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Phytochemical Constituents and Biological Activities of the Unexplored Plant *Rhinanthus angustifolius* subsp. *grandiflorus*

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Abstract: In the present study, a total of 12 extracts of *Rhinanthus angustifolius* subsp. *grandiflorus*, an understudied hemiparasitic species, were obtained using different extraction techniques, namely, homogenizer-assisted extraction (HAE), maceration (MAC), soxhlet (SOX), infusion, and solvents (ethyl acetate, methanol, ethanol, and water), and were evaluated for their in vitro antioxidant and enzyme-inhibiting properties. Additionally, untargeted profiling based on high-resolution mass spectrometry targeted different phytochemical classes, namely, polyphenols, terpenoids, and alkaloids. The highest total phenolic and flavonoid contents were detected using methanol as the extraction solvent. Multivariate statistics following the untargeted profiling revealed that the extraction solvent had a hierarchically higher impact than the extraction method when considering the recovery of bioactive compounds. The methanolic extracts displayed the highest radical-scavenging antioxidant capacity, as provided by CUPRAC and FRAP assays. On the other hand, the water extracts (MAC and HAE) and the infusion extract showed the highest activity as metal chelators (25.66–27.51 mg EDTAE/g). Similarly, the water extract obtained by HAE and the infusion extract revealed the highest phosphomolybdenum activity (3.92 ± 0.14 and 3.71 ± 0.01 mmol TE/g, respectively). The different extracts also exhibited different enzyme inhibition potentials. For instance, HAE and MAC ethanolic extracts inhibited only α-amylase (0.69 ± 0.01 and 0.70 ± 0.01 mmol ACAE/g), while all the other extracts showed a dual inhibition against both carbohydrate-hydrolyzing enzymes tested (i.e., α-amylase: 0.07–0.69 mmol ACAE/g; α-glucosidase: 0.03–1.30 mmol ACAE/g). Nevertheless, the other extracts inhibited acetyl-, butyryl-cholinesterases, or both; MAC–water extract displayed no inhibition against the enzymes. Additionally, all the studied extracts were found to inhibit tyrosinase, ranging from 10.62 to 52.80 mg KAE/g. In general, the water extracts showed weaker inhibition towards the enzymes than the other extracts. This study demonstrated that *R. angustifolius* is an excellent source of natural antioxidants and enzyme inhibitors that could be further investigated and exploited for pharmaceutical purposes.

Keywords: *Rhinanthus angustifolius*; solvent extraction; phenolics/flavonoids; antioxidants; enzyme inhibitors; UHPLC-QTOF-MS; metabolomics

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1. Introduction

The genus *Rhinanthus* (rattle) from the family of Orobanchaceae is comprised of around 30–40 annual species of hemiparasitic annual herbs. Most species are prevalent

in Europe, and about ten species are endemic [1]. Some *Rhinanthus* species are used in folk medicine for treating eye complaints caused by certain bacteria [2], while others are used against stomach diseases or have been ethnobotanically documented to be consumed as a tea against cold [3]. Among the different species reported, *R. angustifolius* has been previously used for external baths for treating eczema [4]. Nevertheless, the flowers of *R. angustifolius* are used as a medicine for treating ear complaints by people in Anatolia [2].

According to scientific literature, *Rhinanthus* species are rich in bioactive compounds such as anthocyanins, flavonoids, anthraquinones, and saponins, characterized by several biological activities [2,3]. For instance, a new iridoid glucoside, namely, 6′-*O*-benzoylshanzhiside methyl ester, together with other known compounds such as aucubin, melampyroside, mussaenoside, shanzhiside methyl ester, 8-epiloganin, gardoside methyl ester, and 2-(4-hydroxyphenyl) ethylalcohol were previously isolated from the aerial parts of *R. angustifolius* subsp. *grandiflorus* [5]. Additionally, the methanolic extract from *R. angustifolius* has been established to possess broad-spectrum activity against Gram-positive and Gram-negative bacteria [6]. Anti-tumor activity was also demonstrated by *R. angustifolius* extracts in the same study [6]. Additionally, the essential oil of *R. angustifolius*, containing major compounds 2,3-dihydro-5-methyl-1H-indene, α-cubebene, 1-hexadecene, and hexadecanoic acid, was found to exhibit antimicrobial activity [2]. Plant species such as *R. angustifolius* are included in the group of hemiparasites. Hemiparasitism is a special form of life whereby the parasite retains a certain degree of independence (being able to photosynthesize) but still depends on its host(s) for maximum performance [7]. Hemiparasitic plants are typically seen as destructive and unpleasant plants, although they have been proposed as ornamental and medicinal plants. In fact, many hemiparasitic plants are used for medicinal purposes in numerous parts of the globe. Many of their preparations in the form of injectable extracts, tinctures, infusions, fluid extracts, or tea bags are extensively used in a variety of cultures and almost every continent for treating and managing health complications, including hypertension, epilepsy, inflammation, diabetes, menopause, irregular menstruations, arthritis, and cancer, amongst others. Some pharmacological studies conducted on such plants' extracts and purified fractions have also revealed their hypoglycemic, hypotensive, antilipidemic, anti-inflammatory, antioxidative, as well as antimicrobial effects, and were safe in experimental animals at the tested doses [8]. However, very little attention has been given to *Rhinanthus*, as a genus of hemiparasitic plants, including *R. angustifolius*.

Therefore, the present study attempted to compare the biological activities of 12 *R. angustifolius* plant extracts prepared using different solvents (i.e., ethyl acetate, methanol, ethanol, and water) and extraction techniques (i.e., homogenizer-assisted extraction, maceration, soxhlet, and infusion). Besides, the untargeted profiling of bioactive metabolites was investigated to find out the best method/solvent of extraction for nutraceutical purposes. Moreover, the antioxidant properties and their antidiabetic, anti-hyperpigmentation, and anti-neurodegenerative effects in terms of their enzyme-inhibitory properties against α-amylase, α-glucosidase, tyrosinase, and acetyl-and butyryl-cholinesterase of *R. angustifolius* were also investigated.

2. Materials and Methods

2.1. Plant Material

Rhinanthus angustifolius subsp. grandiflorus samples were collected in Kastamonu (Taşköprü village, Turkey) in July 2020. The plants were authenticated by a plant taxonomist (Dr. Ismail Senkardes) in Pharmacy Faculty, Marmara University, Istanbul, Turkey, and voucher specimens (voucher number: MARE-22433) were kept at the herbarium of the faculty mentioned above. After that, the aerial parts were separated and dried in the shade for ten days. After the drying process, they were grounded using a laboratory mill.

2.2. Extraction Procedure

The samples were extracted with different solvents (ethyl acetate, ethanol, methanol, and water) in three extraction methods (homogenizer-assisted extraction (HAE), maceration (MAC), and Soxhlet (SOX)). Briefly, in homogenizer-assisted extraction, the plant materials (5 g) were extracted with 100 mL of these solvents using an Ultra-turrax at 6000 g for 5 min. In the maceration, the plant materials (5 g) were macerated with 100 mL of these solvents for 24 h at room temperature. In Soxhlet technique, the plant materials (5 g) were extracted in a Soxhlet apparatus for 6 h. Then, the extracts were filtered and evaporated. Regarding infusion, the plant materials (5 g) were kept in 100 mL of water (boiled) for 15 min. Then, the infusion was filtered and lyophilized. Obtained extracts were stored at 4 °C until experimentation.

2.3. Profiling of Bioactive Compounds by UHPLC-QTOF Mass Spectrometry

The profiling of bioactive compounds of *R. angustifolius* was performed using UHPLC-QTOF mass spectrometry acquired in MS full scan mode through (Agilent Technologies, Santa Clara, CA, USA), as previously optimized [9]. The dry matter of *R. angustifolius* extracts, obtained through three techniques of extraction—i.e., HAE, MAC, and SOX, and four solvents—i.e., EA, MetOH, EtOH, and water, were resuspended in 80% methanol acidified with 0.1% formic acid and finally filtered with 0.2 µm cellulose membrane into vials for UHPLC. The injection volume was set to 6 µL, and each sample was analyzed in triplicate using a randomized pattern of injection.

The raw MS data were processed using an Agilent Profinder (Agilent Technologies) software (version B.06) using a 'find-by-formula' algorithm, according to the parameters previously described [9]. The profiling of bioactive compounds was carried out using the comprehensive database Phenol-Explorer3.6 (version) [10], subsequently integrated with terpenoids and alkaloids obtained from the literature [11]. The putative annotation process was based on level two confidence (i.e., putative identification exploiting the isotopic profile of each compound and a mass accuracy <5 ppm). Finally, the major classes of bioactive compounds were quantified using standard compound solutions analyzed with the same MS conditions. The pure compounds were: cynaropicrin (sesquiterpene lactones), artemisinin (triterpenes), sanguinarine (alkaloids), cyanidin (anthocyanins), luteolin (flavones and other flavonoids), quercetin (flavonols), catechin (flavanols), sesamin (lignans), tyrosol (LMWPs), ferulic acid (phenolic acids), and resveratrol (stilbenes). Compounds were provided by Extrasynthese (Lyon, France), and they were characterized by purity >98%. Linear regression curves were built to quantify the main representative subclasses obtained (R^2 values > 0.98 using the following five concentrations: 0.1, 1, 10, 100, and 1000 mg/L), and the semi-quantitative values were then expressed as mg equivalents/g dry matter (DM).

2.4. In Vitro Antioxidant Potential and Enzyme-Inhibitory Activities

The methodology of determining antioxidant capacity was performed by using different test systems, including phosphomolybdenum, metal chelation, reducing power (FRAP and CUPRAC), and free-radical scavenging (DPPH and ABTS). The results of assays were explained as standard equivalents, namely, Trolox (TE) and EDTAE (EDTA). As for the enzyme inhibition abilities, several enzymes, including cholinesterases, amylase, glucosidase, and tyrosinase, were selected. For each assay, some standard compounds (galantamine, acarbose, and kojic acid) were used to explain the results. All details of the antioxidant and enzyme inhibition assays were reported in our previous paper [12]. All the analyses were performed in triplicate, and the results were reported as mean ± SD.

2.5. Statistics and Chemometrics

The analysis of variance (one-way ANOVA; $p < 0.05$) with Duncan's post hoc test was performed using PASW Statistics 26.0 (SPSS Inc., Chicago, IL, USA) to investigate the significant differences in semi-quantitative values of different bioactive compounds classes, in vitro antioxidants, and in vitro enzymatic inhibitory properties. The Pearson's

correlation coefficients ($p < 0.01$ and $p < 0.05$; two-tailed) were determined to find the significant correlations between phytochemical contents and biological activities (PASW Statistics 26.0, SPSS Inc., Chicago, IL, USA).

The metabolomics-based dataset was obtained by data filtering and data normalization process using Agilent Mass Profiler Professional software (from Agilent Technologies, Santa Clara, CA, USA; version B.05.00) as previously described [9,13]. After normalizing the data, the resulting dataset was further filtered based on an annotation score of 70% and finally processed for multivariate statistical analysis. An unsupervised hierarchical cluster analysis was carried out, setting the similarity measure as 'Euclidean' and 'Wards' as the linkage rule. Afterward, the raw dataset was then interpreted through orthogonal projection to latent structures discriminant analysis using SIMCA 16 (Umetrics, Malmo, Sweden). Cross-validated ANOVA ($p < 0.01$) and permutation testing ($N = 100$) were used for model validation and to exclude overfitting, respectively. Moreover, the OPLS-DA model was investigated for the fitness parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) and outliers, according to Hoteling's T2 test (95% and 99% confidence limit for the suspect and strong outliers, respectively). The variable importance in projection (VIP) was adopted to choose the most discriminant compounds among different solvents used for extraction, selecting those variables with the highest discrimination potentials (VIP score > 1.2), provided with fold-change values obtained by pairwise comparison among different extraction solvents and water. A Venn analysis was finally carried out among VIP markers resulting from different OPLS-DA models.

3. Results

3.1. Profiling of Bioactive Compounds in Rhinanthus Angustifolius Extracts through Untargeted UHPLC-QTOF-MS

The untargeted phytochemical profiling of *R. angustifolius* extracts was carried out through an ultra-high-pressure liquid chromatography quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS), recognized as a powerful analytical tool because of its high sensitivity. Overall, this approach allowed the putative detection of 570 bioactive compounds (BCs), characterized by 309 terpenoids, 248 polyphenols, and 13 alkaloids. The list of BCs, divided among classes and sub-classes, and reported together with their raw abundance values, identification score (>70%), and mass spectra, are provided in Supplementary Materials (Table S1). Regarding terpenoids, 245 sesquiterpene lactones, 44 triterpenes, and 20 limonoids were detected. The most representative terpenoids were ixerisoside-K and -D, taraxacin, and erioflorin acetate belonging to the sesquiterpene lactones class. Additionally, soyasapogenol-A; -E 3-*O*-beta-glucuronate, lupeol dihydrocinnamate, bevirimat, and brusatol were included under the triterpenes group. Finally, we detected amotsangin-A and -B, ohchnolide B, and 11-oxocneorin G, as the most representative limonoids (Table S1). In this work, different phenolic compounds (PCs) were putatively annotated in *R. angustifolius* extracts—i.e., 127 flavonoids (including 38 flavonols, 32 anthocyanins, 32 flavones, and 25 other flavonoids), 50 low-molecular-weight phenolics (LMWPs), 44 phenolic acids, 21 lignans, and 6 stilbenes. The major flavonoid compounds were driven by pelargonidin, cyanidin, peonidin, delphinidin, dihydroquercetin and its 3-*O*-glycoside conjugation form, followed by eriodictyol, genistein, hesperidin, apigenin, luteolin, kaempferol, and isorhamnetin. Meanwhile, for phenolic acids, we found an abundance of hydroxycinnamic acids (i.e., caffeic acid, cinnamic acid, p-coumaroyl malic acid, and p-coumaric acid 4-*O*-glucoside), followed by hydroxybenzoic acids (i.e., benzoic acid, gallic acid, and syringic acid). Regarding lignans, anhydro-secoisolariciresinol, arctigenin, 7-hydroxymatairesinol, dimethylmatairesinol, and pinoresinol were the most representative compounds. Finally, alkyl(methyl)phenols, hydroxybenzaldehydes, hydroxycoumarins, phenolic terpenes, tyrosols derivatives, and stilbenes were also found (Table S1). Finally, other BCs found in R. *angustifolius* extracts were represented by alkaloids, such as schelhammeridine, colchiethine, trigamine, and speciosamine (Table S1).

3.2. Impact of the Solvent and Extraction Method on the Recovery of Phytochemicals

To investigate the effect of different extraction methods, i.e., homogenizer-assisted extraction (HAE), maceration (MAC), Soxhlet (SOX), and infusion; and different solvents, i.e., ethyl acetate (EA), methanol (MetOH), ethanol (EtOH), and water (H_2O) on the untargeted metabolomic profile of *R. angustifolius* extracts; both unsupervised hierarchical cluster analysis (HCA) and supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) were used.

The HCA is reported in Figure 1; as can be observed, three clear clusters were detected. The first cluster was characterized by HAE and MAC extraction methods, using water as the extraction solvent. This cluster suggests a similar extraction capacity among HAE and MAC methods, showing an evident down accumulation of certain metabolites compared to the median intensity of other samples. The second cluster was characterized by the solvent EA, shown as a clear discriminating factor. The third cluster was divided in turn by two different sub-clusters, i.e., the first was characterized by ethanolic extracts clustering together despite the different extraction methods, and the second was driven by MetOH discriminating extraction solvents, showing no differences in the use of different extraction methods. Interestingly, the variable temperature resulted in a critical factor in the recovery of bioactive compounds; indeed, the infusion method showed a phytochemical profile like that of the methanolic extracts (Figure 1).

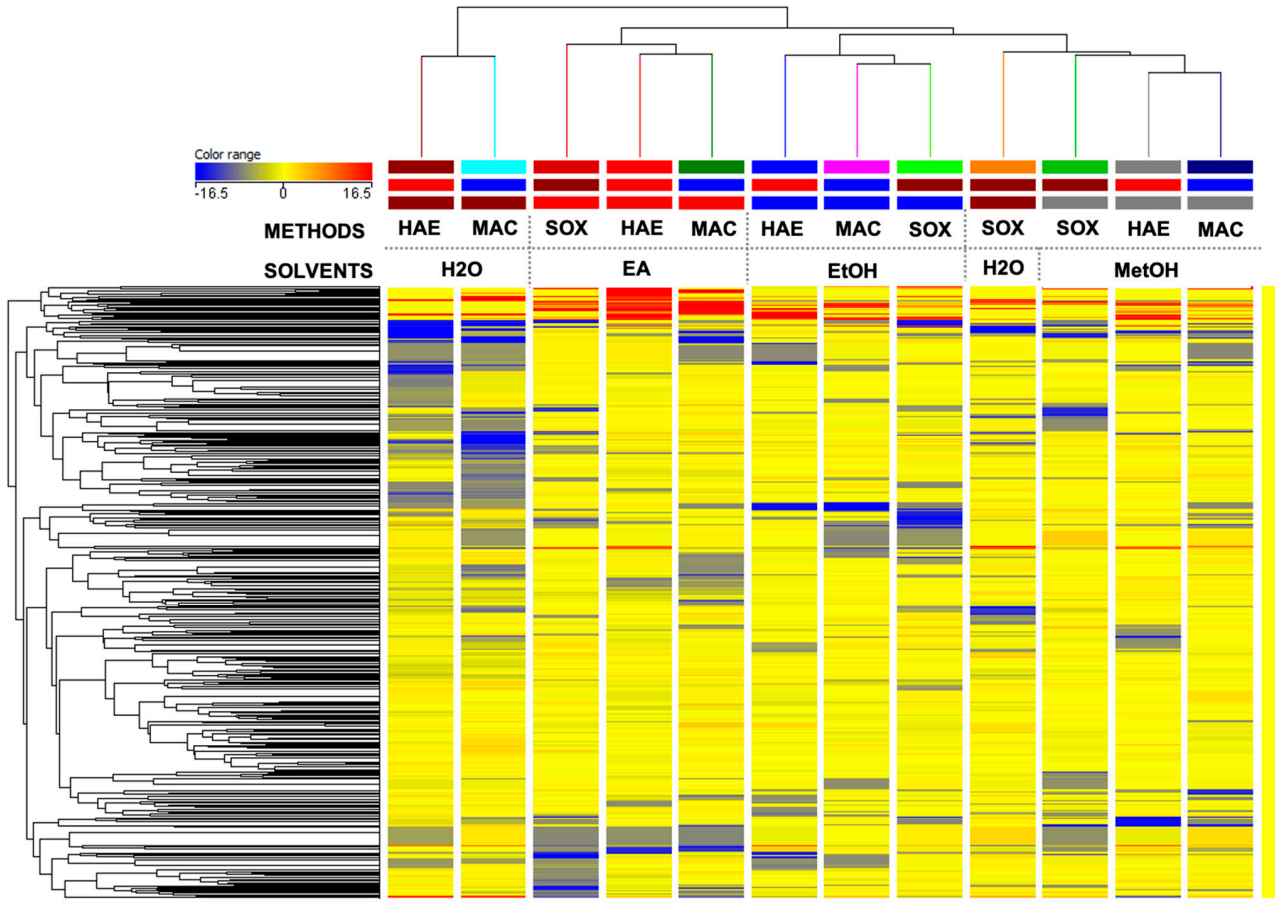

Figure 1. Unsupervised hierarchical cluster analysis of bioactive compounds considering three methods of extraction: homogenizer-assisted extraction (HAE), maceration (MAC), Soxhlet (SOX), and four solvents: ethyl acetate (EA), methanol (MetOH), ethanol (EtOH), and water (H_2O) of metabolites profile of *R. angustifolius*.

Considering that each solvent was able to promote the selective solubilization of different types of chemical compounds based on their polarity index (H_2O > EtOH > MetOH > EA), it was interesting to investigate and compare the impact of different extraction methods

using the same solvent. For this purpose, three supervised OPLS-DA multivariate models were created, one for each technique investigated (Figure S1; (A) HAE, (B) MAC, and (C) SOX), aimed to group and discriminate different solvents employed in accordance with the corresponding phytochemical profiles. The three OPLS-DA models built were characterized by excellent accuracy parameters with goodness-of-fit values (R^2Y) equal to 0.986, 0.987, and 0.986, and goodness-of-prediction values (Q^2Y) equal to 0.886, 0.891, and 0.865, as HAE, MAC, and SOX, respectively. Additionally, the models were cross-validated and inspected for outliers (Figures S2 and S3). For each OPLS-DA model, we selected the variable's importance in projection (VIP) markers used to highlight the BCs most affected by the different extraction solvents. A comprehensive list of VIP markers is reported in Supplementary Materials, divided into classes and sub-classes, including their VIP score (>1.2), cross-validated standard error, and log2 Fold-Change values obtained by pairwise comparison against samples extracted with water.

Interestingly, 58 discriminant compounds were detected for the HAE method, mainly characterized by flavonoids, reporting 33 compounds compared to SOX and MAC, i.e., 19 and 12 flavonoids, respectively (Figure 2A). The main flavonoid biomarkers were represented by anthocyanins (cyanidin 3-*O*-(6″-malonyl-glucoside), vitisin A, cyanidin), flavones (apigenin 7-*O*-glucoside, luteolin 7-*O*-malonyl-glucoside), flavonols (quercetin 3-*O*-xylosyl-rutinoside, kaempferol 3-*O*-glucoside), and isoflavonoids (genistin), owing to the best VIP score ranging from 1.20 to 1.42 (Table S2). According to Log2 Fold-Change (LogFC) analysis, these VIP markers were better extracted in MetOH and EtOH solvents than the aqueous solvent, showing 35.39 and 15.81 sum LogFC values, respectively. Besides the phenolic compounds, terpenoids were also found to possess a high discrimination ability. Particularly, we found triterpenes (lupine, 7-oxo-10alpha-cucurbitadienol acetate) and sesquiterpene lactones (i.e., vernoflexin and crepidiaside E) with VIP scores ranging from 1.32 to 1.36. The best extraction solvents for these terpenoid biomarkers were EtOH (sum LogFC = 33.88) and EA (sum LogFC = 30.87; Table S2).

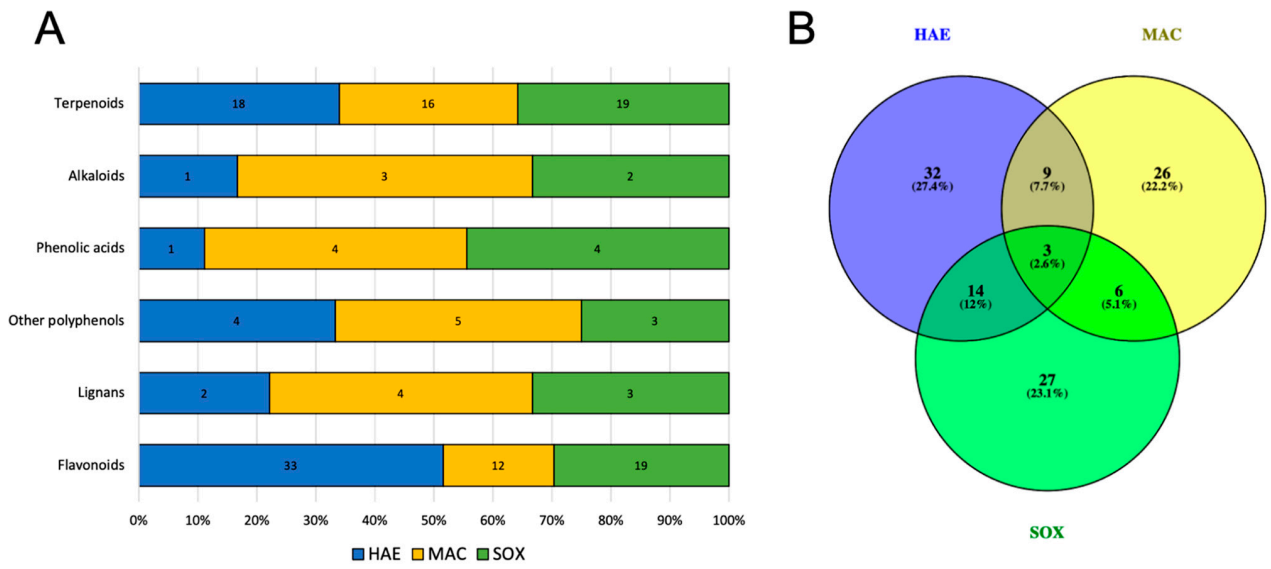

Figure 2. The number, cumulation in classes of compounds, and differences among three methods of extraction represented using variables importance in projection (VIP) markers carried out from three orthogonal projections to latent structures discriminant analysis (OPLS-DA) models such as homogenizer-assisted extraction (HAE), maceration (MAC), and Soxhlet (SOX). (**A**) A cumulative graph of the most representative VIP markers (VIP score > 1.2) resulting from the three OPLS-DA models, according to their compound's classification. The number reported in the bar corresponds to the number of VIP markers belonging to compounds classes, while the bar color represents the different OPLS-DA models: HAE (blue), MAC (yellow), and SOX (green). (**B**) Venn analysis of three lists of VIP markers resulting from OPLS-DA models.

Regarding MAC, we detected 44 discriminant compounds (Table S3). This method was not very effective in extracting BCs, resulting in a lower average number in many classes of compounds compared to HAE and SOX (Figure 2A). The OPLS-DA model allowed detecting 25 polyphenols, reporting flavonoids (dihydroquercetin, luteolin, malvidin 3-*O*-(6″-acetyl-galactoside), kaempferol 7-*O*-glucoside), phenolic acids (caffeic acid 4-*O*-glucoside, 4-hydroxybenzoic acid 4-*O*-glucoside, p-coumaroyl glucose), lignans (isolariciresinol, medioresinol), and other polyphenols (4-hydroxycoumarin, 5-heneicosylresorcinol) as the most discriminant among the different solvents used. Moreover, different sesquiterpene lactones, such as dehydrocostuslactone, ixerin-E or -X, and 10-epi-8-deoxycumambrin were included among the most discriminant compounds for the methanolic extracts (Table S3).

Concerning the SOX extraction method (Table S4), the sesquiterpene lactones were highly discriminant, with some compounds having the highest VIP score, such as 1β,6α-dihydroxycostic acid, ferolide, 11-epiartesin, and xanthumin. Additionally, two alkaloids, namely, trigamine and schelhammeridine, were particularly abundant in the ethanolic extracts. Regarding polyphenols, we found kaempferol 3-*O*-(6″-acetyl-galactoside) 7-*O*-rhamnoside (flavonols), coumestrol (LMWPs), and dihydrocaffeic acid (phenolic acids).

Afterward, a Venn analysis was performed using VIP markers obtained from the three OPLS-DA models (i.e., for each method of extraction) in order to investigate which method was best for providing efficient extraction of BCs in *R. angustifolius* extracts, considering the same extraction solvents (Figure 2B). The three extraction methods considered were shown to share only 2.6% of common BCs, suggesting that the effect of different methods has a greater impact on the isolation of different BCs. The HAE and SOX extraction methods resulted in having the most common isolated BCs (12%) compared to MAC (7.7%). Moreover, HAE was shown to have the highest isolation capacity of the most discriminating unique BCs in *R. angustifolius* extracts (27.4%), compared to SOX and MAC by 23.1% and 22.2%, respectively.

3.3. Characterization and Semi-Quantification of Bioactive Compounds

The semi-quantification of different BCs of *R. angustifolius* extracts was carried out according to a semi-quantitative method focused on the main classes, starting from the UHPLC-QTOF data. The results are reported in Table S5, expressed as mg equivalents/g dry matter (DM). In particular, the main classes of metabolites were quantified using standard curves from pure compounds (purity > 98%). Thus, we quantified alkaloids, triterpenes, sesquiterpene lactones, and polyphenols classes, considering the three methods (HEA, MAC, and SOX) and the four extraction solvents (EA, EtOH, MetOH, and H_2O). Afterward, a cumulative graph for each extraction method was created and reported in Figure 3A–C. The methanolic extract was the best one, allowing recovery of the highest content of BCs, ranging from 182.42 to 239.15 mg equivalents/g DM, highlighting HEA as the most efficient one. On the other hand, water was found to be the worst extraction solvent, recovering 132.10, 139.82, and 151.26 mg equivalents/g DM for MAC, HAE, and SOX, respectively (Figure 3A–C; Table S5).

Regarding the different classes of compounds targeted, polyphenols and sesquiterpene lactones were efficiently extracted in methanol and using HAE, showing semi-quantitative values of 172.57, 59.44, mg equivalents/g DM ($p < 0.05$), respectively (Figure 3A; Table S5). However, triterpenes and alkaloids were statistically abundant in EtOH extracts using the SOX method (7.59 mg equivalents/g DM; Figure 3C; Table S5) and EtOH-MAC (1.18 mg equivalents/g DM; Figure 3B; Table S5), respectively.

Polyphenols are secondary metabolites widely abundant in plants and reported to exhibit health-promoting properties. The semi-quantitative results for the most important phenolic subclasses are shown in Table S6, expressed as mg equivalents/g DM. The cumulative graph of different polyphenols classes is provided in Figure 3D–F as a function of the different extraction methods tested. Overall, the methanolic extracts of HEA resulted in having a statistically higher quantity of the main phenolic classes ($p < 0.05$), showing a greater recovery capacity for anthocyanins (6.96 mg eq./g DM), flavones (30.61 mg eq./g DM), flavonols

(17.80 mg eq./g DM), and LMW phenolics (80.32 mg eq./g DM; Figure 3D; Table S6). Interestingly, stilbenes were efficiently extracted in the methanol solvent coupled to the SOX method (two-fold higher than the other techniques), reconvening 17.5 mg eq./g DM. Concerning lignans and phenolic acids, they were found to be more soluble in EA using MAC (49.68 mg eq./g DM) and SOX (15.92 mg eq./g DM), respectively (Table S6).

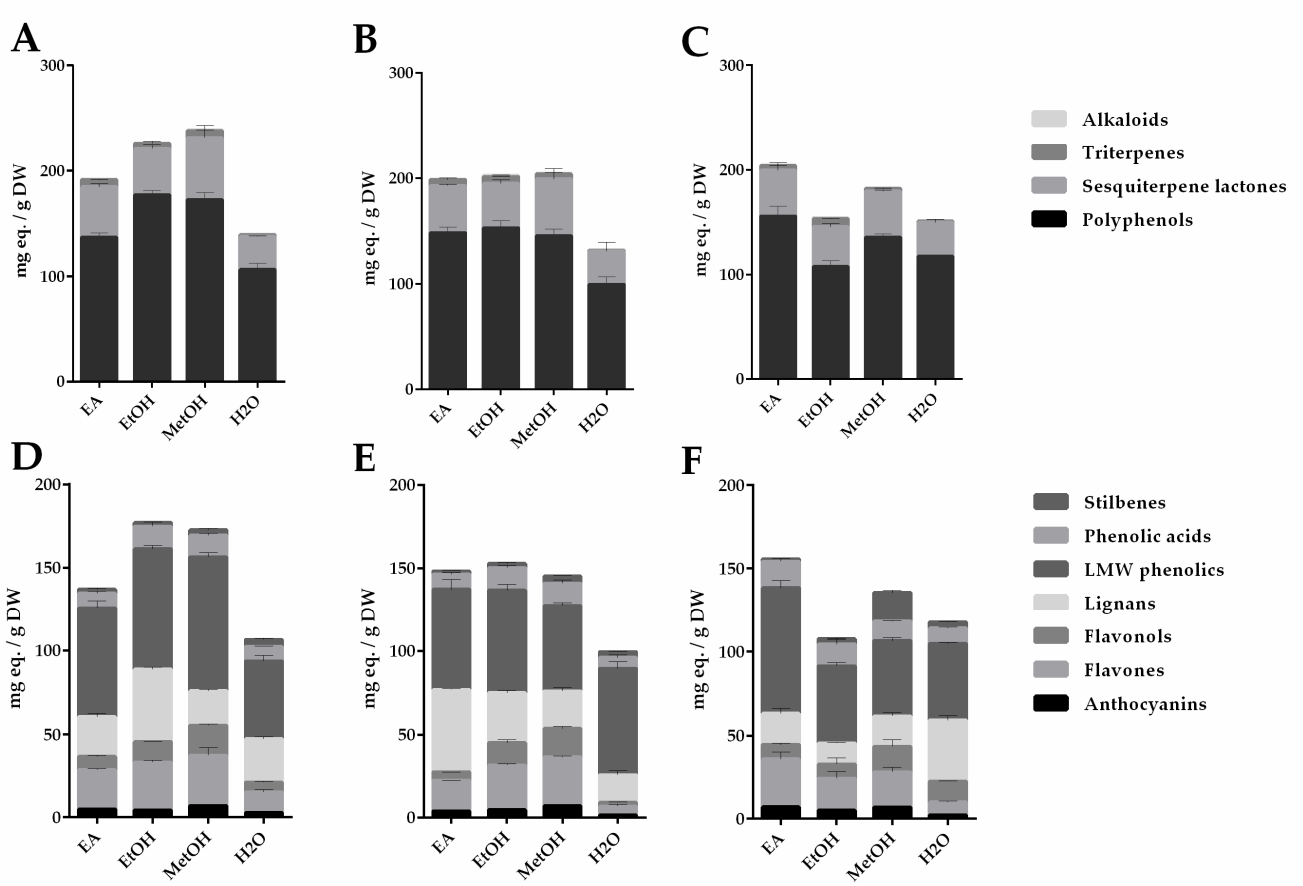

Figure 3. Cumulative graphs of *R. angustifolius* bioactive compounds classes. (**A**–**C**) reported the main classes of bioactive compounds, whereas (**D**–**F**) reported the main classes of polyphenols in *R. angustifolius* extracts. The letters correspond to: (**A**,**D**) = homogenizer-assisted extraction (HAE), (**B**,**E**) = maceration (MAC), and (**C**,**F**) = Soxhlet (SOX) methods of extraction.

3.4. In Vitro Antioxidant Activity

In the present study, six different methods (DPPH, ABTS, CUPARC, FRAP, metal chelating, and phosphomolybdenum assays) were used to evaluate the antioxidant activity of the *R. angustifolius* extracts. The methanolic extracts were noted to exhibit the highest antioxidant capacity, considering different methods of extraction. Indeed, the methanolic extracts have been noted to have the highest DPPH- (45.57–47.20 mg Trolox equivalent (TE)/g) and ABTS (94.68–104.11 mg TE/g)-scavenging properties (Table 1). Regarding other extracts, they were found to have a relatively moderate or lower scavenging ability, ranging from 10.29 to 39.96 mg TE/g and 12.71 to 69.69 mg TE/g in DPPH and ABTS assays, respectively. In particular, the HAE-EA extract demonstrated the lowest radical-scavenging potential in both assays (Table 1). Moreover, the extracts also showed notable reducing power in CUPRAC and FRAP assays. Similarly, the methanolic extracts of *R. angustifolius* were found to exhibit the most significant reducing activity in the CUPRAC assay (144.67–173.03 mg TE/g) and FRAP assay (91.35–103.09 mg TE/g), followed by ethanolic extracts ranging from 130.52 to 140.72 mg TE/g and 67.12 to 79.45 mg TE/g, respectively.

Table 1. Antioxidant properties of the tested extracts.

Methods	Solvents	DPPH	ABTS	CUPRAC	FRAP	MCA	PBD
		(mg TE/g)				(mg EDTAE/g)	(mmol TE/g)
HAE	EA	10.29 ± 0.21 ^{m}	12.71 ± 0.52 ^{l}	77.55 ± 0.47 ^{g}	33.16 ± 0.94 ^{i}	21.71 ± 1.12 ^{c}	2.01 ± 0.09 ^{d}
	EtOH	31.77 ± 0.04 ^{f}	52.28 ± 0.38 ^{f}	140.72 ± 2.00 ^{c}	68.87 ± 0.63 ^{d}	8.90 ± 0.51 ^{g}	1.55 ± 0.02 ^{e}
	MetOH	45.57 ± 0.05 ^{b}	99.17 ± 2.24 ^{b}	173.03 ± 0.90 ^{a}	103.09 ± 1.77 ^{a}	17.12 ± 0.28 ^{d}	1.89 ± 0.03 ^{d}
	Water	33.52 ± 0.49 ^{e}	69.63 ± 0.52 ^{d}	76.85 ± 1.03 ^{g}	60.91 ± 0.31 ^{f}	25.66 ± 0.45 ^{b}	3.92 ± 0.14 ^{a}
MAC	EA	15.67 ± 0.40 ^{l}	24.78 ± 0.59 ^{i}	77.55 ± 0.63 ^{g}	33.11 ± 1.49 ^{i}	21.13 ± 2.49 ^{c}	1.92 ± 0.13 ^{d}
	EtOH	30.49 ± 0.23 ^{g}	51.79 ± 0.50 ^{f}	133.16 ± 0.37 ^{d}	67.12 ± 0.36 ^{e}	7.67 ± 0.57 ^{g}	1.60 ± 0.04 ^{e}
	MetOH	46.94 ± 0.02 ^{a}	104.11 ± 1.09 ^{a}	145.87 ± 3.83 ^{b}	91.76 ± 1.42 ^{b}	12.14 ± 1.21 ^{f}	1.97 ± 0.10 ^{d}
	Water	26.67 ± 0.64 ^{h}	46.43 ± 4.29 ^{g}	71.88 ± 0.32 ^{h}	53.28 ± 0.90 ^{g}	27.51 ± 0.45 ^{a}	2.85 ± 0.04 ^{b}
SOX	EA	23.42 ± 0.93 ^{i}	35.50 ± 0.82 ^{h}	89.24 ± 0.34 ^{f}	43.53 ± 1.23 ^{h}	10.69 ± 0.68 ^{f}	2.34 ± 0.36 ^{c}
	EtOH	39.96 ± 0.18 ^{d}	65.44 ± 0.64 ^{e}	130.52 ± 2.12 ^{d}	79.45 ± 0.50 ^{c}	4.57 ± 1.21 ^{h}	2.30 ± 0.06 ^{c}
	MetOH	47.20 ± 0.06 ^{a}	94.68 ± 1.00 ^{c}	144.67 ± 1.53 ^{b}	91.35 ± 0.80 ^{b}	15.23 ± 0.98 ^{e}	2.98 ± 0.13 ^{b}
	Water (infusion)	44.46 ± 0.17 ^{c}	102.13 ± 1.88 ^{a}	113.78 ± 2.60 ^{e}	79.54 ± 0.71 ^{c}	26.09 ± 0.08 ^{ab}	3.71 ± 0.01 ^{a}

Values are reported as mean ± SD. HAE: homogenizer-assisted extraction; MAC: maceration; PBD: phosphomolybdenum; SOX: Soxhlet; EA: ethyl acetate; EtOH: ethanol; MetOH: methanol; TE: Trolox equivalent; EDTAE: EDTA equivalents. Different letters indicate significant differences in the tested extracts in the same column ($p < 0.05$).

Concerning the metal-chelating activity, the water extracts obtained by MAC, HEA, and infusion showed the highest metal-chelating activity values ranging from 25.66 to 27.51 mg EDTA equivalent/g, especially compared to the ethanolic extracts (4.57–8.90 mg EDTAE/g) (Table 1). Nevertheless, the total antioxidant capacity assessed by the phosphomolybdenum assay highlighted that water extracts, obtained by HAE, MAC, and infusion methods, have the highest antioxidant activity (2.83–3.92 mmolTE/g), while the activity of the other extracts ranged from 1.55 to 2.98 mmol TE/g (Table 1).

3.5. Enzyme Inhibition Activity

Different extracts of *R. angustifolius* were investigated for their inhibition activity against cholinesterase enzymes AChE and BChE. In this regard, out of the 12 investigated extracts, only 4 showed dual inhibitory properties against both cholinesterase enzymes (Table 2). These extracts were mainly characterized by methanolic and ethanolic solvents of extraction, such as HAE-MetOH, MAC-EtOH, SOX-EtOH, and SOX-MetOH, showing inhibition values ranging from 0.90 to 4.73 mg Galantamine equivalent (GALAE)/g. However, the best extraction method was resulted to be MAC coupled with the ethanolic solvent, which had the highest and most significant ($p < 0.05$) inhibition values (2.57 and 4.73 for AChE and BChE, respectively) (Table 2). Concerning other extracts, they have been shown to have a selective inhibition capacity, either AChE or BChE, except for MAC–water extract, which displayed no inhibition against the enzymes. Nevertheless, the alcoholic extracts demonstrated considerable AChE inhibitory activity, considering both HAE and MAC extraction methods, showing the highest activity (2.63 ± 0.02 and 2.57 ± 0.04 mg GALAE/g, respectively). In comparison, none of the ethyl acetate extracts inhibited AChE. On the other hand, the highest BChE activity was observed for HAE-EA and MAC-EtOH extracts (4.94 ± 0.36 and 4.73 ± 0.15 mg GALAE/g, respectively). The other active extracts showed BChE inhibition activity ranging from 0.90 to 3.89 mg GALAE/g (Table 2).

In addition to cholinesterase enzymes inhibition capacity, different extracts of *R. angustifolius* were also investigated against tyrosinase, α-amylase, and α-glucosidase enzymes. All the herein studied extracts were found to inhibit the tyrosinase enzyme. However, SOX-MetOH (52.80 ± 0.85 mg KAE/g) was the most effective tyrosinase inhibitor, followed by SOX-EA and MAC-MetOH (51.34 ± 4.48 and 50.42 ± 0.41 mg KAE/g, respectively), while the other extracts ranged from 10.62 to 49.32 mg KAE/g.

Regarding α-amylase and α-glucosidase enzymes inhibition capacity, all different extracts of *R. angustifolius* showed dual inhibition against both carbohydrate-hydrolyzing enzymes (α-amylase: 0.07–0.69 mmol ACAE/g; α-glucosidase: 0.03–1.30 mmol Acarbose equivalent (ACAE)/g), except for HAE-EtOH and MAC-EtOH extracts, which only inhibited amylase (0.69 ± 0.01 and 0.70 ± 0.01 mmol ACAE/g). Moreover, the high-

est anti-amylase effect was noted by the MAC-EtOH extract, while the most significant anti-glucosidase activity was exhibited by the SOX-EtOH extract (Table 2).

Table 2. Enzyme-inhibiting effects of the tested extracts.

Methods	Solvents	AChE	BChE	Tyrosinase	α-Amylase	α-Glucosidase
		(mg GALAE/g)		(mg KAE/g)	(mmol ACAE/g)	
HAE	EA	-	4.94 ± 0.36 ^{a}	16.92 ± 1.25 ^{f}	0.69 ± 0.01 ^{a}	1.10 ± 0.03 ^{d}
	EtOH	2.63 ± 0.02 ^{a}	-	27.61 ± 2.14 ^{e}	0.69 ± 0.01 ^{a}	-
	MetOH	2.16 ± 0.32 ^{c}	0.95 ± 0.07 ^{e}	38.63 ± 1.03 ^{d}	0.44 ± 0.01 ^{d}	0.49 ± 0.01 ^{e}
	Water	0.73 ± 0.10 ^{e}	-	14.78 ± 1.34 ^{f}	0.07 ± 0.01 ^{h}	0.08 ± 0.01 ^{h}
MAC	EA	-	3.89 ± 0.22 ^{b}	28.76 ± 0.42 ^{e}	0.65 ± 0.03 ^{b}	1.14 ± 0.01 ^{c}
	EtOH	2.57 ± 0.04 ^{a}	4.73 ± 0.15 ^{a}	42.90 ± 2.22 ^{c}	0.70 ± 0.01 ^{a}	-
	MetOH	2.48 ± 0.05 ^{ab}	-	50.42 ± 0.41 ^{ab}	0.40 ± 0.01 ^{e}	0.38 ± 0.02 ^{f}
	Water	-	-	10.62 ± 1.11 ^{g}	0.14 ± 0.01 ^{g}	0.03 ± 0.01 ^{i}
SOX	EA	-	1.94 ± 0.17 ^{d}	51.34 ± 4.48 ^{ab}	0.68 ± 0.02 ^{a}	1.20 ± 0.02 ^{b}
	EtOH	1.84 ± 0.05 ^{d}	3.12 ± 0.03 ^{c}	49.32 ± 0.12 ^{b}	0.48 ± 0.01 ^{c}	1.30 ± 0.01 ^{a}
	MetOH	2.38 ± 0.05 ^{b}	0.90 ± 0.06 ^{e}	52.80 ± 0.85 ^{a}	0.36 ± 0.01 ^{f}	0.35 ± 0.01 ^{g}
	Water (Infusion)	0.42 ± 0.09 ^{f}	-	11.17 ± 0.40 ^{g}	0.08 ± 0.01 ^{h}	1.19 ± 0.01 ^{b}

Values are reported as mean ± SD. HAE: homogenizer-assisted extraction; MAC: maceration; SOX: Soxhlet; EA: ethyl acetate; EtOH: ethanol; MetOH: methanol; GALAE: galantamine equivalent; KAE: Kojic acid equivalent; ACAE: acarbose equivalent; -: not active. Different letters indicate significant differences in the tested extracts in the same column ($p < 0.05$).

3.6. Pearson's Correlation

Pearson's correlation coefficients are used to evaluate the relationship between the different BCs classes of *R. angustifolius* extracts (obtained by semi-quantitative data analysis) and the various biological activity assays. A positive correlation could reflect a direct or indirect molecular interaction between BCs and molecules involved in biological activity. Furthermore, total polyphenols (including their main classes), triterpenes, sesquiterpene lactones, and alkaloids were considered in correlation analysis with antioxidant capacity (DPPH, ATBS, CUPRAC, FRAP, metal-chelating activity, phosphomolybdenum), and enzyme-inhibition activity (AChE, BChE, tyrosinase, α-amylase, and α-glucosidase). The significant correlation coefficients are shown in Table S7. A positive and significant correlation was observed between flavonols and DPPH, ATBS, CUPRAC, and FRAP assays, delivering on average a correlation coefficient of 0.73 ($p < 0.01$). Phosphomolybdenum and metal-chelating activities showed an overall negative correlation, caused by the main group of polyphenols (anthocyanins, flavones, LMWPs, and phenolic acids) and terpenoids (sesquiterpene lactones and triterpenes). Regarding tyrosinase-inhibition activity, anthocyanins, flavones, and phenolic acids exhibited the highest correlation coefficients, i.e., 0.849, 0.664, and 0.746 ($p < 0.01$), respectively. Moreover, α-amylase did not show significant correlations with phytochemical classes, whereas α-amylase inhibition correlated with polyphenols (0.701) and triterpenes (0.761). Finally, AChE and BChE inhibition activity was highly correlated to flavonols (0.729) and triterpenes (0.620), respectively (Table S7).

4. Discussion

Secondary metabolites from plant sources are major bioactive compounds known for their beneficial human health properties. However, these metabolites, initially characterizing the raw plant matrices, could be lost during different food-processing steps. Thus, supplementation of BCs as a dietary additive will help retain the required health-beneficial quantity of BCs in the diet. The extraction process is an important step in the preparation of food additives [14]. For these purposes, the optimal extraction method should be implemented and considered. Indeed, the extraction of BCs could be affected by several factors such as the nature of the sample matrix, type and concentration of extraction solvent, time, temperature, and pH [15,16]. Since the solvent type used is one of the most significant factors, different solvents were tested and used for extracting these value-added

products. Generally, the selection of solvents is based on the chemical nature and polarity of compounds that are required to be extracted. Most BCs, i.e., phenolics, flavonoids, and anthocyanins, are hydro-soluble. For instance, polar and medium-polar solvents such as water, methanol, ethanol, propanol, acetone, and their aqueous mixtures are commonly used for extraction [17]. Polyphenols, terpenoids, and alkaloids are the most prominent secondary metabolites widely present in plants, and their distribution can be observed throughout the whole metabolic process [18]. Remarkably, several reports have shown protective effects of various BCs, from plant matrices, used as prevention against several chronic and degenerative diseases, including neurodegeneration such as Alzheimer's and Parkinson's diseases, cardiovascular disease, type II diabetes, and cancer [19,20].

In the present study, the *R. angustifolius* extracts were extracted through different extraction methods, i.e., HAE, MAC, SOX, and infusion, using different solvents, i.e., EA, MetOH, EtOH, and H_2O. These extracts were analyzed for their bioactive compounds profile using UHPLC-QTOF-MS. Overall, the metabolites profile of *R. angustifolius* extracts has been found to contain reasonable amounts of BCs, characterized by polyphenols, terpenoids, and alkaloids. Indeed, phenolic compounds and terpenoids were the most representative classes extracted in high quantity by HAE coupled with methanol (Figure 2 and Table S5), recovering 172.57 and 65.85 mg eq./g DM, respectively. Other studies have confirmed that methanolic extracts display higher polyphenol and terpenoid contents than other solvents such as distilled water, ethanol, ethyl acetate, acetone, dichloromethane, and hexane [21]. However, alkaloids were reported as more soluble in an ethanolic solvent using the maceration technique. Probably, the timing employed to soak in ethanol leads to a remarkable recovery of alkaloids. Urbanovà and co-worker [22] confirmed that isolation of alkaloids needs a highly labor-intensive and time-consuming process, based on Soxhlet or lengthy maceration extraction.

Besides methanolic extracts using the HAE technique of extraction, the most abundant phenolic classes were LMWPs and flavones, reporting 80.32 and 30.51 mg eq./g DM, respectively. In contrast, lignans were found to be more soluble in EA by maceration (49.68 mg eq./g DM). It is well known that flavonoids and LMWPs are polar compounds, so lower flavonoid contents in the ethanol and ethyl acetate extracts could be expected as they have relatively lower polarity than methanol [23]. These results are in line with the findings of previous studies, which reported that extraction solvents significantly affect the recovery yields of BCs from plant materials [24]. Regarding lignans, Lehraiki et al. [25] reported their solubility in ethyl acetate, extracting two important lignans with relevant biological activity—i.e., secoisolariciresinol and anhydro-secoisolariciresinol with a purity of 97% and 98%, respectively. Accordingly, in our ethyl acetate extracts, there was anhydro-secoisolariciresinol and other important lignans, i.e., pinoresinol, dimethylmatairesinol, 7-hydroxymatairesinol, 7-oxomatairesinol, and isolariciresinol. Interestingly, a wide range of studies reported the estrogenic activity of these lignans through the metabolic activity of intestinal bacteria, producing mammalian lignans such as enterodiol and enterolactone and regulating estrogen receptors in breast tissue [25–27].

The in vitro antioxidant activity of BCs is associated with their capacity to inhibit lipoxygenase, chelate metals, and capture free radicals, even though they can also act as promoters of in vitro oxidation reactions. Accordingly, phenolic compounds act as antioxidants by preventing or delaying auto-oxidation and free radicals' sequestration, producing more stable compounds that cannot undertake subsequent oxidations, which enables them to protect low-density lipoproteins of the human body from oxidation. Antioxidant activity of food matrices, such as vegetables, fruits, and plants in general, have demonstrated multiple beneficial effects in the control of diseases linked to oxidative stress [28]. Indeed, the antioxidant properties of plant foods have mainly been attributed to their rich phenolic contents. In particular, antioxidants play their protective role on cells either by preventing the formation of free radicals or by neutralizing/scavenging free radicals produced in the body, or by reducing/chelating the transitional metal composition of food [29]. The antiradical activity of flavonoids and phenols is principally based on the

structural relationship between various parts of their chemical structure [30]. It has been found that a high and significant ($p < 0.01$) correlation coefficient exists among flavonols and DPPH, ATBS, CUPRAC, and FRAP capacities. Moreover, in the present study, the ethyl acetate extracts showed lower antioxidant activity and contained fewer phenolic compounds and flavonoids than the methanolic extracts, which agreed with the study of [23]. The antioxidant effect of flavonols could be attributed to their chemical structure, indicating that the presence of 3′,4′-catechol, and 4′-OH groups on the B ring were highly correlated with the antioxidant capacity [31]. Accordingly, our findings include many flavonols, particularly in HAE-MetOH extracts, including kaempferol, morin, myricetin, quercetin, and their 3-*O*, 4-*O*, and 7-*O* glycoside groups (Table S1). Other Rhinanthus species, including *R. angustifolius,* possessed notable antioxidant activity [2,32].

In the last years, the food industry is searching and developing healthier and more nutritious foods. In this regard, functional food ingredients have attracted much attention as their dietary intake has been associated with preventing different chronic diseases. Indeed, enzyme inhibitors have become a crucial aspect of the food industries in producing functional foods, as they have a preventive purpose for human health. Enzymes take part in a wide range of human ailments, and several specific enzyme inhibitors have been designed to combat their activities, therefore acting as therapeutic agents [33]. The present work investigated the inhibition properties of *R. angustifolius* extracts against acetyl- and butyryl-cholinesterases, tyrosinase, amylase, and glucosidase enzymes.

Cholinergic deficiency is associated with Alzheimer's disease (AD), and various cholinesterase inhibitors have been developed to treat AD, including naturally derived inhibitors, synthetic analogs, and hybrids. Currently, the available drugs for AD are predominantly cholinesterase inhibitors. However, the efficacy of these drugs is restricted as they may cause adverse effects and cannot fully arrest the progression of the disease [34]. Acetylcholinesterase (AChE) is the most crucial enzyme regulating the acetylcholine level in the healthy brain, while butyrylcholinesterase (BChE) plays a minor role. In patients with AD, the level of AChE activity reduces, and the activity of BChE increases, such that the ratio between BChE and AChE can alter from 0.6 in the normal brain to as elevated as 11 in cortical areas affected by the disease. For that reason, inhibition of AChE and BChE is the most effective therapeutic approach to treat AD symptoms [35]. Regarding our extracts, flavonols were detected to have a high correlation coefficient with the inhibition of AChE enzyme activity. This result was also confirmed by Ademosun et al. [36], who revealed the ability of flavonoid compounds in inhibiting anticholinesterase activity in a concentration-dependent manner, particularly quercetin (flavonols), and its glycosylated conjugation reported the highest capacity. Instead, the inhibition of BChE enzyme activity was observed by the action of triterpenes, which reported a high correlation coefficient. Overall, a wide collection of extracts from medicinal plants used in traditional healing systems to improve cognitive function and their derived phytochemicals have shown promising ChE-inhibiting activities [37]. Besides, in recent decades, several plant extracts have been scrutinized for their in vitro anticholinesterase activity, with plant species producing diverse classes of alkaloids, coumarins, terpenes, and polyphenols demonstrating the most potent results and hence are potential candidates for new anti-AD drugs. Besides, several studies have been carried out to identify and isolate natural molecules applicable for the design and development of new anti-AD drugs [38,39].

Melanin, a major pigment found in mammalian skin, is recognized to protect the skin against the detrimental effects of ultraviolet irradiation, oxidative stress, and DNA damage. However, buildup or excessive production of melanin can result in an esthetic problem and severe diseases allied with hyperpigmentation [40]. In this regard, as the key and rate-limiting enzyme for melanogenesis, tyrosinase has been considered an important target for developing therapeutic agents against pigmentation disorders [41]. Tyrosinase inhibitors are commercially available for cosmetic uses as skin lightening agents. At the same time, they are clinically employed in treating a variety of hyperpigmentation conditions such as senile lentigines, melasma, and freckles [42]. They are equally applicable

as anti-browning agents in the food processing industry. Indeed, this enzyme is responsible for the undesired browning of fruits and vegetables. Therefore, several extensively studied anti-tyrosinase effects have been successfully marketed, including synthetic, semi-synthetic, and natural origins. These compounds are from several chemical classes, i.e., phenolics, terpenes, flavonoids, alkaloids, long-chain fatty acids, coumarins, etc. [43,44]. In this study, the methanolic extracts showed the most potent anti-tyrosinase effect, indicating the presence of key compounds active against tyrosinase. This could be due to their higher phenolic and/or flavonoid contents, which were reported to have a tight correlation with anti-tyrosinase activity. In particular, a strong correlation was observed for anthocyanins, flavones, and phenolic acids, which were also found to be enriched with methanolic extracts. In accordance, previous studies have shown a significant relationship between total phenolic content and anti-tyrosinase activity [45]. The inhibition of tyrosinase activity might depend on the hydroxyl groups of the phenolic compounds of the extracts that could form a hydrogen bond to a site of the enzyme, leading to lower enzymatic activity. Some tyrosinase inhibitors also act through hydroxyl groups that bind to the active site of tyrosinase, inducing steric hindrance or distorted conformation [46,47].

α-glucosidase and α-amylase are the important enzymes involved in the digestion of carbohydrates. α-amylase is involved in the breakdown of long-chain carbohydrates, whereas α-glucosidase breaks down starch and disaccharides into glucose. Thus, they are regarded as the major digestive enzymes involved in intestinal absorption. Accordingly, α-amylase and α-glucosidase inhibitors are the potential targets for the treatment of diabetes [48]. Interestingly, the *R. angustifolius* extracts showed a great inhibition capacity of both enzymes. Specifically, the highest anti-amylase and anti-glucosidase activities were found in ethanolic extracts using maceration and the Soxhlet extraction method, respectively. The inhibition capacity was highly correlated to polyphenols and terpenoids classes. It is well known that plant foods rich in polyphenols have been reported to cause effects comparable to insulin in glucose utilization and can act as good inhibitors of key enzymes such as α-amylase and α-glucosidase associated with type 2 diabetes and lipid peroxidation in tissues [48]. Polyphenolic compounds may diminish the potency of α-amylase and α-glucosidase by interacting or inhibiting specific positions in the enzymes [49]. Studies have also shown that the bioactivity of polyphenols in plants could be linked to their antioxidant activity, and many of these plants have also displayed hypoglycemic properties [50]. Generally, the water extracts in the present study exhibited lower enzyme-inhibition potential, which was in agreement with other previous reports [51], although the water extracts contained relatively moderate phenolics and flavonoids. This could be explained by the complex nature of these extracts and interactions (synergetic or antagonistic) between phytochemicals present therein [51].

5. Conclusions

To the best of our knowledge, this study is the first to investigate the in vitro biological potentials of the extracts of *R. angustifolius*, an understudied species, also providing comprehensive phytochemical profiling by high-resolution mass spectrometry. In this regard, different extraction techniques and solvents yielded varying amounts of bioactive compounds and bioactivity. In particular, the methanolic extracts were found to contain higher levels of total phenolics and flavonoids. Metabolomics allowed identifying 570 compounds, mainly terpenoids, followed by polyphenols and alkaloids. Indeed, the different extracts of *R. angustifolius* revealed antioxidant capacity via different mechanisms, such as radical scavenging, metal chelation, and reducing activity in the various assays used. The methanolic extracts displayed the highest antioxidant capacity in most assays performed, related to the high abundance of polyphenolic compounds. Besides, the extracts showed varying inhibitory potencies against the studied enzymes mediating diseases, such as diabetes, Alzheimer's disease, and skin hyperpigmentation. Therefore, our findings highlighted some of the possible beneficial effects of *R. angustifolius* as sources of bioactive

compounds and showed pertinent activities against key human diseases that could be of interest for herbal drug development applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11199162/s1, Figure S1: OPLS-DA models, Figure S2: Hotelling's T-squared plots, Figure S3: Permutation plots, Table S1: Dataset, Table S2: VIP markers HAE, Table S3: VIP markers MAC, Table S4: VIP markers SOX, Table S5: TBCs, Table S6: TPCs, Table S7: Pearson's correlation.

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