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Chemical Composition of *Clausena lansium* (Lour.) Skeels Leaves and Antifungal Activity

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Abstract – The first study on chemical constituents and biological activities of *Clausena lansium* (Lour.) Skeels (Rutaceae) growing in Vietnam has been done. Phytochemical investigation of *n*-hexane extract led to the isolation of five compounds: dihydroindicolactone (**1**), 8-geranyloxy psoralen (**2**), imperatorin (**3**), heraclenol (**4**) and indicolactone (**5**), in which this is the first report on the presence of dihydroindicolactone (**1**). Their structures were elucidated based on LC/MS/NMR hyphenated techniques as well as comparison with those of literature data. The *n*-hexane extract and its subfractions, ethanol 95% extract and several isolated compounds were evaluated for antifungal activity.
Keywords – Antifungal, *Clausena lansium*, Dihydroindicolactone, Furanocoumarin

Introduction

Clausena lansium (Lour.) Skeels (Wampee) is a tropical species of the Rutaceae family and has a long history of cultivation in Vietnam, China, other Southeastern, and North America. In traditional folk remedies, the leaves of *C. lansium* are used for cough, asthma, viral hepatitis, dermatological and gastro-intestinal diseases. The seed is also used for treating gastro-intestinal diseases like acute and chronic gastro-intestinal inflammation whereas the fruit is used as a vermifuge and for digestive disorders. The halved, sun-dried, immature fruits and slices of dried roots and stems are used as Oriental remedies for bronchitis and malaria in Vietnam.[1] Previous phytochemical investigations of *Clausena* species led to the isolation of coumarins and carbazole alkaloids with antimicrobial, cytotoxic, anti-HIV-1 and anti-inflammatory activities, especially leaves decoction of *Clausena lansium* has used as a shampoo to treating fungal and maintaining hair color.[2-4] Antifungal activity of *Clausena* species has not been reported.

In the continuing research of bioactive constituents, particularly antifungal activity from Vietnamese herbal medicine, this paper describes the isolation and characterization of one new coumarin, dihydroindicolactone (**1**) and four known isolated coumarins, namely 8-geranyloxy psoralen (**2**), imperatorin (**3**), heraclenol (**4**) and indicolactone (**5**) from the leaves of *C. lansium*. Furthermore, the structure of new coumarin **1** was determined by 2D-NMR database and HR-ESI-MS. The ethanol and *n*-hexane extracts, several subfractions of the *n*-hexane extract, and the purified compounds (**1** - **5**) were evaluated for antifungal activity.

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Experimental

General experimental procedures – UV spectra were obtained with UV-2550PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The $[\alpha]_D$ value was determined with P-1020 polarimeter (Jasco Corp., Tokyo, Japan). The IR spectra were measured on a Spectrum One FT-IR Spectrometer (Perkin Elmer Japan Co., Ltd., Kanagawa, Japan). The 1D and 2D-NMR spectra were recorded by using Bruker Avance-600 spectrometer equipped with a cryogenic probe (Bruker BioSpin K.K, Kanagawa, Japan). Chemical shift were expressed in parts per million (δ, ppm) in $CDCl_3$, using TMS as internal reference. HR-ESI-MS was taken on a LTQ Orbitrap Discovery (Thermo Fisher Scientific K. K., Kanagawa, Japan). LC-MS analyses were performed with Bruker Esquire 3000 Ion Trap Mass

Spectrometer equipped with HPLC system.

Flash column chromatography was carried out on Yamazen AI-580 system (Yamazen Corp., Osaka, Japan), a single channel automated flash chromatography system with a pre-packed Inject Column (Yamazen) for sample loading and pre-packed Hi-Flash Column (Yamazen) for separation. After separation, silica gel TLC analyses were performed on TLC Silicagel 60 F_{254} (Merck Ltd., Tokyo, Japan).

Analytical HPLC were performed on the Chromaster, (Hitachi High-Technologies Corp., Tokyo, Japan), a HPLC module, which consisted of chromatographic system (pump, column oven, diode array detector) and an auto sampler. Separation was performed on an Inertsil ODS-3 5 μm column (250 mm × 4.6 mm I.D.) (GL Sciences Inc., Tokyo, Japan).

Preparative HPLC were performed on the PLC761 (GL Sciences Inc.), a preparative HPLC module, which consist of a chromatographic system (with a recycle valve), an auto sampler and a fraction collector. Separation was performed on an Inertsil ODS-3 5 μm column (250 mm × 10 mm i.d. or 250 mm × 14 mm i. d.) (GL Sciences Inc.).

n-Hexane, ethyl acetate, chloroform, methanol, ethanol, 1-butanol, acetonitrile were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Formic acid was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The solvents used for analytical HPLC were of analytical grade. Milli-Q water was used in each experiment. $CDCl_3$ for NMR spectrometry was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of compound **1 - 5** in $CDCl_3$

Position	Compound 2		Compound 3		Compound 4		Compound 5		Compound 1	
	δ_H (ppm); (*J* Hz)	δ_C (ppm)	δ_H (ppm); (*J* Hz)	δ_C (ppm)	δ_H (ppm); (*J* Hz)	δ_C (ppm)	δ_H (ppm); (*J* Hz)	δ_C (ppm)	δ_H (ppm); (*J* Hz)	δ_C (ppm)
2		160.5		160.6		160.2		160.5		160.4
3	6.34, d, 9.6	114.7	6.30, d, 9.5	114.7	6.31; d, 9.76	114.8	6.31, d, 9.4	114.8	6.35, d, 9.4	114.7
4	7.74, d, 9.5	144.3	7.70, d, 9.5	144.4	7.71; d, 9.76	144.3	7.69, d, 9.4	146.8	7.75, d, 9.4	144.4
4a		116.4		116.5		116.4		116.50		116.5
5	7.34, s	113.2	7.29, s	113.2	7.33; s	113.8	7.32, s	113.6	7.36, s	113.5
6		125.8		125.9		126.4		125.9		125.9
7		148.7		148.6		147.9		148.6		148.6
8		131.5		131.7		131.5		130.1		131.3
8a		143.9		143.8		143.3		143.9		143.9
2'	7.66, d, 2.2	146.6	7.62, d, 2.2	146.7	7.63; d 2.3	146.8	7.63, d, 2.3	144.4	7.68,d, 2.3	146.7
3'	6.79, d, 2.3	106.7	6.75, d,2.2	106.7	6.76; d 2.3	106.9	6.76, d, 2.3	106.8	6.80, d, 2.3	106.8
1"	5.01, d, 7.2	70.1	4.94, d, 7.2	70.2	4.68, dd 10.4, 2.6 4.35, dd 10.4, 7.8	72.4	4.98, m	69.6	5.00; dd, 11.6, 6.8 5.03; dd, 11.6, 6.8	69.6
2"	5.57, t, 7.1	119.4	5.54, m	119.8	3.80, dd 7.8, 2.6	75.7	5.65, m	123.9	5.67; br t, 6.8	123.5
3"		143.2		139.8		71.5		137.1		137.7
4"	1.98, s	39.5	1.65, s	18.2	1.22, s	26.7	2.24; dd,13.9, 6.4 2.34; dd,13.9, 6.4	43.3	2.23;dd, 13.7, 6.7 2.41; dd, 13.7, 6.7	44.8
5"	1.99, s	26.3	1.67, s	25.9	1.26, s	25.1	4.84, m	148.4	4.56; ddt, 7.6, 6.7, 4.5	76.4
6"	4.98, m	123.7					6.85, s	79.6	1.84 ;dt, 13.0, 7.6 2.02 ;ddd, 13.0, 8.9, 4.5	34.7
7"		131.7						131.4	2.61; ddq, 8.9, 7.6, 7.4	33.7
8"	1.61, s	25.6						174.0		179.7
9"	1.67, s	16.5					1.71, s	10.7	1.73; s	17
10"	1.54, s	17.6					1.80, s	17.3	1.23; d, 7.4	15.8

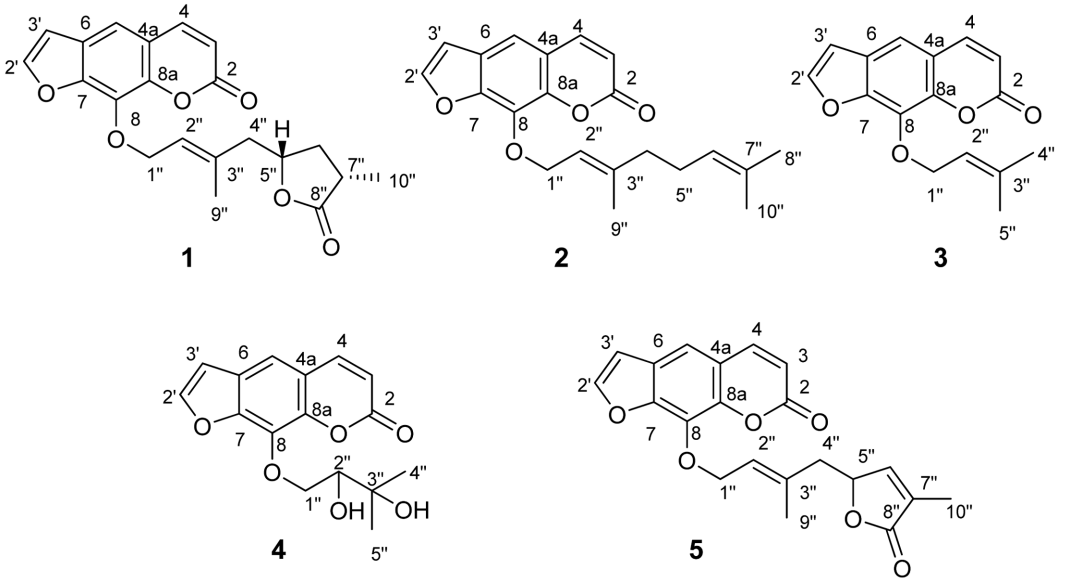

Fig. 1. Structures of compounds **1** - **5**.

Plant materials – Leaves of *Clausena lansium* (Lour.) Skeels were collected from Quan Chu village, Dai Tu district, Thai Nguyen Province, Vietnam in July 2011 and plant materials were identified and authenticated by Association Professor Nguyen Van Tap and Msc. Nguyen Thi Quynh Nga, Department of Natural Resources, National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam. A voucher specimen (No.9976) (Fig. 6) was deposited in the Herbarium of Department of Natural Resources, NIMM, Hanoi, Vietnam.

Extraction and isolation – Air-dried leaves of *Clausena lansium* (CL, 1 kg) were macerated at room temperature in 10 L *n*-hexane for 7 days. The resultant extract was filtered and evaporated to dryness *in vacuo* at 40 °C to give a black residue (CL-H, 24.19 g). The rest of Wampee leaves was dried and macerated in 10 L EtOH 95% for one week, then filtered and concentrated to afford an ethanol 95% residue (CL-E, 48.47 g). The CL-H and CL-E extracts were evaluated for the antifungal activity. A part of the potential antifungal extract, CL-H (7 g) was subjected to flash silica gel column chromatography, eluted with a mixture of *n*-hexane/EtOAc (100:0 - 0:100) to afford 10 fractions (F1-F10). Repeated RP preparative HPLC on an Inertsil ODS-3 5 µm column (250 mm × 10 mm i.d) (ACN-H_2O with 0.1% HCOOH, 60:40; detection at 254 nm, flow rate of 7.5 mL/min, injection volume of 0.5 mL) of a part of F5 (100 mg) to obtain compounds **2** (10 mg) and **3** (20 mg). A part of F7 (100 mg) was separated and purified on preparative HPLC (Inertsil ODS-3 5 µm, 250 mm × 10 mm i.d column; ACN-H_2O with 0.1% HCOOH, 50:50, flow rate of 7.5 mL/min, injection volume of 0.5 ml) to yield heraclenol (**4**) (8 mg). Compound (**5**) (5.8 mg) and (**1**) (5.1 mg) were obtained from part of F8 (100 mg) using a RP preparative HPLC on Inertsil ODS-3 5 µm column (250 mm × 10 mm i.d) (ACN-H_2O with 0.1% HCOOH, 43:57, flow rate of 7.5 mL/min, injection volume of 0.5 mL).

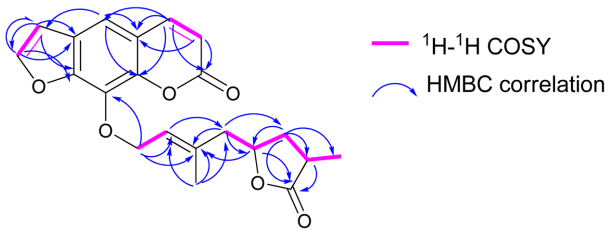

Fig. 2. COSY and HMBC correlation of dihydroindicolactone (**1**).

Dihydroindicolactone (1) – Light yellow gum, $[\alpha]_D^{25}$ –23.6 (*c* 0.264, MeOH); UV λ_{max} (MeOH) nm (log ε) : 218 (4.41), 248 (4.36), 299 (4.07); FT-IR ν_{max} (ATR) cm^{-1} : 1768 (5-membered lactone), 1728 (6-membered lactone), 1586, 1148, 1095; for ^{1}H and ^{13}C-NMR spectroscopic data, see Table 1, for 2D-NMR as COSY, HMBC and NOESY, see Fig. 2-4; HR-ESI-MS *m/z* 369.1333 $[M+H]^+$ (calcd for $C_{21}H_{21}O_6$ 369.1333, Δ = 0.00 mmu).

8-Geranyloxy psoralen (2) – Amorphous powder, for ^{1}H-NMR and ^{13}C-NMR data, see table 2, *m/z* 339 $[M+H]^+$ (supported molecular formula $C_{21}H_{22}O_4$).

Imperatorin (3) – Amorphous powder, for ^{1}H- and ^{13}C-NMR data, see table 2, *m/z* 271 $[M+H]^+$ (supported molecular formula $C_{16}H_{14}O_4$).

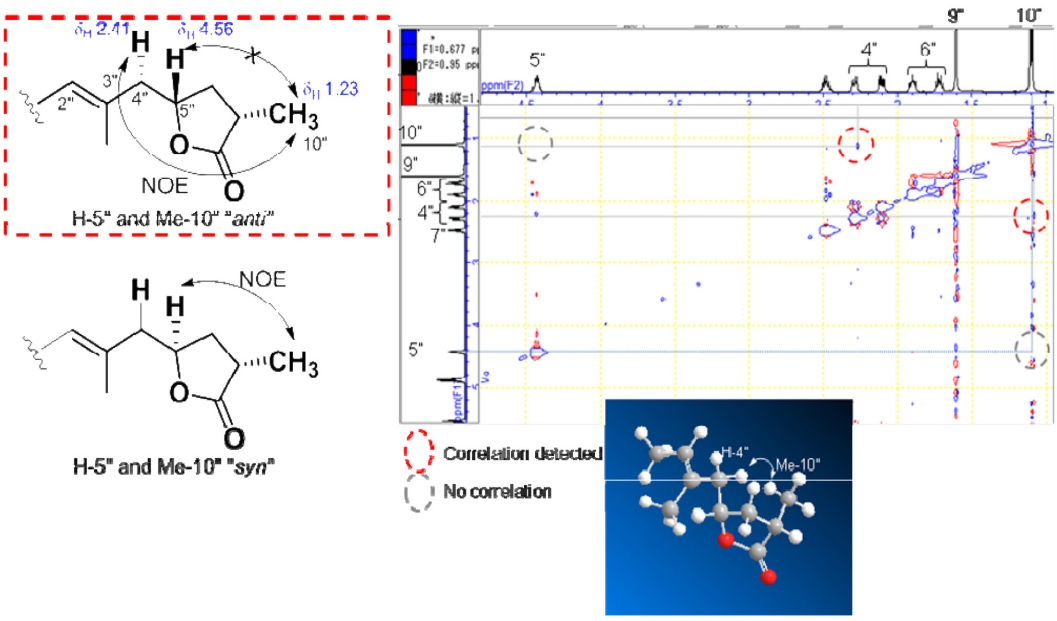

Fig. 3. The relative stereochemistry of dihydroindicolactone (**1**).

Heraclenol (4) – Colourless crystals, for ^{1}H- and ^{13}C-NMR data, see table 2, *m/z* 305 $[M+H]^+$ supported molecular formula $C_{16}H_{16}O_6$).

Indicolactone (5) – Light yellow gum, for ^{1}H- and ^{13}C-NMR data, see table 2, *m/z* 367 $[M+H]^+$ (supported molecular formula $C_{21}H_{18}O_6$).

Antifungal assay – Antifungal assay was carried out using *Malassezia globosa* CBS7874, with Octopirox as a positive control. The strain of *Malassezia* were maintained on modified Leeming and Notman agar[8] composed of (per liter) 10 g of peptone (Oxoid, Basingstoke, United Kingdom), 10 g of glucose, 2 g of yeast extract (Oxoid), 8 g of ox bile (Oxoid), 10 mL of glycerol, 0.5 g of glycerol monostearate, 5 mL of Tween 60, 20 mL of olive oil, and 15 g of agar (Oxoid) at 32 ºC for five days. A loopful of cells on the agar plate was transferred to 5 mL of YM broth (Difco, Detroit, Mich.) supplemented with 0.5% of Tween 60. To evaluate antifungal activities, 90 μL of the cell suspension was added to a well of a 96-well plate (Nunc Black, NY.), containing 1 to 10 μL of the test sample, and the plate was maintained at 32 ºC for 24 hr. ATP contents were determined by Cell Titer-Glo™ Luminescent Assay Kit (Promega).

Result and Discussion

The *n*-hexane extract of leaves of *Clausena lansium* was separated by chromatographic techniques to yield one new furanocoumarin, dihydroindicolactone (**1**) together with four known compounds (**2** - **5**). The chemical structures of isolates (**1** - **5**) were elucidated using spectroscopic data and compared with those reported in the literatures.

Dihydroindicolactone (**1**) was obtained as a light yellow gum, $[\alpha]^{25}_{D}$ –23.6 (c = 0.264 MeOH). A $[M+H]^+$ pseudo-molecular ion peak observed at *m/z*: 369.1333 (Calcd for $C_{21}H_{21}O_6$: 369.1333) in positive HR-electrospray (ESI)-MS, implying 12 degree of unsaturation. The IR spectrum displayed characteristic absorption of 5-membered lactone (1768 cm^{-1}), 6-membered lactone (1728 cm^{-1}) and aromatic ring (1586 cm^{-1}). The UV-Vis spectrum showed an absorption band at 299, 248, 218 nm. Its ^{1}H-NMR spectrum had two distinct pair of doublet at δ_H 6.35 7.75 (1H, *J* 9.4Hz) assignable to H-3, H-4 on a coumarin nucleus and δ_H 7.68 6.80 (1H, *J* 2.3Hz) assignable to H-2' and H-3' as well as a lone singlet at δ_H 7.36 (H-5). The above information coupled with biogenetic considerations and literature references indicated the presence of furanocoumarin skeleton.[7,10] Analysis of the ^{13}C-NMR spectra, including HSQC, suggested a furanocoumarin system with an addition of C_{10} moiety, which consisted of one oxymethylene, one trisubtituted olefin, one methylene and one 5-membered lactone respectively. This phenomenone was illustrated by HMBC and NOESY analysis (Fig. 2). In HMBC spectrum of **1**, correlations for H-5" (δ_H 4.56)/C-7" (δ_C 33.7) and C-8" (δ_C 179.7); H-6" (δ_H 1.84 and 2.02)/C-5" (δ_C 76.4), C-7" (δ_C 33.7) and C-8" (δ_C 179.7); H-7" (δ_H 2.61)/C-6" (δ_C 34.7) and C-8" (δ_C 179.7) suggested that C-5" was connected to the C-8" ester carbonyl to

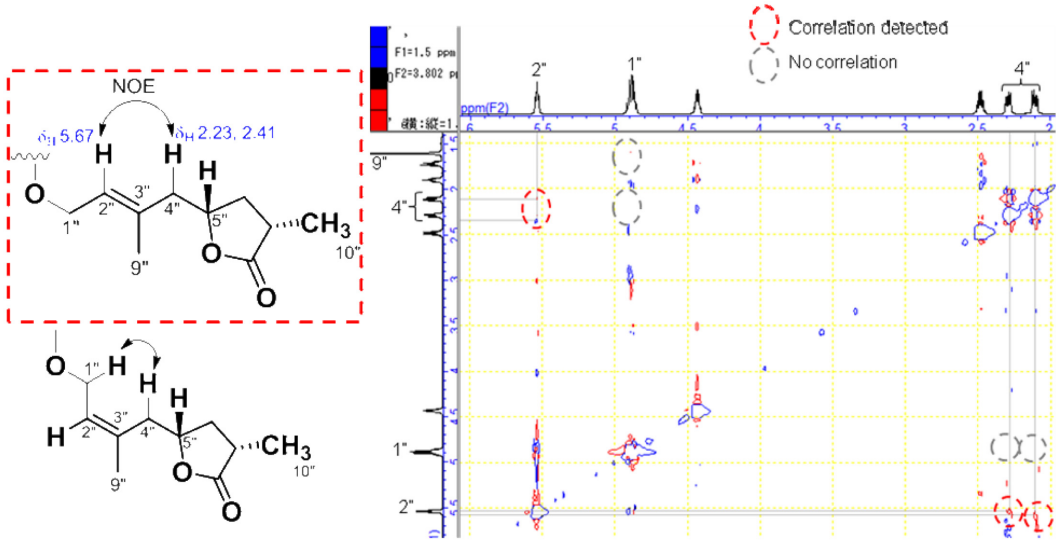

Fig. 4. The geometry of double bond at C-2" and C-3" of dihydroindicolactone (**1**).

form a five-membered lactone ring. HMBC correlations were observed: H-1" (δ_H 5.00 5.03)/C-2" (δ_C 123.5) and C-3" (δ_C 137.7); H-9" (δ_H 1.73)/C-2" (δ_C 123.5) and C-3" (δ_C 137.7) and C-4" (δ_C 44.8); and H-4" (δ_H 2.23 2.41)/C-2" (δ_C 123.5),C-3" (δ_C 137.7), C-5" (δ_C 76.4), C-6" (δ_C 34.7) and C-9" (δ_C 17.0). This indicated that the C-10 side-chain of **1** was as show. In the NOESY experiment, the correlation of H-2" and H-4" indicated a 2"-E configuration for the double bond (Fig. 4). Another correlation between H-4" and H-10" and no interaction of H-5" with H-10" established the H-5" and C-7" methyl group were on the opposite side of the five-membered lactone ring. On the other hand, NMR spectral data of **1** were similar to those of *Clausemarin A.* isolated from *Clausena lansium*[9], except for the position of H-5". This was confirmed by NOESY experiment which showed the long range correlation between methyl proton H-10" [δ_H 1.23 (d, J = 7.4 Hz); δ_C 15.8] and H-4" [δ_H 2.41 (1H, dd, J = 13.7, 6.7) Hz] [δ_H 2.23 (1H, dd, J = 13.7, 6.7) Hz]. In *Clausemarin A.*, there has been a correlation between H-10" [δ_H 1.23] and H-5" [δ_H 4.56], showing that H-10" and H-5" have a *syn* relative orientation whereas in structure of **1**, it hasn't been existed the correlation between H-10" and H-5", illustrating that H-10" and H-5" have an *anti* relative orientation (Fig. 3). Thus the structure of **1** (Fig. 1) was determined to be 9-((*E*)-3-methyl-4-((*2S*,4*S*)-4-methyl-5-oxotetrahydrofuran-2-yl)but-2-enyloxy)-7H-furo[3,2-g]chromen-7-one, named dihydroindicolactone.

Compound **2** was obtained as an amorphous powder. The molecular formula of $C_{21}H_{22}O_4$ was established on the basic of LC-MS analysis (*m/z* 339 $[M+H]^+$) and ^{13}C-NMR (21 signal). The ^1H-NMR spectrum was assigned the common signal of furanocoumarin at δ 6.34 (1H, d, J 9.4Hz, H-3), 7.34 (1H, s, H-5), 7.74 (1H, d, J 9.4Hz, H-4), 6.79 (1H, d, J 2.3Hz, H-3') and 7.66 (1H, d, J 2.3Hz, H-2'). Furthermore, combination data from the DEPT-135, ^1H-NMR and ^{13}C-NMR, we can see three methyl group (CH_3), three methylene group (CH_2) on the branch of molecular **2**. With these assignments, **2** was determined as 8-geranyloxy psoralen.

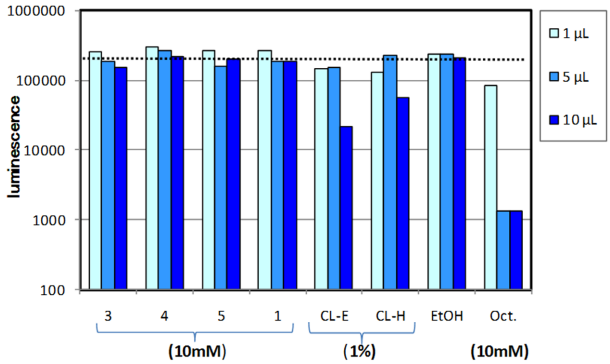

Fig. 5. Antifungal activity of pure compounds and initial extracts.

The remaining three known coumarins included imperatorin (**3**), heraclenol (**4**) and indicolactone (**5**) were determined by 1D and 2D-NMR spectra, LC-MS and comparison with their literature data.[5,6,7] The detail information was showed in Table 1.

All purify compounds and *n*-hexane extracts as well as several subfractions were evaluated for antifungal activity against yeast *Malassezia globosa,* using Octopirox® as positive control. The result of isolated compounds and

some fractions has exhibited weak activity against this yeast except subfraction 6, 7, 8 whereas the *n*-hexane extract of *C. lansium* was showed the moderate active in this activity (Fig. 5). On the other side, the parent compounds like xanthotoxol[11] and xanthotoxin[12] have displayed strong capacity against this yeast. This phenomenon could be explained by the extending of O-substituted branch in these molecular led to reducing antifungal activity.

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