applied sciences

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Comparative Study of the Antibacterial Activity, Total Phenolic and Total Flavonoid Content of Nine *Hypericum* Species Grown in Greece

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Abstract: *Hypericum perforatum* is an herb whose use dates back centuries. Extracts of the plant are available as over-the-counter treatment options for depression. The genus consists of approximately 500 species, most of which have not yet been studied. Antimicrobial resistance has reached alarming levels, indicating a post-antibiotic era as many of the available treatment options become less effective. For this reason, nine *Hypericum* species were studied for their antimicrobial activity and their total phenolic and flavonoid content. Extracts were tested against Gram-negative and Gram-positive bacteria. Extracts inhibited the growth of Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*). The lowest MIC and MBC values were calculated for the extract of *H. perforatum* against both microorganisms tested, reaching 0.06 mg/mL for *S. aureus* and 0.13 mg/mL for *E. faecalis*. Total phenolic content was the highest in the *H. perforatum* extract (86 ± 12.90 mg GAE/g dry plant material). *H. tetrapterum* presented the highest flavonoid content, equal to 1.58 ± 0.4 mg RE/g of dry plant material. The *Hypericum* species studied herein are less common or have not yet been examined compared to *H. perforatum*; therefore, our study adds new data to the knowledge of the genus *Hypericum*.

Keywords: *Hypericum*; total flavonoid content; total phenolic content; antimicrobial activity

Citation: Kakouri, E.; Daferera, D.; Trigas, P.; Charalambous, D.; Pantelidou, M.; Tarantilis, P.A.; Kanakis, C.D. Comparative Study of the Antibacterial Activity, Total Phenolic and Total Flavonoid Content of Nine *Hypericum* Species Grown in Greece. *Appl. Sci.* **2023**, *13*, 3305. https://doi.org/10.3390/app13053305

Academic Editor: Monica Gallo

Received: 8 February 2023
Revised: 27 February 2023
Accepted: 3 March 2023
Published: 5 March 2023

1. Introduction

Antimicrobial resistance towards monotherapy or multiple antibiotic therapy is a spiraling problem because a growing number of infections are becoming harder to treat with current antibiotic treatments, and thereafter serious or even lethal health risks are raised. Multidrug-resistant organisms (MDROs), as reported by official organizations (WHO, ECDC), include, among others, *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* [1,2]. Hence, there is a critical need for new antimicrobial compounds to fight drug-resistant microorganisms. Most antibiotics that are used originate from microbes, which means they are basically naturally derived products. Consequently, plant-derived products are a great alternative to the discovery of new biologically active compounds to use against hospital-acquired infections or community-acquired pathogens. Plant-derived products are mixtures of secondary metabolites that consist of many diverse compounds classified based on structural differences. Among them, phenolic compounds are the largest and best studied group. More than 8000 phenolics with documented molecular structures have been described so far [3–5]. These secondary metabolites are studied with great interest by researchers due to

their multiple biological activities [6,7].Especially, the study of phenolic compounds against antimicrobial resistance is challenging, and several studies confirm theirin vitroefficacy against a vast range of Gram-positive and Gram-negative bacteria [6,8–10].

The genus *Hypericum* belongs to the Hyperiaceae family, which encompasses approximately 500 species divided into 36 sections based mainly on morphological characteristics [11,12]. Historically, the use of the plant as a remedy has its roots in ancient civilizations. Treatment of wounds, burns, and bruises was among the most popular uses of *H. perforatum*, whereas gastrointestinal diseases, dysmenorrhea problems, acute mastitis, and depression were also handled with the herb [13–16]. Nowadays, these healing properties of *Hypericum perforatum* and other species of the genus are attributed to its complex chemical composition. Naphtodianthrones (including hypericin), phloroglucinols (including hyperforin), flavonoids (such as rutin, quercetin, and myricetin glucosides), xanthones (including cis-kielcorin), and phenolic acids (such as coumaroylquinic acid and chlorogenic acid) are the main classes of compounds identified in *Hypericum* extracts [2,17–19].

However, despite the large number of recognized species, the one most studied for its bioactivity remains *H. perforatum*, and many formulations of dietary supplements designed to ease central nervous system disorders are available [20]. In addition, studies on the antimicrobial activity of *H. perforatum* and other species of the genus have been published in the past few years [21–23]. Most focus was given to hypericin and hyperforin as the compounds responsible for the antimicrobial activity [21–23]. Nevertheless, the complex chemical profile of *Hypericum* species in combination with the already known activity of the secondary metabolites produced by the plants indicates that such biological activity is the result of synergistic action between all or some of the compounds that are present in the species [9].

Despite the frequent use of *H. perforatum* in modern society, studies on other species of the genus are lacking. Screening of more *Hypericum* species or species not yet studied is important and can be a strong stimulus for further research on the genus *Hypericum* and the development of new products to introduce to the pharmaceutical market. Therefore, with the aim of providing new data regarding infrequently studied species of the genus *Hypericum*, this study evaluates nine *Hypericum* species, namely *H. perforatum* and *H. tetrapterum* (sect. *Hypericum*), *H. perfoliatum*, *H. rumeliacum* subsp. *apollinis*, *H. vesiculosum* and *H. cycladicum* (sect. *Drosocarpium*), *H. fragile* (sect. *Taeniocarpium*), *H. olympicum* (sect. *Olympia*), and *H. delphicum* (sect. *Adenosepalum*), for their antimicrobial activity, total phenolic content, and total flavonoid content. The present work enhances the knowledge regarding the bioactive compounds of *Hypericum* species and consists of an additional step to further research the biological activity of more species of the genus.

2. Materials and Methods

2.1. Plant Material

Plant material was collected during the flowering period from different localities in Greece. The specimens were deposited at the herbarium of the Agricultural University of Athens (ACA). Precise information about the geographic locations and voucher numbers of the collected plant material is given in Table 1.

2.2. Preparation of Extracts

Two grams of each sample (leaves, flowers, and stems) were weighted and soaked for 2 min in a hydroalcoholic solution (70% v/v). An ultrasonic bath extraction at a stable temperature (25 $\pm$ 1 °C) and frequency (35 kHz) was implemented to isolate secondary metabolites from the plant material. Extraction was repeated three times in the dark. The extract was transferred to a rotary evaporator to remove the organic solvent. The remaining aqueous extract was cooled and lyophilized. The obtained powder was kept at -20 °C until further use.

Table 1. Collection data of the *Hypericum* species examined.

Taxon	Section	Collection Site	Collection Date	Latitude	Longitude	Elevation (m)	Habitat	Voucher Number
H. cycladicum	*Drosocarpium*	Andros Island	12 May2019	37°54′	24°52′	30	Phrygana	12285
H. delphicum	*Adenosepalum*	Evvia Island	14 June2019	38°53′	23°52′	1000	Forest clearings	12281
H. fragile	*Taeniocarpium*	Evvia Island	15 June 2019	38°32′	24°01′	420	Cliffs	12283
H. olympicum	*Olympia*	Evvia Island	14 June 2019	38°36′	23°51′	890	Rocky slopes	12288
H. perfoliatum	*Drosocarpium*	Mt. Parnon	1 June 2019	37°15′	22°39′	1050	Forest	12282
H. perforatum	*Hypericum*	Andros Island	8 June 2019	37°50′	24°53′	560	Rocky slopes	12280
H. rumeliacum subsp. *apollinis*	*Drosocarpium*	Mt. Parnassos	27 May 2019	38°33′	22°34′	1760	Rocky slopes	12286
H. tetrapterum	*Hypericum*	Evvia Island	14 June 2019	38°36′	23°51′	910	Wet places	12287
H. vesiculosum	*Drosocarpium*	Mt. Chelmos	5 June 2022	38°05′	22°10′	910	Woodland	12284

2.3. Quantification of Total Phenolic Content

The total phenolic content of the extracts was estimated with the FolinCiocalteuPhenol Reagent (Sigma-Aldrich, Hamburg, Germany). Crude extracts were dissolved in methanol and water (70% *v*/*v*). The experiment took place in the dark, and the procedure followed the description in our previous study [24]. Extracts were incubated for $1^{1/2}$ h and their absorbance was read at 765 nm. Quantification of phenolic compounds was performed using a standard curve of gallic acid monohydrate (Riedel-de Haën AG, Seelze, Germany) at concentrations ranging from 100–800 μg/mL. Results were expressed as mg of gallic acid equivalents (GAE) per gram of dry plant material using the equation derived from the plot: y = 0.0011x + 0.0148 (r = 0.999, *n* =3).

2.4. Quantification of Total Flavonoid Content

The total flavonoid content of the extracts was estimated using the aluminum chloride ($AlCl_3$ anhydrous crystallized $\geq$ 99.0%, Sigma-Aldrich Germany) colorimetric assay. Crude extracts were dissolved in methanol. Then 200 μL of each extract were added to 1 mL of a 2% $AlCl_3$ methanolic solution, followed by the addition of 3.8 mL of methanol to make a final volume of 5 mL. Solutions were vortexed and incubated for 15 min in the dark. Absorbance was read at 430 nm against a blank solution using methanol. A calibration curve was constructed following the above steps, using rutin dissolved in methanol at concentrations ranging from 200–1700 μg/mL. Results were expressed as mg of rutin equivalents (RE) per gram of dry plant material using the equation derived from the plot: y = 0.0539x + 0.0585 (r = 0.986, *n* =3).

Rutin was chosen as a standard solution, as indicated by the European Pharmacopoeia, Section II, hypericin herba [25], and according to preliminary experiments described as follows: For the total flavonoids assay, preliminary experiments with quercetin and rutin standard solutions (ExtraSynthese, Genay, France) after complexing with $AlCl_3$ showed a maximum absorbance for quercetin at 458 nm, while the maximum absorbance for rutin was read at 435 nm after reaction with $AlCl_3$ [Figure 1]. Afterwards, the reaction of the flavonoids with the $AlCl_3$ complex was performed on the extracts. The results showed that, in the presence of the extracts, absorbance was near 430 nm. According to the literature data, most studies use a range of 410–430 nm to measure TFC [26]. Therefore, for the reasons mentioned above, rutin was chosen for the quantification of TFC, and the selected wavelength was 430 nm.

2.5. Antibacterial Activity

The antibacterial activity of the extracts was evaluated by determining the minimum inhibition concentration (MIC) values and the minimum bactericidal concentration (MBC) values of the extracts. The MIC was determined using the broth micro-dilution method as described in a previous study [27]. *Hypericum* extracts (10 mg/mL starting concentration) were dissolved in DMSO (a final concentration of 4%), and 200 μL of each extract was transferred to a 96-well plate in order to achieve a 2-fold serial dilution with 100 μL

of Mueller Hinton Broth (MHB). Isolated cultures of *E. coli* (NCTC 9001, Sigma Aldrich, Hamburg, Germany), *S. aureus* (NCTC 6571, Sigma Aldrich, Germany), *E. faecalis* (NCTC775, Sigma Aldrich, Hamburg, Germany), and *S. enteritidis* (WDCM 00030, Sigma Aldrich, Hamburg, Germany)were prepared in MHB at a concentration of about 1×10^6 cfu/mL. Onehundred microliters (100 µL) of each bacterial inoculum were added to each well of extract or control. Blank samples of each extract (without bacteria) were subjected to a 2-fold serial dilution with MHB (blank control). Controls with bacteria (100 µL) but no extract were used as growth controls. Also, a solvent control and a sterility control were used with MHB, with no bacteria and no extract, respectively. Ampicillin (0.516 mg/mL, Sigma Aldrich, Gillingham, UK) or Gentamycin (0.064 mg/mL, Molekula, Darlington, UK) were included as positive controls. After 18 h of incubation at 37 °C, 30 µL of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT) (Sigma Aldrich, Gillingham, UK) was added to each plate, followed by incubation at 37 °C for 30 min. The absorbance of each plate was measured at 492 nm with a microplate reader (Sunrise, Tecan Trading Ltd., Zürich, Switzerland). The MIC of each extract was defined as the sample concentration that inhibited bacterial growth as compared with that of the blank control.The MBC of the extracts was also determined by subculturing 2 µL aliquots of the MIC assay preparations in 100 µL MHB and incubating for 24 h at 37 °C. The MBC was defined as the lowest concentration of each sample that did not exhibit a color change after the addition of INT, as described above.

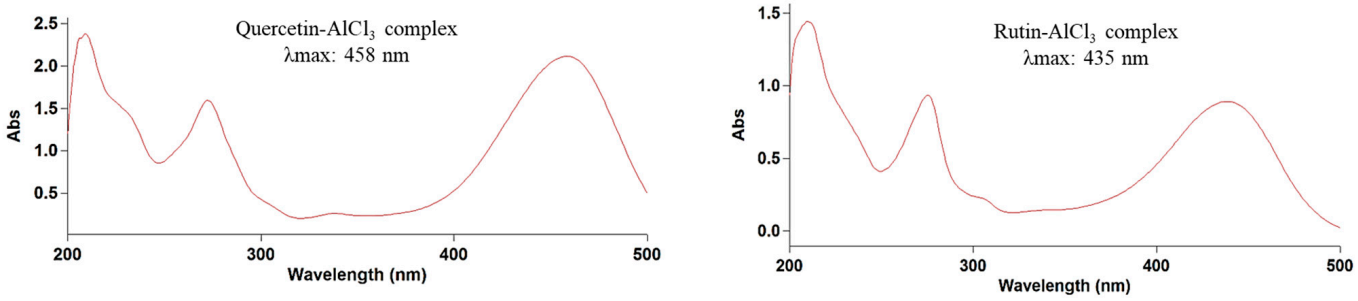

Figure 1. UV-Vis spectrum of quercetin and rutin standard solutions after complexing with $AlCl_3$ reagent.

2.6. Statistical Analysis

All experiments were performed in triplicates. The results were presented as the mean value ± the estimated SD. Significance differences between sample means were determined by a student's *t* test using GraphPad Prism (ver. 8.4.2). The significance level was set to 0.05, and the confidence intervals were at ±95% CI. The data were presented as mean ± standard deviation (SD).

3. Results

3.1. Total Phenolic and Total Flavonoid Content

Results demonstrated that the extract of *H. perforatum* was the most abundant in phenolic compounds (86 ± 13.34 mg GAE/g dry plant material), while the lowest TPC was determined in the extract of *H. vesiculosum* (26.92 ± 10.32 mg GAE/g dry plant material). For the total flavonoid content shown in Table 2, the most abundant extract in flavonoids was that of *H. tetrapterum* (1.58 ± 0.08 mg RE/g dry plant material). Similarly, the lowest TFC was calculated for *H. perfoliatum* (0.21 ± 0.14 mg RE/g dry plant material). The UV-Vis spectra of some of the *Hypericum* species studied are presented in Figure 2. Figure 2a presents the UV-Vis spectra of the less studied species of *Hypericum*, namely *H. cycladicum*, *H. delphicum*, and *H. fragile*, as well as the UV-Vis spectrum of *H. perforatum*, to allow comparison with the other species. Figure 2b presents the UV-Vis spectra of the same species after complexing with $AlCl_3$. As shown in Figure 2a, the UV-Vis spectra of *H. cycladicum* and *H. fragile* present a λmax at 334 nm and 330 nm, respectively. This

range of absorption is typical for flavones and flavonols [28]. To distinguish between these two groups, Marby et al. [29] observed that the UV wavelength of the B ring is near 304–350 nm for flavones, while flavonols absorb at a longer wavelength of 328–357 nm. Therefore, according to the literature data, flavonols are compounds that seem to prevail in the extracts of *H. cycladicum* and *H. fragile*. Moreover, the hypsochromic shift at band I in the case of *H. delphicum* is attributed to the presence of sugar moieties, more probably attached at position 3 of the C ring [29].

Table 2. Total phenolic and total flavonoid content of the *Hypericum* species examined.

Species	TPC ± SD	TFC ± SD
	mg GAE/g dry plant material	mg RE/g dry plant material
H. perfoliatum	59.31 ± 16.47 ^{a,*}	0.21 ± 0.14 ^{A}
H. rumeliacum subsp. *apollinis*	54.87 ± 2.73 ^{a}	1.18 ± 0.22 ^{B}
H. vesiculosum	26.92 ± 10.32 ^{b}	0.30 ± 0.12 ^{C}
H. cycladicum	54.59 ± 8.55 ^{a}	1.34 ± 0.05 ^{D}
H. perforatum	86 ± 13.34 ^{c}	0.76 ± 0.11 ^{E}
H. tetrapterum	51.26 ± 21.36 ^{a}	1.58 ± 0.08 ^{F}
H. fragile	39.72 ± 6.05 ^{d}	0.54 ± 0.12 ^{G}
H. olympicum	32.63 ± 17.42 ^{b}	0.64 ± 0.09 ^{H}
H. delphicum	54.09 ± 4.29 ^{a}	0.6 ± 0.17 ^{I}

* Results are presented as mean ±standard deviation (SD) ($n = 3$) ($p < 0.05$). $^{a–d}$ and $^{A–I}$ values with the same letter are non-significantly different ($p < 0.05$).

As demonstrated in Table 2, there seems to be no proportional relationship between TPC and TFC. The mechanism of action of the Folin Ciocalteu assay is the transfer of electrons from phenolic compounds. Nevertheless, non-phenolic compounds such as ascorbic acid, aromatic amines, and sugars can also reduce the Folin Ciocalteu reagent, resulting in an additive effect on the interpreted results [30]. Consequently, a high TPC is not always accompanied by a high TFC, as in the case of *H. perforatum*. Fewer limitations exist for the $AlCl_3$ colorimetric assay. This method is accepted among researchers for the determination of total flavonoid content because the reagent does not interfere with other subclasses of phenolic compounds. On the contrary, $AlCl_3$ forms stable complexes with flavonoids by binding at position 4 of the cheto-group (C ring) and occupying at the same time hydroxyl groups from positions 5 or 3. The other binding site is at positions 3′ and 4′ [Figure 3]. However, most of the flavonoids are produced as glycosides. A glycosidic moiety or moieties may occupy one or more of the binding sites of $AlCl_3$; therefore, $AlCl_3$ will not bind the positions occupied by the sugar moiety [31].Phenolic compounds are produced mostly in their glycosylated form [32]. Hence, it is possible that the stereochemistry of glycosylated flavonoids presented in the extracts hinders the binding of $AlCl_3$.

3.2. Antimicrobial Activity

According to the results shown in Table 3, none of the extracts exhibited antibacterial activity against Gram-negative bacteria (*E. coli* and *S. enteritidis*). On the contrary, all *Hypericum* extracts demonstrated some degree of inhibition (weak, moderate, or strong) and bactericidal effect against Gram-positive bacteria (*S. aureus* and *E. faecalis*). More specifically, *H. perforatum* demonstrated strong growth inhibition and bactericidal activity against both *S. aureus* (MIC: 0.06 mg/mL; MBC: 0.51 mg/mL) and *E. faecalis* (MIC: 0.13 mg/mL; MBC: 0.51 mg/mL). Similarly, *H. delphicum* also demonstrated a strong growth inhibition against both Gram-positive species. On the other hand, *H. cycladicum* and *H. olympicum* exhibited strong bacterial inhibition activity against *S. aureus* (MIC: 0.31 mg/mL) but moderate inhibition against *E. faecalis* (MIC: 0.63 mg/mL). All the other *Hypericum* species demonstrated moderate bacterial inhibition activity (MIC: 0.13 mg/mL), except the *H. fragile* extract, which exhibited weak inhibition activity against both *S. aureus* and *E. faecalis* (1.75 mg/mL and 3.50 mg/mL, respectively).

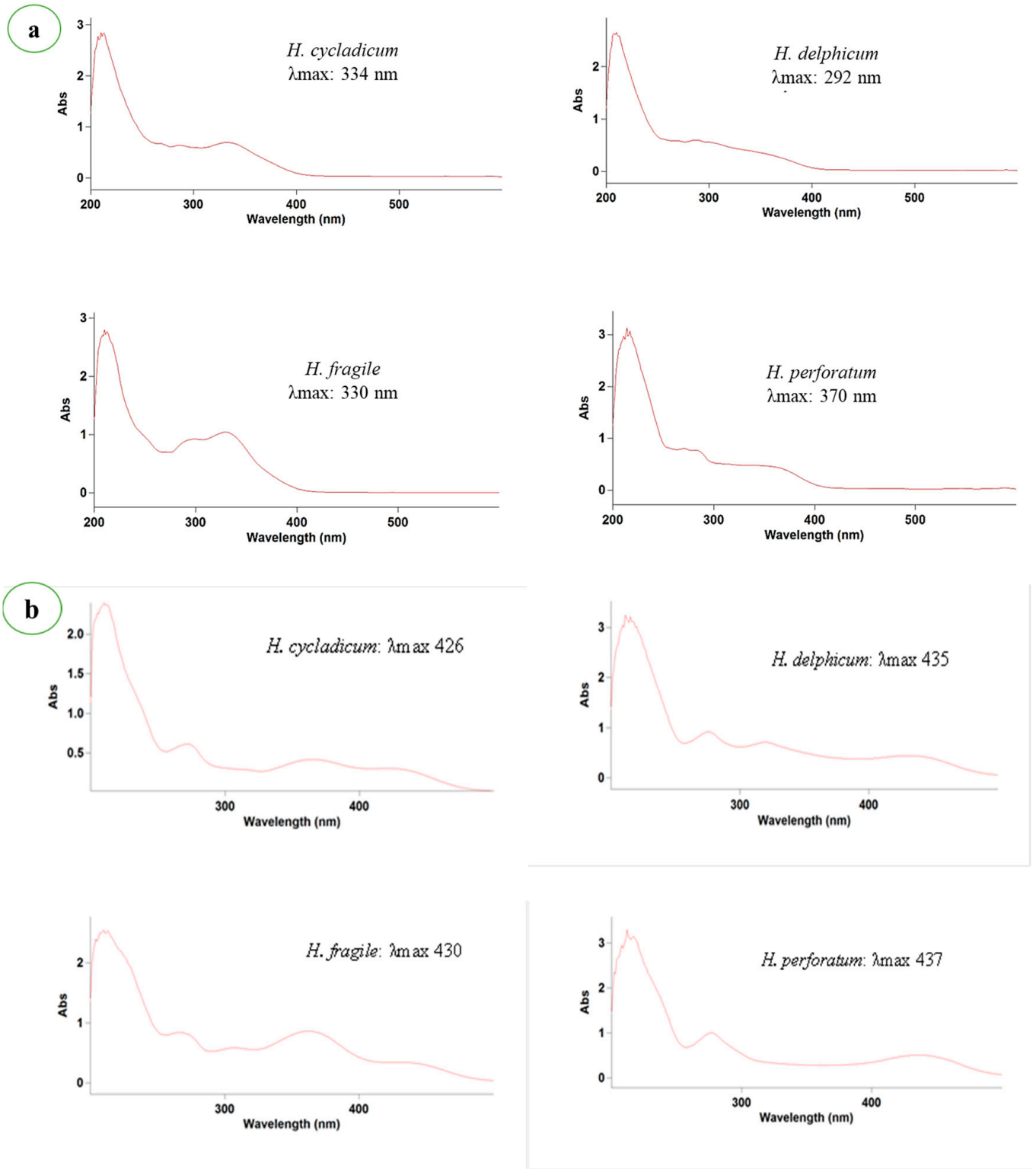

Figure 2. (**a**) UV-Vis spectra of *H. cycladicum*, *H. delphicum*, *H. fragile*, and *H. perforatum* extracts. (**b**) UV-Vis spectra of *H. cycladicum*, *H. delphicum*, *H. fragile*, and *H. perforatum* extracts after complexing with $AlCl_3$ reagent.

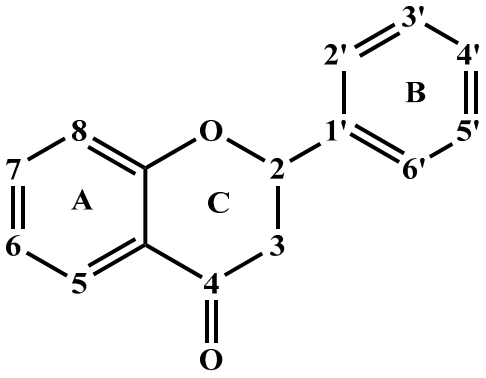

Figure 3. General structure of flavonoids.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the *Hypericum* species against *E. coli*, *S. enteritidis*, *S. aureus*, and *E. faecalis* bacteria.

Hypericum Species Extracts	*E. coli*		*S. enteritidis*		*S. aureus* *		*E. faecalis* **	
	MIC [2] (mg/mL) ± SD	MBC [3] (mg/mL) ± SD	MIC [2] (mg/mL) ± SD	MBC [3] (mg/mL) ± SD	MIC [2] (mg/mL) ± SD	MBC [3] (mg/mL) ± SD	MIC [2] (mg/mL) ± SD	MBC [3] (mg/mL) ± SD
H. perfoliatum	-	-	-	-	1.25 ± 0.02^{a}	1.25 ± 0.02^{A}	1.25 ± 0.02^{a}	1.25 ± 0.02^{A}
H. rumeliacum subsp. *apollinis*	-	-	-	-	1.25 ± 0.02^{a}	1.25 ± 0.02^{A}	1.25 ± 0.02^{a}	5.00 ± 0.04^{B}
H. vesiculosum	-	-	-	-	1.25 ± 0.02^{a}	2.50 ± 0.03^{B}	1.25 ± 0.04^{a}	5.00 ± 0.05^{B}
H. cycladicum	-	-	-	-	0.31 ± 0.04^{b}	1.25 ± 0.04^{A}	0.6 ± 0.05^{b}	1.25 ± 0.02^{A}
H. perforatum	-	-	-	-	0.06 ± 0.01^{c}	0.51 ± 0.06^{C}	0.13 ± 0.02^{c}	0.51 ± 0.05^{C}
H. tetrapterum	-	-	-	-	1.25 ± 0.03^{a}	1.25 ± 0.03^{A}	1.25 ± 0.05^{a}	2.50 ± 0.24^{D}
H. fragile	-	-	-	-	1.75 ± 0.05^{d}	1.75 ± 0.05^{D}	3.50 ± 0.06^{d}	5.00 ± 0.06^{B}
H. olympicum	-	-	-	-	0.31 ± 0.05^{b}	1.25 ± 0.04^{A}	0.63 ± 0.04^{b}	2.50 ± 0.05^{D}
H. delphicum	-	-	-	-	0.16 ± 0.02^{e}	0.63 ± 0.05^{E}	0.31 ± 0.03^{e}	0.63 ± 0.03^{E}
Amp [1]	0.03 ± 0.002	0.03 ± 0.002	0.02 ± 0.001	0.02 ± 0.001	NA	NA	NA	NA
Gen [1]	NA	NA	NA	NA	0.02 ± 00.01	0.02 ± 0.001	0.02 ± 0.003	0.02 ± 0.003

[1] Ampicillin and gentamycin were used as antibacterial control samples against Gram-negative and Gram-positive bacteria, respectively; [2] The smaller the MIC value, the less extract is required for bacterial growth inhibition. Substances with MIC values of <0.6 mg/mL are regarded as strong inhibitors, 0.6–1.6 mg/mL moderate, 1.6–8.0 mg/mL weak, and >8.0 mg/mL low [33,34]; [3] MBC indicates the lowest concentration of the extract that is bactericidal. The smaller the MBC value, the less extract is required to kill the bacteria. -: not active; Amp: Ampicillin; Gen: Gentamycin; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; NA: not available. * $^{a–e}$; $^{A–E}$ values with the same letter are not significantly different ($p < 0.05$). ** $^{a–e}$; $^{A–E}$ values with the same letter are not significantly different ($p < 0.05$).

4. Discussion

Resistance to antibiotics is a spiraling health problem. Current antibiotic therapies gradually become ineffective or are sometimes inadequate to treat infections, an issue that, according to the WHO, prolongs hospitalization and increases costs and mortality rates. In this regard, the discovery of new antibiotics is urgent. Auspicious solutions against antibiotic resistance are naturally derived products [35,36]. Plants have been used to heal numerous infectious diseases since antiquity, and traditional medicine is still widely used to prevent and manage health issues [37,38]. Motivated by traditional medicine and based on scientific knowledge, several medications have been derived from medicinal plants, or naturally derived molecules were the key to the synthesis of new ones [35]. In our study, nine *Hypericum* species were tested for their antibacterial activity against four microorganisms: *E. coli*, *S. enteritidis*, *S. aureus*, and *E. faecalis*. All the tested samples showed selective antibacterial activity against Gram-positive bacteria. *H. perforatum* was the most potent extract against both *S. aureus* and *E. faecalis*, while the extract of *H. fragile* showed the weakest antibacterial activity. Comparing the activities of the species that belong to sect. *Drosocarpium*, namely *H. perfoliatum*, *H. rumeliacum* subsp. *apollinis*, *H. vesiculosum*, and *H. cycladicum*, the latter was the most active to inhibit the growth of *S. aureus* and *E. faecalis* (MIC: 0.31 and 0.63 mg/mL, respectively), while the extracts of *H. perfoliatum*, *H. rumeliacum* subsp. *apollinis*, and *H. vesiculosum* were equally effective. Within the *Drosocarpium* section, non-statistically significant differences were observed

between its TPC and those calculated for *H. perfoliatum* and *H. rumeliacum* subsp. *apollinis* extracts. On the other hand, statistically significant differences were observed between the TFC of the extracts, with *H. cycladicum* being the extract with the highest TFC. Therefore, the better antibacterial activity of the extract can be mainly attributed to its TFC. Regarding *H. rumeliacum* subsp. *apollinis*, the concentration required to inhibit bacterial growth is equal to that of *H. perfoliatum* and *H. vesiculosum*. Within these extracts, non-statistically significant differences were observed regarding their TPC, and all the extracts differed significantly for their TFC.

As for the *Hypericum* section, which includes *H. perforatum* and *H. tetrapterum*, the best antibacterial activity for both of the bacteria tested was observed in the *H. perforatum* extract. The total phenolic content of *H. perforatum* exceeds that of *H. tetrapterum*. This study discusses the phytochemical profile of the extracts based on the TPC and TFC. However, according to the literature, *Hypericum* species also contain other classes of compounds, specifically hyperforin and hypericins. Hyperforin and similar compounds (for example, adhyperforin) are compounds with strong antibacterial activity [39,40]. Therefore, the better antibacterial result observed in the case of *H. perforatum* indicates that, apart from phenolic compounds, the presence of other classes of compounds in this extract contributes to the overall antibacterial activity.

Between the extracts of *H. fragile, H. olympicum*, and *H. delphicum*, the TPC of the latter extract was the highest among the three species, while their TPC and TFC presented statistically significant differences. Naphtodiantrones are also found in *Hypericum* species of the *Adenosepalum* and *Taeniocarpium* sections [41]. Consequently, the strongest antibacterial activity of *H. delphicum* can be attributed not only to its high phenolic content but also to other compounds, such as phenolic acids with interesting antimicrobial activity [42] and the presence of naphtodianthrones and/or phloroglucinols [41].

In general, regarding the results of the antibacterial activity of the extracts, it should be noted that apart from flavonoids such as luteolin [43,44] and kaempferol [43–46], other compounds such as chlorogenic acid and neo-chlorogenic acid are also discussed as occurring in the genus *Hypericum* [44–47]. In addition to flavonoids, it has been demonstrated that chlorogenic acid is a compound with exceptional antibacterial activity [48,49]. Differences regarding the bactericidal activity of the above extracts indicate that not only quantitative but also qualitative differences may produce synergistic effects that potentiate the activity of the extract and contribute to its overall activity.

A broad spectrum of antimicrobial activity has been demonstrated for various *Hypericum* species against Gram-positive and Gram-negative microorganisms. *H. perforatum* is the most studied species, and several solvents, such as ethanol, methanol, and chloroform, have been used by researchers to isolate its secondary metabolites [21,23,50,51]. Similarly, various microorganisms, such as *S. subtilis*, *S. aureus*, *E. faecalis*, and *E. coli*, have been tested for their susceptibility to *Hypericum* extracts [50,51]. For example, in the study by Avato et al. [51], six Gram-positive bacteria were treated with different extracts of *H. perforatum*. It was concluded that the ethanolic and chloroform extracts were the most potent because the MIC values were lower. Nevertheless, all extracts presented antimicrobial activity, and when such activity was compared to that of standard compounds, namely hyperforin and hypericin, it was demonstrated that both standard compounds inhibited the majority of the microorganisms under investigation, while the concentration requested to inhibit growth of the microorganisms was, for most of the bacteria, close to that of the extracts [51]. Similarly, different subspecies of *H. perforatum* and other *Hypericum* species that belong to different sections from Central Italy were examined for their antimicrobial activity [50]. Among all the tested extracts, those of the *H. perforatum* subspecies were the most active, while only the extract of *H. perforatum* subsp. *veronense* managed to inhibit the growth of *E.coli*. Additionally, *H. tetrapterum* inhibited fewer microorganisms, and the zone of inhibition of bacterial growth was smaller than that formed when microorganisms were treated with the extract of *H. perforatum*. In the same study, the antimicrobial activity of *H. hirsutum* (sect. *Taeniocarpium*) was also evaluated. The *H. fragile* reported in our study

belongs to the same section, thus we compared the antimicrobial activity of this extract with *H. hirsutum*, for which no data have been published so far. In contrast to *H. hirsutum*, for which no inhibition against *E. faecalis* was observed, *H. fragile* showed a broader spectrum of antimicrobial activity because the extract hindered both *S. aureus* and *E. faecalis*. It is possible that different extraction procedures resulted in differences in the phytochemical composition of these extracts, which in turn affected the obtained results [50]. *H. delphicum* is another species for which data regarding its antimicrobial activity are not available. However, the above-mentioned study [50] and the study by Dall'Agnol et al. [23] examine *H. montanum* and *H. caprifolium*, respectively, species that belong to the same section (sect. *Adenosepalum*) as *H. delphicum*. The results are partially in accordance with our findings because the extract of *H. caprifolium* was also active against Gram-negative bacteria, a result not observed for *H. delphicum*.

H. olympicum was examined by Radulović et al. [43,52] and, according to their results, a broad spectrum of antimicrobial activity was observed against both Gram-positive and Gram-negative bacteria, including *S. aureus* and *E.coli*. However, in our study, *H. olympicum* did not inhibit Gram-negative bacteria. Nevertheless, new information is now available about its antibacterial activity against *E. faecalis*, a microorganism resistant to many antibiotics [53,54].

There are limited studies that deal with the antibacterial activity of *H. perfoliatum* and *H. rumeliacum* subp. *apollinis* [52,55], while no previous studies were found for *H. vesiculosum* and *H. cycladicum*. All these species are classified within sect. *Drosocarpium*. *H. perfoliatum* was studied for its antimicrobial activity by Del Monte et al. [55]. The methanolic extract did not exhibit antimicrobial activity; on the contrary, chloroform and chloroform-methanol extracts exerted significant antimicrobial activity against all the microorganisms tested. In our study, the *H. perfoliatum* extract inhibited only Gram-positive bacteria, and in fact, the MIC and MBC values were equivalent for both microorganisms. Similar results were reported by Radulović et al. [52] regarding *H. rumeliacum* Boiss. The extract showed strong antimicrobial activity against life-threatening microorganisms, such as *K. pneumonia* and *S. enteritis*. In our study, *H. rumeliacum* subsp. *apollinis* inhibited only Gram-positive bacteria, and the inhibition of *E. faecalis* by *H. perfoliatum* and *H. rumeliacum* subsp. *apollinis* was reported for the first time.

All the tested extracts were active against Gram-positive bacteria; however, considering the literature data, generally, *Hypericum* spp. seem better for combating Gram-positive bacteria. *S. aureus* and *E. faecalis* are two microorganisms that resist some kinds of antibiotics, such as β-lactames [56], tetracyclines, and macrolides [57]. Certainly, the biological activity of an extract is dependent on its phytochemical profile and the extraction technique used to isolate secondary metabolites. Consequently, considering the different geographical origins of the *Hypericum* species used in this study, differences regarding their potency or inability to inhibit microorganisms are expected.

Regarding the total phenolic and total flavonoid content, the most studied *Hypericum* species are those of the *Drosocarpium* section, followed by those of the *Hypericum* section [17,58,59]. For example, Zheleva-Dimitrova et al. [59] studied, among others, some species from the *Drosocarpium* and *Hypericum* sections. Species from the *Hypericum* section, specifically *H. perforatum* and *H. tetrapterum*, were reported to have the highest total tannin content (8.67 ± 0.02 g/100 g DW for *H. perforatum*) and TFC (1.13 ± 0.02 g/100 g DW for *H. tetrapterum*), respectively. Our results demonstrated a similar bioactivity when referring to the *Hypericum* section, as *H. perforatum* contained the highest content of phenolic compounds, while TFC was highest in the *H. tetrapterum* extract. According to Zheleva-Dimitrovaet al. [59], *H. olympicum*, a species of the *Olympia* section, demonstrated a moderate quantity of total tannins (3.28 ± 0.03 g/100 g DW), while the TFC was the lowest (0.20 ± 0.03 g/100 g DW) compared to all the other studied species investigated. In our study, similar results were demonstrated regarding the TPC of *H. olympicum*. However, the TFC of the extract exceeded that of *H. perfoliatum* and *H. vesiculosum*, belonging to

the *Drosocarpium* section. At the same time, for the TFC and the *Drosocarpium* section, *H. cycladicum* and *H. rumeliacum* subsp. *apollinis* were the most abundant.

5. Conclusions

In this study, nine *Hypericum* species collected in Greece were evaluated for their total phenolic and total flavonoid content, as well as for their antibacterial activity. Among the studied species, *H. perforatum* showed the highest TPC, while the TFC was the highest in the case of *H. tetrapterum*, followed by *H. cycladicum* and *H. rumeliacum* subsp. *apollinis*, extracts that belong to the *Drosocarpium* section. The obtained results indicated *Hypericum* species as a good source of secondary metabolites; therefore, their biological activity was evaluated against Gram-positive and Gram-negative bacteria. Extracts demonstrated weak to strong antibacterial activity. Gram-negative bacteria were all resistant to the tested extracts, while Gram-positive bacteria were successfully inhibited. The extracts showed a minor antibacterial potential with respect to the antibiotic gentamycin. However, the need for new antimicrobial agents is continually increasing. In this regard, the study of natural products as potential alternatives to available antibiotic treatments is of critical importance. The findings of this study provide valuable insights into the biological activity of *Hypericum* species. We demonstrate that, apart from *H. perforatum*, screening other species of the genus is also likely to contribute to the field of new drug development.

Author Contributions: Conceptualization, E.K., D.D., P.A.T. and P.T.; methodology, E.K., D.D. and D.C.; investigation, E.K. and D.C.; data curation, E.K., D.D. and D.C.; writing—original draft preparation, E.K. and D.C.; writing—review and editing, D.D., D.C., M.P., P.T., C.D.K. and P.A.T.; supervision, C.D.K., P.A.T. and M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting this study are not publicly available. Please contact eleni.kakouri@aua.gr, ptara@aua.gr.

Conflicts of Interest: The authors declare no conflict of interest.

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