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Research Article

Coumarins and Polar Constituents from *Eupatorium triplinerve* and Evaluation of Their α-Glucosidase Inhibitory Activity

Do Thi Viet Huong,[1] **Phan Minh Giang**,[1] **and Vu Minh Trang**[2]

[1]*Faculty of Chemistry, VNU University of Science, Vietnam National University, 19 Le Thanh Tong Street, Hanoi, Vietnam*
[2]*VNU University of Education, Vietnam National University, 144 Xuan Thuy Street, Hanoi, Vietnam*

Correspondence should be addressed to Phan Minh Giang; giangpm@vnu.edu.vn

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In our study of antidiabetic compounds from the leaves of *Eupatorium triplinerve* Vahl. (Asteraceae), ten compounds were isolated from the methanol leaf extract. They were determined to be β-sitosterol (**1**), stigmasterol (**2**), β-sitosterol 3-*O*-β-D-glucopyranoside (**3**), ayapanin (**4**), ayapin (**5**), thymoquinol 5-*O*-β-D-glucopyranoside (**6**), thyrsifloside (**8**), (*E*)-4-methoxymelilotoside (**9**), and kaempferol 3,7-di-*O*-β-D-glucopyranoside (**10**) by using ESI-MS, 1D (^{1}H-, ^{13}C-, DEPT) and 2D NMR (HSQC, HMBC, and NOESY) techniques. This is the first report of water-soluble compounds from *E. triplinerve* and compounds **6**–**10** were isolated for the first time from *E. triplinerve*. NMR profiling and HPLC analysis are fast and reliable methods to screen phytochemicals in plant samples. Due to their high concentrations in the leaf extracts of *E. triplinerve,* coumarins **4** and **5** could be fast screened by NMR profiling and RP-HPLC-PDA analysis. In the *in vitro* test for α-glucosidase inhibition of compounds **4**–**9,** compounds **4**, **5**, and **7** showed the enzymatic inhibition of 40%, 46%, and 81%, respectively, at 256 μg/mL. An IC_{50} value of 58.65 ± 1.20 μg/mL (302 μM) was calculated for compound **7** which is lower than that of the positive control acarbose (IC_{50} 197.33 ± 2.51 μg/mL; 306 μM).

1. Introduction

The genus *Eupatorium* (family Asteraceae) is a taxonomically complex group of species distributed mainly in Europe, eastern Asia, and North America. Studies on *Eupatorium* species have revealed diversity of secondary metabolites such as sesquiterpene lactones, flavonoids, diterpenes, benzofurans, pyrrolizidine alkaloids, chromenes, and thymol derivatives [1]. A number of *Eupatorium* species are employed in traditional medicine in the treatment of different pathologies and as a consequence bioactive natural compounds with cytotoxic, anti-inflammatory, antifungal, and antibacterial activities have been reported from the species. *E. triplinerve* Vahl. (*syn. E. ayapana* Vent., *Ayapana triplinervis* (Vahl.) R.M. King and H. Robinson) is a perennial and aromatic herb, 35–100 cm tall of the family Asteraceae. The plant was originated in the area from northern Brazil to Suriname and was introduced, cultivated, and naturalized long ago in some Caribbean islands, Africa, India, and South-East Asian countries. The leaves of *E. triplinerve* are used in folk medicine of India and South-East Asian countries as a heart stimulant, laxative, anticoagulant, stimulant, and tonic and for the treatment of yellow fever [2]. In Vietnam, the plant is popularly known as "Bả dột" and the leaves are used to heal wounds, snake bites, and stop bleeding. People also prepare tea from the twigs and leaves [3]. A number of studies investigated essential oils from different parts of *E. triplinerve*, showing variation of chemotypes such as 2,5-dimethoxy-*p*-cymene [4], β-caryophyllene [4], selina-4(15),7(11)-dien-8-one [5], and 2-*tert*-butyl-1,4-methoxybenzene [6]. The leaves emit a distinct coumarin odour, and the isolation of ayapanin (or herniarin, 7-methoxycoumarin) and ayapin (or 6,7-methylenedioxycoumarin) was reported. Ayapanin and ayapin showed toxicity to cancer cells including multidrug

resistant cancer cells [6], haemostatic properties [7], blood coagulation, and phytoalexin activity to inhibit or destroy the invading bacteria, insects, and viruses [8, 9]. Ayapanin reduced the number of abdominal constrictions in mice and decreased the time spent in paw licking and biting response in formalin assay [10]. Kumala and Sapitri reported toxicity test using brine shrimp lethality method that showed all of the fractions of methanol extract had toxicity ($LC_{50} < 1000\,\mu g/mL$). The water extract had no toxicity. The highest LC_{50} was for the ethyl acetate (24,42 μg/mL) and the lowest LC_{50} was for the *n*-hexane (238,66 μg/mL) extracts [11]. Both ayapanin and ayapin are nontoxic and are effective when applied locally or when administered by subcutaneous injections or by mouth. They have no effect on respiration or on blood pressure [12]. In addition, ayapanin did not show antigenotoxic effects on human lymphocyte DNA damage using single-cell gel electrophoresis [13]. Therefore the reason for the toxicity of the *n*-hexane and ethyl acetate extracts is complex and may be related to minor nonpolar or volatile compounds which may be produced by plants either constitutively or in response to different biotic or abiotic stresses [14]. The high contents in *E. triplinerve* enable ayapanin and ayapin to be used as starting materials for pharmacological investigations involving coumarins such as antidiabetic activity [15], affinity to cannabinoid receptors (cannabimimetic ligands) [16] or cognitive enhancing activity through inhibiting oxidative stress and brain inflammation [17, 18]. Till now, there has not been any report on the quantification of the two biologically active coumarins from the leaves of *E. triplinerve*. In this study, the principles **4** and **5** were analysed in the leaves of *E. triplinerve* by using proton NMR profiling and HPLC-PDA analysis.

Type II diabetes is a chronic metabolite disease caused mainly by excess levels of blood glucose and is a major cause of premature death, blindness, kidney failure, heart attack, stroke, and lower limb amputation [19]. Type II diabetes is characterized by hyperglycaemia as a consequence of insulin resistance and affects over 90% of patients diagnosed with this disease. Oral hypoglycemic medications currently used in the treatment of type II diabetes include sulfonylureas, meglitinides, biguanides, thiazolidinediones, α-glucosidase inhibitors, and dipeptidyl peptidase-IV (DPP-IV) inhibitors. The antidiabetic drugs such as metformin, pioglitazone, thiazolidinedione, and acarbose decrease hepatic glucose output and reduce starch digestibility. Due to the side effects of these agents such as severe hypoglycemia, weight gain, and gastrointestinal disturbances medicinal plants with antidiabetic properties have been investigated for finding safer and cost-effective antidiabetic drugs [20]. Coumarins and cinnamic acid derivatives have been reported as antidiabetic agents [15, 21]. Currently, the inhibition of starch-hydrolysing enzymes such as α-amylase and α-glucosidase are one of the approaches to reduce hyperglycemia by retarding glucose uptake. In the present study, coumarins **4** and **5**, thymoquinol glucoside **6**, and *o*-hydroxycinnamic acid derivatives **7–9** were isolated from the leaves of *E. triplinerve* collected in Vietnam and evaluated for the enzymatic inhibitory activity against α-glucosidase.

2. Materials and Methods

2.1. General Procedures. Electron-spray ionization-mass spectrometry (ESI-MS) spectra were measured on an Agilent 1100 LC-MSD-Trap-SL system (Agilent Technologies, USA). ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra were recorded in $CDCl_3$ using a Bruker Avance 500 NMR spectrometer. The chemical shifts are expressed in ppm relative to tetramethylsilane (TMS) as an internal standard. The ^{1}H-NMR spectrum was recorded with spectrometer frequency (SF) 500.20 MHz. The ^{13}C-NMR spectrum was recorded with SF 125.13 MHz. High-performance liquid chromatography (HPLC) analysis was carried out with a Shimadzu 20A HPLC system (Shimadzu corporation, Japan) equipped with a LC-20AD pump, SIL-20AC auto-sampler, DGU-20A5R degassing unit, SPD-M20A photodiode array (PDA) detector, and CBM-20A controller system. Silica gel 40–63 μm (Merck, Germany), reversed-phase C-18 silica gel 40–63 μm (Merck, Germany), and highly porous Diaion HP-20 (Mitsubishi, Japan) were used for column chromatography (CC). Precoated silica gel Merck 60 F_{254} aluminum plates were used for thin-layer chromatography (TLC).

2.2. Plant Material. The leaves of *E. triplinerve* Vahl. were collected in Nghe An province, Vietnam, in July 2016. The plant material was taxonomically identified by Dr. Nguyen Thi Kim Thanh, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi. A voucher sample (ET-616) was deposited at the same institution.

*2.3. Extraction and Isolation of Compounds **1–10**.* The fresh leaves (7.5 kg) were air-dried in the shade, then oven-dried at 45°C, and ground into powder. The dry powder (2 kg) was extracted with MeOH at room temperature for 3 days, and the extraction was repeated for three times. The extracts obtained after filtration were concentrated under reduced pressure to yield a methanol extract. The methanol extract was suspended in water and extracted successively with *n*-hexane and dichloromethane to give the corresponding *n*-hexane- (86 g) and dichloromethane-soluble (50 g) fractions after *in vacuo* evaporation. Separation of a part of the *n*-hexane-soluble fraction (17.2 g) by CC over silica gel with gradient elution of *n*-hexane-acetone 90 : 1, 49 : 1, 19 : 1, 9 : 1, 3 : 1, 1 : 1, 1 : 2 gave 9 fractions. Fractions 4 (0.62 g) and 5 (0.91 g) were purified by CC over silica gel, eluting with *n*-hexane-EtOAc 12 : 1, 9 : 1, 7 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1 to give a mixture of **1** and **2** (110 mg and 38.9 mg, respectively). Fraction 6 (6.1 g) was separated by CC over silica gel, eluting with CH_2Cl_2-MeOH 15 : 1 to give **3** (7 mg) and **4** (109 mg). Fraction 9 (1 g) was separated by CC over silica gel, eluting with *n*-hexane-EtOAc 7 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1, 1 : 2 to give **5** (5.3 mg). A part of the dichloromethane-soluble fraction (1.1 g) was washed with *n*-hexane and then acetone to give **5** (45 mg). The residue was separated by CC over RP-18, eluting with 70% MeOH-H_2O. The fraction eluting with 70% MeOH-H_2O (882.5 mg) was separated by CC over silica gel, eluting with CH_2Cl_2-acetone 90 : 1, 49 : 1, 25 : 1, 15 : 1 to give **4** (13.5 mg). The water phase was concentrated and passed

through a Diaion HP-20 column eluting with MeOH-H_2O 2 : 3, 3 : 2 and MeOH to give three corresponding fractions 1–3. Fr. 1 (6.6 g) was separated by silica gel CC, eluting with EtOAc-MeOH 30 : 1, 25 : 1, 15 : 1, 12 : 1, 9 : 1, 6 : 1, 3 : 1 to give four subfractions 1–4. Subfr. 1 was separated by silica gel CC, eluting with CH_2Cl_2-MeOH 20 : 1, 15 : 1, 10 : 1, 7 : 1, 5, 3 : 1, 3 : 1 and purified by Sephadex LH-20, eluting with MeOH to give **6** (5 mg). Fr. 2 (17.2 g) was separated by silica gel CC, eluting with CH_2Cl_2-MeOH 60 : 1, 50 : 1, 40 : 1, 30 : 1 to give 2 subfractions 1 and 2. Subfr. 1 was purified by silica gel CC, eluting with CH_2Cl_2-EtOAc 45 : 1, 40 : 1, 30 : 1, 25 : 1, 20 : 1 to give **7** (5 mg). Subfr. 2 (3 g) was separated by CC over Sephadex LH-20, eluting with MeOH to give **8** (5 mg), a mixture of **8**/**9** (20 mg), and **10** (5 mg).

2.4. NMR Profiling of the Methanol or Boiling Water Leaf Extracts. The dried leaf powder (240 g) was extracted with methanol at room temperature for three days or with boiling water for 24 h. The extracts were separately filtered and evaporated to dryness under reduced pressure to give the corresponding methanol or water extract. A small part of the methanol or water extract (30 mg) was dissolved in pure methanol and extracted using a Lichrolut® RP-18 SPE cartridge (Merck, 40–60 μm) with a solvent gradient of 70%, 80%, and 100% MeOH-H_2O. The fractions eluting with 70% MeOH-H_2O were concentrated under reduced pressure to afford two NMR samples. 5 mg of each sample was dissolved in CD_3OD and directly analysed by ^{1}H-NMR spectroscopy at 500 MHz frequency.

2.5. HPLC Analysis of the Methanol Leaf Extract. The dried leaf powder (80 g) was extracted with MeOH at room temperature for 1 day. The solution was filtered and then evaporated to dryness under reduced pressure. The methanol leaf extract (220 mg) was dissolved with 2 mL acetonitrile of HPLC grade (Merck, Germany). The reversed-phase solid-phase extraction (RP-SPE) of the solution eluting with MeOH of HPLC grade (Merck, Germany) was performed to remove impurities. The sample was filtered through a Millipore 0.45 μm membrane filter. Two standard solutions (100 μg/mL) were prepared by dissolving compounds **4** and **5** in MeOH of HPLC grade (Merck, Germany). The calibration curves were constructed by plotting the peak areas versus the corresponding concentrations (expressed as μg/mL) of each standard. A Shimadzu 20A HPLC system equipped with a photodiode array detector (PDA) was used for the qualitative and quantitative analysis of samples. An analytical column C-18 (4.6 mm × 250 mm, 5 μm), a gradient mobile phase of 44% acetonitrile-H_2O (15 min.), a flow rate of 1 mL/min., an injection volume of 10 μL, and column temperature 25°C were used for the HPLC analysis. The UV detection wavelength was set at 306 nm. All the measurements were performed in triplicate.

*2.6. α-Glucosidase Inhibitory Activity of Compounds **4–9** and **11**.* The inhibition of α-glucosidase activity was carried out following the method described by Li et al. [22]. A 2.5 mM solution of *p*-NPG (*p*-nitrophenyl α-D-glucopyranoside) (Sigma-Aldrich) and 0.2 U/mL α-glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich) were prepared in 100 mM potassium phosphate buffer pH 6.8. Compounds **4**–**9** and compound **10** previously isolated from the leaves of *E. japonicum* [23] were prepared in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and serially diluted in the concentrations of 1, 4, 16, 64, and 256 μg/mL. 10 μL of sample was added to a reaction mixture consisting of 40 μL of 100 mM phosphate buffer pH 6.8 and 25 μL of 0.2 U/mL α-glucosidase in a 96-well microplate and the reaction mixture was incubated for 10 min at 37°C. Then 25 μL of 2.5 mM *p*-NPG was added and the reaction mixture was further incubated for 20 min at 37°C. After 30 min, 100 mM sodium carbonate solution (100 μL) was added to stop reaction. The absorbance of the mixture was measured at λ 410 nm on an UV-VIS spectrophotometer (Biotek Instruments, USA). To make a control reaction, the tested sample was replaced by 10 μL of 100 mM phosphate buffer (pH 6.8). Acarbose was used as the reference standard. The experiments were repeated three times. The α-glucosidase inhibitory activity was calculated using the following equation, where $A_{control}$ is the absorbance of the control, A_{sample} is the absorbance of the sample: α-glucosidase inhibitory activity (%) = $(A_{control} - A_{sample})/A_{control} \times 100$. IC_{50} (half maximal inhibitory concentration) was calculated using Tablecurve software. All analyses were performed in triplicate and data were reported as mean ± SEM.

3. Results and Discussion

3.1. Isolation and MS/NMR Elucidation of Compounds. The dried powdered leaves of *E. triplinerve* were extracted with MeOH at room temperature to yield a methanol extract. The methanol extract was fractionated into *n*-hexane-, dichloromethane-, and water-soluble fractions. Separation of the *n*-hexane-, dichloromethane-, and water-soluble fractions by repeated CC over different adsorbents such as silica gel, Diaion HP-20, Sephadex LH-20, and RP-18 using stepwise gradient elution gave phytosterols **1**–**3**, coumarins **4** and **5**, thymoquinol glucoside **6**, *o*-hydroxycinnamic acid derivatives **7**–**9**, and kaempferol diglucoside **10**. Structures of the isolated compounds were determined as β-sitosterol (**1**) [24], stigmasterol (**2**) [24], β-sitosterol 3-*O*-β-D-glucopyranoside (**3**) [25], ayapanin (**4**) [26, 27], ayapin (**5**) [28], thymoquinol 5-*O*-β-D-glucopyranoside (**6**) [29], (*E*)-4-hydroxy-2-methoxycinnamic acid (**7**) [30, 31], thyrsifloside (**8**) [32], (*E*)-4-methoxymelilotoside (**9**) [33], and kaempferol 3,7-di-*O*-β-D-glucopyranoside (**10**) [34] (Figure 1) by comparing their spectroscopic data (MS, ^{1}H-, and ^{13}C-NMR) (Figures S1–S30) with the reported literature values. Compound **8** was isolated in its pure form and compound **9** was isolated in a nonseparable mixture of **8** and **9** (molar ratio 1 : 1 as determined by ^{1}H-NMR intensity). The locations of the substituents of compounds **6**, **8**, and **9** were assigned by using heteronuclear single-quantum correlation (HSQC), heteronuclear multibond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) spectra (Figure 2). In compound **6**, key HMBC correlations were

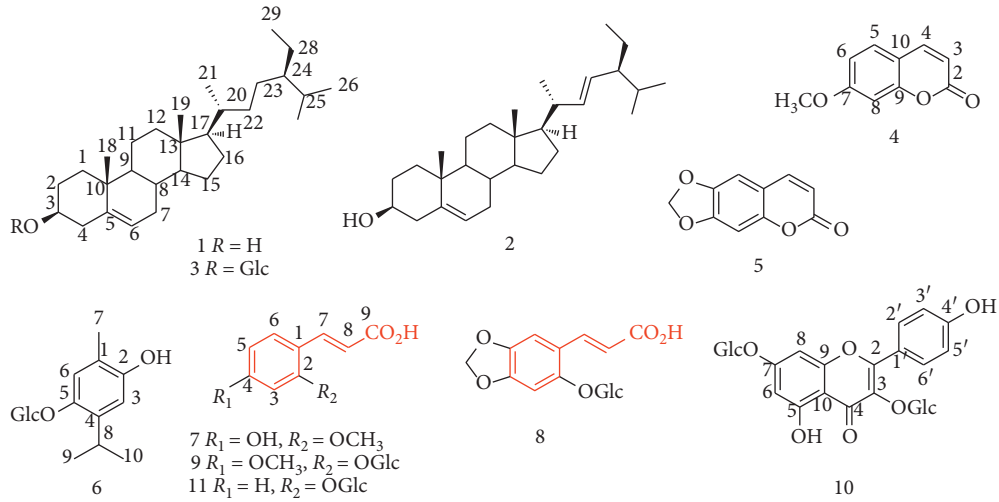

Figure 1: Structures of compounds **1–10** from *E. triplinerve* and **11** from *E. japonicum*.

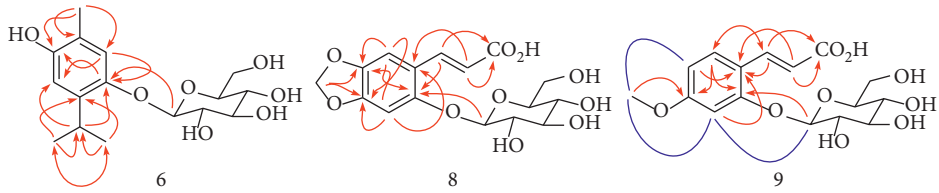

Figure 2: HMBC (⟶) and NOESY (—) correlations of compounds **6**, **8**, and **9**.

observed between CH_3-7 (δ_H 2.15) and C-1 (δ_C 123.3), C-2 (δ_C 151.8), between H-8 (δ_H 3.5) and C-3 (δ_C 113.1), C-5 (δ_H 149.2), and between Glc-1 (δ_H 4.72) and C-5 (δ_C 149.2). In compound **8**, key HMBC correlations for the assignment of the glucopyranosyl moiety were observed between H-7 (δ_H 8.13) and C-2 (δ_C 154.0), between H-6 (δ_H 7.13) and C-2, and between Glc-1 (δ_H 4.85) and C-2. In compound **9**, key HMBC correlations for the assignment of the glucopyranosyl moiety and the methoxy group were observed between H-6 (δ_H 7.57) and C-2 (δ_C 159.0), between 4-OCH_3 (δ_H 3.87) and C-4 (δ_C 164.4), and between Glc-1 (δ_H 4.98) and C-2. Further confirmation was obtained from the NOESY spatial interactions between H-5 (δ_H 6.67) and 4-OCH_3, between 4-OCH_3 and H-3 (δ_H 6.87), and between H-3 and Glc-1. The ^{1}H-NMR spectra of **4** and **5** were also recorded in different NMR solvents ($CDCl_3$, CD_3OD, and DMSO-d_6) and the signals were assigned to ease NMR identification of the compounds in different NMR solvents. Compounds **6–10** were isolated for the first time from *E. triplinerve*. The isolation of compounds has not showed the presence of alkaloids, saponins, triterpenoids, depsides, and depsidones which were detected in the previous phytochemical screening by using color reactions [35]. However, high concentration of coumarins and the presence of sterols in the leaves are in line with most of the phytochemical screenings [35]. In the genus *Eupatorium,* derivatives of 2-*O*-hydroxycinnamic acid and 2-*O*-glucopyranosylcinnamic acid were isolated from *E. micranthum*, while thymols were reported in *E. fortunei* [1]. Compounds **8** and **9** are glycosidic precursors of compounds **5** and **4**, respectively. Under enzymatic conditions, they can be converted into nonglycosidic derivatives 2-hydroxy-6,7-methylenedioxy-*trans*-cinnamic acid (**8a**) and 2-hydroxy-4-methoxy-*trans*-cinnamic acid (**9a**), respectively. Compound **4** would be formed from **9a** and compound **5** from **8a** via photoisomerization of the *trans* double bond to *cis* and subsequent ring closure to the lactones (Figure 3) [30].

3.2. α-Glucosidase Inhibitory Activity. Type II diabetes mellitus is as metabolic disease mainly caused by the accumulation of excess sugar in the body. The enzyme α-glucosidase breaks down large starch polysaccharides into monosaccharides or disaccharides. Thus, inhibiting the enzymatic activity of α-glucosidase may reduce absorption of glucose in the body. In the *in vitro* α-glucosidase inhibitory activity test, the substrate *p*-nitrophenyl α-D-glucopyranoside (pNPG) is hydrolyzed by α-glucosidase to release *p*-nitrophenyl which can be monitored at 405 nm. The results are expressed as percentage inhibition (%) and the concentration of an inhibitor required to inhibit 50% of enzyme activity (IC_{50}) is determined [36]. The inhibitory activity of compounds isolated from *E. triplinerve* against α-glucosidase is reported in Table 1. Compounds **4**, **5**, and **7** showed the highest enzyme inhibitory activity at 256 μg/mL (40%, 46%, and 81%, respectively). An IC_{50} of 58.65 ± 1.20 μg/mL (302 μM) was calculated for compound **7**, while the positive control acarbose showed a higher IC_{50} value of 197.33 ± 2.51 μg/mL (306 μM). The other related *o*-

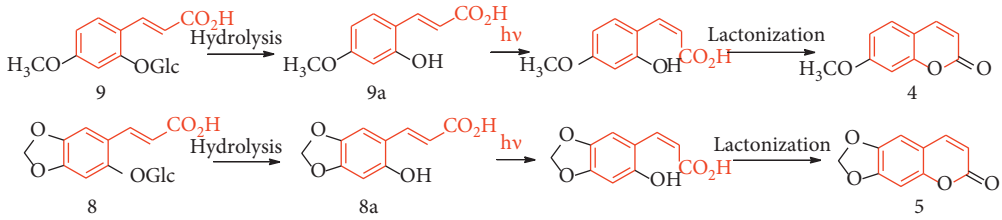

Figure 3: Plausible biosynthesis of **4** from **9** and **5** from **8** in *E. triplinerve*.

Table 1: α-glucosidase inhibitory activity of compounds **4–9** and **11**.

Compound	α-glucosidase inhibitory activity (%)					IC_{50} (μg/mL) (μM)
	256 μg/mL	64 μg/mL	16 μg/mL	4 μg/mL	1 μg/mL	
4	40	38	35	30	25	>256
5	46	41	38	30	19	>256
6	2	0	0	0	0	>256
7	81	53.5	22	16	0	58.65 ± 1.20 (302)
8	2	0	0	0	0	>256
8/9*	2	0	0	0	0	>256
11	2	0	0	0	0	>256
						Acarbose: IC_{50} 197.33 ± 2.51 μg/mL [306 μM]

*^{1}H-NMR: molar ratio 1 : 1.

hydroxycinnamic acids including **8**, a mixture of **8/9** (molar ratio 1 : 1), and **11** (compound **11** was isolated from *E. japonicum* [23]) and thymoquinol glucoside **6** did not show any α-glucosidase inhibitory activity (2% inhibitory activity at 256 μg/mL for all compounds investigated). (*E*)-cinnamic acid does not inhibit α-glucosidase from yeasts, but *o*-hydroxy or *o*-methoxy substituents increase the activity of the derivatives and the potency is higher for the methoxy group [15, 35, 36]. The structure of compound **7** possessing a methoxy group at C-2 and a hydroxy group at C-4 matched well the above-mentioned structural requisite. However, the activity was lost in compounds **8**, **10**, and a mixture of compounds **8/9** (molar ratio 1 : 1) when the 2-hydroxy group was blocked by a glucopyranosyl group in spite of the presence of 4-methoxy group in **9**. Thus, the importance of 2-hydroxy or 2-methoxy group in modulating α-glucosidase inhibitory activity of (*E*)-cinnamic acids was confirmed which is in line with the previously published results [21, 36, 37]. The results of the present investigation are an additional contribution to the development of novel antidiabetic agents derived from (*E*)-cinnamic acid.

*3.3. HPLC Analysis of Coumarins **4** and **5**.* HPLC analysis of the leaf methanol extract revealed coumarins **4** and **5** as the major compounds in the extract. An optimized HPLC condition using a mobile phase of 44% acetonitrile in deionized H_2O in 15 min was used to effectively separate compounds **4** and **5**. With the wavelength of a PDA (photodiode array), detector set at 306 nm the HPLC chromatogram (Figures 4 and S33) showed two main resolved peaks of compound **4** at R_t 5.304 min and compound **5** at R_t 4.628 min. Compounds were identified on the basis of R_t comparison with those of the standard compounds **4** and **5**. Polar compounds including **6–10** were not well separated and they appeared as a broad band at R_t about 2.5 min in the

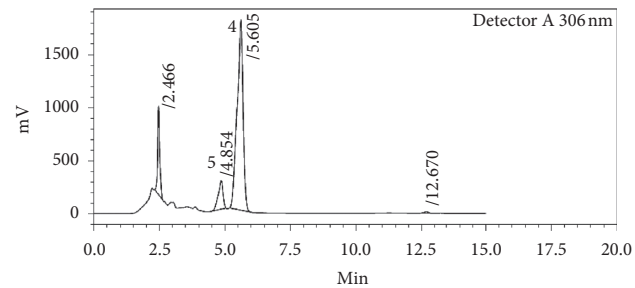

Figure 4: HPLC analysis of compounds **4** and **5** in the methanol leaf extract.

HPLC chromatogram. Thus, the HPLC method was suitable for analysing the contents of compounds **4** and **5**. The calibration curves of compounds **4** and **5** were constructed, injecting 20 μL of each standard compound (5–100 ppm) (Figure S34). Using the Shimadzu LabSolutions HPLC software, each of the peaks was integrated to get the area values. The calibration curves were constructed by plotting area versus concentration with a correlation coefficient $R^2 > 0.990$. Based on the calibration curve, the methanol leaf extract was determined to contain 16.69% of compound **4** and 2.02% of compound **5**. Thus, the amounts of compounds **4** and **5** in the dry leaves of *E. triplinerve* collected in July in Vietnam were calculated as 2.63% and 0.32%, respectively.

*3.4. NMR Profiling of Coumarins **4** and **5**.* At present, ^{1}H-NMR spectroscopy has become a frequently used technique for metabolic fingerprinting. In addition, ^{1}H-NMR spectroscopy has been used in the qualitative and quantitative assessment of secondary plant metabolites as well as quality

control in food science and technology. One of the major advantages of NMR techniques is their reproducibility with rich structure information and applicability to a wide range of plant metabolites [38–40]. Coumarins have been successfully analysed in a few studies by ^{1}H-NMR profiling [41, 42]. Compounds **4** and **5** could be extracted from the leaves of *E. triplinerve* by extraction with an alcohol such as methanol and boiling water. The methanol and boiling water extracts from the dry leaves of *E. triplinerve* were analysed by ^{1}H-NMR spectra in this study. Solid-phase extraction (SPE) of the extracts eluting with 70% MeOH-H_2O afforded the analytical samples. The following NMR parameters were used for the analysis: solvent 0.5 mL CD_3OD in 5-mm NMR tubes, 16 scans, acquisition time 3.2767999 sec, temperature 299.0 K, spectral width 10000 Hz, and line broadening 0.3 Hz. FIDs were Fourier transformed with FIDRES 0.152588, GB 0, and PC 1.0. Figures S31 and S32 showed the representative ^{1}H-NMR spectra of the methanol or water extracts, respectively. Referencing was to the lock solvent. The ^{1}H-NMR spectra of the standards **4** and **5** were prepared in 0.5 mL of CD_3OD in 5 mm NMR tubes. By using high-field 500 MHz NMR spectrometer, the signals of compounds **4** and **5** were resolved in both extracts. The identification match was based on chemical shifts, coupling, peak shape, and peak intensity data for individual compounds. Coumarins **4** and **5** were confirmed to be the abundant compounds in the extracts; their signal intensity ratio was about 15 : 1 in the methanol extract and about 10 : 1 in the water extract. These findings well supported the results obtained via the quantitative HPLC analysis. The concentrations of polar compounds **6–10** may be too low for the reliable detection by NMR techniques. Proton chemical shifts of compounds **4** and **5** in the extracts are summarized in Table S1. The ^{1}H-NMR spectrum of compound **4** in the extracts was in accordance with that of the standard **4** in CD_3OD, showing the signals of a lactone ring of the coumarin structure at δ_H 6.27 (1H, *d*, $J = 9.5$ Hz, H-3) and 7.93 (1H, *d*, $J = 9.5$ Hz, H-4), three aromatic protons with characteristic splitting of a 1,3,4-trisubstituted benzene ring at δ_H 6.93 (1H, br *s*, H-8), 6.96 (1H, dd, $J = 8.5$ Hz, 2.5 Hz, H-6), and 7.57 (1H, *d*, $J = 8.5$ Hz, H-5), and an aromatic methoxy group at δ_H 3.90 (3H, *s*, 7-OCH_3). The characteristic proton signals of compound **5** in the extracts matched the signals of the standard **5** in CD_3OD. The signals of a lactone ring of the coumarin structure at δ_H 6.29 (1H, *d*, $J = 9.5$ Hz, H-3) and 7.90 (1H, *d*, $J = 9.5$ Hz, H-4), a 1,3,4,6-tetrasubstituted benzene ring bearing an *o*-methylenedioxy moiety at δ_H 6.11 (2H, *s*, $-OCH_2O-$), and characteristic singlets of the aromatic protons at δ_H 6.93 (1H, *s*, H-5), 7.07 (1H, *s*, H-8) were observed.

4. Conclusions

The present study isolated phytosterols (**1–3**), coumarins (**4** and **5**) in the *n*-hexane- and dichloromethane-soluble fractions, and polar compounds: thymoquinol glucoside (**6**), *o*-hydroxycinnamic acid derivatives (**7–9**), and kaempferol diglucoside (**10**) in the water-soluble fraction from the leaves of *E. triplinerve* growing in Vietnam. Compounds **6–10** were isolated for the first time from *E. triplinerve*. We provided an efficient approach towards rapid identification of coumarins in *E. triplinerve* by NMR and HPLC analysis. Coumarins **4** and **5** were confirmed as the main constituents in the leaf extracts by proton NMR profiling and quantitative RP-HPLC-PDA analysis. In the inhibitory activity, test against yeast α-glucosidase of compounds **4–9**, coumarins **4** and **5,** and (*E*)-4-hydroxy-2-methoxycinnamic acid (**7**) showed 40%, 46%, and 81% inhibitory activity, respectively, at 256 μg/mL. The half inhibitory concentration (IC_{50}) of compound **7** was determined as 58.65 ± 1.20 μg/mL (302 μM) which is lower than that of the positive control acarbose (197.33 ± 2.51 μg/mL, 306 μM).

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PMG designed the work and performed the research (isolation, structure determination, and NMR profiling) and wrote the manuscript. DTVH performed the HPLC analysis and enzyme inhibitory activity test. VMT contributed to the preparation of analytical samples.

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Supplementary Materials

MS and NMR data and spectra of compounds **1–10**, NMR spectra of the methanol, and boiling water extracts are freely available along with the manuscript as supplementary materials. (*Supplementary Materials*)

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