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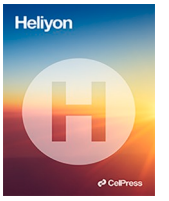

Anti-inflammatory activity of verbascoside- and isoverbascoside-rich Lamiales medicinal plants

Benyakan Pongkitwitoon [a], Waraporn Putalun [b], Kanokporn Triwitayakorn [c], Tharita Kitisripanya [d], Tripetch Kanchanapoom [b], Panitch Boonsnongcheep [c,*]

[a] Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand
[b] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand
[d] Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand

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ABSTRACT

Verbascoside and isoverbascoside are two active phenylethanoid glycosides mainly found in plants of the order Lamiales. This study analyzes the verbascoside and isoverbascoside levels and the total phenolic contents in the water and ethanolic extracts of 20 medicinal plants of the order Lamiales commonly used in Thailand. The related bioactivities, including the antioxidant activity via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reduction activity potential assays and anti-tyrosinase and -inflammatory activities via the cyclooxygenase and nitric oxide assays are also investigated. The extracts of several plant species, including *Barleria prionitis*, *B. lupulina*, *Rhinacanthus nasutus*, *Orthosiphon aristatus*, and *Nicoteba betonica*, exhibit high verbascoside and isoverbascoside content levels. The correlation analysis between the bioactive activities and the active compounds demonstrates a significant association between the verbascoside level in the water extracts and both the DPPH antioxidant activity and the nitric oxide level in the anti-inflammatory assays. This study provides the first report on the verbascoside and isoverbascoside quantification of several plant samples. The findings provide valuable insights for future research on lesser-studied plants possessing high verbascoside and isoverbascoside levels, which exhibit promising anti-inflammatory activities.

1. Introduction

With their abundant secondary metabolites and wide range of biological activities, plants have long been known as valuable sources of medicines. The Lamiales order, which is the mint order of flowering plants, consists of more than 20 families, 1000 genera, and 20,000 plant species [1]. The main Lamiales families include Lamiaceae, Verbenaceae, Plantaginaceae, Scrophulariaceae, and Acanthaceae. Many plant species belonging to Lamiales are used worldwide as herbal medicines and supplementary products. Many Lamiales plants, largely from Lamiaceae and Acanthaceae, are used in Thai traditional medicines and primary health care. Several drug preparations and formulas of these plants are listed in the National List of Essential Medicines for herbal medicines for use in hospitals and public health services across Thailand [2].

Verbascoside and isoverbascoside are water-soluble phenylethanoid glycosides accumulated in various medicinal plants, especially

* Corresponding author.
E-mail address: panitch.boo@mahidol.ac.th (P. Boonsnongcheep).

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Fig. 1. Plant samples used in this study. a) *Ocimum africanum* Lour., b) *Clinacanthus nutans* (Burm.f.) Lindau, c) *Mentha* × *villosa* Huds., d) *Mentha pulegium* L., e) *Mentha canadensis* L., f) *Andrographis paniculata* (Burm.f.) Nees, g) *Ocimum tenuiflorum* L., h) *Ocimum basilicum* L., i) *Clerodendrum indicum* Kuntze, j) *Orthosiphon aristatus* (Blume) Miq., k) *Nicoteba betonica* Lindau, l) *Barleria prionitis* L., m) *Clinacanthus siamensis* Bremek., n) *Acanthus ebracteatus* Vahl, o) *Thunbergia laurifolia* Lindl., p) *Justicia valida* Ridl., q) *Barleria lupulina* Lindl., r) *Rhinacanthus nasutus* (L.) Kurz, s) *Barleria strigosa* Willd, and t) *Leonotis nepetifolia* (L.) R.Br.

those belonging to the Lamiales order [3]. Verbascoside is among the most widespread phenylethanoid glycosides distributed in plants. In 1963, Verbascoside was first isolated from the medicinal plant *Verbascum sinuatum* L. before a compound, called acteoside, was reported in 1968. It was later found to be the same compound as verbascoside [4]. Both the names "verbascoside" and "acteoside" are used in publications to date. Verbascoside has been isolated from different parts of more than 200 plant species [5]. Isoverbascoside, an *iso* derivative of verbascoside, is another active phenylethanoid glycoside accumulated in several plant species. However, compared to verbascoside, reports of isoverbascoside distribution and biological activities are fairly limited.

Plants with high concentrations of verbascoside and other phenylethanoid glycosides have been traditionally used to treat inflammation and microbial infections [6]. Until now, the biological activities of verbascoside and isoverbascoside, including their antimicrobial, neuroprotective, immunomodulatory, anti-inflammatory, antioxidative, and free radical-scavenging activities, are being extensively studied in vitro and in vivo and clinically [3–8]. Li et al. investigated the antioxidative and neuroprotective activities of verbascoside and isoverbascoside, together with two other phenylethanoid glycosides, by triggering the nuclear translocation of the nuclear factor erythroid 2-related factor 2 (Nrf2) in H_2O_2-induced apoptosis in PC12 cells [9]. The strong anti-inflammatory activity of isoverbascoside related to toll-like receptor 4 dimerization was reported in vitro using RAW264.7 cells and in vivo mouse models of xylene-induced ear edema and lipopolysaccharide (LPS)-induced endotoxic shock and endotoxaemia-associated acute kidney injury [10]. In a randomized clinical study, a 2-week oral administration of 100 mg verbascoside significantly decreased the platelet aggregation in patients with cardiovascular risk factors [11]. As phenolic compounds, the phenolic hydroxyl groups of verbascoside and isoverbascoside play critical roles on their antioxidant and related activities.

This study aims to provide qualitative and quantitative data of the verbascoside, isoverbascoside, and phenolic compounds of essential Thai herbs belonging to the Lamiales order. We also evaluated the antioxidant, -tyrosinase, and -inflammatory activities of the water and ethanol extracts of these plants and the relationship of these activities with the quantitative data of the verbascoside, isoverbascoside, and phenolic compounds.

2. Materials and methods

2.1. Chemicals

The verbascoside and isoverbascoside standard compounds, Folin–Ciocalteu reagent, gallic acid, trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and mushroom tyrosinase were purchased from Sigma-Aldrich (USA). The ovine cyclooxygenase (COX)-1 and human COX-2 colorimetric inhibitor screening assay was purchased from Cayman Chemical (USA). High-performance liquid chromatography (HPLC)-grade solvents were used in the analysis. All other chemicals were standard analytical reagent-grade commercial products.

2.2. Plant materials and sample extraction

The plant materials were from Sireeruckhachati Nature Learning Park, Mahidol University, Thailand. Plant samples used in this study are *Ocimum africanum* Lour., *Clinacanthus nutans* (Burm.f.) Lindau, *Mentha* × *villosa* Huds., *Mentha pulegium* L., *Mentha canadensis* L., *Andrographis paniculata* (Burm.f.) Nees, *Ocimum tenuiflorum* L., *Ocimum basilicum* L., *Clerodendrum indicum* Kuntze, *Orthosiphon aristatus* (Blume) Miq., *Nicoteba betonica* Lindau, *Barleria prionitis* L., *Clinacanthus siamensis* Bremek., *Acanthus ebracteatus* Vahl, *Thunbergia laurifolia* Lindl., *Justicia valida* Ridl., *Barleria lupulina* Lindl., *Rhinacanthus nasutus* (L.) Kurz, *Barleria strigosa* Willd, and *Leonotis nepetifolia* (L.) R.Br. Fig. 1a-t shows the plants used in this work. All plant samples were dried at 60 °C for 48 h and pulverized to powder. The powdered samples were extracted thrice using × 30 volume of ethanol or water in a sonication bath at 25 °C. The ethanolic extracted solutions were collected, and then evaporated, while the water extracted samples were freeze-dried. The samples were then appropriately diluted and subjected to further analysis.

2.3. Verbascoside and isoverbascoside quantification from plant extracts and HPLC method validations

Under previously developed HPLC conditions [12], we quantitatively analyzed verbascoside and isoverbascoside by utilizing the Thermo Scientific UltiMate 3000 HPLC system coupled with a diode array detector. The mobile phase was an isocratic 20 % acetonitrile acidified with 2.5 % acetic acid flowing at 0.5 mL/min. The column used was a 4.6 × 150 Nacalai Tesque Cosmosil C18 reverse phase column. Standard curves were plotted using the area under the curve of both compounds at a 1.95–250 μg/mL concentration. The limit of detection and the limit of quantification were obtained by calculating the signal-to-noise ratio equal to 3:1 and 10:1, respectively. The accuracy was obtained by adding both standards of a known amount to the plant extract and expressed as the recovery percentage. The method precision was obtained by analyzing three concentrations of both standards in the same day (intra-day) and three different days (inter-day) and expressed as %RSD. Subsequently, 1 mg of the extracts was dissolved in the mobile phase and

filtered prior injection. The injection volume was 10 μL. sample and standard analyses were performed in triplicate.

2.4. Colorimetric determination of the total phenolic contents

The total phenolic contents of the plant samples were measured using the Folin–Ciocalteu method [13], as previously described [14]. The samples and the standard gallic acid were mixed with a 10 times dilution of the Folin–Ciocalteu reagent. The reaction mixture was set at room temperature for 6 min prior to adding 7 % sodium carbonate. We measured the absorbance at 760 nm after a 30 min incubation. The gallic acid absorbance at 1.5–100.0 μg/mL concentration was plotted as a standard curve. The total phenolic content of the samples is reported here as microgram gallic acid equivalent per gram sample dry weight. All measurements were performed in triplicate.

2.5. Trolox equivalent antioxidant capacity assay

2.5.1. DPPH radical-scavenging assay

We assessed the antioxidant capacity of the samples through the DPPH radical-scavenging assay according to a previously described method [14,15]. Samples at 100 μg/mL, verbascoside and isoverbascoside at 10 μg/mL, and standard trolox at 1.5–25.0 μg/mL were added to a 96-well plate. An equal volume of 0.2 mM DPPH was then added. Absorbance was measured at 490 nm after mixing and incubation at room temperature for 30 min. The results were reported in μg/mL trolox equivalent. All measurements were conducted in triplicate.

2.5.2. Ferric reducing antioxidant power assay

The antioxidant capacity of samples was measured by the ferric reduction activity potential (FRAP) assay according to a previously developed protocol [14]. The FRAP reagent was prepared by mixing 100 mM acetate buffer with 3.6 pH, 10 mM 2,4,6-Tris(2-pyridyl)--striazine in 40 mM HCl, and 20 mM $FeCl_3$ at a 10:1:1 ratio. Each reaction contained 20 μL of 100 μg/mL plant extracts, 10 μg/mL verbascoside, 10 μg/mL isoverbascoside or trolox, and 180 μL of the FRAP reagent. The absorbance was measured at 595 nm after a 30 min incubation at room temperature. The trolox standard curve was constructed using trolox absorbance at 7.8–125 μg/mL concentrations. The results were reported in μg/mL trolox equivalent. All measurements were performed in triplicate.

2.6. Mushroom tyrosinase inhibition assay

We followed the method described in Ref. [16]. Mushroom tyrosinase was prepared at 200 U/mL with 0.05 M phosphate buffer. The sample was prepared in methanol at 100 μg/mL. Verbascoside and isoverbascoside were prepared in methanol at 10 μg/mL. Each well of the 96-well plate contained 50 μL of 2 mM L-DOPA, 50 μL mushroom tyrosinase, and 50 μL of the sample or standard kojic acid at 12.5 and 25 μg/mL concentrations. The reaction was kinetically measured at 450 nm. The results were expressed as %inhibition compared to the control kojic acid.

2.7. Cyclooxygenase inhibitory assay

The COX-1 (ovine) and COX-2 (human) inhibitory activities of the samples were assessed using a COX colorimetric inhibitor screening assay kit (701050, Cayman Chemical, USA). The assay was performed following the manufacturer's recommended protocol. The samples were dissolved in ethanol or water. Enzymes (e.g., either COX-1 or COX-2), hemin, and samples or 10 mM indomethacin were added to the 96-well plate. Arachidonic acid was added to all wells after a 5 min incubation. The absorbance was measured at 590 nm after a 2 min incubation. The results were reported as inhibition percentage.

2.8. Cell culture and anti-inflammatory assay using the nitric oxide level

We used mouse macrophage RAW 264.7 cell lines to test the anti-inflammatory activity. The cell was cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum and 1 % penicillin–streptomycin for 3 days prior seeding. The plant extracts or standard compounds were prepared in dimethyl sulfoxide (DMSO) and dissolved to 100 μg/mL in media. The final DMSO concentration in media was 0.1 %. The cells were trypsinized and seeded in a 96-well plate at a concentration of 2×10^6 per well and grown for 24 h. The culture media were then removed and replaced with treatments and 2 μg/mL LPS. Subsequently, 0.3 mM indomethacin was used as a positive control. The culture media were collected for the nitric oxide analysis after another 24 h. The nitrite level (NO_{2-}), which represented the nitric oxide production, was measured. The media were mixed with an equal amount of freshly prepared Griess reagent (equal volume: 1 % sulphanilamide and 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride in 5 % phosphoric acid). Moreover, 0.78–25.0 μg/mL sodium nitrite was used to create the standard curve. The measurement was made at 562 nm using a microplate reader. The results were reported as nitric oxide levels in μM.

The cell viability assay was performed by adding 10 % PrestoBlue (Thermo Fisher) in the culture media to the remaining cell in the 96-well plate. The absorbance was measured at 570 nm with a 600 nm reference wavelength after a 2 h incubation. The cell viability percentage was calculated according to the PrestoBlue recommended protocol.

Table 1
Validation parameters of HPLC analysis for verbascoside and isoverbascoside.

	Limit of detection (μg/mL)	Limit of quantification (μg/mL)		Accuracy (% recovery)		Precision (%RSD)	
Compound			amount spiked (ng)		Concentration (μg/mL)	Intraday	Interday
Verbascoside	0.56	1.86	50	112.34 ± 1.52	31.25	4.86	3.21
			200	100.72 ± 3.81	62.5	1.23	4.42
			1000	111.40 ± 1.52	250	0.42	3.91
Isoverbascoside	0.55	1.85	50	103.81 ± 2.63	31.25	3.64	4.17
			200	106.37 ± 6.59	62.5	2.79	3.15
			1000	98.77 ± 0.96	250	0.65	3.67

Table 2
Amount of verbacoside and isoverbacoside form ethanol and water extracts of 20 plant samples. The letters indicate a significant difference between plants within the same compound and solvent group at $p < 0.01$.

Scientific name	Family	Part used	Ethanol		Water	
			Verbascoside (μg/g dry weight)	Isoverbascoside (μg/g dry weight)	Verbascoside (μg/g dry weight)	Isoverbascoside (μg/g dry weight)
Ocimum africanum Lour.	Lamiaceae	Aerial parts	0.73 ± 0.59^{a}	17.89 ± 12.42^{ab}	164.79 ± 58.66^{abc}	7059.82 ± 592.80^{g}
Clinacanthus nutans (Burm.f.) Lindau	Acanthaceae	Aerial parts	2032.87 ± 253.78^{f}	14.95 ± 2.05^{ab}	2116.56 ± 86.59f	446.89 ± 60.12^{ab}
Mentha × *villosa* Huds.	Lamiaceae	Aerial parts	20.59 ± 2.52^{a}	67.59 ± 7.54^{abc}	359.79 ± 139.30^{abc}	78.59 ± 75.33^{ab}
Mentha pulegium L.	Lamiaceae	Aerial parts	19.37 ± 0.34^{a}	61.95 ± 25.71^{abc}	503.76 ± 53.65^{abcd}	295.55 ± 303.82^{ab}
Mentha canadensis L.	Lamiaceae	Aerial parts	69.03 ± 15.38^{a}	125.64 ± 4.01^{abcd}	67.32 ± 20.63^{a}	25.35 ± 43.91^{a}
Andrographis paniculata (Burm.f.) Nees	Acanthaceae	Aerial parts	11.78 ± 2.38^{a}	122.94 ± 1.29^{abcd}	28.30 ± 32.94^{a}	1076.78 ± 206.82^{c}
Ocimum tenuiflorum L.	Lamiaceae	Aerial parts	13.23 ± 1.10^{a}	55.40 ± 5.57^{abc}	110.25 ± 27.58^{ab}	2088.23 ± 179.04^{d}
Ocimum basilicum L.	Lamiaceae	Aerial parts	8.34 ± 2.03^{a}	235.57 ± 7.05^{de}	525.36 ± 38.93^{abcd}	5346.83 ± 46.24^{e}
Clerodendrum indicum Kuntze	Lamiaceae	Aerial parts	190.64 ± 8.71^{ab}	150.65 ± 10.40^{bcd}	183.91 ± 32.70^{abc}	55.66 ± 48.36^{a}
Orthosiphon aristatus (Blume) Miq.	Lamiaceae	Aerial parts	15.05 ± 2.72^{a}	184.00 ± 1.37^{cd}	149.20 ± 113.90^{abc}	9671.04 ± 23.08^{i}
Nicoteba betonica Lindau	Acanthaceae	Aerial parts	3180.76 ± 83.62^{h}	61.43 ± 16.32^{abc}	3771.43 ± 54.80^{g}	6148.42 ± 75.83^{f}
Barleria prionitis L.	Acanthaceae	Aerial parts	391.05 ± 36.67^{b}	504.64 ± 7.01^{f}	13273.36 ± 72.86^{i}	7922.90 ± 67.10^{h}
Clinacanthus siamensis Bremek.	Acanthaceae	Aerial parts	1178.41 ± 26.45^{de}	106.69 ± 11.60^{abcd}	737.88 ± 94.04^{bcd}	541.74 ± 96.29^{abc}
Acanthus ebracteatus Vahl	Acanthaceae	Aerial parts	666.64 ± 8.06^{c}	353.78 ± 23.96^{e}	1652.37 ± 129.11^{ef}	677.02 ± 140.45^{bc}
Thunbergia laurifolia Lindl.	Acanthaceae	Aerial parts	1239.55 ± 64.53^{e}	13.12 ± 3.50^{ab}	1022.86 ± 65.09^{de}	ND
Justicia valida Ridl.	Acanthaceae	Aerial parts	95.18 ± 3.08^{a}	11.09 ± 0.21^{ab}	1484.22 ± 37.62^{ef}	99.40 ± 68.29^{ab}
Barleria lupulina Lindl.	Acanthaceae	Aerial parts	4580.89 ± 113.72^{i}	1183.74 ± 40.23^{g}	5700.89 ± 66.96^{h}	483.35 ± 142.84^{abc}
Rhinacanthus nasutus (L.) Kurz	Acanthaceae	Aerial parts	967.42 ± 92.05^{d}	175.12 ± 18.20^{cd}	3976.84 ± 639.66^{g}	160.52 ± 31.50^{ab}
Barleria strigosa Willd	Acanthaceae	Aerial parts	2364.90 ± 34.37^{g}	520.58 ± 21.57^{f}	1994.46 ± 410.36^{f}	4964.13 ± 38.09^{e}
Leonotis nepetifolia (L.) R.Br	Lamiaceae	Flowers	1352.56 ± 108.70^{e}	174.25 ± 165.47^{cd}	766.50 ± 85.52^{cd}	273.12 ± 15.77^{ab}

ND = not detected.

2.9. Statistical analysis

All experiments were performed in triplicate, except for the COX inhibitory assay, which was duplicated. The data were expressed as mean ± SD. The difference between the control and treatment groups was analyzed through one-way analysis of variance and Tukey test at $p < 0.01$ using SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA). The correlations between the activities and the verbascoside,

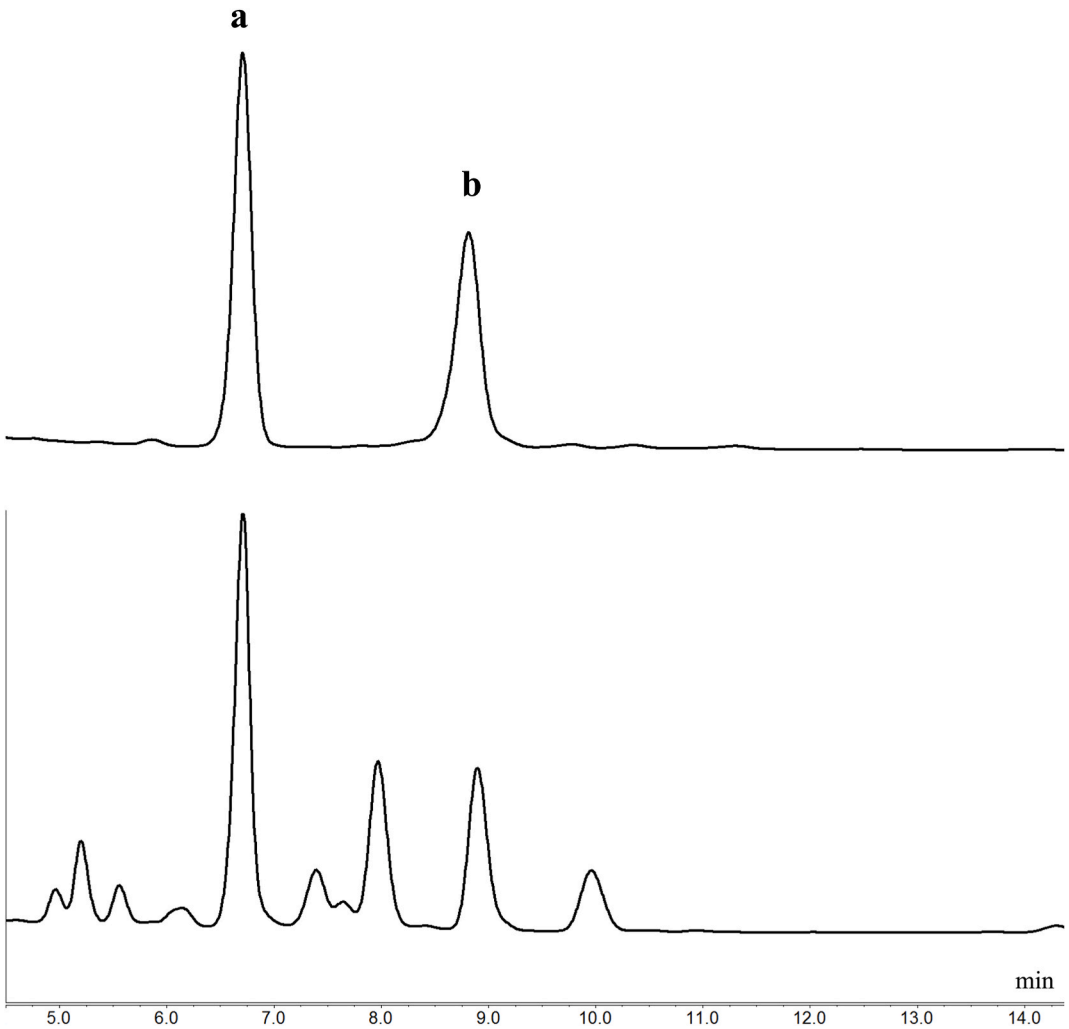

Fig. 2. HPLC chromatograms of standard compound (upper) and *B. prionitis* water extract (lower). (a) verbascoside and (b) isoverbascoside.

isoverbascoside, and total phenolic levels were assessed through Pearson correlation test. The graphs were created using Microsoft Excel 2019.

3. Results and discussion

3.1. Distribution of verbascoside and isoverbascoside in Thai Lamiales plants

The HPLC system for quantifying the amount of verbascoside and isoverbascoside in the plant samples was validated for its calibration curves, precision, and accuracy (Table 1). The calibration curves for verbascoside and isoverbascoside were linear and ranged from 1.9 to 250 μg/mL. The appropriate equations were then formulated, where x is the concentration of either verbascoside or isoverbascoside (μg/mL), and y is the peak area (mAu). The correlation coefficients of both calibration curves were higher than 0.999. The precision was validated at low, mid, and high verbascoside and isoverbascoside concentrations of 31.25, 62.5, and 250 μg/mL, respectively. The obtained RSD values were 0.42–4.86 % (n = 3) for the intra-day precision and 3.15–4.42 % (n = 3) for the inter-day precision. The known amounts of verbascoside and isoverbascoside were spiked to the samples to validate the HPLC system's accuracy. The obtained recovery values were 98.77 ± 0.96–112.34 ± 1.52 %.

We quantitatively analyzed the amounts of verbascoside and isoverbascoside in the ethanolic and water extracts of 20 Lamiales plant species through HPLC. Table 2 presents the results. HPLC chromatogram of both standards and water extract of *Baleria prionitis* are shown in Fig. 2. The verbascoside amount in the ethanolic extracted plant samples ranged from 0.73 ± 0.59 to 4580.89 ± 113.72 μg/g DW, while that in the water extracted samples was between 28.30 ± 32.94 and 13,273.36 ± 72.86 μg/g DW. The highest amounts of verbascoside were obtained from the water extracts of *Barleria prionitis* at 13273.36 ± 72.86 μg/g DW, followed by those of *B. lupulina* and *Rhinacanthus nasutus* at 5700.89 ± 66.96 and 3976.84 ± 639.66 μg/g DW, respectively. The amounts of isoverbascoside

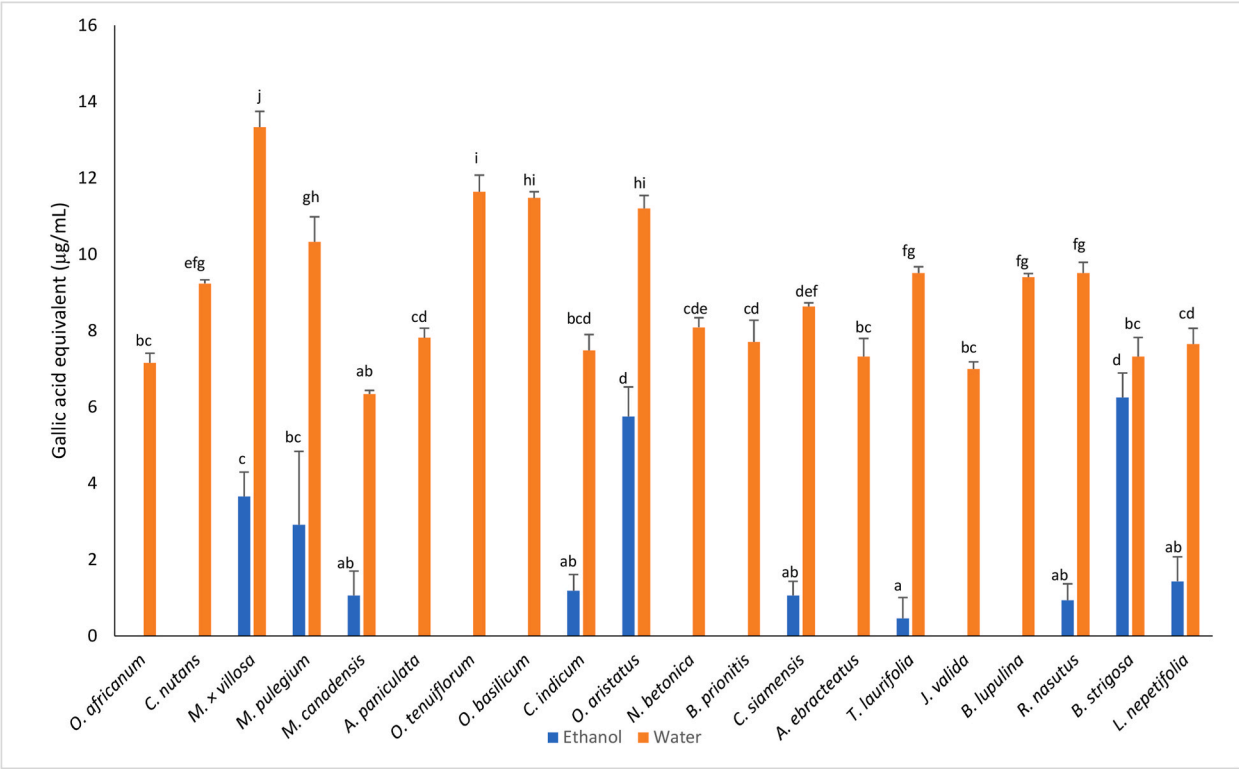

Fig. 3. Total phenolic content form ethanol and water extracts of 20 plant samples. The letters indicate a significant difference between plants within the same solvent group at $p < 0.01$.

in the ethanolic samples ranged from 11.09 ± 0.21 to 1183.74 ± 40.23 μg/g DW, while those in the water samples were between 0 and 9671.04 ± 23.08 μg/g DW. The highest amounts of verbascoside were found in the water extracts of *Orthosiphon aristatus* at 9671.04 ± 23.08 μg/g DW, followed by those of *B. prionitis* and *Nicoteba betonica* at 7922.90 ± 67.10 and 6148.42 ± 75.83 μg/g DW, respectively. Considering the solvents for extraction, the water extracts showed higher amounts of verbascoside and isoverbascoside compared to the ethanolic extracts in most samples. This observation is attributed to the water-soluble nature of phenylethanoid glycosides. However, note that ethanol was a more effective solvent for extracting verbascoside and isoverbascoside in certain samples, such as *Clerodendrum indicum*. A study on *Osmanthus fragrans* also reported that ethanol yields a higher amount of verbascoside when compared to water [17].

Verbascoside and isoverbascoside are mainly detected in the *Verbascum* genus [18] and found in many other species from 23 plant families at widely varying concentrations [3], with some plant species producing high verbascoside and isoverbascoside levels. For example, verbascoside was detected in *Verbascum thapsus* at 30 ± 4 mg/g DW, while its in vitro callus produced 26.27 ± 0.5 to 47.27 ± 0.3 mg/g DW of verbascoside and 29.02 ± 0.3 to 49.69 ± 0.5 mg/g DW of isoverbascoside [19]. The methanolic extracts of *Aloysia polystachya* leaves contained 3.60 ± 0.15 mg/g DW of verbascoside and 0.25 ± 0.01 mg/g DW of isoverbascoside [20]. By contrast, several other species produce much lower levels of these compounds. Interestingly, our study is the first to report on the high verbascoside and isoverbascoside accumulation in *B. prionitis.* Although verbascoside is extensively studied and found in more than 200 plant species, reports on the verbascoside distribution have mainly only tackled some major genera. Additionally, isoverbascoside is even less popular than verbascoside. Hence, the quantitative information on the verbascoside and isoverbascoside distributions of genus *Barleria* are very limited. A previous study on *B. prionitis* reported the isolation of only 0.009 % verbascoside (i.e., 1.0 mg of verbascoside from the methanolic soluble parts of 125 g of the whole plant extract) [21]. For other *Barleria* species, El-Halawany et al. found verbascoside and isoverbascoside as the main active constituents of *B. cristata*. However, the verbascoside and isoverbascoside yields recovered from the 70 % fractionated methanolic extracts of the aerial parts of *B. cristata* were only 16.67 and 6.67 μg/g DW, respectively [22]. Similarly, verbascoside and isoverbascoside were isolated from the methanolic extracts of *B. strigosa* by column chromatography, exhibiting yields of 44.73 and 24.74 μg/g DW, respectively [23]. Only one study performed an HPLC analysis of verbascoside in the methanolic extracts of *B. terminalis*, revealing that verbascoside can be found in the leaves and stems at 273.89 ± 0.6 and 4.13 ± 0.1 μg/g DW concentrations, respectively [24]. Our results suggest that *B. prionitis* and other Thai Lamiales plants could be alternative rich sources for producing verbascoside and isoverbascoside.

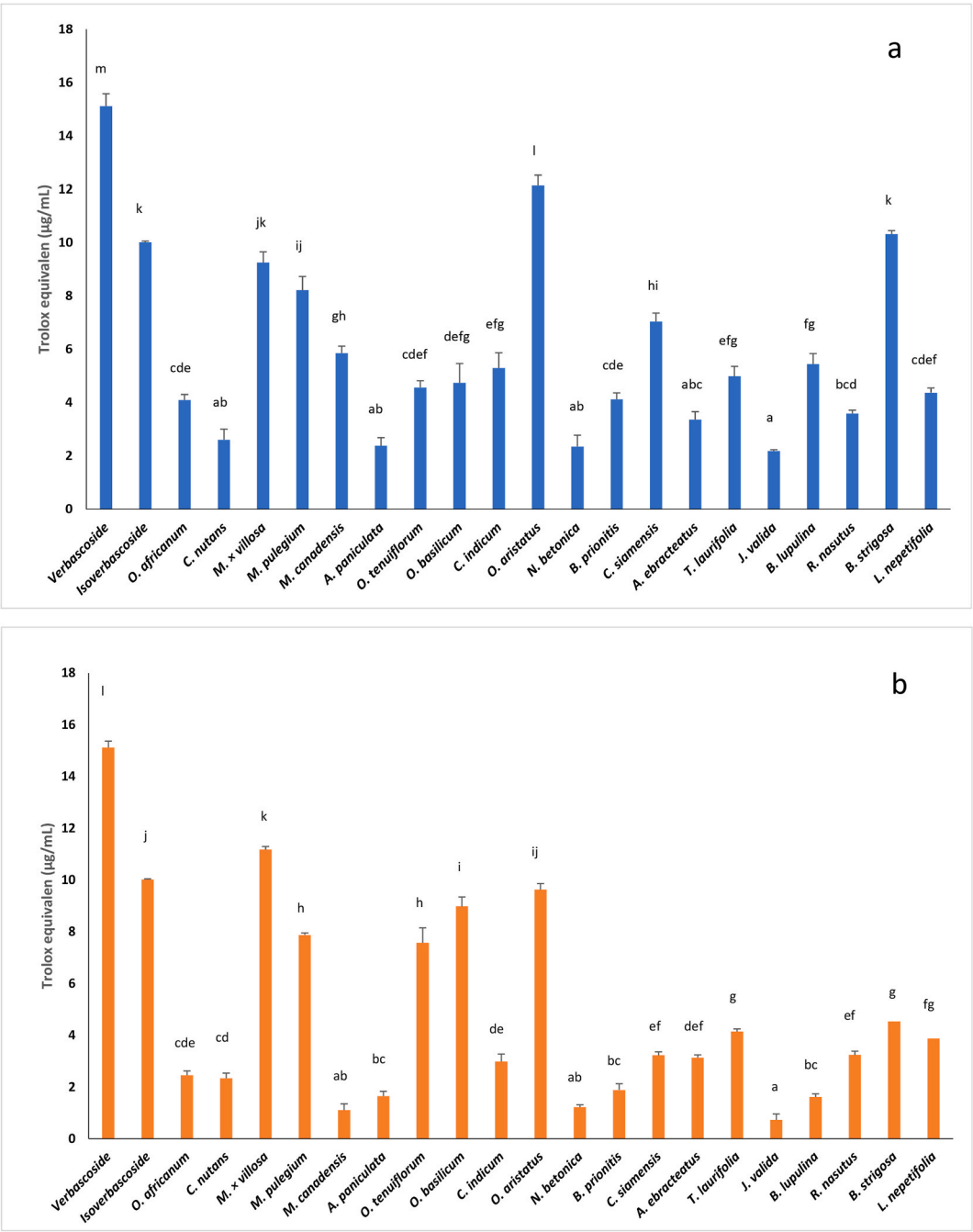

Fig. 4. Antioxidant capacity of 10 μg/mL verbascoside, 10 μg/mL isoverbascoside, and 20 plant samples, extracted with ethanol (a) and water (b), by DPPH assays. The letters indicate a significant difference between plants within the same solvent group at $p < 0.01$.

3.2. Total phenolic contents in the plant extracts

The total phenolic contents in the ethanolic and water extracts of the 20 Lamiales plant species investigated herein were analyzed through the Folin–Ciocalteu colorimetric method. We detected phenolic compounds in only 10 out of 21 samples in the ethanolic extracts, but detected them in every water extracted sample. The water extract of the same plant also showed a higher level of the total phenolic compound when compared to the ethanolic extract (Fig. 3). The total phenolic compounds in the ethanolic extracts ranged from 0 to 6.25 ± 0.64 μg/mL gallic acid equivalent, while those in the water extracts ranged from 5.63 ± 0.19 to 13.33 ± 0.41 μg/mL gallic acid equivalent. The highest amount of the total phenolic content was detected in the water extract of *Mentha* × *villosa* at 13.33

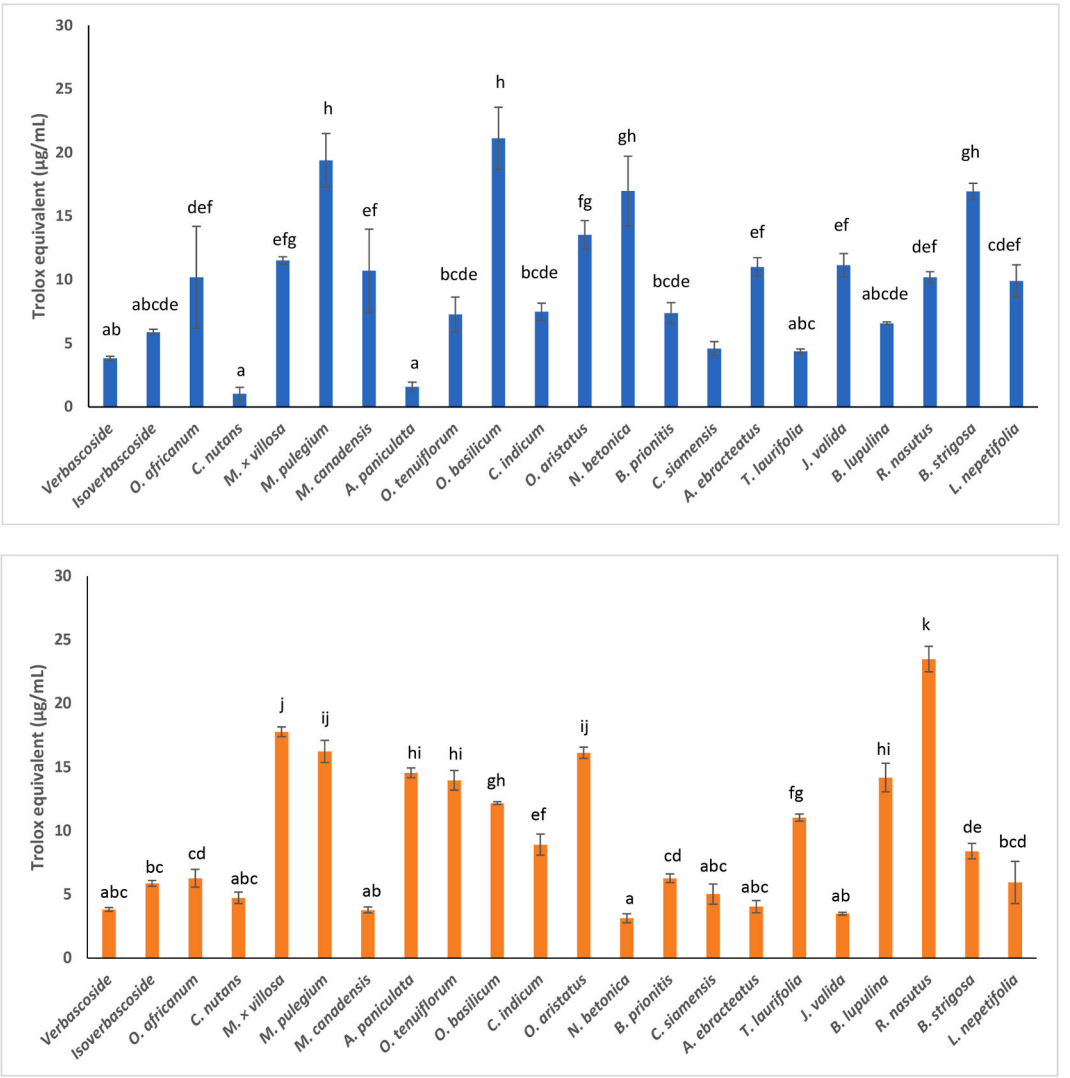

Fig. 5. Antioxidant capacity of 10 µg/mL verbascoside, 10 µg/mL isoverbascoside, and 20 plant samples, extracted with ethanol (a) and water (b) by FRAP assay. The letters indicate a significant difference between plants within the same solvent group at $p < 0.01$.

± 0.41 µg/mL gallic acid equivalent. Previous studies analyzed the total phenolic compounds using various extraction solvents. In contrast to our findings, Khound et al. reported that the phenolic content of *Clerodendrum glandulosum* was the highest when ethanol was used as the extraction solvent in ultrasound-assisted extraction [25]. Similarly, Kirkan reported that the extraction of *Stachys cretica* subsp. *Vacillans* with methanol resulted in higher total phenolic compound yields compared to water extraction [26]. These varying results could be attributed to the variations in the plant materials, extraction methods, and experimental conditions.

3.3. Antioxidant activity of the plant extracts

The antioxidant activity of the plant extracts was compared with that of the standard verbascoside and isoverbacoside by performing the radical-scavenging assay of DPPH and the FRAP assay.

The DPPH radical-scavenging activities of the ethanolic extracts ranged from 2.18 ± 0.05 to 12.14 ± 0.39 µg/mL trolox equivalent, while those of the water extracts ranged from 0.72 ± 0.10 to 11.17 ± 0.16 µg/mL trolox equivalent. Among the ethanolic extracts, the *Orthosiphon aristatus* ethanolic extract showed the highest antioxidant activity at 12.14 ± 0.39 µg/mL trolox equivalent (Fig. 4a). In the water extracted samples, *Mentha* × *villosa* exhibited the highest antioxidant activity at 11.17 ± 0.16 µg/mL trolox equivalent (Fig. 4b). Both samples showed comparable levels of antioxidant activities, which were higher than 10 µg/mL isoverbascoside (10.01 ± 0.05 µg/mL trolox equivalent), but lower than 10 µg/mL verbascoside (15.12 ± 0.46 µg/mL trolox equivalent, Fig. 4a and b). Despite their low or zero total phenolic contents, the ethanolic extracts still showed antioxidant activities, implying that their antioxidant properties in Lamiales plants might be attributed to the presence of verbascoside, isoverbascoside, or other compounds in them.

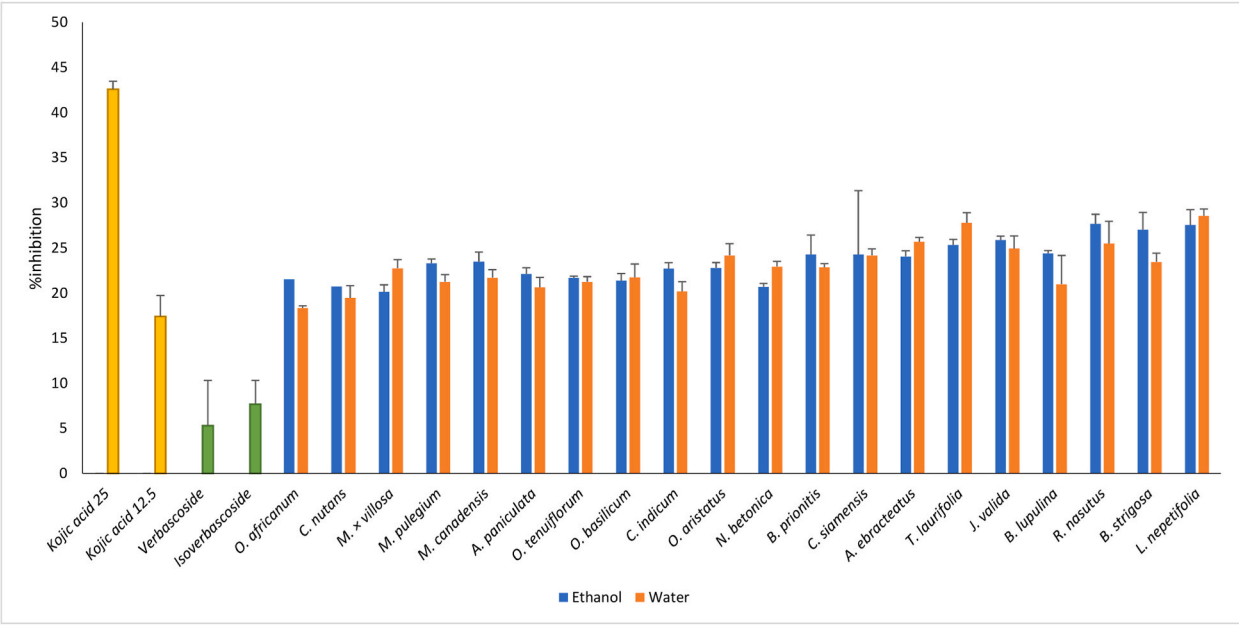

Fig. 6. Mushroom tyrosinase inhibition percentage of 10 μg/mL verbascoside, 10 μg/mL isoverbascoside, and 20 plant samples, extracted with ethanol and water. Kojic acid at 12.5 and 25.0 μg/mL were positive control.

The antioxidant capacities obtained from the FRAP assay of the ethanolic extracts ranged from 1.04 ± 0.51 to 21.11 ± 2.45 μg/mL trolox equivalent, while those of the water extracts ranged from 3.13 ± 0.35 to 23.49 ± 1.01 μg/mL trolox equivalent. *Ocimum basilicum* showed the highest FRAP antioxidant capacity among the ethanolic extracts at 21.11 ± 2.45 μg/mL trolox equivalent (Fig. 5a), while *Rhinacanthus nasutus* depicted the highest activity among the water extracts (23.49 ± 1.01 μg/mL trolox equivalent, Fig. 5b). However, unlike in the DPPH assay, both the verbascoside and isoverbascoside antioxidant capacities from the FRAP assay (3.82 ± 0.16 and 5.88 ± 0.23 μg/mL trolox equivalents, respectively), were lower than most of the extracts (Fig. 5).

In other words, the antioxidant capacities of verbascoside and isoverbascoside were higher in the DPPH assay than in the FRAP assay (Figs. 4 and 5). Each plant contained other antioxidants that contributed to their particularly high antioxidant capacities in both assays.

3.4. Mushroom tyrosinase inhibition assay

We used two concentrations of standard kojic acid (i.e., 12.5 and 25 μg/mL) as the positive controls. The activities of both ethanol and water extracts for each plant were at similar levels. The levels were not different from the inhibition percentage of 12.5 μg/mL kojic acid. Verbascoside and isoverbascoside showed low inhibition percentages at 5.29 ± 0.24 % and 7.66 ± 1.36 %, respectively (Fig. 6). The ethanol extracts demonstrated a tyrosinase inhibition percentage between 20.13 ± 1.03 and 27.65 ± 1.70 %, while the water extracts showed an inhibition percentage between 18.34 ± 0.97 and 28.54 ± 1.15 % (Fig. 6). Our results indicate that verbascoside, isoverbascoside, and the plant extracts tested in this study have no significant mushroom tyrosinase inhibition activity. This result coincided with that of Ref. [27]. The presented activity might be the result of other compounds because verbascoside and isoverbascoside showed lower activities compared to the plant extracts.

3.5. Cyclooxygenase inhibitory assay

The inhibitory activities of the extracts on COX-1 and COX-2 were analyzed through a colorimetric assay. Fig. 7 presents the results. The ethanolic extracts of *Ocimum tenuiflorum* (44.6 %) and *O. basilicum* (38.4 %) exhibited the highest inhibitions of the COX-1 and COX-2 activities, respectively. For the water extracted samples, the water extract of *Leonotis nepetifolia* demonstrated a 46.4 % inhibition of the COX-1 activity, while that of *Rhinacanthus nasutus* showed a 38.4 % inhibition of the COX-2 activity. All samples showed lower inhibitions compared to the positive control of 10 mM indomethacin. Verbascoside also yielded an inhibitory activity that was specifically against the COX-2 isoform, but not against COX-1 (Fig. 7a and b). These findings align with those of the previous reports indicating that verbascoside selectively prevents the COX-2 activation without simultaneously inhibiting the COX-1 enzyme in glioma cells [28]. Another study also reported that herb extracts containing standardized verbascoside do not significantly inhibit COX-1 while effectively suppressing the LPS-induced COX-2 hyperexpression at the mRNA level in human neutrophils [29].

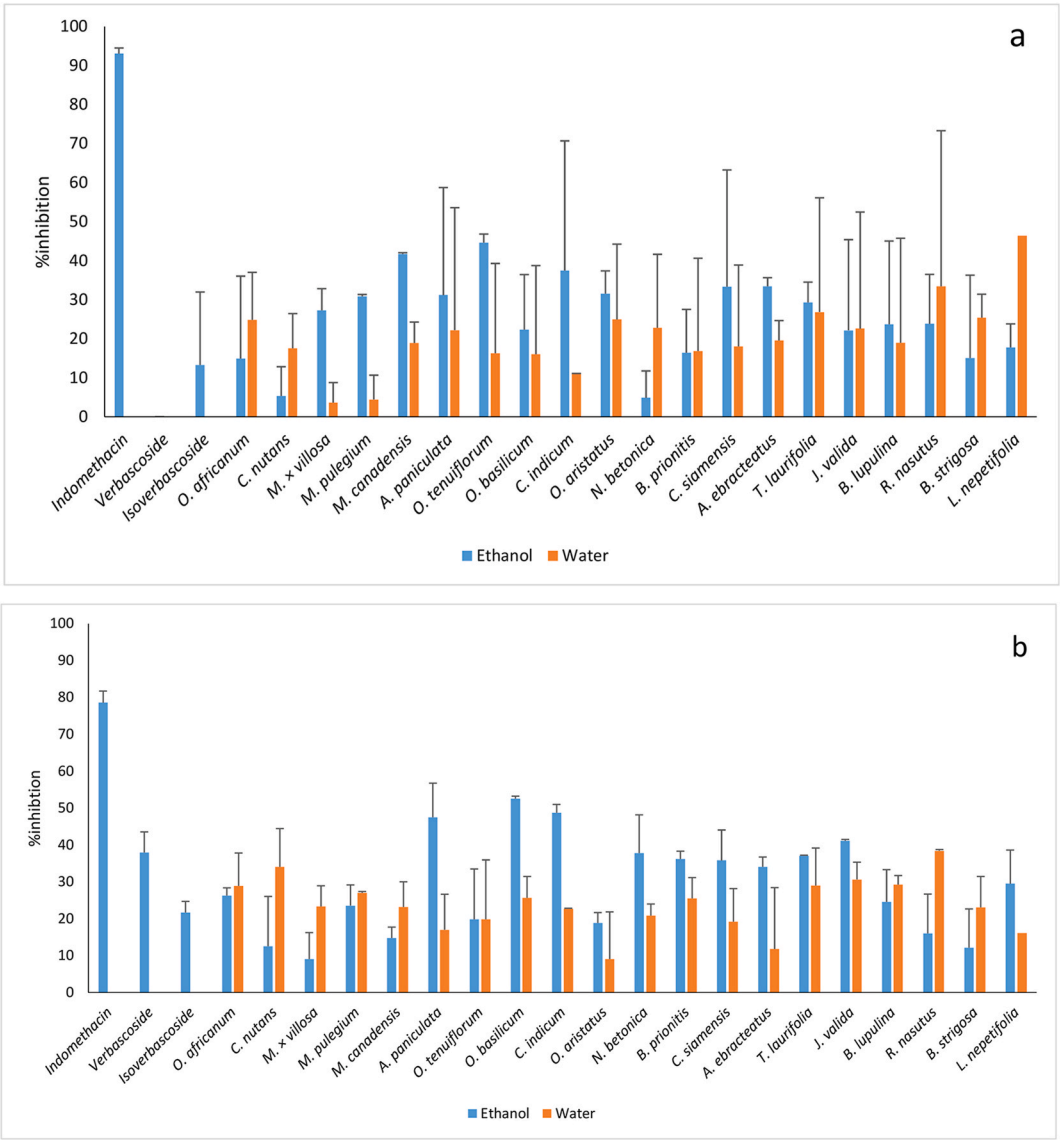

Fig. 7. In vitro inhibitory percentage of COX-1 (a) and COX-2 (b) by 10 μg/mL verbascoside, 10 μg/mL isoverbascoside, and 20 plant samples, extracted with ethanol and water. Indomethacin at 10.0 mM was positive control.

3.6. Anti-inflammatory assay using NO

We tested the anti-inflammatory activity of the plant extracts, verbascoside, and isoverbascoside by co-culturing extracts with LPS inflammation-induced RAW 264.7 cells. The nitric oxide levels were measured as an indicator of the anti-inflammatory activity. Among the tested extracts, the ethanolic extracts of *Mentha canadensis*, *Andrographis paniculata*, *Clerodendrum indicum*, *Rhinacanthus nasutus*, and *Barleria strigosa* significantly reduced the nitric oxide level in the cell line when compared with the LPS-induced group (Fig. 8a). The lowest nitric oxide level was observed in the ethanolic extract of *A. paniculata*, but this extract was toxic to the cells, hence resulting in 0 % cell viability. In contrast, the ethanolic extract of *R. nasutus* showed a significantly low nitric oxide level (1.45 ± 0.20 μM) without toxicity. The water extracts of our samples showed significant anti-inflammatory activities in more samples compared to the ethanolic extracts (Fig. 8b). We observed significantly lower nitric oxide levels in the water extracts of *A. paniculata*, *C. indicum*, *Orthosiphon aristatus*, *Nicoteba betonica*, *B. prionitis*, *Acanthus ebracteatus*, *Thunbergia laurifolia*, *Justicia valida*, *B. lupulina*, *R. nasutus*, *B. strigosa*, and *Leonotis nepetifolia*. Notably, the water extract of *A. paniculata* exhibited the most significantly reduced nitric oxide levels, reaching the lowest level (0.54 ± 0.07 μM) among all the samples while maintaining 84 % cell viability.

The nitric oxide level in the water extracts corresponded to the higher verbascoside concentration in the water extracts compared to the ethanol extracts (Table 2). Both verbascoside and isoverbascoside demonstrated notably low nitric oxide levels of 1.14 ± 0.13 and 1.23 ± 0.07 μM, respectively, which were comparable to that of indomethacin (1.27 ± 0.23 μM). Similarly, Marzocco et al. reported

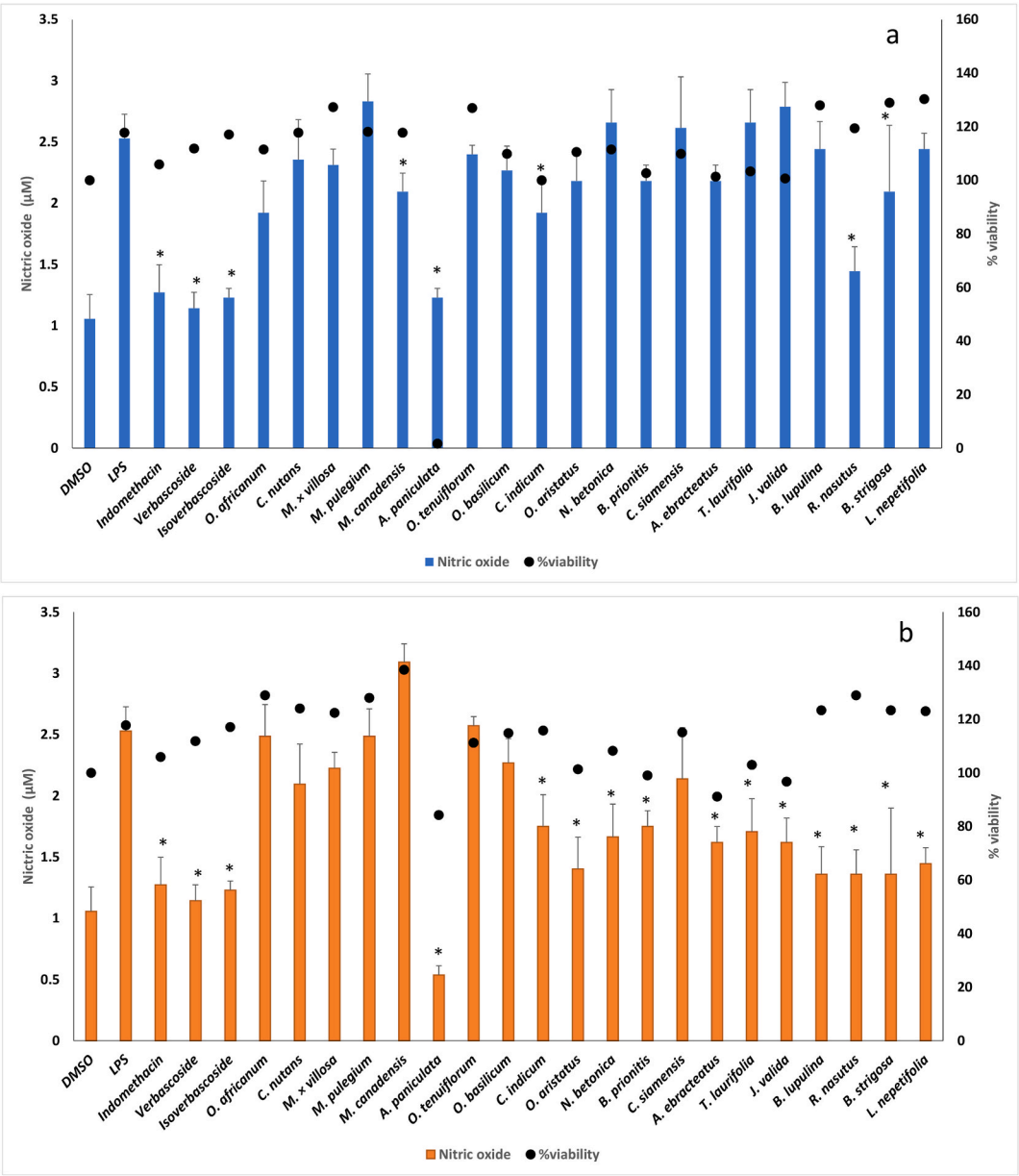

Fig. 8. Nitric oxide level and viability of LPS-induced RAW 264.7 cells after treated with 10 μg/mL verbascoside, 10 μg/mL isoverbascoside, and 20 plant samples, extracted with ethanol (a) and water (b). Indomethacin 0.3 mM was positive control. *Significant difference compared with the untreated group (DMSO) at $p < 0.01$.

Table 3
Pearson's correlation of antioxidant capacities (DPPH), mushroom tyrosinase inhibition, COX-1 inhibition, COX-2 inhibition, and nitric oxide level with and verbascoside, isoverbascoside and total phenolic level in ethanolic extracts.

	DPPH	FRAP	Tyrosinase inhibition	COX-1 inhibition	COX2 inhibition	Nitric oxide
Verbascoside	−.058	−.048	.112	−.342*	−.144	.204
Isoverbascoside	.168	.045	.182	−.058	−.082	−.043
Total phenolic	.874**	.424**	.095	.005	−.446**	.005

**. Correlation is significant at the 0.01 level.
*. Correlation is significant at the 0.05 level.

Table 4
Pearson's correlation of antioxidant capacities (DPPH), mushroom tyrosinase inhibition, COX-1 inhibition, COX-2 inhibition, and nitric oxide level with and verbascoside, isoverbascoside and total phenolic level in water extracts.

	DPPH	FRAP	Tyrosinase inhibition	COX-1 inhibition	COX2 inhibition	Nitric oxide
Verbascoside	−.315**	−.065	−.017	.210	.225	−.213*
Isoverbascoside	.198	−.049	−.206	.115	−.120	−.057
Total phenolic	.849**	.683**	−.051	.045	.156	.147

**. Correlation is significant at the 0.01 level.
*. Correlation is significant at the 0.05 level.

the in vitro and in vivo inhibitions of inducible nitric oxide synthase by the *Wendita calysina* extracts that have verbascoside and isoverbascoside as their active components [30]. Verbascoside became the subject of several early studies that investigated its anti-inflammatory activities, with some papers focusing on the elucidation of its underlying mechanisms [3]. A previous study on the U937 cell line reported that verbascoside enhanced the SHP-1 phosphorylation by attenuating the activation of the TAK-1/JNK/AP-1 signaling, consequently resulting in a decrease in the expression and activity of both cyclooxygenase and nitric oxide synthase [31].

3.7. Correlation between phytochemical constituents and biological activities

The analysis results of the relationships between the activities and the verbascoside, isoverbascoside, and total phenolic contents were assessed through Pearson correlation test. Tables 3 and 4 present the results. The correlation analysis showed that the verbascoside level in the ethanol extracts was significantly correlated with COX-1 at $p < 0.05$, while the total phenolic level was significantly correlated with the DPPH activity at $p < 0.01$. The verbascoside level in the water extract was significantly correlated with DPPH ($p < 0.01$) and the nitric oxide level ($p < 0.05$), while the total phenolic level was significantly correlated with both DPPH and FRAP ($p < 0.01$), implying the role of phenolic compounds in antioxidant activities.

4. Conclusion

We presented herein the first comprehensive analysis of the distributions of verbascoside and isoverbascoside, which are two active phenylethanoid glycosides found in 20 species of Thai medicinal plants belonging to the Lamiales order. Our findings revealed that several species possess high verbascoside and isoverbascoside levels, suggesting their potential as alternative sources for bioactive compound production. We conducted analyses of the total phenolic contents in these plant extracts and evaluated their associated bioactive activities, specifically their antioxidant, anti-tyrosinase, and anti-inflammatory activities.

The correlation analysis between the bioactive activities and the active compounds demonstrated a significant association between the verbascoside level in the water extracts and both the DPPH antioxidant activity and the nitric oxide level in the anti-inflammatory assays. The results indicate that Thai medicinal plants belonging to the Lamiales order (e.g., *B. prionitis* and *B. lupulina*) are rich sources of the phenylethanoid glycosides, verbascoside and isoverbascoside, which exhibit potent anti-inflammatory activities. Further investigations should be performed to explore the potential of scaling up the production of these active compounds from local plant sources. More in-depth studies are also needed to elucidate the precise mechanisms underlying their anti-inflammatory activities.

Data availability statement

The data that has been use is confidential.

CRediT authorship contribution statement

Benyakan Pongkitwitoon: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Waraporn Putalun:** Resources, Conceptualization. **Kanokporn Triwitayakorn:** Conceptualization. **Tharita Kitisripanya:** Resources. **Tripetch Kanchanapoom:** Resources. **Panitch Boonsnongcheep:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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