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ONE NEW PYRONE FROM THE *KNEMA GLOBULARIA* FRUITS**

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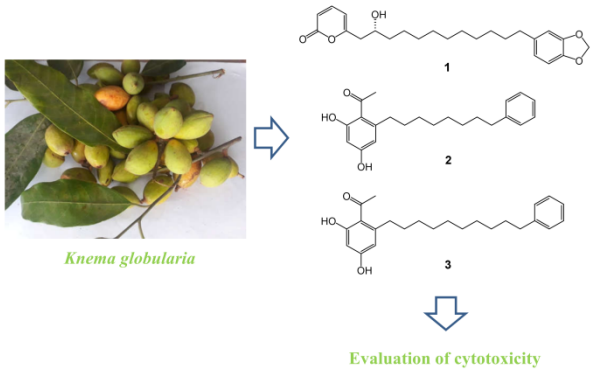
One new pyrone, knecorticosanone I (**1**), was isolated from the fruits of *Knema globularia* collected in Vietnam, together with two known acetophenones, kneglomeratanone A (**2**) and 2,4-dihydroxy-6-(10'-phenyldecyl)-acetophenone (**3**). The chemical structures of the present compounds were identified by means of HRESIMS, 1D and 2D NMR spectroscopy, and by comparisons with the reported data in the literature. The cytotoxicity of three isolated compounds was evaluated against HepG2, MCF-7 and SK-LU-1 cell lines and was tested by a sulforhodamine B assay. Among them, compound **3** had the most potent cytotoxicity against these cancer cell lines with IC_{50} values ranging from 34.76±2.79 to 51.10±2.11 µM. Compounds **1** and **2** exhibited weak inhibitory with IC_{50} values ranging from 81.84±3.57 to 99.25±2.12 µM.



INTRODUCTION

Knema globularia known in Vietnam as "máu chó cầu", is one of the most common trees in warm climates. Its seeds have been used as a treatment for scabies and a blood tonic.[1-2] In the recent years, studies on the chemical constituents and biological activities of *K. globularia*have identified chemical compounds from different plant parts, such as flavonoids, polyketides and steroids.[3-8]Remarkably, most of the new compounds isolated from this plant have showed appreciable cytotoxic activity against Hep-G2, KB, KKU-M156, MCF-7, NCI-H187 and SK-LU-1 cell lines.[6-8]

Previously, we reported the cytotoxicity of components isolated from the *n*-hexane extract of the fruits of *K. globularia*.[7] As part of our continuing study on the phytochemistry of this plant, we report here the isolation, structural elucidation, and evaluation of the cytotoxic activity of one new pyrone, knecorticosanone I (**1**) and two known acetophenones, kneglomeratanone A (**2**) and 2,4-dihydroxy-6-(10'-phenyldecyl)-acetophenone (**3**) from the dichloromethane extract of the fruits of *K. globularia*(Figure 3).[9]

RESULTS AND DISCUSSION

Compound **1** was isolated as an optically active white amorphous powder. From the peak quasimolecular ion at *m/z* 401.2332 $[M+H]^+$ in the positive mode HRESIMS, its molecular formula was established as $C_{24}H_{32}O_5$ (calcd. for $C_{24}H_{33}O_5$,

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401.2328), requiring nine degrees of unsaturation, in conjunction with NMR spectroscopic data. The UV spectrum of **1** in chloroform exhibited maxima at 291 and 343 nm, suggesting an aromatic chromophore. The IR spectrum showed absorption bands of a hydroxyl group (3395 cm^{-1}) and aromatic rings (1634 cm^{-1}).

The ^{1}H NMR spectrum (Table 1) of **1** showed resonances for three aromatic protons belonging to 1,3,4-trisubstituted benzene ring [δ_H 6.67 (1H, d, J = 1.5 Hz, H-2″), 6.71 (1H, d, J = 8.0 Hz, H-5″), 6.61 (1H, dd, J = 8.0, 1.5 Hz, H-6″)], three heteroaromatic methines [δ_H 7.27 (dd, J = 9.5, 6.5 Hz, H-4), 6.19 (d, J = 9.5 Hz, H-3), 6.07 (d, J = 6.5 Hz, H-5)], a methylenedioxy [δ_H 5.91 (2H, s, H_2-1‴)] along with an oxymethine [δ_H 4.04 (1H, m, 2′-OH)]. The high-field region (δ_H 1.28-2.67, 22H) showed methylene resonances attributed to a long hydrocarbon chain.

The ^{13}C NMR and HSQC spectra of **1** (Table 1) revealed resonances for one conjugated carbonyl carbon [δ_C 162.7 (C-2)], three oxygenated sp^2 quaternary carbons [δ_C 163.7 (C-6), 147.5 (C-3″), 145.4 (C-4″)], an sp^2 quaternary carbon [δ_C 136.9 (C-1″)], six sp^2 methine carbons [δ_C 113.7 (C-3), 143.6 (C-4), 104.6 (C-5), 108.9 (C-2″), 108.1 (C-5″), 121.1 (C-6″)], an sp^3 oxygenated methylene carbon [δ_C100.7 (C-1‴)], an sp^3 oxygenated methine carbon [δ_C 69.4 (C-2′)] and nine methylenes (δ_C 25.5-42.0).

The HMBC correlations of H-3 to C-2/C-5, H-4 to C-2/C-6, H-5 to C-3/C-6 as well as an isolated spin system C(3)H-C(4)H-C(5)H in the COSY spectrum (Figure 1) suggested the presence of α-pyrone ring moiety.[10] Next, the HMBC correlations from H-5 to C-1′, H-1′ to C-5/C-6/C-2′/C-3′, and H-3′ to C-1′/C-2′/C-4′ as well as the obvious COSY correlations between H-1′ (δ_H 2.67 and 2.54) and H-2′ (δ_H 4.04) led to the assignment of a α-pyrone ring at C-1′ (δ_C 42.0) and a hydroxyl group at C-2′. The HMBC cross-peaks of H-2″ to C-4″/C-6″, H-5″ to C-1″/C-3″, H-6″ to C-2″/C-4″, H_2-1‴ to C-3″/C-4″ suggested the presence of 1,3,4-trisubstituted benzene ring bearing a methylenedioxy at C-3″ (δ_C 147.5) and C-4″ (δ_C 145.4). This sub-structure was attached to a long straight chain moiety at C-12′ (δ_C 35.7) based on the correlations of H-12′ to C-2″ (δ_C108.9)/C-6″(δ_C121.1), of H-2″/H-6″ to C-12′. In order to determine absolute configuration of C-2′ chiral center, compound **1** was converted to benzoyl derivative (**1a**). The CD spectrum of **1a** exhibited positive, negative Cotton effects at 232 nm (Δε+1.74), 292 nm (Δε-2.49), respectively, indicating the *R* configuration based on the exciton chirality rule (Figure 2). This conclusion was also supported by the fact the optical rotation of **1** had the opposite sign with that of chaetoquadrin F.[11] Consequently, the structure of **1** was elucidated to be 6-(12-(benzo[*d*][1,3]dioxol-5-yl)-2*R*-hydroxydodecyl)-2*H*-pyran-2-one, and was named knecorticosanone I.

The other isolated compounds were identified as kneglomeratanone A (**2**) and 2,4-dihydroxy-6-(10'-phenyldecyl)-acetophenone (**3**).[9] Their chemical structures were established by spectroscopic evidence including 1D, 2D NMR, HRESIMS data and comparisons with those reported in the literature. Compounds **2** and **3** were isolated for the first time from *K. globularia*.

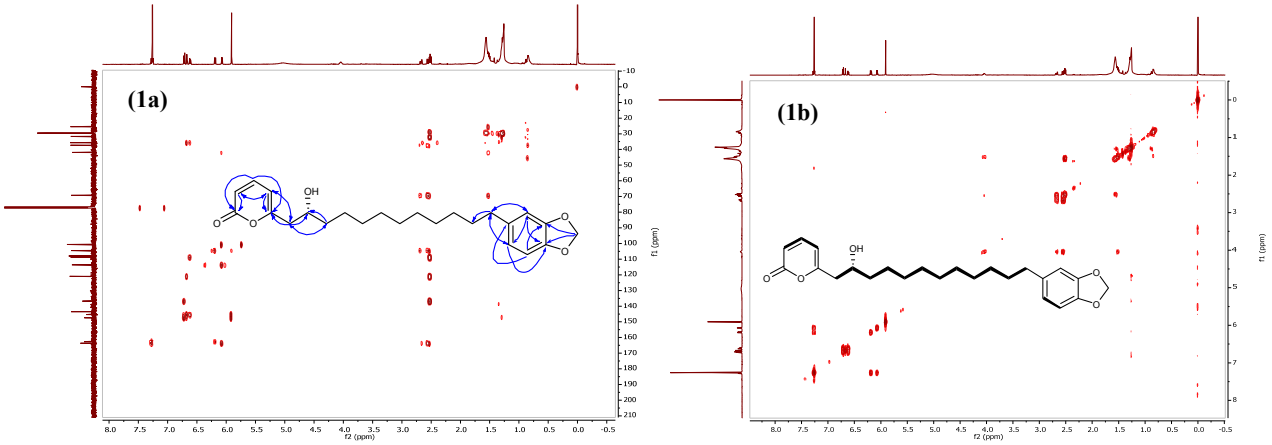

Fig. 1 – Key HMBC (**1a**) and COSY (**1b**) correlations of **1**.

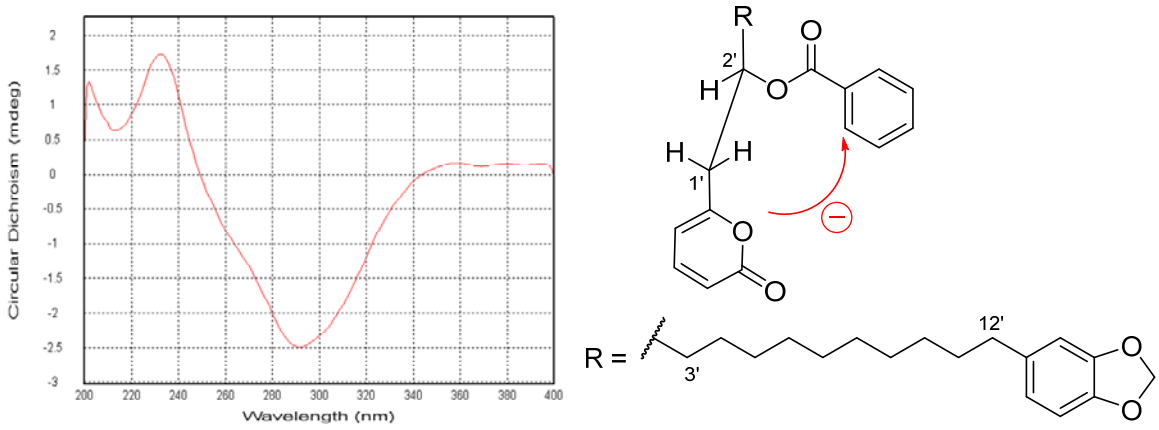

Fig. 2 – CD spectrum of **1a** in methanol.

Table 1

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of **1** in $CDCl_3$ [δ (ppm), J (Hz)]

Position	**1**	
	δ_C	δ_H
1	–	–
2	162.7	–
3	113.7	6.19 d (9.5)
4	143.6	7.27 dd (9.5, 6.5)
5	104.6	6.07 d (6.5)
6	163.7	–
1′	42.0	2.67 dd (14.5, 3.5); 2.54 dd (14.5, 8.5)
2′	69.4	4.04 m
3′	37.4	1.51 t (7.5)
4′	25.5	1.28*
5′	29.8	1.28*
6′	29.6	1.28*
7′	29.6	1.28*
8′	29.5	1.28*
9′	29.5	1.28*
10′	29.2	1.28*
11′	31.8	1.56 t (7.5)
12′	35.7	2.52 t (7.5)
1″	136.9	–
2″	108.9	6.67 d (1.5)
3″	147.5	–
4″	145.4	–
5″	108.1	6.71 d (8.0)
6″	121.1	6.61 dd (8.0, 1.5)
OCH_2O	100.7	5.91 s

Assignments were done by HSQC, HMBC experiments.
*Overlapping signals.

The cytotoxicity of three isolated compounds were evaluated against Hep-G2, MCF-7 and SK-LU-1 cell lines and were tested by a sulforhodamine B assay, at levels comparable to that of ellipticine as the positive control,[7,12-14] and the results are described in Table 2. Among them, compound **3** exhibited the most potent cytotoxicity against these cell lines with IC_{50} values ranging from 34.76 ± 2.79 to 51.10 ± 2.11 μM. Compounds **1** and **2** exhibited weak inhibitory effect against these cell lines with IC_{50} values ranging from 81.84 ± 3.57 to 99.25 ± 2.12 μM. Previous compounds isolated by us also exhibited cytotoxicity against these cell lines, which were more potent than these current compounds.[7] Knecorticosanone B, lacking aryl moiety in the alkyl chain showed stronger activity than compounds **2** and **3**. Comparing the cytotoxicities of **2** and **3** implied that the presence of more two methylene groups in the alkyl chain may be significantly increased the cytotoxic activity. In another way, it is interesting when comparing the activity between knecorticosanone A and malabaricone D, the presence of more two methylene groups in the alkyl chain that bearing a carbonyl group at the terminal reduced the activity.

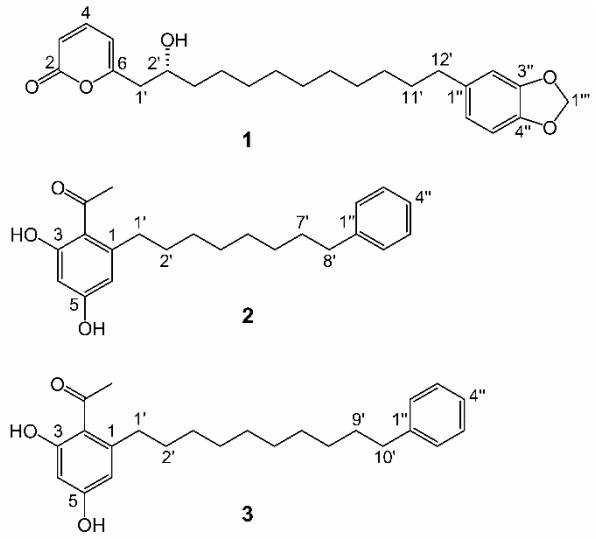

Fig. 3 – Structures of **1–3** isolated from the fruits of *Knema globularia*.

Table 2

Cytotoxicities of **1–3** against human cancer cell lines

Compound	IC_{50}[a] (μM)		
	Hep-G2	MCF-7	SK-LU-1
1	> 100	97.53 ± 1.80	> 100
2	98.20 ± 1.03	81.84 ± 3.57	99.25 ± 2.12
3	51.10 ± 2.11	37.97 ± 2.25	34.76 ± 2.79
Ellipticine[b]	0.33 ± 0.03	0.44 ± 0.03	0.39 ± 0.05

[a] IC_{50} (concentration that inhibits 50% of cell growth).
[b] Positive control.

EXPERIMENTAL

General experimental procedures

Optical rotation was measured with a JASCO-2000 Polarimeter (Hachioji, Japan). UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Infrared spectra were recorded on an IR Prestige-21 spectrometer (Shimadzu, Kyoto, Japan). Circular dichroism spectra were determined on a Chirascan™ CD spectrometer (Applied Photophysics Ltd., Surrey, United Kingdom). A Bruker Avance 500 spectrometer (Bruker, MA, USA), with TMS as an internal reference, was used to measure the NMR spectra. HRESIMS was performed on an Agilent 6545 Accurate-Mass spectrometer (Agilent, CA, USA). Column chromatography was performed using silica gel (60 N, spherical, neutral, 40-50 μm, Kanto Chemical Co., Inc., Tokyo, Japan), Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan), YMC RP-18 (Fuji Silysia Chemical Ltd, Kasugai, Aichi, Japan). Analytical thin-layer chromatography (TLC) was accomplished on pre-coated silica gel $60F_{254}$ and RP-18 F_{254} plates (0.25 or 0.50 mm thickness, Merck KGaA, Darmstadt, Germany). Compounds were visualized under UV radiation (254 and 365 nm) and by spraying the plates with 10% sulfuric acid followed by heating on a hot plate. The human cancer cell lines, Hep-G2, MCF-7 and SK-LU-1, were kindly provided by Prof. Chi-Ying Huang (Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taiwan). Cell culture flasks and 96-well plates were from Corning Inc. (Corning, NY, USA). The ELISA Plate Reader (Bio-Rad, California, USA) was used to measure the absorbance of the cells in the cytotoxicity assay.

Plant material

The fruits of *K. globularia* were collected from Thua Thien Hue province, Vietnam (N16°57'53.0" E106°52'18.5") in July 2019. The plant was identified by Mr. Thao Xuan Hoang, Faculty of Biology, University of Education, Hue University. A voucher specimen (KC-SE-01) was deposited at the Faculty of Chemistry, University of Education, Hue University, Vietnam.

Extraction and isolation

The dried fruits (2.2 kg) of *K. globularia* were powdered with a grinder and extracted successively with *n*-hexane (2.5 L x 6 times) and dichlomethane (DCM) (1.5 L x 4 times) at room temperature. Each of the supernatants was evaporated at under 40°C *in vacuo* to obtain two extracts, crude *n*-hexane (KCH, 700.0 g) and crude DCM (KCD, 55.0 g), respectively.

The KCD extract (55.0 g) was applied to column chromatography on silica gel and subsequently eluted with a gradient system of *n*-hexane-EtOAc (100:0, 90:10, 80:20, 60:40, 50:50, 40:60, 20:80, 10:90, 0:100 v/v, 7.5 L each) to obtain 9 fractions (D1-D9). The D1 (18.0 g) fraction was chromatographed on a normal phase silica gel column, eluted with a gradient of *n*-hexane-acetone (10:1, 5:1, 2.5:1, 1:1, 0:1 v/v, 7.5 L), to obtain 5 subfractions (D1A-D1E), according to their TLC profiles. The subfraction D1C (3.5 g) was subjected to Sephadex LH-20 column chromatography, eluted with

CH_2Cl_2-MeOH (1:1, v/v), and then applied to a YMC RP-18 column, eluted with MeOH-water (6:1, v/v) to yield **2** (26.8 mg) and **3** (32.5 mg). Similarly, fraction D3 (11.0 g) was chromatographed on silica gel column eluted with *n*-hexane-EtOAc (5:1, v/v, 6.6 L) to obtain 9 subfractions (D3A-D3I). The subfraction D3F (175.8 mg) was subjected to a YMC RP-18 column and eluted with MeOH-water (2:1, 1:1, 1:0 v/v, 3.5 L) followed by Sephadex LH-20 column chromatography, eluted with CH_2Cl_2-MeOH (1:1, v/v) and afforded **1** (6.4 mg).

Knecorticosanone I (**1**): White amorphous powder; $[\alpha]_D^{22}$ –4.6° (*c*=0.13, MeOH); UV ($CHCl_3$) λ_{max} (log ε) 291 (6.83), 243 (6.61) nm; IR (KBr) ν_{max} (cm^{-1}): 3395, 2957, 2922, 2851, 1634, 1445, 1234, 1098, 1038, 937, 799, 665; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1; HRESIMS *m/z* 401.2332 $[M+H]^+$ (calcd. for $C_{24}H_{33}O_5$, 401.2328).

Benzoylation of compound 1

Compound **1** (3 mg) was dissolved in the mixture of pyridine (0.5 mL) and benzoyl chloride (0.2 mL) in a sealed tube at room temperature for 30 minutes. The reaction mixture was then diluted with 4 mL water and extracted with EtOAc (2 mL x 3 times). The combined EtOAc extract was washed with 5 mL water (3 times) and then placed in a heating block (37°C), to yield crude product. This was further purified by a silica gel column, eluting with *n*-hexane-EtOAc (10:1, v/v), to afford **1a** (4.7 mg).

Compound (**1a**): Pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.00 (2H, dd, *J* = 1.2, 8.4 Hz, H_{ortho}/OBz), 7.43 (3H, m, H_{para} and H_{meta}/OBz), 7.18 (1H, dd, *J* = 6.6, 9.0 Hz, H-4), 6.71 (1H, d, *J* = 7.8 Hz, H-5″), 6.66 (1H, d, *J* = 1.2 Hz, H-2″), 6.71 (1H, d, *J* = 7.8 Hz, H-5″), 6.60 (1H, dd, *J* = 1.2, 7.8 Hz, H-6″), 6.16 (1H, d, *J* = 9.0 Hz, H-3), 6.02 (1H, d, *J* = 6.6 Hz, H-5), 5.90 (2H, s, OC<u>H</u>$_2$O), 4.22 (1H, m,H-2′), 2.86 (1H, d, *J* = 6.0 Hz, H-1′a), 2.50 (2H, t, *J* = 7.8 Hz, H-12′), 2.51 (1H, d, t, *J* = 7.8 Hz, H-1′b), 1.64 (2H, m, H-11′), 1.54 (2H, m, H-3′), 1.28 (overlapped, H-3′÷H-10′).

SRB Assay for evaluating the cytotoxic activity

Three monolayer human cancer cell lines including LU-1, Hep-G2, and MCF-7 was used in the cytotoxicity assays. Stock cultures were grown in T-75 flasks containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM) with 2 mM -glutamine, 1.5 gL^{-1} sodium bicarbonate and 10% Fetal Bovine Serum (FBS). Media were changed at 48-hours intervals. The cells were dissociated with 0.05% Trypsin-EDTA, sub-cultured every 3-5 days with the ratio of (1:3) and incubated at 37°C under humidified 5% carbon dioxide atmosphere. Tumor cells were cultivated in a humidified atmosphere of 5% CO_2 at 37°C for 48 h. Cell viability was examined by sulforhodamine B (SRB) method for cell density determination, based on the measurement of cellular protein content.[14] Viable cells were seeded in the growth medium (180 µL) into 96-well microplates (4×10^4 cells per well) and allowed to attach overnight. Tested samples were added carefully into each well of 96-well plates and the cultivation was continued under the same conditions for another 72 h. Thereafter, the medium was removed and the remaining cell monolayers are fixed with the cold 20% (w/v) trichloroacetic acid for 1 h at 4°C and stained by 1X SRB staining solution at room temperature for 30 min, after which the unbound dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for optical density determination at 515 nm on an ELISA Plate Reader (Bio-Rad). DMSO 10% was used as blank sample while ellipticine was used as positive control. The cytotoxicity was measured at doses of 100, 20, 4, and 0.8 µM and estimated as a half maximal inhibitory concentration (IC_{50}), which was calculated by the program TableCurve Version 4.0. All experiments were prepared in triplicates. The inhibition rate (IR) of cells was calculated by the following formula IR% = {100% – [(absorbance$_t$ – absorbance$_0$)/(absorbance$_c$– absorbance$_0$)] × 100}, in which: IR: Inhibition rate of cell growth, absorbance$_t$: average optical density value at day 3; absorbance$_0$: average optical density value at time-zero; absorbance$_c$: average optical density value of the blank DMSO control sample.

CONCLUSION

One new pyrone derivative, knecorticosanone I (**1**) has been isolated and structural elucidated by combined spectroscopic data (FT-IR, UV, HRESIMS, CD, 1D and 2D NMR) and by comparison optical rotation with the reported data in the literature, along with two known compounds, kneglomeratanone A (**2**) and 2,4-dihydroxy-6-(10'-phenyldecyl)-acetophenone (**3**) from the fruits of *Knema globularia* (Lam.) Warb. used in Asian ethnomedicine. Their weak to moderate inhibitory effect against three human cancer cell lines (Hep-G2, MCF-7 and SK-LU-1) have been shown in this study.

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