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Research article

First report of piperidone extracts as tyrosinase and α-glucosidase inhibitors from fruits of *Piper retrofractum*

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Abstract

Importance of the work: *Piper retrofractum* is used as a spice. This report is the first to identify tyrosinase and α-glucosidase inhibitors extracted from this plant.

Objectives: To separate and identify the bioactive compounds from the fruits of *P. retrofractum* and to evaluate their tyrosinase and α-glucosidase inhibition activities.

Materials & Methods: The EtOAc extract was isolated and purified using various chromatographic techniques. The structures of isolated compounds were confirmed based on their spectroscopic data. The tyrosinase and α–glucosidase inhibition activities of the isolated compounds were assessed based on a colorimetric method, using kojic acid and acarbose, respectively, as positive controls.

Results: Seven known compounds were identified, named methyl piperate (1), guineensine (2), piperine (3), *N*-feruloyltyramine (4), (3*S*, 4*S*)-4-chloro-3-hydroxy-2-piperidin-2-one (5), 3-chloro-4-hydroxy-2-piperidone (6) and 3α,4α-epoxy-2-piperidone (7). Piperidones 5 and 6 showed tyrosinase inhibition with concentration at 50% inhibition (IC_{50}) values of 4.91 mM and 11.0 mM, respectively, whereas the alkylamides 1–3 and phenylpropanamide 4 were not active. On the other hand, only phenylpropanamide 4 was active against maltase and sucrase with IC_{50} values of 0.28 mM and 0.5 mM, respectively. Piperidone 5 showed weak inhibition against both maltase and sucrase α-glucosidases, while the piperidones 6 and 7 exhibited weak inhibition against sucrase α-glucosidases.

Main finding: The piperidones were more active against tyrosinase than piperine, which is representative metabolite from this plant. Further study on the inhibitory mechanism may lead to suitable applications of the extract in cosmetics.

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Introduction

Piper retrofractum is an herb used for various therapies such as digestive, stimulant, carminative and intestinal disorders (Muharini et al., 2015). Major constituents of this plant include alkyl amides, phenylpropanamides, dimeric amide alkaloids (Matsuda et al., 2009; Luyen et al., 2014; Muharini et al., 2015). Investigations of crude extracts and isolated compounds from this plant demonstrated several biological activities, including antimicrobial, antioxidant, cytotoxic, analgesic, androgenic, aphrodisiac, antihyperlipidemic, antihyperuricemic, leukocyte-reducing, antileishmanial and immunostimulant properties (Wardani and Leliqia, 2021). Yodsawad et al. (2023) reported that the major alkyl amides isolated from the hexane and CH_2Cl_2 extracts of *P. retrofractum* inhibited tyrosinase with weak potency. Thus, the present study further investigated tyrosinase inhibition of the EtOAc extract because it showed a better inhibitory effect against tyrosinase and distinguished the thin-layer chromatography (TLC) and ^{1}H NMR profiles of minor metabolites. Piperidones are minor components isolated from *Piper* species, with 3-chloro-4-hydroxy-2-piperidone being the first piperidone isolated in 1995, from the fruits of *P. hancei* (Narui et al., 1995) and also reported from fruits of *P. retrofractum* (Tang et al., 2019). Furthermore, its structural isomer, named (3*S*, 4*S*)-4-chloro-3-hydroxy-2-piperidin-2-one, was also isolated from the aerial parts of *Leea aequata* (Tun et al., 2019). 3-Chloro-4-hydroxy-2-piperidone and (3*S*, 4*S*)-4-chloro-3-hydroxy-2-piperidin-2-one were tested for platelet inhibition and antimicrobial activity, respectively (Narui et al., 1995; Tang et al., 2019; Tun et al., 2019). However, very little is known about the bioactivity of piperidone extracted from *Piper* species. Hence, the present study investigated the isolation together with the tyrosinase and α-glucosidase inhibition activities of the piperidones.

Materials and Methods

General

One-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded on 400 or 500 MHz Bruker AVANCE spectrometers. High resolution mass spectra were acquired using Bruker micrOTOF mass spectrometers, equipped with an electrospray ionization (ESI) ion source. Chromatography was performed on a Sephadex LH-20, with Merck silica gel 60 (70–230 mesh) and TLC was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer) and visualized using short-wave ultraviolet (UV) light and 5% anisaldehyde reagent.

Plant material

The fruits of *P. retrofractum* were purchased at the Chaokrompoe store, Bangkok, Thailand, in October 2016. A voucher specimen (BCU016033) was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

Extraction and isolation

A sample (4.0 kg) of the fruits of *P. retrofractum* was sequentially exacted with hexane, CH_2Cl_2 and EtOAc to give corresponding extracts. The EtOAc extract (45.0 g) was fractionated over silica gel column chromatography eluted with hexane, hexane-EtOAc (95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, 0:100), EtOAc-MeOH (95:5, 90:10, 80:20) and MeOH to yield seven fractions. Fractions 1, 4 and 5 were further purified based on crystallization to yield methyl piperate (1; 700 mg), guineensine (2; 500 mg) and piperine (3; 25.0 g), respectively. Fraction 6 was subsequently fractionated over Sephadex LH-20 eluted with MeOH to yield six subfractions (A1–A6). The subfraction A4 was fractionated over Sephadex LH-20 eluted with MeOH-CH_2Cl_2 (1:1) to yield three subfractions (A41–A43). Subfraction A42 was purified using flash reverse phase column chromatography with a MeOH-H_2O step gradient, Sephadex LH-20 (MeOH-CH_2Cl_2 (1:1)) and silica gel column chromatography (acetone-hexane, 40:60) to give *N*-feruloyltyramine (4, 10 mg). Subfraction A43 was separated by Sephadex LH-20 (MeOH–CH_2Cl_2 (1:1)) and silica gel column chromatography using a gradient of CH_2Cl_2–MeOH (100:0–20:80) to obtain (3*S*,4*S*)-4-chloro-3-hydroxy-2-piperidin-2-one (5, 8 mg), 3-chloro-4-hydroxy-2-piperidone (6, 5 mg) and subfraction A433. Subfraction A433 was purified using alumina column chromatography with a gradient of CH_2Cl_2-MeOH (100:0–20:80) to yield 3α,4α-epoxy-2-piperidone (7, 4 mg).

Methyl piperate (1): colorless solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.42 (dd, J = 15.2, 10.8 Hz, 1H), 6.99 (d, J = 1.6 Hz, 1H), 6.91 (dd, J = 7.9, 1.6 Hz, 1H), 6.81 (d, J = 15.2 Hz, 1H), 6.78 (d, J = 7.9 Hz, 1H), 6.70 (dd, J = 15.2, 10.8 Hz, 1H), 5.98 (s, 2H, –OCH_2O–), 5.95 (d, J = 15.2 Hz, 1H), 3.75 (s, 3H, –OCH_3) (Kijjoa et al., 1989) (Fig. S1).

Guineensine (2): white solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.21 (dd, J = 15.2, 10.8 Hz, 1H), 6.91 (brs, 1H), 6.77 (m, 2H), 6.31 (d, J = 15.0 Hz, 1H), 6.09 (m, 3H), 5.95 (s, 2H), 5.78 (d, J = 15.0 Hz, 1H), 3.19 (t, J = 6.4 Hz, 2H), 2.20 (m, 4H), 1.82 (m, 1H), 0.95 (d, J = 6.7 Hz, 6H), 1.44 (m, 8H); HRMS m/z 406.2358 $[M+Na]^+$, molecular formula $C_{24}H_{33}NO_3Na$ (Park et al., 2002) (Fig. S2).

Piperine (3): colorless solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.40 (dd, J = 14.5, 9.5 Hz, 1H), 6.98 (brs, 1H), 6.89 (d, J = 8.1 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.76 (m, 1H), 6.74 (d, J = 15.4 Hz, 1H), 6.02 (d, J = 15.0 Hz, 1H) 5.97 (s, 2H, –OCH_2O–), 3.53–3.63 (brs, 2H), 1.59–1.66 (m, 6H) (Narui et al., 1995) (Fig. S3).

N-Feruloyltyramine (4): colorless gum; ^{1}H NMR (CD_3OD, 400 MHz) δ_H 7.40 (d, J = 15.7 Hz, 1H), 7.09 (d, J = 2.0 Hz, 1H), 7.02 (d, J = 8.3 Hz, 2H), 6.92 (dd, J = 8.2, 2.0 Hz, 1H), 6.77 (d, J = 8.2 Hz, 1H), 6.69 (d, J = 8.3 Hz, 2H), 6.37 (d, J = 15.7 Hz, 1H), 3.47 (t, J = 7.0 Hz, 2H), 2.76 (t, J = 7.0 Hz, 2H) (Park, 2009) (Fig. S4).

(3*S*,4*S*)-4-Chloro-3-hydroxy-2-piperidin-2-one (5): colorless gum; $[\alpha]^{24}_D$ -20 (c = 0.2, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ_H 4.09 (m, 1H), 3.90 (d, J = 7.55 Hz, 1H), 3.25 (m, 2H), 2.30 (m, 1H), 2.04 (m, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 172.4, 74.5, 59.96, 39.6, 30.3; HRESIMS m/z: 172.0136 and 174.0102 (cal. for $C_5H_8Cl^{35}NO_2Na$, 172.0244, $[M+Na]^+$ and $C_5H_8Cl^{37}NO_2Na$, 174.0244, $[M+Na]^+$) (Tun et al., 2019) (Figs. S5–S7).

3-Chloro-4-hydroxy-2-piperidone (6):colorless solid; $[\alpha]^{24}_D$+4.37 (c = 1.6, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ_H 4.10 (m, 2H), 3.50 (m, 1H), 3.30 (m, 1H), 2.25 (m, 1H), 1.85 (m, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ_C 170.0, 71.0, 58.6, 38.4, 26.4; HRESIMS m/z: 172.0145 and 174.0110 (cal. for $C_5H_8Cl^{35}NO_2Na$, 172.0244, $[M+Na]^+$ and $C_5H_8Cl^{37}NO_2Na$, 174.0244, $[M+Na]^+$) (Narui et al., 1995) (Figs. S8–S10).

3α,4α-Epoxy-2-piperidone (7): yellow gum; $[\alpha]^{24}_D$+10.5 (c = 2.0, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ_H 3.68 (brt, J = 4.1 Hz, 1H) 3.31 (d, J = 4.1 Hz, 1H), 3.23 (dt, J = 12.7, 4.5 Hz, 1H), 3.04 (m, 1H), 2.29 (m, 1H), 2.01 (m, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 171.3, 54.5, 51.4, 35.9, 24.2 (Lago and Kato, 2007) (Figs. S11–S12).

Tyrosinase inhibition

Anti-tyrosinase activity was conducted as described by Ersoy et al. (2019) using L-DOPA as the substrate and kojic acid as a positive control. Briefly, 50 µL of tyrosinase (20 unit/mL) was mixed with 20 µL of sample or kojic acid. All sample solutions were incubated at 37 °C for 10 min and 300 µL of L-DOPA was added. The reactions were incubated at 37°C for 20 min. The absorbance was measured using spectrophotometry at 475 nm. A blank assay was also conducted. The bioassay was tested in triplicate. The inhibition percentage of tyrosinase activity was calculated using: inhibition (%) = $(A_{control} - A_{sample}) / A_{control} \times 100$, where $A_{control}$ is the absorbance of dimethyl sulfoxide (DMSO) and A_{sample} is the absorbance of the test reaction mixture. The concentration at 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against the sample concentration.

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity against rat intestinal maltase and sucrase was conducted according to the method of Worawalai et al. (2019). The isolated compounds (1 mg/mL in DMSO, 10 µL) were combined with 30 µL of 0.1 M phosphate buffer (pH 6.9), 20 µL of substrate solution (2 mM maltose and 20 mM sucrose in 0.1 M phosphate buffer), 80 µL of glucose assay kit (SU-GLLQ2, Human) and 20 µL of crude enzyme solution. The reaction mixture was incubated at 37°C for 10 min for maltose and 40 min for sucrose. Enzymatic activity was determined by measuring the absorbance of quinoneimine formed at 500 nm using a Bio-Rad 3550 microplate reader. The percentage inhibition was calculated using: inhibition (%) = $(A_{control} - A_{sample}) / A_{control} \times 100$, where A_{sample} is the absorbance with the sample and $A_{control}$ is the absorbance without the sample. The IC_{50} value was determined from a plot of the percentage inhibition against the sample concentration, with acarbose serving as the positive control. Assays were performed in triplicate.

Results and Discussion

The fruits of *P. retrofractum* were sequentially extracted using organic solvents to produce hexane, CH_2Cl_2 and EtOAc extracts. The extraction procedure is summarized in Fig. 1. The EtOAc extract was fractionated and separated using chromatographic techniques until pure compounds had been obtained. Details of isolation are summarized in Fig. 2.

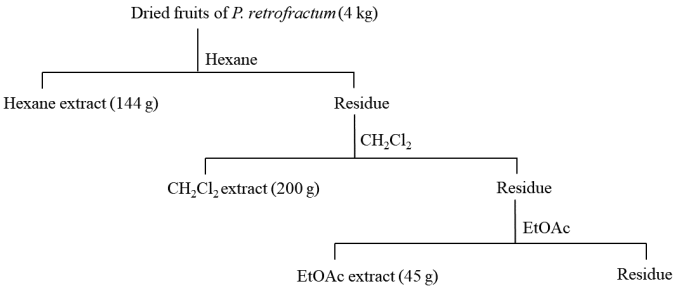

Fig. 1 Extracts from *Piper retrofractum*

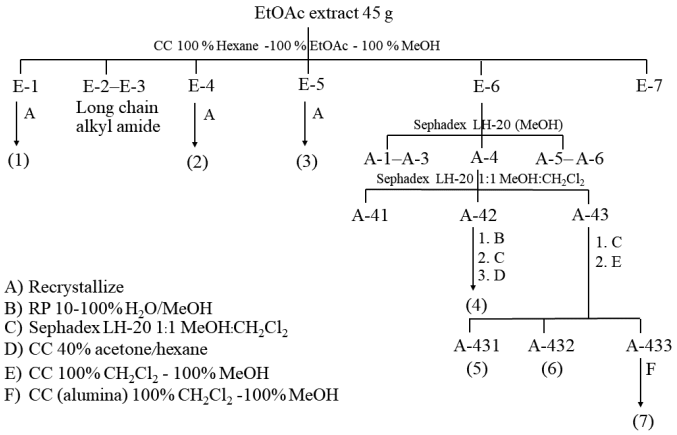

Fig. 2 Chromatographic separation of EtOAc extract

Three known alkylamides (1–3), one known phenylpropanamide (4) and three known piperidones (5–7) were isolated. They were identified as methyl piperate (1) (Kijjoa et al., 1989), guineensine (2) (Park et al., 2002), piperine (3) (Narui et al., 1995), *N*-feruloyltyramine (4) (Park, 2009), (3*S*,4*S*)-4-chloro-3-hydroxy-2-piperidin-2-one (5) (Tun et al., 2019), 3-chloro-4-hydroxy-2-piperidone (6) (Narui et al., 1995) and 3α,4α-epoxy-2-piperidone (7) (Lago and Kato, 2007) based on comparison of their spectroscopic data with those reported in the literature. The structures of all isolated compounds are shown in Fig. 3.

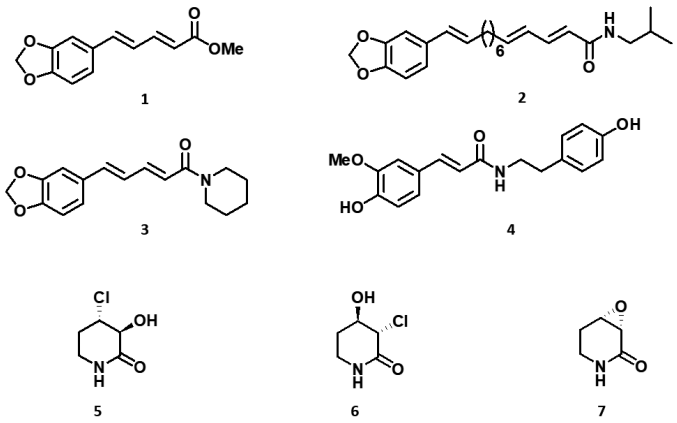

Fig. 3 Chemical structures of isolated compounds (1–7) from fruits of *Piper retrofractum*

All isolated compounds (1–7) were examined for tyrosinase and α–glucosidase inhibitory activities. The results are given in Table 1. Only phenylpropanamide 4 showed potent inhibitory activity against maltase and sucrase with IC_{50} values of 0.28 mM and 0.5 mM, respectively. On the other hand, the piperidones 5–7 weakly inhibited sucrase with IC_{50} values of 44.25 mM, 72.98 mM and 41.25 mM, respectively. Notably, the potent inhibition of 4 was potentially due to the presence of phenolic hydroxyl, which possibly participated

Table 1 α–Glucosidase inhibitory effect and antityrosinase activity of extracted compounds 1–7

Compound	α-Glucosidase inhibitory effect IC_{50}±SD (mM)		Antityrosinase activity
	Maltase	Sucrase	IC_{50}±SD (mM)
1	NI	NI	NI
2	NI	NI	NI
3	NI	NI	NI
4	0.28	0.5	NI
5	34.86±1.61	44.25±1.95	4.91±1.27
6	NI	72.98±0.51	11.00±0.42
7	NI	41.25±0.01	NI
Acarbose	0.0235±0.022	0.0247±0.020	-
Kojic acid	-	-	2.18±0.17

IC_{50} = concentration at 50% inhibition; NI = percentage inhibition less than 30% at highest concentration tested.

in chelating the active or binding site of the targeted enzymes. Upon evaluation against tyrosinase, piperidones 5 and 6 showed good tyrosinase inhibition with IC_{50} values of 4.91 mM and 11.0 mM, respectively, whereas piperidone 7 exhibited no tyrosinase inhibition. These data suggested that the hydroxyl group and chlorine atom in the piperidones 5 and 6 possibly played an important role in inhibiting enzyme function. Piperidone 5 showed two-fold more potent inhibition than that of 6, perhaps because they are structural isomers that differ in the positions of the hydroxy group and chlorine atom. From a biosynthesis point of view, piperidones 5 and 6 were possibly generated from 7 by the nucleophilic addition of chloride ion to epoxide.

Piperidone 5 was first isolated from the aerial parts of *Leea aequata* and was evaluated for antimicrobial activity against four bacteria: *Escherichia coli*, *Staphylococcus aureus* subsp. *aureus*, *Salmonella enterica* subsp. *enterica* and *Pseudomonas aeruginosa*; however, it showed no inhibition (< 30 % inhibition at 125 µg/mL), according to Tun et al. (2019). Piperidone 6 was first separated from *P. hancei* (Narui et al., 1995). However, it showed no inhibitory effect against platelet aggregation (< 20 % inhibition at 100 µM), according to Tang et al. (2019).

In conclusion, the piperidones 5–7 were identified as minor and rare metabolites with relatively more potent inhibitory effects against tyrosinase than the alkylamides 1–3 and phenylpropanamide 4. The presence of the hydroxyl group and chlorine atom in piperidones 5 and 6 likely promoted its better inhibitory effects compared to piperidone 7. This study was the first report on tyrosinase inhibition of piperidones from *Piper* species. Further study on the piperidone series should be undertaken to discover other active components.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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