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Article

Flavonoid Profiles and Antioxidant Potential of *Monochoria angustifolia* (G. X. Wang) Boonkerd & Tungmunnithum, a New Species from the Genus *Monochoria* C. Presl

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Abstract: Plants of the genus *Monochoria* have long been utilized in food, cosmetics, and traditional herbal treatment. Thailand has the highest species diversity of this genus and a new member, *Monochoria angustifolia* (G. X. Wang) Boonkerd & Tungmunnithum has been recently described. This plant is called "Siam Violet Pearl" as a common name or "ไข่มุกสีม่วงแห่งสยาม" as its vernacular name in the same meaning in Thai language. Despite their importance, few researches on *Monochoria* species have been conducted. This study, thus, provided the results to fill in this gap by: i) determining flavonoids phytochemical profiles of 25 natural populations of *M. angustifolia* covering the whole floristic regions in Thailand, and ii) determining antioxidant activity using various antioxidant assays to investigate the probable mechanism. The results revealed that *M. angustifolia* presented a higher flavonoid content than the outgroup, *M. hastata*. Our results also revealed that flavonoids might be used to investigate *Monochoria* evolutionary connections and for botanical authentication. The various antioxidant assays revealed that *M. angustifolia* extracts preferentially act through a hydrogen atom transfer antioxidant mechanism. Pearson correlation analysis indicated significant correlations emphasizing that the antioxidant capacity is most probably the result of a complex phytochemical combinations rather than of a single molecule. Altogether, these results showed that this new species provide an attractive alternative starting material with phytochemical variety and antioxidant potential for the phytopharmaceutical industry.

Keywords: *Monochoria angustifolia*; *Monochoria hastata*; Flavonoid; Antioxidant mechanism; Natural populations; Phytochemical profile; Traditional herbal medicine; Phytopharmaceutical

1. Introduction

Monochoria angustifolia (G. X. Wang) Boonkerd & Tungmunnithum is the newest member of the genus *Monochoria* C. Presl which belongs to the family Pontederiaceae. There are 8 species of *Monochoria* worldwide [1–3], and Thailand is the richest species diversity area of the genus *Monochoria*. There are 4 species reported in Thailand such as *M. elata, M. hastata, M. vaginalis* and *M. angustifolia* [1–4]. Currently, *M. angustifolia* is the new species that described by Tungmunnithum and her research teams in 2020 base on both morphological and molecular (phylogenetic analysis) evidences [1].

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M. angustifolia is an aquatic plant with beautiful blooming violet-pearl perianth and it is the new species from Thailand [1]. Therefore, this plant is called "Siam Violet Pearl" as a common name or "ไข่มุกสีม่วงแห่งสยาม" as its vernacular name in the same meaning in Thai language. The natural habitat of *M. angustifolia* plant is standing water bodies of tropical regions. This new species is annual herb that distributes in several floristic regions in Thailand, and have never been reported in other countries (World Checklist of Selected Plant Families [1–5]. It is possible that the environmental factors play an important role as the limiting factor to limit the distribution of *M. angustifolia* plants as the other endemic species that can be found only in Thailand for example *Hoya siamica* Craib [6]. Even *M. angustifolia* has narrow distribution, but it is abundant individual plants in Thailand. As this plant species is native and well distributed throughout Thailand, as well as it is easy to grow and can be propagated both asexually by seeds and asexually by budding. These characteristics are particularly suitable for research and development as raw plant materials for the phytopharmaceutical and cosmetic industries. Besides, it is also helpful for local people to grow and cultivate the potential population of this plant, so as to provide the quality plant materials for the industrial sector. Furthermore, the *Monochoria* plant group has long been used as food (vegetable and/or cooking ingredient), skin care (leaves and flowers extracts) and traditional medicine (leaves or the aerial part) in Thailand, Japan, India and other countries in Asia since the ancient time. Local people have used its leaves for treatment of asthma and to relieve toothache. Its roots are also used to cure stomach and liver problems [4,7].

Flavonoids and other related phenolic substances are naturally occurring chemicals in plants [8–10]. Their antioxidant activity has been extensively studied, and it is widely assumed that it is connected to their quantity and/or chemical structures, such as the location of hydroxyl groups. However, the majority of information on antioxidant activity is generally based on a small number of plant species or cultivars [11]. Furthermore, antioxidant activity is usually determined using a restricted number of assays. Due to the complex nature of phytochemicals and, in particular, since antioxidant activity is primarily dependent on the reaction mechanism involved, the antioxidant activity of plant extracts cannot be measured using a single approach [12,13]. In addition, both environmental and agricultural factors such as location (e.g., soil conditions) and climate have been shown to have a significant influence on phenolic compounds accumulation and antioxidant activity [11,14,15]. Thailand is located in "Indo-Burma" biodiversity hotspot that is recognized as the world's eighth most bio-diverse area. The country has one of the highest levels of biodiversity per unit area in the world. Thailand, which has a variety of forest types and aquatic environments, supports up to 10,000 plant species and accounts for around 10% of all living organisms on the world [16]. So far, no study has addressed the diversity in flavonoids and other associated phenolic compounds, as well as antioxidant activities measured using different assays capable of accounting for this biological activity in this new species of the world before.

The objective of this study is to complete this knowledge, with natural *M. angustifolia* and the outgroup from the same genus, *M. hastata* populations originating from different floristic regions in Thailand, by determining the total contents of phenolic, flavonoid (including HPLC determination of their flavonoid profile), as well as antioxidant activity determined using five *in vitro* assays based on different mechanisms as well as one yeast cell-based cellular antioxidant assay.

2. Materials and Methods

2.1. Chemicals and Reagents

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For extraction HPLC analysis and biological assays, all solvents and reagents were of analytical grade or of the greatest purity possible (Thermo Fischer Scientific, Illkirch, France). A Milli-Q water-purification system was used to purify ultrapure deionized water (Merck Millipore Fontenay sous Bois, Paris, France). Prior to use, all HPLC solutions were filtered using 0.45 m nylon syringe membranes. Flavonoid standards (apigenin-7-*O*-rutinoside; luteolin-7-*O*-glucoside; apigenin-7-*O*-glucoside (aka apigetrin); luteolin; apigenin) were provided by Extrasynthese (Genay, France). These analytical standards were provided with w/w absolute assay (with purity of at least 97%), to be used for quantitative titration.

2.2. Plant Materials

The living plant specimens were searched and collected from natural habitat cover the whole floristic regions in Thailand such as (1) Northern (2) North-eastern (3) Eastern (4) South-western (5) Central (6) South-eastern and (7) Peninsular. The collected populations were named according to the collected provinces (Table 1). The collected specimens were identified using the key-to-species and description in the existing Floras and previous published works [1,4,7,17–19], as well as compared with the herbarium specimens kept in Forest Herbarium (BKF), the Prof. Kasin Suvatabandhu from Herbarium, Chulalongkorn University (BCU), Kyoto University, Japan (KYO), and Plant Varieties Protection Office, Bangkok, Thailand (BK). The herbarium abbreviations are used according to Thiers [20]. Then, the leaves of each plant populations were air-dried, and then prepared following the World Health Organization [21] recommendations. The total 25 populations of *M. angustifolia* (15 samples/population) collected from every floristic region throughout the country as well as the outgroup (*M. hastata*) following the table 1 were included in this study.

2.3. Extraction

The dried leaf samples [11,22] (100 mg/sample) were placed in 5 mL quartz tubes with a vapor condenser, and were then extracted by ultrasound-assisted extraction in 1 mL of 90 percent (v/v) aqEtOH using the USC1200TH ultrasonic bath (Prolabo, Fontenay-sous-Bois, France) using the following extraction conditions: 30 kHz frequency at 45 °C for 45 minutes. The extract was then cooled down at room temperature, centrifuged at 5,000 g for 15 minutes (Heraeus Biofuge Stratos, Thermo Scientific, Illkirch, France). The resulting supernatant was filtered using 0.45 m nylon syringe membranes, (Merck Millipore, Saint-Quentin Fallavier, France). Flavonoid enrichment was next achieved using the previously reported DAX-8 macroporous resin (Merck Millipore, Saint-Quentin Fallavier, France) purification process [22].

2.4. Determination of Total Phenolic Content (TPC)

The determination of TPC was done using the Folin–Ciocalteu procedure adapted for microplate spectrophotometry as previously described [11]. The absorbance was measured using a spectrophotometer at 650 nm (BioTek ELX800 Absorbance Microplate Reader, BioTek Instruments, Colmar, France). The TPC was then expressed in mg of gallic acid equivalents per 100g dry weight DW (mg GAE/100 g DW) using a standard curve (0-40 g/mL; $R^2 = 0.998$) of gallic acid (Merck, Saint-Quentin Fallavier, France).

2.5. Determination of Total Flavonoid Content (TFC)

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The determination of TFC was done using the colorimetric aluminum trichloride ($AlCl_3$) technique adapted for microplate spectrophotometry as previously described [11]. The absorbance at 415 nm was measured using a microplate reader (Multiskan GO, Thermo Fischer Scientific, Illkirch, France). The TFC was then expressed in mg of quercetin equivalents per 100g dry weight DW (mg QE/100 g DW) using a standard curve (0-40 g/mL; $R^2 = 0.999$) of gallic acid (Merck, Saint-Quentin Fallavier, France).

2.6. High-Performance Liquid Chromatography (HPLC) analysis

The Varian HPLC system controlled by the Galaxie software (Varian v1.9.3.2) was used. The system is composed an autosampler, Varian Prostar 230 pump and Varian Prostar 335 photodiode array detector (PDA). The separation was carried out at 40 °C using a Purospher RP-18 column (250 x 4.0 mm internal diameter; 5 µm) (Merck Chemicals, Molsheim, France). The mobile phase was a methanol (solvent A) and HPLC grade water acidified with 0.05% formic acid (solvent B). A linear gradient was applied: from a 5:95 (v/v) mixture of solvents A and B to a 100:0 (v/v) mixture of solvents A and B, using a flow rate of 0.8 mL/min for 60 minutes. The injection volume was 3 µL, the highest back pressure was 110 bar, and the detection for quantification was done at 320 nm. Quantification was done using commercial authentic flavonoid standards (Extrasynthese, Genay, France).

Validation was performed as described previously by Tungmunnithum et al. [23] and according to the Association of Analytical Communities (AOAC) standards to assure accuracy and reproducibility in quantification [24].

2.7. In Vitro Cell Free Antioxidant Assays

The antioxidant activity of the extract samples was assessed using 3 different *in vitro* cell free antioxidant assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), CUPRAC (cupric reducing antioxidant capacity) and ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), as previously described [12,13].

Briefly, for FRAP assay: 10 µL of extract was mixed with 190 µL of FRAP reagent (10 mM TPTZ; 20 mM $FeCl_3$,$6H_2O$ and 300 mM acetate buffer pH 3.6, in a ratio 1:1:10 (v/v/v)) in a microplate well, and incubated for 15 min in the dark at 25 °C.

For DPPH assay: 20 µL of extract were mixed with 180 µL of DPPH reagent solution (0.1 mM final concentration in methanol) in a microplate well, and incubated for 15 min in the dark at 25 °C.

For CUPRAC assay: 10 µL of an extract was mixed with 190 µL of the CUPRAC solution (10 mM Cu(II), 7.5 mM neocuproine and 1 M acetate buffer pH 7 mixed in 1:1:1 (v/v/v) ratio) in a microplate well, and incubated for 15 min in the dark at 25 °C.

For ABTS assay: 10 µL of extract were mixed with 190 µL of ABTS solution (composed of the solution composed of ABTS salt (7 mM) and potassium persulphate (2.45 mM), incubated in the dark for at least 16 h, with absorbance (734 nm) adjusted at 0.7 prior to its use) in a microplate well, and incubated for 15 min in the dark at 25 °C.

After incubation, absorbance at 590 nm FRAP), 450 nm (CUPRAC), 515 nm (DPPH) and 734 nm (ABTS) was recorded with a microplate reader (BioTek ELX800 Absorbance Microplate Reader, BioTek Instruments, Colmar, France). Antioxidant capacity was expressed as Trolox C equivalent antioxidant capacity (TEAC) with a standard curve (0–500 µM Trolox C; $R^2 = 0.998$-0.999) for each assay.

For ORAC (oxygen radical absorbance capacity assay), 10 µL of extract were combined with 190 µL of fluorescein (0.96 µM) in 75 mM phosphate buffer pH 7.4 and incubated at 37 °C for at least 20 minutes with shaking. Then, 20 µL of 119.4 mM 2,2′-azobis-amidinopropane (ABAP, Sigma Aldrich, Saint-Quentin Fallavier, France) were added and the fluorescence intensity was measured every 5 minutes for 2.5 hours at 37 °C using a fluorescence spectrophotometer (Bio-Rad, Marnes-la-Coquette, France) set

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with excitation wavelength at 485 nm and emission wavelength at 535 nm. Antioxidant capacity was represented as Trolox C equivalent antioxidant capacity in triplicate assays (TAEC).

2.8. Cellular Antioxidant Assays

The protocol described in Nazir et al. [25], employing yeast cells, was used to assess cellular antioxidant activity. Yeast cells (DBY746 (MAT leu2-3,112 his31 trp1-289a ura3-52 GAI+) were cultivated aerobically in full 2.0 percent (w/v) glucose YPD (yeast extract peptone dextrose) medium (Sigma Aldrich, Saint-Quentin Fallavier, France) in an orbital shaker (150 rpm) at 30 °C in an orbital shaker (150 rpm). Each extract was evaporated under nitrogen flow, dissolved in DMSO, and then given to the yeast cells at a final concentration of 1 mg/mL 6 hours before oxidative stress induction. For untreated control yeast cells, the same amount of DMSO was utilized. The final DMSO dose administered to the yeast cells was around 1% (v/v). UV-C irradiation at 106.5 J/m^2 UV-C (254 nm) was used to generate oxidative stress using a Vilber VL-6.C filtered lamp (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The yeast cells were then incubated at 30°C overnight. Dihydrorhodamine-123 (DHR-123) fluorescent dye (Sigma-Aldrich, Saint-Quentin Fallavier, France) to assess the amount of reactive oxygen and nitrogen species (ROS/RNS) generated. Approximately 10^8 yeast cells were rinsed twice with phosphate buffered saline (PBS 1X, pH7.4) before being resuspended in 0.4 M DHR-123 solution prepared in PBS (1X, pH7.4) and incubated in the dark for 10 minutes at 30 °C. The fluorescence intensity was measured with a VersaFluor fluorimeter (Biorad, Marnes-la-Coquette, France) using λex = 505 nm and λem = 535 nm after twice washing with PBS (1X, pH7.4).

2.9. Statistical Analysis

Statistical analyses were done using the XLSTAT 2019 suite (Addinsoft, Paris, France) and the PAST4.0 [26]. The data which composed of at least the three independent replicates were presented in the form of the means and standard deviations. The Student's t-test was performed for statistical comparative analysis. The significant differences at $p < 0.05$, 0.01 as well as 0.001 were presented using *, ** and ***, respectively. Different letters were employed to indicate the significant thresholds at $p < 0.05$.

3. Results and Discussion

3.1. Plant collection and taxonomic description

After the intense searching for the living plant specimens in the natural habitat, the 25 populations of *M. angustifolia* were collected from the different localities cover the whole floristic regions in Thailand as well as the outgroup 6 populations of *M. hastata* from the same genus *Monochoria* following the Table 1 were investigated in this presented study.

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Table 1. The collected 25 populations of the new species, *M. angustifolia* and outgroup (*M. hastata*).

Population No.	Population Names	Floristic Regions	Scientific Name
1	Phichit	Northern (N)	*M. angustifolia*
2	Nakhon Sawan	Northern (N)	*M. angustifolia*
3	Khon Kaen	North-Eastern (NE)	*M. angustifolia*
4	Loei	North-Eastern (NE)	*M. angustifolia*
5	Chaiyaphum	Eastern) E(	*M. angustifolia*
6	Nakhon Ratchasima	Eastern) E(	*M. angustifolia*
7	Buri Ram	Eastern) E(	*M. angustifolia*
8	Ratchaburi	South-Western (SW)	*M. angustifolia*
9	Phetchaburi	South-Western (SW)	*M. angustifolia*
10	Prachuap Khiri Khan	South-Western (SW)	*M. angustifolia*
11	Saraburi	Central)C(	*M. angustifolia*
12	Suphan Buri	Central)C(	*M. angustifolia*
13	Nakhon Pathom	Central)C(	*M. angustifolia*
14	Rayong	South- Eastern) SE(	*M. angustifolia*
15	Prachin Buri	South- Eastern) SE(	*M. angustifolia*
16	Chachoengsao	South- Eastern)SE(	*M. angustifolia*
17	Sa Kaeo	South- Eastern)SE(	*M. angustifolia*
18	Chanthaburi	South- Eastern)SE(	*M. angustifolia*
19	Nakhon Si Thammarat	Peninsular)PEN(	*M. angustifolia*
20	Phatthalung	Peninsular)PEN(	*M. angustifolia*
21	Surat Thani	Peninsular)PEN(	*M. angustifolia*
22	Phangnga	Peninsular)PEN(	*M. angustifolia*
23	Trat	South- Eastern)SE(	*M. angustifolia*
24	Ranong	Peninsular)PEN(	*M. angustifolia*
25	Krabi	Peninsular)PEN(	*M. angustifolia*
Outgroup 1	Nakhon Sawan	Northern (N)	*M. hastata*
Outgroup 2	Loei	North-Eastern (NE)	*M. hastata*
Outgroup 3	Nakhon Ratchasima	Eastern) E(	*M. hastata*
Outgroup 4	Suphan Buri	Central)C(	*M. hastata*
Outgroup 5	Chachoengsao	South- Eastern)SE(	*M. hastata*
Outgroup 6	Ratchaburi	South-Western (SW)	*M. hastata*

Note: The population names come from the collected provinces.

The 25 populations of *M. angustifolia* collected from all the 7 floristic regions of the country showed the semilar trend of morphological characters (Figure 1), and the taxonomic description are provied in the belowed paragraph.

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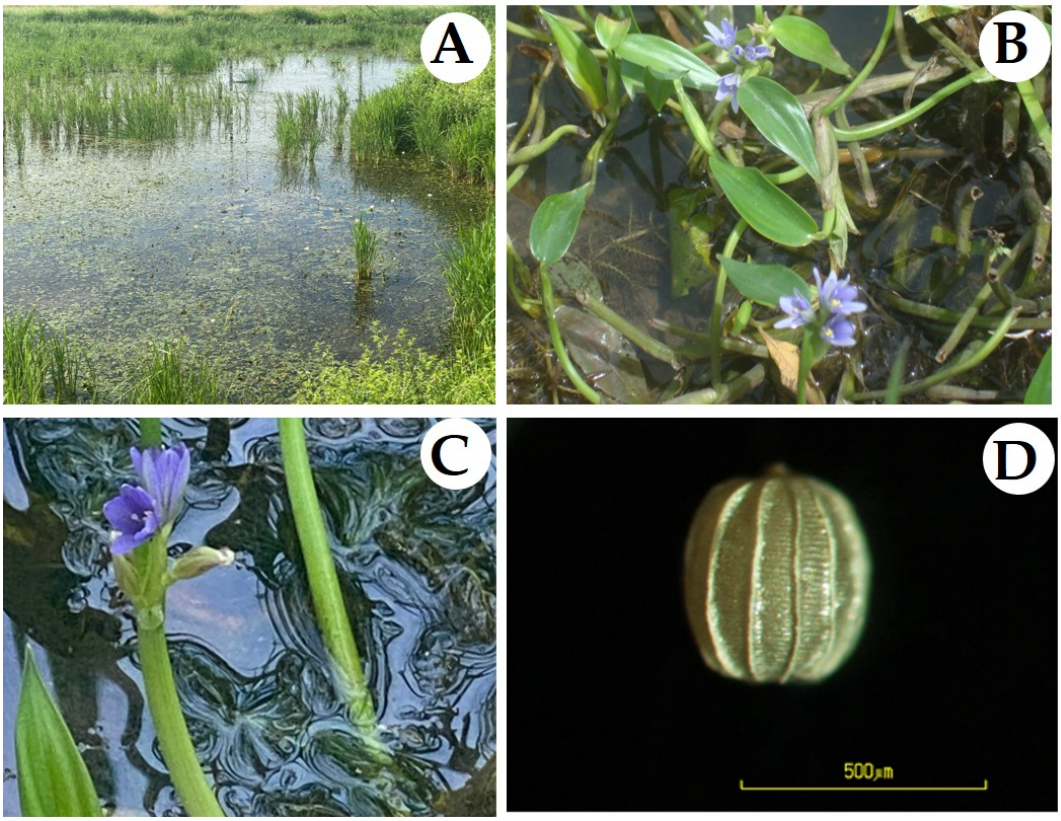

Figure 1. *M. angustifolia* **A:** Natural Habitat, **B:** Habit, **C:** Inflorescence, **D:** Seed, Bar scale = 500 µm

According to the distribution map (Table 1. And Figure 2.), the *M. angustifolia* mainly distribute throughout the Eastern (E), Central (C), South-Eastern (SE), South-Western (SW), Peninsula (PEN) as well as some part of Northern (N) and North-Eastern (NE) floristic regions which is wider than the previous report [1]. During our field study, at least 5 targeted provinces/ a floristic region where are previously reported or consist of the possible aquatic natural habitats were selected and have been seeked for the plant specimens of this new species. However, only 2 locallities (provinces) in the N (Phichit and Nakhon Sawan provinces) and NE (Khon Kaen and Loei provinces) floristic regions that can be found the living plant specimens of *M. angustifolia.* The most abundant *M. angustifolia* can be found in SE floristic regions where the 6 population were found.

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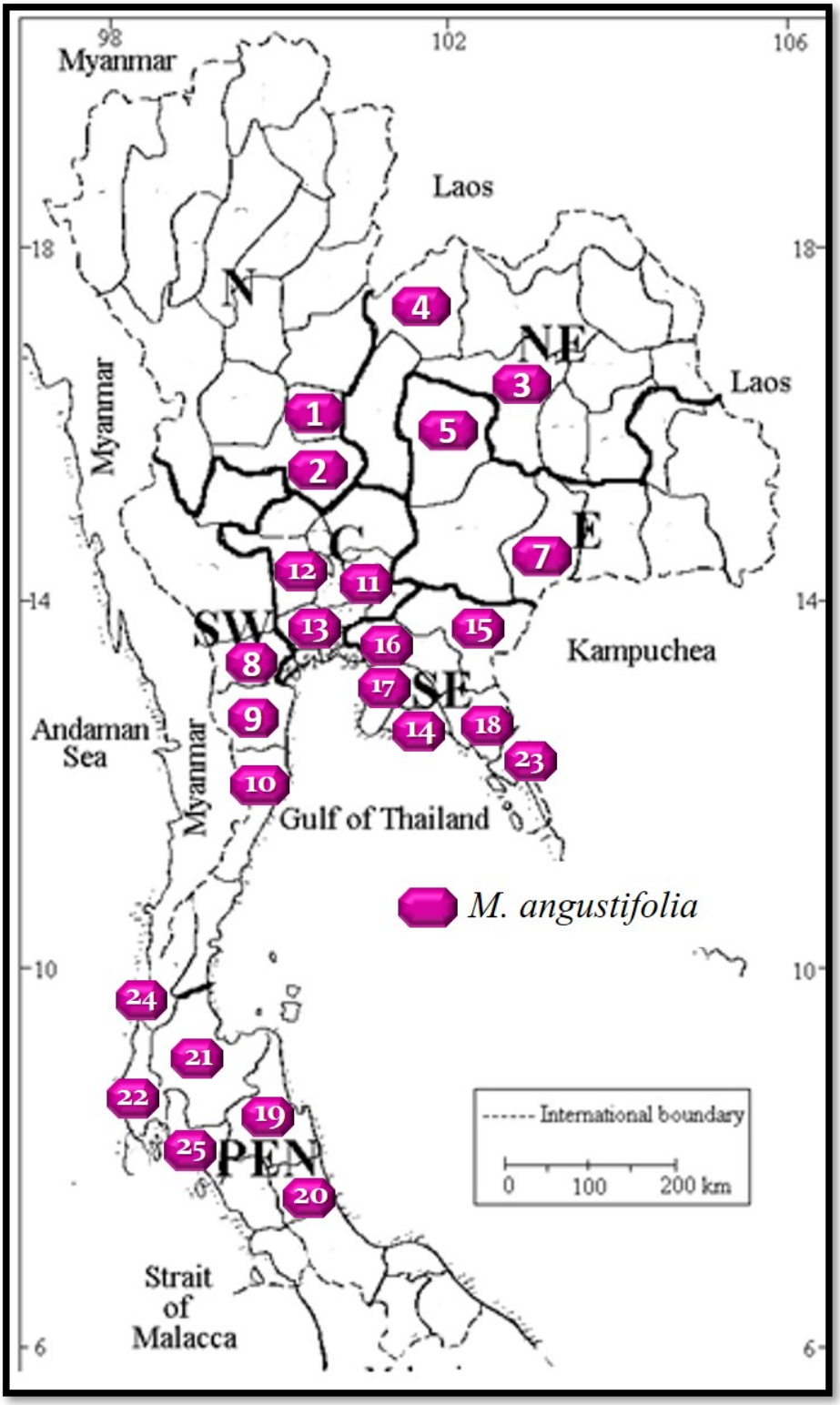

Figure 2. Distribution map of *M. angustifolia* populations throughout the floristic regions in Thailand. The number 1-25 indicates the number of populations.

Taxonomic Description: Annual aquatic herb, Leaf simple, stipulate, green, glabrous; petiole rounded, erect or curved, with broad leaf sheath; leaf blade lanceolate, lanceolate-linear, or ovate-lanceolate, apex abruptlyacuminate, base obtuse, 1.4–2.0 cm wide, 6.0–7.4 cm long, midrib groove on the adaxial surface, leaf blade and petiole forming right angle or acute to each other; Inflorescence racemose, 2–6 flowers; rachis

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5.2–5.4 cm long; peduncle 2.8–3.0 cm long; spathe green, 2.6–2.8 cm long, terminal appendage approximately 0.3 cm long; floral leaf blade lanceolate, adaxial and abaxial surfaces smooth, midrib groove, apex acute, base obtuse, 1.7–1.8 cm wide, 6.3–6.7 cm long; floral leaf petiole green, 4.1–4.5 cm long, level of inflorescence tip higher than that of floral leaf and mature leaf; pedicel glabrous, 1.3–1.4 cm long; outer perianth 3, purple, glabrous, lanceolate, middle of abaxial green, 0.3 cm wide, 0.7–0.8 cm long; inner perianth 3, purple, ovate or elliptic, glabrous, apex obtuse, middle of abaxial green, 0.3 cm wide, 0.8–0.9 cm long; stamen 6; normal stamen 5, filament white unappendage, 0.7–0.8 cm long, anther basifixed, yellow, 0.4–0.5 cm long; largest stamen 1, filament dark purple, appendage, 0.2 cm long, anther basifixed, dark purple, 0.1 cm wide, 0.4–0.5 cm long; ovary superior, style bright purple, 0.4–0.5 cm long. Fruits capsule, glabrous. Seeds barrel, 373–429 μm long, numerous, longitudinal ridges of seed distinct, 7–10 veins.

Specimens examined: *M. angustifolia* Population No. 1 - 25

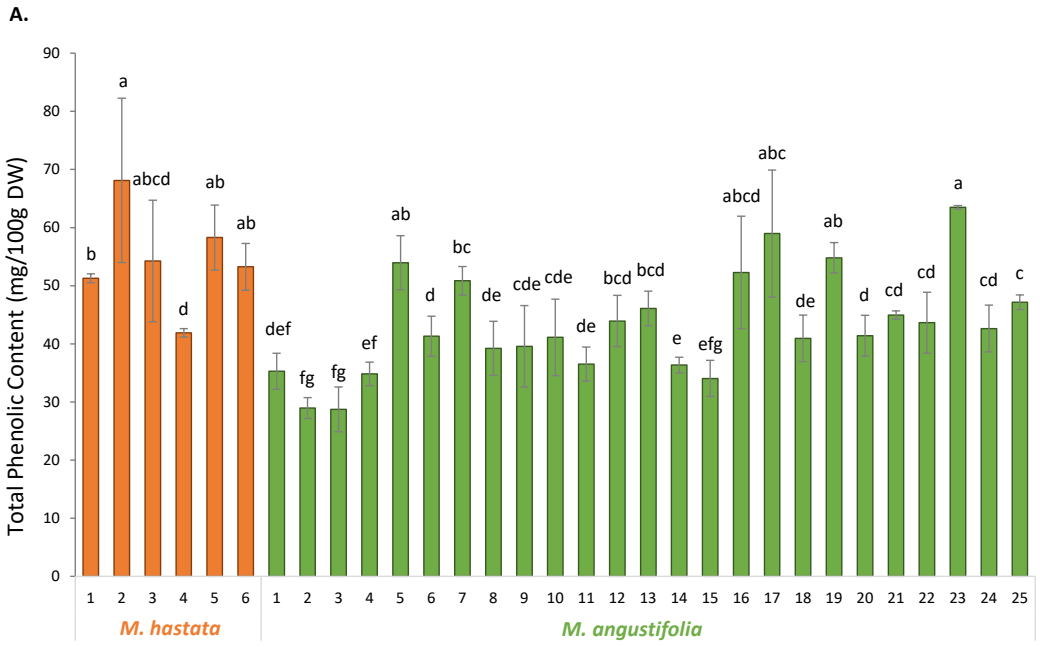
Recent distribution: Northern (N), North-Eastern (NE), Eastern (E), Central (C), South-Eastern (SE), South-Western (SW), Peninsula (PEN).

Ecology: Rice fields and clear aquatic habitats

Flowering Time: Early April to early August

3.2. *Phytochemical Characterization*

The total phenolic and flavonoid contents (TPC and TFC, respectively) found in extracts of the populations of the two *Monochoria* species (*M. angustifolia* and *M. hastata*) are presented in Figure 3 (TPC Figure 3A and TFC Figure 3B)



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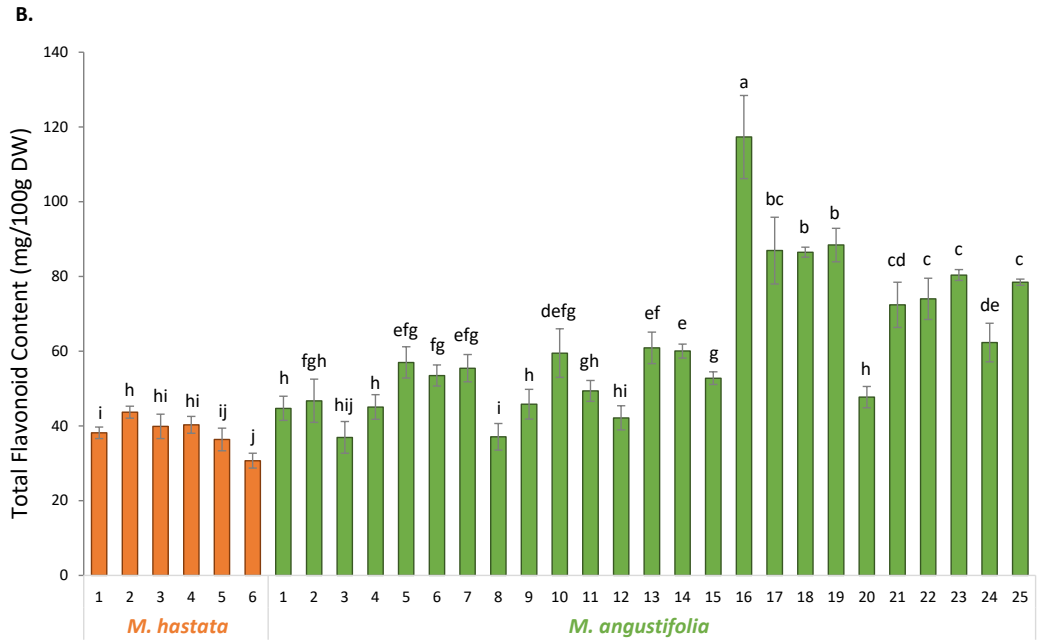

Figure 3. Total phenolic (TPC) and total flavonoid (TFC) contents in different populations of two *Monochoria* species (6 populations of *M. hastata* and 25 populations of *M. angustifolia*) covering the entire floristic regions from Thailand. Different letters indicate significant differences at $p < 0.05$.

The TPC ranged from 28.78 ± 3.86 (Ma#3) to 63.49 ± 0.29 (Ma#23) mg/100 g DW gallic acid equivalent in *M. angustifolia* extracts, and from 41.91 ± 0.72 (Mh#4) to 68.12 ± 14.15 (Mh#2) mg/100 g DW gallic acid equivalent in *M. hastata* extracts. The TFC ranged from 36.92 ± 4.26 (Ma#3) to 117.33 ± 11.18 (Ma#16) mg/100 g DW quercetin equivalent in *M. angustifolia* extracts, and from 30.68 ± 1.99 (Mh#6) to 43.68 ± 1.60 (Mh#2) mg/100 g DW quercetin equivalent in *M. hastata* extracts. These results revealed a significant degree of variability in both TPC and TFC for *M. angustifolia* as compared to *M. hastata*, with TFC playing a critical role in this phytochemical heterogeneity observed within the *M. angustifolia* populations. Noticeably, some populations of *M. angustifolia* appeared to be significantly richer in TFC, which might be of significant relevance given the well-known antioxidative and health-promoting effects of this class of phytochemicals [9,27]. In particular, populations #16-19, #21-23 and #25 of *M. angustifolia* appeared of special interest because of their high TFC. Interestingly, this is the first report on the phytochemical examination of this new species. The TPC and TFC of the outgroup, *M. hastata* extracts, have been reported, and the published ranges of variations are in line with the present results [28,29]. It should be noted, however, that the present study is the first to evaluate TPC and TFC variabilities at the population level.

Because our results indicated TFC as important contributors to the observed variations, high performance liquid chromatography (HPLC) coupled to photodiode array detection (PDA) analyses were carried to provide a thorough understanding of the qualitative and quantitative changes (Figure 4). Compounds were identified using our flavonoid database by comparison with authentic commercial standards based on their retention times and λmax values.

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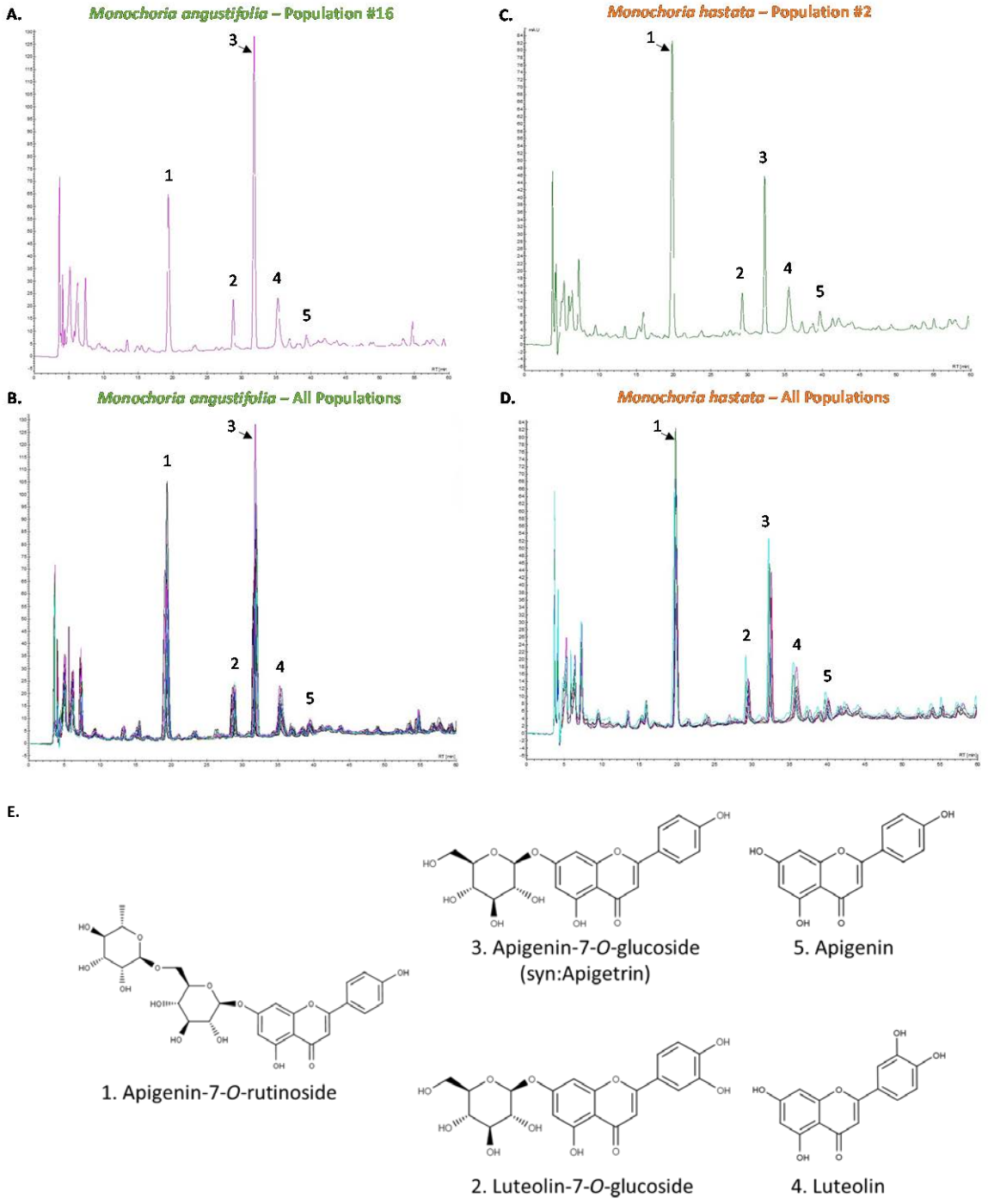

Figure 4. HPLC chromatograms (recorded at 320 nm) of **A.** *M. angustifolia* population #16, **B.** superimposed view of the *M. angustifolia* 25 populations, **C.** *M. hastata* population #2, **D.** superimposed view of the *M. hastata* 6 populations; **E.** Structure of the main identified flavonoids: 1. apigenin-7-*O*-rutinoside, 2. luteolin-7-*O*-glucoside, 3. apigenin-7-*O*-glucoside (aka apigetrin), 4. luteolin, 5. apigenin.

The HPLC-PAD analyses allowed the identification of the five main flavonoids among the distinct *Monochoria* populations extracts: apigenin (compound 5) and two of its glycoside derivatives, apigenin-7-*O*-rutinoside (compound 1) and apigenin-7-*O*-glucoside (aka apigetrin, compound 3), as well as luteolin (compound 4) and one of its glycoside derivatives, luteolin-7-*O*-glucoside (compound 2).

Each compound was separated with great repeatability, as evidenced by the relative standard deviations in their retention time values with enough peak symmetry

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and accurate resolution (Table 2). The LOD and LOQ values were determined using the response standard deviation and slope of the calibration curves, and demonstrated that the proposed technique is adequately sensitive for measuring flavonoids from *M. angustifolia* leaf ethanolic extract (Table 2).

Table 2. Calibration function parameters for the main flavonoids from *M. angustifolia* leave ethanolic extract using UV detection.

Flavonoid	Retention time (t_R)				Calibration curve			LOD	LOQ
	Min	RSD (%)	R_s	Sym Fact	slope	intercept	R^2	(µg/mL)	(µg/mL)
Api-7-Rut	19.90	0.76	3.33	1.07	2291.9	569.6	0.9996	0.08	0.25
Lut-7-Glc	29.19	0.20	3.63	1.01	2553.3	157.1	0.9994	0.02	0.06
Api-7-Glc	32.58	0.51	3.04	1.11	2468.8	642.5	0.9999	0.09	0.26
Lut	36.28	0.41	1.87	1.03	3670.2	285.1	0.9997	0.03	0.08
Api	40.56	0.24	1.67	0.98	3742.3	250.5	0.9992	0.02	0.07

Api: apigenin; Lut: luteolin; Api-7-Rut: apigenin-7-*O*-rutinoside; Lut-7-Glc: luteolin-7-*O*-glucoside; Api-7-Glc: apigenin-7-*O*-glucoside; RSD: relative standard deviation; R_s: resolution value; Sym Fact: symmetry factor; R^2: correlation coefficient; LOD: limit of detection; LOQ: limit of quantification.

The method was then validated using the Association of Analytical Communities (AOAC) standards to assure accuracy and reproducibility in quantification [24]. The validation results are summarized in Table 3, including the Horwitz ratio, accuracy, and intra- and inter-day precision. These results indicated that the used analytical method was adequate for the quantification of the different flavonoids from *M. angustifolia* leave ethanolic extract.

Table 3. Quantification and validation parameters for the simultaneous analysis of the main flavonoids from *M. angustifolia* leave ethanolic extract.

Flavonoid	Concentration	RSD (%)	HortRat	Accuracy		Intra-day precision		Inter-day precision	
	(mg/g DW)			Recovery (%)	RSD	%	RSD	%	RSD
Api-7-Rut	22.04 ± 0.02	0.09	0.03	100.32	1.34	99.15	3.88	99.85	0.67
Lut-7-Glc	18.91 ± 0.13	0.67	0.22	99.90	0.46	99.37	3.35	95.72	3.67
Api-7-Glc	36.84 ± 0.49	1.33	0.39	99.80	0.53	98.80	3.31	97.91	4.81
Lut	25.09 ± 0.15	0.58	0.18	100.11	0.41	99.64	1.43	98.77	4.97
Api	3.41 ± 0.20	5.93	2.47	98.40	3.10	97.99	4.31	96.98	4.37

Api: apigenin; Lut: luteolin; Api-7-Rut: apigenin-7-*O*-rutinoside; Lut-7-Glc: luteolin-7-*O*-glucoside; Api-7-Glc: apigenin-7-*O*-glucoside; RSD: relative standard deviation; HortRat: Horwitz ratio. Concentration values of *M. angustifolia* population #16.

The HPLC profiles (Figure 4) clearly illustrated that the accumulation profile differed qualitatively and quantitatively amongst the two *Monochoria* species. Indeed, while both species accumulated apigenin and luteolin primarily in their conjugated glycoside forms rather than their aglycone forms (compounds 4 and 5 on the HPLC chromatograms), the major conjugated form differs clearly between the two *Monochoria* species, with *M. hastata* samples accumulating apigenin-7-*O*-rutinoside (compound 1) as the main flavonoid as opposed to *M. angustifolia* samples accumulating apigenin-7-*O*-glucoside (compound 3) as the main flavonoid. Plants accumulate glycosidic forms to enhance their solubility and stability [30]. Therefore, this accumulation profile toward glycosidic forms makes sense. Interestingly, in some cases, glycosilation may improve flavonoid biological activities [27,30].

Absolute quantification of the five main flavonoids has been conducted (Figure 5, Table S1).

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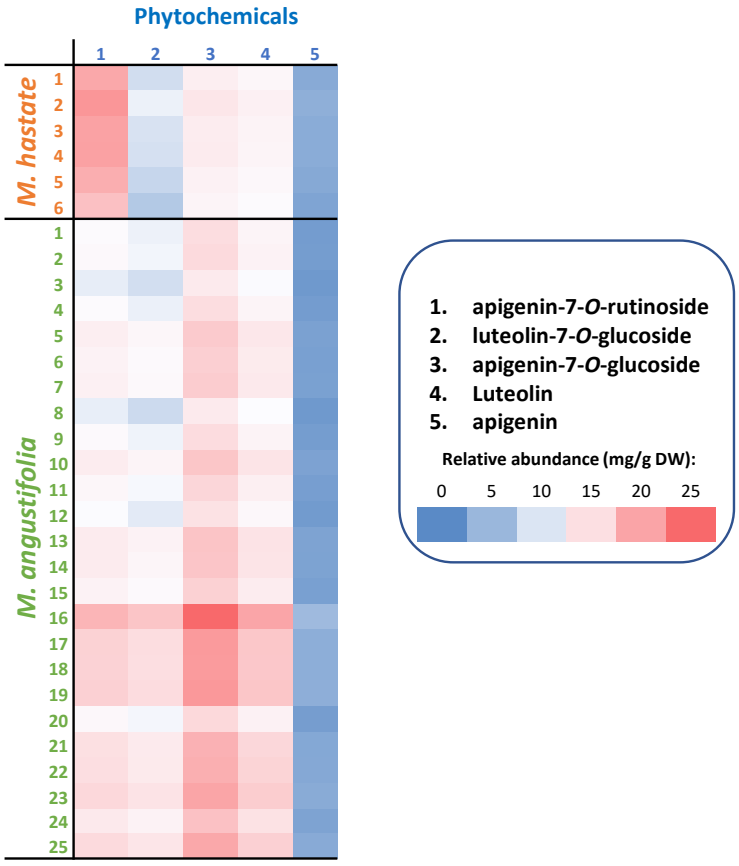

Figure 5. Absolute quantification of the main flavonoids in different populations of two *Monochoria* species (6 populations of *M. hastata* and 25 populations of *M. angustifolia*) covering the entire floristic regions from Thailand. 1. apigenin-7-*O*-rutinoside, 2. luteolin-7-*O*-glucoside, 3. apigenin-7-*O*-glucoside (aka apigetrin), 4. luteolin, 5. apigenin. Means and standard deviations are provided in Table S1.

Individually, 1) apigenin-7-*O*-rutinoside ranged from 6.94 (Ma#3) to 22.04 (Ma#16) mg/100 g DW *M. angustifolia* extracts, and from 19.78 (Mh#6) to 28.15 (Mh#2) mg/100 g DW in *M. hastata* extracts; 2) luteolin-7-*O*-glucoside ranged from 5.62 (Ma#8) to 18.91 (Ma#16) mg/100 g DW *M. angustifolia* extracts, and from 3.43 (Mh#6) to 4.94 (Mh#2) mg/100 g DW in *M. hastata* extracts; 3) apigenin-7-*O*-glucoside ranged from 11.94 (Ma#3) to 36.94 (Ma#16) mg/100 g DW *M. angustifolia* extracts, and from 9.63 (Mh#6) to 12.29 (Mh#2) mg/100 g DW in *M. hastata* extracts; 4) luteolin ranged from 7.86 (Ma#3) to 25.08 (Ma#16) mg/100 g DW *M. angustifolia* extracts, and from 5.77 (Mh#6) to 7.28 (Mh#2) mg/100 g DW in M. hastata extracts; 5) apigenin ranged from 1.07 (Ma#3) to 3.41 (Ma#16) mg/100 g DW *M. angustifolia* extracts, and from 1.83 (Mh#6) to 2.60 (Mh#2) mg/100 g DW in *M. hastata* extracts (Figure 5, Table S1). This is the first work on the HPLC analysis of the flavonoid content of *M. angustifolia* and, to the best of our knowledge, of *M. hastata* as well. However, glycoside derivatives of both apigenin and luteolin have been identified in a variety of species from the pontederiaceae family [31–33], adding validity to our results. Future study will be performed to identify other minor flavonoids using high resolution mass spectrometry. These results confirmed the different accumulation strategies observed with the TPC and TFC analyses for the two species, as well as at the population level in the case of *M. angustifolia*. The present results also revealed the special interest for possible applications of the new species (i.e., *M. angustifolia*), over *M. hastata* due to its higher flavonoid content. Many *Monochoria* species are invasive and considered weeds [34], however, this can be seen as a benefit in terms of obtaining a large and valuable biomass.

The observed variations may be the result of distinct genetic backgrounds, but they may also be the result of the effect of various ecological conditions. Environmental vari-

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ables such as climatic and geographic (including growing conditions) factors, in addition to genetic background, have been demonstrated to have a major impact on the accumulation of phenolic compounds [14,15]. Hierarchical clustering analysis (HCA) was therefore used to discover probable groups among the heterogeneous samples from the various populations in order to examine the structure of the populations (Figure 6).

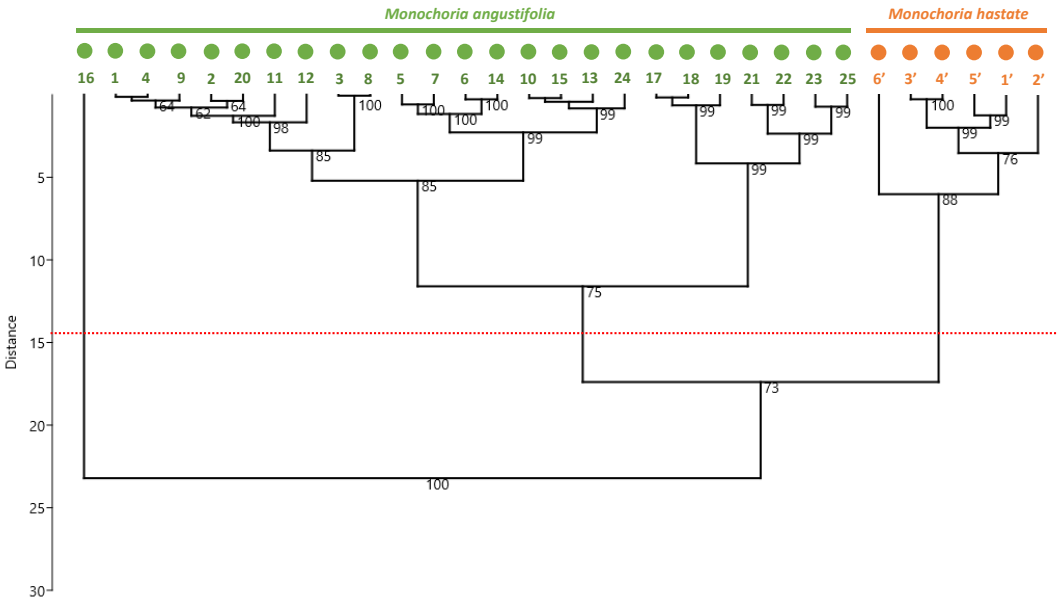

Figure 6. Hierarchical clustering analysis (HCA) of 25 different populations of *M. angustifolia* and 6 different populations of *M. hastata* on the basis of their phytochemical profiles. The percentages of replicate trees in which associated samples cluster together in the bootstrap test (percentage of 5,000 replicates) are indicated next to the branches.

Based on the phytochemical profiles, the HCA indicated that the clustering occurred predominantly at the genetic level, with a clear distinction between the two *Monochoria* species. In good agreement, flavonoids have been successfully employed to analyze evolutionary connections in a variety of angiosperm families, as well as for botanical authentication [35–38]. However, there is no discernible pattern to illustrate the importance of the environmental factor. Given the wide geographic distribution of the different populations across the diverse floristic areas of Thailand. We cannot rule out the possibility that environmental variables may explain at least some of the observed heterogeneity, but it appears that genetics is a major driver of in phytochemical diversity. Overall, the present results give the most comprehensive picture to date of the phytochemical, flavonoid-specific, broad variability found at different population levels of the two *Monochoria* species, including the newly described *M. angustifolia*. Flavonoids, through their antioxidant action, have been demonstrated to have a wide range of health-promoting properties [9]. As a result, we next investigate the impact of this wide flavonoid variability on the antioxidant activity of these extracts.

3.3. Antioxidant Activity

The antioxidant capacity of the leaf extracts from the different populations of *M. angustiolia* and *M. hastata* were assessed for their antioxidant capacity using in vitro assays involving the two major antioxidant mechanisms: the hydrogen atom transfer (HAT) mechanism assessed using the ORAC assay and the single electron transfer (SET) mechanism assessed using the CUPRAX and FRAP assay, whereas the ABTS and DPPH assay allowed for both mechanisms to be assessed [38–40]. The results expressed in μmol of Trolox-C equivalent antioxidant capacity (μmol TEAC) are summarized in Figure 7 and Table S2.

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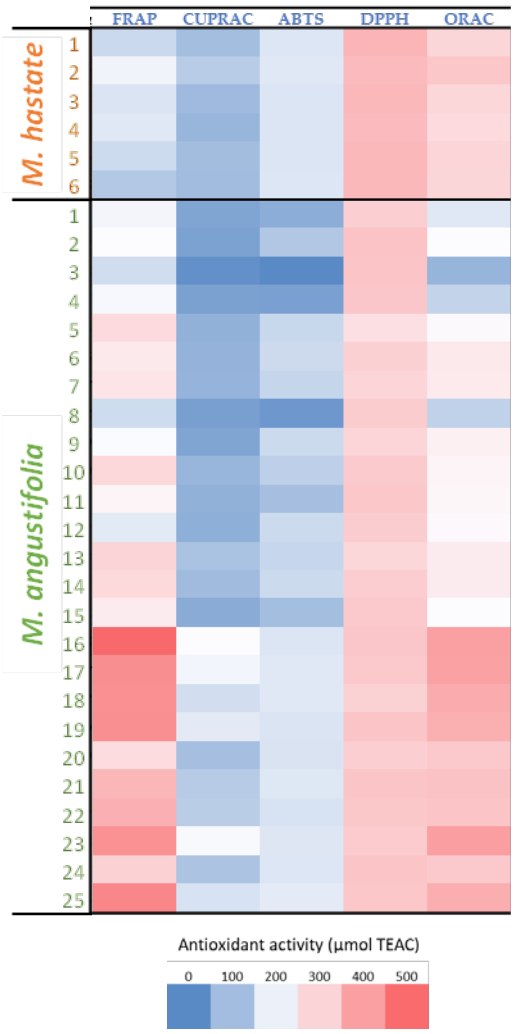

Figure 7. *In vitro* cell-free antioxidant (FRAP, CUPRAC, ABTS and DPPH) of extracts from 25 different populations of *M. angustifolia* and 6 different populations of *M. hastata*. TEAC: TroloxC equivalent antioxidant capacity; ABTS: 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; CUPRAC: cupric reducing antioxidant capacity; ORAC: oxygen radical absorbance capacity. Means and standard deviations are provided in Table S2.

The antioxidant capacity differed substantially for the two species extracts. *M. hastata* showed high ORAC, DPPH and ABTS radical scavenging activities, suggesting the preponderance of a HAT antioxidant mechanism for thes extracts. When compared to *M. hastata* extracts, *M. angustifolia* had a significantly higher FRAP antioxidant activity, in addition to a high DPPH radical scavenging activity, thus implying a greater contribution of the ET-based antioxidant mechanism. Furthermore, the HAT-based antioxidant capacity of *M. angustifolia* extracts is comparable to that of *M. hastata* extracts. As a result by involving the two types of antioxidant mechanisms, *M. angustifolia* extracts appeared more attractive in terms of antioxidant capacity than *M. hastata* extracts.

The higher flavonoid contents of *M. angustifolia* extracts can be related to the largest contribution of the HAT mechanism. When it comes to quenching free radicals, flavonoids preferentially function through HAT-based reactions rather than ET-based reactions [25,41,42]. Other chemicals, notably phenolics, may contribute to this antioxidant effect in synergy via an ET-based mechanism [25,42]. Indeed, flavonoids are known to favor HAT-based antioxidant mechanism over ET-based antioxidant mechanism [25,41,42]. Here, other compounds, particularly phenolics, may also contribute in sinergy with flavonoids through an ET-based mechanism to contribute to

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this antioxidant action [25,42]. For instance, Bai et al. [29] have reported the DPPH radical scavenging capacity of stigmasterol extracted from *M. hastata*.

3.4. Correlation Analysis

A principal component analysis (PCA) was used to identify relevant connections between the different natural populations based on their phytochemical composition and antioxidant activity (Figure 7).

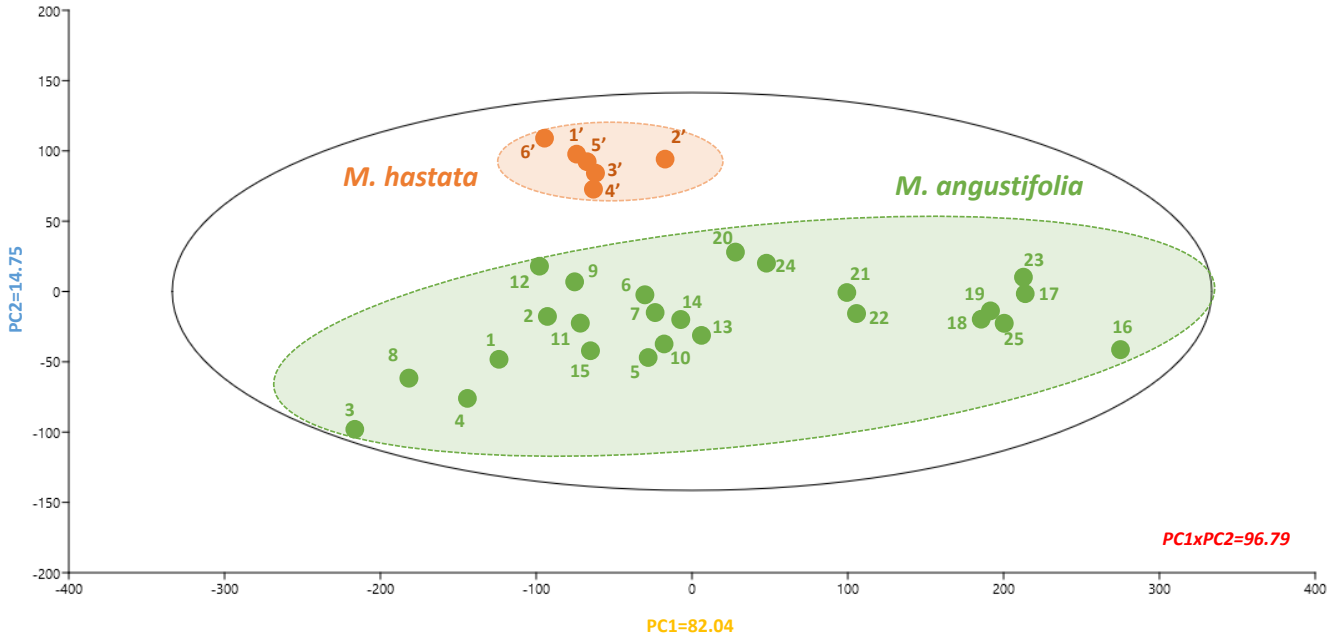

Figure 8. Principal component analysis (PCA) linking the phytochemical profile and antioxidant capacity of the extracts from different populations of the two *Monochoria* species. Variance of component 1 = 82.04% and component = 14.75 %. Each number represents the different populations from *M. angustifolia* (in green) and *M. hastata* (in orange). The corresponding loading score plots for components 1 and 2 are presented in Figure S1.

The biplot representation accounted for 96.79% of the initial variability (Figure 8). The TFC and ET-based antioxidant FRAP and CUPRAC assays were the primary contributors to the discrimination along the component 1 axis, accounting for 82.04% of the initial variability (Figure S1). In contrast, the second component axis accounted for just 14.75% of the initial variability with TPC and the mixed ET/HAT- and HAT-based antioxidant ABTS and ORAC assays (Figure S1). As a result of this PCA, two distinct clusters were discriminated from one another in terms of phytochemical composition and antioxidant activity. Interestingly, these clusters separated *M. hastata* extracts from *M. angustifolia* extracts. The *M. hastata* extracts were grouped, whereas *M. angustifolia* extracts appeared substantially more diverse.

Pearson correlation coefficients (PCC) were used to evaluate the linkage between phytochemical and antioxidant activity (Figure 8, Table S3).

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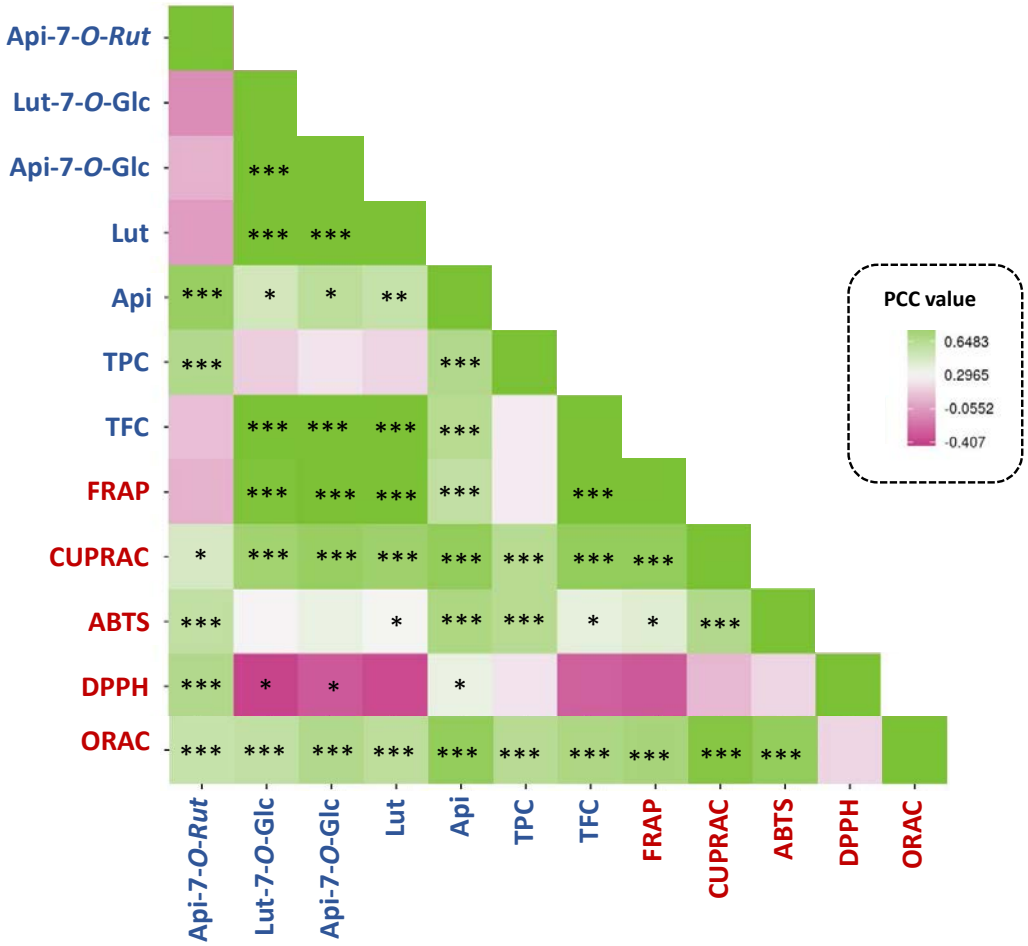

Figure 8. Correlogram analysis (Pearson coefficient correlation) between phytochemical profiles and antioxidant activities of extracts. Api-7-*O*-Rut: apigenin-7-*O*-rutinoside; Lut-7-*O*-Glc: luteolin-7-*O*-glucoside; Api-7-*O*-Glc: apigenin-7-*O*-glucoside (aka apigetrin); Lut: luteolin; Api: apigenin; TPC: total phenolic content; TFC total flavonoid content; ABTS: 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; CUPRAC: cupric reducing antioxidant capacity; ORAC: oxygen radical absorbance capacity. *** significant $p < 0.001$; ** significant $p < 0.01$; * significant $p < 0.05$; PCC values are indicated in Table S3.

The strength of various correlations between the phytochemacals (TPC, TFC and each flavonoid), and the various antioxidant assays was evidenced. The most remarkable were the highly significant correlatione between the various flavonoids and the ET-based antioxidant assays (FRAP and CUPRAC), on the one hand, and the TPC and the HAT-based antioxidant assay, on the other. The correlations are consistent with the antioxidant mechanisms reported for these phytochemicals [25,42]. This correlations also highlighted the fact that the antioxidant capacity of an extract is typically the consequence of complex phytochemical combinations rather than the action of a single molecule [43]. This study proved the great potential of *M. angustifolia* extracts as a starting material for a variety of applications based on their antioxidant flavonoids as previous discussed in the other potential plants species [9,10,13,27,37].

4. Conclusions

To recapitulate, the studied 25 populations of *M. angustifolia* from their natural habitats throughout the floristic regions in Thailand displayed a high heterogeneity in their phenolics/polyphenols accumulations. Furthermore, this analysis showed that flavonoids are the main phytochemical class of these extracts, and pointed out that *M. angustifolia* is richer in flavonoids comparing with the outgroup species from the same genus such as *M. hastata*. The results showed that flavonoids might be employed to examine

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Monochoria's evolutionary connections as well as for botanical authentication. Furthermore, the use of various *in vitro* cell-free antioxidant assays revealed that the antioxidant capacity of *M. angustifolia* extracts is primarily mediated by hydrogen atom transfer mechanism. It was also emphasized that the antioxidant potential of the extracts is the result from the complex phytochemical combinations rather than a single molecule. This study provided a new frontier of knowledge on the phytochemical diversity and antioxidant potential of *M. angustifolia* natural populations from all floristic regions of Thailand, harboring the highest species diversity in the *Monochoria* genus, which will certainly aid phytopharmaceutical industries in their search for potential raw plant material/plant organs to design and develop new bioactive products.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Figure S1.** Loading scores of the component 1 and component 2 of the PCA (presented in Figure 5) linking the phytochemical profile and antioxidant capacity of the extracts of *M. hastata* and *M. angustifolia* populations originating from various floristic regions from Thailand. 1. apigenin-7-*O*-rutinoside; 2. luteolin-7-*O*-glucoside; 3. apigenin-7-*O*-glucoside (aka apigetrin); 4. Luteolin; 5. Apigenin; TPC: total phenolic content; TFC: total flavonoid content; ABTS: 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; CUPRAC: cupric reducing antioxidant capacity.; **Table S1.** HPLC quantification (expressed in mg/100g DW) of the main flavonoids in different populations of two Monochoria species (6 populations of *M. hastata* and 25 populations of *M. angustifolia*) covering the entire floristic regions from Thailand; **Table S2.** *In vitro* cell-free antioxidant (FRAP, CUPRAC, ABTS and DPPH) of extracts from 25 different populations of *M. angustifolia* and 6 different populations of *M. hastata*. **Table S3**. Pearson correlation coefficient linking phytochemicals and antioxidant activity of ethanolic extracts of different populations of two *Monochoria* species (6 populations of *M. hastata* and 25 populations of *M. angustifolia*) covering the entire floristic regions from Thailand.

Author Contributions: Conceptualization, D.T. and C.H.; methodology, D.T., S.D., L.G. and C.H.; software, S.D.; validation, D.T., J.M.L. and C.H.; formal analysis, D.T., J.M.L. and C.H.; investigation, D.T., S.D. and C.H.; resources, D.T. and C.H.; data curation, D.T. and C.H.; writing—original draft preparation, D.T. and C.H.; writing—review and editing, D.T., S.D., L.G., J.M.L. and C.H.; visualization, D.T., S.D., J.M.L. and C.H.; supervision, D.T. and C.H.; project administration, D.T. and C.H.; funding acquisition, D.T. and C.H. All authors have read and agreed to the published version of the manuscript.

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

Sample Availability: Samples of the compounds are available from the authors.

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