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Research article

Isolation, structure elucidation and quantitative analysis of callicarpone and oleanolic acid in the leaves of *Callicarpa candicans* collected from different provinces in Thailand

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Abstract

Callicarpa candicans ('khi-on-don' in Thai) is a plant that has been traditionally recognized for its potential use as a piscicidal agent. Two major compounds were separated from the dichloromethane extract from the leaves of *C. candicans* using solvent-solvent extraction and a column chromatographic technique. These compounds were structurally identified using spectroscopic techniques as callicarpone and oleanolic acid. Quantitative analysis of callicarpone and oleanolic acid using high performance liquid chromatography (HPLC) was developed and validated. The callicarpone and oleanolic acid contents in *C. candicans* leaves collected from different provinces in Thailand were analyzed using the validated HPLC method. The callicarpone and oleanolic acid contents in *C. candicans* leaves were in the ranges 0.04–0.23 g% and 0.44–0.88 g% of dried leaves, respectively. The leaves of *C. candicans* collected from Satun province had the highest callicarpone and oleanolic acid contents (0.23 g% and 0.88 g% of dried leaves, respectively). The HPLC analytical method using callicarpone and oleanolic acid as markers could be used for future standardization and quality control of the extract of *C. candicans* leaves.

Introduction

The genus *Callicarpa* is a large genus in the family Lamiaceae consisting of about 140 species mainly distributed in temperate, subtropical and tropical climates in Asia, America, Australia and the Pacific islands, with 12 species having been reported as endemic plants in Thailand (Ahmad et al., 1976).

Callicarpa candicans (Burm.f.) Hochr. ('khi-on-don' in Thai), one of the plants in the *Callicarpa* species has been found in Thailand. It is a shrub which can grow up to 2–4 m in height. This plant has an ovate to broadly elliptic leaf shape, an attenuated leaf base, pink, violet or pinkish-violet corolla, a glabrous ovary with dense grayish-white stellate hairs on the abaxial surface of leaves and on the outer calyx and the fruits are small and fleshy with a hard, inner layer enclosing a single seed (Anaya et al., 2003).

C. candicans is one of Thai traditional plants that is promoted for its medicinal and agricultural benefits and it has various ethnobotanical uses such as the leaves being used as fish poison and for the treatment of abdominal troubles, amenorrhea, wounds and asthma (Cantrell et al., 2005; Braamley, 2009). The phytochemicals that have been reported in the *Callicarpa* species include diterpenes such as callicarpone, calliphyllin, callicarpenal and calliterpenone (Chatterjee

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et al., 1972; Favel et al., 1994; Gao et al., 2000; Hu et al., 2001; Hu et al., 2002; Jesus et al., 2015), triterpenes such as α-amyrin, β-amyrin, betulinic acid, oleanolic acid and ursolic acid (Kawazu and Mitsui, 1966; Kawazu et al., 1967a; Kawazu et al., 1967b; Jones et al., 2007; Leeratiwong et al., 2009) and some flavonoids and lignans (Hu et al., 2001; Murray et al., 2013). Because of the regulations and limitations on the uses of chemical insecticides nowadays, natural insecticides are promoted to be used due to their effectiveness, low toxicity to humans and the environment, and the availability of raw materials (Ren et al., 2001). Callicarpone, an active component present in the leaves of *C. candicans*, has been reported to exhibit a 10-times stronger toxicity against loach fish (*Misgurnus anguillicaudatus*) than did rotenone (Chatterjee et al., 1972; Favel et al., 1994; Seebacher et al., 2003). However, the isolation and structure elucidation of callicarpone are still unclear. Therefore, this study was set up to isolate two major components in the leaves of *Callicarpa candicans* (callicarpone and oleanolic acid) using column chromatographic techniques. Then the structure elucidation and quantitative analysis of these compounds in *C. candicans* leaf samples, collected from various provinces in Thailand, were conducted using spectroscopic techniques and a validated high performance liquid chromatographic (HPLC) method, respectively.

Materials and Methods

Plant materials and extraction

Five leaf samples of *C. candicans* were collected between December 2010 and February 2011 from Mueang district, Songkhla province, Ao Luek district, Krabi province, Mueang district, Satun province, Phu Kradueng district, Loei province and Pak Chong district, Nakhon Ratchasima province. Plant samples were identified by Dr. Charan Leeratiwong, a botanist from the Department of Biology, Faculty of Science, Prince of Songkhla University, Songkhla, Thailand. Plant specimens were deposited at the Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. All plant samples were dried at 40°C in a hot-air oven and were then finely powdered. Pulverized plant samples (1.6 kg) were extracted three times with dichloromethane (plant:solvent ratio = 1:10 weight per volume) under sonication for 1 hr and then filtered. The combined filtrates were evaporated under a reduced pressure to yield 129.59 g of extract (8.09% weight per weight (w/w) yield).

Chemicals and reagents

Oleanolic acid was obtained from Chromadex Inc. (Genay, France). Silica gel Si-60 for the column chromatography with a particle size of 63–200 µm was purchased from Merck (Darmstadt, Germany) All other reagents and solvents were purchased from Labscan Asia (Bangkok, Thailand) and were either analytical or HPLC grades. Deionized water was used throughout. Polytetrafluoroethylene (PTFE), solid phase extraction cartridges were obtained from Whatman (Kent, UK).

Isolations of compound 1 and compound 2

Dichloromethane extract was defatted to remove non-polar compounds such as oils, waxes and chlorophyll using a solvent-solvent partition method. The extract (116.65 g) was dissolved in 500 mL of 10% aqueous methanol and was extracted three times with an equal volume of hexane. The separated layers were concentrated under reduced pressure to give a hexane soluble fraction of 73.88 g (63.33% w/w yield) and an aqueous methanolic fraction of 30.26 g (25.94% w/w yield). The aqueous methanolic portion (19.25 g) was dissolved in 100 mL methanol and thoroughly mixed with an equal weight of silica gel. The solvent was allowed to evaporate off and the extract impregnated with silica gel was applied onto the top of a silica gel column (200 g Si-60, particle size 63–200 µm, 25.0 cm length × 5.0 cm internal diameter). A step-wise gradient elution using hexane and ethyl acetate was carefully applied (1–3 bed volume) so as not to disturb the silica gel bed. Fractions were collected in round-bottomed flasks, rotary evaporated and then combined according to similar thin layer chromatography (TLC) patterns. Eight fractions (Fr.1–8) were obtained. Fr.5 (2.76 g) was separated on a silica gel Si-60 column (150 g Si-60, particle size 63–200 µm, 25.0 cm length × 5.0 cm internal diameter) using a mixture of hexane-ethyl acetate (55:45) as the mobile phase. A fraction size of 10 mL was collected. Fractions were combined according to similar TLC patterns. A medium-polar, terpenoid-enriched fraction of 735.4 mg was obtained from Fr. 65–78 which was re-chromatographed on a silica gel column (100 g Si-60, particle size 63–200 µm, 25.0 cm length × 5.0 cm internal diameter) using chloroform-diethyl ether (4:1) to produce 252.4 mg of compound 1 enriched fraction. Further purification was done using low pressure column chromatography (LPCC; Lobar RP-18, water-methanol 30:70) to produce 61.2 mg of semi pure compound 1. Re-chromatographing on Lobar RP-18 (water-methanol 40:60) produced 30.9 mg of compound 1. In addition, Fr.4 (4.04 g) was separated on a silica gel Si-60 column (200 g Si-60, particle size 63–200 µm, 29.0 cm length × 5.0 cm internal diameter) using a mixture of hexane:ethyl acetate (70:30) as the mobile phase. Fractions (each of 10 mL fraction size) were collected and combined according to similar TLC patterns. A triterpenoid dominant fraction (491.1 mg), eluting at a retention volume of 170–340 mL, was obtained and further re-chromatographed on a second silica gel column (80 g Si-60, particle size 63–200 µm, 20.0 cm length × 2.0 cm internal diameter) with a mixture of dichloromethane and methanol (98:2) as the mobile phase to give 232.3 mg of the target sub-fraction at a retention volume of 190–350 mL. On a subsequent separation using LPLC with methanol and water (90:10) as the mobile phase with TLC monitoring, a compound 2 enriched fraction (EF2, 105.6 mg) was obtained at a retention volume of 440–540 mL. Furthermore, 36.4 mg of compound 2, was obtained after repeated Lobar chromatography of EF2 using methanol and water (70:30) as the mobile phase.

Structural identification of isolated compounds

The 1D- and 2D-nuclear magnetic resonance (NMR) spectra of

the isolated compounds were investigated, using an Avance 500 (500 MHZ; Bruker; Germany), in $CDCl_3$ with tetramethylsilane (TMS) as the internal standard. A field gradient technique using Proforma 1 equipment, was used for the heteronuclear single quantum correlation and heteronuclear multiple quantum correlation experiments. The NMR spectra of the compound were measured on a Jeol JMN A-500 (JEOL Ltd; Japan), from the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand.

The infrared (IR) spectra (KBr disc) of the isolated compounds were investigated using a Fourier-transform infrared spectrometer (Magna-IRTM spectrometer 550 Nicolet; LabX; Canada) at the Central Laboratory, Faculty of Pharmacy, Mahidol University, Thailand.

ESI mass spectrometry (MS) spectra of the isolated compounds were investigated using a mass spectrometer (LC-10 ADvp, Bruker micro Q-TOF; Bruker; Germany) at the Central Laboratory of the Faculty of Pharmacy, Mahidol University, Thailand.

UV spectral analysis was carried out using an ultraviolet (UV) detector (UA-6; Isco; USA). Each sample solution was prepared in methanol at a concentration of 1 mg/mL.

Analysis of callicarpone and oleanolic contents by HPLC

The HPLC method was performed on a Waters unit, equipped with a Waters 600 Controller pump, a Waters 2996 photodiode array detector, and a Waters 717 Auto-sampler plus with a 250 µL loop. An AX-Bridge Shield RP18 column (5 µm particle size, 150 mm length × 4.6 mm internal diameter) was used for the analysis. The HPLC column was maintained at room temperature. HPLC analysis of the extracts was carried out using the conditions applied from the methods for terpenoid analysis previously reported (Waksmundzka-Hajnos and Sherma, 2010). The gradient mode of the mobile phases with water containing 0.005% trifluoroacetic acid (eluent A) and acetonitrile containing 0.005% trifluoroacetic acid (eluent B) was used. The flow rate was 1.0 mL/min and the injection volumes were 20 µL for plant samples and 5 µL for oleanolic acid and callicarpone. The chromatograms were monitored at 206 nm and 266 nm for oleanolic acid and callicarpone, respectively. An electrospray-ionization mass spectrometer (ES MS) detector was used to identify the peak of callicarpone compared with the information of callicarpone previously reported. Dried *C. candicans* leaf powders (5 g) collected from different provinces were separately extracted with 50 mL of methanol using the sonication method for 30 mins and then filtered. The process was repeated three times. The filtrates were combined and concentrated under vacuum and the final volume was adjusted to 50 mL with methanol. It was filtered through a 0.45 µm PTFE syringe filter and applied to the HPLC analysis as mentioned before (Hu et al., 2005). Standard oleanolic acid purchased from Chromadex Inc. (Genay; France) and isolated callicarpone obtained from the isolation process, were used in the validation and quantitative analysis of oleanolic acid and callicarpone in the *C. candicans* leaf extracts.

Statistical analysis

The results of the quantitative analysis of the callicarpone and oleanolic acid contents in *C. candicans* leaves from different locations in Thailand using HPLC were reported as the mean ± SD of triplicate analysis. Least significant difference was used to compare means ($p < 0.05$). All analyses were performed using the SPSS for Windows software package (version 16.0; SPSS Inc.; USA).

Results and Discussion

Isolation and identification of compound 1 and compound 2

The separation of the dichloromethane extract from the leaves of *C. candicans* from Krabi province, using solvent-solvent extraction and column chromatography, yielded three compounds. The separated compounds were structurally identified using spectroscopic techniques (UV, IR, ^{1}H NMR and ^{13}C NMR) as callicarpone (compound 1, 3.92 mg/100g dried leaves) and oleanolic acid (compound 2, 3.37 mg/100g dried leaves). The amounts of callicarpone and oleanolic acid in the leaf extracts of *C. candicans* collected from different locations in Thailand were quantitatively analyzed using a validated HPLC method. The callicarpone contents in *C. candicans* were in the range 0.04–0.23 g% of dried leaves while oleanolic acid contents were in the range 0.44–0.88 g% of dried leaves.

Compound 1

Compound 1 (30.9 mg, 3.92 mg% of dried leaves) was obtained as a yellow amorphous compound, soluble in chloroform. TLC analysis of compound 1 (silica gel 60 F254, solvent system chloroform-diethyl ether, 4:1) showed a single green spot after being sprayed with 5% vanillin in a concentrated sulfuric acid spraying reagent (R_f 0.38). The ultraviolet absorption spectrum of compound 1 showed maximum absorption (λmax) at 266.5 nm. The electrospray ionization mass spectrum exhibited a peak corresponding to $(M+H)^+$ at m/z 333.17, therefore the molecular mass of compound 1 was deduced to be 332.

IR spectra (KBr disc): The IR spectra showed an IR peak of the alcohol hydrogen group (O-H stretching) at 3,446 cm^{-1}, aldehyde C-H stretching at 2,919 cm^{-1}, ketone group (C=O stretching) at 1,739cm^{-1}, unsaturated alkene group (C=C stretching) at 1,677 cm^{-1}, asymmetric bending and scissoring of CH_3 at 1,444 cm^{-1}, OH bending at 1,370 cm^{-1} and epoxide symmetric ring stretching at 1,238 cm^{-1}.

^{1}H NMR: As shown in Table 1, the 1H NMR spectrum of compound 1 displayed singlet signals of five tertiary methyl protons at 1.20 (3H, H-16), 1.25 (3H, H-17) 0.99 (3H, H-18), 0.99 (3H, H-19) and 1.40 (3H, H-20). Signals at 2.48 (1H, dd, J =20.93 Hz) and 3.37 (1H, dd, J =19.18, 1.22 Hz) were assigned to H-14β and H-14α, respectively, the more de-shielded of which showed long range coupling with protons at 3.75 (1H, d, J =1.22 Hz) assignable to H-12. In addition to the presence of two methylene protons at C-14, four pairs of nonequivalent methylene protons were observed at 1.95 (1H, H-6α, *dd*, J = 15.03, 2.96 Hz), 2.56 (1H, H-6β, *dd*, J = 15.19,

2.01 Hz), 2.74 (1H, H-1β, *dd*, *J* =17.23, 2.91 Hz), 1.40 (1H, H-1α), 1.04 (1H, H-2β, t, *J* =3.52 Hz) and 1.40 (1H, H-2α), 1.66 (2H, H-3). The signal at δ 2.34 (1H, m) was assigned as H-5. Table 1 shows the ^{1}H NMR (500 MHz, $CDCl_3$) correlations of compound 1. The ^{1}H NMR data of compound 1 corresponded to the reported information of callicarpone (Chatterjee et al., 1972; Favel et al., 1994).

^{13}C NMR: As shown in Table 2, the ^{13}C NMR (126 MHz, $CDCl_3$) and the distortionless enhancement by polarization transfer experiments showed 20 signals ascribed to: five methyl carbons (CH_3); 19.61 (C-16), 21.09 (C-17), 20.92 (C-18), 27.13 (C-19), and 22.29 (C-20); five methylene carbons (CH_2); 36.12(C-1), 18.26 (C-2), 35.73 (C-3), 29.84 (C-6), and 23.64 (C-14); two methine carbons (CH); 49.84 (C-5), and 59.78 (C-12); and eight quaternary carbons (C); 40.32 (C-4), 199.71 (C-7), 137.36 (C-8), 151.99 (C-9), 36.82 (C-10), 199.16 (C-11), 62.65 (C-13) and 71.48 (C-15).

^{1}H-^{1}H COSY: The ^{1}H-^{1}H COSY showed cross-peaks from ^{1}H-^{1}H dipolar couplings of H-1β to H-6α, H-1β to H-6β H-3 to H-5, H-6α to H-6β, H-12 to H-14α and H-14α to H-14β, respectively (Table 1).

HSQC and HMBC: The HSQC and HMBC experiments suggested the proton-carbon single bond correlations and multi-bond proton carbon correlations, respectively. The HSQC correlations showed C-H connectivities from H-1α to C-1, H-2α to C-2, H-3 to C-3, H-12 to C-12, H-14α to C-14, H-14β to C-14, H-16 to C-16, H-19 to C-19, and H-20 to C-20, together with their chemical shift values. The HMBC correlation of the proton H-1α (δ 1.40) to C-3 (δ 35.73), C-4 (δ 40.32), C-5 (δ 49.84), H-1β (δ 2.74) to C-9 (δ 151.99), H-2α (δ 1.40) to C-3 (δ 35.73), C-4 (δ 40.32), C-5 (δ 49.84), H-3 (δ 1.66) to C-4 (δ 40.32), H-5 (δ 2.34) to C-6 (δ 29.84), H-6α to (δ 1.95) to C-4 (δ 40.32), C-18 (δ 20.92), H-6β to (δ 1.95) to C-4 (δ 40.32), C-5 (δ 49.84), H-12 (δ 3.75) to C-9 (δ 151.99), C-11 (δ 199.16), C-13 (δ 62.65), H-14α (δ 3.37) to C-9 (δ 151.99), C-11 (δ 199.16), C-13 (δ 62.65), H-14β (δ 2.48) to C-9 (δ 151.99), C-11 (δ 199.16), C-13 (δ 62.65), C-16 (δ 19.61), H-18 (δ 0.99) to C-3 (δ 35.73), C-5 (δ 49.84), H-19 (δ 0.99) to C-3 (δ 35.73), C-5 (δ 49.84), H-20 (δ 1.40) to C-3 (δ 35.73) and C-5 (δ 49.84), respectively (Table 2).

According to the above spectral data, compound 1 was proposed to be 1, 2, 3, 4, 4a, 5, 7, 7a, 8a, 9b-decahydro-7a-(1-hydroxy-1-methylethyl)-4, 4, 9b-trimethylphenanthro [2, 3-b] oxirene-6, 9-dione (callicarpone).

Compound 2

Compound 2 (36.4 mg, 3.37 mg% of dried leaves) was obtained as a white amorphous compound, soluble in chloroform and methanol. TLC analysis of compound 2 (silica gel 60 F254, solvent system chloroform-diethyl ether, 4:1) showed a single pink spot in visible light after spraying with 10% sulfuric acid spraying reagent and appeared as pink band under UV 366 nm (R_f0.35). The ultraviolet absorption spectrum of compound 2 showed the maximum absorption (λmax) at 206 nm. The electrospray ionization mass spectrum exhibited a peak corresponding to (M+Na) + at m/z 479.3; therefore, the molecular mass of compound 2 was deduced to be 456.3.

IR spectrum (KBr disc): The IR spectrum (KBr disc) of compound 2 showed an IR peak of the alcohol hydrogen group (O-H stretching) at 3449 cm^{-1}, methyl C-H stretching at 2946 cm^{-1}, methylene C-H stretching at 2863 cm-^{1},ketone group (C=O stretching) at 1691 cm^{-1}, asymmetric bending and scissoring of CH_3- at 1459 cm^{-1}, CH_2-stretching at 1386 cm^{-1}, C-H bending at 1264 cm^{-1} and C-O- stretching at 1030 cm^{-1}.

Table 1 1H nuclear magnetic resonance correlations of compound 1 (500 MHz, $CDCl_3$).

Proton	Chemical shift (δ_H, ppm) (multiplicity)	J (Hz)	^{1}H-^{1}H COSY	Literature chemical shift (δ_H, ppm) (Kawazu et al., 1967a; 1967b)
1α	1.40 (*s*)*	-	6[b]	-
1β	2.74 (*dd*)	2.91, 17.23	6b, 6[a]	-
2α	1.40 (*s*)*	-	-	-
2β	1.04 (*t*)	3.52	-	-
3	1.66 (*m*)	-	5, 14[a]	-
5	2.34 (*m*)	-	-	-
6α	1.95 (*dd*)	2.96, 15.03	-	-
6β	2.56 (*dd*)	2.01, 15.19	1b, 6[a]	-
12	3.75 (*s*)	1.22	14b	3.71 (1H, J=1.2)
14α	2.48 (*dd*)	5.04, 20.93	14b, 3	3.41 (1H, J=1.2, 19.5)
14β	3.37 (*dd*)	1.22, 19.18	14a, 12	2.43 (1H, J=19.5)
16	1.20 (*s*)	-	-	1.18 (3H)
17	1.25 (*s*)	-	-	1.29 (3H)
18	0.99 (*s*)**	-	-	0.92 (3H)
19	0.99 (*s*)**	-	-	0.90 (3H)
20	1.40 (*s*)*	-	14[b]	1.39 (3H)

ppm = parts per million.

*signal from H-1α overlaps with signals from H-2α and H-20.

**signal from H-18 overlaps with signal from H-19.

Table 2 ^{13}C nuclear magnetic resonance, heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) of compound 1 (126 MHz, $CDCl_3$).

Carbon	Chemical shift (δ_C, ppm)	Type of carbon	HSQC with H	HMBC with H
1	36.12	Methylene	1[b]	-
2	18.26	Methylene	1[a], 2[a], 20	-
3	35.73	Methylene	3, 6[b] 14[a]	2, 16, 18, 19
4	40.32	Quaternary	-	1[a]
5	49.84	Methane	6[a]	-
6	29.84	Methylene	6[b]	17
7	199.71	Quaternary	-	14[b]
8	137.36	Quaternary	-	14[b]
9	151.99	Quaternary	-	12, 14[b],
10	36.82	Quaternary	-	-
11	199.16	Quaternary	-	12
12	59.78	Methane	12	14[b]
13	62.65	Quaternary	-	14[b]
14	23.64	Methylene	14[b]	-
15	71.48	Quaternary	-	-
16	19.61	Methyl	16[a]	-
17	21.09	Methyl	-	-
18	20.92	Methyl	5, 18	-
19	27.13	Methyl	18, 19	-
20	22.29	Methyl	20	-

ppm = parts per million.

^1H NMR: As shown in Table 3, the ^1H NMR spectrum (500 MHz, $CDCl_3$) of compound 2 showed seven singlet signals of tertiary methyl protons (CH_3) at 1.24 (3H, H-23), 1.03 (3H, H-24), 0.91 (3H, H-25), 1.06 (3H, H-26), 1.34 (3H, s, H-27), 0.96 (3H, H-29) and 1.03 (3H, H-30). Signals for methylene protons (CH_2) were found at 1.03 (1H, H-1α), 1.59 (1H, H-1β), 1.83 (2H, H-2), 1.39 (1H, H-6β), 1.58 (1H, H-6α), 1.56 (1H, H-7β), 1.58 (1H, H-7α), 1.97 (2H, H-11), 1.25 (1H, H-15α), 2.30 (1H, H-15β), 1.97 (1H, H-16β), 2.13 (1H, H-16α), 1.35(1H, H-19β), 1.82 (1H, H-19α), 1.25 (1H, H-21β), 1.47 (1H, H-21α),1.82 (1H, H-22α) and 2.03 (1H, H-22β). Methine protons were found at 0.89 (1H, H-5), 1.70 (1H, H-9), 3.31 (1H, dd, H-18), δ 3.47 (1H, dd, H-3) and δ 5.50 (1H, H-12), respectively.

^{13}C NMR: The ^{13}C NMR (126 MHz, $CDCl_3$) signal corresponding to the carboxyl C-28 appeared at 180.23. The spectral data were similar to those reported for oleanolic acid showing 30 signals (from C-1 to C-30) of seven methyl carbons (CH_3); 28.82 (C-23), 16.54 (C-24), 15.57(C-25), 17.54(C-26), 26.17(C-27), 33.30(C-29) and 23.81(C-30). Methylene carbons (CH_2) were found at 38.90 (C-1), 28.11 (C-2), 18.80 (C-6), 33.30 (C-7), 23.82 (C-11), 28.34 (C-15), 23.92 (C-16), 46.57 (C-19), 34.28 (C-21) and 33.59 (C-22). Methine carbons (CH) were found at 78.15 (C-3), 55.83 (C-5), 48.15 (C-9), 42.04 (C-18) and 122.48 (C-12). Seven quaternary carbons (C) were found at 39.42 (C-4), 39.76 (C-8), 37.48 (C-10), 144.93 (C-13), 42.19 (C-14), 46.71 (C-17) and 30.99 (C-20).

HSQC and HMBC: The ^1H-^{13}C HSQC and HMBC spectra of compound 2 suggested proton-carbon single bond correlations and multi-bond proton carbon correlations, respectively. The HSQC correlations showed C-H connectivities from H-12 to C-12, H-18 to C-18 and H-22 to C-22. The HMBC correlations from H-18 at 3.31 showed long range connectivity with a substantial number of carbons, namely C-13 at 144.93, C-17 at δ 46.71 and C-19 at δ 46.57 (two-bonds interaction) and C-12 at 122.48, C-14 at 42.19, C-16 at 23.92 and C-28 at 180.23 (three-bonds interaction), respectively.

According to the above spectral data, compound 2 was proposed to be (3β)-3-hydroxyolean-12-en-28-oic acid (oleanolic acid).

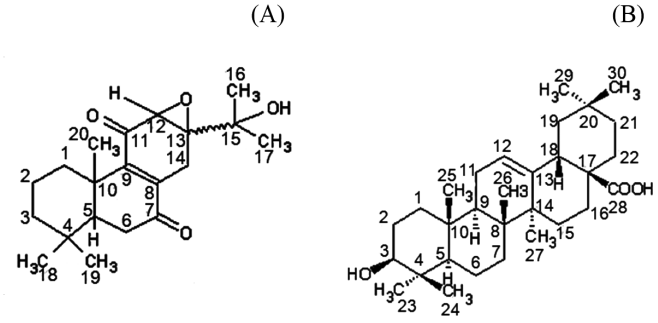

Fig.1 Chemical structures of: (A) callicarpone; (B) oleanolic acid

Table 3 ^{1}H and ^{13}C nuclear magnetic resonance correlations of compound 2 (500 MHz, $CDCl_3$)

Carbon	(δ_C, ppm)	δ_H, ppm (multiplicity)	HMBC from H to C	Literature (Soejarto, 2002)	
				δ_C, ppm	δ_H, ppm (multiplicity)
1α	38.90	1.03*	-	39.0	1α 1.02
1β	-	1.59	-	-	1β 1.57
2α	28.11	1.83	-	28.1	2α 1.82
2 β	-	1.83	-	-	2β 1.82
3	78.15	3.47 (*dd*)	-	78.2	3.44 (*dd*)
4	39.42	-	-	39.4	-
5	55.83	0.89	-	55.9	0.88 (*d*)
6α	18.80	1.58**	-	18.8	6α 1.58
6β	-	1.39	-	-	6β 1.39
7α	33.30	1.58**	-	33.4	7α 1.53
7β	-	1.56	-	-	7β 1.36
8	39.76	-	-	39.8	-
9	48.15	1.70	-	48.2	1.71 (*tr*)
10	37.48	-	-	37.4	-
11α	23.82	1.97	-	23.8	11α 1.96
11β	-	1.97	-	-	11β 1.96
12	122.48	5.50	-	122.6	5.49
13	144.93	-	-	144.8	-
14	42.19	-	-	42.2	-
15α	28.34	1.25	-	28.4	15α 1.22
15β	-	2.30	-	-	15β 2.19
16α	23.92	2.13	-	23.8	16α 2.12 tr
16β	-	1.97	-	-	16β 1.96
17	46.71	-	-	46.7	-
18	42.04	3.31 (*dd*)	C12, C13, C14, C16, C17, C19, C28	42.1	3.30 (*dd*)
19α	46.57	1.82	-	46.6	19α 1.83
19β	-	1.35	-	-	19β 1.32
20	30.99	-	-	31.0	-
21α	34.28	1.47	-	34.3	21α 1.46
21β	-	1.25	-	-	21β 1.23
22α	33.59	1.82	-	33.2	22α 1.82
22β	-	2.03	-	-	22β 2.04
23	28.82	1.24 (*s*)	-	28.8	1.24 (*s*)
24	16.54	1.03 (*s*)*	-	16.5	1.02 (*s*)
25	15.57	0.91 (*s*)	-	15.6	0.93 (*s*)
26	17.54	1.06 (*s*)	-	17.5	1.04 (*s*)
27	26.17	1.35 (*s*)	C13	26.2	1.30 (*s*)
28	180.23	-	-	180.0	-
29	33.30	0.96 (*s*)	-	33.4	0.97 (*s*)
30	23.81	1.03 (*s*)*	-	23.8	1.02 (*s*)

ppm = parts per million; HMBC = heteronuclear multiple bond correlation.

* signal from H-1α overlaps with signals from H-24 and H-30

**signal from H-6α overlaps with signal from H-7α

Analysis of callicarpone and oleanolic contents using high performance liquid chromatography

The validation for the quantitative analysis of callicarpone and oleanolic acid was conducted using HPLC with the analytical conditions as described in the Materials and methods section (Fig. 2A and 2B, respectively). The linearity of the method was evaluated by analyzing a series of varying concentrations of the reference standards. Five microliters of the 10 standard solutions containing 10–1,000 µg/mL of callicarpone or oleanolic acid were analyzed using HPLC. The standard curve of each standard compound was obtained by plotting the concentrations of the standard versus the peak areas. The slope and intercept values were then determined. The correlation coefficient was calculated using the least-square linear regression method. The R^2 values for both compounds were greater than 0.9990, confirming the linearity of the method. Peak purity was investigated using the diode array detector (DAD) and MS data of all peaks of interest and there was no indication of co-elution or impurities.

The intra-day precision was performed by analyzing the reference standards of callicarpone or oleanolic acid at three different concentrations (100, 200 and 300 µg/mL) on the same day (n = 10). Inter-day precision was carried out in the same manner as the intra-day precision but on 10 different days (n = 5). The relative standard deviation (RSD) was calculated. The intra-day precisions (% RSD) of the 100, 200 and 300 µg/mL analysis of standard callicarpone were 0.51, 0.37 and 0.31, respectively, while the %RSD values for the analysis of oleanolic acid were 1.28, 1.03 and 1.02, respectively. The inter-day precision (%RSD) of callicarpone and oleanolic acid were 0.25 ± 0.17 and 1.20 ± 0.34, respectively. The method can therefore be regarded as precise.

The accuracy of the HPLC method was evaluated using the recovery of callicarpone or oleanolic acid in the *C. candicans* leaf extracts. Standard callicarpone and oleanolic acid in the ranges 75 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL were spiked into *C. candicans* leaf extracts. Each concentration was injected into six supinates using the optimized HPLC conditions. The average recoveries were 99.50 ± 0.19% for callicarpone and 103.04 ± 0.08% for oleanolic acid. The results were in compliance with the USP requirement (80–120%) for quantitative analysis of a major ingredient using the chromatographic method. Therefore, this HPLC method was accurate for the analysis of these compounds in *C. candicans* leaf extracts. Limits of detection (LOD) and limits of quantitation (LOQ) of the compounds were calculated from the standard deviation of the y-intercept of the calibration curve and the slope of the calibration curve. The LOD values of callicarpone and oleanolic acid were 0.17 µg/mL and 0.44 µg/mL, respectively while their LOQ values were 0.52 µg/mL and 1.33 µg/mL, respectively.

The HPLC method was successfully utilized for the quantitative analysis of callicarpone and oleanolic acid in extracts from dried leaves of *C. candicans* collected from different provinces in Thailand. The HPLC chromatogram of extract from the leaves of *C. candicans* collected from Krabi province is shown in Fig. 2C. The positive mode ESI-MS spectrum of compounds from peak at RT 21.802 showed *m/z* at 332 and the DAD absorbance spectrum showed λ_{max} at 266 nm corresponding to the chemical characteristics of callicarpone previously reported (Kawazu et al., 1967a; 1967b). The results suggested the presence of callicarpone in *C. candicans* leaf extract. The callicarpone contents in the leaves of *C. candicans* ranged from 0.04 g% to 0.23 g% while the oleanolic acid contents were in the range 0.44–0.88 g%. As shown in Table 4, leaf samples collected from Satun province contained the highest amounts of callicarpone and oleanolic acid (0.23 g% and 0.88 g% of dried leaves, respectively). Callicarpone was previously reported as an active constituent that promoted a piscicidal effect, while oleanolic acid exhibited various biological activities including insecticidal, antibacterial, antifungal, antiviral and as a cytotoxic to cancer cell lines (Spies, 1933; Seebacher et al., 2003; Shao et al., 2006). Therefore, these two compounds have the potential to be used as markers for the quality control of *C. candicans* raw materials and products.

In conclusion, the separation of dichloromethane extract from the leaves of *C. candicans* using solvent-solvent extraction and column chromatography yielded two compounds (callicarpone and oleanolic acid) which could be used as markers. This was the first report of these two compounds in the leaves of *C. candicans* collected in Thailand.

HPLC methods were developed and validated for quantitative analysis of the callicarpone and oleanolic acid contents in leaf extracts of *C. candicans* collected from different provinces in Thailand. The leaves of *C. candicans* collected from Satun province had the highest callicarpone and oleanolic acid contents, suggesting Satun as the preferable source of *C. candicans* leaves with high amounts of active compounds. The validated HPLC method could be beneficial for further standardization and daily routine quality control of *Callicarpa* leaf extract in the future.

Conflict of Interest

The authors declare that there are no conflicts of interest

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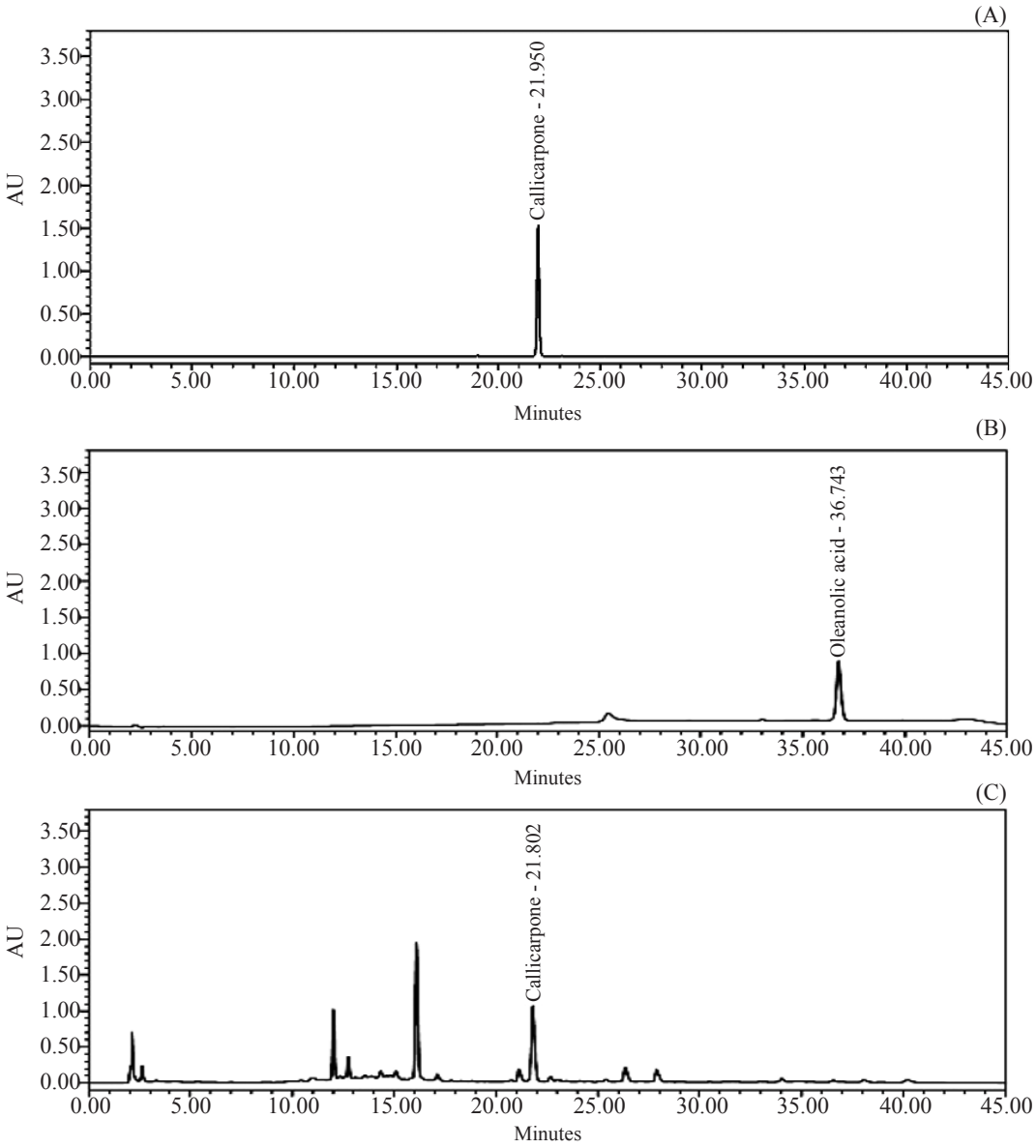

Fig.2 HPLC chromatogram of standard callicarpone (A), standard oleanolic acid (B) and leaf extract of *C. candicans* (C), with high performance liquid chromatography conditions: analytical column was an XBridge Shield RP18 (5 µm, 150 mm × 4.6 mm internal diameter) column. The mobile phases were water containing 0.005% trifluoroacetic acid (eluent A) and acetonitrile containing 0.005% trifluoroacetic acid (eluent B). Gradient flow rate was 1.0 mL/min; ultra violet-visible detection wavelengths at 266 nm (A and C) and 206 nm (B), respectively.

Table 4 Quantitative analysis of callicarpone and oleanolic acid contents in *C. candicans* leaves from different locations in Thailand using high performance liquid chromatography

Collection location	Amount (g per 100 g dry leaves)	
	Callicarpone	Oleanolic acid
Songkhla	$0.04 \pm 0.00^{a,*}$	0.55 ± 0.01^{a}
Krabi	0.14 ± 0.00^{b}	0.64 ± 0.02^{b}
Satun	0.23 ± 0.00^{c}	0.88 ± 0.01^{c}
Loei	0.06 ± 0.00^{d}	0.44 ± 0.00^{d}
Nakhon Ratchasima	0.20 ± 0.00^{e}	0.64 ± 0.01^{b}

Values shown as mean ± SD.

* Different lowercase, superscript letters in the same column are significantly different ($p < 0.05$).

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