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Nguyen Thuy Duong¹, Pham Duc Vinh¹, Phuong Thien Thuong², Nguyen Thi Hoai³, Le Nguyen Thanh⁴, Tran The Bach⁵, Nguyen Hai Nam⁶, Nguyen Hoang Anh¹*

¹Department of Pharmacology, Hanoi University of Pharmacy, 13 Le Thanh Tong, Hoan Kiem, Hanoi, Viet Nam

²Department of Pharmaceutical Analysis and Standardization, National Institute of Medicinal Materials, 3B Quang Trung, Hoan Kiem, Hanoi, Viet Nam

³Department of Pharmacognosy, Hue University of Medicine and Pharmacy, 6 Ngo Quyen, Vinh Ninh, Hue, Viet Nam

⁴Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam

⁵Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam

⁶Department of Medicinal Chemistry, Hanoi University of Pharmacy, 13 Le Thanh Tong, Hoan Kiem, Hanoi, Viet Nam

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ABSTRACT

Objective: To screen Vietnamese medicinal plants for xanthine oxidase (XO) inhibitory activity and to isolate XO inhibitor(s) from the most active plant.

Methods: The plants materials were extracted by methanol. The active plant materials were fractionated using different organic solvents, including *n*-hexane, ethyl acetate, and *n*-butanol. Bioassay-guided fractionation and column chromatography were used to isolate compounds. The compounds structures were elucidated by analysis of spectroscopic data, including IR, MS, and NMR.

Results: Three hundreds and eleven methanol extracts (CME) belonging to 301 Vietnamese herbs were screened for XO inhibitory activity. Among these plants, 57 extracts displayed XO inhibitory activity at 100 µg/mL with inhibition rates of over 50%. The extracts of *Archidendron clypearia* (*A. clypearia*), *Smilax poilanei*, *Linociera ramiflora* and *Passiflora foetida* exhibited the greatest potency with IC₅₀ values below 30 µg/mL. Chemical study performed on the extract of *A. clypearia* resulted in the isolation of six compounds, including 1-octacosanol, docosenoic acid, daucosterol, methyl gallate, quercitrin and (–)-7-O-galloyltricetiflavan. The compound (–)-7-O-galloyltricetiflavan showed the most potent XO inhibitory activity with an IC₅₀ value of 25.5 µmol/L.

Conclusions: From this investigation, four Vietnamese medicinal plants were identified to have XO inhibitory effects with IC₅₀ values of the methanol extracts below 30 µg/mL. Compound (–)-7-O-galloyltricetiflavan was identified as an XO inhibitor from *A. clypearia* with IC₅₀ value of 25.5 µmol/L.

1. Introduction

Xanthine oxidase (XO) is an enzyme catalyzing the formation of uric acid from xanthine and hypoxanthine which in turn is produced from purine catabolism [1]. Therefore, any defect in the purine degradation pathway may result in an increase in the uric acid level recognized as the single most important risk factor for the development of gout [2]. Moreover, reactive oxygen species (ROS) generated from XO-catalyzed reactions may also contribute to a range of pathological conditions including inflammation, metabolic disorders, cellular aging, and carcinogenesis [3]. As a result, inhibition of XO activity is suggested to be one of the therapeutic strategies for not only

First author: Nguyen Thuy Duong, Department of Pharmacology, Hanoi University of Pharmacy, 13 Le Thanh Tong, Hoan Kiem, Hanoi, Viet Nam.

E-mail: duongnt@hup.edu.vn

*Corresponding authors: Nguyen Hai Nam, Department of Medicinal Chemistry, Hanoi University of Pharmacy, 13 Le Thanh Tong, Hoan Kiem, Hanoi, Viet Nam

Nguyen Hoang Anh, Department of Pharmacology, Hanoi University of Pharmacy, 13 Le Thanh Tong, Hoan Kiem, Hanoi, Viet Nam.

E-mails: namnh@hup.edu.vn, anh90tk@yahoo.com

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hyperuricemia but also various other diseases. Although XO inhibitors are currently considered the first-line therapy for long-term management of gout and related disorders, treatment choices are limited to allopurinol and febuxostat [4]. Allopurinol is associated with severe adverse events (e.g., hypersensitivity, hepatitis, interstitial nephritis, and eosinophilia) which reduce the tolerance and long-term adherence with the treatment. Febuxostat is better tolerated, however it also possesses the risk of undesirable effects and access to the drug may be restricted in developing countries due to its cost [5]. These unmet medical needs require more efforts in finding novel XO inhibitors with greater effectiveness and a better safety profile.

In the past two decades, a number of research groups have carried out screening for XO inhibitors from native medicinal plants and this approach was shown to be effective [6–10]. In 2004, Nguyen *et al.* found some plant extracts which exhibited potential XO inhibition through a screening of 96 Vietnamese herbs collected in a mountainous area in the south central provinces of Vietnam [6]. Since Vietnam is a country possessing abundant biological resources and rich knowledge of folk medicines, it is likely that many other indigenous medicinal plants remain potential as a source of potent bioactive compounds, including XO inhibitors. Therefore, focus on the research for novel XO inhibitors from natural products, we have screened 301 plant species used in Vietnamese traditional medicines for treatment of arthropathies, inflammatory disorders, and pain but almost uninvestigated in the earlier study. This paper describes the results obtained from this screening and chemical investigation carried out on *Achidendron clypearia* (Jack) I.C. Neilsen (syn. *Pithecellobium clypearia* Benth), the most active plant in inhibiting XO activity found from the samples screened.

2. Materials and methods

2.1. Chemicals and reagents

XO from bovine milk (X1875-25UN, 7.2 IU/mL), xanthine powder, potassium oxonate and allopurinol biological grade were purchased from Sigma–Aldrich Co (St Louis, MO, USA). Solvents for extraction and fractionation and other chemicals used for preparing buffers were purchased from Merck (Darmstadt, Germany).

2.2. Plant materials

All plant samples used in this study were collected from different provinces in Vietnam and botanically identified by Dr. Nguyen The Cuong (Institute of Ecology and Biological Resources VAST). Voucher specimens were deposited at three herbaria located respectively at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology; the Department of Pharmacognosy, Hue University of Medicine and Pharmacy; and at the Vietnam National Institute of Medicinal Materials.

2.3. Preparation of the methanol extracts

Each dried plant sample (100 g) was ground coarsely and extracted twice with 200 mL of methanol under reflux (2 h each time). The extracts were filtered and combined. The solvent was

evaporated completely under reduced pressure to give a crude methanol extract.

2.4. Extraction and isolation of the active compounds from *A. clypearia*

The dried, powdered plant materials of *A. clypearia* (5 kg) was extracted three times with MeOH by maceration at room temperature (each time 15 L for 1 week). The extracts were combined and solvent was evaporated under reduced pressure to give a MeOH residue (750 g), which was suspended in water and then successively partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Evaporation of solvents under reduced pressure gave *n*-hexane (H, 100 g), chloroform (C, 145 g), ethyl acetate (E, 250 g), *n*-butanol (B 105 g) and water (W, 145 g) residues, respectively. The E extract, which showed the most potent XO inhibition (IC₅₀ value of 4.4 µg/mL) was selected for further chemical study.

The E extract (240 g) was chromatographed on a silica gel column and eluted with a gradient solvent system of *n*-hexane–acetone (100:1–0:1) to afford eight fractions E1–E8, respectively. The fraction E3 was further chromatographed on a silica gel column eluting with chloroform/MeOH (5:1, v/v) to give five fractions E3A–E3E. Fraction E3D was separated by Sephadex LH-20 column chromatography eluted with MeOH/water (4:1, v/v) to give smaller fractions E3D1–E3D4. The fraction E3D2 was subjected to chromatography on a reverse phase silica gel (C-18) column, and eluted with acetone/water/formic acid (6:15:0.5) to afford sub-fractions E3D2A–E3D2E. The fraction E3D2A was purified by Sephadex LH-20 column chromatography, eluted with MeOH followed by column chromatography on silica gel eluting with chloroform/MeOH/water (3:1:0.1, v/v) to give compound 1 (15 mg). The fraction E3D2D was separated with Sephadex LH-20 column chromatography and eluted with MeOH to yield compound 2 (20 mg).

The fraction E4 was separated by column chromatography on silica gel eluting with a chloroform/acetone (10:1) to give five fractions E4A–E4E. Fraction E4B was chromatographed on a reverse phase silica gel column and eluted with acetone/water (3:2) to afford sub-fractions E4B1–E4B4. Repeated column chromatography of fraction E4B3 with a gradient solvent system of chloroform/ethyl acetate/methanol (15/1/0.1, v/v) followed by Sephadex LH-20 column chromatography using MeOH as an eluant yielded compound 3 (25 mg). The fraction E4D was purified by Sephadex LH-20 column chromatography and eluted with MeOH to give four smaller fractions E4D1–E4D4. The fraction E4D2 was chromatographed on reverse phase silica gel column eluting with MeOH/water (8:1) to afford sub-fractions E4D2A–E4D2D. Fraction E4D2B was separated by silica gel preparative TLC eluting with chloroform/MeOH/formic acid (5:1:0.1) to give compound 4 (12 mg).

The fraction E6 was chromatographed on silica gel column eluting with chloroform–acetone (1:1, v/v) to give five fractions E6A–E6E. The fraction E6B was subjected to a reverse phase silica gel column chromatography and eluted with MeOH–water (2:1) to afford sub-fractions E6B1–E6B4. The fraction E6B2 was separated by Sephadex LH-20 column chromatography and eluted with MeOH to give sub-fractions E6B2A–E6B2E. The fraction E6B2A was purified by silica gel column chromatography eluting with chloroform/MeOH/formic acid (6:1:0.1) followed by reverse phase silica gel column chromatography eluting with MeOH/water/formic acid (1:1:0.05) to yield

compound 5 (105 mg). The fraction E6B2C was chromatographed on silica gel column eluting with chloroform/MeOH (3:2, v/v) to give three fractions E6B2C1–E6B2C3. Compound 6 (20 mg) was obtained from fraction E6B2C2 by recrystallization in acetone.

2.5. *In vitro* XO assay

The XO inhibitory activity was assayed spectrophotometrically in 96-well plates, based on a modified protocol described previously by Noro *et al.* with minor modifications [11]. In brief, reagents were 70 nmol/L phosphate buffer pH 7.5, 150 nmol/L xanthine solution dissolved in the phosphate buffer (pH 7.5) and 0.01 IU/mL XO solution (diluted from a stock enzyme solution into the buffer solution). The methanol extracts were prepared initially in dimethyl sulfoxide (DMSO) followed by dilution with the buffer. A mixture consisting of 35 μ L of the phosphate buffer, 50 μ L test solution and 30 μ L of XO solution was pre-incubated for 15 min at 25 °C. Subsequently, the reaction was initiated by the automatic addition of 60 μ L of xanthine solution into the above mixture. The final reaction mixture was incubated for 30 min at 25 °C. The reaction was stopped by adding 25 μ L of HCl 1 mol/L solution prior to measuring the absorbance at 290 nm on a Bio Tek Epoch microplate spectrophotometer. A blank was prepared in the same way, however the order of adding the substrate and HCl solution was reversed.

XO inhibitory activity was expressed as the inhibitory percentage (I) calculated as using the formula:

$$\text{Inhibitory percentage (I)} = (\Delta OD_{\text{control}} - \Delta OD_{\text{sample}}) / \Delta OD_{\text{control}} \times 100.$$

where, $\Delta OD_{\text{control}}$ and $\Delta OD_{\text{sample}}$ are blank-corrected optical density values of control wells (in absence of test materials) and test wells (in presence of test samples). Based on the inhibitory percentage values at various concentrations, IC₅₀ values of potential plant samples were determined by Graphpad Prism version 5.0 Software with the sigmoidal dose–response model.

All experiments were performed in triplicate. Allopurinol, a known XO inhibitory agent, served as positive control. DMSO presented in the final reaction mixture with concentration of below 0.25% at which this solvent was demonstrated to have negligible effect on XO activity.

2.6. Lineweaver–Burk plots

Enzyme kinetic was determined in the absence and presence of compounds isolated from *A. clypearia*, using the XO assay methodology as described above with a range of substrate concentrations between 5 and 25 μ mol/L.

2.7. Evaluation of *in vivo* anti-hyperuricemic effects

2.7.1. Mice

The adult male Swiss mice (8 weeks old, weighing 28–30 g) were obtained from Centre of Experimental Animals, National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). Animals were used and processed according to the suggested ethical guidelines for the care of laboratory animals, and the experimental protocols in this study were approved by the Scientific and Ethical Committee of Hanoi University of Pharmacy (701/QD-QLKH).

The mice were acclimatized one week to adapt to their environment before any experimental manipulation. They were housed in 612 mm \times 345 mm \times 216 mm cages (Tecniplast 2000P) in an animal room (Department of Pharmacology, Hanoi University of Pharmacy) with a temperature of (25 \pm 1) °C, humidity of 55%–60%, regular 12/12 h light/dark cycle, and access to standard laboratory diet and tap water freely until used. All the samples from animals subjected to the treatments were included in the data analysis.

2.7.2. Drug and sample dosage and administration route

The crude methanol extract of *A. clypearia* (CME) was suspended in water. This preparation was given to mice with oral doses of 1 120, 3 730 and 11 180 mg/kg body weight ($n = 9$). Treatment was applied once daily at 8:00 am for 5 consecutive days. In the same period, mice in a negative control group ($n = 10$) were administered with 0.5% sodium carboxymethylcellulose (CMC-Na), while those in a positive control group were administered allopurinol at a dose of 10 mg/kg ($n = 9$).

2.7.3. Protocols

Hyperuricemia in mice was induced by an uricase inhibitor, potassium oxonate, as described by Tung *et al.* with minor modifications [12]. Briefly, a suspension of potassium oxonate prepared in 0.5% CMC-Na was intraperitoneally injected to mice with dose of 500 mg/kg at 1 h before administration of test samples. Whole blood samples were collected from the tail vein of the mice 1 h after the final administration. The blood was allowed to clot for approximately 1 h at room temperature and then centrifuged at 3000 r/min for 10 min and the serum was separated. The serum was stored at –20 C until used. Serum uric acid levels were measured by the phosphotungstic acid method, using standard diagnostic kits purchased from Biosystems S.A (Barcelona, Spain).

2.8. Statistical analysis

Data was expressed as mean \pm standard error (SE). An ANOVA analysis followed by *LSD* or Dunnett's T3 post-hoc test was performed to determine the significance of differences among groups. The values of $P < 0.05$ were considered to be statistically significant. All computations were made with the statistical software SPSS 16.0.

3. Results

3.1. Screening Vietnamese medicinal plants for XO inhibitory activity

Three hundreds and eleven (311) extracts from 301 Vietnamese herbs were assayed for XO inhibition. Among these, 234 extracts showed XO inhibitory activity at 100 μ g/mL (Table 1). At 50 μ g/mL, 123 extracts possessed the activity while 61 were found to be active at 10 μ g/mL (data not shown). Fifty seven extracts which displayed the activity at 100 μ g/mL with inhibition rates of 50% or higher were subsequently evaluated to determine the IC₅₀ values and the results are shown in Table 1. The crude extracts possessing XO inhibitory activity with IC₅₀ values of less than 30 μ g/mL included *Passiflora foetida* (*P. foetida*) (IC₅₀ 25.5 μ g/mL), *Linociera ramiflora* (*L. ramiflora*)

Table 1

The xanthine oxidase (XO) inhibition of the potential herbal extracts.

Scientific name	Local name	Family	Part(s) used	XO inhibition ($\mu\text{g/mL}$)	
				IC ₅₀	95% CI
<i>Archidendron clypearia</i> (Jack) I.C. Nielsen	Man dia	Mimosaceae	Leaf, twig	15.6	14.4–17.4
<i>Smilax poilanei</i> Gagnep.	Kim cang poilane	Smilacaceae	Rhizome	20.0	11.7–28.1
<i>Linociera ramiflora</i> (Roxb.) Wall.	Ho bi	Oleaceae	Leaf, twig	25.4	17.3–33.9
<i>Passiflora foetida</i> L.	Lac tien	Passifloraceae	Aerial part	25.5	17.2–34.3
<i>Clinacanthus nutans</i> (Burm.f.) Lindau	Manh cong	Acanthaceae	Aerial part	30.4	21.7–36.6
<i>Equisetum diffusum</i> D.Don	Co thap but	Equisetaceae	Aerial part	30.8	24.9–37.4
<i>Hibiscus sagittifolius</i> (Kurz) Merr.	Bao sam	Malvaceae	Rhizome	31.1	23.2–40.0
<i>Bridelia retusa</i> (L.) A.Juss.	Dom lom	Euphorbiaceae	Leaf, twig	32.5	23.8–42.8
<i>Dillenia indica</i> L.	So ba	Dilleniaceae	Leaf, twig	33.9	26.0–43.2
<i>Fallopia multiflora</i> (Thunb.) Haraldson	Ha thu o do	Polygonaceae	Rhizome	34.3	21.3–51.1
<i>Anisoptera costata</i> Korth.	Ven ven	Dipterocarpaceae	Leaf, twig	36.4	28.8–45.4
<i>Sabia parviflora</i> Wall.	Hoa khe cay nho goc	Sabiaceae	Root	40.0	30.5–42.8
<i>Leucaena leucocephala</i> (Lam.) De Wit	Keo dau	Mimosaceae	Seed	43.7	32.8–59.7
<i>Buchanania arborescens</i> (Blume) Blume	Chay lon	Anacardiaceae	Leaf, twig	43.8	32.1–56.8
<i>Wikstroemia indica</i> (L.) C.A Mey	Niet do	Thymelaeaceae	Root	44.6	36.3–55.6
<i>Uncaria acida</i> (Hunter) Roxb.	Cau dang	Rubiaceae	Twig	46.3	36.8–59.5
<i>Fernandoa brilletii</i> (Dop) Steenis.	Dinh thoi	Bignoniaceae	Leaf, twig	46.3	29.2–44.7
<i>Elaeocarpus grandiflorus</i> Sm.	Com hoa lon	Elaeocarpaceae	Leaf, twig	48.1	36.2–61.8
<i>Pandanus tectorius</i> Parkinson ex Du Roi	Dua dai	Pandaceae	Stem	49.3	41.0–60.3
<i>Smilax perfoliata</i> Lour.	Chong chong	Similacaceae	Rhizome	49.3	42.1–57.0
<i>Dracaena gracilis</i> Salisb.	Phat du manh	Asparagaceae	Root	50.2	41.0–62.9
<i>Leea aquata</i> L.	Goi hac bang	Leeaceae	Root	50.7	41.4–63.6
<i>Mussaenda cambodiana</i> Pierre ex Pit.	Buom bac Campuchia	Rubiaceae	Twig, leaf, flower	51.6	41.4–63.7
<i>Verbena officinalis</i> L.	Co roi ngua	Verbenaceae	Whole plant	52.4	25.9–114.4
<i>Rauwolfia verticillata</i> (Lour.) Baill.	Ba gac	Apocynaceae	Root	53.1	43.1–67.6
<i>Bauhinia lakhonensis</i> Gagnep.	Mong bo long xam	Caesalpiniaceae	Leaf, stem	53.3	32.6–80.2
<i>Homalomena occulta</i> (Lour.) Schott	Thiên niên kiện	Araceae	Rhizome	58.1	49.8–67.5
<i>Eurya ciliata</i> Merr.	Sum long	Theaceae	Twig, leaf	61.9	53.0–73.1
<i>Alangium chinense</i> (Lour.) Harms	Quang	Alangiaceae	Whole plant	65.0	50.8–90.1
<i>Baeckea frutescens</i> L.	Choi xue	Myrtaceae	Aerial part	66.2	33.1–88.9
<i>Macrosolen cochinchinensis</i> (Lour.) Tiegh.	Tam gui	Loranthaceae	Leaf, stem	68.9	54.3–202.7
<i>Anoetochilus roxburghii</i> (Wall.) Lindl.	Kim tuyen Sapa	Orchidaceae	Whole plant	69.1	57.4–85.2
<i>Allamanda cathartica</i> L.	Day huynh	Apocynaceae	Leaf, stem	71.2	59.6–85.6
<i>Osmanthus matsumuranus</i> Hayata	Vo san la dai	Oleaceae	Twig, leaf	73.6	63.1–87.4
<i>Lagerstroemia loudonii</i> Teysm. & Binn.	N/A	Lythraceae	Leaf, twig	74.2	62.5–88.7
<i>Selaginella uncinata</i> (Desv. ex Poir.) Spring	Quyen ba	Selaginellaceae	Whole plant	75.4	47.1–147.7
<i>Diospyros crumenata</i> Thwaites	Thi den	Ebenaceae	Leaf, twig	77.2	63.6–95.3
<i>Solanum torvum</i> Sw.	Ca dai hoa trang	Solanaceae	Stem, leaf	77.9	46.8–179.9
<i>Callicarpa arborea</i> Roxb.	Tu hu go	Verbenaceae	Leaf, twig	78.3	61.8–102.8
<i>Flueggea spirei</i> Beille	N/A	Euphorbiaceae	Leaf, twig	80.0	37.4–219.8
<i>Cissus triloba</i> (Lour.) Merr.	Chia voi	Vitaceae	Whole plant	80.9	54.6–144.9
<i>Saraca dives</i> Pierre	Vang anh	Fabaceae	Leaf, twig	83.4	47.1–200.0
<i>Elsholtzia penduliflora</i> W.W. Sm.	Kinh gioi ru	Lamiaceae	Aerial part	83.5	64.8–115.9
<i>Persicaria lanigera</i> (R.Br.) Soják	Nghe trang	Polygonaceae	Leaf	84.8	74.3–98.4
<i>Piper thomsonii</i> (C. DC.) Hook. f.	Tieu ba vi	Piperaceae	Aerial part	85.7	69.4–111.4
<i>Elaeocarpus stipularis</i> Blume	Com la kem	Elaeocarpaceae	Leaf, twig	85.9	44.3–269.5
<i>Eriobotrya bengalensis</i> (Roxb.) Hook.f.	Ty Ba Nam A	Rosaceae	Leaf, twig	91.3	73.2–119.7
<i>Triumfetta bartramia</i> L.	N/A	Tiliaceae	Whole plant	91.5	74.2–118.0
<i>Isodon lophanthoides</i> (Buch.-Ham. ex D.Don) H.Hara	Mat gau	Lamiaceae	Whole plant	94.5	69.4–148.2
<i>Helicteres hirsuta</i> Lour.	An dien long	Rubiaceae	Whole plant	94.5	78.9–118.1
<i>Crotalaria assamica</i> Benth.	Luc lac la oi	Fabaceae	Twig, leaf	94.9	64.0–183.3
<i>Homalium dasyanthum</i> Warb.	Cha ran hoa nham	Flacourtiaceae	Leaf, twig	97.3	76.4–132.8
<i>Ardisia gracillima</i> K. Larsen & C.M.Hu	Sang trang	Myrsinaceae	Aerial part	98.2	81.8–123.6
<i>Decaspermum parviflorum</i> (Lam.) A.J.Scott.	Thap tu hoa thua	Myrtaceae	Leaf, twig	100.6	66.5–207.9
<i>Uncaria cordata</i> (Lour.) Merr.	Cau dang	Rubiaceae	Twig with hook	110.3	89.1–156.2
<i>Hedyotis capitellata</i> Wall. ex G.Don	Da cam	Rubiaceae	Aerial part	121.4	108.1–139.8
<i>Prunella vulgaris</i> L.	Ha kho thao	Lamiaceae	Spike	123.0	93.7–199.2

A total of 57 crude methanol extracts which displayed the XO inhibitory activity at 100 $\mu\text{g/mL}$ with inhibition rates of 50% or higher in a screening experiment were subjected to *in vitro* XO assay based on spectrophotometric measurement of uric acid formation from xanthine to determine the IC₅₀ values. These values and their 95% confidence intervals (95% CI) were calculated using Graphpad Prism version 5.0 Software with the sigmoidal dose–response model. The results are sorted in order of decreasing potency.

(IC₅₀ 25.4 µg/mL), *Smilax poilanei* (*S. poilanei*) (IC₅₀ 20.0 µg/mL) and *Archidendron clypearia* (IC₅₀ 15.6 µg/mL). In addition, 17 others also possessed XO inhibitory activity with IC₅₀ values below 50 µg/mL.

3.2. XO inhibitory activity of the fractions from the methanol extract of *A. clypearia*

All fractions obtained from the methanol extract of *A. clypearia* were assayed for XO inhibitory activity. The results (Table 2) showed that fraction E possessed the most potent XO inhibition (IC₅₀ 4.4 µg/mL), which followed by the fraction H (IC₅₀ 22.4 µg/mL), C (IC₅₀ 23.6 µg/mL) and B (IC₅₀ 42.3 µg/mL). The activity remained in aqueous residue was negligible. From these results, the fraction E was chosen for further phytochemical investigation.

3.3. Isolation of compounds from *A. clypearia*

Six compounds were isolated from the E extract of *A. clypearia*. Below are physicochemical parameters and spectroscopic data of the isolated compounds. Based on the analysis of the spectroscopic data and in comparison with those in the literature, the structures of compounds were identified to be 1-octacosanol (1) [13], docosenoic acid (2) [14], daucosterol (3) [13], methyl gallate (4) [15], quercitrin (5) [16] and (–)-7-O-galloylricetiflavan (6) [17].

1-Octacosanol (1). White needles. mp 72–73 °C. ESI-MS *m/z* 411.1 [M+H]⁺ (C₂₈H₅₈O). ¹H-NMR (500 MHz, CDCl₃) δ (ppm): 3.64 (2H, t, *J* = 7.0 Hz, H-1), 1.57 (2H, m, H-2), 1.25–1.34 (50H, H-3 to H-27), 0.88 (3H, t, *J* = 7.0, H-28). ¹³C-NMR (125 MHz, CDCl₃) δ (ppm): 63.1 (C-1), 32.8 (C-2); 31.9 (C-3); 29.7 (C-4 to C-24); 29.4 (C-25); 25.8 (C-26); 22.7 (C-27); 14.1 (C-28) [13].

Docosenoic acid (2). White solid. mp 33–34 °C. ESI-MS *m/z* 337.1 [M-H]⁺ (C₂₂H₄₂O₂). ¹H-NMR (500 MHz, CDCl₃), δ (ppm), *J* (Hz): 5.34 (2H, m, CH=CH), 2.34 (2H, t, *J* = 7.5 Hz, H-2), 2.00 (4H, m), 1.63 (2H, m), 1.14–1.42 (28H, m), 0.88 (3H, t, *J* = 7.5 Hz, H-22). ¹³C-NMR (125 MHz, CDCl₃, δ, ppm): 180.2 (C-1), 129.9 (C-11), 129.6 (C-12), 34.0 (C-2), 31.9 (C-3), 29.7–29.0 (12C), 27.2, 27.1, 24.6, 22.6, 14.0 (C-22) [14].

Daucosterol (3) White crystals. ESI-MS *m/z* 577.1 [M+H]⁺ (C₃₅H₆₀O₆). ¹H-NMR (500 MHz, CDCl₃+ CD₃OD), δ (ppm):

5.37 (1H, d, *J* = 8.0 Hz, H-6), 4.41 (1H, d, *J* = 7.5 Hz, Glc-1), 3.84 (1H, dd, *J* = 12.0; 5.0 Hz, Glc-6), 3.76 (1H, dd, *J* = 12.0; 3.0 Hz, Glc-6), 3.58 (1H, m, H-3), 3.26–3.47 (4H, m, Glc-2; Glc-3, Glc-4, Glc-5), 1.01 (3H, s, H-19), 0.93 (3H, d, *J* = 6.5 Hz, H-21), 0.85 (3H, t, *J* = 8.0 Hz, H-29), 0.83 (3H, d, *J* = 6.5 Hz, H-27), 0.82 (3H, d, *J* = 6.5 Hz, H-26), 0.69 (3H, s, H-18). ¹³C-NMR (125 MHz, CDCl₃+ CD₃OD) δ (ppm): 139.9 (C-5), 121.5 (C-6), 100.7 (Glc-1), 78.6 (C-3), 76.1 (Glc-3), 75.6 (Glc-5), 73.2 (Glc-2), 69.8 (Glc-4), 61.3 (Glc-6), 56.3 (C-14), 55.6 (C-17), 49.8 (C-9), 45.5 (C-24), 41.9 (C-13), 39.3 (C-12), 38.2 (C-4), 36.8 (C-1), 36.2 (C-10), 35.7 (C-20), 33.5 (C-22), 31.5 (C-7), 31.5 (C-8), 29.2 (C-2), 28.7 (C-25), 27.7 (C-16), 25.6 (C-23), 23.8 (C-15), 22.6 (C-28), 20.6 (C-11), 19.1 (C-27), 18.7 (C-18), 18.3 (C-26), 18.2 (C-21), 11.2 (C-19), 11.2 (C-29) [13].

Methyl gallate (4) Yellow oil. ESI-MS *m/z* 185.1 [M+H]⁺. ¹H-NMR (500 MHz, CD₃OD), δ (ppm): 7.06 (2H, s, H-2, H-6), 3.83 (3H, s, OMe). ¹³C-NMR (125 MHz, CD₃OD) δ (ppm): 169.0 (C-7), 146.4 (C-3 and C-5), 139.7 (C-4), 121.5 (C-1), 110.1 (C-2 and C-6), 52.2 (OMe) [15].

Quercitrin (5) Yellow powder; ESI-MS *m/z* 447.1 [M-H]⁺. ¹H-NMR (500 MHz, CD₃OD) δ (ppm): 7.35 (1H, d, *J* = 2.0 Hz, H-2'), 7.32 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 6.93 (1H, d, *J* = 8.0 Hz, H-5'), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 5.31 (1H, d, *J* = 1.5 Hz, H-1''), 4.24 (1H, m, H-2''), 3.77 (1H, m, H-3''), 3.09 (2H, m, H-4'', 5''), 0.96 (d, *J* = 6.5 Hz, H-6''). ¹³C-NMR (CD₃OD) δ (ppm): 176.8 (C-4), 166.2 (C-7), 161.0 (C-5), 156.7 (C-2), 156.2 (C-9), 149.2 (C-4'), 145.7 (C-3'), 133.6 (C-3), 121.9 (C-6'), 120.7 (C-1'), 115.5 (C-2'), 115.4 (C-5'), 102.0 (C-10), 101.7 (C-1''), 99.8 (C-6), 94.2 (C-8), 71.3 (C-4''), 70.9 (C-5''), 70.4 (C-3''), 70.2 (C-2''), 17.5 (C-6'') [16].

(–)-7-O-Galloylricetiflavan (6). brown amorphous powder; [α]_D²⁰ –5.3 (c 0.8, MeOH); ESI-MS *m/z* 443.1443.1 [M+H]⁺ (C₂₂H₁₈O₁₀). ¹H-NMR (500 MHz, CD₃OD) δ (ppm): 7.19 (2H, s, H-2'', H-6''), 6.45 (2H, s, H-2', 6'), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 6.21 (1H, d, *J* = 2.0 Hz, H-8), 4.82 (1H, dd, *J* = 2.0 Hz, 10.0 Hz, H-2), 2.74–2.77 (1H, m, H-4a), 2.64–2.71 (1H, m, H-4b), 2.14–2.18 (1H, m, H-3a), 1.96–2.00 (1H, m, H-3a). ¹³C-NMR (CD₃OD) δ (ppm): 167.1 (C-7''), 157.7 (C-9), 157.2 (C-5), 151.4 (C-7), 146.9 (C-3'', C-5''), 146.6 (C-3', C-5'), 140.4 (C-4''), 134.0 (C-1'), 133.7 (C-4'), 120.8 (C-1''), 110.5 (C-2'', C-6''), 108.6 (C-10), 106.2 (C-2', C-6'), 102.4 (C-8), 101.5 (C-6), 79.0 (C-2), 30.4 (C-4), 20.3 (C-3) [17].

3.4. In vitro XO inhibition of purified compounds from *A. clypearia*

Six compounds isolated from the fraction E of *A. clypearia* were subjected to the XO inhibitory assay. Among them, only two demonstrated remarkable XO inhibitory activity with IC₅₀ values of 25.5 µmol/L ((–)-7-O-galloylricetiflavan, 95% CI 21.5–29.8 µmol/L) and 244.8 µmol/L (methyl gallate, 95% CI 206.1–274.6 µmol/L). Others did not possess the inhibitory activity which is potent enough to determine significant IC₅₀ values.

To elucidate the inhibition mode of the compound 6, which exhibited the most potential activity, Lineweaver–Burk plots were constructed in the presence (3 µg/mL) or absence of this compound (Figure 1). The results indicated this compound to be a noncompetitive inhibitor, which is different from allopurinol, a known competitive inhibitor with respect to XO. Therefore, this compound inhibited XO by binding only to the enzyme–substrate complex.

Table 2

Xanthine oxidase (XO) inhibition of fractions from *A. clypearia*.

No.	Fractions	Amount (g)	XO inhibition (µg/mL)	
			IC ₅₀	95% CI
1	<i>n</i> -Hexane (H)	100	22.4	20.7–24.8
2	Chloroform (C)	145	23.6	20.3–30.8
3	Ethyl acetate (E)	250	4.4	3.6–5.2
4	<i>n</i> -Butanol (B)	105	42.3	26.3–54.1
5	Residue (W)	145	>500.0	

The methanol extract of *A. clypearia* (obtained from 5 kg of dried plant materials) was extracted successively with *n*-hexane, chloroform, ethyl acetate, *n*-butanol under reflux to yield respective fractions. IC₅₀ and 95% confidence interval (95% CI) for XO inhibition of these fractions were calculated on the basis of inhibitory percentage values at various concentrations, using Graphpad Prism version 5.0 Software with the sigmoidal dose–response model.

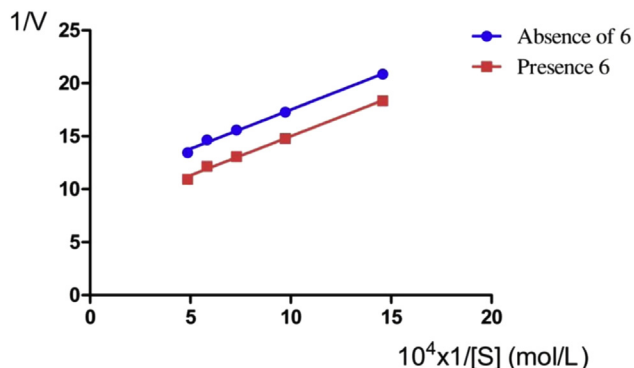


Figure 1. Lineweaver–Burk plots in the absence and in the presence of (–)-7-O-galloyltricetiflavan.

In the absence of (–)-7-O-galloyltricetiflavan, K_m 8.97 $\mu\text{mol/L}$ and V_{max} 0.089; in the presence of (–)-7-O-galloyltricetiflavan, K_m 7.27 $\mu\text{mol/L}$ and V_{max} 0.074. V , μmol substrate metabolized/(mg enzyme·min); S , substrate.

3.5. Anti-hyperuricemic effects of *A. clypearia*

Based on the present screening, the methanol extract of *A. clypearia* exhibited the strongest XO inhibitory activity with the IC_{50} value of 15.6 $\mu\text{g/mL}$. To verify whether this *in vitro* XO inhibitory activity transferred into antiuricemic activity *in vivo*, the methanol extract of *A. clypearia* was evaluated by measurement of serum uric acid levels in potassium oxonate-induced hyperuricemic mice. As shown in Figure 2, compared to the hyperuricemic mice group (140.69 $\mu\text{mol/L}$), serum uric acid concentrations of groups treated with 1120, 3730 and 11180 mg/kg of the methanol extract of *A. clypearia* were reduced by 22.8% ($P = 0.979$), 63.6% ($P < 0.001$) and 68.3% ($P < 0.001$), respectively. The results revealed that the anti-hyperuricemic activity of the methanol extract of *A. clypearia* was dose-dependent and only exhibited significantly with higher doses (3730 and 11180 mg/kg). Also, the anti-hyperuricemic effects of the methanol extract of *A. clypearia* at any dose levels were still inferior to that of allopurinol at 10 mg/kg which

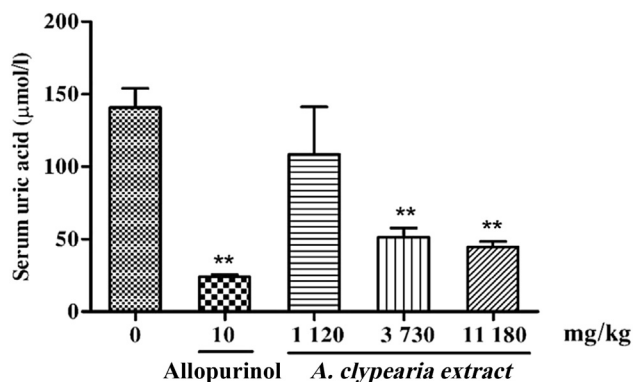


Figure 2. The effects of the methanol extract of *Archidendron clypearia* on serum urate levels in hyperuricemic mice.

Hyperuricemia was induced by a potassium oxonate intraperitoneal injection 1 h before the last drug administration. The methanol extract of *A. clypearia* at 1120, 3730 and 11180 mg/kg and allopurinol at 10 mg/kg were orally administered once a day for 5 consecutive days ($n = 9$ for each treatment group). The control group were orally administered with 0.5% sodium carboxymethylcellulose (CMC-Na) ($n = 10$). The serum uric acid levels of mice were measured by the phosphotungstic acid method. Values are expressed as mean \pm SEM. $^{***}P < 0.01$ vs. control using ANOVA analysis.

displayed an inhibition of 82.9% of the serum uric acid concentration compared to the negative control.

4. Discussion

XO is well known as an important target in the pharmacological approach for the treatment of gout disease and hyperuricemia. Recently, there has been an emerging interest in links between increased XO activity and oxidative stress which may significantly contribute to the development of cardiovascular diseases, cancer, and many other medical conditions [18,19]. From this fact, screening of XO inhibitory activity from medicinal plants might be an effective tool to find new potential candidates for therapy of these major diseases. In the current work, we screened 311 extracts from 301 Vietnamese herbs which had been used empirically for various conditions including inflammation, pain and gout. Four medicinal plants displaying the greatest XO inhibitory activities include *P. foetida*, *L. ramiflora*, *S. poilanei* and *A. clypearia*. As far as our knowledge, this is the first report on the XO inhibitory activity of these four medicinal plants.

The aerial part of *P. foetida* is widely used to treat insomnia, diarrhea and certain inflammatory conditions in Vietnam [20]. Phytochemical studies on this plant species reported several flavonoids (e.g., apigenin, luteolin, quercetin-4'-methyl ether, vitexin, kaempferol) as major constituents, together with alkaloids, glycosides, fatty acid, and some other compounds [21,22]. Flavonoids are well known antioxidants that have been studied extensively as possible medicinal agents for diseases mediated by free radicals. Furthermore, some flavonoids including apigenin, quercetin and their derivatives also exhibited XO inhibitory activity either *in vitro* or in experimental animals [23–26]. Thus, the flavonoid constituents could be responsible for XO inhibitory activity of *P. foetida*.

Limited available data revealed that *L. ramiflora*, also known as *Chionanthus ramiflorus*, has been ethnopharmacological used in a few countries, such as Papua New Guinea and Thailand, for treatment of malnutrition (bark and leaf), uterus infection (root), and cancer (stem) [27,28]. While a decoction from the stem of *L. ramiflora* was reported to have immunosuppressant activity [29], no publication mentioned the XO inhibition-related effects of this plant. Likewise, its phytochemistry remains to be investigated. Another potent herb, identified as *S. poilanei*, appears to have not been previously recorded any bioactivity. The rhizome of this plant, however, was empirically used in management of arthritis in Vietnamese folk medicine. Moreover, anti-hyperuricemic effects of two other *Smilax* species, including *Smilax riparia* and *Smilax china*, were observed in mice [30,31]. These results suggested that *Smilax* genus with more than 300 plant species will be a promising source for screening XO inhibitory activity and therapeutic effects of gout disease.

The most active extract found in the present screening was the methanol extract from leaves and twigs of *A. clypearia*, which is traditionally used as a medicinal plant for detoxicating, cooling, reducing edema, and treating diarrhea in Vietnam, Malaysia, and Indonesia [32,33]. Recently, there have been some studies on the anti-inflammatory potency of *A. clypearia* [34,35]. However to the best of our knowledge, gout-relating activity of this plant has not been reported previously. Thus, further investigations were conducted to examine the anti-hyperuricemic effects of *A. clypearia*. The results demonstrated that

A. clypearia reduced the serum uric levels in a dose-dependent manner in the mouse model of hyperuricemia induced by potassium oxonate, an uricase inhibitor. Based on the *in vitro* assay for XO inhibition and the fact that *A. clypearia* also showed a reduction in hepatic XO activities in another independent experiment (data not shown), it could be suggested that there existed a close correlation between XO inhibition and the hypouricemic effects. Taking into consideration of this relationship, isolation and identification of the active constituents were guided by the XO inhibitory activity.

Since the ethyl acetate-soluble portion (fraction E) was the only fraction of the four fractions displaying superior *in vitro* activity compared to the original extract (methanol extract), the fraction E was selected for chemical investigation. Various chromatographic separation and purification carried out on the fraction E led to the isolation of six compounds, including 1-octacosanol docosenoic acid, daucosterol, methyl gallate, quercitrin, and (–)-7-O-galloyl-tricetiflavan. Among these, two compounds, methyl gallate and 7-O-galloyl-tricetiflavan, were demonstrated to be responsible, at least in part, for the XO inhibitory activity of fraction E. Methyl gallate was also isolated from some other plants and was previously reported to have a relatively weak inhibitory activity against XO [36]. On the contrary, although the conflicting data on XO inhibitory activity of quercitrin were recorded, this flavonoid derivative showed to be almost inactive in the assays [37,38]. Meanwhile, (–)-7-O-galloyl-tricetiflavan was reported to have antiviral activity against respiratory syncytial virus [17] and *in silico* soluble epoxide hydrolase inhibitory activity [39] but its XO inhibition has not been evaluated. Thus, this is a new finding of the biological activity of this compound.

Our present results also demonstrated (–)-7-O-galloyl-tricetiflavan as an uncompetitive inhibitor, which is different from allopurinol, a known competitive inhibitor with respect to XO. Accordingly, this compound inhibited XO by binding only to the enzyme–substrate complex. It should be noted, however, that the potency of (–)-7-O-galloyl-tricetiflavan was not stronger than that of the ethyl acetate portion (IC₅₀ value 11.3 vs. 4.4 µg/mL, respectively). Therefore, a synergistic addition of the XO inhibitory activity from some different constituents present in this portion, including minor constituents that have not yet been identified in our study, might be relevant. Recently, Thao *et al.* reported the isolation of several flavonoids with similar structural features compared to (–)-7-O-galloyl-tricetiflavan [39]. It is likely that these flavonoids are also promising as XO inhibitors.

In conclusion, our systematic *in vitro* screening study of Vietnamese medicinal plants allowed to identify a range of species possessing the XO inhibitory activity. Chemical investigation carried out on *A. clypearia*, the most active plant obtained from the screening, led to the identification of 7-O-galloyl-tricetiflavan as a potential XO inhibitor. Further studies on other active plants should warrant more promising XO inhibitors.

Conflict of interest statement

We declare that we have no conflict of interest.

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