturejoides* (Tablica 1, Slika 2, poglavlje 3.2.) s prevladavajućim spojem kariofilen oksidom (23,65 % u EO i 21,28 % u HY). Među fenolnim spojevima samo je metil eugenol identificiran kao najzastupljeniji spoj, u EO-frakciji s 20,23 %, a u hidrosolnoj frakciji s 23,31 %. Među oksigeniranim monoterpenima kod vrste *V. saturejoides* ssp. *saturejoides* u obje frakcije, *trans*-1(7),8-p-mentadien-2-ol bio je najzastupljeniji spoj (10,62 % u EO i 11,75 % u HY). U hidrosolima KS i PS također je najzastupljeniji *trans*-1(7),8-p-mentadien-2-ol, s postotkom od 31,75 % za uzorak PS i 36,63 % za uzorak KS. Kariofilen oksid također je bio prisutan u obje frakcije *V. saturejoides* DS uzorak (eterično ulje i hidrosol) što je djelomično u skladu s rezultatima za PS i KS uzorak gdje je za PS uzorak EO postotak kariofilen oksida bio 20,25 %, dok je za KS bio tek 2,34 %.

Izolacija i identifikacija FVC-a vrste *V. austriaca* ssp. *jacquinii* je napravljena za četiri uzorka EO i četiri hidrosola, a rezultati su prikazani u Tablicama 1 i 2, poglavlja 3.2. Spojevi u tablici navedeni su redoslijedom njihovog eluiranja s kolone. Prinosi EO s četiri lokacije bili su 0,38 %, 0,47 %, 0,51 % i 0,64 %, redom. Cilj je bio utvrditi sličnosti i razlike u hlapljivim komponentama ovisno o populaciji. U svim uzorcima EO identificirano je više od 90 % ukupnog ulja, a heksahidrofarnezil aceton (23,34–52,56 %) i heksadekanska kiselina (26,71–58,91 %) bili su najzastupljeniji (Slika S1, poglavlje 3.2.). Ove komponente također su identificirane u svim uzorcima hidrosola, ali u znatno nižem postotku u rasponu od 0,48 % do 7,70 % kada se promatraju obje komponente. Osim ove dvije komponente, *(E)*-kariofilen i *(Z)*-metil izoeugenol prisutni su u sva četiri uzorka EO i u sva četiri uzorka hidrosola. Glavne komponente u hidrosolima su metil eugenol (23,35–57,93 %), *trans*-*p*-menta-1(7),8-dien-2-ol (5,24–7,69 %) i timol (3,48–9,45 %) (Tablice 1 i 2, poglavlje 3.2.). Na Slici 2a (poglavlje 3.2.) prikazan je sastav na temelju Tablica 1 i 2 (prema % - relativna površina vrha analize plinskom kromatografijom - masenom spektrometrijom, rezultat je maseni udio određene tvari u ukupnom sastavu, a ne masa tvari; kvalitativno, a ne kvantitativno analiziranje sastava uzorka). Može se vidjeti da je sastav svih EO uzoraka karakteriziran visokim postotkom kategorijom tvari „Oksigenirani seskviterpeni" i kategorijom „Kiselina, alkohola i estera". Ovaj rezultat je u skladu s rezultatima za EO *V. saturejoides* [119].

Heksahidrofarnezil aceton identificiran je u *V. saturejoides* i *V. jacquinii* kao glavni EO spoj, dok je u vrsti *V. officinalis* prevladavajući spoj bio heksadekanska kiselina. Osim heksahidrofarnezil acetona u svim ispitivanim uzorcima EO vrste *V. austriaca* ssp. *jacuinii*, heksadekanska (palmitinska) kiselina je također identificirana u visokom postotku. Sva četiri uzorka hidrosola *V. austriaca* ssp. *jacquinii* imaju sličan sastav s metil eugenolom kao najzastupljenijim spojem (Tablica 2, Slika S2, poglavlje 3.3.). Ovaj bi spoj mogao biti odgovoran za veću antioksidacijsku aktivnost hidrosola u usporedbi s aktivnošću EO (mjereno DPPH metodom). Glavna razlika između sastava EO i hidrosola (Slika 2, poglavlje 3.3.) je u tome što su nepolarni spojevi kao što su masne kiseline i oksigenirani seskviterpeni glavne kategorije spojeva u EO, dok su polarniji spojevi kao što su fenolne kiseline i oksigenirani monoterpeni najzastupljeniji u hidrosolima. Za ovu vrstu istraženi su i drugi specijalizirani metaboliti. Živković i sur. identificirali su flavonoide izvedene iz flavona - luteolin i izoskutelarein i otkrili da je akteozid najdominantniji spoj u *V. austriaca* ssp. *jacquinii*. Također su otkrili derivate kvercetina u ovoj vrsti [77].

U dopunskim materijalima u Tablicama S1 i S2 poglavlja 3.3 prikazani su sastavi hlapljivih spojeva iz EO i hidrosola preračunati prema prinosima iz suhog biljnog materijala (u

mg po g suhog materijala biljke). Glavne kategorije prikazane su na Slici 2b poglavlja 3.3. Ako usporedimo podatke sa Slike 2a, glavna razlika je u tome što je na Slici 2b dobro prikazano da je koncentracija hlapljivih tvari u hidrosolima niža nego u EO, ali distribucija glavnih kategorija ostaje ista kao što je raspoređena na Slici 2a. Može se vidjeti da su u EO spojevi koji pripadaju skupinama "Oksigeniranim seskviterpenima" i "Kiseline, alkoholi i esteri" najzastupljeniji (1,44-2,01 odnosno 1,34-4,00 mg/g), a spojevi koji pripadaju "Fenolnim spojevima" su najzastupljeniji u hidrosolima (1,20–2,24 mg/g).

Pregledom literature utvrđeno je da nije provedeno mnogo istraživanja o sastavu hlapljivih spojeva drugih vrsta čestoslavica. Sastav EO *V. spicata* je prethodno analiziran i studija je pokazala da je najzastupljeniji spoj fitol (21,13 %) [20]. Valyova i sur. proučavali su ekstrakte bugarske vrste *Veronica officinalis* i pronašli su sljedeći sastav u etanolnom ekstraktu nadzemnih dijelova: terpeni, zasićene i nezasićene masne kiseline i esteri, steroidi, *p*-hidroksifeniletil alkohol, maltol i loliolid. Po sadržaju je najzastupljeniji *β*-sitosterol. U svojoj studiji također su identificirali terpinen-4-*ol*, neofitadien, heksahidrofarnezil aceton, vitamin E, fitol i skvalen po prvi put u rodu *Veronica* [82]. Ostala istraživanja EO roda *Veronica* provedena su na *V. thymoides* subsp. *pseudocinerea, V. linariifolia* i *Veronica* sp.. Ertas i sur. utvrdili da je najzastupljeniji sastojak eteričnog ulja *Veronica thymoides* subsp. *pseudocinerea* bio je heksatriakonten (21 %) koji pripada skupini ugljikovodičnih spojeva [80]. U drugom istraživanju sastava eteričnog ulja vrste *Veronica linariifolia* Pall. ex Link glavni sastojci bili su cikloheksen (25,83 %), *β*-pinen (11,61 %), 1S-α-pinen (10,65 %), *β*-felandren (10,49 %), *β*-mircen (10,42 %) i germakren D (4,99 %) (monoterpenski i seskviterpenski ugljikovodici). Çelik i sur. proučavali su eterična ulja ekstrahirana iz *Veronica* sp. i utvrdili da su glavne komponente uglavnom linalol (4,18 %) i karvakrol (7,28 %) [42].

Fenilpropanoidi kao što su metil eugenol i identificirani *Z*-metil izoeugenol (1,25 % i 4,16 %) pojavljuju se u biljkama u uvjetima stresa, kao što je ultraljubičasto zračenje i napad patogena [120], pa ne čudi njihova identifikacija u uzorcima iz ove disertacije, a posebno kod vrste *V. saturejoides* koja raste na velikim visinama, u sušnim uvjetima. U prethodno istraživanoj vrsti *Veronica spicata* L. fitol je bio dominantan spoj (21,13 %) u ukupnom EO [9]. U istraživanju u okviru ove disertacije fitol je bio prisutan samo u eteričnom ulju PS (Tablica 1, poglavlje 3.1.) (2,82 %). Daljnja usporedba spojeva pronađenih u *V. spicata* L. i *V. saturejoides* pokazuje da je devet spojeva pronađeno u obje biljne vrste: *(E)*-kariofilen, spatulenol, kariofilen oksid, *γ*-eudesmol, fitol, dokosan, trikosan, tetrakosan i pentakosan. Ako se usporedi sve ispitane uzorke, može se reći da su vrste *V. jacquinii* i *V. officinalis* bogatije heksadekanskom kiselinom od vrste *V. saturejoides* ssp. *saturejoides*. Zapravo za sve uzorke

se može zaključiti, zbog identificiranog heksahidrofarnezil acetona i heksadekanske kiseline u višim postotcima, da su najbogatije kategorijom FVC-a „Oksigenirani seskviterpeni“ ili kategorijom „Masne kiseline, aldehidi, alkoholi, esteri i ketoni“, ovisno o tome u koju kategoriju je smještena tvar heksahidrofarnezil aceton.

Za rad unutar poglavlja 3.3. provedena je preliminarna PCA (principal component analyses) analiza za hlapljive spojeve iz EO i hidrosola u količini većoj od 2 % (Slika 3, poglavlje 3.3.) u koju su uključeni spojevi vrste *V. austriaca* ssp. *jacquinii* te *V. saturejoides* kako bi se istražilo hoće li se dvije vrste razlikovati jedna od druge prema sadržaju FVC-a i mogu li hlapljivi spojevi biti kemofenetski marker za ovaj rod (Slika 3a, poglavlje 3.3.). PC1 i PC2 za hlapljive spojeve iz EO i hidrosola objasnili su 50,04 % varijance i razlikovali *V. saturejoides* od *V. austriaca* ssp. *jacquinii*. Osim toga, hidrosoli su se razlikovali od EO. To je bilo za očekivati, kao što je ranije spomenuto, sastav hlapljivih spojeva u EO je nešto drugačiji nego u hidrosolima. Komponente koje razlikuju EO od hidrosola nalaze se u negativnom području PC1 i PC2 za obje vrste. Glavne komponente koje razlikuju *V. austriaca* ssp. *jacquinii* od *V. saturejoides* za hidrosole su trans-*p*-1(7),8-menta-*dien*-*2*-*ol*, *allo*-aromadendren, *(Z)*-metil izoeugenol, germakren D i *(E)*-kariofilen (Slika 3b, poglavlje 3.3.). Glavne komponente koje razlikuju *V. austriaca* ssp. *jacquinii* od *V. saturejoides* za EO su 1-heksadekanol, *α*-muurolol, trikosan, pentakosan, 2-metoksi-4-vinilfenol, oktadekanol acetat, *β*-kariofilen oksida i heksadekanska kiseline (Slika 3b, poglavlje 3.3.). Iz Slike 3. vidljivo je da se te dvije vrste najbolje razlikuju na temelju hlapljivih komponenti hidrosola.

Istraživanje FVC-a u okviru četvrtog rada iz disertacije (poglavlje 3.4.) rađeno je prvenstveno u svrhu izrade klastera temeljenih na slobodnim hlapljivim spojevima roda *Veronica*. Osim izolacije klasičnom Clevenger aparaturom korištena je i nova aparatura mikrovalne ekstrakcije te su uspoređeni rezultati ove dvije ekstrakcije. U ovoj studiji proučavani su FVC-ovi u vrstama *Veronica* kako bi se utvrdilo mogu li se neki od spojeva koristiti kao kemofenetski markeri za buduća istraživanja i kako bi se utvrdilo utječu li okolišni uvjeti na različitim staništima na sastav tih spojeva. Studijom je utvrđeno da su neki glavni sastojci prisutni u svim vrstama bez obzira na stanište u kojem vrste obitavaju. Ovi FVC-ovi su heksahidrofarnezil aceton, fitol i heksadekanoična kiselina. Gledajući klastere za hidrodestilaciju Clevengerom (HD) i mikrovalnom pećnicom, može se vidjeti da staništa temeljena na vlažnosti ne utječu na sastav hlapljivih spojeva, jer su sva staništa zastupljena u većini klastera. Ovakav sličan rezultat su dobili Mehrvarz i sur. u analizi četiri vrste čestoslavica (*V. persica, V. polita, V. francispetae* M. A. Fisch. i *V. siaretensis* E. Lehm.). Studija je pokazala kvalitativno konstantan sastav uzoraka iridoida kod svih vrsta, neovisno o okolišnim uvjetima

[98]. Brojni drugi pokusi pokazali su da se FVC mogu koristiti za razlikovanje vrsta i kultivara [121]–[124].

Što se tiče relativnog sadržaja hlapljivih spojeva u vrstama *Veronica* prikupljenim iz suhih staništa, oksigenirani seskviterpeni čine glavnu klasu klasično izoliranih spojeva. Iznimka je endemična vrsta *V. dalmatica*, kod koje je udio oksigeniranih seskviterpena bio sličan za obje metode izolacije. Kod vrste *V. dalmatica* oksigenirani diterpeni bili su najzastupljeniji u HD, a seskviterpenski ugljikovodici bili su dominantni u mikrovalnoj ekstrakciji (Slika 2, poglavlje 3.4.). U skupini biljaka sakupljenih s vlažnih staništa postotci klasa spojeva ekstrahiranih objema metodama bili su isti. Najveće varijacije utvrđene su u postotku identifikacije oksigeniranih monoterpena u *V. beccabunga* (Slika 4, poglavlje 3.4.). Spojevi u sastavu monoterpenskih ugljikovodika općenito su najmanje identificirani, a najviše izolirani kod vrste umjerenih staništa *V. hederifolia* objema metodama izolacije (Slika 6, poglavlje 3.4.). Utvrđeno je da u sastavu *V. officinalis* dominira skupina spojeva koja se sastoji od kiselina, alkohola i estera. Jedan od tih spojeva bio je alkohol *3*-heksen-*1*-*ol*, koji je izoliran zelenom ekstrakcijom. Poznato je da je ova komponenta jedna od najvažnijih u sastavu FVC-a [125]. Ova se vrsta često koristi u biljnim čajevima, pa je prisutnost *3*-heksen-*1*-*ol*-a bila očekivana budući da je ovaj spoj široko zastupljen u svježim listovima čaja [125]. S ovom studijom o slobodnim VC-ima, proširenо je znanje o specijaliziranim metabolitima koji čine osnovu za daljnja biološka istraživanja.

Uspoređujući rezultate ekstrakcije FVC-a klasičnom hidrodestilacijom i mikrovalnom destilacijom, može se vidjeti da su iste glavne komponente izolirane objema metodama, ali u različitim relativnim postocima. Neki spojevi izolirani su samo hidrodestilacijom ili mikrovalnom destilacijom. To je i logično jer je poznato da proces hidrodestilacije može negativno utjecati na neke od spojeva koji se zbog visokih temperatura i dugog vremena ekstrakcije razgrađuju. S druge strane, mikrovalna destilacija ponekad može rezultirati izolacijom manjeg broja komponenti kao što je navedeno u studiji Wu i sur. [126]. U svojem su istraživanju zaključili da hidrodestilacija ostaje bolja opcija za ekstrakciju FVC-a jer ekstrahira najveći broj komponenata. Gledajući rezultate za izolirane FVC za rod *Veronica* i činjenicu da su svi glavni spojevi ekstrahirani objema metodama, treba uzeti u obzir mikrovalnu ekstrakciju pri ekstrakciji FVC iz manje količine (laboratorijska ekstrakcija) uzorka jer je to ekološki prihvatljiviji izbor te koristi manje vode i energije i neće pregrijati uzorak.

Polifenoli

Biljni polifenoli prirodni su biološki aktivni spojevi koji se mogu sintetizirati i u laboratoriju. Zbog svojih dobrih antioksidativnih, antineurodegenerativnih i antikancerogenih svojstava, često se konzumiraju u prehrani [127]. Također, pomažu biljci da se zaštiti od ultraljubičastog zračenja ili napada patogena [128]. U istraživanju u okviru ove disertacije, kao što je prikazano u Tablici 3 (poglavlje 3.1.), najveći sadržaj ukupnih fenolnih kiselina utvrđen je kod *V. saturejoides* ssp. *saturejoides* (Kamešnica-KS; 1; A525 nm, 86.9±1.4 mg/g), dok je prinos ukupnih flavonoida (TF) bio vrlo nizak i isti za oba istraživana primjerka *V. saturejoides* ssp. *saturejoides* (Tablica 3, poglavlje 3.1.). Harput i sur. utvrdili su da je ukupni sadržaj fenola bio 200,20 mg/g u *V. officinalis* L., 139,92 mg/g kod *V. peduncularis* M. Bieb., 127,64 mg/g kod *V. orientalis* Mill. i 83,15 mg/g kod *V. baranetzkii* Bordz. [84]. Uspoređujući ove rezultate s rezultatima dobivenim u okviru ove disertacije, može se vidjeti da *V. saturejoides* ima sličan ukupni sadržaj fenola kao *V. baranetzkii*. Ertas i sur. otkrili su da je ukupni sadržaj fenola u metanolnim ekstraktima *V. thymoides* subsp. *pseudocinerea* iznosio 248,37 ± 3,68 mg/g, a ukupni sadržaj flavonoida 47,02 ± 0,21 mg/g [80].

Tablica 4 unutar poglavlja 3.3. prikazuje rezultate analiza polifenola *V. austriaca* ssp. *jacquinii* s četiri lokacije u Hrvatskoj. Najveći sadržaj ukupnih fenolnih kiselina (TPA, total phenolic acids) utvrđen je kod *V. austriaca* ssp. *jacquinii* s lokaliteta Br (78.79±1.30 mg/g) dok je prinos ukupnih flavonoida (TF) vrlo nizak i sličan za sve istraživane jedinke ispitivane vrste. Za ovaj uzorak s najvišim ukupnim fenolima možemo reći da je sličnog sastava kao i uzorak *V. saturejoides*, ali rezultati iz pregleda literature pokazuju da *V. officinalis* ima najveći sadržaj ukupnih polifenola, ranije navedenih [84]. Vrsta *V. jacquinii* ima veći sadržaj ukupnih tanina od vrste *V. saturejoides*, te veći sadržaj ukupnih flavonoida.

4.2. Trihomi

Istraživanja slobodnih hlapljivih spojeva roda *Veronica* vrlo su oskudna, stoga je važno da je ovo istraživanje potkrijepljeno mikromorfologijom trihoma kao vidljivim dokazom mjesta sinteze slobodnih hlapljivih spojeva. Žljezdani trihomi su „bio-tvornice“ hlapljivih spojeva koji stvaraju komponente eteričnih ulja. Imaju važnu ulogu u zaštiti biljaka od biljojeda i patogena te u privlačenju oprašivača [43].

Općenito, mikromorfološka istraživanja čestoslavica su rijetka, pa je stoga odlučeno provesti mikromorfološka istraživanja biljaka odabranih za ovu disertaciju kroz prva tri znanstvena rada, kako bi se pronašlo mjesto proizvodnje eteričnog ulja. Na stabljikama,

listovima i čaškama *V. saturejoides* mogu se uočiti nežljezdani i žljezdani trihomi. Prema istraživanju skenirajućeg elektronskog mikroskopa (SEM), nežljezdani trihomi (Slika 2a, 2b, 2g, 2h, poglavlje 3.1.) su nerazgranati, dvostanični do višestanični, jednostruki i presavijeni na različitim razinama. Mogli bi se definirati kao utanjeni trihomi [129]. Duljina ovih trihoma varirala je od vrlo kratkih do dugih trihoma (Slika 2g, 2h, poglavlje 3.1.). Ovi trihomi štite biljku od gubitka vode i održavaju pozitivnu mikroklimu (na površini zadržavaju vlagu, održavaju umjerenu temperaturu pri površini listova, štite od prezagrijavanja). Površine ovih trihoma pokazale su bradavičast izgled zbog pojave kutikularnih mikropapila (Slika 2g, 2h, poglavlje 3.1.). Listovi i cvjetovi su rijetko prekriveni nežljezdanim trihomima (Slika 2a–f, poglavlje 3.1.), dok stabljiku karakterizira relativno gusti raspored ovih trihoma (Slika 2g, 2h, poglavlje 3.1.). Kurer je prije 100 godina spomenuo postojanje nežljezdanih trihoma na dijelovima cvijeta vrste *Veronica* [46]. Dodatno, Kraehmer i Baur opisali su te trihome u *V. persica* Poir [47]. Ista vrsta nežljezdanih trihoma uobičajena je u mnogim drugim vrstama Lamiaceae [48]–[50].

Osim nežljezdanih trihoma, na stabljikama, listovima i čaškama *V. saturejoides* ssp. *saturejoides* mogu se uočiti i žljezdani glavičasti trihomi. Ti se trihomi sastoje od jedne stanice stapke i dvije eliptično oblikovane stanice glave (Slika 2e, poglavlje 3.1.). Nisu uspravne i mogu se opisati kao pripijene uz površinu. Svi istraživani dijelovi biljaka su prekriveni rijetko raspoređenim glavičastim trihomima. Isti tip glavičastih trihoma uočen je i kod *V. beccabunga* L. [51]. Isto tako, tip nagnutog trihoma s dvostaničnom glavom zabilježen je u *Stachys recta* L. ssp. *recta* u studiji koju su proveli Vundać i sur. [52]. Usporedivi glavičasti trihomi sa samo jednom eliptično oblikovanom stanicom glave mogu se uočiti na SEM mikrofotografijama *Marrubium vulgare* L. (Lamiaceae) u istraživanju Haratyma i Weryszko-Chmielewske [48]. Također, Hanlidou i sur. opisali su sličan tip trihoma ("kratak i obično savijen") u *Calamintha menthifolia* Host. [130]. Kremer i sur. također su identificirali tip nagnutog trihoma s jednom stanicom glave kod vrste *Micromeria croatica* (Pers.) Schott [55], kao i u novijem istraživanju kod 9 vrsta roda *Micromeria* te pet vrsta roda *Clinopodium* [56]. Iz podataka o prinosu EO iz PS i KS uzoraka za *V. saturejoides* ssp. *saturejoides* (poglavlje 3.1.), prinos je znatno veći u PS (0,07 %) nego u KS (0,03 %), ali mikrografije nisu pokazale nikakvu značajnu razliku između uzoraka u broju glavičastih trihoma na čašicama i listovima (Tablica 2, Slika 2c–f, poglavlje 3.1.). Nešto veći broj glavičastih trihoma pronađen je na stapkama iz PS (Slika 2g, poglavlje 3.1.). Dobivena razlika u prinosu EO između uzoraka mogla bi biti posljedica drugih razloga, poput klimatskih uvjeta ili mogućeg mehaničkog oštećenja žljezdanih trihoma.

Na površini proučavane biljke *V. officinalis* mogu se vidjeti dvije vrste žljezdanih trihoma: štitasti i glavičasti (Slika 3, poglavlje 3.2.). Štitasti žljezdani trihomi sastoje se od bazalne stanice, vrlo kratke stapke i višestanične glave s prilično velikim subkutikularnim prostorom (Slika 3c, poglavlje 3.2.). Ovi trihomi su vrlo rijetki i uočeni su samo na čašci *V. officinalis.* Ova vrsta trihoma nije identificirana kod vrste *V. saturejoides* ssp. *saturejoides* ni kod *V. austriaca* ssp. *jacquinii.* S druge strane, štitasti trihomi česti su u nekim drugim biljnim vrstama, posebice u Lamiaceae [48], [131]. Štitastu vrstu žljezdanih trihoma opisali su Kremer i sur. kod vrsta roda *Micromeria* i *Clinopodium* gdje su poprilično zastupljene [55], [56]. Glavičasti žljezdani trihomi ljekovite čestoslavice mogu se dalje podijeliti u dva podtipa. Glavičasti trihomi podtipa 1 (C1) sastoje se od stanice drške i dviju stanica glave eliptičnog oblika sa subkutikularnim prostorom (Slika 3g, poglavlje 3.2.). Prisutni su na adaksijalnoj i abaksijalnoj strani lista, na čaški i na stabljici. Glavičasti trihomi podtipa 2 (C2) jednostruki su, nerazgranati, višestanični, dugi i presavijeni na različitim razinama (Slika 3e, 3h, poglavlje 3.2.). Sastoje se od nekoliko (najčešće četiri do pet) stanica stabljike i stanice glave sa subkutikularnim prostorom. Prisutni su na stabljici i čaški *V. officinalis*. Na površini lista prisutni su samo C1 trihomi i čini se da su gušći nego na stabljici i čaški. C1 trihomi također su uočeni na površini stabljike, lišća i čaške *V. saturejoides* ssp. *saturejoides*. S druge strane, C2 trihomi nisu pronađeni u *V. saturejoides* ssp. *saturejoides*. Kao što je i ranije spomenuto, glavičasti trihomi tipa C1 prethodno su uočeni u vrste *V beccabunga* L. [51]. Usporedni, ali više ili manje uspravni glavičasti trihomi s kratkom peteljkom i dvostaničnom glavom uočeni su u *Marrubium vulgare* L. [48] i u endemskoj *Salvia smyrnea* L. iz Turske [131].

Nežljezdani trihomi također imaju zaštitnu funkciju u biljkama. Mogu zaštititi biljke od biljojeda i spriječiti veći gubitak vode transpiracijom [43]. Ovi trihomi uočeni su na čaškama, listovima i peteljkama *V. officinalis*. Prema istraživanju provedenom SEM–om, nežljezdani trihomi su nerazgranati i jednostruki. Također se može primijetiti da su to kratki (dvostanični) ili duži (višestanični) trihomi (Slika 3b, 3f, poglavlje 3.2.). Ovi trihomi su presavijeni na različitim razinama. Nežljezdani trihomi gušći su na površini stabljike nego na listovima i čaškama (Slika 3, poglavlje 3.2.). Prisutnost istog tipa trihoma identificirana je i na nadzemnim dijelovima *V. saturejoides,* na dijelovima cvijeta *Veronica* sp. [46] i kod vrste *V. persica* Poir. [47].

Za treću vrstu odabranu za istraživanje kroz ovu disertaciju, *V. austriaca* ssp. *jacquinii*, nežljezdani i jedan tip žljezdanih trihoma uočeni su na čaškama, listovima i stabljici. Prema istraživanju provedenom SEM–om, nežljezdani trihomi variraju od dvostaničnih do višestaničnih (Slika 4a, 4f, 4h, poglavlje 3.3.). Nerazgranate su, jednostruke i presavijene na

različitim razinama, a duljina je varirala od kratkih do dugih dlaka. Njihova je površina bila bradavičastog izgleda zbog prisutnosti kutikularnih mikropapila (Slika 4a, poglavlje 3.3.). Ove vrste trihoma mogu se uočiti kao utanjene dlake [129]. Čaške, stabljike i adaksijalna strana lista su umjereno gusto prekriveni nežljezdanim trihomima (Slika 4a, 4e, 4h), dok su na abaksijalnog strani lista ti trihomi bili uglavnom raspoređeni duž glavne žile i ruba lista (Slika 4c, poglavlje 3.3.). Ovaj tip nežljezdanih dlačica uočen je kod *V. saturejoides* ssp. *saturejoides* i *V. officinalis.* Utanjeni, nežljezdani trihomi općenito su poznati iz drugih biljnih vrsta [132]–[134].

Žljezdani trihomi vrste *V. austriaca* ssp. *jacquinii* pripadaju glavičastom tipu trihoma i sastoje se od jedne stanice unutar drške i dvije glavičaste stanice eliptičnog oblika (Slika 4b, poglavlje 3.3.). Ovi trihomi nisu uspravni, već bi se mogli opisati kao prilijepljeni za površinu. Uočeni su na čaškama, listovima i stabljici. Svi istraživani dijelovi biljaka bili su srednje gusti prekriveni glavičastim trihomima. Ova vrsta žljezdanih trihoma također je identificirana kod *V. saturejoides* ssp. *saturejoides* i *V. officinalis*. Osim već ranije spomenutih istraživanja, usporedivi žljezdani trihomi, ali sa samo jednom eliptično oblikovanom stanicom glave, pronađeni su u vrstama *Satureja thymbra* L., *Thymus capitatus* (L.) Hoffmanns, *Majorana syriaca* (L.) Rafin. [33], *Calamintha menthifolia* Host. [39], *Geranium macrorrhizum* L. i *G. dalmaticum* (Beck) Rech. f. [135]. U općoj usporedbi s *V. saturejoides*, možemo reći da su trihomi *V. austriaca* ssp. *jacquinii* gušće raspoređeni na svim dijelovima nadzemnih dijelova (stabljika, listovi i čaške) dok vrsta *V. officinalis* ima sličnu gustoću trihoma kao i *V. austriaca* ssp. *jacquinii*.

4.3. Biološka aktivnost

Za rod *Veronica* različite biološke aktivnosti ispitivane su najviše na nekoj vrsti fenolnih ekstrakata (metanolni, etanolni, acetonski i vodeni) ili na ekstraktima iridoidnih ili feniletanoidnih glikozida. Istraživanja biološke aktivnosti ekstrakata slobodnih hlapljivih spojeva roda *Veronica* do sada nije ni bilo, stoga je na tri vrste odabrane za istraživanje u okviru ove doktorske disertacije testirano antioksidativno, antiproliferativno (citotoksično) i antifitovirusno djelovanje. S obzirom da za ove ekstrakte FVC-a nema prijavljenih istraženih aktivnosti drugih vrsta roda *Veronica*, ovi rezultati uspoređuju se s drugim relevantnim EO i hidrosolima koji se obično koriste u konzerviranju hrane, prehrani, farmaciji i kozmetici.

Antioksidativno djelovanje

Ispitivanje antioksidativne aktivnosti često je jedan od prvih koraka u procjeni pozitivnog djelovanja odabranih ekstrakata s obzirom da je stvaranje ROS–a (reactive oxygen

species) posljedica svakog normalnog metabolizma, a njihovo nakupljanje uzrokuje bolesti. Antioksidativna aktivnost je najčešće ispitivana na različitim vrstama hrane te medicinskom i začinskom bilju koje također koristimo kao dodatke prehrani. S obzirom na sve navedeno, u okviru ove doktorske disertacije istraženo je antioksidativno djelovanje svih ekstrakata dvjema metodama, ORAC (oxygen radical absorbance capacity) i DPPH (2,2-difenil-1-pikrilhidrazil).

Ako promotrimo aktivnost prve istražene vrste, u Tablici 5 (poglavlje 3.1.) može se vidjeti da je KS uzorak vrste *Veronica saturejoides* pokazao nešto veću aktivnost u ORAC i DPPH metodi nego uzorak PS, i u slučaju EO i hidrosola. U usporedbi s izvješćima o drugim biljkama, Aazza et al. [136] istraživali su antioksidacijsku aktivnost prema ORAC metodi hidrosola nekoliko ljekovitih biljaka (*Lavandula officinalis*, *Origanum majorana*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thymus vulgaris*, *Cinnamomum verum* i *Syzgium aromaticum*). Hidrosol KS ispitan u našem istraživanju ima veću vrijednost ORAC od vrste *Salvia officinalis* i sličnu kao *Rosmarinus officinalis* [136]. Bentayeb i sur. [137] istražili su antioksidativno djelovanje za 10 EO biljaka koje se često koriste kao začini. Vrijednost ORAC vrste *V. saturejoides* uzorak KS, prema njihovim utvrđenim vrijednostima, ima sličnu aktivnost kao EO ružmarina i bosiljka. Viuda-Martos i sur. [138] izvijestili su o antioksidativnom djelovanju za pet biljaka koje se koriste na Mediteranu, a EO *V. saturejoides* pokazao je sličnu vrijednost DPPH kao ulje ružmarina, što je u skladu s prethodno spomenutim rezultatima druge studije. Antioksidativno djelovanje metabolita roda *Veronica* većinom je ispitano na različitim ekstraktima fenolnih spojeva i na iridoidnim glukozidima. Mocan i sur. [81] potvrdili su antioksidativnu aktivnost za *V. officinalis* s TEAC metodom 157,99 ± 6,58 mg TE/g. Živković i sur. [77] testirali su metanolne ekstrakte *V. teucrium* i *V. jacquinii* i bili su jači nego što su prethodno izvijestili Harput et al. [139] za *V. officinalis*, s IC50 vrijednostima od 28.49 ± 0.6, za *V. teucrium* i 37.63 ± 0.6 µg/mL za *V. jacquinii*, iako oba imaju nižu aktivnost od standarda (BHT-butilirani hidroksitoluen i BHA-butilirani hidroksianizol). Kwak i sur. izvijestili su o antioksidativnom djelovanju iridoidnih glukozida u kojima je etanolni ekstrakt pokazao veću aktivnost od Troloxa [83]. Dunkić i sur. [20] testirali su antioksidativnu aktivnost prema DPPH metodi različitih ekstrakata vrste *V. spicata*, a IC50 vrijednost za DPPH metodu pokazala je najveću aktivnost u metanolnim ekstraktima cvjetova i listova s vrijednostima od 8,21 ± 0,06 µg/mL i 8,69 ± 0,06 µg/mL, tj. više od vrijednosti za standarde BHT i BHA prema vrijednosti u studiji Živkovića i sur. [77]. Uspoređujući rezultate za antioksidativnu aktivnost vrste *V. officinalis* s izvješćima za druge vrste biljkama, čini se da *V. officinalis* hidrosol ima veću vrijednost aktivnosti prema ORAC metodi od *Salvia officinalis* hidrosola [136]. Valyova i sur. proučavali su fenolne ekstrakte *V. officinalis* i otkrili da ekstrakti etil acetata pokazuju izvrsnu

antioksidativnu aktivnost na temelju DPPH i ABTS testova [82]. Harput i sur. istraživali su antioksidativno djelovanje za četiri vrste čestoslavica (*V. orientalis, V. baranetzkii, V. officinalis* i *V. peduncularis*) i utvrdili najjače antioksidativno djelovanje za vodene ekstrakte *V. officinalis* (IC50 54,19 µg/mL). To bi moglo biti zbog najvećeg utvrđenog ukupnog sadržaja fenola za ovu vrstu (200,20 mg/g) [84]. U drugoj studiji, Harput i sur. izvijestili su da je antioksidacijska aktivnost protiv SO (superoksida) za *V. chamaedrys* (IC50 za inhibiciju superoksidnog radikala) veća od standarda (BHA i kvercetin), a najviša vrijednost IC50 za *V. officinalis* protiv DPPH radikala iznosi 40,93 µg/mL [139]. U njihovoj drugoj studiji antioksidativna aktivnost prema DPPH metodi otkrivena je za vodene ekstrakte vrsta *V. cymbalaria, V. hederifolia, V. pectinata, V. persica* i *V. polita*. Najveća aktivnost otkrivena je za *V. polita* [86]. Živković i dr. istraživali su antioksidacijsku aktivnost triju vrsta čestoslavica i izvijestili o najvećoj antioksidativnoj aktivnosti za *V. teucrium* za 70 % vodeni acetonski ekstrakt (DPPH IC50 vrijednost 12,58 µg/mL) [140]. Dunkić i sur. izvijestili su o čak većoj antioksidativnoj aktivnosti metanolnih ekstrakata cvjetova *V. spicata* s IC50 8,21 µg/mL [20]. Sharifi-Rad i sur. izvijestili su da je antioksidativna vrijednost DPPH metanolnog ekstrakta nadzemnih dijelova *V. persica* IC50 30 µg/mL [141]. Svi ovi rezultati pokazuju da bi čestoslavice trebalo dodatno istražiti u pogledu njihove *in vivo* antioksidativne aktivnosti ili potencijalne upotrebe u konzerviranju hrane.

Eterično ulje *V. saturejoides* ssp. *saturejoides* s treće lokacije DS (poglavlje 3.2.) pokazalo je veću antioksidacijsku aktivnost od EO *V. officinalis* objema metodama. To bi moglo biti posljedica puno većeg sadržaja oksigeniranih seskviterpena, posebice spoja kariofilen oksida. Hidrosoli su pokazali malo drugačije rezultate. Kod DPPH metode veću aktivnost pokazao je hidrosol *V. saturejoides*, a kod ORAC metode hidrosol *V. officinalis* (Tablica 4, poglavlje 3.2.). Hidrosoli *V. saturejoides* također imaju veći sadržaj oksigeniranih seskviterpena. Prethodno je objavljeno da seskviterpeni posjeduju brojne biološke aktivnosti [142], [143]. Uspoređujući ove rezultate s onima za *V. saturejoides* ssp. *saturejoides* s druge dvije lokacije (Kamešnica i planina Prenj), može se vidjeti da je antioksidativna aktivnost EO *V. saturejoides* (uzorak s Dinare) pokazala sličnu aktivnost prema ORAC metodi kao druga dva prethodno prijavljena EO, ali se čini da je aktivnost po DPPH metodi niža nego EO *V. saturejoides* KS i PS. Može se zaključiti da KS uzorak ima najveće antioksidativno djelovanje, što je pokazano i za antiproliferativno djelovanje (dalje u raspravi). Eterično ulje vrste *V. officinalis* ima nižu antioksidativnu aktivnost od uzorka EO *V. saturejoides* iz istraživanja (uzorak Dinara, objavljeno u radu, poglavlje 3.2.) i također nižu od dva prethodno navedena EO (objavljeno u radu, poglavlje 3.1.). Stoga se može zaključiti da *V. officinalis* EO ima slabiju

antioksidativnu aktivnost nego *V. saturejoides* EO. Uspoređujući rezultate za hidrosole, vidljivo je iz Tablice 4. (poglavlje 3.2.) i rezultata za *V. saturejoides* (poglavlje 3.1.), da hidrosoli *V. officinalis* i *V. saturejoides* DS imaju nižu antioksidativnu aktivnost od prethodno prijavljenih hidrosola s dvije lokacije za *V. saturejoides* (KS i PS).

Na antioksidativnu aktivnost ispitan je i najzastupljeniji spoj u EO vrsta čestoslavica, heksahidrofarnezil aceton, s DPPH i ORAC metodama i on nije pokazao nikakvo antioksidativno djelovanje, pa možemo pretpostaviti da antioksidativno djelovanje dolazi od drugog spoja u EO ili je vjerojatnije rezultat sinergijskog djelovanja između različitih spojeva. Sinergijsko djelovanje spojeva dokazano je u nekim studijama. U svojim eksperimentima Huang i sur. potvrdili su sinergistički učinak manje zastupljenih komponenti EO cimeta protiv *Salmonella pullorum* [144]. Amorati i sur. testirali su i usporedili antioksidacijsku aktivnost za 5 različitih EO, njihove ugljikovodične i oksigenirane frakcije te također za dva spoja karakteristična za te EO, timol i karvakrol. Rezultati su pokazali da u 4 od 5 uzoraka ukupno ulje (ne pojedine komponente ulja, nego cijelo eterično ulje dobiveno ekstrakcijom iz biljke) ima veću antioksidacijsku aktivnost od svojih frakcija, a u 3 od 5 uzoraka ukupno ulje ima veću aktivnost od izoliranih pojedinačnih aktivnih spojeva, tako da se može zaključiti da sinergija između različitih spojeva u eteričnom ulju igra ključnu ulogu u njegovom djelovanju [59]. Iz rezultata u okviru ove disertacije također možemo zaključiti da aktivnost ne potječe od najzastupljenijeg spoja, već od međusobnog djelovanja svih spojeva iz EO. U studiji Dunkić i sur. za vrstu *V. spicata* pokazano je da postoji negativna korelacija između količine fenolnih spojeva i antioksidativnog djelovanja, pa je zaključeno da druge tvari poput terpenoida i proteina mogu utjecati na antioksidacijsku aktivnost [20].

Promatrajući rezultate antioksidativnog djelovanja za uzorke vrste *V. austriaca ssp. jacquinii,* iz Tablice 3 (poglavlje 3.3.) vidljivo je da se rezultati antioksidativnog djelovanja za EO i hidrosole razlikuju usporedbom dviju metoda (ORAC i DPPH). Ako pogledamo rezultate za ORAC metodu, najveću aktivnost pokazao je uzorak St (Stupačinovo) (6,6±0,47 µmol/g EO) što nije slučaj za rezultate dobivene DPPH metodom. U ovoj metodi uzorak Mr (Mrkopalj) pokazao je najveću aktivnost (IC50 246,55±14,19 mg/mL). Rezultati za hidrosole pokazali su da je uzorak GJ (Gornje Jelenje) imao najveću aktivnost primjenom ORAC metode (1,41±0,149 µmol/g hidrosola). Najveću aktivnost DPPH metodom pokazao je uzorak Br (Brezovac) hidrosola (7,263 ± 0,593 mg/mL). Kada se usporede EO i hidrosoli, vidi se da EO pokazuju veću aktivnost u ORAC metodi, dok hidrosoli pokazuju veću aktivnost u DPPH metodi. Ovo se može objasniti različitim mehanizmom djelovanja reakcija ovih testova, zbog čega je i bitno u istraživanjima koristiti više od jedne metode, kao što je naznačeno i u uvodu disertacije. U

usporedbi s istraživanjem na ekstraktima *V. saturejoides* ssp. *saturejoides,* EO *V. jacquinii* imaju nižu antioksidacijsku aktivnost u obje metode. Uzimajući u obzir studiju Aazza et al. [136] koji su istraživali ORAC aktivnost hidrosola nekoliko ljekovitih biljaka, istraživani hidrosoli čestoslavica u koncentraciji hlapivih tvari od 10 mg/mL sa sva četiri lokaliteta imaju veću ORAC antioksidativnu aktivnost od hidrosola prije spomenutih vrsta *S. officinalis, L. officinalis, R. officinalis* i *C. verum*. Pregled istraživanja antioksidansa drugih specijaliziranih metabolita iz roda *Veronica* (fenolnih spojeva i iridoidnih glikozida) pokazuje da mnogi od njih imaju značajno antioksidativno djelovanje.

Kao što je ranije spomenuto, u testiranju antioksidacijske aktivnosti najzastupljenijeg spoja u EO, heksahidrofarnezil acetona, ovaj spoj nije pokazao antioksidativno djelovanje, pa je zaključeno da vjerojatno antioksidativno djelovanje dolazi od drugog spoja u EO ili je rezultat sinergističkog učinka između različitih spojeva. Rezultati antioksidativne aktivnosti za sve tri vrste iz ove disertacije u skladu su s ovim nalazima, jer se može vidjeti da postoje velike razlike u aktivnosti EO *V. austriaca* ssp. *jacquinii* i *V. saturejoides,* iako su iste glavne komponente prisutne u oba ulja. S druge strane, iz ovog i prethodnih istraživanja može se zaključiti da jedan spoj koji je zastupljeniji u hidrosolima nego u EO može imati utjecaj na antioksidacijsku aktivnost ovih ekstrakata. Spoj *trans*-*p*-menta-1(7),8-dien-2-ol prisutan je u sva četiri uzorka hidrosola *V. jacquinii*. Ovaj spoj nije prisutan u EO *V. jacquinii* pa bi to mogao biti razlog veće aktivnosti hidrosola u DPPH metodi. Ovaj spoj je također prisutan u EO uzorku *V. saturejoides,* koji je pokazao veću antioksidativnu aktivnost od svih ostalih testiranih uzoraka. Također, zanimljiva je činjenica da je antioksidativna aktivnost po DPPH metodi za dvije lokacije vrste *V. jacquinii* (Mr i Br) veća od svih ostalih istraženih hidrosolnih ekstrakata. Ovi hidrosoli imaju visoke sadržaje metil eugenola, timola i spomenute tvari *trans*-*p*-menta-1(7),8-dien-2-ol. Gledajući kemijsku strukturu *trans*-*p*-menta-1(7),8-dien-2-ola, to je izomer karveola koji prema Kaur i sur. ima visok potencijal antioksidativnog djelovanja [145]. Ovaj tim ispitao je antioksidacijsku aktivnost karveola pomoću četiri metode i zaključeno je da visoka antioksidativna aktivnost karveola vjerojatno dolazi od nezasićene hidroksilne skupine. Gledajući ponovno kemijsku strukturu trans-*p*-menta-1(7),8-dien-2-ola [47], može se vidjeti da ovaj spoj također ima ovakvu hidroksilnu skupina. Stoga bi u budućim istraživanjima ovaj spoj trebao biti testiran na njegovu antioksidacijsku aktivnost kako bi se vidjelo je li ova hipoteza točna. Drugi spoj koji bi mogao biti razlog boljeg antioksidativnog djelovanja hidrosola je metil eugenol koji je glavni spoj za sva četiri uzorka hidrosola vrste *V. austriaca* ssp. *jacquinii*, posebno u uzorku Br koji je pokazao najveću antioksidativnu aktivnost u DPPH metodi i u GJ uzorku koji je pokazao najveću aktivnost u ORAC metodi.

Osim zaključka o sinergističkom djelovanju komponenti ekstrakata, rezultati također pokazuju važnost korištenja različitih metoda pri procjeni antioksidativne aktivnosti biljnih ekstrakata jer su različite metode pokazale za različite ekstrakte posjedovanje najveće aktivnost. To je očekivano jer različiti spojevi imaju različite reakcije sa spojevima i radikalima [136].

Antifitovirusno djelovanje

Većina današnjih istraživanja neke vrste biološke aktivnosti prirodnih ekstrakata i spojeva izvodi se u svrhu pronalaženja prirodnijih rješenja za primjenu u poljoprivredi, medicini, farmaciji, prehrani i kozmetici. Ista je situacija i s antifitoviralnim djelovanjima koja se izvode u potrazi za prirodnim rješenjima protiv različitih biljnih patogena s obzirom na sve češću prijavljivanu štetnost umjetno sintetiziranih pesticida.

Rezultati aktivnosti na biljne viruse, odabranih vrsta u okviru ove disertacije, pokazuju da su biljke tretirane hidrosolom *V. officinalis* i *V. saturejoides* prije infekcije TMV-om značajno smanjile broj lokalnih lezija u usporedbi s kontrolnim biljkama. Kod biljaka tretiranih eteričnim uljem (EO) broj lokalnih lezija smanjio se samo na listovima biljaka tretiranih EO *V. officinalis* (Tablica 3, poglavlje 3.2.), dok su biljke tretirane EO *V. saturejoides* u preliminarnom pokusu razvile slične, odnosno čak teže infekcije od kontrolnih biljaka. Trećeg dana nakon inokulacije, inhibicija lezija na lišću biljaka tretiranih s hidrosolima *V. officinalis* i *V. saturejoides* bila je 84,69 % odnosno 74,11 %, dok je na lišću tretiranom s EO *V. officinalis* iznosila 59,43 % (Slika 4, poglavlje 3.2.). Sve do sedmog dana nakon inokulacije, inhibicija lokalnih lezija bila je još uvijek izražena u svim tretiranim skupinama, sa 79,09 % i 68,38 % na listovima biljaka tretiranih s hidrosolima *V. officinalis* i *V. saturejoides*, odnosno 62,70 % na listovima biljaka tretiranih EO vrste *V. officinalis*. Pregledom literature mogu se usporediti ovi rezultati s antifitovirusnim djelovanjem hlapljivih tvari biljaka objavljenim u prethodnim studijama. Tako su eterična ulja izolirana iz aromatične vrste *Satureja montana* ssp. *variegata* i *Teucrium arduini* inhibirala infekciju TMV-om za 29,2 % odnosno 25,7 %, a infekciju virusom mozaika krastavca (CMV) za 24,1 % odnosno 21,9 % [146]. Eterična ulja vrsta iz roda *Micromeria* (*M. graeca, M. fruticulosa* i *M. croatica*) pokazala su antifitovirusno djelovanje na biljke zaražene satelitskim RNA virusom mozaika krastavca (satCMV) s aktivnošću od 59,3 %, 43,6 % i 34,5 %, tim redoslijedom [147]–[149]. Eterična ulja ekstrahirana iz četiri vrste roda *Teucrium* (*T. polium, T. flavum, T. montanum* i *T. chamaedrys*) su pokazali da smanjuju broj lezija u biljkama domaćinima zaraženim CMV-om, pri čemu je eterično ulje *T. polium* imalo najjači učinak s aktivnošću od 41,4 % [150]. Eterična ulja *Hypericum perforatum* ssp. *veronense*, *Eryngium alpinum* i *E. amethystinum* pokazale su obećavajuće razine antivirusnog

djelovanja od 88 %, 77,8 % odnosno 80,5 % [151], [152]. Osim toga, među eteričnim uljima ekstrahiranima iz 29 autohtonih kineskih aromatičnih biljaka, ulja đumbira, limuna, čajevca, kore mandarine, artemizije i limunske trave uzrokovala su više od 50 % inhibicije TMV-a pri testiranim koncentracijama [153], [154]. Osim biljnih ekstrakata, inhibitori biljnih virusa dobiveni iz metabolita mikroba također se smatraju potencijalnom alternativom kemijskim pesticidima [155]. Ningnanmicin izoliran iz fermentacijske smjese *Streptomyces noursei* var. *xichangensis* pokazuje sveobuhvatnu antifitoviralnu aktivnost i karakterizirana je povećanom otpornošću, izvrsnom učinkovitošću i niskom toksičnošću u biljkama domaćinima djelujući kroz ekspresiju proteina povezanih s patogenezom, povećavajući biosintezu salicilne kiseline i izazivajući sistemsku otpornost u biljkama domaćinima [156]–[158]. Ova i prethodna istraživanja koje se bave eteričnim uljima i hidrosolima aromatičnih biljaka pokazuju da hlapljivi biljni spojevi mogu povećati otpornost biljaka na virusne patogene. Djelovanje hidrosola obiju vrsta čestoslavica još više obećava od aktivnosti eteričnih ulja, a prikazani rezultati upućuju na potrebu daljnjih istraživanja antivirusnog djelovanja hlapljivih tvari drugih vrsta čestoslavica. Djelovanje testiranih ekstrakata vjerojatno je povezano s njihovim kemijskim sastavom, gdje bi sinergistički učinak hlapljivih sastojaka mogao aktivirati signalne puteve biljaka i dovesti do povećane otpornosti na virusne infekcije. Među glavnim sastojcima eteričnog ulja (Tablica 2, poglavlje 3.2.), *β*-ionon i heksadekanska kiselina su zastupljeni u EO *V. officinalis* (17,88 % odnosno 20,62 %), za razliku od EO *V. saturejoides,* gdje su ovi sastojci manje zastupljeni (heksadekanska kiselina) ili uopće nisu identificirani (*β*-ionon). Osim toga, vrijedni su pažnje *δ*-selinen (3,32 %), benzil benzoat (4,56 %), dokosan (2,13 %) i pentakosan (2,71 %), koji su također otkriveni samo u EO *V. officinalis*, ali ne i u EO *V. saturejoides*. Uspoređujući sastav eteričnog ulja sa sastavom hidrosola, *α*-terpineol, *allo*-aromadendren, benzen acetaldehid, n-nonanal i heksil 2-metilbutanoat navedeni su u sličnoj količini u hidrosolima *V. saturejoides* i *V. officinalis*, dok su identificirani u manjoj količini ili ih nema u EO *V. saturejoides*. Navedene sličnosti i razlike u sastavu ispitivanih ekstrakata mogu biti korisne za buduća predviđanja njihove učinkovitosti u zaštiti bilja. Antifitovirusno djelovanje vrsta roda *Veronica* trebalo bi detaljnije analizirati u budućnosti, uključujući daljnja istraživanja za procjenu njihove učinkovitosti protiv virusnih bolesti u terenskim uvjetima.

Antiproliferativno djelovanje

Potraga za prirodnim ekstraktima i na ovom području istraživanja je jako zastupljena oduvijek, jer su neki od najboljih lijekova protiv tumora izolirani iz biljaka [67]. Stoga je uz antioksidativno i antifitovirusno, u okviru istraživanja za ovu disertaciju, analizirano i

antiproliferativno djelovanje eteričnog ulja (EO) i hidrosola za sve tri vrsta čestoslavica (*V. saturejoides, V. austriaca ssp. jacquinii* i *V. officinalis*). Ovo djelovanje je bilo od posebnog interesa jer u literaturi nema podataka o antiproliferativnom djelovanju ovih vrsta. Rezultati ovog istraživanja pokazali su značajno antiproliferativno djelovanje ulja i hidrosola za neke vrste. Eterično ulje i hidrosol *V. saturejoides* prikupljeni s dvije različite lokacije (uzorak s Kamešnice (KS) i uzorak s Dinare (DS)) i *V. officinalis* (uzorak s Kamešnice (KS)) ispitani su na tri stanične linije raka: HeLa (stanična linija ljudskog cervikalnog raka), HCT116 (stanična linija raka debelog crijeva) i U2OS (stanična linija osteosarkoma). Najbolju antiproliferativnu aktivnost na sve tri testirane stanične linije pokazalo je eterično ulje vrste *V. saturejoides* KS (Slika 5a, poglavlje 3.2.). Osobito značajna razlika u antiproliferativnoj moći *V. saturejoides* KS u usporedbi s *V. saturejoides* DS i *V. officinalis* KS uočena je u staničnim linijama HeLa i U2OS. Zanimljivo je da je hidrosol pokazao nešto drugačije rezultate u odnosu na eterično ulje (Slika 5b, poglavlje 3.2.). Hidrosol *V. saturejoides* KS i DS pokazao je sličan antiproliferativni učinak na sve ispitivane stanice raka. Hidrosol *V. officinalis* DS pokazao je najjače antiproliferativno djelovanje na staničnim linijama HeLa i HCT116 s IC50 vrijednostima od 64,93 % odnosno 45,93 %. Hidrosol *V. saturejoides* KS imao je najbolju inhibiciju rasta na staničnoj liniji U2OS s IC50 vrijednošću od 48,23 %.

Citotoksični učinak eteričnog ulja i hidrosola *V. austriaca* ssp. *jacquinii* testiran je na tri stanične linije raka (HeLa, U2OS i HCT116) i dvije zdrave stanične linije (HaCaT i RPE1). Rezultati su pokazali da ni EO ni hidrosol nisu pokazali toksičnost ni za stanice raka ni za zdrave ljudske stanične linije (Slika 5a, 5b, poglavlje 3.3.). Vrijednosti IC50 bile su iznimno visoke, preko 1,5 mg/mL za ulje i preko 3 mg/mL za hidrosol. HeLa stanice su pokazale najveću otpornost, zahtijevajući 3,47 mg/mL EO i 4,55 mg/mL hidrosola za inhibiciju rasta stanica za 50 %. Stanice debelog crijeva (HCT116) i osteosarkoma (U2OS) pokazale su nešto manju, ali još uvijek značajnu otpornost na učinke EO i hidrosola, s IC50 vrijednostima od 1,9 mg/mL i 2,7 mg/mL za EO te 3,65 i 4,54 mg/mL za hidrosol, tim rasporedom. EO i hidrosol vrste *V. austriaca* ssp. *jacquinii* također nisu spriječili diobu zdravih stanica. Stanice RPE1, koje predstavljaju model epitelnih stanica, nisu bile osjetljive na učinak ulja i hidrosola s IC50 vrijednostima od 2,82 odnosno 4,61 mg/mL. Osim toga, ulje i hidrosol nisu pokazali toksičnost za stanice ljudskih keratinocita (HaCaT) (vrijednosti IC50 bile su 1,93 mg/mL za EO i 3,72 mg/mL za hidrosol). Dva glavna spoja EO-a, heksahidrofarnezil aceton i palmitinska kiselina, također su testirani na antiproliferativno djelovanje. Nijedan od testiranih spojeva nije pokazao citotoksičnu aktivnost protiv raka i zdravih staničnih linija. Vrijednosti IC50 za heksahidrofarnezil aceton bile su iznad 4 mg/mL, a za palmitinsku kiselinu znatno niže, ali još

uvijek dovoljno visoke da ne inhibiraju proliferaciju i raka i zdravih stanica (IC50 > 1 mg/mL) (Slika 5c, poglavlje 3.3.).

Nadalje, metanolni i vodeni ekstrakti nekoliko vrsta čestoslavica testirani su na različitim staničnim linijama raka. Citotoksično djelovanje metanolnih ekstrakata pet vrsta roda *Veronica* (*V. cymbalaria, V. hederifolia, V. pectinata var. glandulosa, V. persica* i *V. polita*) ispitano je na stanice KB (humani epidermoidni karcinom) i B16 (mišji melanom). Sve su vrste pokazale slične učinke ovisne o dozi protiv testiranih stanica [86]. Metanolni ekstrakt *V. americana* uspio je zaustaviti diobu dviju stanica raka HF-6 (debelo crijevo) i PC-3 (prostata) staničnih linija raka [87]. Feng i sur. pokazali su da flavonoidi izolirani iz *V. sibirica* induciraju apoptozu ovisnu o dozi u stanicama raka dojke MCF-7 s IC50 od 42 µg/mL [159]. Vodeni ekstrakti *V. cuneifolia* subsp. *cuneifolia* i *V. cymbalaria* pokazale su umjerenu citotoksičnu aktivnost protiv Hep-2 (humani epidermoidni karcinom), RD (ljudski rabdomiosarkom) i L-20B (transgene mišje L-stanice) [88]. Vodene frakcije metanolnih ekstrakata *V. persica* i *V. crista-galli* učinkovito su inhibirale proliferaciju HeLa i MCF-7 stanica, ali nisu pokazale toksičnost protiv normalne stanične linije fibroblasta. Ove vodene frakcije sadržavale su visoku koncentraciju flavonoida i fenola, zbog čega su uz citotoksičnu aktivnost pokazale i značajno antioksidativno djelovanje [160].

Vrste iz roda *Veronica* odavno se koriste u tradicionalnoj medicini za liječenje brojnih bolesti uključujući rak, gripu, herniju, kašalj, bolesti dišnog sustava i mnoge druge [161]–[163]. Istraživanja su pokazala da su ove biljke izvor specijaliziranih metabolita koji su uvelike odgovorni za njihovu izvrsnu biološku aktivnost, poput antimikrobnih, antioksidativnih i protuupalnih svojstava. Iako su se različiti ekstrakti čestoslavica koristili u narodnoj medicini za liječenje raka, vrlo je malo vrsta proučavano zbog njihove citotoksične i antikancerogene aktivnosti [31]. Prethodna istraživanja na raznim vrstama čestoslavica uglavnom su dovela do izolacije biološki aktivnih spojeva kao što su iridoidni glukozidi, za koje je utvrđeno da su izvrsna prirodne tvari s djelovanjem protiv raka [86], [164]–[168].

Rezultati ovog istraživanja po prvi put pokazuju da i eterično ulje i hidrosol vrste *V. saturejoides* i *V. officinalis* imaju značajno antiproliferativno djelovanje, a time i moguća kemoterapijska svojstva, zbog čega zaslužuju daljnje studije. Potrebno je provesti daljnja istraživanja ovih ekstrakata i njihovih glavnih sastojaka na različitim staničnim linijama raka kako bi se utvrdio mehanizam antiproliferativnog djelovanja i procijenila mogućnost njihove primjene u medicini i farmakologiji.

4.4. Molekularna analiza i kemofenetski markeri

Ovaj posljednji dio rasprave temelji se na rezultatima dva rada prikazana u poglavljima 3.4. i 3.5. Analiza FVC-a prikazana u radu u poglavlju 3.4. preuzeta je u svrhu izrade klaster analize vrsta na temelju ovih spojeva detektiranih u eteričnim uljima 18 vrsta odabranih za molekularnu analizu. Dobiveni klasteri usporedili su se s klasterima dobivenim molekularnom analizom ITS regija za odabrane vrste roda *Veronica* (Tablica 1, poglavlje 3.5.).

Iako je bilo moguće identificirati većinu analiziranih vrsta *Veronica* koje su proučavane iz podataka o njihovim ITS2 sekvencama generiranim NGS sekvenciranjem, točnija identifikacija postignuta je korištenjem duljih ITS1-5.8S-ITS2 sekvenci dobivenih klasičnim Sangerovim sekvenciranjem. Osim toga, bolji rezultati filogenetske analize i klasteriranja vrsta *Veronica* u 8 podrodova dobiveni su na temelju ITS1-5.8S-ITS2 sekvenci nego s podacima temeljenim na ITS2 sekvencama. To je vjerojatno zbog činjenice da su dulje sekvence generirane Sangerovom metodom sekvenciranja (~580 bp) dale više varijabilnih i informativnih pozicija od kraćih NGS sekvenci (~360 bp). Međutim, obilje ITS2 sekvenci dobivenih NGS tehnologijom, zajedno s ITS1 sekvencama također sekvenciranim NGS-om za 18 vrsta roda *Veronica*, koje nisu prikazane u ovom radu jer su manje informativne od ITS2 sekvenci, koristit će se za detaljnu analizu raznolikosti kontiga/ribotipova (kontige – konsenzusne sekvence, skup DNA segmenata ili sekvenci koji se preklapaju na način da pružaju kontinuirani prikaz genomske regije) unutar svake vrste i njihove molekularne evolucije i strukture genoma (ove analize su u tijeku).

Rezultati dviju filogenetskih analiza (na temelju ITS2 i ITS1-5.8S-ITS2) uvelike su u skladu s prethodnim publikacijama. Prema ovim i ranije objavljenim podacima o molekularno-filogenetskim analizama vrste *Veronica* [3], [4], [10], [93]–[95], [103], [169], vrste iz Hrvatske odabrane za ovo istraživanje pripadaju u osam podrodova (Slike 2 i 3, poglavlje 3.5.): *Pseudolysimachium* (*V. longifolia*), *Beccabunga* (*V. acinifolia, V. anagallis-aquatica, V. beccabunga, V. catenata, V. serpyllifolia, V. anagalloides*), *Veronica* (*V. montana, V. officinalis*), *Chamaedrys* (*V. arvensis, V. chamaedrys*), *Pentasepalae* (*V. austriaca, V. dalmatica*), *Stenocarpon* (*V. saturejoides*), *Pocilla* (*V. persica, V. polita*) i *Cochlidiosperma* (*V. cymbalaria, V. hederifolia*).

Uspoređujući dva klastera stvorena na temelju slobodnih hlapljivih spojeva (Slike 4 i 5, poglavlje 3.5.) i na temelju molekularnih podataka za ITS regije (Slika 2, poglavlje 3.5), može se reći da je klasteriranje na temelju hlapljivih spojeva iz mikrovalne ekstrakcije rezultiralo klasterima koji se bolje uklapaju u molekularne klastere. Prvi klaster (Slika 5, poglavlje 3.5.)

sastoji se od vrsta koje pripadaju podrodovima *Chamaedrys, Pocilla, Stenocarpon* i *Beccabunga*. *Chamaedrys* i *Pocilla* srodniji su u molekularnim ITS klasterima nego s ostalim podrodovima. Drugi klaster uključuje vrste iz podrodova *Cochlidiosperma, Veronica* i *Pentasepalae*. Treći klaster čine vrste iz podrodova *Veronica* i *Beccabunga*. Na Slici 2 ova su dva podroda, *Veronica* i *Beccabunga*, blisko srodna. FVC-ovi koji su bili prisutni u svim vrstama bez obzira na stanište i uvjete u kojima su rasle uključuju heksahidrofarnezil aceton, heksadekanska kiselina, kariofilen oksid i *(E)*-kariofilen. Drugi spojevi također prisutni u uzorcima gotovo svih vrsta su fitol, germakren D i pentakosan. Različiti relativni postotci i njihov međusobni omjer vjerojatno su posljedica ekoloških uvjeta na njihovim staništima. Budući da nije pronađena korelacija između ITS klastera i hlapljivog klastera, može se zaključiti da ovi spojevi nisu dobri interspecijski (koji pripadaju istom rodu) kemofenetski markeri, ali se neki od njih mogu definirati kao kemofenetski markeri za cijeli rod *Veronica*. U prilog ovom rezultatu, uspoređeni su hlapljivi spojevi proučavani u drugim rodovima koji pripadaju porodici Plantaginaceae i otkriveno je da se sastavi hlapljivih tvari razlikuju u glavnim spojevima, ali imaju neke iste spojeve kao vrste *Veronica*. Hammami i sur. proučavali su eterično ulje vrste *Plantago afra* i otkrili da su glavni sastojci timol (14,3 %), 3-[4-(t-Butil) fenil]furan-2,5-dion (12,7 %), heksadekanska kiselina (8,9 %) i eudezman [170]. Al-Mazroa i sur. proučavali su eterična ulja vrsta *Plantago amplexicaulis* i *Plantago boissieri* i rezultati su pokazali da su glavne komponente EO vrste *P. amplexicaulis* heksadekanska kiselina i 3-metil undekan [171]. Utvrđeno je da su glavni spojevi EO vrste *P. boissieri* biciklo-2,2, 1-heptan, 2-(2-propenil i l-dodekan-3-ol. U studiji de Lima i sur. utvrđeno je da su glavni sastojci EO *Conobea scoparioides* timol metil eter (62 %), timol (16 %) i *α*-felandren (14 %) [172]. U drugoj studiji, Brandao i sur. otkrili su da su glavni sastojci eteričnog ulja *Dizygostemon riparius* endo-fenhilacetat i endo-fenhol, a poslije tih tvari, *(E)*-kariofilen i kariofilen oksid u manjim relativnim postocima [173]. Bajer i sur. proučavali su sastav EO lišća *Plantago lanceolata* i pronašli ove glavne spojeve: heksadekansku kiselinu, linalol i pentil vinil keton. Također su identificirali heksahidrofarnezil aceton, ali u malim relativnim postocima (1,81-2,99 %) [174]. U drugoj studiji o EO vrsta *Plantago lanceolata* i *Plantago major* identificirani su drugačiji glavni spojevi nego u istraživanju Bajer i sur.: metaraminol, bifemelan, metozamin i pterin-6-karboksilna kiselina u vrsti *P. lanceolata* i 2-dodecen-1-il (-) jantarni anhidrid, benzenmetanol, *α*-(1-aminoetil)-2,5-dimetoksi, dl-fenilefrin i nortriptilin u vrsti *P. major* [175]. Fons i sur. također su proučavali eterično ulje vrste *Plantago lanceolata* i otkrili da su glavni spojevi eteričnog ulja lišća: oct-1-en-3-ol i (E)-4(3-okso-2,6,6-trimetilcikloheks-2-en-1-il)-3-buten-2-ol [176]. Pronašli su heksahidrofarnezil aceton kao glavni spoj, ali samo u plodovima

ove biljke [176], [177]. Još jedna studija o porodici Plantaginaceae identificirala je heksahidrofarnezil aceton kao spoj u sastavu FVC-a. Roudbaraki i sur. proučavali su hlapljive spojeve lišća *Digitalisa nervosa* i otkrili da su glavni spojevi bili trans-pinokamfon i heksadekanska kiselina, te nakon njih u nešto manjem postotku, kariofilen oksid i fitol. Heksahidrofarnezil aceton bio je identificiran u nešto manjem postotku od navedenih glavnih tvari (4.6 %) . Uspoređujući sve ove rezultate s identificiranim slobodnim hlapljivim tvarima vrste *Veronica* i njihovim relativnim postotkom, može se zaključiti da identificirani hlapljivi spojevi roda *Veronica* nisu pronađeni izvan roda u ovoj kombinaciji, barem ne do ovog trenutka. Neki glavni spojevi spomenuti ranije u ovim identificiranim postotnim omjerima mogu se definirati kao kemofenetski markeri za rod *Veronica*. Osobito je vrijedan pažnje heksahidrofarnezil aceton, koji se pojavljuje u visokim postotcima kod svih proučavanih vrsta *Veronica* i pojavljuje se u drugim rodovima porodice Plantaginaceae u mnogo manjim relativnim postocima nego kod čestoslavica. Druge prijavljene identifikacije ovog spoja proučavane su u sjemenkama ili plodovima, tako da nisu usporedive sa studijom ove doktorske disertacije [176], [178]. Slična je situacija i s kariofilen oksidom, *(E)*-kariofilenom, fitolom, germakrenom D i pentakosanom. Pregledom svih ovih hlapljivih tvari porodice Plantaginaceae može se zaključiti da heksadekanska kiselina nije dobar kemofenetski marker jer se pojavljuje u mnogim drugim vrstama izvan roda *Veronica*, te čak i izvan ove porodice. Naša studija nije prva koja je pronašla spojeve koji su kemofenetski markeri za međurodne, ali ne i za relacije unutar roda. Mehrvarz i sur. pronašli su slične rezultate u svojoj studiji, u kojoj su otkrili stalne i karakteristične iridoidne i flavonoidne profile u odabranim vrstama roda *Veronica*, što je korisno u analizi taksonomskih problema na specifičnoj razini (razina između rodova) [98], ali ne i za razlikovanje vrsta koje pripadaju istom rodu. Pregledom glikozida koji su otkriveni u vrstama roda *Veronica*, Taskova i sur. otkrili su aukubin i katalpol u svim vrstama koje su istraživali. Mnoge od njih su bile dio studije unutar ove doktorske disertacije kroz četvrti rad pod poglavljem 3.4: *V. polita, V. persica, V. chamaedrys, V. cymbalaria, V. montana, V. officinalis, V. longifolia, V. anagallis-aquatica, V. peregrina, V. beccabunga, V. serpyllifolia i V. acinifolia* [97]. U svom istraživanju Taskova i sur. također nisu pronašli korelaciju između pripadnosti podrodovima i fitokemijskih komponenti zbog varijabilnosti unutar roda što je slučaj i sa slobodnim hlapljivim spojevima iz istraživanja u okviru ove disertacije. U svom drugom istraživanju novozelandske snježne hebe i njenih glikozida, Taskova i sur. su zaključili da kemijski profili mogu pružiti vrijedne podatke za taksonomske probleme na rangu pododsjeka [179]. Daljnja istraživanja slobodnih hlapljivih spojeva kao kemofenetskih markera mogla bi uključiti neke dijelove roda *Veronica* koji rastu na južnoj hemisferi kao što su *Hebe*,

Parahebe, Heliohebe, Detzneria i *Derwentia*, budući da su se nekada smatrali različitim rodovima.

Mantelov test (statistički postupak koji ispituje korelaciju između dviju matrica udaljenosti) za usporedbu između euklidskih udaljenosti hlapljivih komponenata i Kimura genetskih udaljenosti podataka sekvenci DNA pokazao je da nema korelacije između grupa kemijskih i genetskih podataka (Kimura genetska udaljenost – matematički model za računanje genetskih udaljenosti između sekvenci [180]). Genetske udaljenosti obično se temelje na molekularnim markerima koji se sporo razvijaju tijekom vremena i podložni su nasumičnim mutacijama. Također, uočeni slučajevi paralelne evolucije u rodu *Veronica* mogu dodatno otežati korelaciju genetskih podataka s kemijskim sastavom hlapljivih komponenti [3], [34]. Hlapljive komponente kao i drugi biljni metaboliti mogu biti pod utjecajem bržih promjena kao što su promjene u ekspresiji gena, čimbenici okoliša ili epigenetske modifikacije. Stoga je važno uzeti u obzir više linija dokaza kada se proučavaju evolucijski odnosi između organizama, uključujući genetske, morfološke i biokemijske podatke.

5. ZAKLJUČCI

- Mikromorfološkim istraživanjem u sve tri vrste obuhvaćene ovom disertacijom (*V. saturejoides* ssp. *saturejoides*, *V austriaca* ssp. *jacquinii* i *V. officinalis*) identificirani su žljezdani trihomi u kojima se primarno događa sinteza istraživanih spojeva. U sve tri istražene vrste žljezdani trihomi se sastoje od jedne stanice koja čini stapku i dvije stanice koje čine glavu žlijezde. Kod vrste *V. officinalis* identificiran je i drugi tip glavičastih trihoma sa dužom stapkom te štitasti trihomi.
- Osim žljezdanih trihoma, na površini listova, stabljike i čaške uočeni su i nežljezdani trihomi koji su nerazgranati i jednostruki. Također se može primijetiti da se ovi trihomi pojavljuju kao kratki (dvostanični) ili duži (višestanični). Uočeni su u sve tri istražene vrste.
- Prevladavajući hlapljivi spojevi vrste *V. saturejoides* ssp. *saturejoides* u eteričnim uljima bili su heksahidrofarnezil aceton i heksadekanska kiselina. Glavni hlapljivi spoj u hidrosolima bio je *trans*-*p*-menta-1(7),8-dien-2-ol.
- Heksadekanska kiselina i *p*-vinil gvajakol bili su najzastupljeniji spojevi u eteričnom ulju i hidrosolima ljekovite čestoslavice (*V. officinalis*).
- Za vrstu *V. austriaca* ssp. *jacquinii* uspoređeni su hlapljivi spojevi ekstrahirani u eteričnim uljima i hidrosolima s četiri lokacije. Heksahidrofarnezil aceton i heksadekanska kiselina bile su glavne komponente eteričnih ulja bez obzira na lokaciju sakupljanja, a prevladavajuće komponente hidrosola bile su metil eugenol, timol te *trans*-*p*-menta-1(7),8-dien-2-ol.
- Heksadekanska kiselina, heksahidrofarnezil aceton i kariofilen oksid pojavljuju u sve tri istraživane vrste.
- Sastavi ukupnih polifenola utvrđen u okviru ove disertacije u skladu je s dosadašnjim literaturnim podacima.
- Eterična ulja *V. saturejoides* i *V. officinalis* pokazuju jače antioksidativno djelovanje od hidrosola, dok u slučaju *V. austriaca* ssp. *jacquinii* hidrosoli imaju jaču antioksidativnu aktivnost. Iz ovog rezultata se može zaključiti da hidrosoli, iako u pravilu sadrže manje otopljenih hlapljivih molekula u svom sadržaju, mogu pokazati jače antioksidativno djelovanje od eteričnih ulja.
- Antioksidativno djelovanje eteričnog ulja vrste *V. saturejoides* ssp. *saturejoides* bilo je jače od onog kod vrste *V. officinalis* prilikom testiranja obje metode (ORAC i DPPH). Suprotno ovom rezultatu, hidrosoli vrste *V. officinalis* pokazali su veću aktivnost ORAC metodom, a hidrosoli vrste *V. saturejoides* DPPH metodom.

- Rezultati za antifitoviralnu aktivnost pokazuju da je hidrosol vrsta *V. officinalis* i *V. saturejoides* smanjio broj lokalnih lezija na biljkama zaraženim virusom TMV u usporedbi s kontrolnim biljkama. Eterično ulje vrste *V. officinalis* također je pokazalo ovakav rezultat, dok ono izolirano iz vrste *V. saturejoides* nije pokazao antifitovirusno djelovanje.
- Najbolje antiproliferativno djelovanje na sve tri ispitivane stanične linije pokazalo je eterično ulje vrste *V. saturejoides* s lokaliteta Kamešnica.
- Najveća antiproliferativna aktivnost hidrosola protiv HeLA i HCT116 staničnih linija bila je kod vrste *V. officinalis.*
- Izolati vrste *V. austriaca* ssp. *jacquinii* nisu pokazale toksičnost prema zdravim stanicama, te bi se kao takvi mogli koristiti kao siguran proizvod u kozmetici ili za konzerviranje hrane. Također, izolati ove vrste nisu pokazali antiproliferativnu aktivnost ni prema odabranim staničnim linijama raka (HeLA – stanična linija cervikalnog raka, U2OS – stanična linija osteosarkoma i HCT116 – stanična linija raka debelog crijeva).
- Heksahidrofarnezil aceton, heksadekanska kiselina, fitol, *(E)*-kariofilen i kariofilen oksid su identificirani kao glavne komponente uporabom klasične (Clevenger hidrodestilacija), ali i zelene (mikrovalna) metode ekstrakcije svih istraživanih vrsta roda *Veronica* bez obzira na lokaciju skupljanja. Dokazano je da se metoda mikrovalne ekstrakcije može koristiti kao održiva i „zelena“ metoda umjesto klasične hidrodestilacije jer nije zabilježena bitna razlika u detekciji različitih hlapljivih spojeva.
- Nije pronađena korelacije između srodstvenih odnosa dobivenih analizom sekvenci DNA (pripadnosti podrodovima roda *Veronica*) i sastava slobodnih hlapljivih spojeva. Ovaj rezultat se može objasniti činjenicom da hlapljive komponente kao i drugi biljni metaboliti mogu biti pod većim utjecajem okoliša kroz različitu ekspresiju gena uslijed epigenetskih modifikacija.
- Heksahidrofarnezil aceton, heksadekanska kiselina, fitol, *(E)*-kariofilen i kariofilen oksid identificirani su u svim istraživanim vrstama roda *Veronica*. Budući da ovi spojevi nisu identificirani kao glavni spojevi i u istim relativnim postotnim omjerima ni u jednom rodu unutar porodice Plantaginaceae, mogli bi se koristiti kao kemofenetski markeri za rod, ali ne i za međuvrsne odnose unutar roda *Veronica.*

6. LITERATURA

[1] T. Reynolds, "The evolution of chemosystematics," *Phytochemistry*, vol. 68, no. 22–24, pp. 2887–2895, 2007, doi: 10.1016/j.phytochem.2007.06.027.

[2] F. Loreto, M. Dicke, J. P. Schnitzler, and T. C. J. Turlings, "Plant volatiles and the environment," *Plant Cell Environ*, vol. 37, no. 8, pp. 1905–1908, 2014, doi: 10.1111/pce.12369.

[3] D. C. Albach, M. M. Martínez-Ortega, and M. W. Chase, "*Veronica*: Parallel morphological evolution and phylogeography in the Mediterranean," *Plant Syst*, vol. 246, no. 3–4, pp. 177–194, 2004, doi: 10.1007/s00606-004-0148-9.

[4] D. C. Albach, M. M. Martínez-Ortega, M. A. Fischer, and M. W. Chase, "A new classification of the tribe Veroniceae-problems and a possible solution," *Taxon*, vol. 53, no. 2, pp. 429–452, 2004, doi: 10.2307/4135620.

[5] J. C. M. Barreira *et al.*, "Phenolic profiling of *Veronica* spp. grown in mountain, urban and sandy soil environments," *Food Chem*, vol. 163, pp. 275–283, 2014, doi: 10.1016/j.foodchem.2014.04.117.

[6] T. G. Tutin *et al.*, *Flora Europaea*, Volume 3. London, UK: Cambridge University Press, 1972.

[7] T. Nikolić, "Flora Croatica Database." http://hirc.botanic.hr/fcd

[8] M. H. A. AL-Hadeethi, S. N. Hawar, A. T. AL-Taie, and L. Q. Ahmed, "Morphological and anatomical review of some *Veronica* species from the Plantaginaceae Family," *J Pharm Negat Results*, vol. 13, pp. 192–197, 2022, doi: 10.47750/pnr.2022.13.S07.029.

[9] "Čestoslavica." https://www.plantea.com.hr/cestoslavica/ (accessed Mar. 15, 2023).

[10] D. C. Albach, M. Von Sternburg, R. Scalone, and K. E. Bardy, "Phylogenetic analysis and differentiation of *Veronica* subgenus *Stenocarpon* in the Balkan Peninsula," *Bot J Linn*, vol. 159, no. 4, pp. 616–636, 2009, doi: 10.1111/j.1095-8339.2009.00958.x.

[11] Č. Šilić, *Endemične biljke*, 1st ed. "Svjetlost", OOUR Zavod za udžbenike i nastavna sredstva, Sarajevo; Zavod za udžbenike i nastavna sredstva, Beograd, 1984.

[12] R. Domac, *Flora Hrvatske*, 2nd ed. Zagreb: Školska knjiga, 2002.

[13] W. Hensel, *Medicinal plants of Britain and Europe*. London, UK: A&C Black Publishers Ltd, 2008.

[14] D. Kremer, *Ljekovito bilje Farmaceutskog botaničkog vrta "Fran Kušan,"* 1st ed. Zagreb, Croatia: Javna ustanova "Maksimir," 2018.

[15] J. Č. Živković, " Morfološka, hemijska i farmakološka karakterizacija odabranih vrsta roda *Veronica* L. (Plantaginaceae)," doktorska disertacija, Farmaceutski fakultet, Sveučilište u Beogradu, Beograd, 2014.

[16] B. M. Rojas-Andres and M. M. Martinez-Ortega, "Taxonomic revision of *Veronica* subsection Pentasepalae (*Veronica*, Plantaginaceae sensu APG III)," *Phytotaxa*, vol. 285, pp. 1–100, 2016, doi: 10.11646/phytotaxa.285.1.1.

[17] B. B. Buchanan, G. Wilhelm, and R. L. Jones, *Biochemistry & Molecular Biology of Plants*, Second Edi. The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK: Wiley-Blackwell, 2015.

[18] N. E. Thomford *et al.*, "Natural products for drug discovery in the 21st century: Innovations for novel drug discovery," *Int J Mol Sci*, vol. 19, p. 1578, 2018, doi: 10.3390/ijms19061578.

[19] B. Pevalek-Kozlina, *Fiziologija bilja*, 1st ed. Zagreb, Croatia: PROFIL, 2003.

[20] V. Dunkić *et al.*, "Antioxidant and antimicrobial properties of *Veronica spicata* L. (Plantaginaceae)," *Curr Drug Targets*, vol. 16, no. 14, pp. 1660–1670, 2015, doi: 10.2174/1389450116666150531161820.

[21] N. Dudareva, E. Pichersky, and J. Gershenzon, “Biochemistry of plant volatiles,” *Plant Physiol*, vol. 135, pp. 1893–1902, 2004.
[22] A. Hamedi, A. Pasdaran, Z. Zebarjad, and M. Moein, “A survey on chemical constituents and indications of aromatic waters soft drinks (hydrosols) used in Persian nutrition culture and folk medicine for neurological disorders and mental Health,” *J Evid Based Complementary Altern Med*, vol. 22, no. 4, pp. 744–752, 2017, doi: 10.1177/2156587217714145.
[23] T. S. Cid-Pérez, R. Ávila-Sosa, C. E. Ochoa-Velasco, B. E. Rivera-Chavira, and G. V. Nevárez-Moorillón, “Antioxidant and antimicrobial activity of mexican oregano (*Poliomintha longiflora*) essential oil, hydrosol and extracts fromwaste solid residues,” *Plants*, vol. 8, no. 1, 2019, doi: 10.3390/plants8010022.
[24] S. D’Amato, A. Serio, C. C. López, and A. Paparella, “Hydrosols: Biological activity and potential as antimicrobials for food applications,” *Food Control*, vol. 86, pp. 126–137, 2018, doi: 10.1016/j.foodcont.2017.10.030.
[25] B. R. R. Rao, “Hydrosols and water-soluble essential oils: Their medicinal and biological properties,” in *Recent Progress in Medicinal Plants Essential Oils*, J. N. Govil and S. Bhattacharya, Eds., 1st ed. Houston, USA: Studium Press LLC, 2013, pp. 120–140.
[26] V. Ninkuu, L. Zhang, J. Yan, Z. Fu, T. Yang, and H. Zeng, “Biochemistry of terpenes and recent advances in plant protection,” *Int J Mol Sci*, vol. 22, no. 11, 2021, doi: 10.3390/ijms22115710.
[27] J. Sharifi-Rad *et al.*, “Biological activities of essential oils: From plant chemoecology to traditional healing systems,” *Molecules*, vol. 22, no. 1, p. 70, 2017, doi: 10.3390/molecules22010070.
[28] P. Noriega, “Terpenes in essential oils: Bioactivity and applications,” in *Terpenes and Terpenoids*, S. Perveen and A. M. Al-Taweel, Eds., 1st ed. London, UK: IntechOpen, 2020. doi: 10.5772/intechopen.93792.
[29] Y. Li *et al.*, “Essential oils: From conventional to green extraction,” in *Essential Oils as Reagents in Green Chemistry*, S. K. Sharma, Ed., 1st ed. eBook: Springer Cham, 2014, pp. 9–20. doi: 10.1007/978-3-319-08449-7_2.
[30] M. Blekić, A. R. Jambrak, and F. Chemat, “Mikrovalna ekstrakcija bioaktivnih spojeva,” *CJFST*, vol. 3, no. 1, pp. 32–47, 2011.
[31] B. Salehi *et al.*, “Veronica plants—drifting from farm to traditional healing, food application, and phytopharmacology,” *Molecules*, vol. 24, no. 13, 2019, doi: 10.3390/molecules24132454.
[32] R. Lone, R. Shuab, and A. N. Kamili, *Plant Phenolics in Sustainable Agriculture*, 1st ed., vol. 1. Singapore: Springer, 2020.
[33] M. Ganzera and S. Strum, “Recent advances on HPLC/MS in medicinal plant analysis—An update covering 2011–2016,” *J Pharm Biomed Anal*, vol. 147, pp. 211–233, 2018.
[34] D. C. Albach, R. J. Grayer, S. R. Jensen, F. Özgökce, and N. C. Veitch, “Acylated flavone glycosides from *Veronica*,” *Phytochemistry*, vol. 64, no. 7, pp. 1295–1301, 2003, doi: 10.1016/j.phytochem.2003.08.012.
[35] D. C. Albach, R. J. Grayer, G. C. Kite, and S. R. Jensen, “*Veronica*: Acylated flavone glycosides as chemosystematic markers,” *Biochem Syst Ecol*, vol. 33, no. 11. pp. 1167–1177, 2005. doi: 10.1016/j.bse.2005.01.010.
[36] S. R. Jensen, D. C. Albach, T. Ohno, and R. J. Grayer, “*Veronica*: Iridoids and cornoside as chemosystematic markers,” *Biochem Syst Ecol*, vol. 33, no. 10, pp. 1031–1047, Oct. 2005, doi: 10.1016/j.bse.2005.03.001.
[37] U. S. Harput, I. Saracoglu, M. Inoue, and Y. Ogihara, “Phenylethanoid and iridoid glycosides from *Veronica persica*,” *Chem Pharm Bull (Tokyo)*, vol. 50, no. 6, pp. 869–871, 2002, doi: 10.1248/cpb.50.869.

[38] J. Hong-Young, “Phenolic constituents of *Veronica linariifolia*,” *Chin J Nat Med*, vol. 6, no. 2, pp. 126–129, 2008.
[39] I. Beara *et al.*, “Phenolic profile and anti-inflammatory activity of three *Veronica* species,” *Ind Crops Prod*, vol. 63, pp. 276–280, 2015, doi: 10.1016/j.indcrop.2014.09.034.
[40] J. Živković *et al.*, “Phenolic profile, antibacterial, antimutagenic and antitumour evaluation of *Veronica urticifolia* Jacq.,” *J Funct Foods*, vol. 9, no. 1, pp. 192–201, 2014, doi: 10.1016/j.jff.2014.04.024.
[41] F. Li, “Analysis of chemical constituents of essential oil in *Veronica linariifolia* by gas chromatography-mass spectrometry,” *Chinese J Anal Chem*, no. 30, pp. 822–825, 2002.
[42] E. Çelik, G. Yuvali Çelik, and A. Meysun, “Essential oil composition and antibacterial activity of some plant species,” *J Appl Biol*, vol. 1, no. 4, pp. 45–48, 2010, doi: 10.1089/jmf.2013.0079.
[43] Z. Hazzoumi, Y. Moustakime, and K. Amrani Joutei, “Essential oil and glandular hairs: Diversity and roles,” in *Essential Oils - Oils of Nature*, H. A. El-Shemy, Ed., 1st ed.eBook: IntechOpen, 2019, pp. 1–16. doi: 10.5772/intechopen.86571.
[44] X. Wang, C. Shen, P. Meng, G. Tan, and L. Lv, “Analysis and review of trichomes in plants,” *BMC Plant Biol*, vol. 21, no. 1, pp. 1–11, 2021, doi: 10.1186/s12870-021-02840-x.
[45] “https://www.bartleby.com.” https://www.bartleby.com/questions-and-answers/i.-identification-2-1.-what-specific-type-of-trichomes-is-a-2.-what-specific-plant-part-is-b/acac7cdc-101d-49cf-9f7e-bd2c25047b39 (accessed Mar. 12, 2023).
[46] G. A. Kurer, *Kutikularfalten und Protuberanzen an Haaren und Epidermen und ihre Verwendung zur Differenzialdiagnose offizineller Blätter*, 1st ed. Dresden, Germany: Druck, Gebr. Leeman, 1917.
[47] H. Kraehmer and P. Baur, *Weed Anatomy*, 1st ed. Oxford, UK: Wiley-Blackwell, 2013.
[48] W. Haratym and E. Weryszko-Chmielewska, “Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare* L. (Lamiaceae),” *Flora*, vol. 231, pp. 11–20, 2017, doi: 10.1016/j.flora.2017.04.001.
[49] C. Giuliani, R. Pellegrino, B. Tirillini, and L. M. Bini, “Micromorphological and chemical characterisation of *Stachys recta* L. subsp. *serpentini* (Fiori) Arrigoni in comparison to *Stachys recta* L. subsp. *recta* (Lamiaceae),” *Flora*, vol. 203, no. 5, pp. 376–385, 2008, doi: 10.1016/j.flora.2007.07.001.
[50] V. Dunkić *et al.*, “Micromorphological and phytochemical traits of four *Clinopodium* L. species (Lamiaceae),” *S Afr J Bot*, vol. 111, pp. 232–241, 2017, doi: 10.1016/j.sajb.2017.03.013.
[51] U. Kristen and J. Lockhausen, “The leaf glands of *Veronica beccabunga* L .: Ultrastructure and a possible pathway of secretion,” *Isr J Bot*, vol. 34, pp. 147–156, 2013, doi: 10.1080/0021213X.1985.10677018.
[52] V. Bilušić Vundać, E. Stabentheiner, A. Brantner, and M. Plazibat, “Morphology and distribution of trichomes on leaves in seven Croatian taxa of the genus *Stachys* (Lamiaceae),” *Phyton (B Aires)*, vol. 51, no. 1, pp. 161–176, 2011.
[53] L. Fabijanić, “Usporedba sastava hlapljivih komponenti vrsta *Veronica saturejoides* Vis. ssp. *saturejoides* i *Veronica austriaca* L. ssp. *jacquinii* (Baumg.) Eb. Fisch,” diplomski rad, Prirodoslovno-matematički fakultet, Sveučilište u Splitu, 2020.
[54] D. Kremer, E. Stabentheiner, M. Nazlić, M. Randić, S. Srečec, and V. Dunkić, “Barrelier’s speedwell (*Veronica barrelieri* Schott ex Roem. et Schult., Plantaginaceae)—Potential of free volatile compounds for horticulture,” *Horticulturae*, vol. 8, no. 9, 2022, doi: 10.3390/horticulturae8090777.

[55] D. Kremer *et al.*, "Micromorphological and chemotaxonomical traits of *Micromeria croatica* (Pers.) Schott," *Chem Biodivers*, vol. 9, no. 4, pp. 755–768, 2012, doi: 10.1002/cbdv.201100121.
[56] D. Kremer *et al.*, "Micromorphological traits of Balcanic *Micromeria* and closely related *Clinopodium* Species (Lamiaceae)," *Plants*, vol. 10, no. 8, pp. 1–21, 2021, doi: 10.3390/plants10081666.
[57] M. Olszowy and A. L. Dawidowicz, "Essential oils as antioxidants: their evaluation by DPPH, ABTS, FRAP, CUPRAC, and β-carotene bleaching methods," *Monatshefte für Chemie - Chemical Monthly*, vol. 147, no. 12, pp. 2083–2091, 2016, doi: 10.1007/s00706-016-1837-0.
[58] S. A. Sinenko, T. Y. Starkova, A. A. Kuzmin, and A. N. Tomilin, "Physiological signaling functions of reactive oxygen species in stem cells: From flies to man," *Front Cell Dev Biol*, vol. 9. Frontiers Media S.A., 2021. doi: 10.3389/fcell.2021.714370.
[59] R. Amorati, M. C. Foti, and L. Valgimigli, "Antioxidant activity of essential oils," *J Agric Food Chem*, vol. 61, no. 46, pp. 10835–10847, 2013, doi: 10.1021/jf403496k.
[60] S. Dutta, S. Mahalanobish, S. Saha, S. Ghosh, and P. C. Sil, "Natural products: An upcoming therapeutic approach to cancer," *FCT*, vol. 128, no. February, pp. 240–255, 2019, doi: 10.1016/j.fct.2019.04.012.
[61] R. Amorati and M. C. Foti, "Mode of antioxidant action of essential oils," in *Essential Oils in Food Processing: Chemistry, Safety and Applications*, S. M. Bagher Hashemi, A. M. Khaneghah, and A. de Souza Sant'Ana, Eds., 1st ed.Oxford, UK: Wiley-Blackwell, 2017, pp. 267–291. doi: https://doi.org/10.1002/9781119149392.ch9.
[62] J. Yeo and F. Shahidi, "Revisiting DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as a useful tool in antioxidant evaluation: A new IC100 concept to address its limitations," *J Food Bioact*, vol. 7, 2019, doi: 10.31665/JFB.2019.7196.
[63] J. Borlinghaus, J. Reiter, M. Ries, and M. C. H. Gruhlke, "Screening procedures and tests for antioxidants," in *Pathology*, V. R. Preedy, Ed., 1st ed.Oxford, UK: Academic Press, Elsevier, 2020, pp. 389–395. doi: https://doi.org/10.1016/B978-0-12-815972-9.00037-8.
[64] M. N. Alam, N. J. Bristi, and M. Rafiquzzaman, "Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity," *SPJ*, vol. 21, no. 2, pp. 143–152, 2013, doi: 10.1016/j.jsps.2012.05.002.
[65] R. L. Prior, X. Wu, and K. Schaich, "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements," *J Agric Food Chem*, vol. 53, no. 10, pp. 4290–4302, May 2005, doi: 10.1021/jf0502698.
[66] K. Blowman, M. Magalhães, M. F. L. Lemos, C. Cabral, and I. M. Pires, "Anticancer properties of essential oils and other natural products," *eCAM*, vol. 2018, pp. 1–12, 2018, doi: 10.1155/2018/3149362.
[67] S. B. Ateba *et al.*, "Natural Terpenoids Against Female Breast Cancer: A 5-year Recent Research," *Curr Med Chem*, vol. 25, no. 27, pp. 3162–3213, 2018, doi: 10.2174/0929867325666180214110932.
[68] N. Gautam, A. K. Mantha, and S. Mittal, "Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View," *Biomed Res Int*, vol. 2014, pp. 1–23, 2014, doi: 10.1155/2014/154106.
[69] J. F. Lesgards, N. Baldovini, N. Vidal, and S. Pietri, "Anticancer activities of essential oils constituents and synergy with conventional therapies: A review," *Phytotherapy Research*, vol. 28, no. 10, pp. 1423–1446, 2014, doi: 10.1002/ptr.5165.
[70] T. M. Buttke, J. A. Mccubrey, T. C. Owen, T. M. Bunke, and C. Owen, "Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines," *J Immunol Methods*, vol. 157, pp. 233–240, 1993.

[71] J. A. Barltrop, T. C. Owen, A. H. Cory, and J. G. Cory, "5-(3-carboxymethoxyphenyl)-2-(4,5-dimenthylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicat," *Bioorg Med Chem Lett*, vol. 1, no. 11, pp. 611–614, 1991.

[72] K. Ishibashi and M. Ishikawa, "Replication of Tobamovirus RNA," *Annu Rev Phytopathol*, vol. 54, no. May, pp. 55–78, 2016, doi: 10.1146/annurev-phyto-080615-100217.

[73] B. A. Othman and S. A. Shoman, "Antiphytoviral activity of the *Plectranthus tenuiflorus* on some important viruses," *IJAB*, vol. 6, no. 5, pp. 844–849, 2004, [Online]. Available: http://www.ijab.org

[74] G. Rusak, M. Krajačić, M. Krsnik-Rasol, and H. O. Gutzeit, "Quercetin influences response in *Nicotiana megalosiphon* infected by satellite-associated cucumber mosaic virus," *JPDP*, vol. 114, no. 4, pp. 145–150, Aug. 2007.

[75] E. Krcatović, G. Rusak, N. Bezić, and M. Krajačić, "Inhibition of tobacco mosaic virus infection by quercetin and vitexin," *Acta Virol*, vol. 52, no. 2, pp. 119–24, 2008.

[76] A. Moeini *et al.*, "α-costic acid, a plant sesquiterpenoid from *Dittrichia viscosa*, as modifier of Poly (lactic acid) properties: a novel exploitation of the autochthone biomass metabolite for a wholly biodegradable system," *Ind Crops Prod*, vol. 146, no. July 2019, p. 112134, 2020, doi: 10.1016/j.indcrop.2020.112134.

[77] J. Č. Živković *et al.*, "Chemical profiling and assessment of antineurodegenerative and antioxidant properties of *Veronica teucrium* L. and *Veronica jacquinii* Baumg," *Chem Biodivers*, vol. 14, no. 8, 2017, doi: 10.1002/cbdv.201700167.

[78] M. Sharifi-Rad, G. S. Tayeboon, J. Sharifi-Rad, M. Iriti, E. M. Varoni, and S. Razazi, "Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts," *Cell Mol Biol*, vol. 62, no. 6, pp. 80–85, 2016, doi: 10.14715/cmb/2016.62.6.15.

[79] H. Xue, K. X. Chen, L. Q. Zhang, and Y. M. Li, "Review of the ethnopharmacology, phytochemistry, and pharmacology of the genus *Veronica*," *Am J Chin Med*, vol. 47, no. 6, pp. 1193–1221, 2019, doi: 10.1142/S0192415X19500617.

[80] A. Ertas *et al.*, "Chemical profile and biological activities of *Veronica thymoides* subsp. *pseudocinerea*," *Pharm Biol*, vol. 53, no. 3, pp. 334–339, 2015, doi: 10.3109/13880209.2014.919326.

[81] A. Mocan, D. C. Vodnar, L. Vlase, O. Crișan, A. M. Gheldiu, and G. Crișan, "Phytochemical characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea crantz* from Romania and their antioxidant and antimicrobial properties," *Int J Mol Sci*, vol. 16, no. 9, pp. 21109–21127, 2015, doi: 10.3390/ijms160921109.

[82] M. Valyova, V. Hadjimitova, S. Stoyanov, Y. Ganeva, and I. Petkov, "Free radical scavenging activity of extracts from Bulgarian *Veronica officinalis* L. and GC-MS analysis of ethanol extract," *IJAAM*, vol. 2, no. 1, pp. 2–6, 2008, doi: 10.5580/2394.

[83] J. H. Kwak, H. J. Kim, K. H. Lee, S. C. Kang, and O. P. Zee, "Antioxidative iridoid glycosides and phenolic compounds from *Veronica peregrina*," *Arch Pharm Res*, vol. 32, no. 2, pp. 207–213, 2009, doi: 10.1007/s12272-009-1137-x.

[84] U. S. Harput, I. Saracoglu, and Y. Genc, "Comparative Bioactivity Studies on Four *Veronica* species," *Fabad J Pharm Sci*, vol. 34, no. 2, pp. 67–72, 2009.

[85] D. S. Stojković *et al.*, "Antibacterial activity of *Veronica montana* L. extract and of protocatechuic acid incorporated in a food system," *FCT*, vol. 55, pp. 209–213, 2013, doi: 10.1016/j.fct.2013.01.005.

[86] U. S. Harput, I. Saracoglu, M. Inoue, and Y. Ogihara, “Anti-inflammatory and cytotoxic activities of five *Veronica* species,” *Biol Pharm Bull*, vol. 25, no. 4, pp. 483–486, 2002, doi: 10.1248/bpb.25.483.
[87] J. A. Moreno-Escobar, S. Bazalda, M. L. Villarreal, J. R. Bonilla-Barbosa, S. Mendoza, and V. Rodríguez-López, “Cytotoxic and antioxidant activities of selected Lamiales species from Mexico,” *Pharm Biol*, vol. 49, no. 12. pp. 1243–1248, 2011. doi: 10.3109/13880209.2011.589454.
[88] I. Saracoglu, F. H. Oztunca, A. Nagatsu, and U. S. Harput, “Iridoid content and biological activities of *Veronica cuneifolia* subsp. *cuneifolia* and *V. cymbalaria*,” *Pharm Biol*, vol. 49, no. 11, pp. 1150–1157, 2011, doi: 10.3109/13880209.2011.575790.
[89] M. Ginovyan, M. Petrosyan, and A. Trchounian, “Antimicrobial activity of some plant materials used in Armenian traditional medicine,” *BMC Complement Altern Med*, vol. 17, no. 1, p. 50, 2017, doi: 10.1186/s12906-017-1573-y.
[90] J. Sharifi-Rad *et al.*, “*Veronica persica* Poir. extract - antibacterial, antifungal and scolicidal activities, and inhibitory potential on acetylcholinesterase, tyrosinase, lipoxygenase and xanthine oxidase,” *Cell Mol Biol (Noisy-le-grand)*, vol. 64, no. 8, pp. 50–56, 2018.
[91] J. Sharifi-Rad, M. Iriti, W. N. Setzer, M. Sharifi-Rad, A. Roointan, and B. Salehi, “Antiviral activity of *Veronica persica* Poir. on herpes virus infection.,” *Cell Mol Biol (Noisy-le-grand)*, vol. 64, no. 8, pp. 11–17, 2018.
[92] D. M. Pinto-Zevallos, H. Hellén, H. Hakola, S. Van Nouhuys, and J. K. Holopainen, “Induced defenses of *Veronica spicata*: Variability in herbivore-induced volatile organic compounds,” *Phytochem Lett*, vol. 6, no. 4. pp. 653–656, 2013. doi: 10.1016/j.phytol.2013.08.015.
[93] D. C. Albach, H. M. Meudt, and B. Oxelman, “Piecing together the ‘new’ Plantaginaceae,” *Am J Bot*, vol. 92, no. 2, pp. 297–315, 2005, doi: 10.3732/ajb.92.2.297.
[94] D. C. Albach, S. R. Jensen, F. Özgökce, and R. J. Grayer, “*Veronica*: Chemical characters for the support of phylogenetic relationships based on nuclear ribosomal and plastid DNA sequence data,” *Biochem Syst Ecol*, vol. 33, no. 11, pp. 1087–1106, 2005, doi: 10.1016/j.bse.2005.06.002.
[95] R. M. Taskova, D. C. Albach, and R. J. Grayer, “Phylogeny of *Veronica* - A combination of molecular and chemical evidence,” *Plant Biol*, vol. 6, no. 6, pp. 673–682, 2004, doi: 10.1055/s-2004-830330.
[96] C. Zidorn, “Plant chemophenetics − A new term for plant chemosystematics/plant chemotaxonomy in the macro-molecular era,” *Phytochemistry*, vol. 163, pp. 147–148, 2019, doi: 10.1016/j.phytochem.2019.02.013.
[97] R. Taskova, D. Peev, and N. Handjieva, “Iridoid glucosides of the genus *Veronica* s.l. and their systematic significance,” *Plant Systematics and Evolution*, vol. 231, no. 1–4, pp. 1–17, 2002, doi: 10.1007/s006060200008.
[98] S. Saeidi Mehrvarz, N. O. Mahmoodi, R. Asadian, and G. Bakhshi Khaniki, “Iridoid and flavonoid patterns of the genus *Veronica* sect. *Alsinebe* subsect. *Agrestis* (Benth.) Stroh (Lamiales) and their systematic significance,” *Aust J Crop Sci*, vol. 1, no. 1, pp. 1–5, 2008.
[99] G. Crișan *et al.*, “LC /MS analysis of aucubin and catalpol of some *Veronica* species,” *Farmacia*, vol. 58, no. 2, pp. 237–242, 2010.
[100] R. Tundis, M. Loizzo, F. Menichini, G. Statti, and F. Menichini, “Biological and pharmacological activities of iridoids: Recent developments,” *Mini Rev Med Chem*, vol. 8, no. 4, pp. 399–420, 2008, doi: 10.2174/138955708783955926.

[101] D. C. Albach, C. Held Gotfredsen, and S. R. Jensen, “Iridoid glucosides of *Paederota lutea* and the relationships between *Paederota* and *Veronica*,” *Phytochemistry*, vol. 65, no. 14, pp. 2129–2134, 2004, doi: 10.1016/j.phytochem.2004.05.022.
[102] I. Saracoğlu, Ü. Ş. Harput, and Y. Ogihara, “Acylated flavone glycosides from *Veronica pectinata* var. *glandulosa* and *V. persica*,” *Turk J Chem*, vol. 28, no. 6, pp. 751–759, 2004.
[103] D. C. Albach, Ma. M. Martınez-Ortega, M. A. Fischer, and M. W. Chase, “Evolution of Veroniceae: A phylogenetic perspective,” *Ann Mo Bot Gard*, vol. 91, no. 2, pp. 275–302, 2004.
[104] D. C. Albach, M. M. Martínez-Ortega, L. Delgado, H. Weiss-Schneeweiss, F. Özgökce, and M. A. Fischer, “Chromosome numbers in Veroniceae (Plantaginaceae): Review and several new counts,” *Ann Mo Bot Gard*, vol. 95, no. 4, pp. 543–566, 2008, doi: 10.3417/2006094.
[105] B. M. Rojas-Andrés, E. Low, D. C. Albach, J. M. Prebble, H. M. Meudt, and P. J. Garnock-Jones, “Is genome downsizing associated with diversification in polyploid lineages of *Veronica*?,” *Bot J Linn*, vol. 178, no. 2, pp. 243–266, 2015, doi: 10.1111/boj.12276.
[106] D. C. Albach and H. M. Meudt, “Phylogeny of *Veronica* in the Southern and Northern Hemispheres based on plastid, nuclear ribosomal and nuclear low-copy DNA,” *Mol Phylogenet Evol*, vol. 54, no. 2, pp. 457–471, 2010, doi: 10.1016/j.ympev.2009.09.030.
[107] K. E. Bardy, D. C. Albach, G. M. Schneeweiss, M. A. Fischer, and P. Schönswetter, “Disentangling phylogeography, polyploid evolution and taxonomy of a woodland herb (*Veronica chamaedrys* group, Plantaginaceae s.l.) in southeastern Europe,” *Mol Phylogenet Evol*, vol. 57, no. 2, pp. 771–786, 2010, doi: 10.1016/j.ympev.2010.06.025.
[108] K. E. Bardy, P. Schönswetter, G. M. Schneeweiss, M. A. Fischer, and D. C. Albach, “Extensive gene flow blurs species boundaries among *Veronica barrelieri*, *V. orchidea* and *V. spicata* (Plantaginaceae) in southeastern Europe,” *Taxon*, vol. 60, no. 1, pp. 108–121, 2011, doi: 10.1002/tax.601010.
[109] J. Batovska, N. O. I. Cogan, S. E. Lynch, and M. J. Blacket, “Using next-generation sequencing for DNA barcoding: Capturing allelic variation in ITS2,” *G3: Genes, Genomes, Genetics*, vol. 7, no. 1, pp. 19–29, 2017, doi: 10.1534/g3.116.036145.
[110] R. Matyášek *et al.*, “Next generation sequencing analysis reveals a relationship between rDNA unit diversity and locus number in *Nicotiana* diploids,” *BMC Genomics*, vol. 13, no. 1, 2012, doi: 10.1186/1471-2164-13-722.
[111] A. Delgado-Tejedor, P. Leekitcharoenphon, F. M. Aarestrup, and S. Otani, “Evaluating the usefulness of next-generation sequencing for herb authentication,” *Food Chem*, vol. 3, p. 100044, 2021, doi: 10.1016/j.fochms.2021.100044.
[112] S. Chen *et al.*, “Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species,” *PLoS One*, vol. 5, no. 1, pp. 1–8, 2010, doi: 10.1371/journal.pone.0008613.
[113] O. S. Balogun, O. S. Ajayi, and A. J. Adeleke, “Hexahydrofarnesyl acetone-rich extractives from *Hildegardia barteri*,” *J Herbs Spices Med Plants*, vol. 23, no. 4, pp. 393–400, 2017, doi: 10.1080/10496475.2017.1350614.
[114] R. O. Silva *et al.*, “Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress,” *Fundam Clin Pharmacol*, vol. 28, no. 4, pp. 455–464, 2014, doi: 10.1111/fcp.12049.
[115] D. Saikia *et al.*, “Antitubercular potential of some semisynthetic analogues of phytol,” *Bioorg Med Chem Lett*, vol. 20, no. 2, pp. 508–512, 2010, doi: 10.1016/j.bmcl.2009.11.107.

[116] J. P. Costa, P. B. Ferreira, D. P. De Sousa, J. Jordan, and R. M. Freitas, "Anticonvulsant effect of phytol in a pilocarpine model in mice," *Neurosci Lett*, vol. 523, no. 2, pp. 115–118, 2012, doi: 10.1016/j.neulet.2012.06.055.
[117] U. Pongprayoon, P. Baeckström, U. Jacobsson, M. Lindström, and L. Bohlin, "Antispasmodic activity of *β*-Damascenone and *E*-Phytol isolated from *Ipomoea pes-caprae*," *Planta Med*, vol. 58, no. 1, pp. 19–21, 1992.
[118] B. Pejin, V. Kojic, and G. Bogdanovic, "An insight into the cytotoxic activity of phytol at *in vitro* conditions," *Nat Prod Res*, vol. 28, no. 22. pp. 2053–2056, 2014. doi: 10.1080/14786419.2014.921686.
[119] M. Nazlić *et al.*, "Endemic *Veronica saturejoides* Vis. ssp. *saturejoides*–Chemical composition and antioxidant activity of free volatile compounds," *Plants*, vol. 9, p. 1646, 2020.
[120] K. H. Tan and R. Nishida, "Methyl eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination," *J Insect Sci*, vol. 12, no. 56, 2012, doi: 10.21273/hortsci.43.1.51.
[121] M. Popović *et al.*, "Essential oil volatile fingerprint differentiates Croatian cv. Oblica from other *Olea europaea* L. cultivars," *Molecules*, vol. 26, no. 12, p. 3533, 2021, doi: 10.3390/molecules26123533.
[122] J. D. Craft, P. Satyal, and W. N. Setzer, "The chemotaxonomy of common sage (*Salvia officinalis*) based on the volatile constituents," *Medicines*, vol. 4, no. 47, p. 12, 2017, doi: 10.3390/medicines4030047.
[123] Y. Wang *et al.*, "GC-MS analysis of the volatile constituents in the leaves of 14 Compositae plants," *Molecules*, vol. 23, no. 1, p. 166, Jan. 2018, doi: 10.3390/molecules23010166.
[124] C. Giuliani *et al.*, "A volatolomic approach for studying plant variability: the case of selected *Helichrysum species* (Asteraceae)," *Phytochemistry*, vol. 130, pp. 128–143, 2016, doi: 10.1016/j.phytochem.2016.07.013.
[125] B. Niederbacher, J. B. Winkler, and J. P. Schnitzler, "Volatile organic compounds as non-invasive markers for plant phenotyping," *J Exp Bot*, vol. 66, no. 18, pp. 5403–5416, 2015, doi: 10.1093/jxb/erv219.
[126] C. Wu, F. Wang, J. Liu, Y. Zou, and X. Chen, "A comparison of volatile fractions obtained from *Lonicera macranthoides* via different extraction processes: ultrasound, microwave, Soxhlet extraction, hydrodistillation, and cold maceration," *Integr Med Res*, vol. 4, no. 3, pp. 171–177, 2015, doi: 10.1016/j.imr.2015.06.001.
[127] S. Quideau, D. Deffieux, C. Douat-Casassus, and L. Pouységu, "Plant polyphenols: Chemical properties, biological activities, and synthesis," *Angewandte Chemie - International Edition*, vol. 50, no. 3, pp. 586–621, 2011, doi: 10.1002/anie.201000044.
[128] K. B. Pandey and S. I. Rizvi, "Plant polyphenols as dietary antioxidants in human health and disease," *Oxid Med Cell Longev*, vol. 2, no. 5, pp. 270–278, 2009, doi: 10.4161/oxim.2.5.9498.
[129] W. W. Payne, "A glossary of plant hair thermonology," *Brittonia*, vol. 30, no. 2, pp. 239–255, 1978.
[130] E. Hanlidou, S. Kokkini, A. M. Bosabalidis, and J. M. Bessière, "Glandular trichomes and essential oil constituents of *Calamintha menthifolia* (Lamiaceae)," *Plant Systematics and Evolution*, vol. 177, no. 1–2, pp. 17–26, 1991, doi: 10.1007/BF00937823.
[131] P. Baran, K. Aktaş, and C. Özdemir, "Structural investigation of the glandular trichomes of endemic *Salvia smyrnea* L.," *S Afr J Bot*, vol. 76, no. 3, pp. 572–578, 2010, doi: 10.1016/j.sajb.2010.04.011.

[132] E. Werker, U. Ravid, and E. Putievsky, “Structure of glandular hairs and identification of the main components of their secreted material in some species of the Labiatae,” *Isr J Bot*, vol. 34, no. 1, pp. 31–45, 1985, doi: 10.1080/0021213X.1985.10677007.
[133] C.-L. Xiang, Z.-H. Dong, H. Peng, and Z.-W. Liu, “Trichome micromorphology of the East Asiatic genus *Chelonopsis* (Lamiaceae) and its systematic implications,” *Flora*, vol. 205, no. 7, pp. 434–441, Jan. 2010, doi: 10.1016/j.flora.2009.12.007.
[134] Z. Seyedi and Y. Salmaki, “Trichome morphology and its significance in the systematics of *Phlomoides* (Lamiaceae; Lamioideae; Phlomideae),” *Flora*, vol. 213, pp. 40–48, 2015, doi: 10.1016/j.flora.2015.04.003.
[135] D. Kremer, D. Vitali Čepo, V. Dunkić, I. Dragojević Müller, Ivna Kosalec, N. Bezić, and E. Stabentheiner, “Phytochemical and micromorphological traits of *Geranium dalmaticum* and *G. macrorrhizum* (Geraniaceae),” *Nat Prod Commun*, vol. 8, no. 5, pp. 645–650, 2013, doi: 10.1177/1934578X1300800526.
[136] S. Aazza, B. Lyoussi, and M. Miguel, “Antioxidant activity of some Morrocan hydrosols,” *J Med Plant Res*, vol. 5, no. 30, 2011, doi: 10.5897/jmpr11.1176.
[137] K. Bentayeb, P. Vera, C. Rubio, and C. Nerín, “The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils,” *Food Chem*, vol. 148, pp. 204–208, 2014, doi: 10.1016/j.foodchem.2013.10.037.
[138] M. Viuda-Martos, Y. Ruiz Navajas, E. Sanchez Zapata, J. Fernandez-Lopez, and J. A. Perez-Alvarez, “Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet,” *Flavour Fragr J*, vol. 25, pp. 13–19, 2010, doi: 10.1002/ffj.1951.
[139] U. Ş. Harput, Y. Genç, N. Khan, and I. Saracoglu, “Radical scavenging effects of different *Veronica* species,” *Rec Nat Prod*, vol. 5, no. 2, pp. 100–107, 2011.
[140] J. Živković, T. Ćebović, and Z. Maksimović, “In vivo and in vitro antioxidant effects of three *Veronica* species,” *Cent Eur J Biol*, vol. 7, no. 3, pp. 559–568, 2012, doi: 10.2478/s11535-012-0041-4.
[141] J. Sharifi-Rad *et al.*, “In vitro and in vivo assessment of free radical scavenging and antioxidant activities of *Veronica persica* Poir,” *Cell Mol Biol*, vol. 64, no. 8, pp. 57–64, 2018.
[142] A. Assaeed, A. Elshamy, A. E.-N. El Gendy, B. Dar, S. Al-Rowaily, and A. Abd-ElGawad, “Sesquiterpenes-rich essential oil from above ground parts of *Pulicaria somalensis* exhibited antioxidant activity and allelopathic effect on weeds,” *Agronomy*, vol. 10, no. 3, p. 399, 2020, doi: 10.3390/agronomy10030399.
[143] J. Chappell and R. M. Coates, “Sesquiterpenes,” in *Comprehensive Natural Products II: Chemistry and Biology*, H.-W. (Ben) Liu and L. Mander, Eds., 1st ed.Elsevier Science: Elsevier, 2010, pp. 609–641. doi: 10.1016/b978-008045382-8.00005-8.
[144] Z. Huang *et al.*, “Synergistic effects of cinnamaldehyde and cinnamic acid in cinnamon essential oil against *S. pullorum*,” *Ind Crops Prod*, vol. 162, p. 113296, 2021, doi: 10.1016/j.indcrop.2021.113296.
[145] N. Kaur, K. K. Chahal, A. Kumar, R. Singh, and U. Bhardwaj, “Antioxidant activity of *Anethum graveolens* L. essential oil constituents and their chemical analogues,” *J Food Biochem*, vol. 43, no. 4, pp. 1–11, 2019, doi: 10.1111/jfbc.12782.
[146] V. Dunkić, N. Bezić, E. Vuko, and D. Cukrov, “Antiphytoviral activity of *Satureja montana* L. ssp. *variegata* (host) P. W. Ball essential oil and phenol compounds on CMV and TMV,” *Molecules*, vol. 15, no. 10, pp. 6713–6721, 2010, doi: 10.3390/molecules15106713.
[147] E. Vuko, V. Dunkić, N. Bezić, M. Ruščić, and D. Kremer, “Chemical composition and antiphytoviral activity of essential oil of *Micromeria graeca*,” *Nat Prod Commun*, vol. 7, no. 9, pp. 1227–30, 2012.

[148] E. Vuko *et al.*, "Inhibition of aatellite RNA associated Cucumber Mosaic Virus infection by essential oil of *Micromeria croatica* (Pers.) Schott," *Molecules*, vol. 24, no. 7, p. 1342, 2019, doi: 10.3390/molecules24071342.
[149] M. Ruščić, E. Vuko, N. Bezić, D. Kremer, and V. Dunkić, "Croatian *Micromeria fruticulosa*-essential oil composition and antiphytoviral activity," *Nat Prod Commun*, vol. 12, no. 7, pp. 1133–1135, 2017, doi: 10.1177/1934578x1701200733.
[150] N. Bezić, E. Vuko, V. Dunkić, M. Ruščić, I. Blažević, and F. Burčul, "Antiphytoviral activity of sesquiterpene-rich essential oils from four Croatian *Teucrium* species," *Molecules*, vol. 16, no. 9, pp. 8119–8129, 2011, doi: 10.3390/molecules16098119.
[151] V. Dunkić, E. Vuko, N. Bezić, D. Kremer, and M. Ruščić, "Composition and antiviral activity of the essential oils of *Eryngium alpinum* and *E. amethystinum*," *Chem Biodivers*, vol. 10, no. 10, pp. 1894–1902, 2013, doi: 10.1002/cbdv.201300061.
[152] E. Vuko *et al.*, "Chemical composition and new biological activities of essential oil and hydrosol of *Hypericum perforatum* L. ssp. *veronense* (Schrank) H. Lindb," *Plants*, vol. 10, no. 5, p. 1014, May 2021, doi: 10.3390/plants10051014.
[153] M. Lu, Z. Han, Y. Xu, and L. Yao, "*In vitro* and *in vivo* anti-Tobacco Mosaic Virus activities of essential oils and individual compounds," *J Microbiol Biotechnol*, vol. 23, no. 6, pp. 771–778, 2013, doi: 10.4014/jmb.1210.10078.
[154] C. D. Bishop, "Antiviral activity of the essential oil of *Melaleuca alternifolia* (Maiden amp; Betche) cheel (tea tree) against Tobacco Mosaic Virus," *J Essent Oil Res*, vol. 7, no. 6, pp. 641–644, 1995, doi: 10.1080/10412905.1995.9700519.
[155] M. An, T. Zhou, Y. Guo, X. Zhao, and Y. Wu, "Molecular regulation of host defense responses mediated by biological anti-TMV agent ningnanmycin," *Viruses*, vol. 11, no. 9, 2019, doi: 10.3390/v11090815.
[156] Y. Han, Y. Luo, S. Qin, L. Xi, B. Wan, and L. Du, "Induction of systemic resistance against tobacco mosaic virus by Ningnanmycin in tobacco," *Pestic Biochem Physiol*, vol. 111, no. 1, pp. 14–18, 2014, doi: 10.1016/j.pestbp.2014.04.008.
[157] H. Fan, B. Song, P. S. Bhadury, L. Jin, D. Hu, and S. Yang, "Antiviral activity and mechanism of action of novel thiourea containing chiral phosphonate on tobacco mosaic virus," *Int J Mol Sci*, vol. 12, no. 7, pp. 4522–4535, 2011, doi: 10.3390/ijms12074522.
[158] D. Gao, D. Wang, K. Chen, M. Huang, X. Xie, and X. Li, "Activation of biochemical factors in CMV-infected tobacco by ningnanmycin," *Pestic Biochem Physiol*, vol. 156, no. October 2018, pp. 116–122, 2019, doi: 10.1016/j.pestbp.2019.02.012.
[159] K. Feng, R. Jiang, and L. W. Sun, "Studies on the anti-tumor activity in vitro of the flavonoid extract in round leaf speedwell," in *Proceeding of 2010 First International Conference on Cellular, Molecular Biology, Biophysics and Bioengineering*, 2010.
[160] M. Mohadjerani and S. Asadollahi, "*Veronica crista-galli* Steven and *Veronica persica* Poir. as anticancer and antioxidant plants *in-vitro*," *Trends Phytochem. Res*, vol. 3, no. 1, pp. 61–66, 2019.
[161] L. Tomassini, D. Brkic, M. Serafini, and M. Nicoletti, "Constituents of *Veronica hederifolia* and *Veronica polita*," *Fitoterapia*, vol. 66, p. 382, 1995.
[162] B. N. Su, Q. X. Zhu, and Z. J. Jia, "Aquaticol, a novel bis-sesquiterpene from *Veronica anagallis-aquatica*," *Tetrahedron Lett*, vol. 40, no. 2, pp. 357–358, 1999, doi: 10.1016/S0040-4039(98)02303-X.
[163] J. G. Graham, M. L. Quinn, D. S. Fabricant, and N. R. Farnsworth, "Plants used against cancer - An extension of the work of Jonathan Hartwell," *J Ethnopharmacol*, vol. 73, no. 3, pp. 347–377, 2000, doi: 10.1016/S0378-8741(00)00341-X.
[164] V. Mohan Chari, R. J. Grayer-Barkmeijer, J. B. Harborne, and B.-G. Osterdahl, "An acylated allose-containing 8-hydroxyflavone glycoside from *Veronica filiformis*,"

Phytochemistry, vol. 20, no. 8, pp. 1977–1979, 1981, doi: 10.1016/0031-9422(81)84048-4.
[165] R. Taskova, N. Handjieva, D. Peev, and S. Popov, "Iridoid glucosides from three *Veronica* species," *Phytochemistry*, vol. 49, no. 5, pp. 1323–1327, 1998, doi: 10.1016/S0031-9422(98)00090-9.
[166] I. Saracoglu, U. S. Harput, M. Inoue, and Y. Ogihara, "New phenylethanoid glycosides from *Veronica pectinata* var. *glandulosa* and their free radical scavenging activities," *Chem Pharm Bull (Tokyo)*, vol. 50, no. 5, pp. 665–668, 2002, doi: 10.1248/cpb.50.665.
[167] I. Saracoğlu, U. Ş. Harput-Hudaverdi, A. Nagatsu, and M. Varel, "Cytotoxic Activity and Structural Aspects of Iridoids Isolated from *Veronica thymoides* subsp. *pseudocinerea*," in *Natural Products with Pharmaceutical, Nutraceutical, Cosmetic, and Agrochemical Interest, 7th Joint Meeting of AFERP, ASP, GA, and PSE (49th Annual Meeting of the American Society of Pharmacognosy)*, Athens: Planta Med, 2008, p. 1046.
[168] I. Saracoğlu and U. Ş. Harput-Hudaverdi, "*In vitro* anticancer activity and structure-activity relationships of iridoid glucosides," in *9th International Symposium on Pharmaceutical Sciences*, Ankara, 2009.
[169] M. Doostmohammadi, F. Bordbar, D. C. Albach, and M. Mirtadzadini, "Phylogeny and historical biogeography of *Veronica* Subgenus *Pentasepalae* (Plantaginaceae): Evidence for its origin and subsequent dispersal," *Biology (Basel)*, vol. 11, no. 5, 2022, doi: 10.3390/biology11050639.
[170] S. Hammami, H. Debbabi, I. Jlassi, R. K. Joshi, and R. El Mokni, "Chemical composition and antimicrobial activity of essential oil from the aerial parts of *Plantago afra* L. (Plantaginaceae) growing wild in Tunisia," *S Afr J Bot*, vol. 132, pp. 410–414, 2020, doi: 10.1016/j.sajb.2020.05.012.
[171] S. A. Al-Mazroa, L. H. Al-Wahaibi, A. A. Mousa, and H. Z. Al-Khathlan, "Essential oil of some seasonal flowering plants grown in Saudi Arabia," *Arab J Chem*, vol. 8, no. 2, pp. 212–217, 2015, doi: 10.1016/j.arabjc.2011.06.014.
[172] E. J. S. P. de Lima *et al.*, "Essential oil from leaves of *Conobea scoparioides* (Cham. & Schltdl.) Benth. (Plantaginaceae) causes cell death in HepG2 cells and inhibits tumor development in a xenograft model," *Biomed Pharmacother*, vol. 129, no. March, p. 110402, 2020, doi: 10.1016/j.biopha.2020.110402.
[173] C. M. Brandão *et al.*, "Composition and larvicidal activity of the oil of *Dizygostemon riparius* (Plantaginaceae), a new aromatic species occurring in Maranhão, Brazil," *Chem Biodivers*, vol. 17, no. 11, 2020, doi: 10.1002/cbdv.202000462.
[174] T. Bajer, V. Janda, P. Bajerová, D. Kremr, A. Eisner, and K. Ventura, "Chemical composition of essential oils from *Plantago lanceolata* L. leaves extracted by hydrodistillation," *J Food Sci Technol*, vol. 53, no. 3, pp. 1576–1584, 2016, doi: 10.1007/s13197-015-2083-x.
[175] S. Rahamouz Haghighi, A. Yazdinezhad, K. Bagheri, and A. Sharafi, "Volatile constituents and toxicity of essential oils extracted from aerial parts of *Plantago Lanceolata* and *Plantago Major* growing in Iran," *PBR*, vol. 8, no. 3, pp. 205–224, Oct. 2022, doi: 10.18502/pbr.v8i3.11035.
[176] F. Fons, S. Rapior, A. Gargadennec, C. Andary, and J. M. Bessière, "Volatile components of *Plantago lanceolata* (Plantaginaceae)," *Acta Bot Gall*, vol. 145, no. 4, pp. 265–269, 1998, doi: 10.1080/12538078.1998.10516306.
[177] S. J. Roudbaraki and D. Nori-Shargh, "The volatile constituent analysis of *Digitalis nervosa* Steud. & Hochst. ex Benth. from Iran," *Russ Chem Bull*, vol. 65, no. 4, pp. 1148–1150, 2016, doi: 10.1007/s11172-016-1428-6.
[178] H. Seifi, S. Masoum, S. Seifi, and E. H. Ebrahimabadi, "Chemometric resolution approaches in characterisation of volatile constituents in *Plantago ovata* seeds using gas

chromatography-mass spectrometry: Methodology and performance assessment," *Phytochemical Analysis*, vol. 25, no. 3, pp. 273–281, 2014, doi: 10.1002/pca.2503.
[179] R. M. Taskova, T. Kokubun, K. G. Ryan, P. J. Garnock-Jones, and S. R. Jensen, "Phenylethanoid and iridoid glycosides in the New Zealand snow hebes (*Veronica*, Plantaginaceae)," *Chem Pharm Bull (Tokyo)*, vol. 58, no. 5, pp. 703–11, 2010, doi: 10.1248/cpb.58.703.
[180] M. Kimura, "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences," *J Mol Evol*, vol. 16, no. 2, pp. 111–120, 1980, doi: 10.1007/BF01731581.
[181] M. Nazlić *et al.*, "Wild Species *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides*—Biological potential of free volatiles," *Horticulturae*, vol. 7, p. 295, 2021.
[182] M. Nazlić *et al.*, "Free volatile compounds of *Veronica austriaca* ssp. *jacquinii* (Baumg.) Eb. Fisch. and their biological activity," *Plants*, vol. 10, no. 11, p. 2529, 2021, doi: 10.3390/plants10112529.
[183] U. S. Harput, I. Saracoglu, A. Nagatsu, and Y. Ogihara, "Iridoid glucosides from *Veronica hederifolia*.," *Chem Pharm Bull*, vol. 50, no. 8, pp. 1106–1108, 2002, doi: 10.1248/cpb.50.1106.
[184] R. P. Adams, *Identification of essential oil components by gas chromatography/mass spectrometry*, 4th ed. Allured Publishing Co, Carol Stream, 2007.
[185] "NIST Chemistry WebBook." https://webbook.nist.gov/ (accessed Mar. 12, 2021).
[186] S. Ulusoy, G. Boşgelmez-TInaz, and H. Seçilmiş-Canbay, "Tocopherol, carotene, phenolic contents and antibacterial properties of rose essential oil, hydrosol and absolute," *Curr Microbiol*, vol. 59, no. 5, pp. 554–558, 2009, doi: 10.1007/s00284-009-9475-y.
[187] P. Vlachou, N. Stathopoulou, K Georgousaki, K Lemonakis, N. Aligiannis, A. Skaltsounis, and N. Fokialakis, "Exploitation of agricultural by-products for the recovery of bioactive compounds with applications in cosmetic industry," in *Planta Medica*, 2015, p. 81. doi: DOI: 10.1055/s-0035-1565771.
[188] National Center for Biotechnology Information PubChem, "Compound Summary for CID 6428442, trans-1(7),8-p-Menthadien-2-ol," 2021. https://pubchem.ncbi.nlm.nih.gov/compound/trans-1_7_8-p-Menthadien-2-ol. (accessed Jul. 30, 2021).
[189] V. I. Babushok, P. J. Linstrom, and I. G. Zenkevich, "Retention indices for frequently reported compounds of plant essential oils," *J Phys Chem Ref Data*, vol. 40, no. 4, 2011, doi: 10.1063/1.3653552.
[190] D. Kremer *et al.*, "Chemical traits and antimicrobial activity of endemic *Teucrium arduini* L. from Mt Biokovo (Croatia)," *Cent Eur J Biol*, vol. 7, no. 5, pp. 941–947, 2012, doi: 10.2478/s11535-012-0077-5.
[191] M. Jukić Špika *et al.*, "Differentiation between unfiltered and filtered Oblica and Leccino cv. virgin olive oils," *J Food Sci*, vol. 84, no. 4, pp. 877–885, 2019, doi: doi.org/10.1111/1750-3841.14494.
[192] R. J. Grubešić, J. Vuković, D. Kremer, and S. Vladimir-Knežević, "Spectrophotometric method for polyphenols analysis: Prevalidation and application on *Plantago* L. species," *J Pharm Biomed Anal*, vol. 39, no. 3–4, pp. 837–842, 2005, doi: 10.1016/j.jpba.2005.05.004.
[193] J. V. Rodríguez, R. J. Grubešić, D. Kremer, and V. Kokot, "Quality assessment of two spectrophotometric procedures for polyphenol determination and application in *Moltkia petraea* species," *JCCS*, vol. 63, no. 8, pp. 677–687, 2016, doi: 10.1002/jccs.201600024.

[194] Council of Europe, *European Pharmacopoeia*, 4th Ed. Strasbourg: European directorate for the quality of medicines and health care (EDQM), 2014.
[195] Ž. Fredotović *et al.*, "Phytochemical characterization and screening of antioxidant, antimicrobial and antiproliferative properties of *Allium* × *cornutum clementi* and two varieties of *Allium cepa* l. peel extracts," *Plants*, vol. 10, no. 5, pp. 1–15, 2021, doi: 10.3390/plants10050832.
[196] L. L. Mensor *et al.*, "Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method," *Phytother Res*, vol. 15, no. 2, pp. 127–130, 2001, doi: 10.1002/ptr.687.
[197] B. Payet, A. S. C. Sing, and J. Smadja, "Assessment of antioxidant activity of cane brown sugars by ABTS and DPPH radical scavenging assays: Determination of their polyphenolic and volatile constituents," *J Agric Food Chem*, vol. 53, no. 26, pp. 10074–10079, 2005, doi: 10.1021/jf0517703.
[198] N. S. Schneider, C. R. Desper, and R. E. Singler, "The thermal transition behavior of polyorganophosphazenes," *J Appl Polym Sci*, vol. 20, pp. 3087–3103, 1976, doi: 10.1002/app.1976.070201114.
[199] B. Christ and K. H. Müller, "Zur serienmaSigen bestimmung des gehaltes an flavonol-derivaten in drogen," *Arch Pharm (Weinheim)*, vol. 293./65., no. 12, pp. 1033–1042, 1960.
[200] G.-C. Yen and P.-D. Duh, "Scavenging effect of methanolic extracts of peanuts hulls on free-radical and active-oxygen species," *J Agric Food Chem*, vol. 42, pp. 629–632, 1994, doi: 10.1021/jf00039a005.
[201] R. J. Grayer-Barkmeijer, "A chemosystematic study of *Veronica*: Iridoid glucosides," *Biochem Syst Ecol*, vol. 1, no. 2, pp. 101–110, 1973, doi: 10.1016/0305-1978(73)90023-9.
[202] R. J. Grayer, M. W. Chase, and M. S. J. Simmonds, "A comparison between chemical and molecular characters for the determination of phylogenetic relationships among plant families: An appreciation of Hegnauer's 'Chemotaxonomie der Pflanzen,'" *Biochem Syst Ecol*, vol. 27, no. 4, pp. 369–393, 1999, doi: 10.1016/S0305-1978(98)00096-9.
[203] N. Bezić, I. Šamanić, V. Dunkić, V. Besendorfer, and J. Puizina, "Essential oil composition and internal transcribed spacer (ITS) sequence variability of four South-Croatian *Satureja* species (Lamiaceae)," *Molecules*, vol. 14, no. 3, pp. 925–938, 2009, doi: 10.3390/molecules14030925.
[204] T. J. White, T. Bruns, S. Lee, and J. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds., New York: Academic Press Inc., 1990, pp. 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1.
[205] K. Tamura, G. Stecher, and S. Kumar, "MEGA11: Molecular evolutionary genetics analysis Version 11," *Mol Biol Evol*, vol. 38, no. 7, pp. 3022–3027, 2021, doi: 10.1093/molbev/msab120.
[206] D. C. Albach and M. W. Chase, "Incongruence in Veroniceae (Plantaginaceae): Evidence from two plastid and a nuclear ribosomal DNA region," *Mol Phylogenet Evol*, vol. 32, no. 1, pp. 183–197, 2004, doi: 10.1016/j.ympev.2003.12.001.
[207] L. M. Muñoz-Centeno, D. C. Albach, J. A. Sánchez-Agudo, and M. M. Martínez-Ortega, "Systematic significance of seed morphology in *Veronica* (Plantaginaceae): A phylogenetic perspective," *Ann Bot*, vol. 98, no. 2, pp. 335–350, 2006, doi: 10.1093/aob/mcl120.

[208] S. J. Wagstaff and P. J. Garnock-Jones, “Evolution and biogeography of the hebe complex (scrophulariaceae) inferred from ITS sequences,” *N Z J Bot*, vol. 36, no. 3, pp. 425–437, 1998, doi: 10.1080/0028825X.1998.9512581.
[209] B. Surina, S. Pfanzelt, H. J. R. Einzmann, and D. C. Albach, “Bridging the Alps and the Middle East: Evolution, phylogeny and systematics of the genus *Wulfenia* (Plantaginaceae),” *Taxon*, vol. 63, no. 4, pp. 843–858, 2014, doi: 10.12705/634.18.
[210] S. Andrews, “FastQC: A quality control tool for high throughput sequence data.” 2018.
[211] M. A. Bogdan *et al.*, “Chemical profile, antioxidant capacity, and antimicrobial activity of essential oils extracted from three different varieties (Moldoveanca 4, Vis Magic 10, and Alba 7) of *Lavandula angustifolia*,” *Molecules*, vol. 26, no. 14, p. 4381, 2021, doi: 10.3390/molecules26144381.
[212] R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight, “UCHIME improves sensitivity and speed of chimera detection,” *Bioinformatics*, vol. 27, no. 16, pp. 2194–2200, 2011, doi: 10.1093/bioinformatics/btr381.
[213] K. Abarenkov *et al.*, “UNITE general FASTA release for eukaryotes 2.” UNITE Community.
[214] E. Kopylova, L. Noé, and H. Touzet, “SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data,” *Bioinformatics*, vol. 28, no. 24, pp. 3211–3217, 2012, doi: 10.1093/bioinformatics/bts611.
[215] B. Chevreux *et al.*, “Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs,” *Genome Res*, vol. 14, no. 6, pp. 1147–1159, 2004, doi: 10.1101/gr.1917404.
[216] M. J. Lim Ah Tock, G. P. P. Kamatou, S. Combrinck, M. Sandasi, and A. M. Viljoen, “A chemometric assessment of essential oil variation of three *Salvia* species indigenous to South Africa,” *Phytochemistry*, vol. 172, p. 112249, 2020, doi: https://doi.org/10.1016/j.phytochem.2019.112249.
[217] J. S. Nikolić, B. K. Zlatković, S. Č. Jovanović, G. S. Stojanović, P. D. Marin, and Z. S. Mitić, “Needle volatiles as chemophenetic markers in differentiation of natural populations of *Abies alba*, *A. x borisii-regis*, and *A. cephalonica*,” *Phytochemistry*, vol. 183, p. 112612, 2021, doi: 10.1016/j.phytochem.2020.112612.
[218] J. Oksanen *et al.*, “vegan: Community Ecology Package. R package.” 2022.
[219] Y. J. Ramos *et al.*, “Advanced chemophenetic analysis of essential oil from leaves of *Piper gaudichaudianum* Kunth (Piperaceae) using a new reduction-oxidation index to explore seasonal and circadian rhythms,” *Plants*, vol. 10, no. 10, p. 2116, 2021, doi: 10.3390/plants10102116.

Ostala literatura

Ostala literatura koja je navedena samo u radovima (reference vezane za metode rada) je sljedeća:

[181]–[219]

Životopis autorice

Marija Nazlić rođena je u Splitu. Diplomirala je 2010. godine na Prirodoslovno-matematičkom fakultetu u Splitu s temom rada: "Mikroskopska građa probavnog sustava škrpuna *Scorpaena porcus* L.". Od 2017. godine zaposlena je na Prirodoslovno – matematičkom fakultetu u Splitu kao asistentica na Odjelu za Biologiju kada je upisala i poslijediplomski studij Biologija na Prirodoslovno-matematičkom fakultetu u Zagrebu.

Izvodi vježbe iz praktikuma obaveznih kolegija Fiziologija bilja i Opća botanika, te izbornog kolegija Izolacija i primjena eteričnih ulja, i seminare izbornih predmeta Začinsko i aromatsko bilje i Osnove mediteranske prehrane na Preddiplomskom studiju Biologija i kemija i Biologija na Prirodoslovno-matematičkom fakultetu u Splitu. Na integriranom studiju Farmacije na Medicinskom fakultetu u Splitu izvodi vježbe u praktikumu iz kolegija Farmaceutska botanika.

U koautorstvu i autorstvu objavila je 19 znanstvenih radova u časopisima citiranim u bazi Web of Science, 1 poglavlje u knjizi te je sudjelovala na 21 znanstvenom skupu. Bila je neposredna voditeljica na 14 završnih i diplomskih radova.

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Za vrijeme studija bila je dobitnica Dekanove nagrade za izvrstan uspjeh na Preddiplomskom sveučilišnom studiju Biologije i kemije, te Diplomskom sveučilišnom studiju Biologije i kemije. Dobitnica je Nagrade Prirodoslovno-matematičkog fakulteta u Splitu za znanost za 2021. i 2022. godinu u kategoriji Mladi znanstvenici te godišnje Nagrade za znanost Sveučilišta u Splitu za 2022. godinu, također u kategoriji Mladi znanstvenici.

Proširenisažetak

Rod *Veronica* (čestoslavice) je najveći rod porodice Plantaginaceae, bogat biološki aktivnim specijaliziranim metabolitima. Istraživanje u okviru ove doktorske disertacije obuhvaća odabrane vrste roda *Veronica* prikupljene na prirodnim staništima u Republici Hrvatskoj – endemičnu vrstu *V. saturejoides* ssp. *saturejoides* – vriskova čestoslavica, *V. austriaca* ssp. *jacquinii* – tankolisna čestoslavica i *V. officinalis* – ljekovita čestoslavica. Čestoslavice su kozmopolitske biljke rasprostranjene na mnogo različitih staništa, uključujući planine, travnjake, obrađene površine, površine uz vodu, prilagođavajući se na različite uvjete staništa. Ove biljke poznate su od davnina po svojim blagotvornim djelovanjima te se stoga koriste u narodnoj medicini te su privlačne za istraživanje bioloških aktivnosti koje pokazuju njihovi ekstrakti. Cilj ove doktorske disertacije prvenstveno je bio istražiti sastav slobodnih hlapljivih spojeva (FVC) vrsta *V. saturejoides* ssp. *saturejoides*, *V. officinalis* i *V. austriaca* ssp. *jacquinii* i njihovu biološku aktivnost. Vrsta *V. officinalis* L. je najčešće korištena u narodnoj medicini i time čini dobar materijal za usporedbu biološke aktivnosti. Sastav FVC-a analiziran je plinskom kromatografijom-masenom spektrometrijom za obje vrste ekstrakata, eterična ulja i hidrosole. Rezultati pokazuju da su u eteričnim uljima više zastupljene nepolarnije komponente kao što su seskviterpeni i ugljikovodici, a u hidrosolima prevladavaju polarnije komponente kao što su fenoli, fenilpropanoidi i oksigenirani monoterpeni. Prevladavajući spojevi za eterična ulja su heksahidrofarnezil aceton i heksadekanska kiselina te nakon njih kariofilen oksid i *(E)*-kariofilen. U hidrosolima glavne identificirane komponente su: metil eugenol, timol, *trans-p*-menta-1(7),8-dien-2-ol i benzaldehid. Ekstrakti vrsta *V. saturejoides* i *V. officinalis* pokazale su jače antioksidativno i antiproliferativno djelovanje u većini testova od ekstrakata vrste *V. jacquinii*. Iako, hidrosoli *V. jacquinii* su pokazali jače antioksidativno djelovanje od hidrosola ostalih dviju vrste, što bi moglo biti zbog visokog sadržaja metil eugenola i timola. Mikromorfološkim istraživanjem u sve tri vrste identificirani su žljezdani trihomi u kojima se primarno događa sinteza istraživanih spojeva. U sve tri istražene vrste žljezdani trihomi se sastoje od jedne stanice koja čini stapku i dvije stanice koje čine glavu žlijezde. Kod vrste *V. officinalis* identificiran je i drugi tip glavičastih trihoma sa dužom stapkom te štitasti trihomi. U sve tri istraživane vrste na površini listova, stabljike i čaške uočeni su i nežljezdani trihomi koji su nerazgranati i jednostruki, duži i kraći. Uz analiziranje sastava FVC-a, cilj ove doktorske disertacije bio je i genetički analizirati ITS (*internal transcribed spacer*) regije i usporediti ih s klasterima dobivenim temeljem analize FVC-a 18 vrsta čestoslavica. Osim klasične Sanger metode sekvenciranja ITS regija, napravljena je i NGS

analiza ovih sekvenci. Bolji rezultati filogenetske analize i klasteriranja vrsta *Veronica* u 8 podrodova dobiveni su na temelju ITS1-5.8S-ITS2 sekvenci nego s podacima temeljenim na ITS2 sekvencama. To je vjerojatno zbog činjenice da su dulje sekvence generirane Sangerovom metodom sekvenciranja (~580 bp) dale više varijabilnih i informativnih pozicija od kraćih NGS sekvenci (~360 bp). Međutim, veliki broj ITS2 sekvenci dobivenih NGS tehnologijom, zajedno s ITS1 sekvencama također sekvenciranim NGS-om za 18 vrsta *Veronica* koristit će se za detaljnu analizu raznolikosti kontiga/ribotipova unutar svake vrste i njihove molekularne evolucije i strukture genoma (ove analize su trenutno u tijeku). Mantelov test za usporedbu između euklidskih udaljenosti hlapljivih komponenata i Kimura genetskih udaljenosti od podataka sekvenci DNA pokazao je da nema korelacije između grupa kemijskih i genetskih podataka. Ali, pregledom FVC-a unutar drugih vrsta iste porodice donesen je zaključak da se komponente koje se pojavljuju u svim vrstama roda *Veronica,* u takvom omjeru ne pojavljuju u drugim vrstama porodice Plantaginaceae te mogu biti kemofenetski markeri za rod *Veronica*. Svi ovi rezultati pokazuju da su čestoslavice vrijedan izvor biološki aktivnih spojeva. Daljnja bi se istraživanja mogla usmjeriti na *in vivo* studije različitih ekstrakata ovih raznolikih, kozmopolitskih, i često podcijenjenih vrsta.

Extended abstract

The genus *Veronica* is the largest genus of the Plantaginaceae family, rich in biologically active specialized metabolites. The research within the framework of this doctoral dissertation includes selected species of the genus *Veronica* collected in natural habitats in the Republic of Croatia – endemic *V. saturejoides* ssp. *saturejoides*, *V. austriaca* ssp. *jacquinii* – broadleaf speedwell, and *V. officinalis* – common speedwell. Speedwells are cosmopolitan plants distributed in many different habitats, including mountains, grasslands, cultivated areas, waterside areas, adapting to different habitat conditions. These plants have been known since ancient times for their beneficial effects and are therefore used in folk medicine and are attractive for research into the biological activities shown by their extracts. The goal of this doctoral dissertation was primarily to investigate the composition of free volatile compounds (FVC) of *V. saturejoides* ssp. *saturejoides*, *V. officinalis* and *V. austriaca* ssp. *jacquinii* and their biological activity. The composition of FVC was analyzed by gas chromatography-mass spectrometry for both types of extracts, essential oils, and hydrosols. The results show that more non-polar components such as sesquiterpenes and hydrocarbons are isolated in essential oils, and more polar components such as phenols, phenylpropanoids and oxygenated monoterpenes prevail in hydrosols. The predominant compounds for essential oils are hexahydrofarnesyl acetone and hexadecanoic acid, followed by caryophyllene oxide and *(E)*-caryophyllene. In hydrosols, the main identified components are methyl eugenol, thymol, *trans*-*p*-mentha-1(7),8-dien-2-ol and benzaldehyde. Extracts of the species *V. saturejoides* and *V. officinalis* showed stronger antioxidant and antiproliferative activity in most tests than extracts of the species *V. jacquinii*. Although, hydrosols of *V. jacquinii* showed stronger antioxidant activity than the other two species, which could be due to the high content of methyl eugenol and thymol. Through micromorphological research in all three species, glandular trichomes were identified, where the synthesis of the studied compounds primarily takes place. In all three researched species, glandular trichomes consist of one cell that forms the stalk and two cells that form the head of the gland. Another type of head trichomes with a longer stalk and peltate trichomes were also identified in the species *V. officinalis*. In all three studied species, non-glandular trichomes were observed on the surface of the leaves, stems, and calyx, which are unbranched and single, longer and shorter. In addition to the analyses FVC composition, the goal of this doctoral dissertation was to genetically analyze the ITS (*internal transcribed spacer*) region and compare it with the clusters obtained based on the FVC analysis of 18 species of speedwells. In addition to the classic Sanger analysis of the ITS regions, an NGS analysis of these sequences

was also performed. Better results of phylogenetic analysis and clustering of *Veronica* species into 8 subgenera were obtained based on ITS1-5.8S-ITS2 sequences than with data based on ITS2 sequences analyzed with NGS. This is probably because longer sequences generated by Sanger sequencing (~580 bp) yielded more variable and informative positions than shorter NGS sequences (~360 bp). However, the abundance of ITS2 sequences obtained by NGS technology, together with ITS1 sequences also sequenced by NGS for 18 *Veronica* species will be used for detailed analysis of contig/ribotype diversity within each species and their molecular evolution and genome structure (these analyzes are currently ongoing). A Mantel test comparing Euclidean distances of volatile components and Kimura genetic distances from DNA sequence data showed no correlation between groups of chemical and genetic data. But, by reviewing the FVCs within other species of the same family, the conclusion was reached that the components that appear in all species of *Veronica*, do not appear in such proportions in other species of the Plantaginaceae family and may be chemophenetic markers for the genus *Veronica*. All these results show that speedwells are a valuable source of biologically active compounds. Further research could be focused on *in vivo* studies of different extracts of these diverse, cosmopolitan, and often underestimated species.

POPIS KRATICA

AAPH – *2,2'-azobis(2-amidinopropane) dihydrochloride*; 2,2-azobis(2-amidopropan) dihidroklorid

ABTS – *2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)*; 2,2′-azino-bis(3-etilbenzotiazolin-6-sulfonska kiselina)

Br – Brezovac, uzorak s lokacije Brezovac

BHA – *butylated hydroxyanisole*; butilirani hidroksianizol

CAT – *catalase*; katalaza

DNA – *deoxyribonucleic acid*; deoksiribonukleinska kiselina

DPPH – *2,2-diphenyl-1-picrylhydrazyl*; 2,2-difenil-1-pikrilhidrazil

EO – *essential oil*; eterično ulje

FRAP – *ferric-reducing activity of plasma*; snage krvne plazme da reducira željezo

FVC – *free volatile compounds*; slobodne hlapljive tvari

GC-MS – *gas chromatography-mass spectrometry*; plinska kromatokrafija-masena spektrometrija

GSH – *glutathione*; glutation

GR – *glutathione reductase*; glutation reduktaza

GJ – Gornje Jelenje (lokacija uzorka)

GSt – *glutathione S-transferases*; glutation S-transferaze

Hep-2 – *human epithelial cells*; epitelne stanice

HF-6 – *human fibroblast cells*; fibroblasti

HSV1/HSV2 – *Herpes simplex virus*; virus Herpex siplex

HY – *hydrosols*; hidrosoli

IC50 – *half-maximal inhibitory concentration*; koncentracija potrebna za 50 % inhbicije, kvantitativna mjera koja pokazuje koliko je određene inhibitorne tvari (npr. lijeka ili biljnog ekstrakta) potrebno za inhibiciju, *in vitro*, određenog biološkog procesa ili biološke komponente za 50 %

ITS1/ITS2 – *internal transcribed spacer*; unutarnji transkribirani razmaknici

LDH – *lactate dehydrogenase*; laktat dehidrogenaza

L-20B – *mouse L cells*; mišja stanična linija genetski modificirana za ekspresiju ljudskog poliovirusnog receptora

KS – *Kamešnica sample*; uzorak s planine Kamešnica

MCF-7 – *Michigan Cancer Foundation-7*; stanična linija za rak dojke

MDA-MB-231 – *epithelial, human breast cancer cell line*; epitelna stanična linija humanog raka dojke

MIC – *minimum inhibitory concentration*, minimalna koncentracije inhibije

Mr – Mrkopalj, uzorak s lokacije Mrkopalj

MTT – *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide*

NGS – *next generation sequencing*; sekvenciranje sljedeće generacije

PS – *Prenj sample*; uzorak s planine Prenj

PARP – *poly(ADP ribose) polymerase*, poli(ADP-ribozil) polimeraza

PC-3 – *prostate cancer cell line*; stanična linija raka prostate

rDNA – *ribosomal DNA*; ribosomska DNA

RNA – *ribonucleic acid*; ribonukleinska kiselina

RD – *rhabdomyosarcoma cells*; stanice rabdomiosarkoma

ROS – *reactive oxygen species*; reaktivne kisikove čestice

RNS – *reactive nitrogen species*; reaktivne dušikove čestice

SOD – *superoxide dismutase*; superoksid dismutaza

SO – *superoxide*; superoksid

SEM – *scanning electron microscopy*; skenirajuća elektronska mikroskopija

SRB – *sulforhodamine B assay*; sulforodamin B proba

St – Stupačinovo, uzorak s lokacije Stupačinovo

TEAC – *Trolox equivalent antioxidant capacity*; antioksidativni kapacitet ekvivalenta Troloxa

TE – *Trolox equivalents*; ekvivalenti Troloxa

TMV – *Tobaco mosaic virus*; mozaični virus duhana