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# Identification of Flavonoids from Marrubium and Ballota Species (Lamiaceae) and Determination of Chemotaxonomic Markers Using High Performance Liquid Chromatography Mass Spectrometer

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## Abstract

*Marrubium* and *Ballota* are known to be important medicinal plants belonging to the Lamiaceae family. Their aerial parts have been widely used in traditional medicine. The present study, for the first time, aimed to investigate flavonoid constituents and chromatographic pattern of the methanolic extract of leaf from *Marrubium* and *Ballota* species. The technique was performed on High Performance Liquid Chromatography (HPLC)-Micromass Quattro micro Atmospheric Pressure Ionization (API) Mass Spectrometer in six taxa. A total of 59 chemical compounds were recognized, among which 49 flavonoids, three polyphenols, and one methoxyphenol were identified. In addition, five chemical groups were recognized in *Marrubium* and *Ballota* species. It is noteworthy that *Marrubium* and *Ballota* species provide a major source of apigenin, kaempferol, and quercetin glycosides. The flavonoid compounds, such as licoricidin, sophoraflavanone G, and methyl robustone were highly frequent considering qualitative markers for the genus *Marrubium*. Despite the striking similarity between *Marrubium* and *Ballota* species, they were accurately separated using chemical markers, particularly the mass (MS/MS) spectra of flavonoid compounds, which can develop the functional products and pharmaceutical, chemotaxonomic, and chemo diversity purposes for *Marrubium* and *Ballota* genera.

**Keywords**: Flavone; Liquid chromatography; Mass spectrometer; Lamiaceae; *Marrubium*.

## Introduction

Lamiaceae is known as a medicinal aromatic plant family with high chemical compounds, which has been applied in traditional medicine. It is represented by 240 genera and 11species in Iran with different life forms, such as herbaceous, shrub, perennial, and annual forms [1]. It comprises different kinds of chemical metabolites that mainly contain phenolic acids, essential oils, and phenolic compounds. Moreover, this family has been reported to have certain chemical compounds with biological activities (for example, anti-viral, anti-bacterial, anti-inflammatory, antioxidant, anti-depressive, anti-cancer, anti-angiogenic, and anti-

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hepatotoxic effects) [2].

The genus *Marrubium* L., belonging to Lamiaceae family and Lamioideae subfamily, is one of the useful medicinal plants [3]. Its species are distributed in the Mediterranean, Russia, Asia, and North Africa [4]. This genus has 97 species throughout the world and 11 species in Iran. The plant grows in perennial, annual, suffruticose, and herbaceous forms [1, 5]. *Marrubium* species have been used for pharmaceutical effects including anti-inflammatory, anti-microbial, anti-hypertensive, anti-bronchitis, anti-cancer, anti-spasmodic, anti-diabetes mellitus, analgesic, dyslipidemia, expectorant, and antioxidant properties [6].

The genus *Ballota* L., belonging to Lamiaceae family and Lamioideae subfamily, has been mainly distributed in the Mediterranean and Eurasia. It is represented by 33 species throughout the world and three species in Iran. Its species grow as a small perennial shrub [7]. It has been demonstrated that the genus *Ballota* is of different pharmacological properties, including anti-tumor, anti-ulcer, anti-cancer, anti-fungal, anti-spasmodic, anti-malarial, anti-inflammatory, anti-nociceptive, anti-hemorrhoid, diuretic, and antioxidant activities [8-10].

Some previous investigations on phytochemical constituents have been presented in *Marrubium* genus. This genus is rich in phenolic compounds. Based on previous literature, certain chemical compounds, such as flavonoid glycosides and phenolic acids have been identified in *M. vulgare* L. and *M. velutinum* Sm. [11-12]. Moreover, terpenes and non-terpenes in *M. parviflorum* Fisch. & C.A. Mey. [13] have been separated using GC-MS (Gas Chromatography–Mass Spectrometry), H-NMR (*Hydrogen*-1 *Nuclear Magnetic Resonance)*, and C-NMR (*Carbon*-13 *NMR)*. Flavonoid classes and their diversity have been also reported in *Marrubium* species employing TLC (Thin Layer Chromatography) [3].

Some of the secondary metabolites have also been identified in *Ballota* species, including methoxy flavonoids in *B. glandulosissima* Hub.-Mor. & Patzak and *B. inaequidens* Hub.-Mor. & Patzak. In addition, the following substances have been recognized in *Ballota* species via GC-MS and HPLC: sesquiterpenes, oxygenated sesquiterpenes and monoterpenes in *B. undulata* (Sieber ex Fresen.) Benth., and *B. saxatilis* Sieber ex. C. Presl, diterpenoids, Phenylpropanoids, forsythosides, verbascosides, verminosides, and iridoids in *B. inaequidens*, *B. acetabulosa* (L.) Benth., *B. antalyense* F. Tezcan & H. Duman, *B. cristata* P. H. Davis, *B. larendana* Boiss. & Heldr., *B. latibracteolata* P. H. Davis & Doroszenko, *B. macrodonta* Boiss. & Bal., *B. pseudodictamnus* (L.) Benth., *B. rotundifolia* K. Koch, and *B. saxatilis* [8, 10].

Flavonoids contain polyphenolic compounds distributed in plant organs. It has been revealed that this metabolite possesses high biological and pharmacological activities, such as anti-cancer, anti-viral, anti-bacterial, and anti-oxidant properties. Different substitutions in flavonoid structures illustrate wide derivative compounds in the plant kingdom [14].

Over the recent years, a wide range of phytochemical analyses have been performed using different chromatography techniques. High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS/MS) is believed to be one of the powerful techniques for detecting the chemical constituents of natural products. It can illustrate low and high molecular weights of unknown compounds. Moreover, liquid chromatography coupled with atmospheric pressure ionization (API) technique is an ionization method for evaluating small, large, polar, and nonpolar compounds. Thus, a reliable analysis of an unknown compound could be represented utilizing this technique [15]. Different kinds of chromatography have been reported in Lamiaceae family, including UPLC (Ultra-Performance Liquid Chromatography) and LC-MS/MS in *Origanum majorana* L., *Lavandula officinalis* L., and *Phlomis* L. species [14, 16-17].

For note, the identification of the chemical constituents of *Marrubium* and *Ballota* species with powerful chemical techniques is of great necessity. To the best of our knowledge, no studies have been previously conducted on chemical compounds in both taxa from Iran using HPLC-MS/MS technique. Hence, the present work was conducted to separate and investigate the flavonoid constituents of *Marrubium* and *Ballota* species and characterize specific flavonoid derivatives and chemotaxonomic markers for both genera via HPLC-MS/MS technique. All the data were initially reported for Iran.

## Materials and Methods

***Plant material***

In this section, 15 accessions from five *Marrubium* species (*M. cuneatum* Banks & Sol., *M. vulgare* L., *M. crassidens* Boiss., *M. astracanicum* Jacq., and *M. anisodon* K. Koch) and three accessions from one *Ballota* species (*B. aucheri* Boiss.; endemic for Iran) were collected from their natural habitats of the center and south-west of Iran during flowering periods in March, May and July 2013-2014 (Table 1). All the specimens were identified using Flora Iranica and Flora of Iran [1, 5]. The authenticity of plant species was

**Table 1.** The list of collected *Marrubium* and *Ballota* species from their natural habitats, voucher numbers, altitude and geographical position

| Species/voucher no. | Location | Altitude (m) | Geographical position |
|---|---|---|---|
| ***M. cuneatum* 27,101,102** | Isfahan- Bordekan, Mehrgerd | 1833-1900 | 51°31'E,31°34'N |
| ***M.anisodon* 49,156,157** | Koykilouye va BoyerAhmad- Sisakht, Kouhgole | 1950-2150 | 51°33'E,30°47'N |
| ***M. crassidens* 88,164,165** | Chaharmahal va Bakhtiari- Chaleshtor | 2020-2085 | 50° 47'E, 32°22'N |
| ***M. vulgare* 43,134,135** | Koykilouye va BoyerAhmad- Sisakht | 1820-1950 | 51° 27'E,30°51'N |
| ***M. astracanicum* 89,146,147** | Isfahan- Khansar, Golestan Kouh | 1850-1965 | 50° 24'E, 33° 9'N |
| ***B. aucheri* 50, 51,52** | Koykilouye va BoyerAhmad- Yasouj, Kouh-e Mishi | 1805-1830 | 51°27'E, 30°51'N |

verified by Dr. N. Kharazian, Botany Department of Shahrekord University. The identified specimens were deposited in Herbarium of the Shahrekord University.

***Extraction method***

The total flavonoid was extracted from air-dried leaves (10 g) of five *Marrubium* species and *B. aucheri* using 85% MeOH at 40°C [18]. The total solvent was evaporated under reduced pressure (70°C; rotary evaporator, Eyela Co., Tokyo, Japan). Subsequently, flavonoid solution was isolated from carotene via n-butanol. The fractionation of flavonoid was accomplished by thin-layer chromatography (TLC; 3 µm, 20 × 20 cm) on plates coated with silica gel 60F 254 (17 mg, 70 mL $H_2O$). A solvent system was developed in AcOET–HCOOH–$CH_3COOH$–$H_2O$ (48:11:11:30). Spot detection with natural product identifiers ($H_2SO_4$ in MeOH and $C_{14}H_{16}BNO$ in 1:1 MeOH-$H_2O$) was carried out under ultraviolet-366 nm [3]. Moreover, column chromatography with Sephadex $LH_{20}$ (65 × 3 cm, with 98% MeOH) was done eluting with MeOH in $H_20$ (20%-100% MeOH). In this process, a total of 14 aqueous methanol fractions were obtained (50 mL each).

***HPLC-MS/MS method***

The extracted fractions of each species were checked with UV-absorption 200-400 nm. Consequently, a total of 10 aqueous methanol fractions were subjected to HPLC-MS/MS. The liquid chromatography analysis was used under the following conditions:

The technique was performed on Waters Alliance 2695 HPLC-Micromass Quattro micro API (Atmospheric Pressure Ionization) Mass Spectrometer. HPLC/MS was run on an Atlantis T3-C18 column (3µ, 2.1×100 mm, flow rate of 0.25 mL/min) at 30℃. The mobile phase included acetonitrile, ultra-pure water, methanol, and formic acid (98%). It consisted of two sections: A) 0.1% (v/v) formic acid in water and B) 0.1% formic acid in acetonitrile. The gradient elution was programmed as follows: 0 min, 5% B; 25 min, 95% B; 35 min, 95% B; injection volume 5 µL [14, 16].

***The mass spectrometer (MS) conditions***

The mass spectrometer (MS) was implemented on negative ionization mode with full scanning conducted by a mass range of 250 to 700 m/z. The mass spectra were performed in total ion chromatogram (TIC) and extracted electrospray (ES). Nitrogen (grade 5.0) was applied as nebulizer with gas flow 200 L/h. The desolvation and ion source temperatures were adjusted at 300°C and 120°C, respectively. The ion spray voltage was arranged as capillary voltage 3.5 kV, extractor 2 V, RF Lens 0.2 V, and sampling cone voltage 30V. The collision energy ranged from 30 to 40 eV. The extractions were refined over a 0.2 µm filter. The molecular ions of MS/MS were investigated using collision- induced dissociation (CID).

Commercial standards were not used for all the flavonoid compounds. Some of the analytical-grade standards purchased from Sigma-Aldrich Chemical Co. (St Louis, MO) included kaempferol, apigenin, and catechin with 98% purity. Methanol was used for preparing the stock solution (1 mg $mL^{-1}$) and filtration [14].

***Statistical analysis***

In order to estimate the statistical significance of the measured values, ANOVA test (Analysis of variance) was performed utilizing SPSS V. 20. Cluster analysis with Neighbor-Joining method, Dice similarity index, and PAST 3.0 software were employed to provide chemical groups in *Marrubium* and *Ballota* species.

## Results and Discussion

HPLC-MS/MS analysis of the *Marrubium* and *Ballota* species indicated a total of 59 chemical compounds, out of which 49 flavonoid compounds, three polyphenols, and one methoxyphenol were identified. The rest of the chemical compounds were related to two steroid derivatives, one tannin and one quinic acid, in addition to one cinnamic acid derivative. In this work, different flavonoid classes including flavones (15), flavonols (13), isoflavonoids (7), flavanones (4), flavans (4), bioflavonoids (2), arylbenzofuran flavonoid (1), dihydrochalcone (1), and two unknown compounds were distinguished (Table 2; peaks no. 1-102). The molecular mass of the chemical compounds was estimated via ion electrospray mass spectra (ESI-MS). The identification of each compound was done using MS/MS in fragments. The negative ionization mode $[M-H]^-$ with different collision energies (30-40 eV) was employed for MS/MS data. It has been previously revealed that negative ionization mode has a high sensitivity in the detection of the site of substitution in flavonoid compounds and provides evidence for compound structure [19]. TIC analysis was performed at time curve versus relative abundance. Moreover, mass baseline with different ions (m/z) was investigated. Utilizing a chromatogram, MS and MS/MS spectra were investigated to identify the structure of the proposed flavonoid compound (Figures

**Table 2.** The identified chemical compounds with molecular weight ($M_r$), m/z value, Retention time (RT), MS/MS spectrum and classification.

| Species | Peak no. | RT | $M_r$ | m/z $[M-H]^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. anisodon*** | 1 | 1.191 | 290 | 289 | 289 246 230 228 213 204 199 179 175 165 163 159 147 143 136 131 125 107 119 111 | Catechin | Flavan |
| | 2 | 1.45 | 378 | 377 | 378 359 348 332 326 276 194 193 183 178 155 | 12-gingerol | Phenol (methoxyphenol) |
| | 3 | 5.92 | 600 | 599 | 590 479 447 316 301 271 194 179 155 152 136 106 | Quercitrin-*O*-gallate | Flavonol |
| | 4, 9 | 6.72, 6.87 | 523 | 522 | 522 177167 161 148 | Cinnamic acid derivatives | Cinnamic acid |
| | 5 | 6.85 | 616 | 615 | 301 229 271 201 185 153 133 | Quercetin-7-*O*-galloyl-glucoside | Flavonol |
| | 6 | 7.00 | 434 | 433 | 447 436 433 421 418 404 395 383 364 355 342 322 313 304 271 261 252 242 228 164 152 151 145 120 117 | Naringenin-7-*O*-glucoside (prunin) | Flavanone |
| | 7 | 9.19 | 654 | 653 | 653 501 477 301 175 | Quercetin-di-glucuronide | Flavanol |
| | 8 | 14.51 | 562 | 561 | 554 531 429 399 278 267 252 245 | Formononetin-7-*O*-apiofuranosyl-*O*-glucopyranoside | Isoflavone |
| | 10 | 6.94 | 446 | 445 | 445 431 349 272 269 240 213 195 184 179 166 151 143 135 122 113 110 | Baicalein-7-*O*-glucuronide (baicalin) | Flavone |
| | 11 | 7.09 | 512 | 511 | 445 431 415 351 348 271 270 269 241 | Baicalein-7-*O*-glucopyranosyl sulfate | Flavone |
| | 12 | 7.11 | 442 | 441 | 289 169 135 | Catechingallate | Flavan |
| | 13 | 8.57 | 556 | 555 | 555 445 431 403 375 324 295 271 244 | Uknown | - |
| | 14 | 9.10 | 698 | 697 | 681 661 651 619 367 | Steroid derivatives | Steroid |
| | 15 | 9.33 | 655 | 654 | 652 489 473 330 | Malvidin-3,5-di-*O*-hexoside | Polyphenol (anthocyanidin) |
| | 16 | 10.15 | 508 | 507 | 507 345 330 302 286 273 269 242 229 113 | Syringetin-3-*O*-hexoside | Flavonol |
| | 17 | 11.92 | 460 | 459 | 458 444 429 297 267 266 252 209 171 141 113 | wistin | Isoflavone |
| | 18 | 11.95 | 462 | 461 | 447 299 253 209 153 | Chrysoeriol-7-*O*-glucoside | Flavone |

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z [M-H]$^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. vulgare*** | 19 | 8. 35 | 586 | 585 | 584 424 423 379 261 258 246 240 233 227 203 189 175 160 142 | 3-Hydroxyhispidin-3,4'-di-*O*-glucoside | Polyphenol |
| | 20 | 9.95 | 568 | 567 | 436 405 273 | Phloretin-3-*O*-xyloglucoside | Dihydrochalcone |
| | 21 | 10.94 | 490 | 489 | 488 447 327 285 | Kaempferol-3-*O*-acetylglucoside | Flavonol |
| | 22 | 11.44 | 378 | 377 | 377 362 349 343 333 331 320 316 315 305 292 284 270 260 243 230 225 216 213 208 201 200 182 172 156 137 132 115 | Methyl robustone | Isoflavan |
| | 23 | 12.85 | 424 | 423 | 423 380 377 361 348 336 231 189 173 163 137 123 | Licoricidin | Isoflavan |
| | 24 | 12.96 | 378 | 377 | 378 359 193 182 178 | Uknown | - |
| | 25 | 14.00 | 424 | 423 | 405 379 355 301 299 285 283 270 255 243 242 227 224 217 | Sophoraflavanone G | Flavanone |
| | 26 | 14.45 | 328 | 327 | 327 326 320 317 314 312 300 299 297 284 282 270 254 247 246 234 225 211 171 165 183 137 | 3-Hydroxy-trimethoxyflavone | Flavone |
| ***M. crassidens*** | 27 | 10.08 | 568 | 567 | 436 273 | Phloretin-3-*O*-xyloglucoside | Dihydrochalcone |
| | 28 | 12.15 | 698 | 697 | 681 661 651 619 367 | Steroid derivatives | Steroid |
| | 29 | 12.62 | 652 | 651 | 609 489 430 285 266 243 241 217 175 | Isoscutellarein-7-*O*-(6-*O*-acetylallosyl) glucoside | Flavone |
| | 30 | 12.87 | 424 | 423 | 424 391 380 365 347 233 218 203 192 188 177 173 146 137 123 109 | Licoricidin | Isoflavan |
| | 31 | 13.88 | 506 | 505 | 505 463 343 301 299 271 255 242 228 226 178 164 151 147 107 | Quercetin-3-*O*-glucoside-6"-acetate | Flavonol |
| | 32 | 14.01 | 424 | 423 | 405 379 340 300 299 271 243 217 227 | Sophoraflavanone G | Flavanone |
| | 33 | 8.59 | 586 | 585 | 585 557 423 261 246 242 202 233 159 175 144 | 3-Hydroxyhispidin-3,4'-di-*O*-glucoside | Polyphenol |
| | 34 | 9.98 | 566 | 565 | 445 440 433 415 328 301 265 | Quercetin-3-*O*-pentosyl-pentoside | Flavonol |

1-3). In the identification process, all the mass spectra of this research and MS/MS data of standard references were investigated [19-20]. In addition, the database of different mass banks, ChemSpider, NIST.8 MS search, and METLIN were applied in this process [21-23]. The m/z values in 10 fractions ranged from 269 to 700. The retention times of MS/MS fragments in chemical compounds ranged from 1.19 to 23.95 min. All the information about TIC analysis with their MS/MS fragmentation, retention times, and high-intensity peaks are presented in Table 2. Statistical significance was evaluated using mass to charge and frequency of ions for each species. ANOVA test detected statistical significance at $P<0.05$ ($F=1.57$, sig. 0.001).

There are certain characteristic of some compounds in each species which could be employed as chemotaxonomic markers, including quercitrin-*O*-gallate, catechin, syringetin-hexoside, formononetin-apiofuranosyl-glucopyranoside, malvidin-di-hexoside, quercetin-galloyl-glucoside, quercetin-diglucuronide, prunin, baicalein-glucuronide, baicalein-glucopyranoside, and wistin for *M. anisodon*, quercetin-pentoside, isoscutellarein-acetylallosyl, quercetin-glucoside-acetate, quercetin-glucuronide, baicalein-glucuronopyranoside, and kaempferol-glucuronide for *M. crassidens*, quercetin-di-rhamnoside, isorhamnetin-diglucoside, and kaempferol-trimethyl ether for *M. astracanicum*, kaempferol, hydroxy-dimethoxyflavanone, vicenin II, biapigenin, maxima-isoflavone, artocarpin, zapotin, jaceidin, derrusnin,

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z $[M-H]^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. crassidens*** | 35 | 10.63 | 458 | 457 | 440 415 310 293 273 253 222 191 187 163 127 | 6-*O*-acetyldaidzin | Isoflavone |
| | 36 | 10.71 | 525 | 524 | 525 446 444 348 269 | Baicalein-6-*O*- -glucuronopyranosyl-7-*O*-sulfate | Flavone |
| | 37 | 10.87 | 630 | 629 | 629 477 465 459 455 313 295 211 186 163 146 124 | 6-*O*-coumaroyl-digalloylglucose | Tannin |
| | 38 | 11.15 | 556 | 555 | 559 556 445 432 429 403 389 375 361 324 269 271 243 223 | Fukugetin | Flavone |
| | 39 | 11.21 | 442 | 441 | 441 413 318 291 289 287 269 179 177 170 169 161 | Epicatechin-3-*O*-gallate | Flavan |
| | 40 | 11.29 | 478 | 477 | 477 371 301 283 266 259 247 240 228 192 177 166 152 121 118 106 | Quercetin-3-*O*-glucuronide | Flavonol |
| | 41 | 11.33 | 490 | 489 | 488 447 327 285 | Kaempferol-3-*O*-acetylglucoside | Flavonol |
| | 42 | 11.56 | 378 | 377 | 377 362 350 344 333 331 324 315 313 302 298 292 284 260 243 201 185 168 163 155 150 137 131 120 | Methyl robustone | Isoflavan |
| | 43 | 11.57 | 493 | 492 | 492 463 331 330 329 303 283 272 268 259 243 221 205 193 186 180 164 138 | Malvidin-3-*O*-hexoside | Polyphenol (anthocyanidin) |
| | 44 | 12.19 | 525 | 524 | 525 444 348 269 | Baicalein-6-*O*-glucuronopyranosyl-7-*O*-sulfate | Flavone |
| | 45 | 12.65 | 458 | 457 | 456 331 305 287 270 243 219 201 185 169 125 | Epigallocatechin-3-gallate | Flavan |
| | 46 | 12.94 | 424 | 423 | 424 379 365 352 334 307 295 279 268 245 231 215 200 205 191 189 177 163 137 124 109 | Licoricidin | Isoflavan |
| | 47 | 13.89 | 506 | 505 | 505 463 343 301 299 272 226 216 200 179 151 | Quercetin-3-*O*-glucoside-6"-acetate | Flavonol |
| | 48 | 14.05 | 424 | 423 | 379 355 313 301 286 271 259 260 283 270 243 255 241 227 217 213 | Sophoraflavanone G | Flavanone |

apigenin-rhamnosyl-xylo-hexose-uloside, and proanthocyanidin for *B. aucheri*, and Isorhamnetin-glucoside, and apigenin-7-*O*-glucoside for *M. cuneatum*. Based on MS/MS spectra, *M. vulgare* did not have any specific flavonoid compounds. Accordingly, five chemical groups were certainly identified (Figure 4).

According to the published literature, flavonoid derivatives are the main chemical compounds in *Marrubium* and *Ballota* species [8, 12]. The identified chemical compounds are introduced and discussed in the following sections.

***Flavone derivatives***

It has been previously revealed that the majority of flavonoid classes in Lamiaceae family belonged to flavone derivatives [10]. Fifteen flavones were recognized in *Marrubium* and *Ballota* genera.

The myricetin derivatives have been previously proposed in *Schotia brachypetala* Sond., Fabaceae [20, 24]. The approved myricetin-3-*O*-hexoside-6''-*O*-gallate (m/z 631; m/z 317 [M-H-314], galloyl-hexoside moiety) was primarily reported for *M. astracanicum*, and *B. aucheri*. Myricetin glycosides were found to have antioxidant activity [20].

Three identified baicalein derivatives, namely baicalein-7-*O*-glucuronide (m/z 445; m/z 269 [M-H-176] or glucuronide moiety), baicalein-7-*O*-glucopyranosyl sulfate (m/z 511; m/z 269 (242 AMU) or 7-*O*-glucopyranose moiety and sulfate conjugate), and baicalein-6-*O*-glucuronopyranosyl-7-*O*-sulfate (m/z 524; m/z 269 [M-H-255], glucuronopyranosyl unit and sulfate moiety) were first attributed to *M. anisodon* and

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z $[M-H]^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. crassidens*** | 49 | 14.48 | 328 | 327 | 327 326 316 314 299 284 282 270 254 247 246 226 211 198 183 171 167 165 155 137 117 106 | 3-Hydroxy-trimethoxyflavone | Flavone |
| | 50 | 16.79 | 462 | 461 | 460 285 267 241 229 213 196 185 175 164 159 150 135 113 106 | Kaempferol-3-glucuronide | Flavonol |
| ***M. astracanicum*** | 51 | 8.45 | 586 | 585 | 583 557 423 261 246 242 233 175 143 | 3-Hydroxyhispidin-3,4'-di-*O*-glucoside | Polyphenol |
| | 52 | 10.04 | 566 | 565 | 546 486 456 446 445 433 366 313 271 | Pinobankasin-glucopyranosyl-arabinopyranoside | Flavanone |
| | 53 | 10.46 | 458 | 457 | 440 416 295 274 253 221 203 219 191 163 155 140 126 | 6-*O*-acetyldaidzin | Isoflavone |
| | 54 | 10.83 | 566 | 565 | 547 487 457 447 445 433 367 337 313 271 | Pinobankasin-glucopyranosyl-arabinopyranoside | Flavanone |
| | 55 | 11.11 | 556 | 555 | 555 445 432 428 403 389 322 295 270 244 240 | Fukugetin | Flavone |
| | 56 | 11.17 | 490 | 489 | 488 447 327 285 | Kaempferol-3-*O*-acetylglucoside | Flavonol |
| | 57 | 11.43 | 378 | 377 | 377 358 362 348 345 333 332 331 323 316 315 293 284 281 270 268 261 243 230 212 209 201 183 137 114 116 | Methyl robustone | Isoflavan |
| | 58 | 12.44 | 458 | 457 | 456 331 305 287 270 243 219 201 185 169 125 | Epigallocatechin-3-gallate | Flavan |
| | 59 | 12.76 | 424 | 423 | 423 392 380 379 364 355 352 347 321 279 244 230 220 215 205 191 177 173 149 137 | Licoricidin | Isoflavan |
| | 60 | 13.12 | 378 | 377 | 378 359 193 182 178 | Uknown | - |
| | 61 | 14.00 | 424 | 423 | 379 355 313 300 286 271 260 283 271 243 217 255 241 227 | Sophoraflavanone G | Flavanone |
| | 62 | 14.50 | 328 | 327 | 313 306 292 285 284 281 265 230 220 212 200 194 185 171 166 155 | Kaempferol-trimethyl ether | Flavonol |

*M. crassidens.* Such flavone derivatives were in accordance with the reference standards reported for *Scutellaria baicalensis* Gerogi [25]. Baicalein derivatives are the most well-known components of *Sc. baicalensis*. Nevertheless, they were demonstrated in *Marrubium* species.

Based on the fragmentation patterns of *M. crassidens*, and *M. cuneatum*, the MS/MS analysis of deprotonated molecules was indicative of isoscutellarein-7-*O*-(6-*O*-acetylallosyl) glucoside (m/z 651; m/z 285 [M-H-324-42] or *O*-acetylallosyl glucoside residue) [26]. According to previous reports, isoscutellarein derivatives have been mostly attributed to *Lamium album* L., Lamiaceae [19]. Chrysoeriol-7-*O*-glucoside (m/z 461; m/z 299 [M-H-162] or glucoside moiety), tricin-7-*O*-glucuronide (m/z 505; m/z 329 [M-H-176]), and zapotin (m/z 341) were first observed in *M. anisodon* and *B. aucheri* previously reported in *Scutellaria schachristanica* Juz., *Casimiroa pubescens* Ramirez, and *Phlomis* L. species [14, 27-28].

Hydroxyl-methoxylated flavones have been previously isolated from *Scutellaria* L., Lamiaceae [10, 24- 25]. According to the results, 3-hydroxy-trimethoxyflavone (m/z 327; m/z 254 [M-H-73] or successive loss of methoxy and hydroxyl groups) was characterized in *M. crassidens, M. vulgare*, and *B. aucheri*.

Isorhamnetin-3,4'-diglucoside (m/z 639; m/z 315 [324 AMU] or successive cleavage of the hexose moiety) and isorhamnetin-3-*O*-glucoside (m/z 477; m/z 315 [M-H-162]) were observed in *M. astracanicum* and *M. cuneatum*. In previous published reports, isorhamnetin-3-*O*-*β*-D-rutinoside and isorhamnetin-3-*O*-*β*-D-glucoside have been identified in *M. velutinum,*

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z $[M-H]^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. astracanicum*** | 63 | 16.60 | 462 | 461 | 460 285 241 200 213 183 175 167 159 151 113 | Kaempferol-3-glucuronide | Flavonol |
| | 64 | 16.81 | 392 | 391 | 391 255 247 227 214 194 177 163 121 114 | Deoxycholic acid derivatives | Steroid |
| | 65 | 23.71 | 266 | 265 | 265 251 120 | Conocarpan | 2-arylbenzofuran flavonoid |
| | 66 | 8.57 | 586 | 585 | 557 421 261 246 233 204 187 176 160 | 3-Hydroxyhispidin-3,4'-di-*O*-glucoside | Polyphenol |
| | 67 | 10.36 | 640 | 639 | 624 477 431 354 369 339 315 285 271 255 243 225 192 | Isorhamnetin-3,4'-diglucoside | Flavone |
| | 68 | 10.41 | 594 | 593 | 593 447 432 356 315 301 269 259 255 244 242 230 228 214 211 187 163 152 151 | Quercetin-3,7-di-*O*-rhamnoside | Flavonol |
| | 69 | 10.55 | 632 | 631 | 479 317 298 287 271 269 241 211 180 169 151 | Myricetin-3-*O*-hexoside-6''-*O*-gallate | Flavone |
| | 70 | 13.05 | 424 | 423 | 424 390 379 365 353 351 311 263 245 203 191 173 163 148 138 | Licoricidin | Isoflavan |
| | 71 | 14.07 | 424 | 423 | 379 355 313 300 271 283 260 243 240 227 217 | Sophoraflavanone G | Flavanone |
| | 72 | 16.70 | 462 | 461 | 460 285 255 240 227 211 189 174 163 143 135 107 113 | Kaempferol-3-glucuronide | Flavonol |
| ***M. cuneatum*** | 73 | 8.30 | 478 | 477 | 315 302 299 284 272 270 259 254 242 199 150 | Isorhamnetin-3-*O*-glucoside | Flavone |
| | 74 | 8.61 | 586 | 585 | 585 557 423 261 246 241 233 159 143 | 3-Hydroxyhispidin-3,4'-di-*O*-glucoside | Polyphenol |
| | 75 | 10.08 | 566 | 565 | 486 456 446 445 433 366 337 313 271 | Pinobankasin-glucopyranosyl-arabinopyranoside | Flavanone |
| | 76 | 11.29 | 432 | 431 | 432 403 387 374 328 269 254 224 164 162 137 107 | Apigenin-7-*O*-glucoside | Flavone |

and *Jatropha tanjorensis* J.L. Ellis & Saroja, Euphorbiaceae [12, 29].

The presence of prenylated flavone or artocarpin (m/z 435) was initially reported for *B. aucheri*. According to the published works, this flavone derivative was isolated from *Artocarpus altilis* (Parkinson ex. F.A. Zorn) Fosberg, Moraceae. This prenyl flavone has been found to possess anti-malarial property [30].

According to earlier studies, the successive cleavage of some produced ions in *B. aucheri* contributes to determination of the flavone compounds, such as vicenin II (apigenin-6,8-di-*C*-glycoside, m/z 593; m/z 383 ([M-H-90] and [M-H-120] cleavage of hexosyl moiety at the 6- and 8-*C* positions), and apigenin-8-*C*-[6-deoxy-2-*O*-rhamnosyl]-xylo-hexos-3-uloside (m/z 559; m/z 395 [164 mass unit] or 2-*O*-glycosylation with a deoxyhexose). Apigenin-6,8-di-*C*-glycoside has been previously described in *B. nigra* [31]. The molecular ions of *M. cunetaum* were characterized as apigenin-7-*O*-glucoside (m/z 431; m/z 269 [M-H-162] or glucoside moiety). Additionally, other apigenin derivatives including apigenin-7-*O*-*β*-D-glucoside, apigenin-7-*O*-*β*-D-(3''-*p*-coumaryl)-glucoside, and apigenin-4'-*O*-*β*-D-glucopyranoside have been presented in *M. velutinum* and *M. anisodon* [12, 32]. In conclusion, flavone glycosides are commonly found in *Marrubium* and *Ballota* species.

***Flavonol derivatives***

Different flavonol derivatives were characterized in *Marrubium* and *Ballota* species.

*M. astracanicum, M. vulgare, M. crassidens*, and *B. aucheri* exhibited typical fragments recognized as kaempferol-3-glucuronide (m/z 461; m/z 285 [176 AMU] or glucuronide moiety), kaempferol-3-*O*-acetylglucoside (m/z 489; m/z 285 [M-H-204] or loss of hexosyl and acetyl moiety), and kaempferol-trimethylether (m/z 327; m/z 285 [M-H-$CH_3$-O-$CH_3$]) compared with the reference standards [24, 33-34]. Four

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z [M-H]$^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***M. cuneatum*** | 77 | 11.37 | 442 | 441 | 441 332 318 303 289 270 259 244 215 192 187 178 168 145 137 125 109 | Epicatechin-3-*O*-gallate | Flavan |
| | 78 | 11.60 | 378 | 377 | 377 362 357 349 334 333 331 320 315 313 298 283 269 260 247 243 231 207 201 183 167 137 113 | Methyl robustone | Isoflavans |
| | 79 | 12.31 | 652 | 651 | 591 489 448 285 257 217 175 | Isoscutellarein-7-*O*-(6-*O*-acetylallosyl) glucoside | Flavone |
| | 80 | 12.99 | 424 | 423 | 423 391 380 348 263 243 231 206 123 | Licoricidin | Isoflavan |
| | 81 | 14.14 | 424 | 423 | 379 355 301 284 243 227 217 | Sophoraflavanone G | Flavanone |
| ***B. aucheri*** | 82 | 6.64 | 286 | 285 | 284 270 266 254 238 225 220 197 194 185 173 166 157 146 139 133 121 118 108 | Kaempferol | Flavonol |
| | 83 | 6.96 | 300 | 299 | 299 297 284 271 269 266 259 250 241 216 190 184 175 151 149 137 131 122 117 108 | 4'-hydroxy-5,7-dimethoxyflavannone | Flavanone |
| | 84 | 11.01 | 632 | 631 | 630 479 317 300 288 270 241 179 169 149 123 | Myricetin-3-*O*-hexoside-6''-*O*-gallate | Flavone |
| | 85 | 18.28 | 436 | 435 | 435 377 366 350 333 326 307 298 296 266 227 179 109 | Artocarpin | Flavone |
| | 86 | 23.95 | 266 | 265 | 265 250 247 184 159 133 119 | Conocarpan | 2-arylbenzofuran flavonoid |
| | 87 | 6.76 | 342 | 341 | 297 179 161 132 | Caffeic acid-*O*-glucoside | phenolic acid |
| | 88 | 6.87 | 286 | 285 | 285 270 254 243 214 197 180 171 165 157 146 139 133 126 117 112 108 | Kaempferol | Flavonol |
| | 89 | 7.70 | 342 | 341 | 341 338 327 325 305 282 279 260 255 245 238 221 216 217 197 185 181 184 172 164 120 115 | Zapotin | Flavone |

different kaempferol derivatives, namely kaempferol-3-*O*-*β*-D-rutinoside, kaempferol-3-*O*-acetylglucoside, kaempferol-3-*O*-*β*-D-glucoside, and kaempferol-3-*O*-*β*-D-glucopyranoside have been already identified in *M. anisodon, Allium kurrat* Schweinf. ex K. Krause, Amaryllidaceae, and edible flowers in medicinal plants [32]. The presence of kaempferol (m/z 285) is consistent with the reference standards in *Ballota nigra* [9, 21].

Based on the MS/MS spectra of *M. anisodon* and previous reports, further flavonol compounds were suggested to be syringetin-3-*O*-hexoside (m/z 507; m/z 345 [M-H-162] or cleavage of hexose moiety at the *O*-position) [24]. Syringetin has been also identified in *Mentha pulegium* L. and *Origanum majorana*, Lamiaceae [18].

Based on the cleavage of produced ions in *M. anisodon, M. astracanicum,* and *M. crassidens*, different quercetin derivatives were suggested as quercetin-3-*O*-glucuronide (m/z 477; m/z 301 [M-H-176]), quercetin-di-glucuronide (m/z 653; m/z 301 [M-H-352] as successive loss of glucuronide moieties), quercetin-3-*O*-glucoside-6"-acetate (m/z 505; m/z 301 [M-H-204] or acetyl-glucosyl moiety), quercetin-3-*O*-pentosyl-pentoside (m/z 565; m/z 301 [264 AMU] or successive loss of *O*-pentosyl units), quercetin-7-*O*-galloyl-glucoside (m/z 615; m/z 301 [M-H-314]), quercetin-3,7-di-*O*-rhamnoside (m/z 593; 301 [M-H-292] or successive loss of *O*-rhamnosyl units), and quercitrin-*O*-gallate (m/z 599; 447 [M-H-152]), in line with the reference standards [21, 24]. Other quercetin glycosides, such as quercetin-3-*O*-*β*-D-rutinoside, quercetin-3-*O*-*β*-D-glucoside, and quercetin-3-O-α-rhamnosyl-glucoside, have been identified in *M. vulgare* [11]. The presence of quercetin-3-*O*-glucuronide is in line with the reports by Krauze-Baranowska et al. (2014), on *Rubus* L. species, Rosaceae [35]. Farag et al. (2015) and Figueiredo-Gonzalez et al. (2016) reported a similar quercetin derivative as quercetin-3-*O*-pentosyl-rutinoside in *Bauhinia* L. (Fabaceae) species and *Lycopersicon*

**Table 2.** Continued

| Species | Peak no. | RT | $M_r$ | m/z [M-H]$^-$ | MS/MS fragment ions (MS2) | Proposed compounds | Compound class |
|---|---|---|---|---|---|---|---|
| ***B. aucheri*** | 90 | 8.58 | 594 | 593 | 503 486 473 455 413 383 353 297 | Vicenin II (apigenin 6,8-di-*C*-glycoside) | Flavone |
| | 91 | 8.99 | 338 | 337 | 337 222 191 190 173 163 145 119 108 | 3-coumaroyl quinic acid | Quinic acid |
| | 92 | 9.59 | 338 | 337 | 337 222 191 173 163 145 119 109 | 3-coumaroyl quinic acid | Quinic acid |
| | 93 | 10.46 | 538 | 537 | 451 443 417 371 343 332 308 294 268 239 224 213 210 195 167 152 119 117 109 | 3,8'-Biapigenin | Flavone |
| | 94 | 11.62 | 310 | 309 | 293 291 276 273 256 247 216 208 193 185 165 | Maxima-Isoflavone A | Isoflavone |
| | 95 | 14.05 | 360 | 359 | 359 344 341 331 328 322 315 314 302 287 284 270 259 256 243 241 230 213 203 187 175 161 157 147 135 121 119 109 | Jaceidin | Flavonol |
| | 96 | 14.65 | 328 | 327 | 327 326 314 299 297 284 282 270 254 247 246 230 217 211 185 183 177 174 171 165 163 158 149 137 121 | 3-Hydroxy-trimethoxyflavone | Flavone |
| | 97 | 15.23 | 506 | 505 | 504 329 300 271 113 | Tricin-7-*O*-glucuronide | Flavone |
| | 98 | 18.02 | 392 | 391 | 391 321 315 247 240 214 194 | Deoxycholic acid derivatives | Steroid |
| | 99 | 19.40 | 356 | 355 | 354 341 322 309 300 282 270 267 241 225 212 209 198 181 168 153 130 120 | Derrusnin | Isoflav-3-ene |
| | 100 | 20.31 | 562 | 561 | 544 435 289 | Proanthocyanidin derivative | Polyphenol |
| | 101 | 23.11 | 560 | 559 | 395 394 321 269 | Apigenin-8-*C*-[6-deoxy-2-*O*-rhamnosyl]-xylo-hexos-3-uloside | Flavone |

*esculentum* Mill. (Solanaceae) leaves. Quercetin-3-*O*-*β*-D-rutinoside and quercetin-3-*O*-*β*-D-glucoside revealed great anti-bacterial activity [36-37]. This activity was attributed to aglycone moiety.

Through the MS/MS spectrum, the precursor ions of *B. aucheri* were primarily identified as jaceidin (m/z 359; m/z 230 [M-H-129] or successive loss of hydroxyl and methoxy groups), approved by Taamalli et al. (2015) in *Mentha pulegium* and *Origanum majorana*, Lamiaceae [18].

In previous papers, a few flavonols had been isolated from *Marrubium* species [12]. Nevertheless, it is important to consider that *Marrubium* and *Ballota* species present a major source of flavonol compounds, such as kaempferol and quercetin glycosides.

***Isoflavonoid derivatives***

A total of 7 isoflavonoid compounds were specified in *Marrubium* and *Ballota* species.

In MS/MS spectrum of *M. anisodon*, an isoflavone derivative was first assigned to formononetin-7-*O*-apiofuranosyl-*O*-glucopyranoside (m/z 561; m/z 267 [294 AMU] or apiofuranosyl and *O*-glucopyranoside units). In previous published works, this compound has been reported in *Millettia nitida* var. *hirsutissima* Z. Wei, Fabaceae [38].

Another isoflavone derivative was first observed in *M. anisodon*. It was assigned to wistin (m/z 459; m/z 297 [M-H-162] or glucoside unit) and supported by the reference standards [24].

The produced ions of *M. vulgare, M. crassidens, M. astracanicum*, and *M. cuneatum* were first attributed to methyl robustone (m/z 377; m/z 362 [M-H-CH3] or methoxy group) as an isoflavan [24]. It has been previously isolated from *Kigelia africana* (Lam.) Benth., Bignoniaceae with anti-cancer activity [39].

Further isoflavan derivatives were initially found in *M. vulgare, M. crassidens, M. astracanicum*, and *M. cuneatum*. The fragment ions displayed the exact dissociation pathway for known licoricidin (m/z 423), which is approved by the reference standards [24]. According to previous reports, licoricidin compound has been isolated from *Paeoniae* L. (Paeoniaceae) and *Glycyrrhizae* L. (Fabaceae) [40]. It has been found that

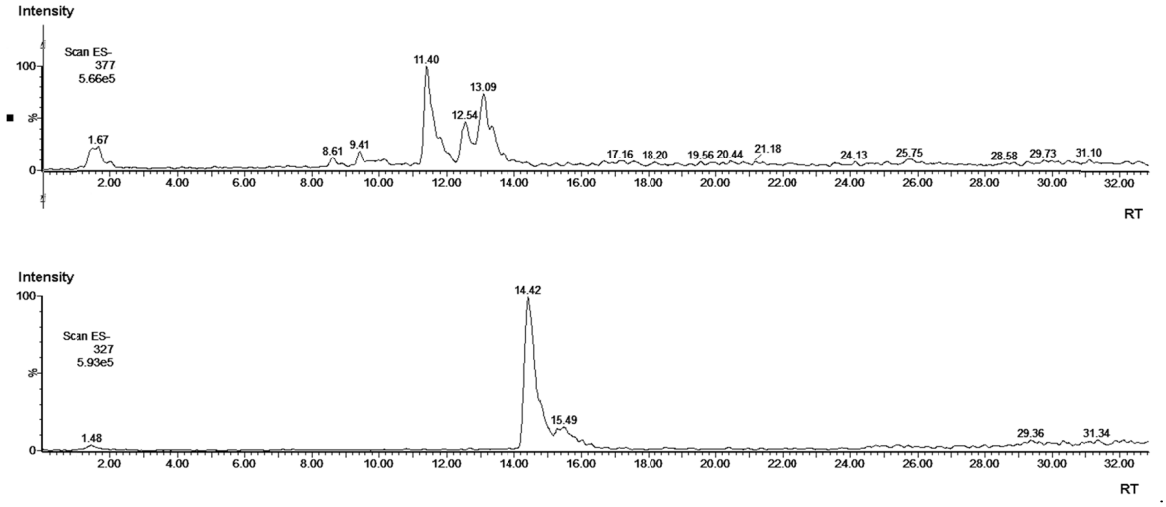


**Figure 1.** Representative of some chromatograms at m/z 377 and 327 (*M. vulgare*), RT: Retention time

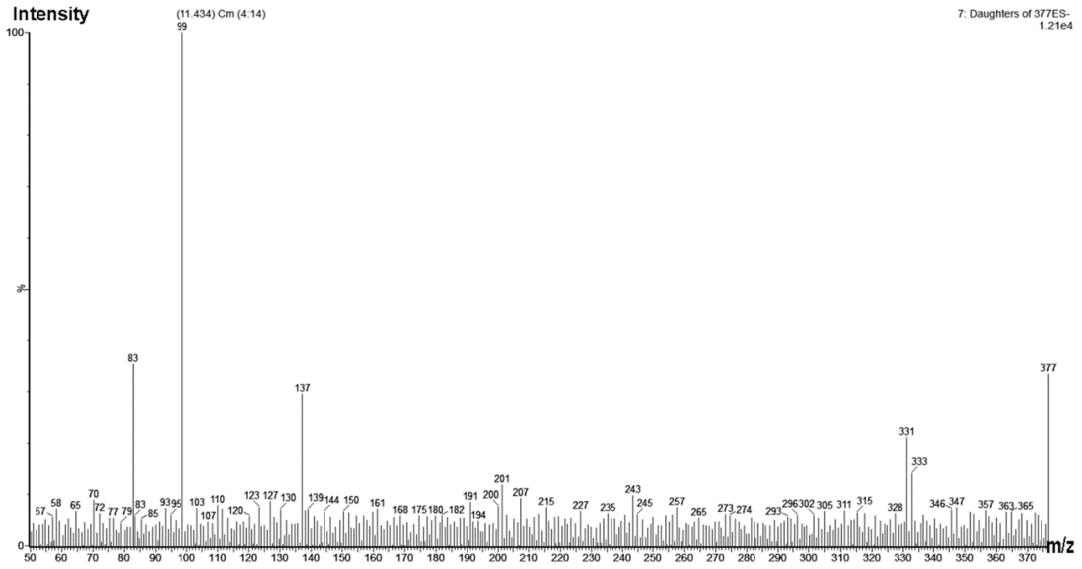


**Figure 2.** MS/MS spectrum of some flavonoid compounds in *Marrubium* species; methylrobustone

methyl robustone, and licoricidin are mostly present in *Marrubium* species.

In MS/MS spectrum of *M. crassidens, M. astracanicum*, and *B. aucheri*, two new isoflavonoids including 6-*O*-acetyldaidzin (m/z 457; m/z 253 [M-H-162-42] or acetyl-glycoside unit), and maxima-isoflavone A (m/z 309) were recognized. According to previous reports, both derivatives have been recognized in *Glycine max* (L.) Merr. and *Arachis hypogaea* L., Fabaceae [33, 41-42].

The produced ions of *B. aucheri* were proposed as derrusnin (m/z 355; Isoflav-3-ene derivative) based on the reference standards [24, 43]. The anti-viral effect of derrusnin has been previously reported [43].

***Flavanone derivatives***

The mass chromatogram of each fraction displayed different flavanone derivatives in *Marrubium* and *Ballota* species. Four flavanone compounds were mostly found in *M. anisodon, M. cuneatum, M. astracanicum,* and *B. aucheri*.

Naringenin-7-*O*-glucoside or prunin (m/z 433; m/z 271 [162 AMU] or *O*-glucoside unit) was recognized in *M. anisodon*. Based on previous reports, naringenin and its glycosides have been commonly isolated from Lamiaceae family with a high anti-bacterial activity

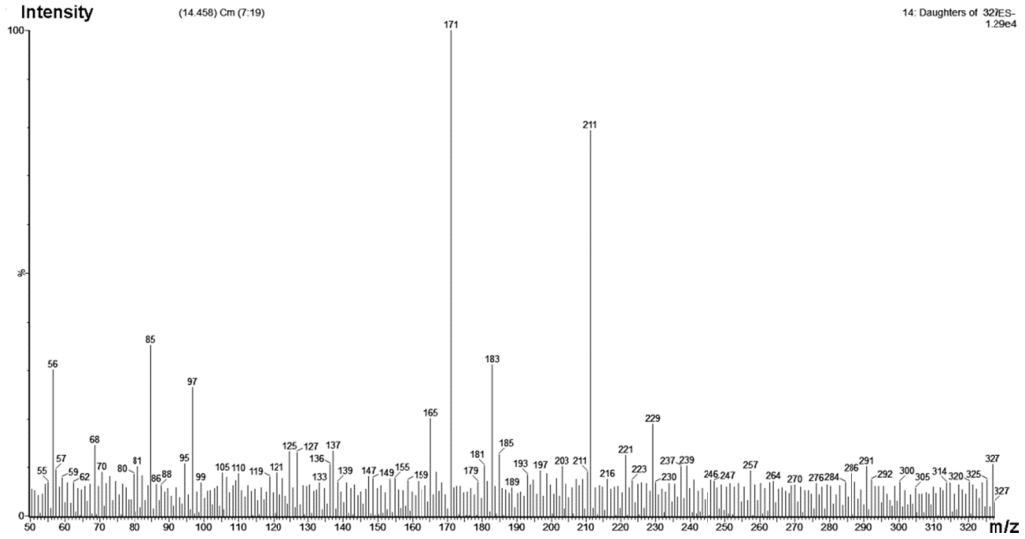


**Figure 3.** MS/MS spectrum of some flavonoid compounds in *Marrubium* species; hydroxy-trimethoxyflavone

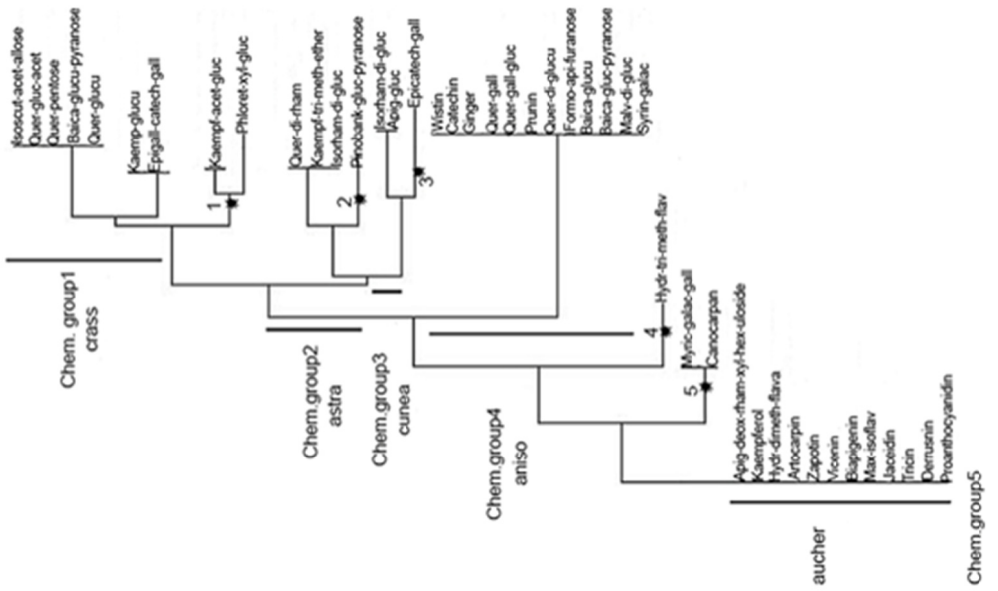


**Figure 4.** Representative of chemical groups in *Marrubium* and *Ballota* species using flavonoid data, NJ method and Dice similarity index. All chemical data are mentioned in Table 2. Crass: *M. crassidens*, astra: *M. astracanicum*, cunea: *M. cuneatum*, aniso: *M. anisodon*, aucher: *B. aucheri*. *1: vulg (*M. vulgare*), astra and crass, *2: cunea, astra, *3: cunea, crass, *4: crass, vulg, and auch, *5: astra and aucher

[14].

Pinobankasin-glucopyranosyl-arabinopyranoside (m/z 565; m/z 271 [M-H-294] or glucopyranosyl and arabinopyranosyl units) and Sophoraflavanone G (m/z 423) were observed in *M. astracanicum, M. crassidens, M. vulgare,* and *M. cuneatum* for the first time. Pinobankasin has been previously reported in *Scutellaria baicalensis*, Lamiaceae [25]. The present 4'-hydroxy-5,7-dimethoxyflavannone (m/z 299; m/z 241 [M-H-30] and [M-H-28] or OH-substitute) is also consistent with those in a previous study on *Phlomis* species, Lamiaceae [14].

***Flavan derivatives***

Based on the data spectrum and reference standards, four flavan derivatives were recognized, namely

epicatechin-3-*O*-gallate (Rt=11.21, 11.37, m/z 441; m/z 289 [M-H-152] or gallate moiety), epigallocatechin-3-gallate (m/z 457), catechingallate (Rt=7.11, m/z 441), and catechin (m/z 289; m/z 179 [M- H-110] or dihydroxybenzene moiety) [20, 24]. These compounds were recognized in *M. astracanicum*, *M. anisodon, M. crassidens*, and *M. cuneatum*. It is of note that gallocatechin and catechin compounds have been previously isolated from *Phlomis aucheri* Boiss., *Mentha pulegium* and *Origanum majorana*, Lamiaceae [14, 18].

***Bi-flavonoid derivatives (bi-flavones)***

Two bi-flavonoid derivatives were first recognized in *M. crassidens, M. astracanicum*, and *B. aucheri*. The produced ions helped the identification of the bi-flavone derivatives, including fukugetin (m/z 555; morelloflavone) and 3,8'-biapigenin (m/z 537), which is supported by the reference standards [24, 34]. Based on previous studies, the derivatives of bi-apigenin have also been identified in *Hypericum perforatum* L., Hypericaceae [44].

***Dihydrochalcone, Arylbenzofuran and methoxyphenol derivatives***

The fragment ions obtained from *M. vulgare* and *M. crassidens* were identified as phloretin-3-*O*-xyloglucoside (dihydrochalcone class, m/z 567; m/z 273 [294 AMU], xylose and hexose moieties), which is consistent with the reports by Sobeh et al. (2016) [20]. The hexoside derivative of phloretin is an aglycone reported in *Malus* Mill., Rosaceae [20].

An Arylbenzofuran derivative was first identified in *M. astracanicum* and *B. aucheri*. According to the MS/MS spectra, this derivative was suggested to be conocarpan (m/z 265) [24]. Published reports have indicated that conocarpan is formerly isolated from *Piper* solmsianum C. DC. var. solmsianum, Piperaceae**.** Anti-nociceptive property has been attributed to conocarpan compound [45]**.**

The fragment ions of *M. anisodon* were first characterized as 12-gingerol (phenolic compound, m/z 377) previously assigned to *Zingiber officinale* Roscoe, Zingiberaceae [24, 46]. The gingerol-related compounds have revealed anti-inflammatory and anti-nausea effects [46].

***Polyphenol derivatives***

There is some evidence on glycosylated polyphenolic derivatives in *M. crassidens* and *M. anisodon* (*O*-methylated anthocyanidin). The obtained fragment ions were characterized malvidin. The compound was assigned to malvidin-3-*O*-hexoside (m/z 492; m/z 330 [M-H-162] or *O*-hexose residue) and Malvidin-3,5-di-*O*-hexoside (m/z 654; m/z 330 [M-H-324] or di-*O*-hexoside unit), in line with the results reported in the reference standards [21, 24]. Malvidin-3-*O*-glucoside is the main source of anthocyanidins in Grape skin with an anti-cancer activity [47]. In addition, proanthocyanidin derivative (m/z 561) was identified in *B. aucheri* [21, 24].

Further polyphenols were attributed to hispidin. Based on MS/MS spectra, this compound was identified as 3-hydroxyhispidin-3,4'-di-*O*-glucoside (m/z 585; m/z 233 [M-H-352], di-*O*-glucoside and OH-substitute) compared with the work by Francescato et al. (2013) on *Equisetum giganteum* L., Equisetaceae [48]. The presence of this compound was first reported for *M. vulgare, M. crassidens, M. astracanicum*, and *M. cuneatum*.

The fragment ions of *B. aucheri* were determined as caffeic acid-*O*-glucoside (phenolic acid, m/z 341; m/z 179 [162 AMU] or glucoside moiety), which is consistent with the reference standards of *Rosmarinus officinalis* L., *Origanum vulgare* L., *Salvia officinalis* L., *Ocimum basilicum* L., and *Thymus* L. species, Lamiaceae [16]. The obtained results implied that caffeic acid was not found in *M. vulgare*. It is known as a major source of phenolic acid in different medicinal plants.

***Additional compounds***

In this process, some additional compounds were identified in *Marrubium* and *Ballota* species, including steroid, quinic acid, tannin, and cinnamic acid derivatives.

A steroid derivative (m/z 697) was proposed in *M. anisodon* and *M. crassidens* [24]. However, further experiments have to be conducted in order to identify its structure.

Further steroid compounds were identified in *M. astracanicum* and *B. aucheri,* such as deoxycholic acid (m/z 391; steroids derivatives) [24].

The MS/MS spectrum of *B. aucheri* revealed a quinic acid molecule. This compound was determined as 3-coumaroyl quinic acid (m/z 337; m/z 163 [174 AMU] or coumaroyl moiety), also confirmed by Taamalli et al. (2012) results on *Origanum majorana*, Lamiaceae [18].

The precursor ions of *M. anisodon* were found to have cinnamic acid derivative (m/z 522). This attribution was confirmed by the reference standards in *Mentha pulegium* and *Origanum majorana*, Lamiaceae [18, 24].

The molecular ions of *M. crassidens* were assigned to 6-*O*-coumaroyl-digalloylglucose (tannin derivatives,

m/z 629; m/z 146 [M-H-483] or digalloylglucose residue) [24]. Coumaroyl hexose derivatives have been previously identified for *Salvia officinalis*, Lamiaceae [49].

***Chemotaxonomic markers and botanical perspective***

Based on the chemotaxonomic point of view, the presence of high gene flow, intermediate species, and hybridization at inter-specific levels was recognized for *Marrubium* species [3]. Previous reports have provided evidence on the similarity of pollen characters with color spots of flavonoid data in *Marrubium* and *Ballota* species [3-4]. However, additional research is needed for discriminating the two recent genera. Nonetheless, the present flavonoid compositions are more appropriate to elucidate taxonomic status compared to the color spots in flavonoids. It was observed that a total of 15 chemical compounds of *B. aucheri* were significantly different from *Marrubium* species. The chemical markers found in *Marrubium* and *Ballota* species showed the presence of chemo type in both genera. Despite the striking morphological similarity between *Marrubium* and *Ballota* species, they were accurately separated using chemical markers. As reported in earlier published works, similar flavonoids were observed in *Marrubium* and *Ballota* genera [26]. In addition, the presence of five chemical groups could provide distinctive chemical markers for both genera and its species.

According to previous published works, *M. crassidens* and *M. vulgare* showed morphological similarities and intermediate characters [3]. In our investigation, both species revealed similar compounds, including 3-hydroxy-trimethoxyflavone, phloretin-xyloglucoside, and kaempferol-acetylglucoside. Nevertheless, *M. crassidens* presented seven differentiated chemical compounds. As mentioned above, *M. vulgare* did not show any specific flavonoid compounds. *M. cuneatum* and *M. anisodon* indicated similar characters, including calyx size, equatorial and polar axis of pollen, and seed form [3-5]. Despite the similarities reported, a total of 14 differentiated chemical compounds were identified in *M. anisodon* and *M. cuneatum*. *M. cuneatum* and *M. crassidens* revealed intermediate shape of calyx teeth [3] and a related compound such as epicatechin-gallate. Meanwhile, two species were separated using nine chemical compounds. In *M. astracanicum,* the presence of conocarpan, pinobankasin-gluco-arabino-pyranose, kaempferol-acetylglucoside, and malvidin-hexoside was probably related to polymorphism in its morphological characteristics [1].

It is of note that most flavonoid compounds identified were first reported for *Marrubium* (36 compounds) and *Ballota* species (14 compounds) grown in Iran. Moreover, the phenolic acid, methoxyphenol, tannin, steroid, quinic acid, cinnamic acid, and deoxycholic acid were first identified for *Mrrubium* and *Ballota* species. The flavonoid compounds such as licoricidin, sophoraflavanone G, and methyl robustone were in highly frequent considering qualitative markers for the genus *Marrubium*. In addition, the majority of flavonoid classes belonged to flavones (15), flavonols (13), and isoflavonoids (7).

Micromass Quattro micro API Mass Spectrometer using a number of different types of ionization has allowed the development of HPLC-MS/MS. This ionization technique has greatly extended the range of analysis by mass spectrometry (MS) to compounds with high molecular weight and polar characteristic [50]. This technique is a powerful diagnostic method for separating the constituents of a mixture through the mass to charge ratio. The application of different collision-induced dissociations and negative ionization mode revealed the structures of different chemical compounds, such as flavonoids.

***Conclusions***

The newly presented data concerning *Marrubium* and *Ballota* species could provide critical background for pharmaceutical, phytochemical, and chemo taxonomical fields. The characterization of flavonoid composition in *Marrubium* and *Ballota* species is due to chemical diversity. The identified chemical markers herein certainly facilitate the taxonomy of both genera. In order to elucidate the detailed structure of certain chemical compounds with similar molecular weights or glucoside and galactoside substitutions, other analytical techniques such as NMR method, with a large amount of raw material could be considered in future works.

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