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# Phenolic Profile of *Artemisia alba* Turra

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The aim of this study was to evaluate flower and leaf methanol extracts of *Artemisia alba* Turra for their total phenolic and flavonoid contents, antioxidant capacity and to investigate their phenolic composition. The flower extract was richer in total phenolics and flavonoids and possessed higher antioxidant activity through DPPH and ABTS assays. The UHPLC-PDA-MS analysis of the flower and leaf methanol extracts revealed similar phenolic profile and allowed identification of 31 phenolic compounds (flavonoids, coumarins, and phenolic acids) by comparison with the respective reference compounds or tentatively characterized by their chromatographic behavior, UV patterns, and MS fragmentations. The presence of hispidulin, jaceosidin, desmethoxycentaureidin, and dicaffeoyl esters of quinic acid in *A. alba* is reported herein for the first time. The distribution of flavonoids in *A. alba* from different origins was discussed from chemotaxonomic point of view.

**Keywords:** *Artemisia alba* Turra, UHPLC-PDA-MS, flavonoids, phenolic compounds, antioxidant activity.

## Introduction

*Artemisia alba* Turra (synonyms – *A. lobelii* All., *A. biasolettiana* Vis., *A. suavis* Jord., *A. incanescens* Jord., and *A. camphorata* Vill.) is an essential oil bearing plant distributed widely in the southern and south-eastern parts of Europe.[1] Its decoction has found ethnomedicinal application as a stomach digestive and a tonic in the countries of the Mediterranean region.[2] *A. alba* is considered as an example of a problematic taxon because of its significant morphological variability.[3] Literature survey on the chemical constituents of this species has also shown a great diversity among the populations of the plant. Various sesquiterpenoids,[4 – 9] flavonoids and coumarins[5 – 7][9 – 13] have been detected in *A. alba* from different localities as well as in the species with synonym names *A. lobeli* and *A. incanescens* Jord. It is well-known that phenolic compounds (flavonoids and phenolic acids) possess antioxidant properties and they contribute to reducing the risk of cancer and cardiovascular disease.[14][15] Therefore, in continuation of our previous investigation on the content of sesquiterpenoid compounds in this plant,[8] the present work is focused on the evaluation of antioxidant capacity and the characterization of phenolic compounds in *A. alba*.

## Results and Discussion

Initially, the leaf and flower methanol extracts of *A. alba* were analyzed for their total phenolic and flavonoid contents (*Table 1*). The results were expressed as mg gallic acid and mg catechin equivalents, respectively. Flowers were richer in phenolic compounds and flavonoids (10.7 ± 0.4 mg GAE/g DM and 2.9 ± 0.3 mg CE/g DM, respectively) than leaves (5.4 ± 0.3 mg GAE/g DM and 1.8 ± 0.1 mg CE/g DM, respectively). $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays were used to estimate antioxidant capacity of the extracts and the obtained results were expressed as μM Trolox equivalents per gram of dry plant material (μM TE/g DM). As shown in *Table 1*, the *A. alba* flower extract possessed higher radical scavenging activity (101.6 ± 0.8 and 102.2 ± 0.7 μM TE/g DM, respectively) than the leaf extract in both assays. The results in the present work showed that *A. alba* samples possess a moderate radical scavenging capacity, corresponding to the total phenolic and flavonoid content of the two types of extracts assayed. Therefore, further research was performed in order to elucidate the phytochemical constituents with phenolic and flavonoid structure present in the plant.

UHPLC-PDA-MS studies of leaf and flower methanol extracts of *A. alba* did not show any significant

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**Table 1.** Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity of *A. alba* extracts (values are means ± SD)

| Sample | TPC | TFC | Antioxidant capacity [μM TE/g DM] | |
|---|---|---|---|---|
| | [mg GAE/g DM] | [mg CE/g DM] | ABTS | DPPH |
| Flowers | 10.7 ± 0.4 | 2.9 ± 0.3 | 102.2 ± 0.7 | 101.6 ± 0.8 |
| Leaves | 5.4 ± 0.3 | 1.8 ± 0.1 | 70.5 ± 0.8 | 63.4 ± 0.9 |

qualitative differences (*Figure 1*) and resulted in the identification of 31 compounds, comprising caffeoyl and feruloyl quinic acids, coumarins, and flavonoids (*Table 2*). Caffeoylquinic acids (peaks **1**, **3**, and **11** – **14**) exhibited the UV absorption maximum at 324.9 or 326.1 nm, and based on their parent ions in the negative-ion mode ($[M-H]^-$ at *m/z* 353.3 and 515.4), were classified into two groups: mono- (peaks **1** and **3**), and dicaffeoyl (peaks **11** – **14**) esters of quinic acid. Fragment ions at *m/z* 181.1 ($[C_9H_8O_4+H]^+$) and 163.1 ($[M+H-C_7H_{12}O_6]^+$), corresponding to the protonated caffeoyl acid group and a cleavage of quinic acid, were also detected in the positive-ion mode of mono- and dicaffeoylquinic acids (DCQA). In addition, the sodium adduct at *m/z* 539.2 ($[M+Na]^+$) and the ion at *m/z* 499.3, which was formed by water elimination from the protonated molecule $[M+H-H_2O]^+$, were the most intensive peaks in the spectra of dicaffeoylquinic acids. The presence of chlorogenic acid (peak **3**), 3,4-DCQA (peak **11**), 3,5-DCQA (peak **12**), and 4,5-DCQA (peak **14**) was confirmed by comparison of their retention times, UV absorption maxima, and mass spectra with that of commercial standards. Furthermore, two feruloylquinic acid isomers (peaks **2** and **4**) were tentatively identified by their deprotonated ion $[M-H]^-$ at *m/z* 367.4 in the negative-ion mode as well as by fragment ions at *m/z* 195.1 ([ferulic acid + H]$^+$) and 177.1 ([M + H – quinic acid]$^+$) in the positive-ion-mode MS.[16][17]

The presence of umbeliferone and scopoletin (peaks **5** and **6**) was deduced from their UV maxima and corresponding pattern ions $[M-H]^-$ and $[M+H]^+$ in the respective negative- and positive-ion modes (*Table 2*) and their comparison with authentic standards and literature data.[17][18] The characteristic absorption maximum at 330 – 360 nm observed by the PDA detector indicated the presence of flavonoids. They were divided in two groups: flavonoid glycosides (peaks **7** – **10**) and aglycones (**15** – **31**) according to their parent ions in the negative-ion mode. Thus, the

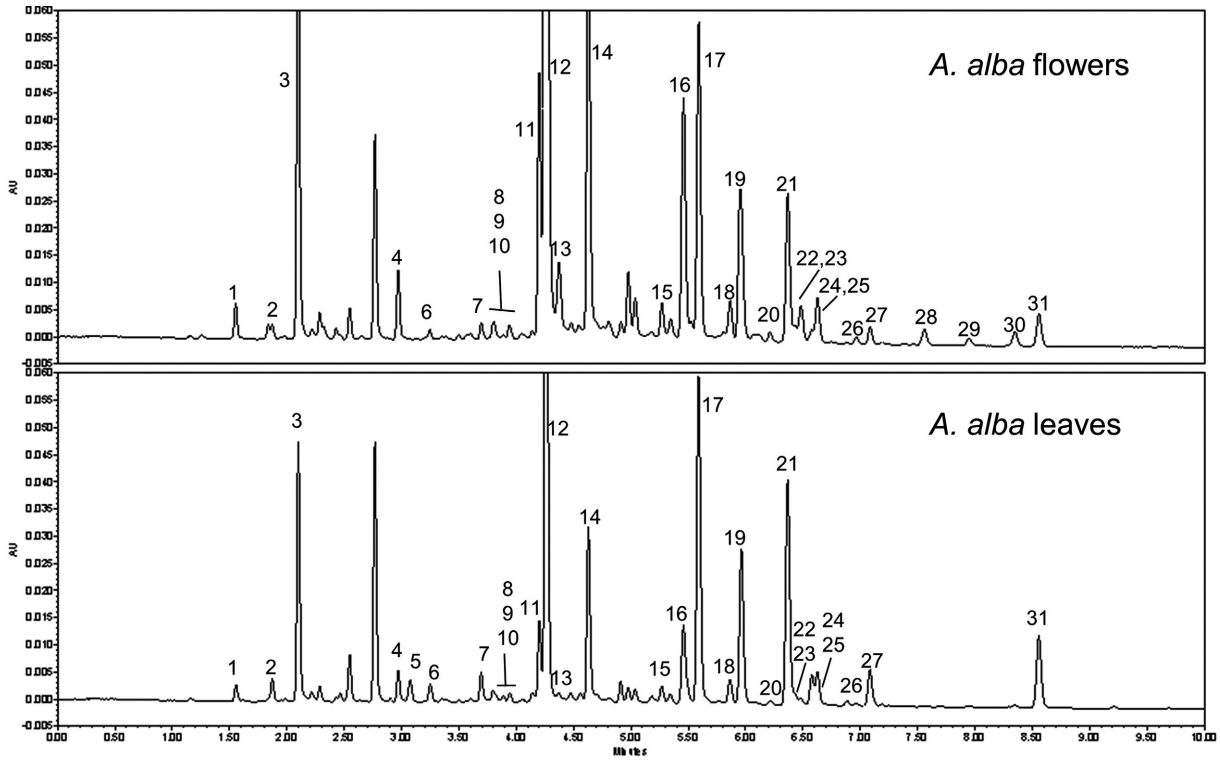



**Figure 1.** UHPLC-DAD chromatograms at 340 nm of *A. alba* extracts. Peak numbers correspond to those of the compounds listed in *Table 2*.


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**Table 2.** Phenolic components in *A. alba* leaf and flower extracts identified by UHPLC-PDA-MS

| Peak | $t_R$ [min] | Compound[a] | Molecular formula | UV max [nm] | (−) MS fragment ions [*m/z*] | (+) MS fragment ions [*m/z*] |
|---|---|---|---|---|---|---|
| 1 | 1.55 | Chlorogenic acid – isomer[b] | $C_{16}H_{18}O_9$ | 324.9 | 353.3 ($[M-H]^-$) | 181.1 ($[C_9H_8O_4+H]^+$), 163.1 ($[M+H-C_7H_{12}O_6]^+$) |
| 2 | 1.84 | Feruloylquinic acid – isomer[b] | $C_{17}H_{20}O_9$ | 324.9 | 367.4 ($[M-H]^-$) | 369.4 ($[M+H]^+$) |
| 3 | 2.1 | Chlorogenic acid | $C_{16}H_{18}O_9$ | 324.9 | 353.3 ($[M-H]^-$) | 377.3 ($[M+Na]^+$), 181.1, 163.1 |
| 4 | 2.97 | Feruloylquinic acid – isomer[b] | $C_{17}H_{20}O_9$ | 326.1 | 367.4 ($[M-H]^-$) | 391.4 ($[M+Na]^+$), 195.1 ([ferulic acid + H]$^+$), 177.1 ($[M-C_7H_{12}O_6O]^+$) |
| 5 | 3.08 | Umbeliferone[b] | $C_9H_6O_3$ | 323.7 | 161.1 ($[M-H]^-$) | 163.1 ($[M+H]^+$) |
| 6 | 3.25 | Scopoletin[b] | $C_{10}H_8O_4$ | 336.8 | 191.3 ($[M-H]^-$) | 193.0 ($[M+H]^+$) |
| 7 | 3.70 | Rutin | $C_{27}H_{30}O_{16}$ | 254.7, 353.1 | 609.3 ($[M-H]^-$) | 633.4 ($[M+Na]^+$), 465.3 ($[M+H-C_6H_{10}O_4]^+$), 303.2 ([quercetin + H]$^+$) |
| 8 | 3.80 | Quercetin hexoside[b] | $C_{21}H_{19}O_{12}$ | 253.5, 342.5 | 463.3 ($[M-H]^-$) | 487.2 ($[M+Na]^+$), 303.1 ([quercetin + H]$^+$) |
| 9 | 3.88 | Apigenin glucuronide[b] | $C_{21}H_{18}O_{11}$ | 252.3, 347.1 | 447.2 ($[M-H]^-$) | 449.4 ($[M+H]^+$), 271.3 ([apigenin + H]$^+$) |
| 10 | 3.94 | Quercetin dimethyl ether hexoside[b] | $C_{23}H_{24}O_{12}$ | 254.7, 342.5 | 493.4 ($[M-H]^-$) | 517.2 ($[M+Na]^+$), 333.1 ([M + H – hexoside]$^+$) |
| 11 | 4.20 | 3,4-Dicaffeoylquinic acid | $C_{25}H_{24}O_{12}$ | 324.9 | 515.4 ($[M-H]^-$) | 539.2 ($[M+Na]^+$), 181.2, 163.1 |
| 12 | 4.26 | 3,5-Dicaffeoylquinic acid | $C_{25}H_{24}O_{12}$ | 326.1 | 515.4 ($[M-H]^-$), 353.2 ($[M-C_9H_6O_3]^-$) | 539.2 ($[M+Na]^+$), 499.3 ($[M+H-H_2O]^+$), 181.2, 163.1 |
| 13[b] | 4.38 | Dicaffeoylquinic acid – isomer | $C_{25}H_{24}O_{12}$ | 324.9 | 515.4 ($[M-H]^-$), 353.2 ($[M-C_9H_6O_3]^-$) | 539.2 ($[M+Na]^+$), 499.3 ($[M+H-H_2O]^+$), 181.2, 163.1 |
| 14 | 4.63 | 4,5-Dicaffeoylquinic acid | $C_{25}H_{24}O_{12}$ | 326.1 | 515.4 ($[M-H]^-$), 353.2 ($[M-C_9H_6O_3]^-$) | 539.2 ($[M+Na]^+$), 499.3 ($[M+H-H_2O]^+$), 181.2, 163.1 |
| 15 | 5.28 | Eriodictyol[b] | $C_{15}H_{12}O_6$ | 289.1, 324.9 | 287.2 ($[M-H]^-$) | 289.1 ($[M+H]^+$) |
| 16 | 5.46 | Luteolin | $C_{15}H_{10}O_6$ | 348.3 | 285.2 ($[M-H]^-$) | 287.1 ($[M+H]^+$) |
| 17 | 5.59 | Nepetin | $C_{16}H_{12}O_7$ | 346 | 315.1 ($[M-H]^-$) | 317.1 ($[M+H]^+$) |
| 18 | 5.87 | Quercetin 3-methyl ether | $C_{16}H_{12}O_7$ | 254.7, 356.7 | 315.2 ($[M-H]^-$) | 317.1 ($[M+H]^+$) |
| 19 | 5.96 | Axillarin | $C_{17}H_{14}O_8$ | 348.3 | 345.3 ($[M-H]^-$) | 347.1 ($[M+H]^+$) |
| 20 | 6.21 | Apigenin | $C_{15}H_{10}O_5$ | 334.5 | 269.3 ($[M-H]^-$) | 271.3 ($[M+H]^+$) |
| 21 | 6.37 | Hispidulin | $C_{16}H_{12}O_6$ | 335.7 | 299.2 ($[M-H]^-$) | 301.1 ($[M+H]^+$) |
| 22 | 6.43 | Diosmetin | $C_{16}H_{12}O_6$ | 335.7 | 299.3 ($[M-H]^-$) | 301.1 ($[M+H]^+$) |
| 23 | 6.48 | Chrysoeriol | $C_{16}H_{12}O_6$ | 339.15 | 299.2 ($[M-H]^-$) | 301.1 ($[M+H]^+$) |
| 24 | 6.58 | Desmethoxycentaureidin | $C_{17}H_{14}O_7$ | 342.5 | 329.1 ($[M-H]^-$) | 331.1 ($[M+H]^+$) |
| 25 | 6.63 | Jaceosidin | $C_{17}H_{14}O_7$ | 344.8 | 329.1 ($[M-H]^-$) | 331.1 ($[M+H]^+$) |
| 26 | 6.97 | Quercetin 3,3′-dimethyl ether | $C_{17}H_{14}O_7$ | 257.1, 354.3 | 329.3 ($[M-H]^-$) | 331.1 ($[M+H]^+$) |
| 27 | 7.08 | Centaureidin | $C_{18}H_{16}O_8$ | 254.7, 348.3 | 359.3 ($[M-H]^-$) | 361.2 ($[M+H]^+$) |
| 28 | 7.56 | Methoxyquercetin[b] | $C_{16}H_{12}O_7$ | 255.9, 360.3 | 315.3 ($[M-H]^-$) | |
| 29 | 7.95 | Dimethoxyquercetin[b] | $C_{17}H_{14}O_7$ | 255.9, 355.5 | 329.4 ($[M-H]^-$) | 331.1 ($[M+H]^+$) |
| 30 | 8.34 | Methoxyapigenin[b] | $C_{16}H_{12}O_5$ | 334.5 | 283.1 ($[M-H]^-$) | 285.1 ($[M+H]^+$) |
| 31 | 8.56 | Dimethoxyapigenin[b] | $C_{17}H_{14}O_6$ | 273.7, 333.3 | 313.3 ($[M-H]^-$) | 315.2 ($[M+H]^+$) |

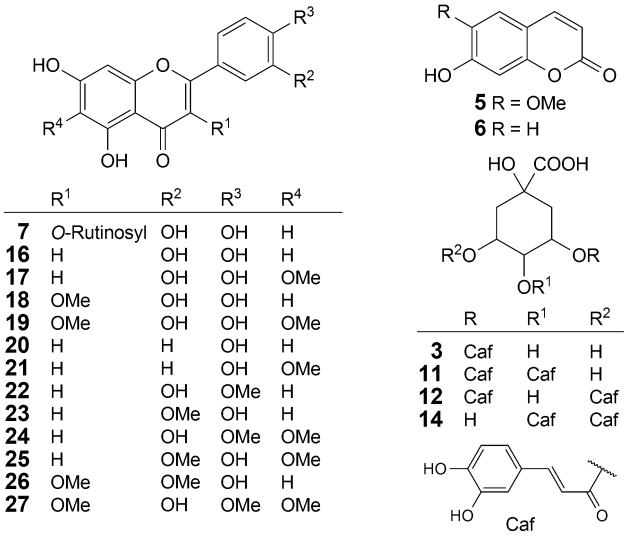
[a] All identified compounds were detected in both *A. alba* extracts. [b] Compounds were identified and/or tentatively assigned by comparison of their data with literature data as follows: **1**, **2**, **4**, and **13**[16][17]; **8** – **10**[17][18]; **15**[19]; **28** – **31**.[20][21]

pseudo-molecular ion $[M-H]^-$ at *m/z* 609.3 (peak **7**), and sodium adduct $[M+Na]^+$ at *m/z* 633.4 revealed the presence of flavonoid diglycoside. The ions $[M+H-146]^+$ and [aglycone + H]$^+$ at *m/z* 465.3 and 303.2 were a result from subsequent elimination of rhamnosyl (146 Da) and hexosyl (162 Da) units. The ion at *m/z* 303.2 ($[M+H]^+$) clearly indicated a pentahydroxyflavone (quercetin) structure. Comparison with authentic standard allowed the identification of rutin (peak **7**). The deprotonated molecular ions $[M-H]^-$ at *m/z* 463.3, 447.2, and 493.4 (peaks **8** – **10**) indicated the presence of flavonoid glycosides. The intensive fragment ions at *m/z* 303.1, 271.3, and 333.1 corresponded to pentahydroxy-, trihydroxy-,


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and dimethoxytrihydroxy-flavonoid aglycones, respectively. These ions were formed by the loss of hexosyl (162 Da; peaks **8** and **10**) or glucuronyl (176 Da; peak **9**) units from the corresponding protonated molecular ions. Thus, peaks **8** – **10** were tentatively identified as quercetin-*O*-hexoside, apigenin-*O*-glucuronide, and dimethoxyquercetin-*O*-hexoside, respectively.[17][18] The identification of the flavonoid aglycones was partly hampered, as some of them possessed similar UV and MS characteristics. Thus, the parent ions $[M-H]^-/[M+H]^+$ at *m/z* 315/317 (peaks **17**, **18**, and **28**), *m/z* 299/301 (peaks **21** – **23**), and *m/z* 329/331 (peaks **24** – **26** and **29**) indicated the presence of positional isomers. For the correct determination of peaks **16** – **27**, the corresponding components were isolated and thereafter used as reference standards. In addition, five compounds (peaks **15** and **28** – **31**) were tentatively assigned by comparison of their UV and MS data with reported data.[19 – 21]

The compounds, corresponding to the peaks **3**, **5** – **7**, **12**, **14**, and **16** – **27** (*Table 2*) were isolated from the methanol extract of *A. alba* by column chromatography (CC) on Sephadex LH-20 and subsequent CC and PTLC of the obtained fractions (see Experimental Section). The identification of chlorogenic acid,[16] 3,5- and 4,5-dicaffeoylquinic acids,[16][17][22] scopoletin,[18][23] umbeliferone,[18][23] luteolin,[24][25] nepetin,[26] quercetin 3-methyl ether,[10][27] axillarin,[10][28] apigenin,[21][25] hispidulin,[29][30] diosmetin,[24][29] chrysoeriol,[24][29] desmethoxycentaureidin,[30] jaceosidin,[31] quercetin 3,3′-dimethyl ether,[20] centaureidin,[10][32] and rutin[25] was achieved by comparison of their $^1$H-NMR, MS, and UV spectral data with those published in the literature and/or with authentic standards. Their structures are presented in *Figure 2*. As can be seen, *A. alba* extracts contained predominately 3′- or 4′-methoxyflavones and 3-methoxy or glycosyl substituted flavonols. The lack of 3′,4′-dihydroxy groups in the B ring in most components and/or 3-OH group, which is important for the effective radical scavenging activity[33] could explain the moderate activity of the extracts against DPPH and ABTS radicals (*Table 1*). Chlorogenic acid (**3**) has been detected recently in *A. alba* from Serbia[13] and Italy,[9] while the presence of dicaffeoylquinic acids in this species is described now for the first time. Dicaffeoylquinic acids are widely distributed through the representatives of the Asteraceae family and probably play significant role for their antioxidant activity.[14] The coumarins umbeliferone (**5**) and scopoletin (**6**) have been previously isolated from *A. alba* from Italy[7] as well as from *A. lobeli*[5] and *A. incanescens* Jord.[10]




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **7** | *O*-Rutinosyl | OH | OH | H |
| **16** | H | OH | OH | H |
| **17** | H | OH | OH | OMe |
| **18** | OMe | OH | OH | H |
| **19** | OMe | OH | OH | OMe |
| **20** | H | H | OH | H |
| **21** | H | H | OH | OMe |
| **22** | H | OH | OMe | H |
| **23** | H | OMe | OH | H |
| **24** | H | OH | OMe | OMe |
| **25** | H | OMe | OH | OMe |
| **26** | OMe | OMe | OH | H |
| **27** | OMe | OH | OMe | OMe |

| | R | $R^1$ | $R^2$ |
|---|---|---|---|
| **3** | Caf | H | H |
| **11** | Caf | Caf | H |
| **12** | Caf | H | Caf |
| **14** | H | Caf | Caf |

**Figure 2.** Structures of isolated and identified compounds. Compound numbers correspond to those of the peaks listed in *Table 2*.

Flavonoids are the most thoroughly studied compounds in *A. alba*. The data obtained in this study were compared with previously reported data for the species from different localities as well as from the species with synonym name *A. incanescens* Jord. (*Table 3*). As can be seen, the *A. alba* extract investigated in the present work was characterized by a domination of aglycones rather than glycosides, similarly to that originating from Macedonia.[12] Both samples contained flavone (luteolin) and flavonol (quercetin) types of compounds. Apigenin, luteolin, and rutin were common for Bulgarian and Serbian[13] samples. Quercetin 3-methyl ether, centaureidin, and axillarin have been detected in *A. incanescens* Jord,[10] while chrysoeriol – in *A. alba* from Sicily.[7] Hispidulin, jaceosidin, and desmethoxycentaureidin have been now found for the first time in *A. alba*. The presence of apigenin, luteolin, kaempferol, and quercetin types of flavonoids in *A. alba* is not surprising, as they have been observed in many species of the genus *Artemisia*.[34] Further, the accumulation of glycosides distinguished the Serbian *A. alba*[13] from the other Balkan samples (Macedonia and Bulgaria) and made it slightly closer to those from Spain[10][11] and Italy.[7][9] However, the presence of flavone besides flavonol glycosides differentiated the Serbian sample from those from Mediterranean area, which contained only flavonol glycosides. Moreover, kaempferol glycosides dominated in Serbian sample, while quercetin glycosides seemed to be typical for the samples from Spain and Italy. In fact, the taxons from Mediterranean region produced flavonol type of compounds only.


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**Table 3.** Distribution of flavonoids in *A. alba* from different localities (literature data and present results)

| Compound | Type[a] | Sample | | | | | |
|---|---|---|---|---|---|---|---|
| | | *A. incanescens*, Spain[10][11] | *A. alba*, Sicily, Italy[7] | *A. alba*, Verona, Italy[9] | *A. alba*, Macedonia[12] | *A. alba*, Serbia[13] | *A. alba*, grown in Bulgaria (present results) |
| Flavones | | | | | | | |
| Apigenin | Api | | | | | +[b] | + |
| Hispidulin | Api | | | | | | + |
| Luteolin | Lu | | | | | + | + |
| Chrysoeriol | Lu | | + | | + | | + |
| Diosmetin | Lu | | | | + | | + |
| 6-Methoxyluteolin | Lu | | | | + | | + |
| Jaceosidin | Lu | | | | | | + |
| Desmethoxycentaureidin | Lu | | | | | | + |
| Flavonols | | | | | | | |
| Kaempferol | K | + | | + | | | |
| 6-Methoxykaempferol | K | + | | | | | |
| Santin | K | + | | | | | |
| Penduletin | K | + | | | | | |
| Quercetin | Qu | + | + | | | | |
| Quercetin 3-methyl ether | Qu | + | | | + | | + |
| Isorhamnethin | Qu | + | + | + | | | |
| Quercetin 3,3′-dimethyl ether | Qu | | | | + | | + |
| Quercetin 3,4′-dimethyl ether | Qu | + | | | | | |
| 3,6,7-Trimethoxyquercetagetin | Qu | | | | + | | |
| Centaureidin | Qu | + | | | | | + |
| Axillarin | Qu | + | | | | | + |
| Casticin | Qu | + | | | | | |
| Flavone glycosides | | | | | | | |
| Genkwanin-5-*O*-glucoside | Api | | | | | + | |
| Luteolin-5-*O*-glucoside | Lu | | | | | + | |
| Luteolin-5-*O*-(6″-*O*-malonylglucoside) | Lu | | | | | + | |
| Flavonol glycosides | | | | | | | |
| Kaempferol-3-*O*-glucopyranoside | K | + | | + | | | |
| Kaempferol-3-*O*-rhamnoside | K | | | | | + | |
| Kaempferol-7-*O*-rhamnoside | K | | | | | + | |
| Kaempferol-3,7-*O*-diglucoside | K | | | | | + | |
| Kaempferol-3-*O*-rhamnoside | | + | | | | | |
| Kaempferol-3-*O*-(6″-*O*-malonylglucoside) | K | | | | | + | |
| Kaempferol-3-*O*-(6″-*O*-malonylglucoside)-7-*O*-rhamnoside) | K | | | | | + | |
| Quercetin-3-*O*-glucoside | Qu | + | | + | | | |
| Quercetin-3-*O*-galactoside | Qu | + | | | | | |
| Rutin | Qu | + | | + | | + | + |
| Isorhamnetin-3-*O*-glucopyranoside | Qu | + | | + | | | |
| Isorhamnetin-3-*O*-rutinoside | Qu | | | + | | | |

[a] Api, Lu, K, and Qu – apigenin, luteolin, kaempferol, and quercetin type, respectively. [b] +, detected in the sample.

## Conclusions

In this study, 31 phenolic compounds (coumarins, flavonoids, and esters of quinic acid) were identified in *A. alba* by UHPLC-PDA-MS analysis. Although the differences in the methods of isolation and analysis pointed out in the literature, comparison of the flavonoid profiles of *A. alba* studied so far revealed that the samples from the Balkan Peninsula produced both flavones and flavonol types of compounds in contrary to those from the Mediterranean countries, which contained only flavonol type. Therefore, a deeper study of the flavonoids in *A. alba* in the future will contribute to the knowledge on the


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intraspecific variability of this taxonomically doubtful species.

## Experimental Section

### *Solvents, Reagents, and Samples*

Acetonitrile (LC-MS grade, VWR International GmbH, Switzerland), formic acid (ACS reagent Carlo Erba, Thommen–Furler AG, Switzerland), and $H_2O$ purified by installed purification system Sartorius stedim, arium pro VF, 0.055 μS/cm were used for the UHPLC analyses. All other solvents were analytical grade and purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent (Merck KGaA, Darmstadt, Germany), DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), potassium peroxodisulfate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and Natural Product Reagent (NP, diphenylboric acid 2-aminoethyl ester) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA, USA), polyethylene glycol 4000 (PEG), anhydrous $AlCl_3$, NaOH and anhydrous $Na_2CO_3$ (Carl Roth GmbH, Karlsruhe, Germany), and $NaNO_2$ (Merck, Darmstadt, Germany). Rutin, chlorogenic acid, 3,5-dicaffeoylquinic acid (3,5-DCQA), 3,4-dicaffeoylquinic acid (3,4-DCQA), 4,5-dicaffeoylquinic acid (4,5-DCQA), luteolin, and apigenin were obtained from Phytolab (Vestenbergsgreuth, Germany). Scopoletin, umbelliferon, nepetin, quercetin 3-methyl ether, axillarin, hispidulin, diosmetin, chrysoeriol, desmethoxycentaureidin, jaceosidin, quercetin 3,3′-dimethyl ether, and centaureidin were isolated from *A. alba* as described in the part of Extraction and Isolation of Individual Compounds.

### *Plant Material*

*A. alba* was cultivated in the Experimental Field of the Institute of Biodiversity and Ecosystem Research near Sofia (Bulgaria) using seeds originating from Turkey. The aerial parts were collected in August 2016 in full flowering stage. The species was provided by Dr. L. Evstatieva. A voucher specimen (SOM-167590) has been deposited with the Herbarium of the Institute of Biodiversity and Ecosystem Research, Sofia, Bulgaria.

### *Sample Preparation*

The air-dried and ground leaves and flowers of *A. alba* were separately extracted with methanol (2 × 25 mL) at room temperature for 30 min in an ultrasonic bath. The obtained methanol extracts were concentrated under reduced pressure and kept at 4 °C. Samples of leaf and flower extracts were dissolved in MeOH to form a solution of 1 mg/mL and the corresponding solutions were used for total phenolic and flavonoid determinations, for DPPH and ABTS assays as well as for UHPLC study, Solutions of the pure compounds were prepared at a concentration of 0.2 mg/mL in MeOH. The sample solutions were filtered through a 0.45 μm filter prior UHPLC analysis.

### *Determination of Total Phenolic Content* (TPC) *and Total Flavonoid Content* (TFC)

Total phenolic content (TPC) was measured using Folin–Ciocalteu method.[35] Gallic acid was used as a standard compound and TPC was expressed as mg gallic acid equivalents (GAE) per 1 g of dry plant material. Total flavonoid content (TFC) was measured using a colorimetric assay developed previously.[35] (+)-Catechin was used as a standard compound and TFC was expressed as mg catechin equivalents per 1 g of dry plant material.

### *Determination of Antioxidant Capacity*

DPPH and ABTS radical scavenging activities were determined according to the methods described previously.[36] Results were expressed as Trolox equivalent antioxidant capacity (μM Trolox equivalents per gram plant material, μM TE/g DM), using calibration curve (absorption *vs.* concentration) of Trolox dissolved in methanol at different concentrations.

### *Ultra High Performance Liquid Chromatography* (UHPLC)

Chromatography was performed on 2 μL samples on a 2.1 × 50 mm Acquity BEH $C_{18}$ (Waters, Milford, USA) column filled with 1.7 μm particles on an Acquity UPLC® (Waters, Milford, USA) system equipped with an Acquity eLambda PDA (UV/VIS) detector and an Acquity QDa (SQ-ESI-MS) detector (both Waters, Milford, USA). An Acquity ISM (Waters, Milford, USA) isocratic pump was used to introduce a makeup solvent before the MS detector. The gradient separation was performed at 30 °C with 0.1% formic acid in water and acetonitrile as the mobile phases A and B, respectively, and a flow rate of 0.6 mL/min. The separation was done by the following linear gradient (min/% B): 0/1; 22.50/99.9, followed by a suitable equilibration step on initial conditions. The effluent after the column was split by a ratio of 9/1 and the bigger fraction directed to the PDA, the lower one towards the MS detector. The UV/VIS signal was recorded in the range of 200 – 800 nm with a scan


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rate of 20 Hz. Before entering the ESI source, the effluent was combined with 0.2 ml/min of a mixture of 0.05% formic acid in acetonitrile/water 50/50 (v/v) to enhance the ionization. The MS capillary was heated to 600 °C and a voltage of ± 800 V was applied, whereas the cone voltage was set to ± 15 V for both positive and negative recording in the range of 100 – 1000 Da.

*Extraction and Isolation of Individual Compounds*

The air-dried aerial parts (70 g) from *A. alba* were extracted exhaustively with methanol (2 × 1 L) at room temperature for 24 h each. After filtration, the solvent from the combined extracts was evaporated under vacuum to give the crude methanol extract (3.25 g). A portion of the methanol extract (2.5 g) was dissolved in MeOH (15 mL) and subjected to a Sephadex LH-20 column (equilibrated with MeOH) to give 12 fractions, Frs. 1 – 12. TLC comparison was performed on silica gel ($CHCl_3$/MeOH, 10:1; toluene/dioxane/AcOH, 90:25:4 and AcOEt/HCOOH/AcOH/$H_2O$, 100:11:11:26), spraying with NP/PEG reagent and UV visualization at 366 nm. Frs. 4 – 12, containing phenolics, were further worked-up for isolation of the individual compounds. MPLC on LiChroprep RP-18 and eluted with increasing concentrations of MeOH in $H_2O$ (from 20 to 80%) of Fr. 4 (0.145 g) afforded a mixture of 3,5- and 4,5-dicaffeoylquinic acid (3.8 mg) and chlorogenic acid (2.5 mg). Prep. TLC (silica gel, $CHCl_3$/MeOH, 10:1) of Fr. 5 (0.036 g) gave scopoletin (1 mg) and umbeliferone (1.8 mg). Centaureidin (2.8 mg) and axillarin (2.3 mg) were obtained from Frs. 6 (0.038 g) and 7 (0.025 g), respectively, by prep. TLC (silica gel, $CHCl_3$/MeOH, 10:1, two developments). Fr. 8 (0.083 g) was applied to MPLC on LiChroprep RP-18 and eluted with increasing concentrations of MeOH in $H_2O$ (from 20 to 80%) to give subfrs. 8.1 – 8.4. Further prep. TLC (silica gel, $CHCl_3$/MeOH, 10:1, two developments) of subfrs. 8.1 (0.022 g) and 8.3 (0.010 g) yielded desmethoxycentaureidin (1.2 mg), jaceosidin (1.2 mg), hispidulin (1.0 mg), and nepetin (2.2 mg). Prep. TLC (silica gel, $CHCl_3$/MeOH, 10:1, two developments) of Fr. 9 (0.015 g) gave diosmetin (0.8 mg) and chrysoeriol (1.0 mg). Prep. TLC (silica gel, $CHCl_3$/MeOH, 10:1, two developments) of Fr. 10 (0.023 g) yielded quercetin 3,3′-dimethyl ether (1.1 mg) and quercetin 3-methyl ether (2.6 mg). Prep. TLC (RP-18, $H_2O$/MeOH, 1:1, two developments) of Fr. 11 (0.043 g) afforded apigenin (1.1 mg) and luteolin (4.3 mg). All isolated compounds were identified by comparison of their spectral data ($^1$H-NMR, MS, and UV) with literature data.

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## Author Contribution Statement

*A. T.* – identification of compounds, interpretation of all available data (NMR, MS, UV), total phenolic and flavonoid content determinations and antioxidant activity, discussion, preparation and writing the manuscript. *M. T.* supervising all experiments on extraction and isolation of individual compounds, identification of compounds, and discussion on the manuscript. *V. I.* extraction and isolation of individual compounds. *S. P.*, and *E. W.* UHPLC analysis. *K. D.* total phenolic and flavonoid content determinations, discussion on the manuscript. *L. E.* providing plant material.

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