รายงานวิจัยฉบับสมบูรณ์

โครงการ องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเนื้อไม้จันทน์ชะมด

โดย ศาสตราจารย์ ดร. อุดม ก๊กผล และคณะ

28 กันยายน 2545

สัญญาเลขที่ BGJ/21/2544

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

ในการศึกษาองค์ประกอบทางเคมีจากต้นจันทน์ชะมด สามารถแยกสารได้ 16 ชนิด จากสิ่งสกัดไดคลอโรมีเทน เอทิลแอซีเตต และเมทานอลของเนื้อไม้ โดยอาศัยสมบัติทางกายภาพ และเทคนิคทางสเปกโทรสโกปี สามารถพิสูจน์ทราบสูตรโครงสร้างของสารเหล่านี้ คือ mansorin A (**1**), mansorin B (**2**), mansorin C (**3**), mansonone N (**4**), mansonone O (**5**), mansonone P (**6**), mansonone Q (**7**), mansonone C (**8**), mansonone E (**9**), mansonone G (**10**), mansonone H (**11**), dehydrooxoperezinone (**12**), 3-methoxy-4,5-dihydroxybenzaldehyde (**13**), mansoxetane (**14**), mansonone R (**15**) และ mansonone S (**16**) mansonones N, O, P, Q, R, S และ mansoxetane เป็นสารใหม่ที่พบในธรรมชาติ เมื่อทำการทดสอบฤทธิ์ชีวภาพของสารที่แยกได้ พบว่า mansonone C แสดงฤทธิ์ทางชีวภาพได้ดีที่สุด คือ มีความเป็นพิษในระดับสูงต่อไรสีน้ำตาล *Artemia salina* Linn. ลูกน้ำยุงที่ก่อให้เกิดโรคไข้เหลือง *Aedes egypti* และเซลล์มะเร็งหลายชนิด อีกทั้งแสดงฤทธิ์ต้านเชื้อรา *Cladosporium cucumerinum* และ *Candida albicans* สำหรับ mansonone E แสดงฤทธิ์เช่นเดียวกับ mansonone C ยกเว้นไม่แสดงฤทธิ์ฆ่าลูกน้ำยุงที่ก่อให้เกิดโรคไข้เหลือง นอกจากนี้ mansorins A และ B แสดงฤทธิ์ต้านเชื้อรา *C. cucumerinum* mansonone N และ 3-methoxy-4,5-dihydroxybenzaldehyde แสดงฤทธิ์ต้านอนุมูลอิสระ DPPH ส่วน mansonones G, H และ dehydrooxoperezinone แสดงฤทธิ์ต้านการแข็งตัวของเลือดในระดับที่น่าสนใจ

ABSTRACT

The investigation of chemical constituents from the dichloromethane, ethyl acetate and methanolic extracts of the heartwoods of *Mansonia gagei* Drumm. to furnish sixteen compounds. By means of their physical properties and spectroscopic data, these compounds were firmly elucidated their strutures as mansorin A (**1**), mansorin B (**2**), mansorin C (**3**), mansonone N (**4**), mansonone O (**5**), mansonone P (**6**), mansonone Q (**7**), mansonone C (**8**), mansonone E (**9**), mansonone G (**10**), mansonone H (**11**), dehydrooxoperezinone (**12**), 3-methoxy-4,5-dihydroxybenzaldehyde (**13**), mansoxetane (**14**), mansonone R (**15**) and mansonone S (**16**). Among them, mansonones N, O, P, Q, R and S together with mansoxetane were characterized as new naturally occurring compounds. Moreover, those compounds were tested for their biologically activities. Mansonone C displayed the most potency activities. It showed high toxicity against brine shrimp *Artemia salina* Linn., larvae *Aedes egypti* and a variety of cancer cell lines. In addition, it revealed antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*. Mansonone E exhibited the same activities as mansonone C except for larvicidal activity. Mansorins A and B were found to be active against *C. cucumerinum*. Mansonone N and 3-methoxy-4,5-dihydroxybenzaldehyde possessed radical scavenging properties toward DPPH. Furthermore, mansonones G, H and dehydrooxoperezinone displayed significant activity in antithrombin assay.

KEY WORD: *Mansonia gagei* / MANSONONE / COUMARIN / BIOACTIVE COMPOUND

EXECUTIVE SUMMARY

According to the literature survey of plants in *Mansonia* genus, a series of mansonones has been isolated from these plants. Moreover, the ethanolic extract of the heartwoods of *Mansonia gagei* Drumm. gave attractive results, high toxicity against both brine shrimp *Artemia salina* Linn. and a variety of cancer cell lines, on the preliminary study with the aim of screening for bioactive compounds. Therefore, the heartwoods of this plant was selected for further investigation of the chemical constituents and their bioactivities.

The dried heartwoods of *Mansonia gagei* Drumm. were collected and extracted with the appropiate solvents: hexane, ethyl acetate and methanol, to yield Fractions I, II and III, respectively. All fractions were then preliminarily screened for their biological activities. Each fraction was further fractionated and separated using column chromatography, LPLC, MPLC and HPLC to furnish sixteen compounds. These compounds were characterized their structures on the basis of chemical and physical properties including spectroscopic evidence. Among them, seven compounds named mansonones N, O, P, Q, R and S together with mansoxetane were characterized as new naturally occurring compounds. Moreover, those compounds were tested for their biologically activities. Mansonone C displayed the most potency activities. It showed high toxicity against brine shrimp *Artemia salina* Linn., larvae *Aedes egypti* and a variety of cancer cell lines. In addition, it revealed antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*. Mansonone E exhibited the same activities as mansonone C except for larvicidal activity. Mansorins A and B were found to be active against *C. cucumerinum*. Mansonone N and 3-methoxy-4,5-dihydroxybenzaldehyde possessed radical scavenging properties toward DPPH. Furthermore, mansonones G, H and dehydrooxoperezinone displayed significant activity in antithrombin assay.

These results call for further work on synthesizing additional mansonone-type derivatives including studying the chemical constituents in other parts of *M. gagei* with the aim of obtaining the lead compounds that are more potent and selective to above-mentioned bioactivities.

OBJECTIVES

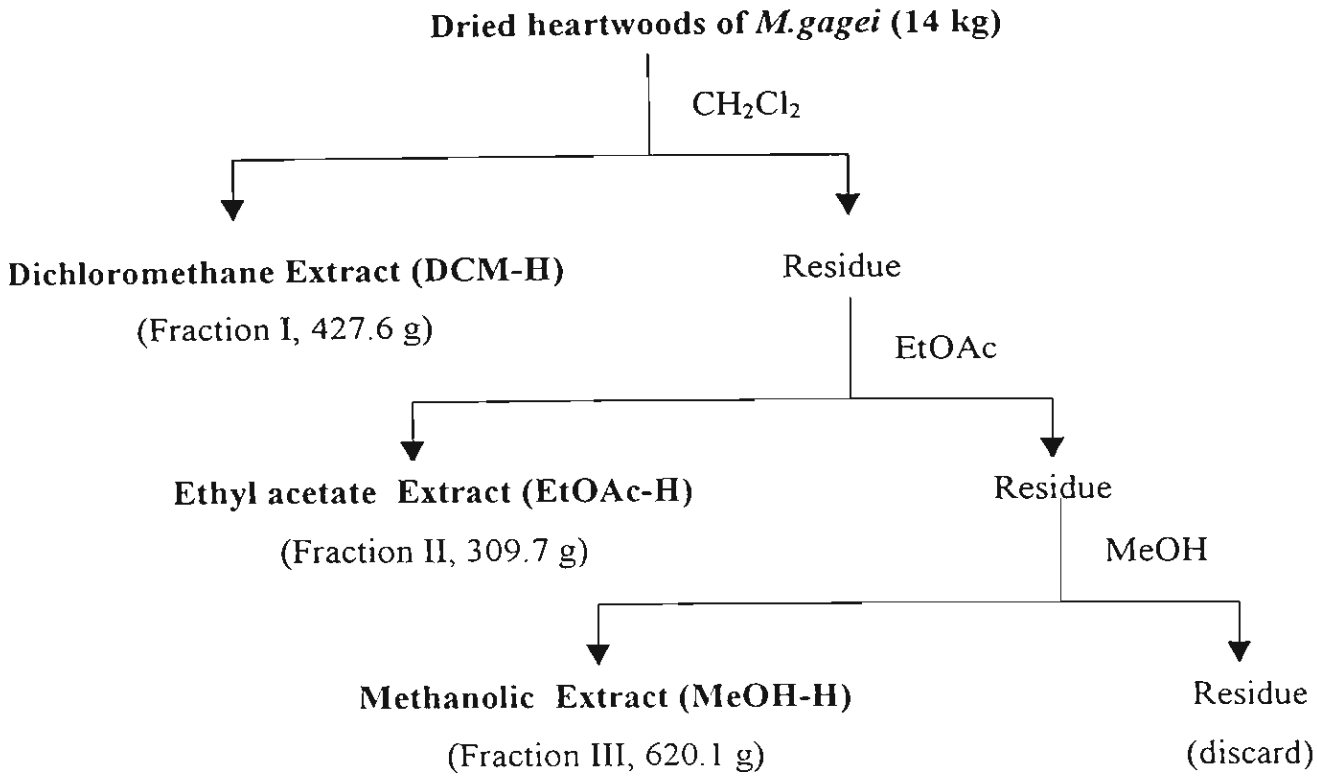
1. To extract and isolate the chemical constituents from the heartwood extract of *Mansonia gagei* which exhibited significant bioactivities.
2. To elucidate the structures of the isolated substances.
3. To search for biologically active principles.

THE RESEARCH WORK PERFORMED DURING 28 SEPT 2001 - 28 SEPT 2002

The dried heartwoods of *Mansonia gagei* Drumm. were collected from Saraburi province, Thailand. The identity of this plant has been compared with a voucher specimen no. 43281 at the herbarium of the Royal Forestry Department of Thailand.

The dried heartwoods of *Mansonia gagei* Drumm. (14 kg) were milled and extracted with dichloromethane three times at room temperature. The dichloromethane extract was evaporated under a vacuum to yield a black extract, Fraction I (DCM-H, 427.6 g, 3.08 % yield of the dried heartwood). The residue was successively extracted with ethyl acetate and methanol as the same above mentioned procedure to give the ethyl acetate extract, Fraction II (EtOAc-H, 309.7 g, 2.23 % yield of the dried heartwood) and methanolic extract, Fraction III (MeOH-H, 620.1 g, 4.46 % yield of the dried heartwood), respectively. All extracts, Fractions I, II and III, were then preliminarily screened for their cytotoxicity against brine shrimp *Artemia salina* Linn., as well as for their antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*, larvicidal activity against *Aedes aegypti*, radical scavenging properties in a DPPH assay, antithrombin and anticancer activities. The results derived from those activities could be utilized as a guidance to determine the interest fractions for further isolating the lead compounds.

A part of each extract was fractionated by silica gel quick column chromatography. Each fraction was then separated and purified using silica gel and sephadex LH-20 column chromatography, LPLC, MPLC and HPLC. The isolated compounds were characterized their structures on the basis of chemical and physical properties including spectroscopic evidence. In addition, these compounds were reassayed for brine shrimp cytotoxicity, antifungal, larvicidal, antioxidant, antithrombin and anticancer activities in order to confirm those biological activities.



Scheme 1 The extraction procedure of heartwoods of *M. gagei*

RESULTS AND DISCUSSION

PART 1:

1.1 Preliminary Bioassay Screening Results of Extracts from the Heartwoods

The preliminarily screening results for the cytotoxicity against brine shrimp *Artemia salina* Linn., as well as for their antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*, larvicidal activity against *Aedes aegypti*, radical scavenging properties in a DPPH assay, antithrombin and anticancer activities of Fractions I, II and III are shown in Tables 1.1-1.3 and Figures 1.1-1.2.

Table 1.1 Brine shrimp cytotoxicity test of extracts from the heartwoods of *M. gagei*

Extract	LC_{50}	Activity
DCM-H	3.53	High
EtOAc-H	12.56	Medium
MeOH-H	120.80	Low

Note High activity (LC_{50} < 10 μg/mL)
Medium activity ($10 \leq LC_{50} \leq 100$ μg/mL)
Low activity (LC_{50} > 100 μg/mL)

Table 1.2 Antifungal, larvicidal and radical scavenging activities of extracts from the heartwoods of *M.gagei*.

Extract	*Cladosporium cucumerinum*[a]	*Candida albicans*[a]	*Aedes aegypti*[b]	DPPH[a]
DCM-H	+	+	-	+
EtOAc-H	+	+	-	+
MeOH-H	-	-	-	+

[a] : tested amount: 100 μg of crude extract
[b] : tested amount: 500 ppm of crude extract
+ : active
- : not active

Table 1.3 The anticancer activity of extracts from the heartwoods of *M.gagei*

Extract	Mouse Leukemia	Human Myeloma	Human Colon Adenocarcinoma	Human Leukemia	Human Breast Carcinoma
DCM-H	98.7	90.2	86.8	94.5	80
EtOAc-H	99.5	85.8	82.6	97.5	89.5
MeOH-H	100	96.4	84.8	93.9	91.8
Methotraxate	97.6	89.1	61.8	65.1	17.5

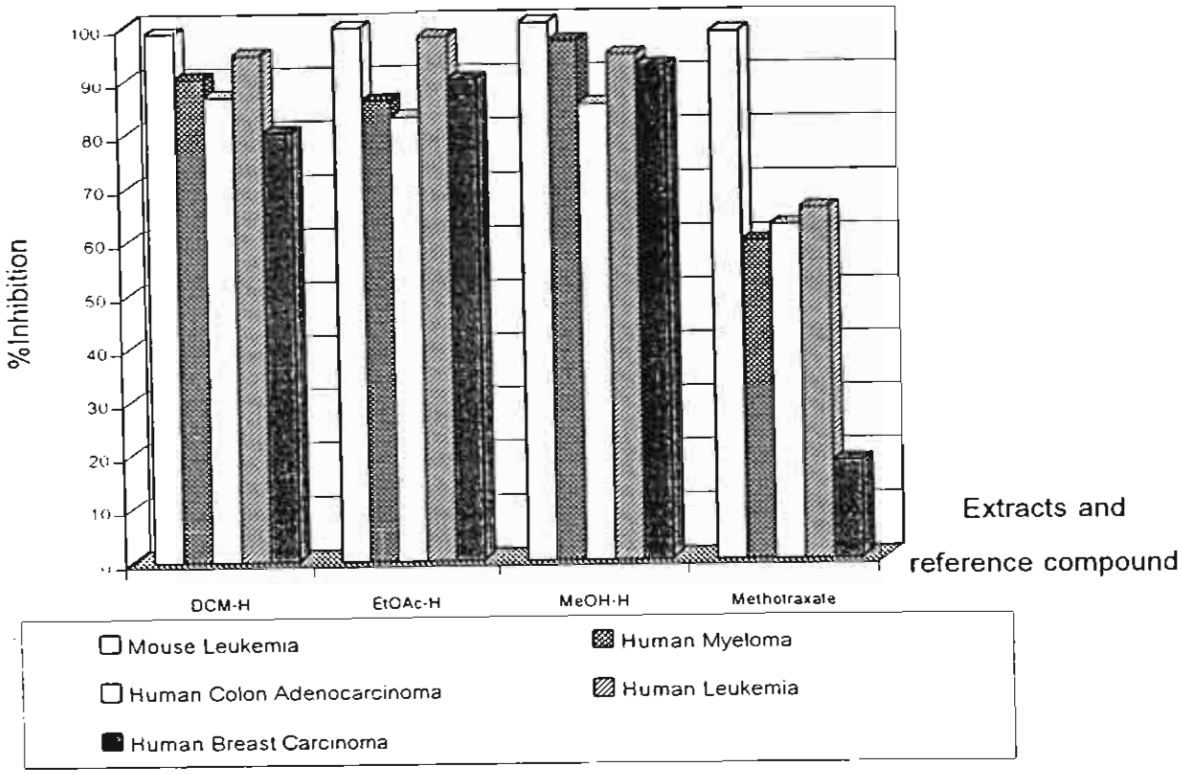


Fig 1.1 The anticancer activity of extracts from the heartwoods of *M. gagei*

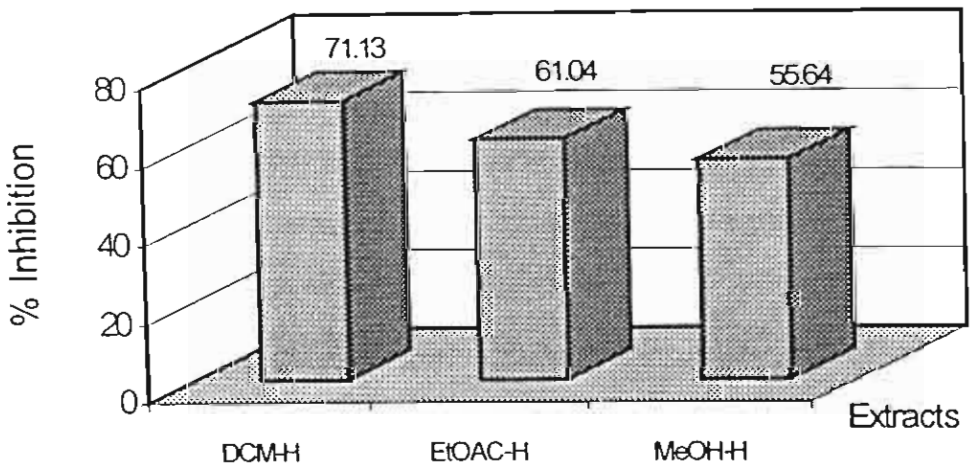


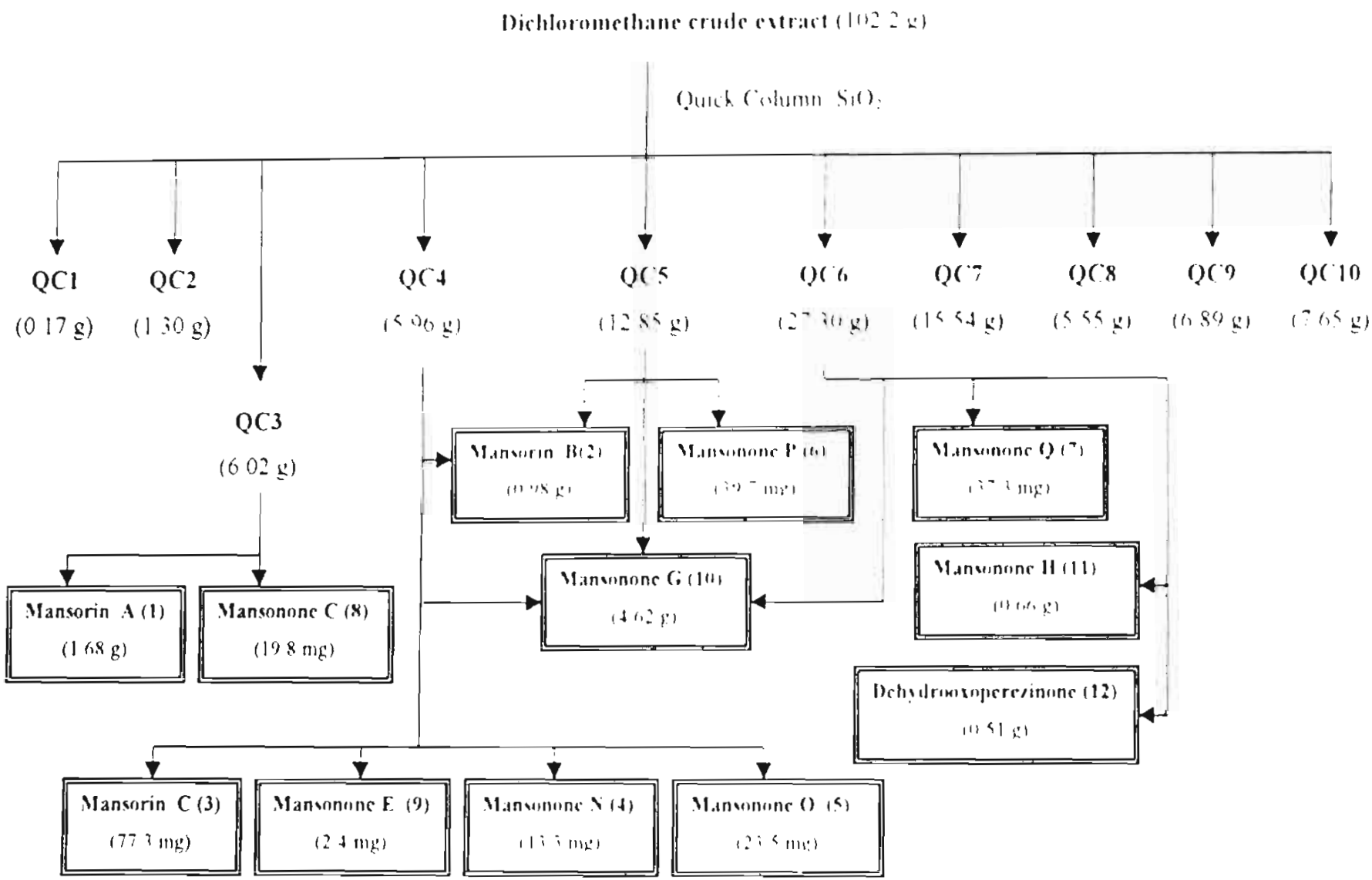
Fig. 1.2 The antithrombin activity of extracts from the heartwoods of *M. gagei*

The bioactivity screening results revealed that the most promising extract for further investigation was dichloromethane extract (DCM-H). It exhibited high cytotoxicity activity against brine shrimp, positive test for antifungal and radical scavenging assays, high activity in antithrombin and anticancer tests. Based on these

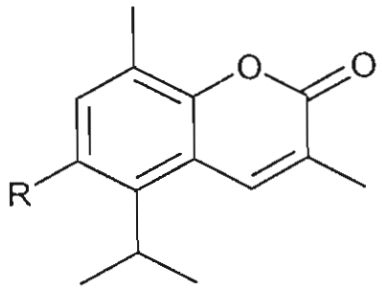
mentioned activities, the dichloromethane extract was the lead extract to be undertaken to examine its constituents along with biological activity.

1.2 Separation of Dichloromethane Extract of the Heartwoods

A part of the dichloromethane crude extract (102.2 g) was subjected to silica gel quick column chromatography using a step gradient of hexane-CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH as a solvent system. The fractions were collected and combined according to TLC results. Ten fractions were obtained. Each fraction, QC1-QC8, was subjected to silica gel column chromatography using mixtures of hexane-CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH of increasing polarity as eluents. Twelve pure compounds were obtained after further separation of fractions on silica gel column chromatography. Mansorin A (1.68 g), mansonone C (19.8 mg) were isolated from QC3. Mansorin C (77.3 mg), mansonones E (2.4 mg), N (13.3 mg) and O (23.5 mg) were obtained from QC4. Mansonone P (39.7 mg) could be separated from QC5. Mansonones H (0.66 g), Q (37.3 mg) and dehydrooxoperezinone (0.51 g) were derived from QC6. Mansorin B (0.98 g) was fruitfully isolated from both QC4 and QC5. Mansonone G (4.62 g), the major compound of this extract, was obtained from QC3-QC6. Among these twelve compounds, mansonones N, O, P and Q have been characterized as new naturally occurring compounds. The identification of eight known compounds, mansorins A, B and C including mansonones C, E, G, H and dehydrooxoperezinone, was carried out by comparing their physical and spectral data with previously reported values.[1-3] The concise diagram of isolation and the structures of the isolated compounds were presented in Scheme 1.1 and Figure 1.3, respectively.

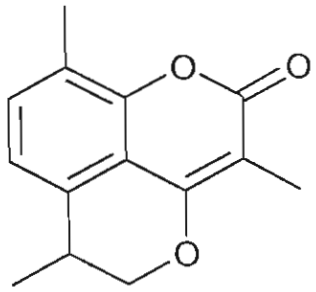


Scheme 1.1 Isolation diagram of the dichloromethane extract from the heartwoods of *M. gagei*

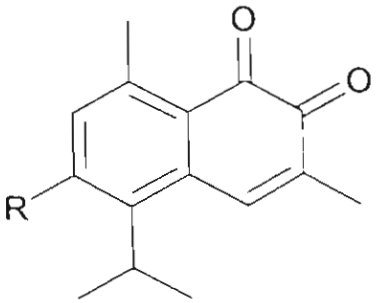


R = OH : MANSORIN A

R = OCH_3 : MANSORIN B

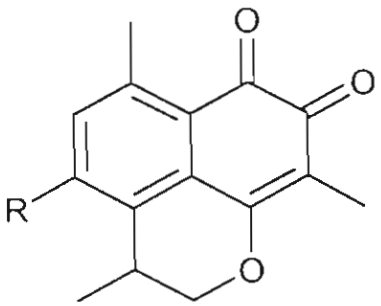


MANSORIN C



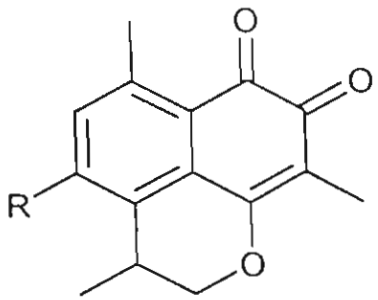
R = H : MANSONONE C

R = OH : MANSONONE G



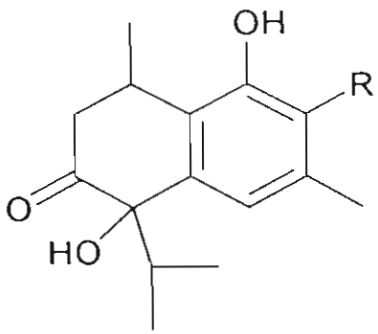
R = H : MANSONONE E

R = OH : MANSONONE H



R = H : MANSONONE E

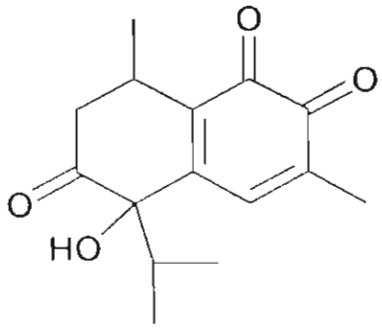
R = OH : MANSONONE H



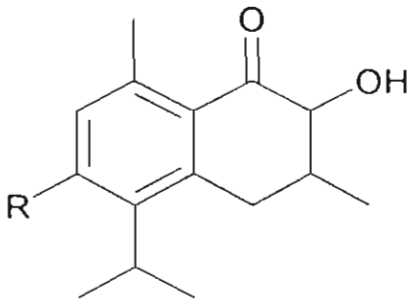
R = OCH_3 : MANSONONE N

R = OH : MANSONONE Q

Fig 1.3 The structures of the isolated compounds from the dichloromethane extract

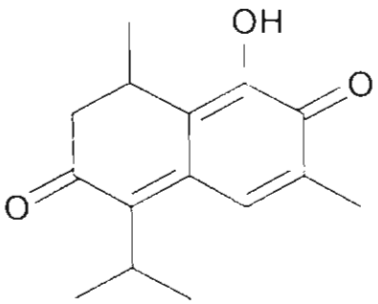


MANSONONE O

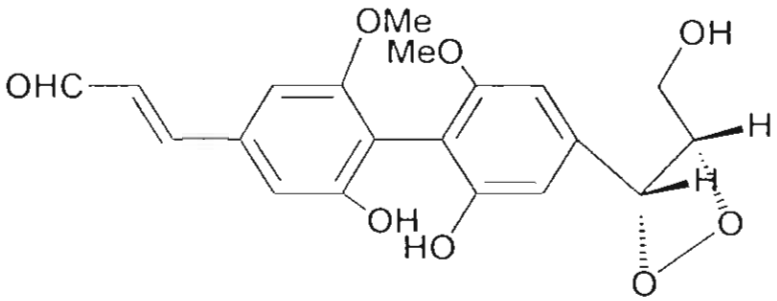


R = OH : MANSONONE P

R = H : MANSONONE R



MANSONONE S



MANSOXETANE

Fig 1.3 Cont.

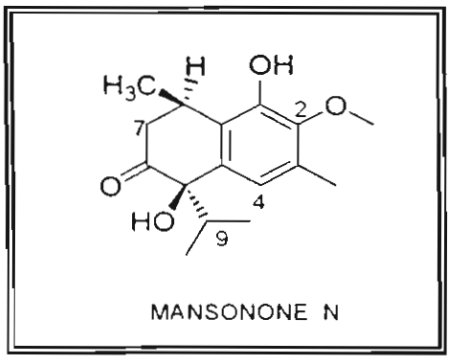
1.3 Structural Elucidation of New Compounds

1.3.1 *Compound 4:* Mansonone N

Compound **4** was isolated as a colorless crystal; $[\alpha]_D^{20}$ +30.0 (c = 0.20, $CHCl_3$), m.p. 144-145 °C. A molecular ion at m/z 278 in the EIMS, ^1H NMR and ^{13}C NMR spectra of Compound **4** suggested a molecular formula of $C_{16}H_{22}O_4$. Two different kinds of chemical shifts were noticed in the ^{13}C NMR spectrum: six resonances between 120-145 ppm, typical of an aromatic moiety and high field signals from 15 to 80 ppm, characteristic of an aliphatic unit. A ketonic carbonyl was also identified in the low-field region of the ^{13}C NMR spectrum by a resonance at δ_C 212.1 (C-6). This signal was positioned on the aliphatic unit based on the informative data from the HMBC correlations with ^1H NMR signals at δ_H 3.79 (H-8), 2.49 (H-7), 3.21 (H-7), and 2.15 (H-9). An isopropyl group could be noticeable from the ^1H NMR spectrum by the presence of two methyl groups at δ_H 0.79 (d, J = 6.9 Hz, 9-CH_3) and δ_H 0.99 (d, J = 6.7 Hz, 9-CH_3) which correlated in the COSY spectrum with a methine resonance centered at δ_H 2.15 (sept, J = 6.9 Hz, H-9). This isopropyl unit also showed long-range heteronuclear correlations to the aliphatic group between the protons of the methyl units located at δ_H 0.79 (9-CH_3) and δ_H 0.99 (9-CH_3) in the ^1H NMR spectrum with the ^{13}C NMR resonance at δ_C 80.6 (C-5). The chemical shift of C-5 was typical of a quaternary oxygenated carbon. Other two methyl signals were detected in the ^1H NMR spectrum. The doublet at δ_H 1.16 (d, J = 6.9 Hz, 8-CH_3) was correlated in the COSY spectrum with the aliphatic proton at δ_H 3.79 (m, H-8). Further analysis of the aliphatic area of the same spectrum gave evidence for the presence of a CH_3-CH-CH_2 unit. The second methyl group was a singlet located at δ_H 2.30 (s, 3-CH_3) suggesting an attachment to the aromatic system of the molecule. This hypothesis was supported by HMBC correlations between the δ_H 2.30 and the ^{13}C NMR resonances at δ_C 120.1 (C-4), 128.8 (C-3), and 144.4 (C-2).

The only proton in the aromatic region of the ^1H NMR spectrum was positioned on C-4 as a result of the long-range heteronuclear correlations observed between the ^1H NMR signal at δ_H 6.86 (H-4) and the ^{13}C NMR resonances located at δ_C 80.6 (C-5), 16.0 (3-CH_3), 124.6 (C-8a), and 144.4 (C-2). This assignment was

supported by the ^{1}H-^{1}H NOESY spectrum observed between proton at δ_H 6.86 (H-4) and the ^{1}H NMR signals at δ_H 2.15 (H-9), 2.30 (3-CH_3) and 0.99 (9-CH_3). Substitution of a methoxy group (δ_H 3.79) at the C-2 position of the aromatic moiety was also confirmed through NOE correlations with the methyl group at δ_H 2.30 (3-CH_3). Spectrometric data permitted to establish the structure of Compound **4** as a new sesquiterpenoid derivative named mansonone N and their NMR data is shown in Table 1.4. Definitive evidence for this structure was obtained from a single-crystal X-ray analysis. The molecular structure and crystallographic numbering scheme are illustrated in Figure 1.4. Unfortunately, the measured crystal was an inversion twin and, therefore, the absolute configuration could not be determined.



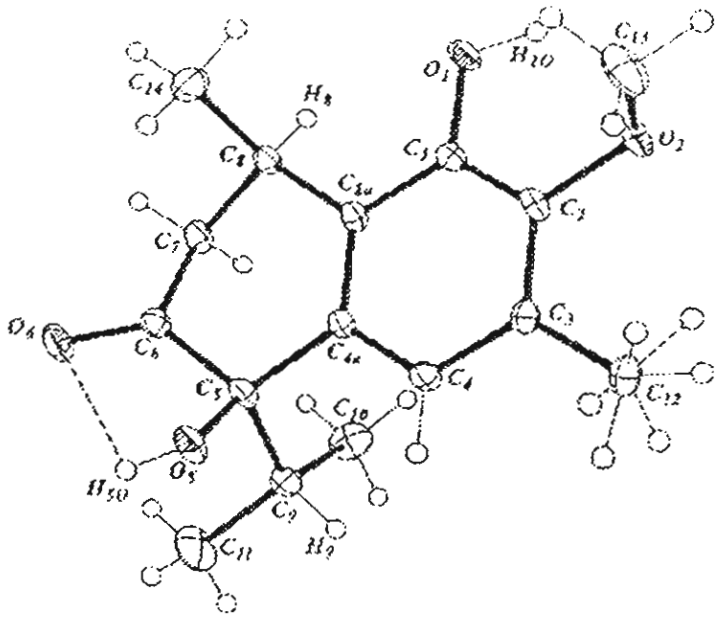


Fig. 1.4 ORTEP of Compound **4**

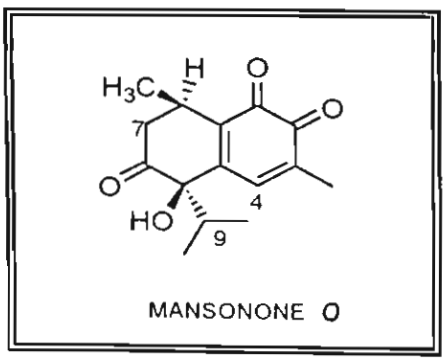
Table 1.4 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **4**

Position	Chemical Shift (ppm)[a]	
	δ_H (J in Hz)	δ_C
1	-	145.1
2	-	144.4
3	-	128.8
4	6.86, s, 1H	120.1
4a	-	136.5
5	-	80.6
6	-	212.1
7	2.49, dd, 1H (1.7, 12.5) 3.21, dd, 1H (7.8, 12.6)	43.8
8	3.79, m, 1H	32.4
8a	-	124.6
9	2.15, sept, 1H (6.9)	39.6
1-OH	5.84, s, 1H	-
2-OCH_3	3.79, s, 3H	60.6
3-CH_3	2.30, s, 3H	16.0
5-OH	4.05, s, 1H	-
8-CH_3	1.16, d, 3H (6.9)	22.0
9-$(CH_3)_2$	0.79, d, 3H (6.9) 0.99, d, 3H (6.9)	18.2 16.3

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 500 and 125 MHz, respectively

1.3.2 *Compound 5:* Mansonone O

Compound 5 was isolated as a violet orthorhombic; $[\alpha]_D^{20}$ –76.0 (c = 0.10, $CHCl_3$), m.p. 125-128°C. A molecular ion at m/z 262 in the EI-MS, ^1H NMR and ^{13}C NMR spectra of Compound 5 suggested a molecular formula of $C_{15}H_{18}O_4$. Comparison of the ^1H NMR and ^{13}C NMR spectra of this compound with those of Compound 4 (Table 1.5), it was manifestly observed that the non-aromatic moiety of these two molecules were identical. The ^1H NMR singlet signal at δ_H 7.06 was positioned at C-4 after the observation of a long-range heteronuclear correlation between this signal and the ^{13}C NMR resonance at δ_C 79.3 (C-5). Measurement of a NOE effect between the same ^1H NMR singlet at δ_H 7.06 and the signal at δ_H 2.00 (3H) permitted to locate of this methyl group at C-3. This result was confirmed by an HMBC correlation observed between the ^{13}C NMR resonance at δ_C 15.5 (3-CH_3) and the ^1H NMR singlet at δ_H 7.06 (H-4). Finally, two ketonic carbonyl carbon signals seen in the ^{13}C NMR spectrum at δ_C 179.3 and 180.1 were located at C-1 and C-2, respectively, in agreement with the HMBC correlation observed between the ^{13}C NMR signal at δ_C 180.1 (C-2) and the ^1H NMR singlet at δ_H 7.06 (H-4). Compound 5 was identified as a new natural product and named mansonone O. Definitive evidence of this structure was obtained from a single-crystal X-ray analysis. The molecular structure and crystallographic numbering scheme are illustrated in Figure 1.5.



MANSONONE O

Table 1.5 ^{1}H-NMR and ^{13}C-NMR spectral data of Compounds **4** and **5**

Position	Chemical Shift (ppm)			
	4[a]		**5**[a]	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	-	145.1	-	179.3
2	-	144.4	-	180.1
3	-	128.8	-	137.2
4	6.86, s, 1H	120.1	7.06, dd, 1H (1.5, 3.4)	136.0
4a	-	136.5	-	148.8
5	-	80.6	-	79.3
6	-	212.1	-	208.8
7	2.49, dd, 1H (1.7, 12.5) 3.21, dd, 1H (7.8, 12.6)	43.8	2.47, dd, 1H (1.2, 12.8) 3.10, dd, 1H (7.9, 13.1)	42.6
8	3.79, m, 1H	32.4	3.54, m, 1H	32.0
8a	-	124.6	-	137.0
9	2.15, sept, 1H (6.9)	39.6	2.21, sept, 1H (6.7)	38.4
1-OH	5.84, s, 1H	-	-	-
2-OCH_3	3.79, s, 3H	60.6	-	-
3-CH_3	2.30, s, 3H	16.0	2.00, d, 3H (1.5)	15.5
5-OH	4.05, s, 1H	-	3.95, s, 1H	-
8-CH_3	1.16, d, 3H (6.9)	22.0	1.06, d, 3H (6.7)	21.7
9-$(CH_3)_2$	0.79, d, 3H (6.9) 0.99, d, 3H (6.9)	18.2 16.3	0.89, d, 3H (7.0) 1.08, d, 3H (7.0)	17.7 16.9

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 500 and 125 MHz, respectively

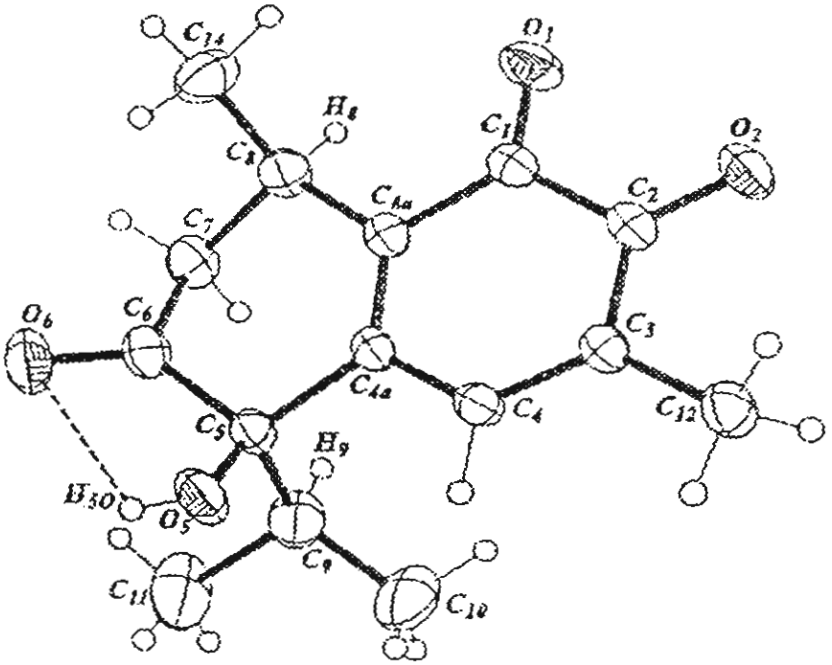
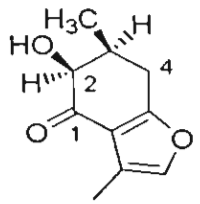


Fig. 1.5 ORTEP of Compound 5

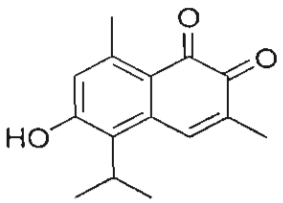
1.3.3 *Compound 6:* Mansonone P

Commpound **6** was accomplishly isolated as pale yellow powder, $[\alpha]_D^{20}$ -13.5 (c = 0.20, $CHCl_3$), m.p. 175-176 °C. A molecular formula of $C_{15}H_{20}O_3$ was deduced for Compound **6** from the EIMS (m/z 248 $[M]^+$), ^1H NMR and ^{13}C NMR spectra. As Compound **4**, the ^{13}C NMR spectrum of Compound **6** suggested the presence of an aromatic moiety with six signals located between 120 and 160 ppm as well as an aliphatic portion of the molecule represented by resonances found from 15 to 40 ppm. An isopropyl group was identified from the ^1H NMR spectral data by the presence of two methyl groups at δ_H 1.36 (d, J = 6.7 Hz, 9-$(CH_3)_2$) and a methine resonance centered at δ_H 3.32 (sept, J = 6.7 Hz, H-9). This unit was assigned to C-5 from HMBC correlations observed between the septet at δ_H 3.32 and ^{13}C signals observed at δ_C 143.8 (C-5), 158.5 (C-4a) and 129.2 (C-6). Other long-range heteronuclear correlations between the ^1H NMR signals of the methyl unit at δ_H 1.31 (3-CH_3) and the ^{13}C NMR resonances found at δ_C 77.9 (C-2), 36.9 (C-3), and 35.1 (C-4) allowed the placement of the latter methyl group at C-3. The fourth methyl group was positioned on the aromatic ring by HMBC correlations between its ^1H NMR signals at

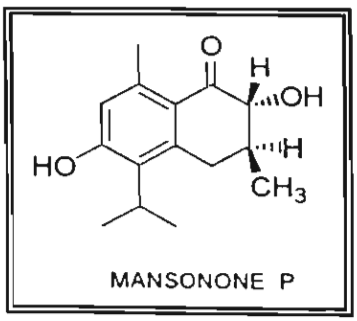
δ_H 2.57 (8-CH_3) and the ^{13}C NMR resonances at δ_C 118.8 (C-7), 122.4, (C-8a) and 141.8 (C-8). A ketonic carbonyl was also identified in the low-field region of the ^{13}C NMR spectrum at δ_C 199.5 (C-1). This signal was shown to be linked to the aliphatic part of the molecule based upon the information derived from HMBC correlations with ^1H NMR signals at δ_H 3.89 (H-2), 4.29 (2-OH), and 2.00 (H-3). The structure was finally confirmed by comparison of ^1H and ^{13}C NMR data with desacetylcalaminthone[4] and mansonone G[3] (Table 1.6). Desacetylcalaminthone is a monoterpene derivative isolated from *Calamintha ashei* (Lamiaceae), with an aliphatic moiety closely related to that of Compound **6** while mansonone G, isolated for the first time from the heartwood of *Mansonia altissima*,[2] shares a common aromatic pattern with Compound **6**. The relative chiralities of carbons C-2 and C-3 were determined different from those of Compound **4** and **5** on the basis of the *trans* pseudo-diaxial coupling constants calculated between the ^1H NMR signals H-2 and H-3 (J = 12.5 Hz) as well as between H-3 and H-4 (J =11.8 Hz). The relative stereochemistry of Compound **6** was confirmed by a NOESY correlation observed between ^1H NMR resonances at δ_H 3.89 (H-2) and 2.61 (H-4). Compound **6** was characterized as a novel natural product, named mansonone P.



DESACETYCALAMINTHONE



MANSONONE G



MANSONONE P

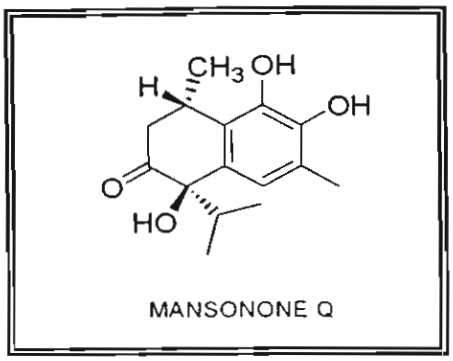
Table 1.6 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **6**, desacetylcalaminthone and mansonone G

Position	6[a]		Descetylcalaminthone[b]		Mansonone G[c]
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	-	199.5	-	194.7	
2	3.89, dd, 1H (2.4, 12.5)	77.9	4.37, dd, 1H (2.0, 4.6)	75.9	
3	2.00, m, 1H (12.5, 11.7)	36.9	2.81, dddd, 1H (1.9, 4.6, 5.5, 7.1)	35.4	
4	3.16, dd, 1H (4.9, 17.1) 2.61, dd, 1H (11.9, 17.4)	35.1	3.16, dd, 1H (5.5, 17.6) 2.77, dd, 1H (1.9, 17.6)	30.0	
4a	-	129.2			
5	-	143.8			
6	-	158.2			
7	6.49, s, 1H	118.8			6.49, s, 1H
8	-	141.8			
8a	-	122.4			
9	3.32, sept, 1H (6.7)	27.2			3.48, sept, 1H (7.0)
2-OH	4.29, d, 1H (2.2)	-			
3-CH_3	1.31, d, 3H (6.4)	18.8			
6-OH	5.67, s, 1H	-			
8-CH_3	2.57, s, 3H	22.8			2.47, s, 3H
9-$(CH_3)_2$	1.36, d, 6H (6.7)	20.1 (2C)			1.38, d, 6H (7.0)

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 500 and 125 MHz, respectively
[b] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 and 100 MHz, respectively
[c] ^{1}H NMR spectra was measured in $CDCl_3$-CD_3OD (1:1) at 60 MHz

1.3.4 *Compound 7:* Mansonone Q

Commpound 7 was obtained as pale yellow powder, $[\alpha]_D^{20}$ +9.5 (c = 0.10, $CHCl_3$), m.p. 105-106 °C. The ^1H and ^{13}C NMR spectra of this compound were very similar to those of Compound **4** (Table 1.7). The two compounds differed by the absence of signals for the methoxy group of δ_H 3.79 in the ^1H NMR spectrum, and δ_C 60.6 in the ^{13}C NMR spectrum. This methoxy group was replaced by a hydroxyl group firmly characterized by the appearance of a ^1H NMR singlet signal at δ_H 4.65 ppm. The position of the hydroxyl unit was confirmed by long-range heteronuclear correlations, with the ^{13}C NMR resonances at δ_C 141.2 (C-1), 140.1 (C-2), and 122.1 (C-3). The EIMS (m/z 264 $[M]^+$) of Compound 7 as well confirmed the substitution. Compound 7 was identified as a new natural product named mansonone Q. The relative chiralities of carbons C-5 and C-8 were determined different with mansonone N and P. They were attributed on the basis of differences observed in the coupling constants of H-7 (2H) and H-8 (J = 10.1 and 4.6 Hz) of mansonone Q compared with those of the structurally-related mansonones N (J = 1.7 and 7.8 Hz) and O (J = 1.2 and 7.9 Hz) as well as after observation of a lower-field chemical shift of the 8-CH_3 group at δ_H 1.52 for mansonone Q, which was at δ_H 1.16 and 1.06 for mansonones N and O, respectively. This attribution was in good agreement with the NOE effects noticed between the 8-CH_3 group at δ_H 1.52 and the signal at δ_H 0.93 belonging to one of the methyl groups of the isopropyl moiety (9-CH_3) and the NOE between the proton signal at δ_H 2.50 and the 8-CH_3 and 9-CH_3 groups positioned at δ_H 1.52 and 0.86, respectively. The relatively large coupling constant obtained between ^1H NMR signals at δ_H 3.04 and 3.60 (J = 10.1 Hz) was attributed to a *cis* arrangement of these two protons, due to the ring tension resulting from the steric hindrance between the isopropyl moiety and the 8-CH_3 group. If this compound was isolated more, further studies are planned for the determination the absolute configuration of the isolated compounds by LC/NMR analysis of derivatives obtained by the Mosher esterification.



MANSONONE Q

Table 1.7 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound 7

Position	Chemical Shift[a]	
	δ_H (J in Hz)	δ_C
1	-	141.2
2	-	140.1
3	-	122.1
4	6.88, s, 1H	119.9
4a	-	133.0
5	-	80.4
6	-	212.5
7	2.50, dd, 1H (4.6, 16.8) 3.04, dd, 1H (10.1, 16.8)	40 5
8	3.60, m, 1H	29.3
8a	-	123.7
9	2.10, sept, 1H (6.9)	38.0
1-OH	5.46, s, 1H	-
2-OH	4.65, s, 1H	-
3- CH_3	2.25, s, 3H	15.6
5-OH	3.88, s, 1H	-
8- CH_3	1.52, d, 3H (7.3)	23.4
9-$(CH_3)_2$	0.86, d, 3H (6.7) 0.86, d, 3H (6.7)	16.7 15.6

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 500 and 125 MHz, respectively

1.4 HPLC Analysis of the Dichloromethane Extract and Isolated Compounds

Dichloromethane extract and ten isolated compounds were detected by HPLC Column (Nova-Pak RP-18) using acetonitrile:H_2O = 20:80 → 100:0 in 30 minutes. The chromatograms of dichloromethane extract and those isolated compounds are shown in Fig 1.6 and 1.7, respectively

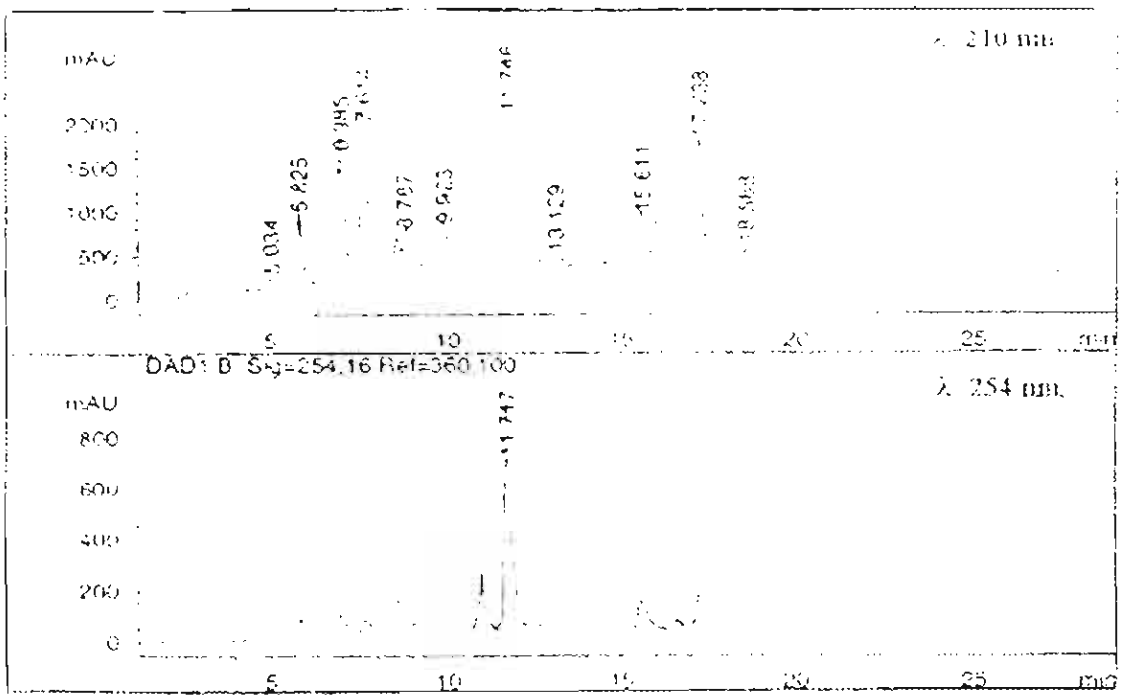


Fig 1.6 The HPLC Chromatogram of the dichloromethane extract

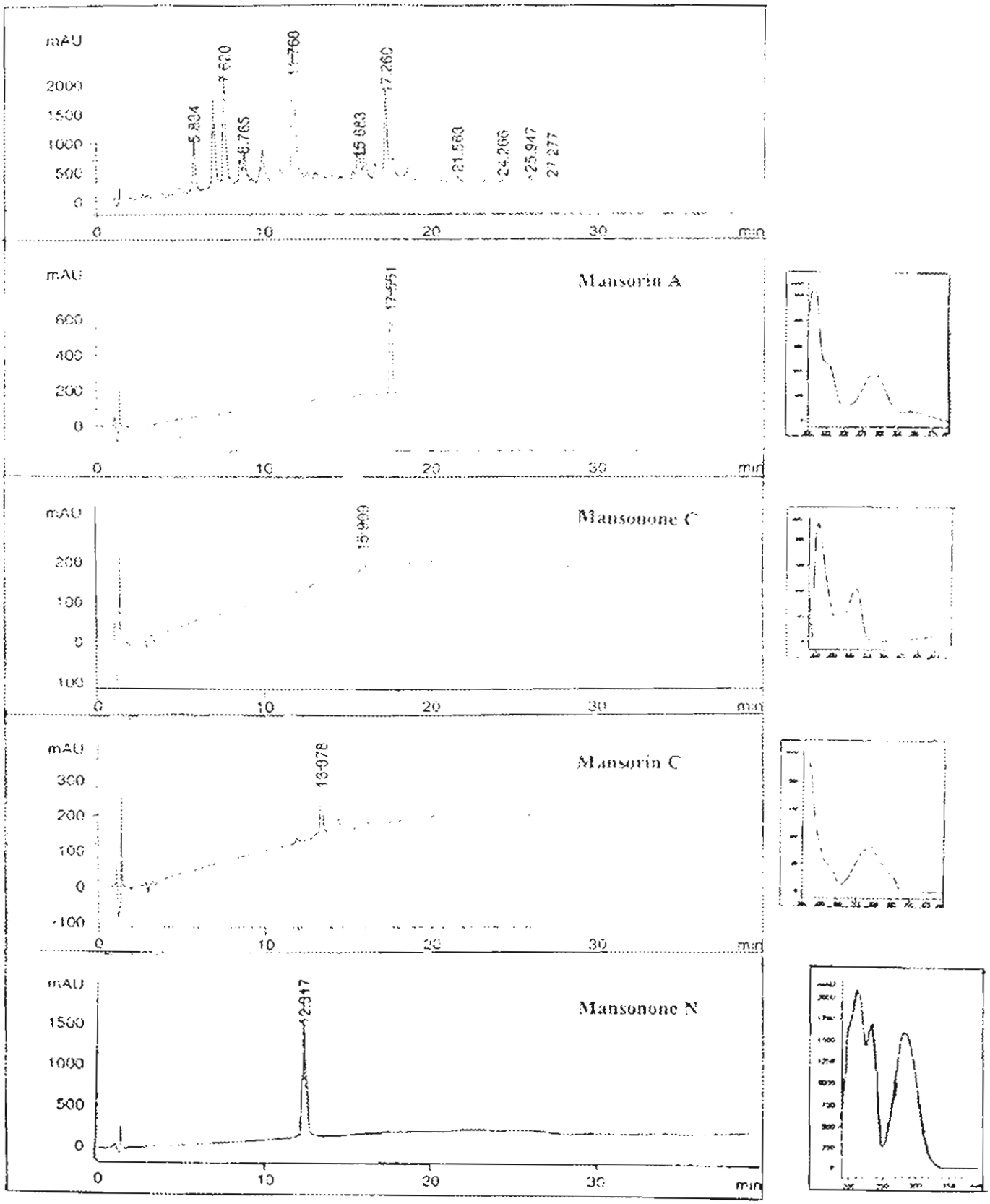


Fig 1.7 The HPLC Chromatogram of ten compounds isolated from the dichloromethane extract

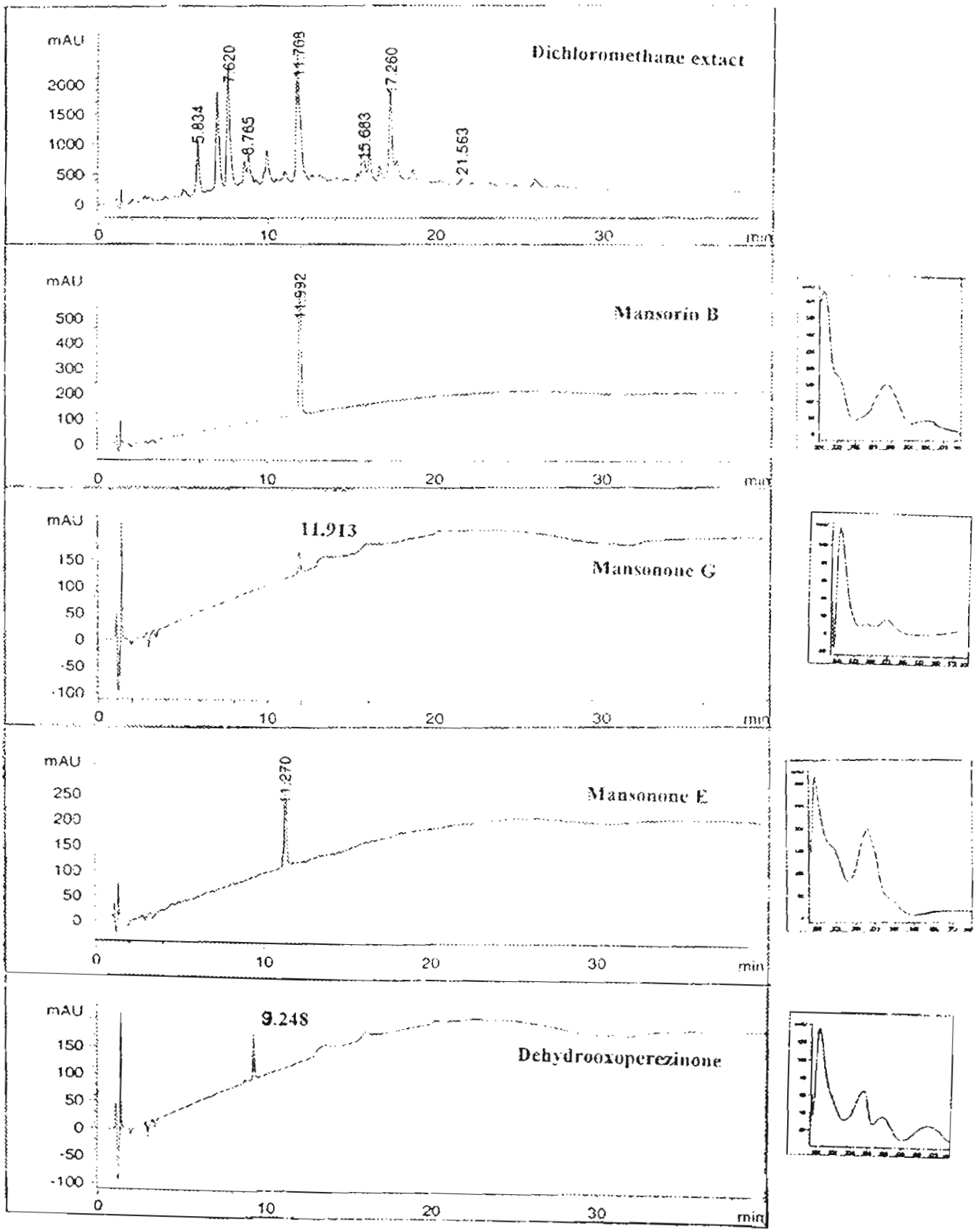

mAU
Dichloromethane extact
5.834
7.620
8.765
11.708
15.683
17.260
21.563
min
mAU
Mansorin B
11.992
min
mAU
11.913
Mansonone G
min
mAU
11.270
Mansonone E
min
mAU
9.248
Dehydrooxoperezinone
min

Fig 1.7 (Cont)

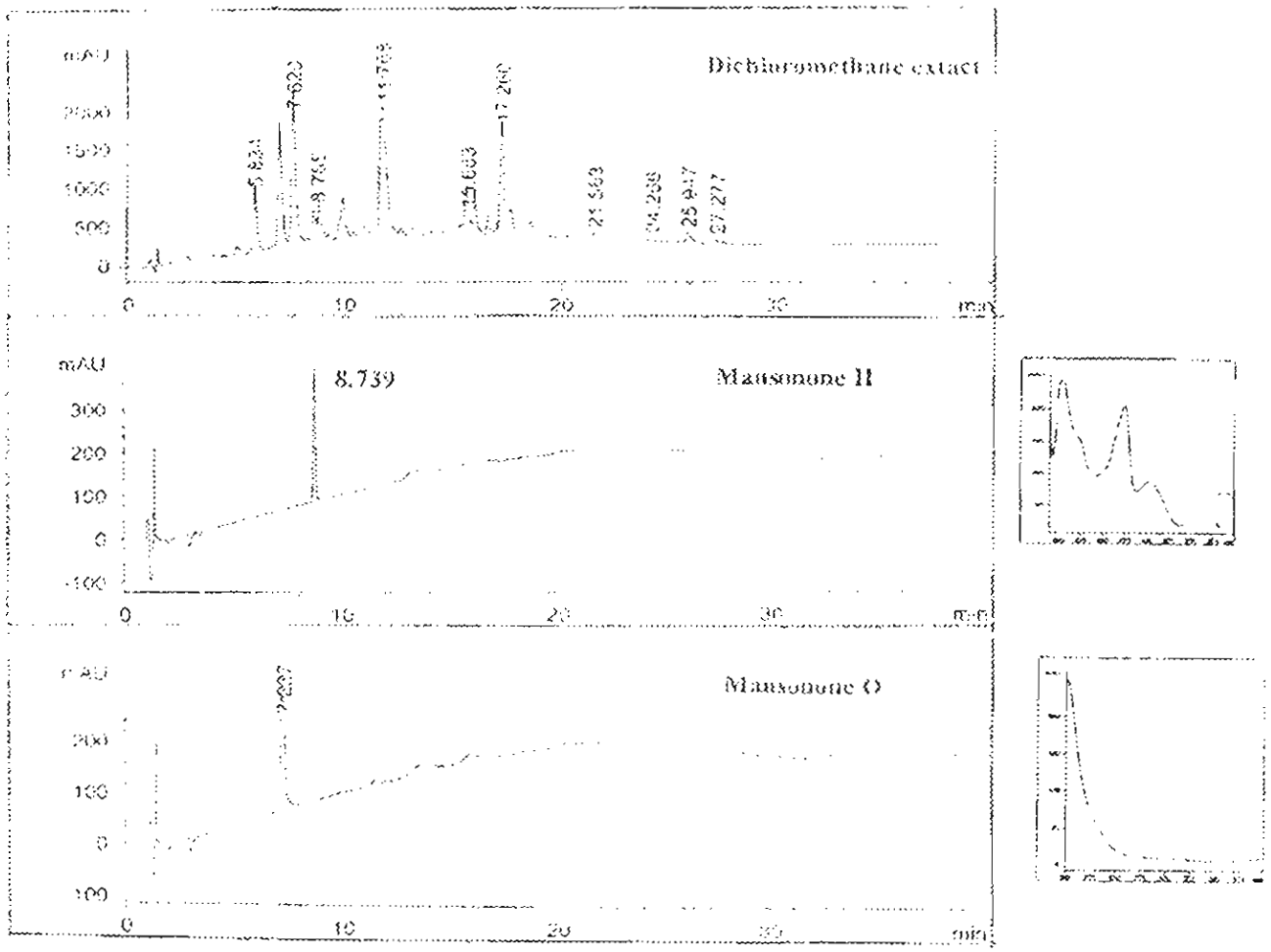


Fig 1.7 (Cont.)

From HPLC Chromatogram (Fig 1.6), it lucidly showed the remaining high intensity peaks at R_t 5.8, 6.9 and 7.6 mins. This maybe due to the high polarity of compounds that can not isolated by silica gel column chromatography.

3.5 Biological Activities of Isolated Compounds of the Dichloromethane Extract

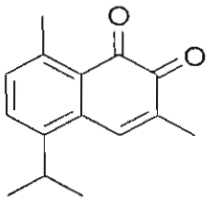
As it has been aforementioned, the dichloromethane extract displayed the most intriguing results for preliminary screening test (see Tables 1.1-1.3 and Figs 1.1-1.2). As a consequent the constituents have been thoroughly investigated. All isolated compounds from this extract were reassayed for brine shrimp cytotoxicity, antifungal, larvicidal, antioxidant, antithrombin and anticancer activities in order to confirm those biological activities. The biological activity studies are tabulated as shown in Tables 1.8-1.11

Table 1.8 Brine shrimp cytotoxicity test of the isolated compounds from the dichloromethane extract of the heartwoods of *M.gagei*

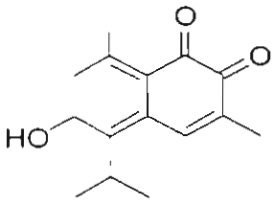
Compound	LC_{50}	Activity
Mansorin A	88.58	Medium
Mansorin C	201.79	Low
Mansonone C	2.47	High
Mansonone G	2.24	High
Mansonone H	58.52	Medium

Note
High activity ($LC_{50} < 10$ µg/mL)
Medium activity ($10 \leq LC_{50} \leq 100$ µg/mL)
Low activity ($LC_{50} < 1000$ µg/mL)

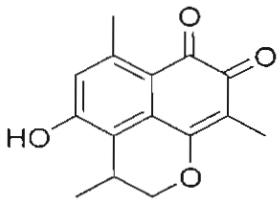
From Table 1.8, these compounds can be categorized into two classes; 1,2-naphthoquinones (mansonones C, G and H) and coumarins (mansorins A and C).



MANSONONE C



MANSONONE G



MANSONONE H

Based upon the biological activity results of a series of 1,2-naphthoquinones, mansonones C, G and H, the influence of substituent on the activity could be observed. To illustrate this, mansonones C and G had the same main structure except for the substituent at 6-position. Both compounds revealed the same trend of high toxicity activity against brine shrimp whatever the substituent at 6-position was either H or OH. Comparison of mansonones G and H, the results of brine shrimp cytotoxicity test manifestly revealed that a pyran ring in mansonone H decreased this activity.

As clearly demonstrated the results in Table 1.8, it seemed that the isolated 1,2-naphthoquinones exhibited higher toxicity than the isolated coumarins. These observations, however, could not draw any final conclusion. More compounds

in these two types were needed for further studying the structure-activity relationship (SAR).

Table 1.9 Antifungal, larvicidal and radical scavenging activities of the isolated compounds from the dichloromethane extract of the heartwoods of *M. gagei*

Compounds	*C. cucumerinum*[a]	*C. albicans*[a]	*A. aegypti*[b]	DPPH[c]
Mansorin A (**1**)	2.5	> 10	> 50	> 10
Mansorin B (**2**)	0.6	> 10	> 50	> 10
Mansorin C (**3**)	> 10	> 10	> 50	> 10
Mansonone N (**4**)	> 10	> 10	> 50	2.5
Mansonone O (**5**)	> 10	> 10	n.t.	> 10
Mansonone P (**6**)	> 10	> 10	> 50	> 10
Mansonone C (**8**)	0.6	0.15	6.25	> 10
Mansonone E (**9**)	0.6	2.5	> 50	> 10
Mansonone G (**10**)	> 10	> 10	> 50	> 10
Mansonone H (**11**)	> 10	> 10	> 50	> 10
Dehydrooxoperezinone (**12**)	> 10	> 10	> 50	> 10
Nystatin	0.2	1	n.t.	n.t.
Rotenone	n.t.	n.t.	3	n.t.
Quercetin	n.t.	n.t.	n.t.	0.5

[a] Minimal amount (μg) of compound to inhibit growth on a silica gel TLC plate

[b] Minimal concentration (ppm) of compound required to kill all the larvae after 24 hours

[c] Minimal amount (μg) of compound required to show a radical scavenging activity on a silica gel TLC plate

n.t. not tested

From the previous study on the bioactivities of mansonones, many reports[5-8] indicated the antifungal properties of mansonones as phytoalexins, especially against Dutch elm disease (DED) by the fungi *Ophiostoma ulmi* (Buism.) Nannf. and

Ophiostoma novo-ulmi Brasier. In the plant genus *Ulmus*, mansonones E, F and G were found to be the most potent antifungal compounds.

From this examination (Table 1.9), the isolated compounds from *M. gagei* were tested for antifungal activity against *Cladosporium cucumerinum* and *Candida albicans*. Mansonones C and E were found to be the only active products against *C. albicans* with a minimal amount of 0.15 µg, and 2.5 µg, respectively to ensure inhibition of the yeast growth. Mansonones C and E as well as mansorins A and B were found to be active against *C. cucumerinum* with minimal inhibitory amount of 0.6, 0.6, 2.5 and 0.6 µg, respectively. The antifungal potency of mansorin B and mansonones C and E was close to that of nystatin employed as a control.

Mansonone C was the only isolated compound to possess toxic property against the larvae of the yellow fever-transmitting mosquito *Aedes aegypti* at 50 ppm. Dilution tests were performed and the minimal amount of compound to kill all the larvae after 24 hours was calculated as 6.25 ppm. This activity could not be detected in the raw extract probably due to the low concentration of the active compound in the dichloromethane extract (0.0046 %). In comparison, rotenone used as the reference compound was twice more active.

Mansonone N was finally the only isolated compound to exhibit radical scavenging properties in the DPPH, the other compounds being inactive on the TLC assay at a tested amount of 10 µg. A limit of activity at 2.5 µg was determined for mansonone N in the same assay. Quercetin used as a reference substance was 5 times more active.

Table 1.10 The antithrombin activity of the isolated compounds from the dichloromethane extract of the heartwoods of *M. gagei*

Compounds	% Inhibition
Mansorin A (**1**)	24.31
Mansorin B (**2**)	19.84
Mansorin C (**3**)	45.61
Mansonone N (**4**)	25.18
Mansonone O (**5**)	44.94
Mansonone P (**6**)	39.09
Mansonone Q (**7**)	n.t.
Mansonone C (**8**)	45.9
Mansonone E (**9**)	52.84
Mansonone G (**10**)	96.37
Mansonone H (**11**)	97.57
Dehydrooxoperezinone (**12**)	99.65

n.t. : not tested

On comparing the activities of various 1,2-naphthoquinones obtained from dichloromethane extract (Table 1.10), antithrombin property was significant enhanced by hydroxy group at the C-6 position as in mansonones G, H and dehydrooxoperezinone.

Mansorins A-C and mansonones N-Q exhibited with %inhibition values ranging between 19.84 and 45.61 %inhibition which were lower than those for mansonones C-H and dehydrooxoperezinone and had no activity influence by substituents. The fact maybe that the activity mainly depends on the 1,2-naphthoquinone basic skeleton.

Table 1.11 The anticancer activity of the isolated compounds from the dichloromethane extract of the heartwood of *M.gagei*.

Compounds	% Activities				
	Mouse Leukemia	Human Myeloma	Human Colon Adenocarcinoma	Human Leukemia	Human Breast Carcinoma
Mansorin A (**1**)	70.8	87.5	18.2	29.3	66.9
Mansorin B (**2**)	67.0	64.5	0.0	87.0	95.3
Mansorin C (**3**)	76.8	71.5	9.3	17.2	79.4
Mansonone N (**4**)	4.0	48.0	0.0	17.1	0.0
Mansonone O (**5**)	n.t	n.t	n.t.	n.t.	n.t.
Mansonone P (**6**)	82.3	66.2	34.9	28.8	96.0
Mansonone Q (**7**)	n.t.	n.t	n.t.	n.t.	n.t.
Mansonone C (**8**)	98.8	100.0	98.7	100.0	100.0
Mansonone E (**9**)	100.0	100.0	96.5	99.3	98.3
mansonone G (**10**)	96.0	100.0	96.8	100.0	80.7
Mansonone H (**11**)	n.t.	n.t	n.t	n.t	n.t.
Dehydrooxopere-zinone (**12**)	n.t.	n.t	n.t	n.t.	n.t
Methotrexate	97.1	65.1	69.8	69.2	24.3

n.t. : not tested

According to the results in Table 1.11, mansonones C, G and H demonstrated very potent inhibitors against a number of cancer cell lines. Mansonone P displayed selective inhibition toward Mouse Leukemia and Human Breast Carcinoma with 82.3 and 96.0 %inhibition, respectively. Mansorin A exhibited the selective activity against Human Myeloma with 87.5 %inhibition as well as mansorin B displayed the certain inhibitory activity against Human Leukemia and Human Breast Carcinoma with 87.0 and 95.3 %inhibition.

PART II

2.1 Separation of the Ethyl Acetate Extract of the Heartwoods

The constituents of the ethyl acetate extract were examined by HPLC analysis using the same chromatographic conditions as those of the dichloromethane extract The chromatogram of the ethyl acetate extract (Fig 2.1) was compared with that of dichloromethane extract. The results (Fig 2 2) revealed the similarity of the constituents in both extracts. To confirm the constituents of ethyl acetate extract, this extract was further fractionated and identified their constituents by HPLC analysis

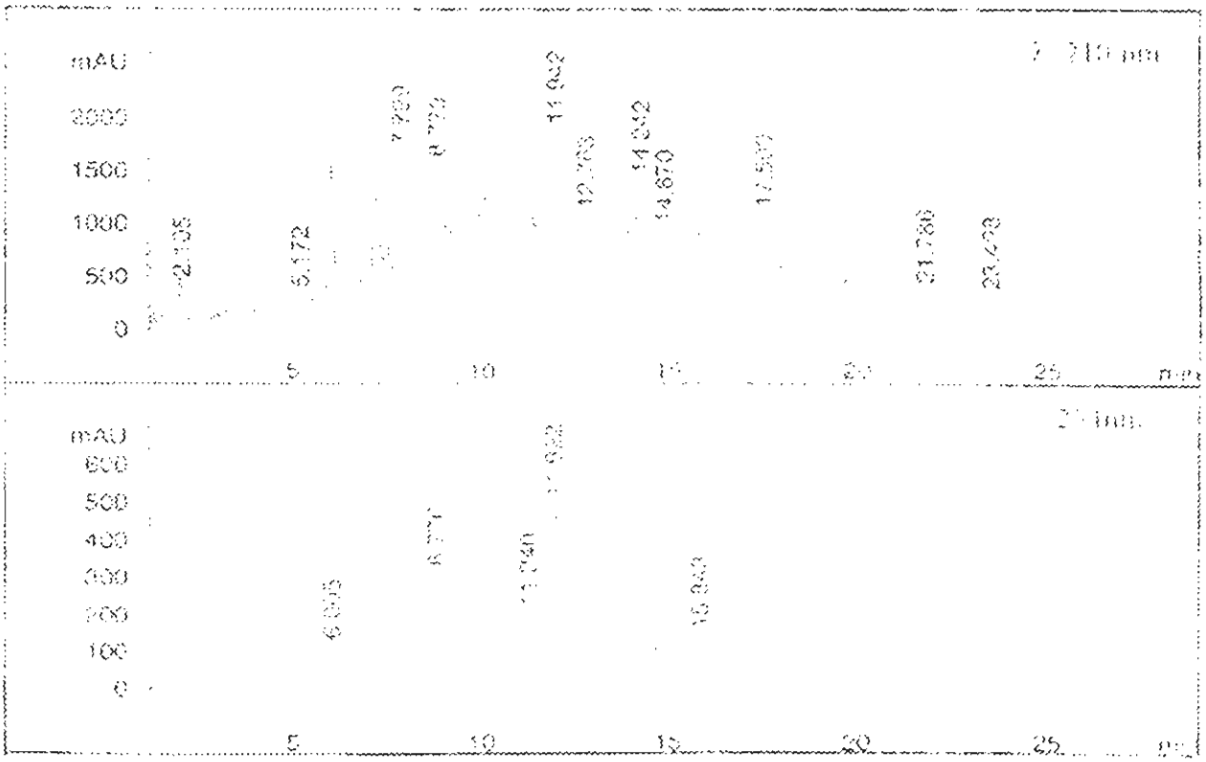


Fig 2.1 The HPLC chromatogram of the ethyl acetate extract

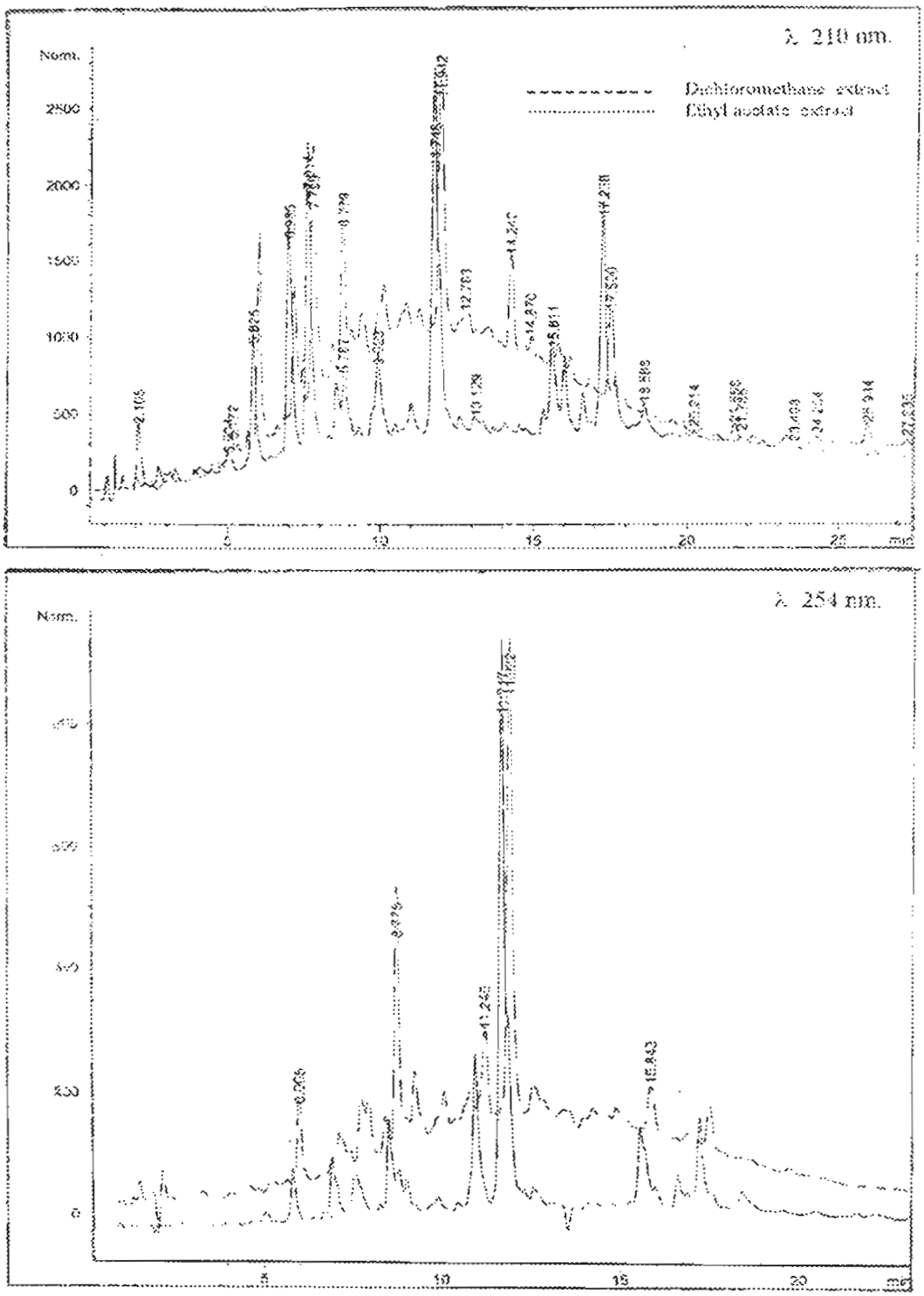


Fig 2.2 The HPLC chromatogram of the dichloromethane and ethyl acetate extracts

2.1.1 Fractionation

A part of the crude ethyl acetate extract (30.1 g) was fractionated by quick column chromatography using silica gel 60G Art. 7731 as an adsorbent. The column was eluted by using step gradients of hexane-CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH. The fractions were collected and combined according to TLC results in order to obtain eight fractions (E1-E9).

2.1.2 HPLC Analysis of Fractions of the Ethyl Acetate Extract

Fractions E1-E9, were checked the constituents by HPLC with a Nova-Pak RP-18 column using the same procedure as the ethyl acetate extract and then compared the chromatograms with those of the isolated compouds from the dichloromethane extract. The results are presented as follows:

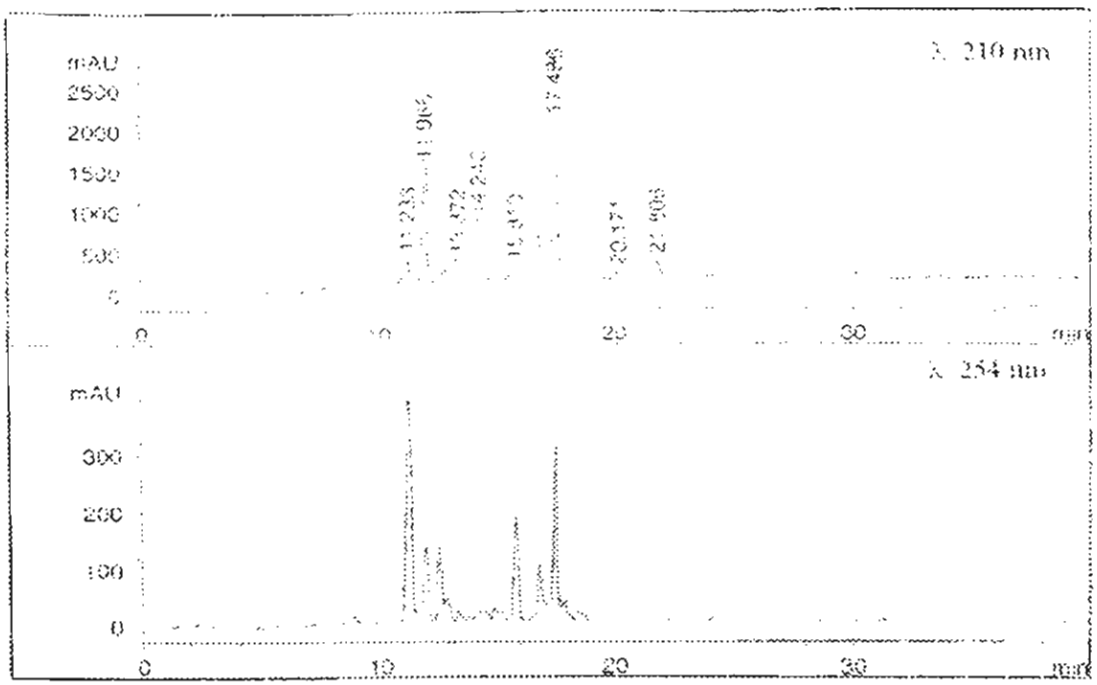


Fig 2.3 The HPLC chromatogram of E1

a) The major peaks of E1 were mansorin B and/or mansonone G (R_t 11.9 min), mansorin C (R_t 13.4 min), mansonone C (R_t 15.8 min) and mansorin A (R_t 17.49 min)

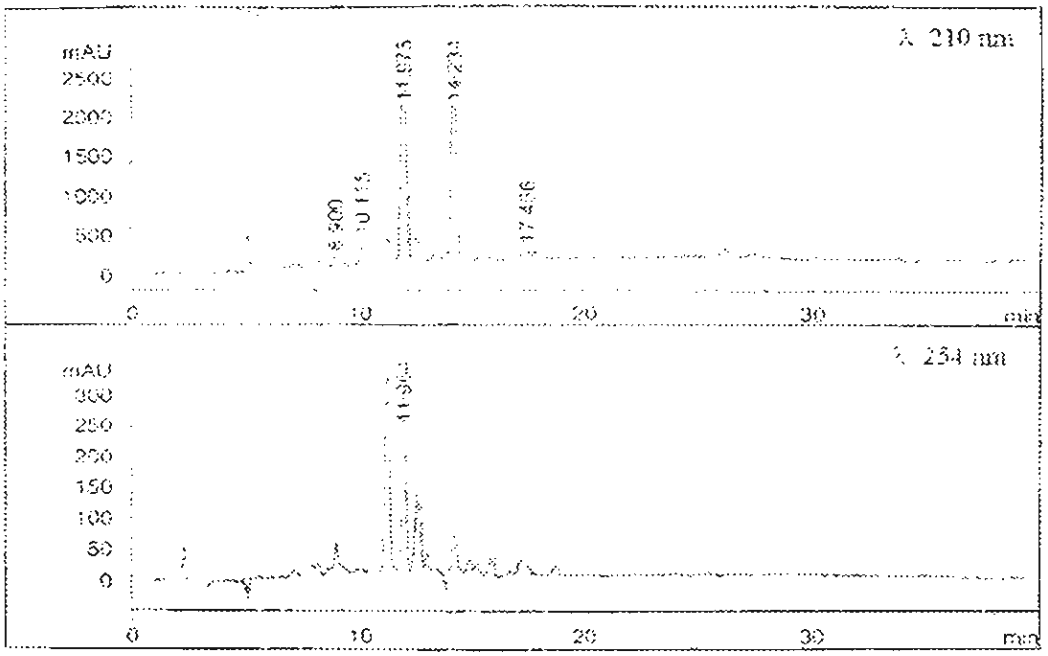


Fig 2.4 The HPLC chromatogram of E2

b) The major peaks of E2 were mansorin B and/or mansonone G (R_t 11.9 min), including two unidentified peaks at R_t 11.3 and 14.2 mins.

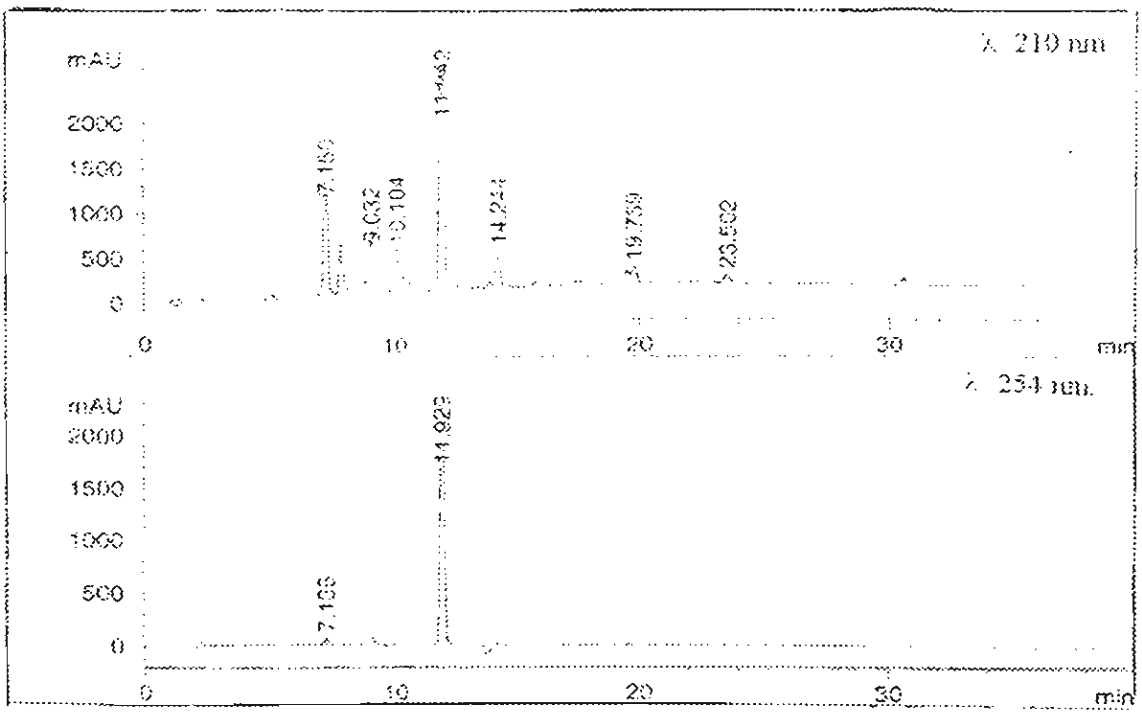


Fig 2.5 The HPLC chromatogram of E3

c) The major peaks of E3 were mansorin B and/or mansonone G (R_t 11.9 min) and an unidentified peak at R_t 7.1 min.

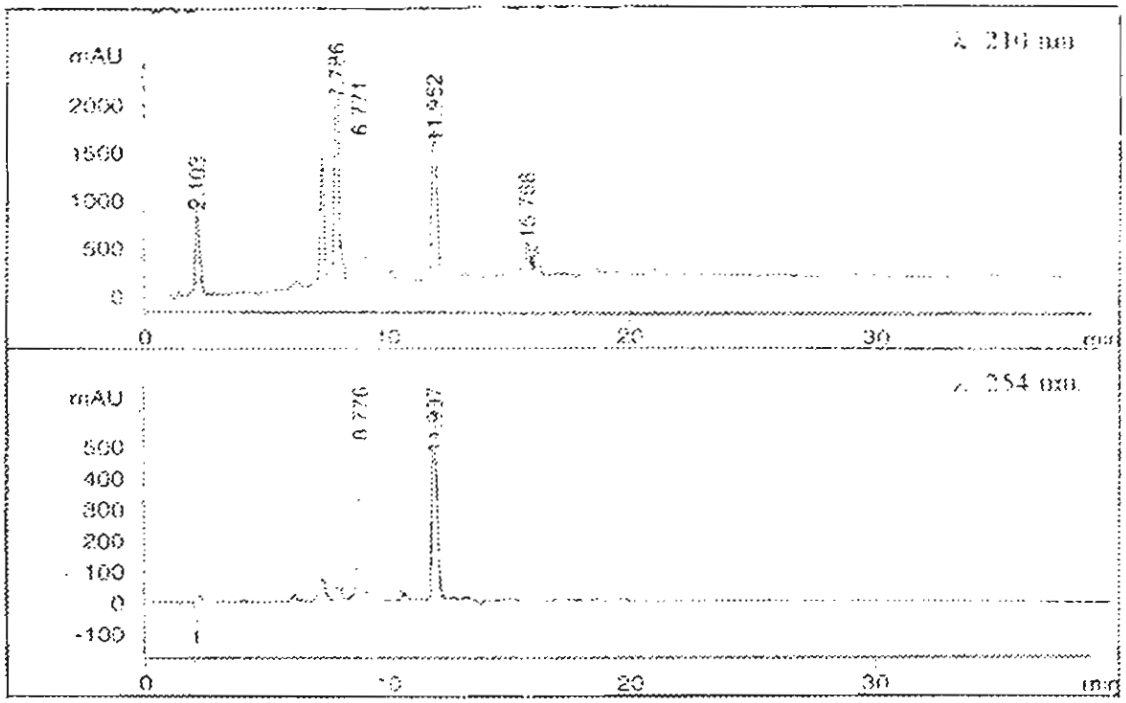


Fig 2.6 The HPLC chromatogram of E4

d) The major peaks of E4 were mansonone H (R_t 8.7 min), mansorin B and/or mansonone G (R_t 11.9 min) and three unidentified peaks at R_t 2.1, 7.1 and 7.8 mins.

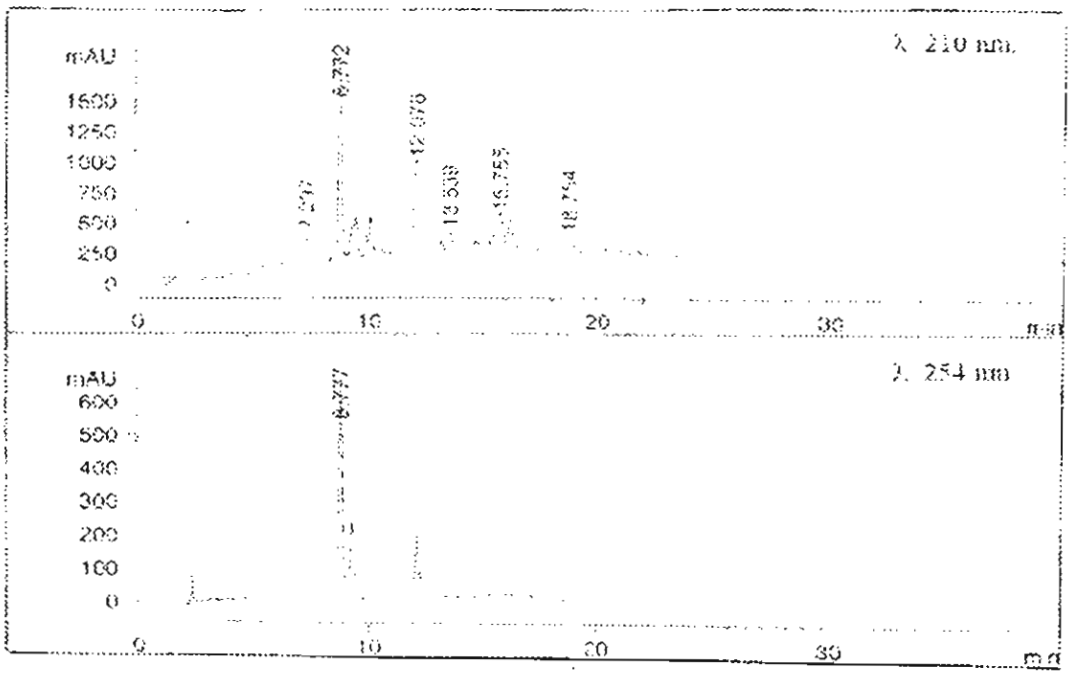


Fig 2.7 The HPLC chromatogram of E5

e) The major peaks of E5 were mansonone H (R_t 8.7 min), dehydrooxoperezinone (R_t 9.2 min) and an unidentified peak at R_t 12.1 min.

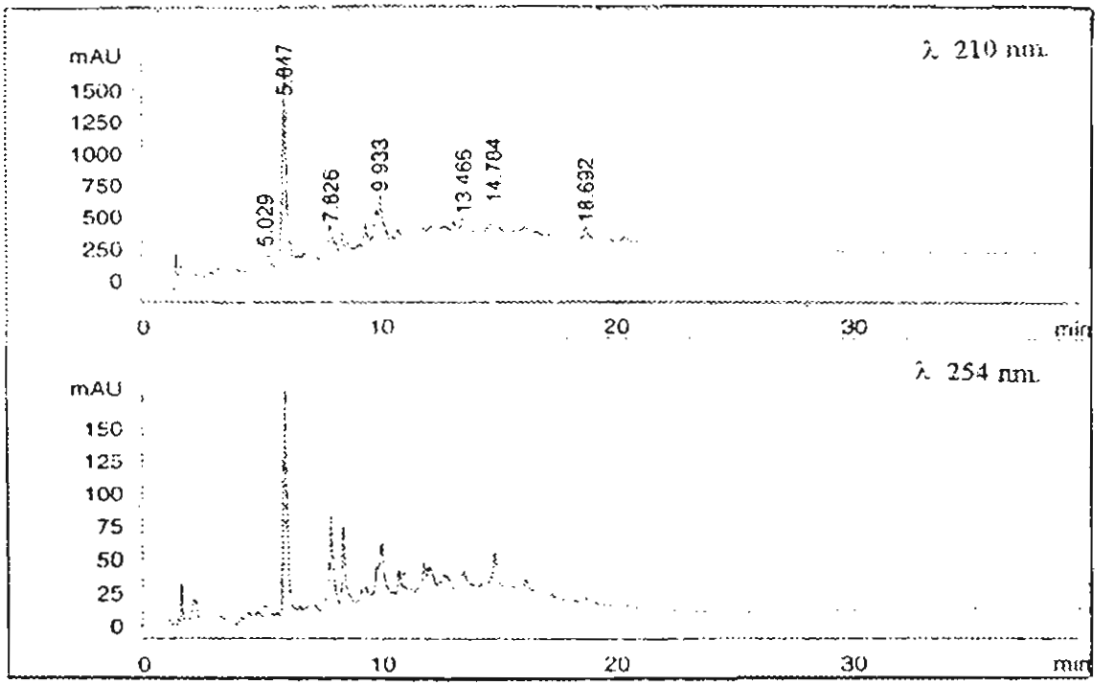


Fig 2.8 The HPLC chromatogram of E6

f) E6 showed only one unidentified major peak at R_t 5.9 min.

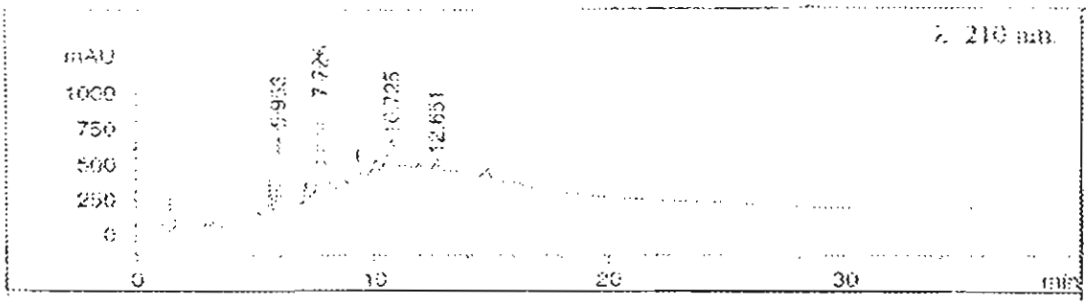


Fig 2.9 The HPLC chromatogram of E7

g) E7 showed the same peak as E5 (R_t 5.9 min) as well as a complex peak at R_t 7.8 and 8.8 mins.

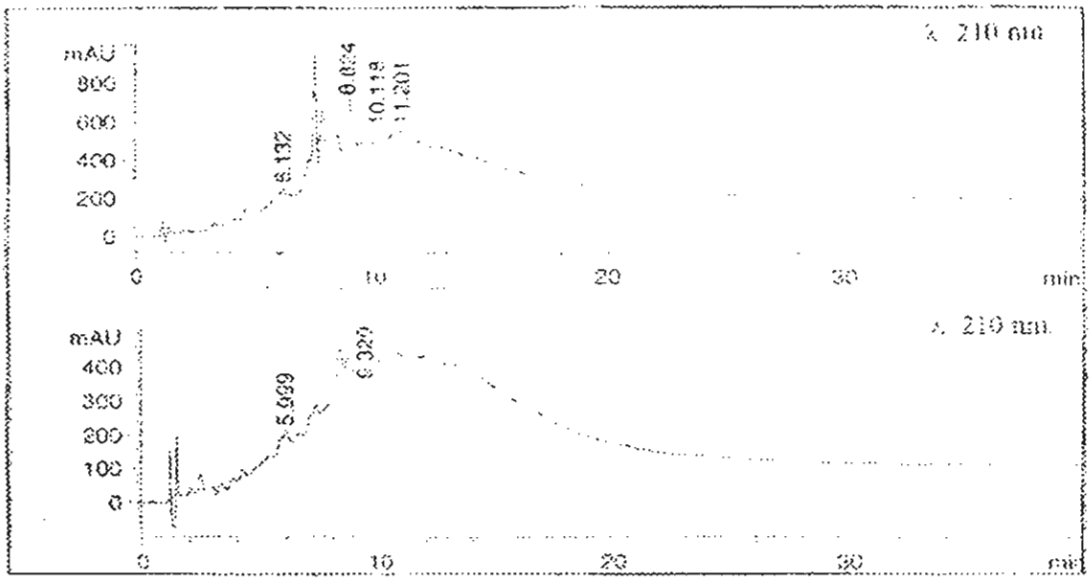


Fig 3.99 The HPLC chromatogram of E8-E9

h) E8 and E9 did not show any interesting peak.

In conclusion, the major peaks of each fraction were almost identified by comparing with the isolated compounds from dichloromethane extract, except for the unidentified peaks at R_t. 2.1, 5.9, 7.1, 7.8, 8.8, 11.3, 12.1 and 14.2 mins.

Fractions E2 and E3 were not further separated due to their limited amount. E5 was further subjected to LPLC with the aim to try to purify the compound with R_t 12.1 min with Sephadex LH-20 column chromatography using $CHCl_3$:MeOH = 1:1. The separation was repeated for several times ; however, the separation did not give satisfied results. The peaks at R_t 7.8 and 8.8 mins of E7 were not interesting to isolate due to the fact that they overlapped with other minor peaks. Thus, only E4 and E6 were further separated to isolate the compounds with R_t 2.1 and 12.1 mins, respectively.

2.1.3 Separation

2.1.3.1 E4

E4, 700 mg, was fractionated by LPLC with Lobar LiChroprep RP-18 column using MeOH-H_2O gradient = 4:6 → 9:1. The fractions were collected and combined by monitoring with analytical TLC (RP-18 WF_{254s}) and HPLC analysis to obtain six

fractions (E4-A to E4-F). E4-A was further purified by Sephadex LH-20 column chromatrography using $CHCl_3$: MeOH = 1 : 1 as eluent to yield Compound **13** (3-methoxy-4,5-dihydroxybenzaldehyde, 9.5 mg). Moreover, mansonone H, dehydrooxoperezinone and mansonone G were obtained from E4-D to F, respectively, by comparing R_f on TLC and retention times (R_t) of HPLC analysis.

2.1.3.2 E6

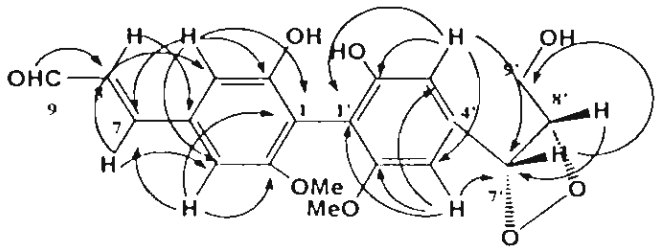
E6, 21.5 mg, was fractionated by LPLC with Lobar LiChroprep RP-18 column using MeOH-H_2O gradient = 1:1 → 9:1. The fractions were collected and combined by monitoring with analytical TLC (RP-18 WF_{254s}) and HPLC analysis to obtain four fractions (E6-A to E6-D). E6-C was further purified by Sephadex LH-20 column chromatrography using $CHCl_3$: MeOH = 1 : 1 as eluent to yield Compound **14** (mansoxetane, 9.5 mg).

2.2 Structural Elucidation of New Compound

Compound 14: mansoxetane

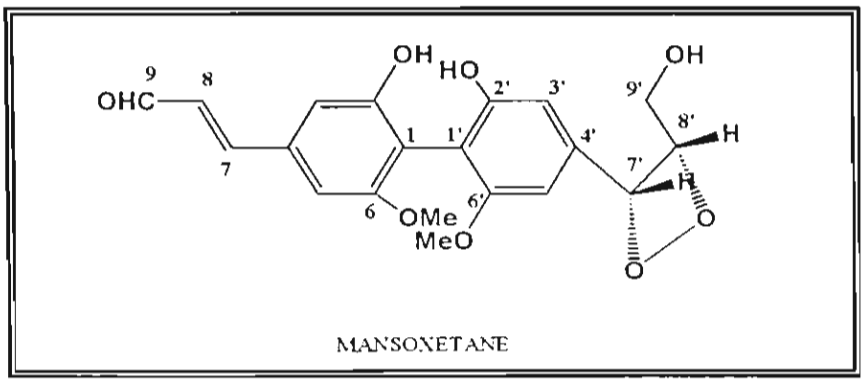
Compound **14**, named mansoxetane, was obtained as a pale brown solid, $[\alpha]_D^{15}$ -12.8 (c = 0.27, $CHCl_3$). High-resolution FABMS analysis gave m/z 389.1199 $(M+H)^+$ and established the molecular formula as $C_{20}H_{20}O_8$, which indicated Compound **14** to have the unsaturation number of 11. The presence of a *trans*-cinnamaldehyde residue in the molecule was suggested based on the observation of the characteristic signals at δ_H 9.66 and δ_C 193.5 ascribing to an aldehyde moiety and at δ_H 7.35 (d, J = 15.9 Hz) and δ_H 6.60 (dd, J = 15.9 and 7.6 Hz) arising from a *trans*-olefin, as well as on the HMBC correlations between these olefinic protons and the aromatic carbons on the benzene ring. A set of *meta*-coupled protons was observed at δ_H 6.90 and δ_H 6.76 in the ^{1}H-NMR spectrum, indicating that this benzene ring had four substituents, two of which were methoxy (δ_H 3.92 and δ_C 149.2) and hydroxy (δ_C 144.6). From both of the aromatic protons just mentioned above, HMBC correlations with the β-carbon (δ_C 152.8) of the acrylaldehyde function were observed, revealing that this part existed at the *ortho* position toward both of two *meta*-coupled protons. Furthermore, the HMBC correlations between both

of these aromatic protons and the sp^2 quaternary carbon (δ_C 135.9) on the benzene ring were observed, indicating that this benzene ring had linkage with other unit at the *para* position of the acrylaldehyde group. All HMBC correlations are shown below.



From the ^1H and ^{13}C NMR spectral data, the presence of one more C6-C3 unit in Compound **14** was suggested. In analogy with the phenylpropanoid unit described above, the benzene ring in this residue had four substituents, two of which were methoxy (δ_H 3.90 and δ_C 147.2) and hydroxy (δ_C 144.2). From both of two *meta*-coupled aromatic protons (δ_H 6.70 and 6.57) on this benzene ring, HMBC correlations between the sp^3 carbon (δ_C 76.3) in one of the C3 unit were observed, indicating that the propane unit was present at the *ortho* position towards both of two *meta*-coupled protons. The chemical shifts of three carbons in the propane unit were observed at δ_C 76.3, 78.8 and 61.3, revealing that they were the aliphatic carbons bearing oxygen functions, respectively. Further investigation of the structure of Compound **14** was performed by acetylation of this compound. Compound 14 (4.8 mg) was dissolved in dry pyridine (0.5 mL) was treated with dist. Acetic anhydride (0.25 mL) under reflux at room temperature for 10 hours. After the reaction was completed, dryness the products by flowing N_2 gas to take off the residue of pyridine and acetic anhydride. A triacetyl derivative, Compound **14a**, was obtained upon purification using silica gel column chromatography and confirmed by observation the molecular peak at m/z 514 in mass spectrum, three singlet signals of acetyl groups at δ_H 2.05, 2.29 and 2.31 (3H each) in ^1H NMR spectrum and three carbonyl signals at δ_C 167.5, 167.9 and 170.3 as well as three methyl carbon signals at δ_C 20.3, 20.6 and 20.7 detected in ^{13}C NMR spectrum. Based on the result of acetylation of Compound **14**, the terminal carbon of the propane unit was proved to be a primary alcohol. Considering the

molecular formula, the remaining two carbons constituted oxetane ring. A clear NOE observation between two protons (δ_H 4.90 and 4.05) on the oxetane ring revealed the stereochemical relation of the primary alcohol and the benzene ring to be *cis*. Furthermore, HMBC correlations between both of the aromatic protons and the sp^2 quaternary carbon (δ_C 133.2) on the benzene ring were observed, indicating that this benzene ring had linkage with another residue at the *para* position of the functionalized propane group. All the above spectroscopic analyses enabled to construct the dimeric structure Compound **14** that had a bridge between the *para* positions of each phenylpropanoid unit. To the best of our knowledge, this is the first example of biphenylneolignan[9] possessing an oxetane ring. The NMR data of Compound **14** are shown in Table 2.1.



MANSOXETANE

Table 2.1 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **14**

Position	Chemical Shift (ppm)[a]	
	δ_H (J in Hz)	δ_C
1	-	135.9
2	-	144.6
3	6.90, d, 1H (1.8)	111.3
4	-	126.7
5	6.76, d, 1H (1.8)	104.0
6	-	149.2
7	7.35, d, 1H (15.6)	152.8
8	6.60, dd, 1H (7.9, 15.6)	127.3
9	9.66, d, 1H (7.6)	193.5
1′	-	133.2
2′	-	144.2
3′	6.70, d, 1H (1.8)	108.2
4′	-	127.4
5′	6.57, d, 1H (1.8)	102.3
6′	-	147.2
7′	4.90, d, 1H (8.2)	76.3
8′	4.05, ddd, 1H (3.3, 3.3, 8.2)	78.8
9′	3.60, m, 1H 3.90, overlapped, 1H	61.3
2- and 2′-OH	5.56, br s, 2H	-
6-OMe	3.92, s, 3H	56.3
6′-OMe	3.90, s, 3H	56.2
9′-OH	2.30, s, 1H	-

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 and 100 MHz, respectively

2.3 The Biological Activity of Isolated Compounds from the Ethyl Acetate Extract

Two additional compounds were isolated from ethyl acetate extract: 3-methoxy-4,5-dihydroxybenzaldehyde (**13**) and mansoxetane (**14**). The former was examined for its bioactivities and the results are shown in Tables 2.2-2.3. The latter, nevertheless, was unfortunately isolated in an inadequate amount, thus the bioactivity is impossible to be evaluated.

Table 2.2 Antifungal, larvicidal and radical scavenging activities of 3-methoxy-4,5-dihydroxybenzaldehyde (**13**)

Compound	*Cladosporium Cucumerinum*[a]	*Candida Albicans*[a]	**Aedes aegypti**[b]	DPPH[a]
3-methoxy-4,5-dihydroxybenzaldehyde	>10	>10	>50	5.0

[a] : tested amount: 10 µg of pure compound
[b] : tested amount: 50 ppm of pure compound

Table 2.3 The anticancer activity of 3-methoxy-4,5-dihydroxybenzaldehyde (**13**)

Compounds	% Activities				
	Mouse Leukemia	Human Myeloma	Human Colon Adenocarcinoma	Human Leukemia	Human Breast Carcinoma
3-methoxy-4,5-dihydroxybenzaldehyde	70.8	87.5	18.2	29.3	66.9

From the result in above table, this compound possessed the cytotoxicity activity against various cancer cell-lines as well as the radical scavenging property towards DPPH.

PART III

3.1 Separation of the Methanolic Extract from the Heartwoods

To extend the investigation of chemical composition of the heartwoods of *M.gagei*, the methanolic extract was throughly analyzed by HPLC compared with those of dichloromethane and ethyl acetate extracts (Fig 3.1). It was noticed that the major components of methanolic extract were in fact similar to those of dichloromethane and ethyl acetate extracts, except that the peaks of polar compounds (R_t 5-10 min) in methanolic extract were more intense than those of less polar compounds.

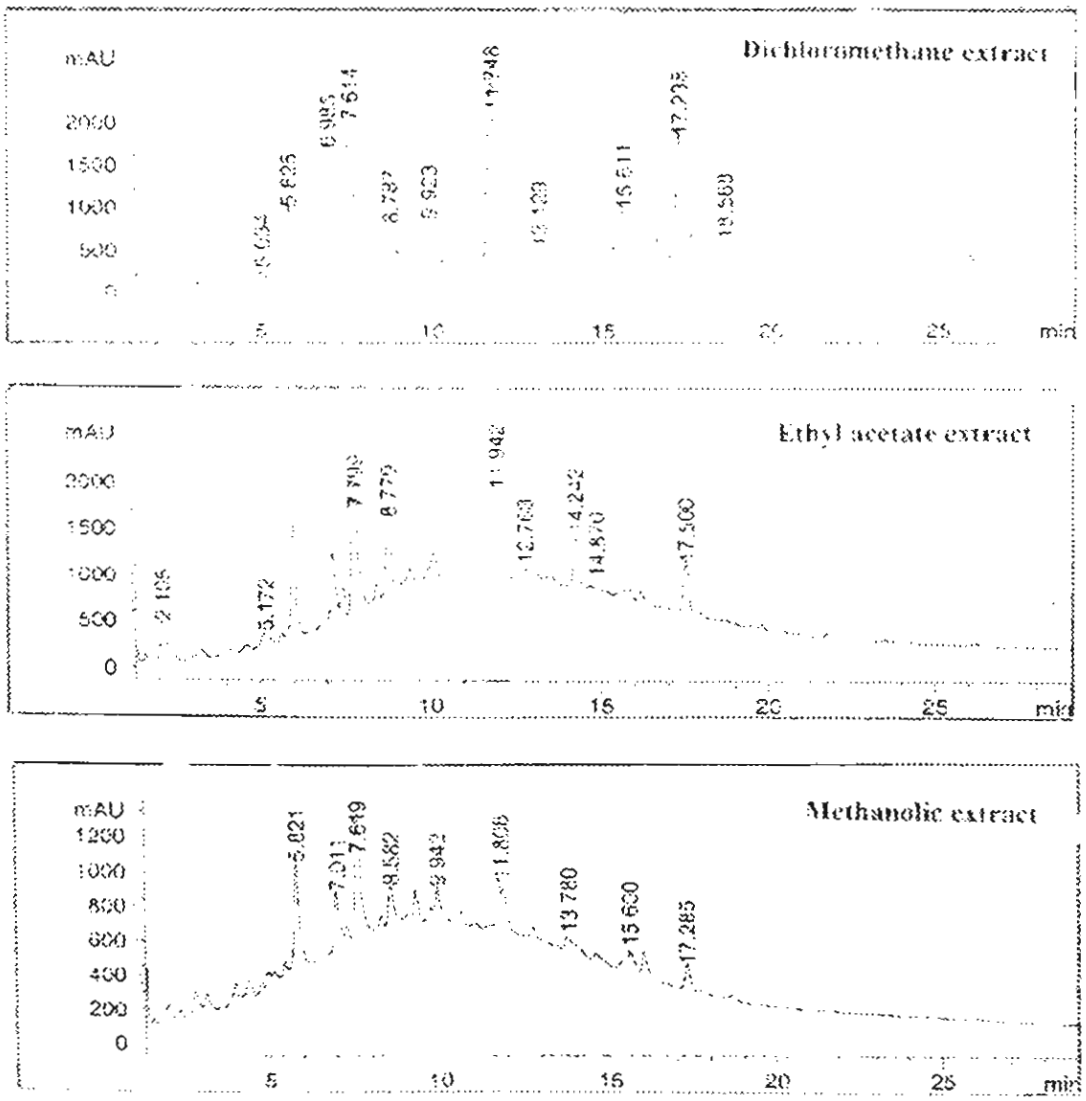


Fig 3.1 HPLC chromatogram of dichloromethane, ethyl acetate and methanolic Extracts (λ 210 nm)

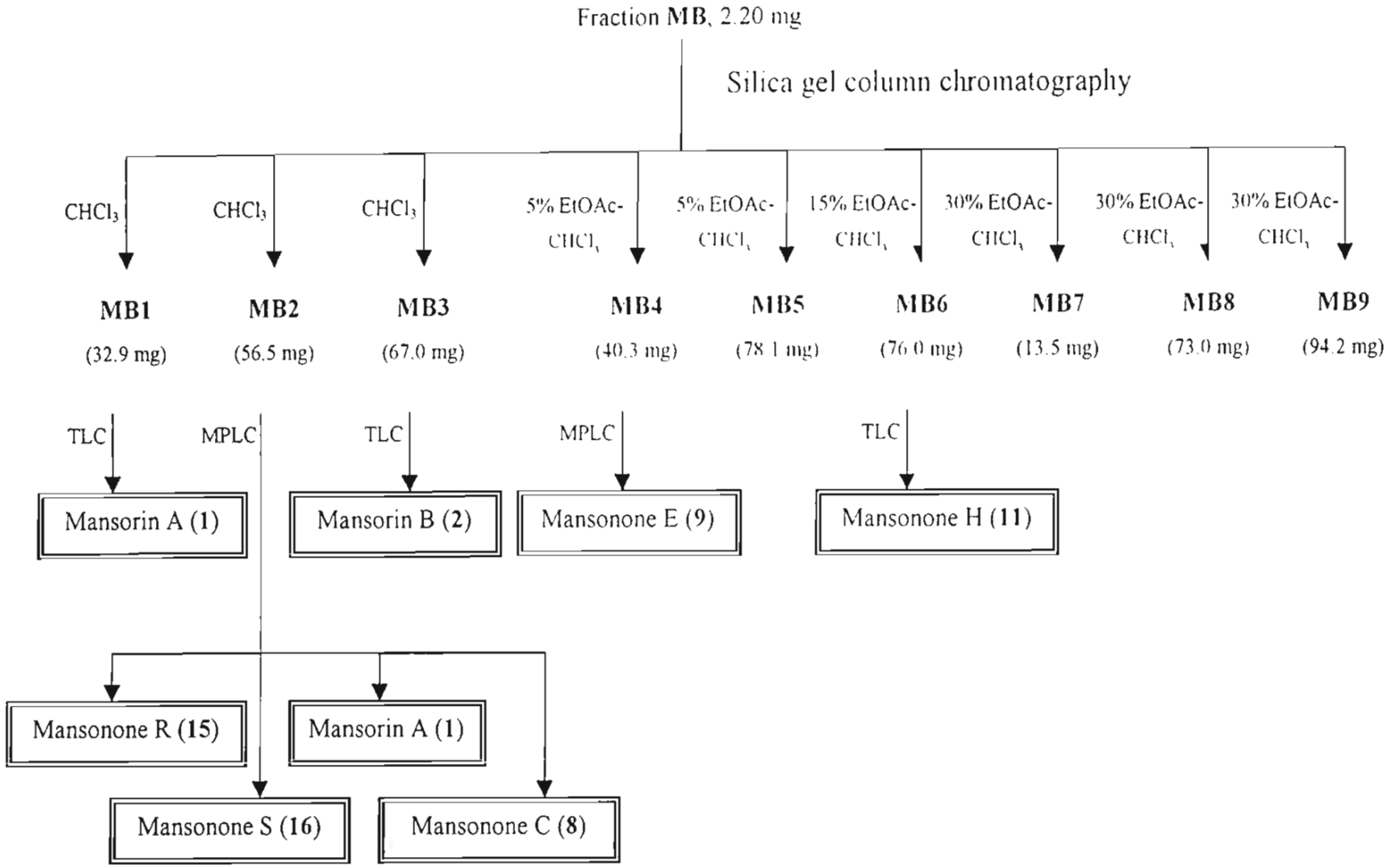
3.1.1 Fractionation

The MeOH extract (20.10 g) was first subjected to fractionation using silica gel column chromatography eluting by the gradient of $CHCl_3$ and methanol. The fractions were collected and combined according to TLC results to furnish four fractions, MA-MD.

3.1.2 SEPARATION

3.1.2.1 Fraction MB

Fraction MB was further separated by subjection to silica gel column using the gradient of Hexane-EtOAc and EtOAc-MeOH giving nine fractions, MB1-MB9, monitoring by analytical TLC. Comparing R_f of major constituents of fractions MB1,MB3 and MB6 with the isolated compounds from the dichloromethane extract on analytical TLC plates revealed that fraction MB1,MB3 and MB6 contained mansorin A (**1**), mansorin B (**2**) and mansonone H (**11**), respectively. Fractions MB2 (56.5 mg) and MB4 (40.3 mg) were further subjected to MPLC with silica gel prepacked column using Hexane:EtOAc = 85:15 as eluent. Mansonones R (**15**) (2.7 mg) and S (**16**) (27.4 mg), mansorin A (**1**) (11.9 mg), mansonone C (**8**) (3.3 mg) were gained from fraction MB2 and Mansonone E (4.8 mg) was derived from fraction MB4. Mansonones R and S had not been isolated from dichloromethane and ethyl acetate extracts, and characterized as new naturally occurring compounds The results of separation are summarized as shown in Scheme 3.1.



Scheme 3.1 Isolation diagram of fraction **MB** of the methanolic extract from the heartwoods of *M. gagei*

3.1.2.2 Fraction MC

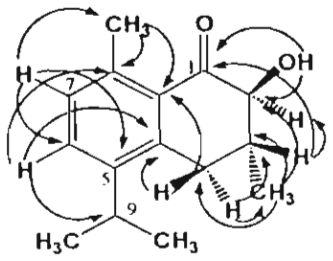
Fraction MC was first fractionated on a silica gel column using a gradient of $CHCl_3$-MeOH giving nine fractions, MC1-MC-9, monitoring by analytical TLC (silica gel) plates. A part of fraction MC3 (56.0 mg) was further separated by MPLC with silica gel prepacked column using the gradient of $CHCl_3$-EtOAc and EtOAc-MeOH as eluent, yielding 6.3 mg of mansoxetane (**14**), previously obtained from ethyl acetate extract.

3.2 Structural Elucidation of New Compounds

3.2.1 *Compound 15:* Mansonone R

Compound **15**, as pale yellow powder, was established as $C_{15}H_{20}O_2$ based on EIMS and NMR data. The ^1H NMR spectrum of Compound **15** disclosed the presence of a set of *ortho*-coupled protons, an isopropyl group, two methyl groups and two methylene protons. The ^{13}C NMR spectrum of Compound **15** also indicated the presence of these mentioned groups and an α-hydroxy ketone moiety.

The HMBC spectrum of Compound **15** showed the correlations of the set of *ortho*-coupled proton signals in an aromatic ring at δ_H 7.14 and 7.38 with the methyl carbon at δ_C 22.6 (8-CH_3) and the methine carbon of the isopropyl group at δ_C 28.3, respectively. In addition, this methyl group (8-CH_3) was correlated to an aromatic carbon at δ_C 130.5. Furthermore, one of two methylene protons (δ_H 3.21) showed correlation with two aromatic carbons at δ_C 140.6 and 130.5, and the other (δ_H 2.63) was coupled with a methine carbon at δ_C 37.4 as well as another methyl group at δ_C 19.0 (3-CH_3). HMBC correlations were also observed between the methyl group (3-CH_3) and a carbon bearing a hydroxy group (δ_H 78.5). All HMBC correlations of Compound **15** are shown below:



The relative stereochemistry of Compound **15** was determined primarily on the basis of J value obtained from the ^{1}H NMR spectrum. The large coupling constant observed between H-2 and H-3 (J = 12.8 Hz) implied a *trans*-diaxial orientation for this proton pair. Compound **15** was thus identified as a new natural product and named mansonone R. The NMR data of this compound are presented in Table 3.1.

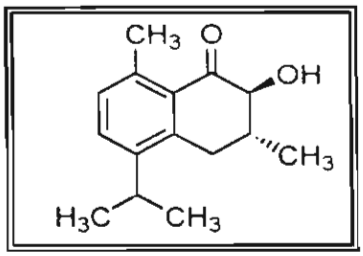


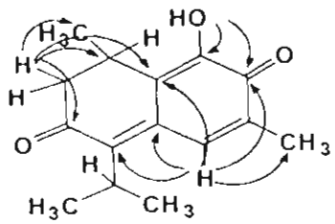
Table 3.1 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **15**

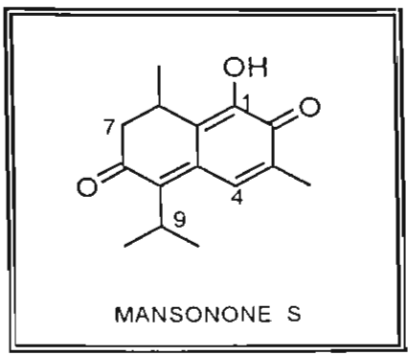
Position	Chemical Shift (ppm)[a]	
	δ_H (J in Hz)	δ_C
1	-	201.4
2	3.95, dd, 1H (2.7, 12.8)	78.5
3	2.10, m, 1H	37.4
4	2.63, dd, 1H (11.9, 17.1) 3.21, overlapped, 1H	33.7
4a	-	140.6
5	-	144.4
6	7.38, d, 1H (7.9)	128.8
7	7.14, d, 1H (7.9)	130.0
8	-	139.0
8a	-	130.5
9	3.19, m, 1H	28.3
2-OH	4.15, d, 1H (2.7)	-
3-CH_3	1.34, d, 3H (6.4)	19.0
8-CH_3	2.62, s, 3H	22.6
9-$(CH_3)_2$	1.24, d, 3H (6.4) 1.25, d, 3H (6.4)	22.9 23.5

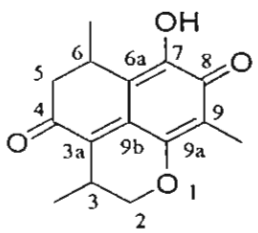
[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 and 100 MHz, respectively

3.2.2 *Compound 16:* Mansonone S

Compound **16** was obtained as orange amorphous, and the molecular formula was determined to be $C_{15}H_{18}O_3$ from the molecular ion at m/z 247.1316 $[M+H]^+$ in the high-resolution FAB$^+$-mass spectrum. The ^{13}C-NMR spectrum showed fourteen carbon signals. The signals at δ_C 180.8 (C-2) and 200.0 (C-6) were clearly ascribed to two carbonyl carbon signals. Comparison of the ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **16** with those of mansonones in literature found that those data of Compound **16** were very similar to those of 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtho [1,8-b,c]pyran-4,8-dione,[10] (Table 3.2), except for the absence of the naphtho[1,8-b,c]pyran signals, and the presence of the additional isopropyl group signals at δ_H 1.28 (d, 3H, J = 7.0 Hz), 1.38 (d, 3H, J = 7.0 Hz) and 3.45 (m, 1H) in Compound **16**. The complete structure of Compound **16**, a new natural product named mansonone S, was well-confirmed by HMQC, HMBC and COSY spectra. The HMBC correlations of this compound are shown below.







7-hydroxy-2,3,5,6-tetrahydro-

3,6,9-trimethylnaphtho[1,8-b,c]pyran-4,8-dione

Table 3.2 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **16** and 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtho[1,8-b,c]pyran-4,8-dione[10]

	Chemical Shift (ppm) of Compound **16**[a]			Chemical Shift (ppm) of 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtho[1,8-b,c] pyran-4,8-dione[b]	
Position	δ_H (J in Hz)	δ_C	Position	δ_H (J in Hz)	δ_C
1	-	144.6	7	-	143.6
2	-	180 8	8	-	181.3
3	-	136 1	9	-	115.0
4	7.55, s, 1H	132.4	9a	-	157.3
4a	-	135.3	9b	-	131.0
5	-	150.2	3a	-	139.4
6	-	200.0	4	-	197.1
7	2 50, d, 1H (15 0) 2 80, dd, 1H (6.4, 14 7)	46 0	5	2 60, dd, 1H (1.5, 16.3) 2 78, dd, 1H (6.6, 16.3)	44.5
8	3 55, m, 1H	28 0	6	3.55, m, 1H	27.5
8a	-	123 0	6a	-	115.1
9	3.45, m, 1H	28.5	3	3.12, dq, 1H (3.5, 7.1)	26.4
1-OH	6.86, s, 1H	-	2	-	71.9
3-CH_3	2.13, s, 3H	16.2	9-CH_3	1.94, s, 3H	8.0
8-CH_3	1.20, d, 3H (7.3)	20.5	6-CH_3	1.19, d, 3H (7.1)	20.6
9-$(CH_3)_2$	1.38, d, 3H (7.0) 1.28, d, 3H (7.0)	21.0 22.7	3-CH_3	1.16, d, 3H (7.1)	16.2

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 and 100 MHz, respectively
[b] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 500 and 125.8 MHz, respectively

CONCLUSION

Previous research of the heartwoods results in the isolation of three new coumarins, mansorin A-C, as well as three known mansonones, C, G and H. This work involves continuing studies on the chemical constituents from *Mansonia gagei* and determination of their biological activities. Studies on the heartwood extracts have now led to the isolation of ten compounds that had not been isolated previously from this plant. Twelve compounds were obtained from the dichloromethane extract and their structures were established on the basis of physical properties and spectroscopic experiments as mansorin A **(1)**, mansorin B **(2)**, mansorin C **(3)**, mansonone N **(4)**, mansonone O **(5)**, mansonone P **(6)**, mansonone Q **(7)**, mansonone C **(8)**, mansonone E **(9)**, mansonone G **(10)**, mansonone H **(11)** and dehydrooxoperezinone **(12)**. Among them, mansonones N, O, P and Q are new naturally occurring compounds. In addition, the ethyl acetate extract furnished 3-methoxy-4,5-dihydroxybenzaldehyde **(13)**, mansoxetane **(14)** together with the same compounds as isolated from dichloromethane extract. Two additional new compounds, mansonones R **(15)** and S **(16)**, were obtained from the methanolic extract.

As part of on going investigation of biologically active compounds, some isolated compounds were tested in a variety of bioactivity assays. The results indicated that mansonones or 1,2-naphthoquinones showed higher activity than coumarins in Brine Shrimp Cytotoxicity test. The same trend was also visualized for anticancer test. Mansonones C **(8)** and G **(10)** showed high toxicity in Brine Shrimp Cytotoxicity test and high % inhibition value for a number of cancer cell lines whereas mansorinsA **(1)** and C **(3)** showed medium and low activities, respectively, for Brine Shrimp Cytotoxicity test and showed low %inhibition value for the same cancer cell lines except for mansorin A that had high %inhibition specifically for Human Myeloma. In addition, mansonones C **(8)** and E **(9)** showed fungitoxicity against *Cladosporium cucumerinum* and *Candida albicans* which were previously reported in the literature.[5-8] Mansorins A **(1)** and B **(2)** also contained antifungal activity against *C. cucumerinum*. This is the first report of larvicidal activity (*Aedes egypti*) of mansonone C **(8)**.

For the antioxidatnt properties of 1,2-naphthoquinones, it is very surprising that only mansonone N **(4)** including 3-methoxy-4,5-dihydroxybenzaldehyde **(13)** showed radical scavenging properties toward DPPH. Moreover, mansonones G **(10)**, H **(11)** and dehydrooxoperezinone **(12)** showed the potent tendency for antithrombin agent.

FUTURE WORKS

1. Synthesizing additional mansonone-type derivatives to examine the structure activity relationship.
2. Studying the chemical constituents in other parts of *M. gagei* such as with the aim of obtaining the lead compounds.

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Phytotherapy Research

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Antifungal, antioxidant and larvicidal activities of compounds isolated from the heartwood of *Mansonia gagei*

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Eleven compounds isolated from the heartwood of *Mansonia gagei* were tested for their antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*, as well as for their larvicidal activities against *Aedes aegypti* and radical scavenging properties in a DPPH assay. Mansonone C (**4**) was found to be the most interesting compound with antifungal activities against *Cladosporium cucumerinum* and *Candida albicans* as well as for its larvicidal properties against *Aedes aegypti*. Mansonone E (**5**) was active against *Cladosporium cucumerinum* and *Candida albicans*. Two coumarin derivatives, mansorin A (**1**) and mansorin B (**2**) were also found to be active against *Cladosporium cucumerinum*, while mansonone N (**9**) was the only isolated product to show radical scavenging properties.

Keywords: *Mansonia gagei*; antifungal activity; antioxidant activity; larvicidal activity; mansonones; coumarins

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INTRODUCTION

Mansonia gagei Drumm. is a large tree belonging to the Sterculiaceae family. It is the only species of this family to be found in Thailand where it grows in dry evergreen forests. This plant is known as chan-cha-mod, chan-hom chan-khao or chan-pha-ma and, according to folklore beliefs, its heartwood is locally used as a cardiac stimulant, antiemetic, antidepressant and refreshment agent (Pongboonrod, 1976).

In our search for new antifungal, larvicidal and antioxidant lead compounds from higher plants, the dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and methanol (MeOH) extracts from the heartwood of *Mansonia gagei* showed activities against the phytopathogenic fungus *Cladosporium cucumerinum* (Homans and Fuchs, 1970), the human pathogenic yeast *Candida albicans* (Rahalison *et al.*, 1991) and the larvae of the yellow fever-transmitting mosquito *Aedes aegypti* (Cepleanu, 1993). These extracts were also found to possess radical scavenging activities in a DPPH test (Cuendet *et al.*, 1997). Subsequent activity-guided fractionations of these raw extracts allowed the isolation of several compounds - mainly coumarins and mansonones (Tiew *et al.*, in press; Tiew *et al.*, accepted), the respective activities of which were evaluated in the different tests mentioned.

MATERIALS AND METHODS

Plant Material. The dried heartwood of *Mansonia gagei* Drumm. was collected from Saraburi province, Thailand in 1997. The identify of this plant has been confirmed by a comparison with a voucher specimen no. 43281 at the Herbarium of the Royal Forestry Department of Thailand.

Extraction and isolation. Dried heartwood of *Mansonia gagei* Drumm. (14 kg) was milled and extracted three times with CH_2Cl_2 at room temperature. Evaporation of the solvent under vacuum yielded 427.6 g of crude CH_2Cl_2 extract. Following the same procedure, this residue of extraction was successively extracted with EtOAc and MeOH to give 309.7 g of EtOAc extract and 620.1 g of MeOH extract.

A part of the CH_2Cl_2 crude extract (102.2 g) was fractionated by quick column chromatography using silica gel 60G (Art. 7731, Merck). The column was eluted by using a step gradient of hexane-CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH. The fractions were collected and combined according to TLC results in order to obtain ten fractions (1-10). Eleven pure compounds were obtained after further separation of fractions on silica gel columns using a step gradient of hexane-CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH. Mansorin A (**1**) (1.68 g), mansonone C (**4**) (19.8 mg) were isolated from fraction 3. Mansonones E (**5**) (2.4 mg), N (**9**) (13.3 mg), O (**10**) (23.5 mg) and mansorin C (**3**) (77.3 mg) were obtained from fraction 4. Mansonone P (**11**) (39.7 mg) was isolated from fraction 5. Mansonones H (**7**) (0.66 g), dehydrooxoperezinone (**8**) (0.51 g) were obtained from fraction 6. Mansorin B (**2**) (0.98 g) was isolated from both fractions 4 and 5. Mansonone G (**6**) (4.62 g), the major compound of the extract, was obtained from fractions 3-6. Among these eleven compounds, mansorins A, B and C, mansonones N, O and P have been characterized as new naturally occurring compounds (Tiew *et al.*, in press; Tiew *et al.*, accepted).

Sample preparation for bioautographic assays. Geometric dilutions were obtained from freshly prepared stock solutions of isolated and reference compounds at a concentration of 1 mg/ml in an appropriate solvent. 10 µl of these solutions were applied on the TLC plates using graduated capillaries.

Bioautographic assays. Direct bioautography with *Cladosporium cucumerinum*: after application of the samples on silica gel 60 F_{254} Al-backed sheets (Merck), the TLC plates were developed using appropriate solvent systems and thoroughly dried for complete removal of solvents. The plate was then sprayed with a suspension of spores of *C. cucumerinum* in a nutritive medium and incubated for 2-3 days in polystyrene boxes with a moist atmosphere. Clear inhibition zones appeared against a dark grey background. Nystatin (Sigma) was used as a reference compound.

Direct bioautography with *Candida albicans*: after application of the samples on a silica gel 60 F_{254} glass-backed plate (Merck), the TLC plate was developed using appropriate solvent systems and thoroughly dried for complete removal of solvents. An inoculum of yeast in malt agar was prepared and spread over the TLC plate. The plates were incubated overnight at 30°C and then sprayed with methylthiazolyltetrazolium chloride (MTT). Active compounds appeared as clear spots against a purple-coloured background. Nystatin (Sigma) was used as a reference compound.

Larvicidal assays. Geometric dilutions of the isolated and reference compounds were freshly prepared from stock solutions at 1 mg/100 µl in DMSO. Aliquots of these dilutions were added to a graduated tube containing approximately 10 larvae of *Aedes aegypti* in tap water and the final volume was adjusted to 10 ml. The tubes were incubated in darkness at 26-28°C for 24 hours. Larvae lethality was observed under lab light. All samples were measured in duplicate. Rotenone (Sigma) was used as a reference compound.

Radical scavenging assay. This test was performed on thin-layer chromatography. Plant extracts and pure compounds were applied on aluminium-backed silica gel 60 F_{254} plates (Merck). The plates were developed in appropriate solvent systems before being dried out. For free radical scavenging assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (2 mg/mL in MeOH) was used as spray reagent (Cuendet *et al.*, 1997). The active compounds were seen as clear spots against a purple background. Quercetin was used as a reference compound.

RESULTS AND DISCUSSION

The CH_2Cl_2, EtOAc and MeOH extracts from the heartwood of *M. gagei* were preliminary screened for their antifungal, larvicidal and radical scavenging activities (Table 1). All the three extracts showed antioxidant activity based on scavenging activity towards the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical. In addition, the CH_2Cl_2 and EtOAc extracts showed antifungal activities against *Clasdosporium cucumerinum* and *Candida albicans* in TLC bioautographic assays. No larvicidal activity against the larvae of *Aedes aegypti* could be determined for these three extracts.

As both CH_2Cl_2 and EtOAc extracts showed similar biological activities and very closely related HPLC/UV chromatograms, it was decided to proceed to the isolation of the bioactive compounds of the CH_2Cl_2 extract, available in a larger quantity.

Eleven pure compounds were obtained and then tested in the same bioassays. Results are shown in Table 2. Mansonones C and E were found to be the only active products against *C. albicans* with a minimal amount of 0.15 µg, and 2.5 µg, respectively to ensure inhibition of the yeast growth. Mansonones C and E as well as mansorins A and B were found to be

active against *C. cucumerinum* with minimal inhibitory amount of 0.6, 0.6, 2.5 and 0.6 µg, respectively. Mansorin B and mansonones C and E were slightly less potent than nystatin employed as a control.

Mansonone C was the only isolated compound to possess toxic properties against the larvae of the yellow fever-transmitting mosquito *Aedes aegypti* at 50 ppm. Dilution tests were performed and the minimal amount of compound to kill all the larvae after 24 hours was calculated as 6.25 ppm. This activity could not been detected in the raw extract probably due to the low concentration of the active compound in the CH_2Cl_2 extract (0.0046 %). In comparison, rotenone used as the reference compound, was twice more active.

Mansonone N was finally the only isolated compound to exhibit radical scavenging properties in the DPPH, all the other compounds being inactive on the TLC assay at a tested amount of 10 µg. A limit of activity at 2.5 µg was determined for mansonone N in the same assay. Quercetin used as a reference was 5 times more active.

In this work we reported the promising antifungal, larvicidal and (or) antioxidant properties of several compounds isolated from the heartwood of *Mansonia gagei*. Further toxicological and pharmacological studies should now been performed to assess the therapeutical interest of these compounds.

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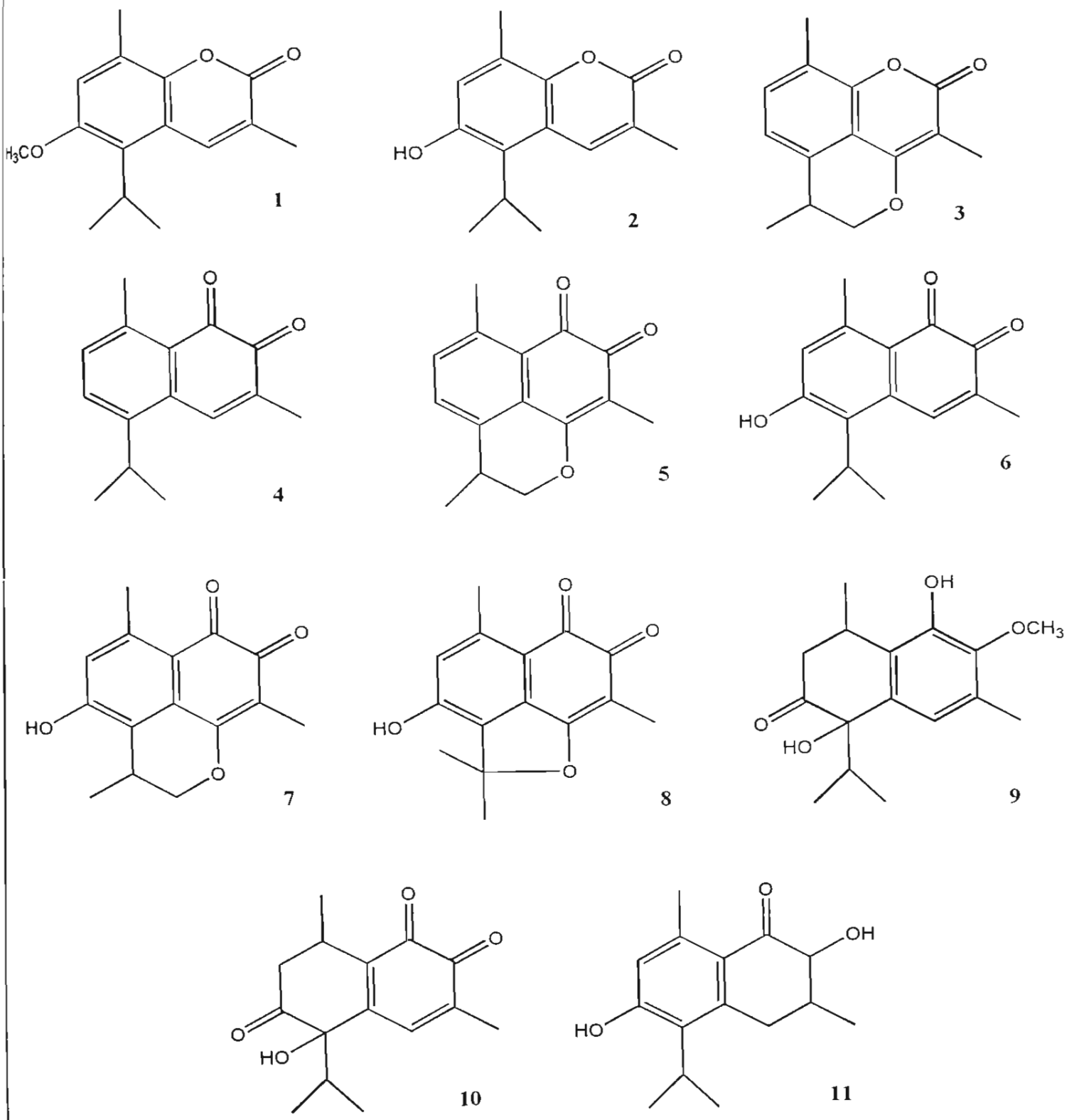

Figure 1. Structures of the isolated compounds

Table 1. Antifungal, larvicidal and radical scavenging activities of extracts from the heartwood of *Mansonia gagei*

Extract	*Clasdosporium cucumerinum*[a]	*Candida albicans*[a]	*Aedes aegypti*[b]	DPPH[a]
DCM	+	+	-	+
EtOAc	+	+	-	+
MeOH	-	-	-	+

[a] : tested amount : 100 µg of crude extract
[b] : tested amount : 500 ppm of the extract
+ : active
- : not active

Table 2. Antifungal, larvicidal and radical scavenging activities of the isolated compounds from the heartwood of *Mansonia gagei*

n°	Compounds	*C. cucumerinum*[a]	*C. albicans*[a]	*A. aegypti*[b]	DPPH[c]
1	mansorin A	2.5	> 10	> 50	> 10
2	mansorin B	0.6	> 10	> 50	> 10
3	mansorin C	> 10	> 10	> 50	> 10
4	mansonone C	0.6	0.15	6.25	> 10
5	mansonone E	0.6	2.5	> 50	> 10
6	mansonone G	> 10	> 10	> 50	> 10
7	mansonone H	> 10	> 10	> 50	> 10
8	dehydrooxoperezinone	> 10	> 10	> 50	> 10
9	mansonone N	> 10	> 10	> 50	2.5
10	mansonone O	> 10	> 10	n.t.	> 10
11	mansonone P	> 10	> 10	> 50	> 10
	nystatin	0.2	1	-	-
	rotenone	-	-	3	-
	quercetin	-	-	-	0.5

[a] Minimal amount (µg) of compound to inhibit growth on a silica gel TLC plate
[b] Minimal concentration (ppm) of compound required to kill all the larvae after 24 hours
[c] Minimal amount (µg) of compound required to show a radical scavenging activity on a silica gel TLC plate
n.t. not tested