CHAPTER V

RESULTS & DISCUSSION

4.1 Analysis of degradation products of DHA

Chemical stability of DHA was studies using stability indicating assay procedure that differentiates between the intact molecule and degradation products. Suitable HPLC with UV detection method was developed for the chemical stability study of DHA.

Figure 18 showed HPLC chromatogram of crude DHA. The isolated compounds were reported in Table 1.

Table 1 The isolated compounds from crude DHA.

Compound	Retention time (min)
Compound 1	5.49
Compound 2	6.09
α-DHA	6.46
β-DHA	9.73

HPLC chromatogram indicated that two epimer, α- and β-epimer, of DHA was eluted at 6.46, 9.73 min, respectively. Whereas, DHA exists only as β-form in the solid crystalline state. Melendez, et al[53]. who proposed that the first peak must be the α-form based on consideration of the conformation of the two epimers. They suggested that the α-epimer is more polar than the β-epimer so it must elute earlier than the β-epimer on a reversed phase column. The solid, β-DHA changed rapidly to the α-DHA in aqueous solution. Therefore the α-DHA is the form that should exist in

physiological medium. The rate of epimerization and the equilibrium α/β-epimer ratio are dependent on the polarity of solvent[54]. Batty, et al[55]. who concluded that both α- and β-DHA exist in plasma and both epimers were equally active.

Since the presence of peroxide bridge, DHA is unstable. It was found that degradation products of DHA developed when it was storage for a long time. In addition, under stress condition as high temperature, DHA could be generated degradation products. These products could be followed by HPLC with UV detector using 50% (v/v) acetonitrile-water as mobile phase. Chromatogram showed that the retention time of two major compounds of these products, compound 1 and 2, are 5.49 and 6.09 min., respectively. It was indicated that they were eluted earlier than DHA, thus being more polar than too.

For toxicity test, samples, which are the large amounts of degradation products, were fractionated by silica gel chromatography and then analyzed by HPLC. Figure 19 showed that these samples consist of the two major compounds of degradation products. The ratio of compound 1 to compound 2 was 1.0:1.2.

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4.2 Structure elucidation of isolated compounds from degradation products

4.2.1 Structure elucidation of compound 1

The UV spectra data of compound 1 dissolved in acetonitrile showed a maximum absorption wavelength at 235 nm (Figure 20).

IR spectrum of compound 1 showed in Figure 21 and the absorption peaks were assigned in Table 2. Its IR spectrum showed important absorption bands at 2925, 2875 cm^{-1} (C-H stretching vibration) and 1727 cm^{-1} (C=O stretching vibration of carbonyl group).

Table 2 The IR absorption band assignment of compound 1

Wave number (cm^{-1})	Intensity	Tentative assignment
2925, 2875	Medium	C-H stretching vibration of CH_3-, CH_2-
1727	Strong	C=O stretching vibration of carbonyl group

The ^{1}H-NMR spectrum (Figure 22, Table 3) of compound 1 showed the presence of an aldehyde proton at 9.74 ppm, a sharp singlet at 2.11 ppm for methyl function attach with carbonyl group and two methyl groups at 0.74 and 1.06 ppm.

The ^{13}C-NMR spectrum (Figure 23, Table 3) showed 14 signals. One signal of aldehydic carbon appeared at 203.9 ppm and two ketonic carbon at 208.9 and 211.8 ppm.

From HSQC (Figure 24-25), compound 1 showed four methine carbons at 37.4, 45.1, 51.9 and 53.8, four methylene carbons at 21.3, 26.5, 32.2 and 41.6, and three methyl carbons at 11.1, 13.3 and 29.9.

From HMBC correlation (Figure 26-29), COSY correlation (Figure 30) and NOESY correlation (Figure 31-32) were used to assist the interpretation the structure of compound 1.

Table 3 The ^{1}H, ^{13}C-NMR and 2D long range ^{1}H-^{13}C correlation in the HMBC spectrum data of compound 1

Position	1H-NMR	13C-NMR	HMBC (H to C)
1	-	211.8 q	
2	2.57 (1H, m)	53.8 s	C-1, C-3, C-9, C-10, C-14
3	2.40 (1H, m)	37.4 s	
4	1.75 (1H, d, *J*=2.3 Hz)	32.2 d	C-3, C-5
	2.02 (1H, t, *J*=3.9 Hz)		
5	1.65 (1H, d, *J*=1.6 Hz)	26.5 d	C-1, C-7
	1.97 (1H, m)		
6	2.73 (1H, m)	45.1 s	C-1, C-5, C-7, C-8
7	2.71 (1H, m)	51.9 s	C-6, C-8, C-12
8	9.74 (1H, s)	203.9 q	
9	1.40 (1H, m)	21.3 d	C-1, C-2, C-10, C-11,
	1.97 (1H, m)		C-1, C-2, C-3, C-10, C-11,
10	2.30 (1H, m)	41.6 d	C-2, C-9, C-11, C-13
	2.52 (1H, d, *J*=2.1 Hz)		C-2, C-9, C-11
11	-	208.9 q	
12	1.06 (1H, d, *J*=7.1 Hz)	11.1 t	C-6, C-7, C-8
13	2.11 (1H, s)	29.9 t	C-10, C-11
14	0.74 (1H, d, *J*=7.0 Hz)	13.3 t	C-2,C-3, C-4

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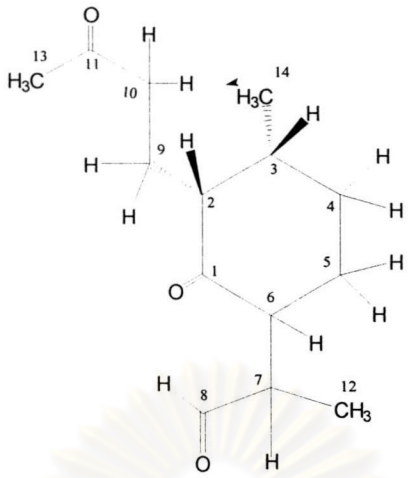

Figure 10 The structure of compound 1

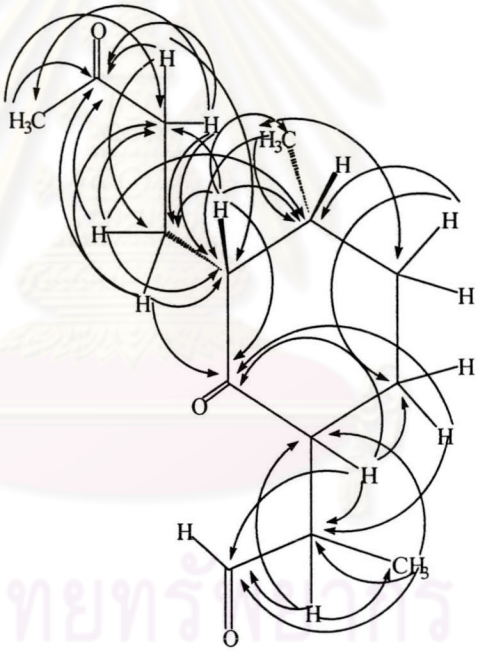

Figure 11 The HMBC correlation of compound 1

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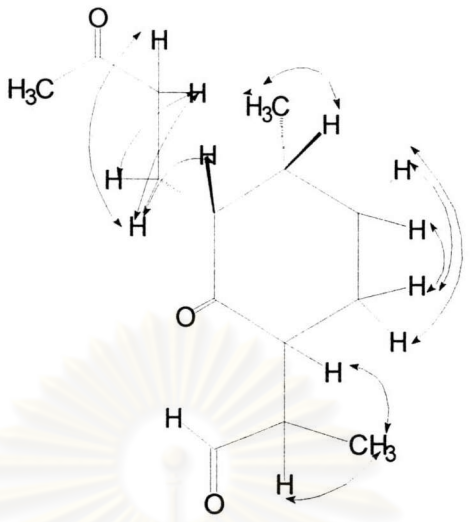

Figure 12 The COSY correlation of compound 1

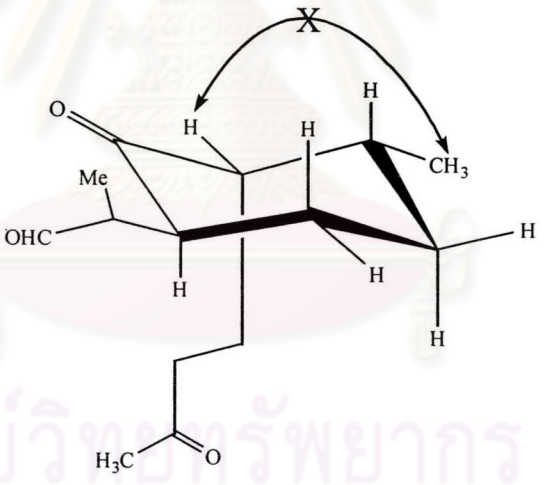

Figure 13 The NOESY correlation of compound 1

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The information from 1D-, 2D-NMR technique were used to assist the interpretation the structure of compound 1. This compound contains only carbons, protons and oxygens, then the molecular formula of $C_{14}H_{22}O_3$ can be established. The molecular formula,$C_{14}H_{22}O_3$ (M.W. 238 Da), of this compound indicated four degrees of unsaturation; therefore, compound 1 must consist of one ring and three carbonyl groups.

Although, ESI mass spectrum of compound 1 showed no molecular ion(*m/z* of 239). However, it was found that molecular ion is presented peak at *m/z* of 261 (Figure 33). This peak may be caused from sodium adduct of compound 1, $[M+Na]^+$. From these results, they showed that the result of compound 1 analyzed by MS confirmed the result from NMR which described above.

4.2.2 Structure elucidation of compound 2

The UV spectra data of compound 2 dissolved in acetonitrile showed a maximum absorption wavelength at 235 nm (Figure 34).

IR spectrum of compound 2 showed in Figure 35 and the absorption peaks were assigned as in Table 4. Its IR spectrum showed important absorption bands at 2933, 2867 cm^{-1} (C-H stretching vibration) and 1707 cm^{-1} (C=O stretching vibration of carbonyl group).

Table 4 The IR absorption band assignment of compound 2

Wave number (cm^{-1})	Intensity	Tentative assignment
2933, 2867	Medium	C-H stretching vibration of CH_3-, CH_2-
1707	Strong	C=O stretching vibration of carbonyl group

The ^{1}H-NMR spectrum (Figure 36, Table 5) of compound 2 indicated that the presence of an aldehyde proton at 9.74 ppm , a sharp singlet at 2.13 ppm for methyl function attach with carbonyl group, and two methyl groups at 1.10 and 1.17 ppm.

The ^{13}C-NMR spectrum (Figure 37, Table 5) showed 14 signals. One signal of aldehydic carbon appeared at 204.7 ppm and two ketonic carbon at 209.1 and 211.3 ppm.

From HSQC (Figure 38), compound 2 showed four methine carbons at 39.3, 45.4, 52.4 and 56.3, four methylene carbons at 20.0, 29.5, 34.4 and 41.1, and three methyl carbons at 11.0, 20.6 and 30.0.

Table 5 The ^{1}H, ^{13}C-NMR and 2D long range ^{1}H-^{13}C correlation in the HMBC spectrum data of compound 2

Position	1H-NMR	13C-NMR	HMBC (H to C)
1	-	211.3 q	-
2	2.05 (1H, s)	56.3 s	C-1, C-3, C-6
3	1.59 (1H, m)	39.3 s	C-4
4	1.53 (1H, m)	34.4 d	C-2, C-6
	1.93 (1H, m)		
5	1.53 (1H, m)	29.5 d	C-1, C-3, C-6
	2.03 (1H, m)		C-4
6	2.76 (1H, m)	52.4 s	C-1, C-5, C-7, C-8, C-12
7	2.52 (1H, m)	45.4 s	C-1, C-6, C-8, C-12
8	9.74 (1H, s)	204.7 q	-
9	1.86 (1H, m)	20.0 d	C-1, C-2,C-3, C-10, C-11
	1.73 (1H, m)		
10	2.38 (1H, m)	41.1 d	C-2, C-9, C-11, C-14
	2.53 (1H, m)		C-2, C-9, C-11, C-14
11	-	209.1 q	-
12	1.17 (1H, d, *J*=7.1 Hz)	11.0 t	C-6, C-7
13	2.13 (1H, s)	30.0 t	C-10, C-11
14	1.10 (1H, d, *J*=5.4 Hz)	20.6 t	C-2, C-3, C-4

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From HMBC correlation (Figure 39-42) and COSY correlation (Figure 43) were used to assist the interpretation the structure of compound

It could be concluded that compound 2 exhibited the ^{13}C-NMR chemical shifts similar to (2S, 3R, 6S)-2-(3-oxobutyl)-3-methyl-6-[(R)-2-propanol]-cyclohexane [40] Table 6.

Table 6 ^{13}C-NMR spectra of compound 2 and (2S, 3R, 6S)-2-(3-oxobutyl)-3-methyl-6-[(R)-2-propanol]-cyclohexane

Position	^{13}C-NMR Chemical shifts (ppm)	
	Compound 2	(2S, 3R, 6S)-2-(3-oxobutyl)-3-methyl-6-[(R)-2-propanol]-cyclohexane [40]
C-1	211.3 q	211.8
C-2	56.3 s	56.5
C-3	39.3 s	40.2
C-4	34.4 d	34.4
C-5	29.5 d	29.8
C-6	52.4 s	51.7
C-7	45.4 s	45.4
C-8	204.7 q	203.6
C-9	20.0 d	20.0
C-10	41.1 d	41.1
C-11	209.1 q	208.7
C-12	11.0 t	11.0
C-13	30.0 t	30.2
C-14	20.6t	20.4

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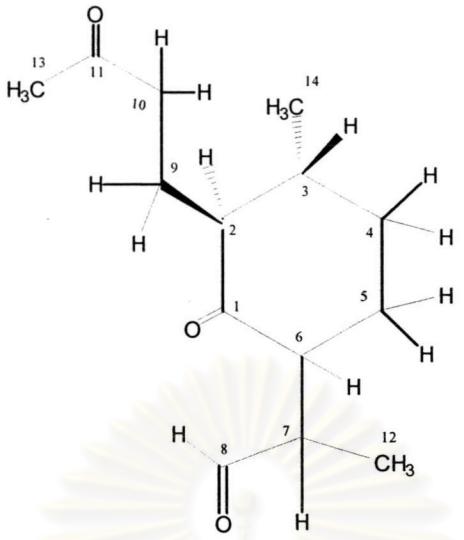

Figure 14 The structure of compound 2

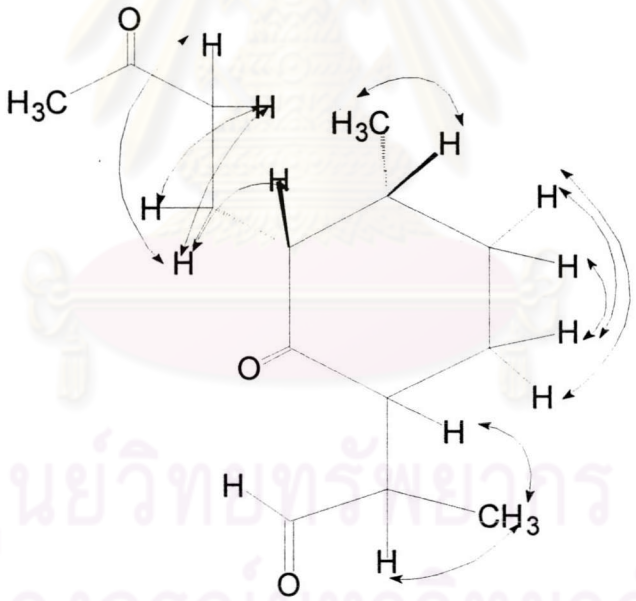

Figure 15 The HMBC correlation of compound 2

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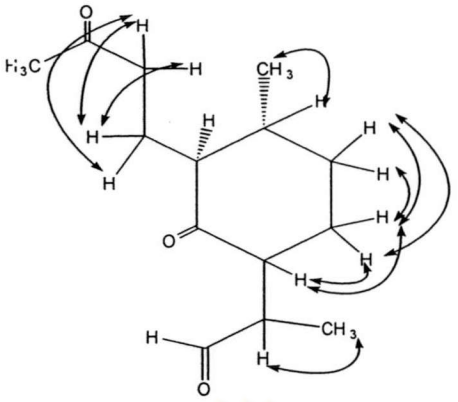

Figure 16 The COSY correlation of compound 2

From previous reported and information from 1D-, 2D-NMR technique were used to assist the interpretation the structure of compound 2. This compound contains only carbons, protons and oxygens, then the molecular formula of $C_{14}H_{22}O_3$ can be established. The molecular formula,$C_{14}H_{22}O_3$ (M.W. 238 Da), of this compound indicated four degrees of unsaturation; therefore, compound 2 must consist of one ring and three carbonyl groups.

Although, ESI mass spectrum of compound 2 showed no molecular ion (*m/z* of 239). However, it was found that molecular ion is presented peak at *m/z* of 261 (Figure 43). This peak may be caused from sodium adduct of compound 2, $[M+Na]^+$. From these results, they showed that the result of compound 2 that analyzed by MS confirmed the result from NMR which described above.

Degradation products of DHA could separated from HPLC. Two major products, compound 1 and 2, were found that ESI-MS and IR data are similar, but NMR data of these compounds were slightly difference. Compound 1 and 2 were proposed to be (2R, 3R, 6S)-2-(3-oxobutyl)-3-methyl-6-[(R)-2-propanol]-cyclohexane and (2S, 3R, 6S)-2-(3-oxobutyl)-3-methyl-6-[(R)-2-propanol]-cyclohexane, respectively.

4.3 Toxicity test

4.3.1 Cytotoxicity test

In vitro activity of DHA and its degradation products against 8 cell lines such as 3T3 (Embryo tissue, Mouse), L929 (Connective tissue, Mouse), BHK21(C13) (Kidney, Hamster (baby)), IEC-6 (Small intestine, Rat), Vero (Kidney, African green monkey), HepG2 (Hepatoma (liver), Human), Caco2 (Adenocarcinoma (Colon), Human) and MCF7 (Adenocarcinoma (breast), Human) were reported in Table 7 [46].

Table 7 Cytotoxic activity against 8 cell lines of degradation products and DHA itself

Sample	IC_{50} (μg/ml)							
	3T3	IEC 6	Vero	L929	BHK21	HepG2	Caco2	MCF7
Fresh DHA	11±3	**5±0**	**6±0.4**	32±6	57±2	21±2	51±3	38±3
Old DHA	10±1	**5±0**	**6±0**	13±3	60±4	26±5.5	46.5±7	36±5.5
Degradation products of DHA	53±17	66±11	68±9	45±11	49±14	37±13.5	93±6	41±9

Fresh DHA and old DHA, storaged for 20 months at RT gave very similar IC_{50}'s. it seems that DHA was toxic to cell lines regardless of age of drug. Both DHA were more cytotoxic to 2 cell lines (IEC-6, Small intestine, Rat and Vero, Adenocarcinoma (Colon), Human). It was not surprising because of previous work[56] showed that oral DHA appeared to cause gastric intestinal irritation in rat.

Previous reports showed that DHA could produce degradation products[40-42]. In this report we found that degradation products showed no toxicity to all cell lines. There was no cell death with dose of these products form 0 to 6.25 μg/ml and only a slight increase in cell death up to 12.5 μg/ml. Based on the lack of toxicity for degradation products, we would not expect an increase in cytotoxicity of the old DHA. However, if the additional degradation products, in effect, reduce the concentration of DHA, cytotoxicity of old DHA might decrease.

4.3.2 Acute toxicity test

Acute toxicity of degradation products and DHA itself were tested in mice ICR stain given oral administration were reported in Table 8 [48].

Any signs of toxic effect presented within 15 minute after oral administration.

Clinical observation

Respiratory : Dyspnea

Motor activities : Ataxia

: Prostation

Convulsion : Found

The result suggested that degradation products caused CNS suppression, while DHA caused gastro intestinal tract irritation[56].

Table 8 LD_{50} of degradation products and DHA itself given p.o. in mice

Sample	LD_{50} (g/kg)	95 % CI
DHA[56]	1.17	0.64-1.65
Degradation products	7.54	5.51-10.46

Degradation products of DHA was more safety than DHA itself with LD_{50}' s is 7.54 g/kg which is higher than LD_{50}' s DHA about 6 fold.

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4.4 **Anti-malarial activity test**

In vitro activity of Mefloquine, Chloroquine, Quine, DHA and degradation products of DHA against W2; Chloroquine-resistant, Mefloquine-sensitive and D6; Chloroquine-sensitive, Mefloquine- resistant of *P. falciparum* were reported in Table 9 [50].

Table 9 *In vitro* response of drugs against *P. falciparum*

Drug	IC_{50} (nM)	
	W2	D6
Mefloquine	10.0	42.0
Chloroquine	90.4	39.4
Quinine	160.6	438.3
Old DHA	1.465	2.92
Fresh DHA	1.123	2.36
Degradation products of DHA	98.0	235.8

Conventional malaria chemotheraphy; Mefloquine, Chloroquine and Quinine, were low ability for killing malaria, whereas both old and fresh DHA, were the most potent antimalarial drug which IC_{50}'s was within a narrow range from 1.1-2.9 nM. In addition, DHA was equally active against the Chloroquine-senstive and Chloroquine-resistant parasite. Degradation products of DHA showed the lower activity against both stains than other antimalarial drugs. From these results, it seems that the degradation products diluted the concentration of DHA, leading to the lower activity of DHA. However, the long time storage for 20 months at room temperature showed that DHA had been active to malaria.