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Chemical constituents and antimicrobial activity of *Lisotrigona cacciae* propolis collected in Hoa Binh province

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Abstract:

A chemical study on the propolis of the stingless bee *Lisotrigona cacciae* collected from Hoa Binh province led to the isolation of four compounds including cycloartenol (1), cochinchinone A (2), *α*-mangostin (3), and isomangiferolic acid (4). *Mangifera indica* (Xoài) and *Cratoxylum cochinchinense* (Thành Ngạnh Nam) were suggested to be the resin sources of the propolis. The propolis EtOH extract showed good antimicrobial activity against the Gram (+) strains *B. cereus* with an MIC value of 8 µg/ml. Among the isolated compounds, *α*-mangostin (3) was the most active and displayed strong activity against the five strains *B. cereus*, *E. feacalis, S. aureus*, *P. aeruginosa,* and the fungus *C. albicans* with MIC values of 1-2 µg/ml.

Keywords: **antimicrobial activity, *Lisotrigona cacciae*, propolis, stingless bee, *α*-mangostin.**

Classification number: **3.4**

Introduction

Propolis is a bee product that originates from plant resin and exhibits various biological activities and phytochemical compositions. Propolis products have been used as medicinal agents for centuries. Propolis has numerous health benefits stemming from its many pharmacological activities due to its antioxidant, antimicrobial, antiviral, anti-inflammatory, and anticancer properties. Previous chemical studies of propolis has led to the isolation of flavonoids, terpenoids, phenolic acids and their esters, lignans and coumarins [1]. However, information regarding the chemical composition of Vietnamese bee propolis remains limited. Cycloartane-type triterpenes and alkyl phenols were isolated from the propolis of the stingless bees *Trigona minor* and *Lisotrigona cacciae* [2-4] while xanthones and homoisoflavonoids were found from the *Lisotrigona sp.* propolis [4, 5]. Herein, four compounds were isolated from the propolis of the stingless bee *Lisotrigona cacciae* in the Hoa Binh province and were identified as cycloartenol (**1**), cochinchinone A (**2**), *α*-mangostin (**3**), and isomangiferolic acid (**4**). The activity of these compounds and EtOH extract against several microbial strains are also described.

Experimental

Propolis sample

Stingless bee propolis was collected from beehives in the Tan Lac district, Hoa Binh province, in November of 2018. The stingless bee species was determined to be *Lisotrigona cacciae* by Ms. Tran Thi Ngat and Dr. Nguyen Thi Phuong Lien of the Institute of Ecology and Biological Resources (VAST).

General procedures

The NMR spectra were taken using a Bruker AM500 FT-NMR spectrometer with TMS as an internal standard. The electrospray ionization mass spectra (ESI-MS) were obtained using Agilent 1260 series single quadrupole LC/MS system. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 40-63 µm, Merck). Analytical and preparative thin layer chromatography were performed using precoated silica gel plates (Merck $60F_{254}$).

Extraction and isolation

The propolis of *Lisotrigona cacciae* (158 g) was extracted with EtOH (1 l x 4 times, 1 day/time) at room temperature.

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The EtOH solvent was evaporated *in vacuo*. The residue (126 g) was suspended in H_2O and was then extracted with ethyl acetate (3 times x 500 ml/time). The organic solvents were removed *in vacuo* to obtain ethyl acetate residue (108 g). The ethyl acetate residue was subjected to a silica gel column chromatography (CC) and was eluted with a gradient solvent of *n*-hexane-EtOAc (100:0-0:100) to afford 12 fractions (F1-F12). Fraction F2 (600 mg) was fractionated by silica gel CC and eluted with *n*-hexane/EtOAc (9/1, v/v) to yield compound **1** (15 mg). Fraction F7 (1 g) was purified by silica gel CC and was eluted with *n*-hexane/acetone (98/2, v/v) to afford compound **2** (63 mg). Fraction F10 (1.28 g) was chromatographed on silica gel CC and eluted with *n*-hexane/EtOAc (8/2, v/v) to yield 5 fractions (F10.1-F10.5). The F10.4 (50 mg) was purified by preparative TLC using *n*-hexane/EtOAc (9/1, v/v) as the eluant to afford compound **3** (4 mg) and compound **4** (21 mg).

Cycloartenol (**1**): white solid, ESI-MS *m/z* 427 $[M+H]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.10 (1 H, t, *J*=7.0 Hz, H-24), 3.28 (1 H, m, H-3), 1.68 (3 H, s, H-27), 1.60 (3 H, s, H-26), 0.97 (3 H, s, H-28), 0.87 (3 H, s, H-18), 0.88 (3 H, d, *J*=7.0 Hz, H-21), 0.81 (3 H, s, H-30), 0.56 (1 H, d, *J*=4.5 Hz, H-19), 0.57 (1 H, d, *J*=4.5 Hz, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 130.9 (C-25), 125.3 (C-24), 78.9 (C-3), 52.3 (C-17), 48.8 (C-14), 48.0 (C-8), 47.1 (C-5), 45.3 (C-13), 40.5 (C-4), 36.3 (C-22), 35.9 (C-20), 35.6 (C-15), 32.9 (C-12), 32.0 (C-1), 30.4 (C-2), 29.9 (C-19), 28.1 (C-16), 26.5 (C-11), 26.1 (C-10), 26.0 (C-7), 25.7 (C-27), 25.4 (C-28), 24.9 (C-23), 21.1 (C-6), 20.0 (C-9), 19.3 (C-30), 18.2 (C-21), 18.0 (C-19), 17.6 (C-26), 14.0 (C-29).

Cochinchinone A (**2**): yellow solid, ESI-MS *m/z* 449 $[M+H]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 13.06 (s,1-OH), 7.61 (1 H, d, *J*=3 Hz, H-8), 7.33 (1 H, d, *J*=9 Hz, H-5), 7.24 (1 H, dd, *J*=3 Hz, 9 Hz, H-6), 6.46 (1 H, s, OH), 6.19 (1 H, s, OH), 5.27 (2 H, m, H-2', H-2''), 5.05 (1 H, t, *J*=7 Hz, H-6''), 3.55 (2 H, d, *J*=7.5 Hz, H-1''), 3.45 (2 H, d, *J*=7 Hz, H-1'), 2.09 (2 H, m, H-5''), 2.05 (2 H, m, H-4''), 1.87 (3 H, s, H-9''), 1.84 (3 H, s, H-4'), 1.76 (3 H, s, H-5'), 1.63 (3 H, s, H-10''), 1.57 (3 H, s, H-8''). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 180.9 (C-9), 161.1 (C-3), 158.3 (C-1), 153.0 (C-4a), 152.4 (C-7), 150.5 (C-4b), 137.8 (C-3''), 135.0 (C-3'), 131.8 (C-7''), 123.9 (C-6''), 123.9 (C-6), 121.6 (C-2'), 121.6 (C-2''), 120.7 (C-8a), 119.0 (C-5), 109.1 (C-8), 109.1 (C-2), 105.1 (C-4), 103.2 (C-9a), 39.7 (C-4''), 26.4 (C-5''), 25.8 (C-5'), 25.6 (C-10''), 21.8 (C-1''), 21.6 (C-1'), 17.9 (C-4'), 17.7 (C-8''), 16.3 (C-9'').

α-Mangostin (**3**): yellow solid, ESI-MS *m/z* 411 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.70 (1 H, s, H-5), 6.25 (1 H, s, *J*=9Hz, H-4), 5.25 (2 H, m, H-2', H-2''), 4.09 (2 H, d, *J*=6.5 Hz, H-1''), 3.77 (3 H, s, 7-OMe), 3.30 (2 H, d, *J*=7.5 Hz, H-1'), 1.84 (3 H, s, H-5''), 1.79 (3 H, s, H-4''), 1.69 (3 H, s, H-4'), 1.67 (3 H, s, H-5'). ^{13}C NMR (125 MHz, CD_3OD) δ: 183.1 (C-9), 163.6 (C-3), 161.6 (C-1), 157.9 (C-4b), 156.7 (C-4a), 156.2 (C-6), 144.8 (C-7), 138.5 (C-8), 131.8 (C-3'), 131.7 (C-3''), 125.1 (C-2'), 123.8 (C-2''), 112.2 (C-8a), 111.4 (C-2), 103.8 (C-9a), 102.8 (C-5), 93.1 (C-4), 61.3 (7-OMe), 27.1 (C-1'), 26.0 (C-4'), 25.9 (C-4''), 22.2 (C-1''), 18.3 (C-5'), 17.9 (C-5'').

Isomangiferolic acid (**4**): white solid, ESI-MS *m/z* 457 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.79 (1 H, t, *J*=7 Hz, H-24), 3.33 (1 H, br s, H-3), 2.28 (1 H, m, H-23a), 2.18 (1 H, m, H-23), 1.83 (3 H, s, H-27), 1.04 (3 H, s, H-18), 0.98 (3 H, s, H-28), 0.96 (3 H, d, *J*=6.5 Hz, H-21), 0.95 (3 H, s, H-30), 0.89 (3 H, s, H-29), 0.55 (1 H, d, *J*=4 Hz, H-19), 0.38 (1 H, d, *J*=4 Hz, H-19). ^{13}C NMR (125 MHz, CD_3OD) δ: 173.6 (C-26), 145.9 (C-24), 126.8 (C-25), 77.7 (C-3), 53.5 (C-17), 50.1 (C-14), 48.5 (C-8), 46.4 (C-13), 42.2 (C-5), 40.6 (C-4), 37.2 (C-20), 36.6 (C-15), 36.1 (C-22), 34.1 (C-12), 30.7 (C-19), 29.5 (C-2), 29.1 (C-16), 28.6 (C-1), 27.8 (C-10), 27.3 (C-11), 26.9 (C-23), 26.5 (C-7), 26.5 (C-28), 22.2 (C-29), 21.8 (C-6), 21.9 (C-6), 19.8 (C-30), 18.6 (C-21), 18.5 (C-18), 12.4 (C-27).

Antimicrobial activity

The antimicrobial activity was determined by multi-concentration dilution method [6] and expressed as MIC (minimal inhibitory concentration) values. The isolated compounds were diluted in dimethylsulfoxide (DMSO) at the following concentrations: 256 µg/ml, 128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, and 1 µg/ml, which were used for the antimicrobial test. The positive controls were streptomycin for bacterial strains and cyclohexamide for fungus. Three strains of Gram-positive (*Enterococcus faecalis ATCC299212*, *Staphylococcus aureus ATCC25923*, *Bacillus cereus ATCC13245)*; three strains of Gram-negative bacteria (*Escherichia coli ATCC25922*, *Pseudomonas aeruginosa ATCC27853*, *Salmonella enterica ATCC13076*), and the fungus *Candida albicans ATCC10231* were used for the test.

Results and discussion

Compound **1** was isolated as a white solid. The ESI-MS spectrum showed a protonated molecular ion peak *m/z* of 427 $[M+H]^+$, which corresponds to a molecular formula of $C_{30}H_{50}O$ (M=426). The ^{1}H-NMR spectrum displayed the characteristic signals of a cycloartane-type triterpene with

two H-19 proton signals at δ_H of 0.79 (1 H, d, *J*=4.0 Hz) and 0.57 (1 H, d, *J*=4.0 Hz) and seven methyl signals at δ_H values of 1.68 (s, H-27), 1.60 (s, H-26), 0.97 (s, H-28), 0.87 (s, H-18), 0.88 (d, *J*=7.0 Hz, H-21) and 0.81 (s, H-30) (Fig. 1). An oxymethine group signal was observed at δ_H 3.28 (1 H, m, H-3). The ^{13}C-NMR and DEPT spectra of **1** revealed 30 carbon signals including 7 methyl groups at δ_C of 25.4 (C-28), 19.3 (C-29), 18.1 (C-21), 18.0 (C-18), 13.9 (C-30), and 9.2 (C-27), with 2 olefinic carbons at δ_C of 130.9 (C-25) and 125.3 (C-24) and an oxymethine group at δ_C of 78.9 (C-3). Therefore, compound **1** was determined as cycloartenol. The NMR data of **1** were in accordance with published values [7].

Compound **2** was obtained as a yellow solid. The ESI-MS showed a *quasi*-molecular ion peak *m/z* of 449 $[M+H]^+$, which corresponds to a molecular formula of $C_{28}H_{32}O_5$ (M=448). The ^{1}H-NMR spectrum revealed a hydrogen-bonded OH proton at δ_H of 13.06 (s) and three aromatic protons in an ABX system at δ_H 7.61 (1 H, d, *J*=3 Hz, H-8), 7.33 (1 H, d, *J*=9 Hz, H-5) and 7.24 (1 H, dd, *J*=3 Hz; 9 Hz, H-6). The characteristic signals of protons in an isoprenyl group were displayed at δ_H 5.27 (m), 3.45 (2 H, d, H-1'), 1.84 (3 H, s, H-4'), and 1.76 (3 H, s, H-5'). In addition, the presence of a geranyl group was indicated from the signals at δ_H 5.27 (m), 5.05 (1 H, t, H-6''), 2.09 (2 H, m, H-5''), 2.05 (2 H, m, H-4''), 1.87 (3 H, s, H-9''), 1.63 (3 H, s, H-10'') and 1.57 (3 H, s, H-8''). In the ^{13}C-NMR spectrum of **2**, 28 carbon signals were observed including a signal of a carbonyl group at δ_C 180.9 (C-9), signals of prenyl group at δ_C 135.0 (C-3'), 121.6 (C-2'), 25.8 (C-5'), 21.6 (C-1'), and 17.9 (C-4'), and signals of a geranyl group at δ_C 137.8 (C-3''), 131.8 (C-7''), 123.9 (C-6''), 121.6 (C-2''), 39.7 (C-4''), 26.4 (C-5''), 25.6 (C-10''), 21.8 (C-1''), 17.7 (C-8''), and 16.3 (C-9''). Compound **2** was determined to be cochinchinone A. The NMR data of **2** agreed with reported literature [8].

Compound **3** was obtained as a yellow solid. The ESI-MS revealed a *quasi*-molecular ion peak *m/z* of 411 $[M+H]^+$ suggesting that the molecular formula of **3** is $C_{24}H_{26}O_6$ (M=410). The ^{1}H-NMR spectrum showed the presence of two aromatic singlet protons at δ_H values of 6.70 and 6.25. In addition, there was one methoxy group found at δ_H 3.77 (s). The presence of two olefinic protons were found at δ_H 5.25 (2 H, m), then 2 methylene groups at δ_H 4.09 (d) and 3.30 (d), and four methyl singlets at δ_H 1.84 (s, H-5''), 1.79 (s, H-4''), 1.69 (s, H-4') and 1.67 (s, H-5') confirmed the presence of two isoprenyl groups.

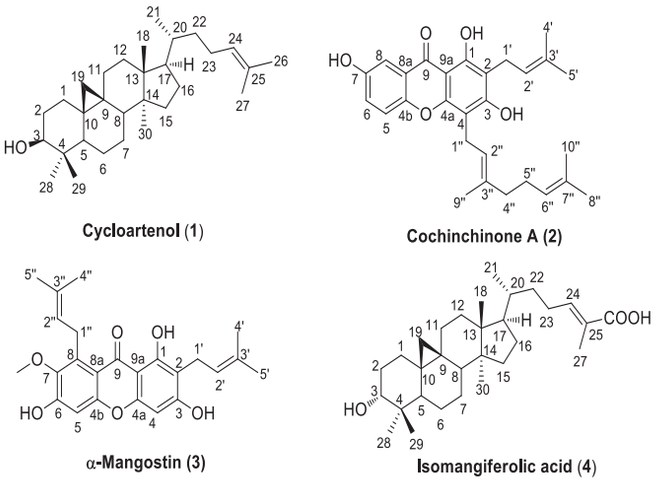

Fig. 1. Chemical structures of compounds 1-4.

The ^{13}C-NMR spectrum showed the presence of a carbonyl group at a δ_C of 183.1 (C-9), a methoxy group at a δ_C of 61.3 (7-OMe), and four methyl groups at δ_C values of 26.0 (C-4'), 25.9 (C-4''), 18.3 (C-5') and 17.9 (C-5''). Based on the spectral analysis, compound **3** was identified as *α*-mangostin. The analytical NMR data of **3** are identical with those previously published [9].

Compound **4** was isolated as a white solid. The ESI-MS showed a protonated molecular ion peak *m/z* of 457 $[M+H]^+$, which suggested the molecular formula of **4** is $C_{30}H_{48}O_3$ (M=456). The ^{1}H-NMR spectrum of **4** is similar to that of compound **1** and showed the signals of a cycloartane-type triterpene with 2 protons at δ_H of 0.55 (1 H, d, H-19) and 0.38 (1 H, d, H-19). However, in the NMR spectrum, only six methyl groups were displayed at δ_H 1.04 (3 H, s, H-18), 0.98 (3 H, s, H-28), 0.96 (3 H, d, *J*=6.5 Hz, H-21), 0.95 (3 H, s, H-30) and 0.89 (3 H, s, H-29). The ^{13}C-NMR and DEPT spectra of **4** showed 30 carbon signals including the signal of a carboxylic acid group at δ_C 173.0 (C-26), signals of 2 olefinic carbons at δ_C 145.7 (C-24) and 126.6 (C-25), a signal of an oxymethine group at δ_C 78.8 (C-3) and signals of 6 methyl groups at δ_C values of 25.4 (C-28), 19.3 (C-30), 18.1 (C-21), 18.0 (C-18), 14.0 (C-29), and 11.9 (C-27). Therefore, compound **4** was determined as isomangiferolic acid. The NMR data of **4** agreed with the values in the reported literature [10].

The cycloartan triterpenes and xanthones from the propolis of *Lisotrigona cacciae* collected in Hoa Binh were also found from the propolis in Binh Dinh province [4]. Cycloartenol and isomangiferolic acid were found in the *Mangifera indica* tree (Xoài), which is a common plant source of bee propolis [2, 4, 10, 11]. Cochinchinone A is only isolated from the plant *Cratoxylum cochinchinense* (Thành Ngạnh Nam) while *α*-mangostin is usually found

in *Cratoxylum cochinchinense* and the *Garcinia* species [8, 9, 12, 13]. Therefore, *Mangifera indica* and *Cratoxylum cochinchinense* trees are possibly the plant sources of this *Lisotrigona cacciae* propolis.

Table 1. Antimicrobial activity of EtOH extract and isolated compounds.

Samples	MIC (µg/ml)						
	E. faecalis	*S. aureus*	*B.cereus*	*E.coli*	*P. aeruginosa*	*S. enterica*	*C.albicans*
EtOH extract	32	32	8	na	64	na	128
Compound **1**	64	128	256	na	128	na	256
Compound **2**	16	32	16	na	64	na	128
Compound **3**	1	1	1	16	2	32	1
Compound **4**	1	64	128	na	2	na	16
Streptomycin	256	256	128	32	256	128	-
Cyclohexamide	-	-	-	-	-	-	32

na: not active; - : not tested.

The propolis EtOH extract and isolated compounds were tested for antimicrobial activity. As shown in Table 1, the EtOH extract displayed selective antimicrobial activity against Gram (+) strains over Gram (-) strains and the *C. albicans* fungus. The EtOH extract exhibited good activity on *B. cereus* with an MIC value of 8 µg/ml. Among the isolated compounds, *α*-mangostin **(3)** displayed the strongest activity against three Gram (+) strains, *P. aeruginosa,* and *C. albicans* with MIC values ranging between 1-2 µg/ml. *α*-Mangostin also had moderate activity on *E. coli* and *S. enterica*. Isomangiferolic acid (**4**) showed strong activity against *E. faecalis* and *P. aeruginosa* with MIC values of 1 and 2 µg/ml, respectively.

Conclusions

The phytochemical investigation on the propolis of the stingless bee *Lisotrigona cacciae* from the Hoa Binh province led to the isolation of four compounds including cycloartenol (**1**), cochinchinone A (**2**), *α*-mangostin (**3**), and isomangiferolic acid (**4**). The plants *Mangifera indica* and *Cratoxylum cochinchinense* were possible resin sources of the *L. cacciae* propolis. The EtOH extract showed good antimicrobial activity on Gram (+) strains *B. cereus* with an MIC value of 8 µg/ml. *α*-Mangostin (**3**) was the most active compound displaying strong activity against the five strains *B. cereus*, *E. feacalis, S. aureus*, *P. aeruginosa,* and the fungus *C. albicans* with MIC values ranging between 1-2 µg/ml. Isomangiferolic acid (**4**) also exhibited strong antimicrobial activity against *E. faecalis* and *P. aeruginosa.*

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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