CHAPTER IV
RESULTS AND DISCUSSION

The dried flowers of *Melodorum fruticosum* Lour. (2.3 kg) were successively extracted with petroleum ether, ethyl acetate, and 70% aqueous acetone. The petroleum ether extract was then separated by extensive repeated chromatography to afford one compound (MF-6). The ethyl acetate extract was fractionated by various chromatographic techniques to afford four compounds (MF-1, MF-3, MF-4, and MF-5). Finally, one pure compound (MF-2) was crystallized from the 70% aqueous acetone extract. All structures were determined through the interpretation of their spectroscopic data (UV-vis, IR, MS, and NMR), and subsequently confirmed by comparison with reported values.

Structure Determination of Isolated Compounds

1. Structure Elucidation of Compound MF-1

The methane CIMS of compound MF-1 exhibited MH^+ at *m/z* 259 indicating the molecular weight as 258. The ^{13}C NMR spectrum of this compound showed 14 carbon signals at 12 different frequencies, while its ^1H NMR displayed integration for 10 protons, suggesting the molecular formula of $C_{14}H_{10}O_5$. Elemental analysis data of this compound supported the proposed formula (calcd. for $C_{14}H_{10}O_5$: C 65.12%, H 3.88%; found: C 65.15%, H 3.87%). In the IR spectrum, three carbonyl absorption bands at 1790, 1731, and 1704 cm^{-1} could be observed. The strong IR absorption bands at 1790 and 1731 cm^{1} were consistent with the carbonyl ester of an α,β-unsaturated-γ-lactone ring[4] and a carbonyl ester linkage, respectively. ^{13}C and ^1H NMR data together with 2D-NMR experiments allowed the complete structure of compound MF-1 to be established.

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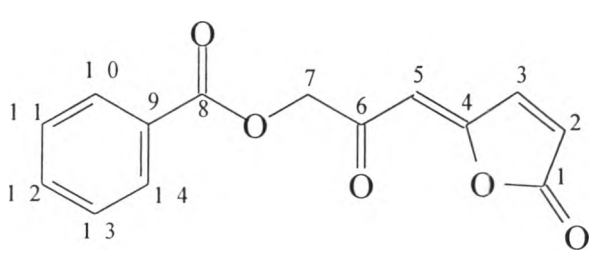
The ^{1}H NMR spectral data (Table 2) indicated that compound MF-1 possesses methylene protons (δ 5.38), three olefinic protons (δ 5.69, 6.49, and 7.54), and five aromatic protons (δ 7.44, 7.57, and 8.09). The ^{13}C NMR spectral data (Table 2) showed one methylene carbon (δ 69.3), eight sp^2 carbons (δ 107.3, 124.2, 128.4, 129.9, 133.3, and 145.0), and five quaternary carbons (δ 129.3, 155.9, 165.8, 166.9, and 190.8). The presence of a benzoyl moiety was deduced by comparison of ^{13}C and ^{1}H NMR spectral data with those of acetylmelodorinol[4] (Table 3). Two olefinic protons were coupled to each other with J=5.5 Hz indicating that the double bond is part of α,β-unsaturated-γ-lactone ring[4]. The methylene carbon appeared at rather downfield chemical shift (δ 69.3) than typical methylene carbon and the COLOC experiment (Table 2) displayed long range coupling between the methylene protons and the carbonyl carbon of the benzoyl moiety, so the benzoyl moiety can be extended to methylene benzoyloxy moiety. One olefinic proton (δ 5.69) showed long range coupling to the β-carbon of α,β-unsaturated-γ-lactone ring, indicating the presence of sp^2 carbon at γ-carbon of the lactone moiety. Another carbonyl carbon at 190.8 ppm represented the ketonic carbon of α,β-unsaturated ketone. This carbon showed two-bond coupling with the methylene protons, therefore the methylene benzoyloxy moiety and the exocyclic sp^2 carbon of the γ-lactone ring could be connected via this carbonyl. The structure of compound MF-1 could be either cisoid or transoid configuration. However, the NOE difference data, in which irradiation of H-3 signal (δ 7.54) enhanced the signal of H-5 (δ 5.69), comfirmed the cisoid configuration. Thus, compound MF-1 was assigned the structure (4*Z*)-7-benzoyloxy-2,4-heptadiene-6-one-4-olide (**19**) trivially named melodorinone-A, a novel heptene derivative.



(**19**)

Table 2: ^{13}C NMR (75 MHz in $CDCl_3$), ^{1}H NMR (300 MHz in $CDCl_3$), C-H COSY (75 MHz in $CDCl_3$) and COLOC spectral data of compound MF-1

Carbons Position	**δ-^{13}C (ppm)**	**δ-^{1}H (ppm)**	**COLOC Correlation**
C-1	166.9 s	-	H-3
C-2	124.2 d	6.49 (1H, d, *J*=5.5 Hz)	-
C-3	145.0 d	7.54 (1H, d, *J*=5.5 Hz)	H-5
C-4	155.9 s	-	H-2; H-3
C-5	107.3 d	5.69 (1H, s)	-
C-6	190.8 s	-	H-7
C-7	69.3 t	5.38 (2H, s)	H-5
C-8	165.8 s	-	H-7
C-9	129.3 s	-	-
C-10, 14	129.9 d	8.09 (2H, m)	H-14; H-10
C-11, 13	128.4 d	7.44 (2H, m)	H-13; H-11
C-12	133.3 d	7.57 (1H, m)	-

Chemical shifts are relative to the solvent signal ($CDCl_3$).

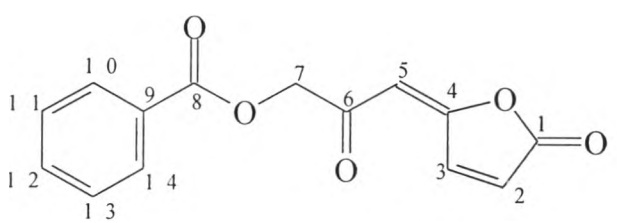
Multiplicities were established from C-H COSY and DEPT spectra.

Table 3: ^{13}C chemical shifts of benzoyl moiety of compound MF-1 compared with the data of acetylmelodorinol.

Carbons Position	**Compound MF-1**	**Acetylmelodorinol**
C-8	165.8	165.94
C-9	129.3	129.48
C-10, 14	129.9	129.64
C-11, 13	128.4	128.43
C-12	133.3	133.23

2. Structure Determination of Compound MF-2

Compound MF-2 was isolated as colorless crystals from the 70% aqueous acetone extract and then was recrystallized from ethyl acetate. Its molecular formula was established from elemental analysis, CIMS, and NMR data (Tables 4). Most of the ^{1}H and ^{13}C NMR data of compound MF-2 were similar to those of compound MF-1, except that the chemical shifts of H-3 (δ 8.32) and C-4 (δ 160.5) of this compound were downfield than those of compound MF-1. Thus, the structure of compound MF-2 could possibly be proposed as in transoid configuration. NOE difference experiments were used to confirm the assignment in which irradiation of H-3 signal at δ 8.32 enhanced the signal of H-2 (δ 6.51) but not H-5 (δ 6.33). Hence, the transoid configuration was confirmed and compound MF-2 was assigned the structure (4*E*)-7-benzoyloxy-2,4-heptadiene-6-one-4-olide (**20**), trivially named melodorinone-B, a new derivative of heptene group.



(**20**)

Table 4: ^{13}C NMR (75 MHz in $CDCl_3$), ^{1}H NMR (300 MHz in $CDCl_3$), C-H COSY (75 MHz in $CDCl_3$) and COLOC spectral data of compound MF-2

Carbons Position	**δ-^{13}C (ppm)**	**δ-^{1}H (ppm)**	**COLOC Correlation**
C-1	167.4 s	-	H-3
C-2	125.9 d	6.51 (1H, d, *J*=5.7, 1.3 Hz)	-
C-3	142.7 d	8.32 (1H, d, *J*=5.7 Hz)	H-5
C-4	160.5 s	-	H-2
C-5	103.1 d	6.33 (1H, d, *J*=1.3 Hz)	-
C-6	192.8 s	-	H-7
C-7	69.2 t	4.99 (2H, s)	H-5
C-8	165.8 s	-	H-7
C-9	128.9 s	-	-
C-10, 14	129.9 d	8.09 (2H, m)	H-14; H-10
C-11, 13	128.6 d	7.47 (2H, m)	H-13; H-11
C-12	133.7 d	7.60 (1H, m)	-

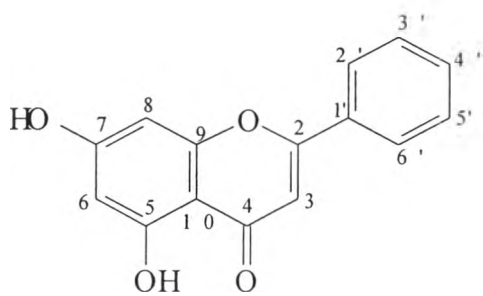
Chemical shifts are relative to the solvent signal ($CDCl_3$).

Multiplicities were established from C-H COSY and DEPT spectra.

3. Identification of Compound MF-3

The EI mass spectrum of compound MF-3 showed the molecular ion at *m/z* 254. Fifteen carbons appeared as signals at 13 different chemical shifts in its ^{13}C NMR spectrum while the ^{1}H NMR spectrum indicated at least 9 protons in the structure. These NMR spectral data are shown in Tables 5 and 6. The mass spectrum and NMR data indicated the molecular formula of MF-3 to be $C_{15}H_{10}O_4$. The NMR spectral pattern of this compound appeared to be similar to those of the flavone compounds. A sharp singlet signal at δ 12.80 indicated the chelated hydroxy proton at position 5 of the flavone

structure. The aromatic proton signals at δ 6.21 and 6.50 were meta-coupled to each other with a *J* value of 1.6 Hz, the EIMS fragment at *m/z* 124 supported another hydroxyl substitution of the flavone structure at position 7. On the other hand the EIMS fragment at m/z 77 indicated the unsubstituted ring B of this structure. Therefore, compound MF-3 was assigned the structure of chrysin (**21**)[14,15], of which comparison of the MS and NMR spectra of this compound with those previously reported (Table 6) confirmed the identification.



(**21**)

Table 5: ^{1}H NMR (300 MHz in DMSO-d_6) spectral data and C-H COSY (75 MHz in DMSO-d_6) data of compound MF-3

Protons Position	**δ-^{1}H (ppm)**	**Correlated Carbon (δ: ppm)**
H-3	6.94 (1H, s)	105.4
H-6	6.21 (1H, d, *J*=1.6 Hz)	99.2
H-8	6.50 (1H, d, *J*=1.6 Hz)	94.3
H-2', 6'	8.04 (2H, d, *J*=7.0 Hz)	126.6
H-3', 5'	7.58 (2H, m)	129.3
H-4'	7.58 (1H, m)	132.2
5-OH	12.80 (1H, s)	-
7-OH	10.88 (1H, br s)	-

Chemical shifts are relative to the solvent signal.

Table 6: NMR spectral data comparing of compound MF-3 with chrysin.

Position	Proton Chemical Shift (ppm)		Carbon-13 Chemical Shift (ppm)	
	MF-3	Chrysin	MF-3	Chrysin
2	-	-	163.4	163.0
3	6.94 s	6.94 s	105.4	105.0
4	-	-	182.0	181.6
5	-	-	161.6	161.5
6	6.21 d (1.8)	6.23 d (1.9)	99.2	98.9
7	-	-	164.6	164.3
8	6.50 d (1.4)	6.53 d (1.9)	94.3	94.0
9	-	-	157.6	157.3
10	-	-	104.1	103.9
1'	-	-	130.9	131.7
2'	8.04 d (7.01)	8.06 d (7.9)	126.6	126.1
3'	7.58 m	7.58 m	129.3	128.9
4'	7.58 m	7.58 m	132.2	130.6
5'	7.58 m	7.58 m	129.3	130.6
6'	8.04 d (7.01)	8.06 d (7.9)	126.6	126.1
5-OH	12.80 s	12.85 s	-	-
7-OH	10.88 br s	-	-	-

4. Identification of Compound MF-4

The molecular ion peak of this compound was shown in EI mass spectrum at *m/z* 282. ^{13}C NMR and ^{1}H NMR data (Table 8) indicated the presence of 17 carbons at 15 different frequencies and 14 protons, respectively, suggesting the molecular formula of $C_{17}H_{14}O_4$. The proton NMR pattern of this compound was similar to that of compound MF-3. The difference was the appearance of two methoxy signals while the hydroxy proton signal could not be observed, indicating the dimethoxy substitution at positions 5

and 7 of the flavone structure. Comparison of its spectral information with those previously reported (Table 7) indicated that MF-4 was 5,7-dimethoxyflavone (**22**)[16]. Two-dimensional NMR experiments were used to study the chemical shift assignments and the data are shown in Table 8.

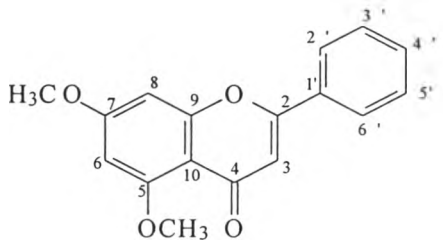


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Table 7: NMR spectral data comparison of compound MF-4 with 5,7-dimethoxyflavone.

	Proton Chemical Shift (ppm)	
Position	**MF-4**	**5,7-dimethoxyflavone**
2	-	-
3	6.66 s	6.65 s
4	-	-
5	-	-
6	6.35 d (2.1)	6.38 d (2.5)
7	-	-
8	6.55 d (2.1)	6.57 d (2.5)
9	-	-
10	-	-
1'	-	-
2', 6'	7.85 m	7.88 m
3', 4', 5'	7.48 m	7.48 m
5-OCH_3	3.89 s*	3.92 s
7- OCH_3	3.93 s*	3.97 s

*Interchangeable

Table 8: ^{13}C NMR (75 MHz in $CDCl_3$), ^{1}H NMR (300 MHz in $CDCl_3$), C-H COSY (75 MHz in $CDCl_3$) and COLOC spectral data of compound MF-4

Carbons Position	**δ-^{13}C (ppm)**	**δ-^{1}H (ppm)**	**COLOC Correlation**
C-2	160.9 s	-	H-2'; H-6'
C-3	109.0 d	6.66 (1H, s)	-
C-4	177.6 s	-	-
C-5	160.6 s	-	5-OCH_3
C-6	92.8 d	6.35 (1H, d, *J*=2.1 Hz)	H-8
C-7	164.0 s	-	7-OCH_3
C-8	96.2 d	6.55 (1H, d, *J*=2.1 Hz)	H-6
C-9	159.9 s	-	H-8
C-10	109.1 s	-	H-3; H-6; H-8
C-1'	131.5 s	-	H-3'; H-4'; H-5'
C-2', 6'	125.9 d	7.85 (2H, m)	H-2'; H-6'
C-3', 5'	128.9 d	7.48 (2H, m)	H-3'; H-4'; H-5'
C-4'	131.1 d	7.48 (1H, m)	-
5-OCH_3	55.7 q*	3.89 (3H, s)*	-
7-OCH_3	56.4 q*	3.93 (3H, s)*	-

Chemical shifts are relative to the solvent signal ($CDCl_3$)

Multiplicities were established from C-H COSY and DEPT spectra.

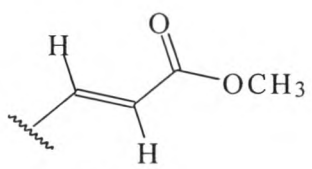
*Interchangeable

5. Structure Determination of Compound MF-5

The ^{1}H and ^{13}C NMR data of compound MF-5 (Table 9) indicated 14 protons and 15 carbons appearing at 13 different frequencies, respectively. The EI mass spectrum of this compound exhibited MH^+ at *m/z* 291 and M^+ at 290, suggesting the molecular formula of $C_{15}H_{14}O_6$, which was confirmed by the elemental analysis data (calcd. for

$C_{15}H_{14}O_6$: C 62.07, H 4.86; found: C 62.06, H 4.85). IR spectrum showed two strong carbonyl absorptions at 1722 and 1589 cm^{-1} consistent with carbonyl ester and carbonyl of tautomeric keto and enol forms, respectively. A broad shallow band at 3500-2700 cm^{-1} represented the enolic O-H stretching absorption, suggesting the β-diketones exist as mixtures of tautomeric keto and enol forms in this structure.[17] ^{13}C NMR and ^{1}H NMR spectra together with two-dimensional NMR experiments allowed the complete structure of compound MF-5 to be established.

The ^{1}H NMR spectrum of compound MF-5 displayed a methoxy proton singlet at δ 3.77, a methylene proton singlet at δ 4.93, three olefinic proton signals at δ 5.86, 6.75, and 6.92, and five aromatic proton signals at δ 7.47, 7.60, and 8.09. The ^{13}C NMR spectrum indicated one methoxy carbon (δ 52.2), one methylene carbon (δ 66.6), and eight sp^2 carbons (δ 100.2, 128.1, 128.6, 129.9, 133.6, and 198.2). Comparison of ^{13}C and ^{1}H NMR spectral data with those of melodienone[4] suggested the presence of a benzoyl moiety. Two olefinic proton doublets at δ 6.75 and 6.91 were coupled to each other with a coupling constant of 15.6 Hz indicating their trans-configuration. One of these doublets at δ 6.91 exhibited long range coupling to a carbonyl carbon at δ 165.9, which, in turns showed long range coupling with the methoxy protons at δ 3.77, forming the fragment (**23**). The downfield methylene carbon at δ 66.6 implicated its connection with an oxygen atom. The COLOC experiment (Table 9) showed long range coupling between these methylene protons and the carbonyl carbon (δ 165.7) of the benzoyl moiety, extending the benzoyl moiety into the methylene benzoyloxy moiety fragment (**24**). Another carbonyl carbon signal at 198.2 ppm exhibited two-bond coupling with both the methylene protons and the olefinic proton at 5.86 ppm. Finally, the sp^2 carbon at 170.7 ppm exhibited two-bond correlation with both olefinic protons at 6.91 and 5.86 ppm, connecting fragment (**23**) and (**24**) via the β-diketone part into the structure (*E*)-7-benzoyloxy-4-hydroxy-1-methoxy-2,4-heptadien-1,6-dione (**25**). This compound also possesses a novel structure belonging to the heptene class and was named tautomelodienone.



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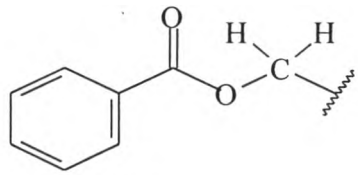

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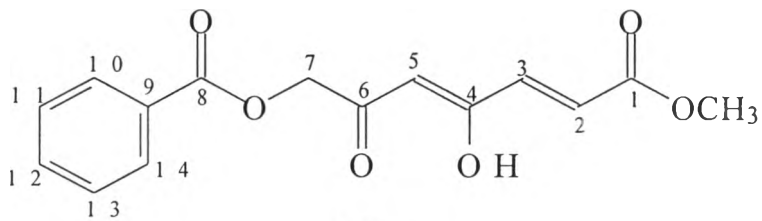

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Table 9: ^{13}C NMR (75 MHz in $CDCl_3$), ^{1}H NMR (300 MHz in $CDCl_3$), C-H COSY (75 MHz in $CDCl_3$) and COLOC spectral data of compound MF-5

Carbons Position	**δ-^{13}C (ppm)**	**δ-^{1}H (ppm)**	**COLOC Correlation**
C-1	165.9 s	-	1-OCH_3; H-3
C-2	128.1 d	6.75 (1H, d, *J*=15.6 Hz)	-
C-3	137.4 d	6.91 (1H, d, *J*=15.6 Hz)	-
C-4	170.7 s	-	H-3; H-5
C-5	100.2 d	5.86 (1H, s)	-
C-6	198.2 s	-	H-5; H-7
C-7	66.6 t	4.93 (2H, s)	-
C-8	165.7 s	-	H-7; H-10; H-11
C-9	128.9 s	-	-
C-10, 14	129.9 d	8.09 (2H, m)	-
C-11, 13	128.6 d	7.47 (2H, m)	-
C-12	133.6 d	7.56 (1H, m)	-
1-OCH_3	52.2 q	3.77 (3H, s)	-
6-OH	-	13.84 (1H, br s)	

Chemical shifts are relative to the solvent signal ($CDCl_3$).

6. Identification of Compound MF-6

The EI mass spectrum of compound MF-6 indicated the molecular ion at *m/z* 302. Proton signals integrated for 14 protons and 16 carbon signals at 14 different chemical shifts could be observed in the ^{1}H and ^{13}C NMR spectral data (Table 10), suggesting the molecular formula of $C_{16}H_{14}O_6$. Using the same approach as compound MF-1, a benzoyl moiety and a lactone part could be inferred. The proton signals at δ 4.50 and δ 4.43 displayed geminal coupling with *J*=11.8 Hz and were also vicinal-coupled to a doublet of doublets of doublets at δ 6.07 with *J*=4.1 and 6.1 Hz, respectively. A pair of signals, a one-proton ddd signal at δ 6.07 and an olefinic proton at δ 5.30, were coupled to each other with *J*=8.1 Hz. Two proton signals at δ 4.50 and δ 4.43, C-H correlated to a methylene carbon signal at δ 64.4, displayed long range coupling with the carbonyl carbon signals (δ 165.6) of the benzoyl moiety. Furthermore, the COLOC experiment (Table 10) displayed long range coupling between the proton signal at δ 6.07 and another carbonyl carbon at δ 169.5, while the same carbonyl carbon also showed long range coupling with the methyl proton signal at δ 2.02, indicating the presence of an acetyl moiety at position 6. One olefinic proton (δ 5.30) showed long range coupling to the β-carbon of α,β-unsaturated-γ-lactone ring, confirming the position of a double bond adjacent to the α,β-unsaturated-γ-lactone ring. The structure of compound MF-6 was thus identified as acetylmelodorinol (**26**). This structure was confirmed by comparison of its ^{1}H and ^{13}C NMR spectral data with those of acetylmelodorinol[4] (Table 11) previously reported.

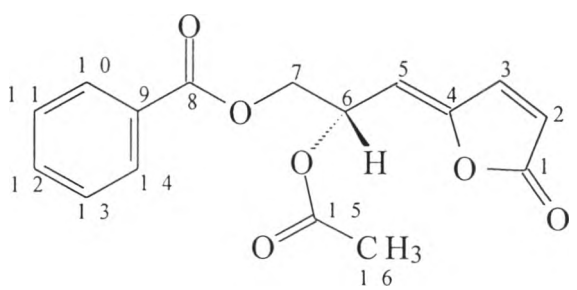

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Table 10: ^{13}C NMR (75 MHz in $CDCl_3$), ^{1}H NMR (300 MHz in $CDCl_3$), C-H COSY (75 MHz in $CDCl_3$) and COLOC spectral data of compound MF-6

Carbons Position	**δ-^{13}C (ppm)**	**δ-^{1}H (ppm)**	**COLOC Correlation**
C-1	168.2 s	-	H-2; H-3
C-2	121.2 d	6.20 (1H, d, *J*=5.4 Hz)	H-3
C-3	143.2 d	7.36 (1H, d, *J*=5.8 Hz)	H-2; H-5
C-4	150.3 s	-	H-2; H-3
C-5	108.6 d	5.30 (1H, d, *J*=8.1 Hz)	-
C-6	67.0 d	6.07 (1H, ddd, *J*=8.1, 6.1, 4.1 Hz)	-
C-7	64.4 t	4.50 (1H, dd, *J*=11.8, 4.1 Hz) 4.43 (1H, dd, *J*=11.8, 6.1 Hz)	-
C-8	165.6 s	-	H-7; H-10; H-14
C-9	129.2 s	-	H-10; H-14
C-10, 14	129.3 d	7.95 (2H, m)	-
C-11, 13	128.2 d	7.37 (2H, m)	-
C-12	132.9 d	7.50 (1H, m)	H-10; H-14
C-15	169.5 s	-	H-16
C-16	20.8 q	2.02 (3H, s)	-

Chemical shifts are relative to the solvent signal ($CDCl_3$).

Table 11: NMR spectral data of compound MF-6 compared with acetylmelodorinol[4].

	Proton Chemical Shift (ppm)		Carbon-13 Chemical Shift (ppm)	
Position	**MF-6**	**Acetylmelodorinol**	**MF-6**	**Acetylmelodorinol**
1	-	-	168.2	168.4
2	6.20 d (5.4)	6.26 dd (5.5, 0.5)	121.2	121.4
3	7.36 d (5.8)	7.36 d (5.5)	143.2	143.3
4	-	-	150.3	150.6
5	5.30 d (8.1)	5.31 dd (8.1, 0.5)	108.6	108.8
6	6.07 ddd (8.1, 6.1, 4.1)	6.12 ddd (8.1, 6.1, 4.1)	67.0	67.2
7a	4.50 dd (11.8, 4.1)	4.55 dd (11.7, 4.1)	64.4	64.6
7b	4.43 dd (11.8, 6.1)	4.50 dd (11.7, 6.1)	64.4	64.6
8	-	-	165.6	165.9
9	-	-	129.2	129.5
10, 14	7.95 m	8.01 ddd (8.2, 1.3, 0.9)	129.3	129.6
11, 13	7.37 m	7.43 tt (8.2, 1.3)	128.2	128.4
12	7.50 m	7.56 tt (8.2, 1.3)	132.9	133.2
15	-	-	169.5	169.7
16	2.02 s	2.08 s	20.8	20.9

Compound MF-1, MF-2, and MF-5 have been tested for their in vitro cytotoxicity against Vero cell, P388, KB, and BC cell cultures. Their cytotoxicity data were tabulated in Table 12.

Table 12: Cytotoxicity data of Compound MF-1, MF-2, and MF-5

	Cytotoxicity IC_{50} (μg/ml)			
Compound	**Vero Cell**	**P388**	**KB**	**BC**
MF-1	15	4.54	>20	5.0
MF-2	5	-	>20	>20
MF-5	7.5	0.60	>20	7.5

From a previous report[6], melodorinol (**9**), (4*E*)-7-benzoyloxy-6-hydroxy-2,4-heptadien-4-olide (**10**), acetylmelodorinol (**11**), and (4*E*)-7-benzoyloxy-6-acetoxy-2,4-heptadien-4-olide (**12**) were evaluated for their cytotoxic potential using established procedures.[18-21] These compounds exhibited more cytotoxicity against P388, KB, and BC cell cultures than compounds MF-1, MF-2, and MF-5. It should be noted that the position 6 of MF-1, MF-2, and MF-5 each possessed a ketone functionality while at position 6 of compounds (**9**), (**10**), (**11**), and (**12**) a hydroxy or an acetoxy groups was present. It appeared that the substitution at position 6 of these compounds is important for cytotoxic activity. While, MF-1 and MF-2 contained a lactone moiety, MF-5 was an open chained heptene, demonstrated greater cytotoxic activity than MF-1 and MF-2 compounds.