

DITERPENOIDS FROM THE ROOT EXTRACT OF *CHIOCOCCA ALBA*

EUGENIA V. DZIB-REYES^a, KARLINA GARCÍA-SOSA^a, PAULINO SIMÁ-POLANCO^b,
LUIS M. PEÑA-RODRÍGUEZ^{a,*}

(Received September 2012; Accepted October 2012)

This paper is dedicated to Professor Pedro Joseph-Nathan in recognition of his 50 years of outstanding scientific trajectory.

ABSTRACT

Two new *ent*-kaurane diterpenes, 1-hydroxy-18-nor-kaur-4,16-dien-3-one (**1**) and 15-hydroxy-kaur-16-en-3-one (**2**), along with the four known metabolites kaur-16-en-19-ol (**3**), kaurenoic acid (**4**), merilactone (**5**), and ribenone (**6**), and a mixture of stigmasterol and β -sitosterol were isolated from the ethanolic root extract of *Chiococca alba*. Chemical structures of the different metabolites were established through the interpretation of their spectroscopic data and/or by comparing them with those reported in the literature. www.relaquim.com

Keywords: Rubiaceae, *Chiococca alba*, kaurane, pimarane, labdane, diterpenes.

RESUMEN

Dos nuevos diterpenos tipo *ent*-kaurano, 1-hidroxi-18-nor-kaur-4,16-dien-3-ona (**1**) y 15-hidroxi-kaur-16-en-3-ona (**2**), además de cuatro metabolitos ya conocidos, kaur-16-en-19-ol (**3**), ácido kaurenoico (**4**), merilactona (**5**), y ribenona (**6**), y una mezcla de estigmasterol y β -sitosterol, fueron aislados del extracto etanólico de la raíz de *Chiococca alba*. Las estructuras químicas de los diferentes metabolitos fueron establecidas mediante la interpretación de sus datos espectroscópicos y/o por comparación con los datos reportados en la literatura.

Palabras clave: Rubiaceae, *Chiococca alba*, kaurano, labdano, pimarano, diterpenos.

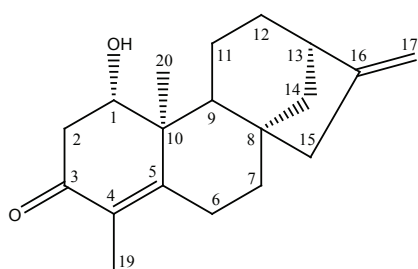
^aUnidad de Biotecnología and ^bUnidad de Recursos Naturales, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, 97200 Mérida, Yucatán, México.

* Corresponding autor. Tel.: +52-999-9428330 ext. 159; Fax: +52-999-9813900; email: Imanuel@cicy.mx

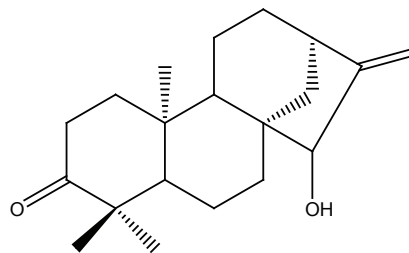
INTRODUCTION

Chiococca alba (L.) Hitchc. (Rubiaceae), commonly known as “t’unché” or “kan-chakché” in the Yucatán peninsula (Mendieta and Del Amo, 1981), is a tropical and sub-tropical shrub distributed all over the American continent (Standley and Williams, 1975). The root decoction of this plant is used to cure dysentery, as a diuretic and cathartic, and against snake bites (Mendieta and Del Amo, 1981; Argueta *et al.*, 1994), while the leaf infusion is used to alleviate various ailments such

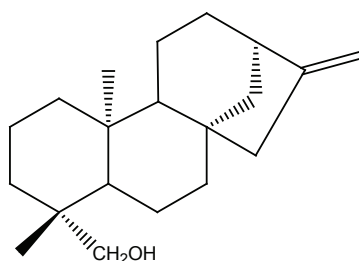
as asthma, headaches and diarrhea, and a decoction of the whole plant is reported to be effective as a laxative, against gonorrhea, to cure skin infections, and to treat rheumatism (Mendieta and Del Amo, 1981; Del Amo, 1980). Although the *Chiococca* genus includes more than twenty species found throughout the American continent (Lopes *et al.*, 2004), its phytochemical and pharmacological knowledge is limited to a number studies carried out on *C. alba* which have reported the isolation of alkaloids (El Abbadi *et al.*, 1989), flavonoids (Lopes *et al.*, 2004), iridoids (Carbonezi *et al.*,



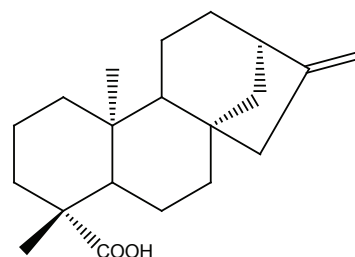
1



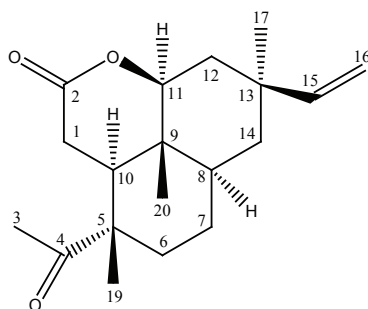
2



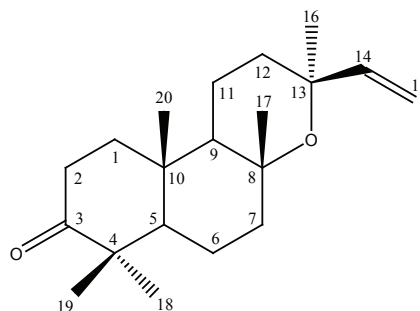
3



4



5



6

1999), saponins (Borges *et al.*, 2009), and terpenoids (Lopes *et al.*, 2004; Carbonezi *et al.*, 1999; Borges *et al.*, 2009; Bhattacharyya and Cunha, 1992; Borges-Argaez *et al.*, 1997, 2001) from the roots of the plant, and lignans, coumarins and ketoalcohols from the leaves (El Hafiz *et al.*, 1991). As part of our search for novel metabolites from medicinal plants of the Yucatan peninsula, we wish to report herein on the isolation and identification of the novel *ent*-kauranes 1-hydroxy-18-nor-kaur-4, 16-dien-3-one (**1**) and 15-hydroxy-kaur-16-en-3-one (**2**), together with the known diterpenes kaur-16-en-19-ol (**3**), kaurenoic acid (**4**), merilactone (**5**) and ribenone (**6**), and a mixture of stigmasterol and β -sitosterol, from the root extract of *C. alba*.

EXPERIMENTAL

General experimental procedures.

Analytical and preparative TLC were carried out using precoated Si gel aluminum and glass plates respectively (Merck 60F₂₅₄, 0.25 and 0.50 mm thickness). Chromatograms were examined under UV light in a UV-viewing cabinet and visualized by dipping in 4% phosphomolybdic acid solution containing a trace of ceric sulfate in 5% sulfuric acid, followed by drying and gentle heating. GC/MS analyses were performed on an Agilent Technologies 6890N gas chromatograph [1.0 μ L of 3% sample in CHCl₃, HP 5MS (30 m x 0.25 mm i.d. x 30 μ m), flow rate 1 mL/min, temperature program: T_1 150 °C, T_2 280 °C, gradient 10 °C/min, injector temperature 280 °C, detector temperature 280 °C] coupled to an Agilent Technologies 5975B mass selective detector. IR spectrum was recorded in CHCl₃ (film) using a FT-IR Nicolet Magna 750 spectrophotometer, and ¹H-NMR (400 MHz) and ¹³C-NMR (100 MHz) spectra were obtained on a Bruker Avance 400, Variant Unity Plus-300 and Bruker DRX400, using the residual CHCl₃ signal (7.26 and 77.00 ppm for ¹H and ¹³C,

respectively) as reference.

Plant material. The roots of *C. alba* were collected during march 2008 and 2009, from plants growing in a field located at km 7 of Yaxcabá highway in Yucatán, México. A voucher specimen has been deposited in the herbarium of the Unidad de Recursos Naturales of the Centro de Investigación Científica de Yucatán (CICY) under the collection number 3200. The plant material was washed with tap water and dried, first for a week at room temperature, and then for 72 h in an oven at 50 °C. The dried roots were ground using a Pagani Dycomet 1520 mill and a No. 6 sieve.

Extraction and isolation. The dry roots (3.95 Kg) were extracted four times with EtOH (6 L) at room temperature. The extracts were combined and the solvent was removed under reduced pressure to produce 209.5 g of crude ethanolic extract. The crude extract was suspended in 1725 ml of a 3:2 mixture of H₂O:MeOH and the resulting aqueous suspension was subjected to a liquid-liquid partition procedure with hexane and ethyl acetate to produce the corresponding low (19.5 g) and medium (55.6 g) polarity fractions, respectively. Successive purifications of the low polarity fraction, using a combination of vacuum liquid chromatography, flash column chromatography, gravity column chromatography and preparative-TLC, led to the isolation of metabolites **1** (45.5 mg), **2** (3.0 mg), **3** (22.3 mg), **4** (10.9 mg), **5** (11.6 mg), and **6** (2.1 mg) in pure form, and 3.7 mg of an inseparable mixture of stigmasterol and β -sitosterol.

1-hydroxy-18-nor-kaur-4, 16-dien-3-one (**1**), colorless oil, soluble in CH₂Cl₂, single spot on TLC visible under UV light, R_f =0.48 (Et₂O/Hx 6:4); $[\alpha]_D^{25}$ = -20.0 (c 0.0116, CHCl₃); GC-MS: t_R = 12.9 min, m/z 286.2 [M^+]; IR (CHCl₃ 2%): 3477.7, 1669.9, 1613.7 cm⁻¹ ¹H-NMR (CDCl₃, 400 MHz): see data in Table 1.

15-hydroxy-kaur-16-en-3-one (**2**), obtained as a crystalline solid, soluble in CH_2Cl_2 , $R_f=0.37$ ($\text{Et}_2\text{O}/\text{Hx}$ 6:4); GC-MS: $t_R=14.0$ min, m/z 302.2 [M^+]; IR (CHCl_3): 3457.0, 1705.8 cm^{-1} . ^1H -NMR (CDCl_3 , 400 MHz): 1.55 (H-1a, m, 1H); 2.02 (H-1b, ddd, 3.9, 6.4, 13.0, 1H); 1.53 (H-2, m, 2H); 1.45 (H-5/6a, m, 2H); 1.64 (H-6b, m, 1H); 2.48 (H-7, ddd, 3.6, 6.4, 8.8, 2H); 1.13 (H-9, d, 7.9, 1H); 1.64 (H-11, m, 2H); 1.43 (H-12a, d, 2.5, 1H); 1.79 (H-12b, dd, 3.3, 8.7, 1H); 2.78 (H-13, bs, 1H), 1.40 (H-14a, d, 4.0, 1H); 1.87 (H-14b, dd, 1.4, 11.8, 1H); 3.82 (H-15, bs, 1H); 5.10 (H-17a, bs, 1H); 5.23 (H-17b, bs, 1H); 1.10 (H-18, s, 3H); 1.05 (H-19, s, 3H); 1.08 (H-20, s, 3H); ^{13}C -NMR (CDCl_3 , 100 MHz): 39.4 (C-1, t); 32.6 (C-2, t); 207.3 (C-3, s); 47.2 (C-4, s); 54.4 (C-5, d); 20.8 (C-6, t); 34.1 (C-7, t); 47.6 (C-8, s); 53.0 (C-9, d); 38.7 (C-10, s); 18.7 (C-11, t); 34.2 (C-12, t); 42.3 (C-13, d); 36.0 (C-14, t); 82.6 (C-15, d); 160.0 (C-16, s); 108.7 (C-17, t); 27.5 (C-18, q); 21.1 (C-19, q); 18.1 (C-20, q) ppm.

RESULTS AND DISCUSSION

The ethanolic root extract of *C. alba* was first fractionated using a liquid-liquid partition procedure with hexane and ethyl acetate. Purification of the resulting low-polarity (hexane) fraction using different chromatographic techniques produced diterpenes **1-6** in pure form, together with a mixture of stigmasterol and β -sitosterol.

The molecular formula of **1**, established as $\text{C}_{19}\text{H}_{26}\text{O}_2$ from its MS data (parent ion peak at m/z 286.2), implied the presence of seven unsaturation sites in its structure. The IR spectrum of **1** exhibited strong absorption bands at 3477 and 1669 cm^{-1} , indicating that the two oxygen atoms in the molecular formula were in the form of hydroxyl and carbonyl groups, respectively. The presence of these two functional groups in the structure of **1** was further supported by the signals at δ 200.5 and 68.2 in its

^{13}C -NMR spectrum (Table 1), while the four additional sp^2 -carbon signals at δ 154.4, 104.6, 166.8, 124.0, corresponding to the carbon atoms of two double bonds, strongly suggested that the four remaining unsaturation sites were in the form of a tetracyclic structure. The combined analysis of the ^{13}C -NMR spectrum of **1**, together with the data from its HSQC, DEPT-135 and DEPT-90 experiments, allowed the identification of all carbon atoms in the structure and indicated the presence of two methyl groups, eight methylenes, three methines and six quaternary carbons.

The ^1H -NMR spectrum of **1** (Table 1) showed the presence of an axial-oriented carbinol proton at δ_H 4.09 (ddd, $J=13.8$, 6.1, 2.0 Hz), together with two vinylic protons of an exocyclic double bond at δ_H 4.81 and 4.86, and a vinylic methyl group at δ_H 1.85. In the ^1H - ^1H COSY experiment, the clear correlation observed between the carbinol proton at δ_H 4.09 and the signals corresponding to two methylene protons at δ_H 2.53 (dd, $J=13.8$, 6.1 Hz) and δ_H 1.34 (d, $J=13.9$ Hz) indicated their being an isolated system. However, a detailed analysis of the HMBC experiment (Table 1) allowed the construction of the full structure; the 2J and 3J correlations observed between the protons of the methylene group at δ_H 2.53/1.34 and the carbonyl carbon at δ_C 200.5 and the sp^2 carbon at δ_C 166.8, respectively, together with the 3J and 2J correlations observed between these two carbons and the vinylic methyl group at δ_H 1.85, respectively, and the 3J correlation observed between the carbinol proton at δ_H 4.09 and the methyl group at δ_C 27.4 indicated that they were all located in the same ring. Similar correlations allowed the construction of the remaining tetracyclic structure; a through literature search indicated that metabolite **1** is a new natural product with a nor-diterpene *ent*-kaurane skeleton.

Table 1. Spectroscopic data [^1H -NMR (100 MHz, CDCl_3), ^{13}C -NMR (100 MHz, CDCl_3), ^1H - ^1H COSY and HMBC correlations] of 1-hydroxy-18-nor-kaur-4,16-dien-3-one (**1**)

C	^1H-NMR (δ_{H})	^{13}C-NMR (δ_{C})	^1H-^1H COSY	HMBC ^2J	^3J
1	4.09 (1H, ddd, 13.8, 6.1, 2.0)	68.2 (d)	H2	C2	C3
2a	1.34 (1H, bd, 13.9)	40.7 (t)	H1	C1	C4
2b	2.53 (1H, dd, 13.8, 6.1)			C3	C4, C10
3		200.5 (s)			
4		166.8 (s)			
5		124.0 (s)			
6a	2.42 (1H, ddd, 13.3, 13.1, 3.5)	27.4 (t)	H7a, H7b	C5	C4
6b	2.62 (1H, ddd, 12.6, 3.5, 3.5)		H7a, H7b	C5	C4, C10
7a	1.52 (1H, ddd, 13.5, 13.5, 3.4)	41.7 (t)	H6a, H6b		
7b	1.67 (1H, dd, 3.5, 3.5)		H6a, H6b		
8		44.6 (s)			
9	1.40 (1H, d, 10.7)	48.1 (d)		C8 C10 C11	C7, C14
10		43.1 (s)			
11a	1.40 (1H, d, 10.7)	40.8 (t)		C9	
11b	2.16 (1H, d, 9.4)				
12	1.60 (2H, d, 3.8)	33.0 (t)	H13		C16
13	2.75 (1H, bs)	43.3 (d)	H12		
14a	1.70 (1H, dd, 3.5, 3.5)	20.5 (t)			
14b	1.74 (1H, dd, 4.0, 3.9)				
15	2.11 (2H, s)	48.2 (t)		C16	C9, C17
16		154.4 (s)			
17a	4.81 (1H, bs)	104.6 (t)			C13, C15
17b	4.86 (1H, bs)				
19	1.85 (3H, s)	11.8 (c)		C4	C3, C5
20	1.30 (3H, s)	27.4 (c)		C10	C1, C9

Metabolite **2** showed a parent ion peak at m/z 302.2 in its mass spectrum, which indicated a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_2$ that, in turn, implied the presence of six unsaturation sites in the structure. The IR spectrum of **2** exhibited a strong hydroxyl group absorption band at 3457 cm^{-1} and another one in the carbonyl region at 1705 cm^{-1} , indicating that, as in the case of **1**, the two oxygen atoms in the molecular formula were in the form of hydroxyl and carbonyl groups, respectively; this was confirmed by

the signals corresponding to a carbonyl and to an sp^3 -oxygenated carbon at δ_{C} 207.3 and 82.6, respectively, in the ^{13}C -NMR spectrum of **2**. Additionally, the signals corresponding to the two sp^2 -carbons of an exocyclic double bond at δ_{C} 160.0, 108.7 (Wehrli and Nishida, 1979), identified the second insaturation in the structure and suggested that the four remaining unsaturation sites corresponded to a tetracyclic *ent*-kaurane diterpenoid structure. The nature of all carbon atoms was confirmed

through a combined analysis of the ^{13}C -NMR spectrum and the HSQC experiment and indicated the expected presence of three methyl groups, nine methylenes, three methines and five quaternary carbons. The ^{13}C and ^1H chemical shift values of both protons and carbons were compared to those reported in the literature (Wehrli and Nishida, 1979; Baptista *et al.*, 2007; Pacheco *et al.*, 2009); the search indicated that metabolite **2** represents a new natural *ent*-kaurane.

Metabolites **3**, **4**, **5** and **6** were found to be identical with kaur-16-en-19-ol (Wehrli and Nishida, 1979; Baptista *et al.*, 2007; Pacheco *et al.*, 2009), kaurenoic acid (Pacheco *et al.*, 2009; Da Silva *et al.*, 2001; Dang *et al.*, 2005; Batista *et al.*, 2007; Lacruz, 2008; Lee, *et al.*, 2008), merilactone (Borges-Argaez *et al.*, 2001), and ribenone (Wehrli and Nishida, 1979; Da Silva *et al.*, 2001; Almquist, *et al.*, 1975; Konishi *et al.*, 1996; Wantanee *et al.*, 2003), by comparing their spectroscopic data (MS, IR, ^1H and ^{13}C NMR) with those reported in the literature. Although metabolites **3**, **4** and **6** have been found in several plant families

such as *Euphorbiaceae*, *Aristolochiaceae* and *Asteraceae*, respectively, this is the first report of their occurrence in the *Rubiaceae* family and in *C. alba* in particular. Merilactone (**5**) is the only metabolite that has been previously isolated from the root extract of the plant.

CONCLUSIONS

The results obtained in this investigation confirm the root extract of *Chiococca alba* as an important source of novel diterpenoids, particularly of those having labdane, pimarane and *ent*-kaurane skeletons.

ACKNOWLEDGEMENTS

EVDR gratefully acknowledges the support of The American Society of Pharmacognosy (ASP), through their Undergraduate Research Award Programme, which enabled her to work on this project. This work was supported by Project FOMIX-Yucatán (66262).

REFERENCES

- Almqvist, S.O., Enzell, C.R., Wehrli, F.W. (1975) Carbon-13 NMR studies of labdane diterpenoids. *Acta Chemica Scandinavica* **B 29**: 695-702
- Argueta-Villamar, A. (1994) Atlas de la Medicina Tradicional Mexicana. Vol II. Edición del Instituto Nacional Indigenista, México D.F., p1082
- Baptista, G.J., Monsalve, M., Alonso, M.E., Ávila, J.L., Usubillaga, A. (2007) Ensayos de actividad antialimentaria sobre *Tribolium castaneum* y *Sitophilus oryzae* de algunos derivados del *ent*-kaureno. *Scientific Journal of the Experimental Faculty of Sciences* **15**: 248-258
- Batista, R., García, P.A., Castro, M.A., Miguel del Corral, J.M., San Feliciano, A., De Oliveira, A.B. (2007) New oxidized *ent*-kaurane and *ent*-norkaurane derivatives from kaurenoic acid. *Journal of the Brazilian Chemical Society* **18**: 622-627
- Bhattacharyya, J., Cunha, E.V. (1992) A triterpenoid from the root-bark of *Chiococca alba*. *Phytochemistry* **31**: 2546-2547
- Borges, R.M., Tinoco, L.W., Filho, J.D., Barbi, N.S., Da Silva, A.J. (2009) Two new oleanane saponins from *Chiococca alba* (L) Hitchc. *Journal of the Brazilian Chemical Society* **2**: 1738-1741

- Borges-Argáez, R.L., Medina-Baizabál L., May-Pat, F., Peña-Rodríguez, L.M. (1997) A new *ent*-kaurane from the root extract of *Chiococca alba*. *Canadian Journal of Chemistry* **75**: 801-804
- Borges-Argáez, R.L., Medina-Baizabál, L., May-Pat, F., Waterman, P.G., Peña-Rodríguez, L.M. (2001) Merilactone, an unusual C₁₉ metabolite from the root extract of *Chiococca alba*. *Journal of Natural Products* **64**: 228-231
- Carbonezi, C.A., Martins, D., Young, M.C., Lopes, M.N., Burlan, M., Filho, E.R., Bolzani, V.S. (1999) Iridoids and seco-iridoids from *Chiococca alba* (Rubiaceae). *Phytochemistry* **51**: 781-785
- Da Silva, A., Barbosa, P.S., Muller, A.H., Guilhon, G.M. (2001) Constituintes químicos do caule e das cascas do caule de *Croton pullei* var. *Glabior* (Euphorbiaceae). *Revista Virtual de Iniciação Acadêmica da UFPA* **1**: 1-9
- Dang, N.H., Zhang, X.F., Zheng, M.S., Son, K.H., Chang, H.W., Kim, H.P., Bae, K.H., Kang, S.S. (2005) Inhibitory constituents against cyclooxygenases from *Aralia cordata* Thunb. *Archives of Pharmaceutical Research* **28**: 28-33
- Del Amo S. (1980) Plantas medicinales del Estado de Veracruz. Instituto Nacional de Investigaciones sobre Recursos Bióticos, Veracruz, México, p. 76
- El Abbadi, N., Weniger, B., Lobstein, A., Quirion, J.C., Anton, R. (1989) New alkaloids of *Chiococca alba*. *Planta Medica* **55**: 603-604
- El-Hafiz, M.A., Weniger, B., Quirion, J.C., Anton, R. (1991) Ketoalcohols, lignans and coumarins from *Chiococca alba*. *Phytochemistry* **30**: 2029-2031
- Konishi, T., Azuma, M., Itoga, R., Kiyosawa, S., Fujiwara, Y., Shimada, Y. (1996) Three new labdane-type diterpenes from the wood of *Excoecaria agallocha*. *Chemical and Pharmaceutical Bulletin* **44**: 229-231
- Lacruz, E.K. (2008) Estudio fitoquímico de *Simsia pubescens* Triana. Tesis, Universidad de Los Andes, Mérida, Venezuela
- Lee, I., Kim, H., Youn, U., Min, B., Jung, H.J., Yoo, J., Seong, R., Bae, K. (2008) Absolute configuration of a diterpene with an acyclic 1,2-diol moiety and cytotoxicity of its analogues from the aerial parts of *Aralia cordata*. *Bulletin of the Chemical Society of Japan* **29**: 1839-1842
- Lopes, M.N., Oliveira, A.C., Young, M.C., Bolzani, V.S. (2004) Flavonoids from *Chiococca braquiata* (Rubiaceae). *Journal of the Brazilian Chemical Society* **15**: 468-471
- Mendieta, R.M., Del Amo S. (1981) Plantas medicinales del Estado de Yucatán. Editorial C.E.C.S.A, México D.F., p. 98
- Pacheco, A.G., Machado, P., Piló, D., de Carvalho, A.F. (2009) ¹³C-NMR data of diterpenes isolated from *Aristolochia* species. *Molecules* **14**: 1245-1262
- Standley, P.C., Williams, L.O. (1975) Flora de Guatemala Vol. 24. Museum of Natural History, New York, USA., pp 33, 34
- Wantanee, S., Chavasiri, W., Kokpol, U. (2003) Chemical constituents from the heartwood of *Xylia xylocarpa* Taub var. *kerrii* Nielsen. 29th Congress on Science and Technology of Thailand
- Wehrli, F.W., Nishida, T. (1979) The use of carbon-13 nuclear magnetic resonance in natural products chemistry. In Herz, H., Grisebach, G.W., Kirby (eds) Progress in the chemistry of organic natural products. Springer-Verlag, New York, USA, pp 69-75