PHD DISSERTATION

PHYTOCHEMICAL INVESTIGATION OF INDONESIAN PLANT, *CURCUMA HEYNEANA* AND ITS CHEMICAL CONVERSION STUDY

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CHAPTER 1.

PHYTOCHEMICAL INVESTIGATION OF *CURCUMA HEYNEANA*

1.1 Introduction

Curcuma heyneana Valeton & Zijp is one of the medicinal plants native to Indonesia and widely found in Java Island. It belongs to the Zingiberaceae family and includes of the species of the Curcuma genus, which has local name Temu giring. [1] It is a perennial, herbaceous plant, with 0.3-0.5 m height, producing pseudo stems from a much branched and elongated rhizome. The pseudo stems are made up of several leaves whose blades can be 17-42 cm long and 7-13 cm wide. The rhizomes are aromatic, pale yellow and taste bitter. It grows down with square branches. The leaves are all in green. The flower comes out from the side of the pseudo stem with red color on the edge of the crown. Its protective leaves have sharp edges. [2] The picture of *C. heyneana* as portrayed in fig. 1

Figure 1. *Curcuma heyneana*
(https://www.biodiversitylibrary.org/name/Curcuma_heyneana#)

It is used traditionally as anthelmintic, and also to treat diseases such as cancer, inflammatory conditions, and skin diseases. For preventive purpose, it's also applied for skin treatment as skin scrub, to make women skin's color brighter.

The rhizomes of *C. heyneana* contained ca.0.43% oil, classified as sesquiterpenes (87.3%), diterpenes (4.8%), and monoterpenes (3.0%). [3]. Isolation of the essential oil from the rhizomes of *C. heyneana* has been described by Firman *et al.* [4]. It consisted

sesquiterpenoids and also a diterpene, (*E*)- labda-8(17), 12-diene-15,16-dial. While Azis Saifudin *et al.*, obtained germacranes, guaianes, spirolactones together with other sesquiterpenes and also labdane-type diterpenes. [5]

In this study, we reported phytochemical investigation of ethyl acetate extract of *C.heyneana* through bioassay guided isolation procedure. It leaded to the isolation of 15 known compounds including terpenoids (guaiane-type sesquiterpenes and labdane-type diterpenes), two compounds fatty acids, two compounds curcuminoids and also adenosine, a purine nucleoside. All isolated compounds were separated by assorted of chromatographic methods, whereas the structures of these compounds were determined by spectroscopic analyses. We also examined all isolated compounds against parasite *Leishmania major.*

1.2 Extraction and Isolation of Chemical Constituent

C. heyneana rhizomes were attained from a cultivated region in East Java, Indonesia. A voucher specimen was deposited in the Herbarium of Hiroshima University (KLN CH032015). The rhizomes (6 kg) were washed and cut into pieces. It was dried using at 40 °C for 72 hours, followed by milling it into powder. Ethanolic extract was obtained by macerating 100 g powder using 70% ethanol (1:10, w/v) to give extract (606.3 g). About 266.0 g of them was suspended in water and partitioned with ethyl acetate three times producing ethyl acetate extract (167.5 g). After that, the water layer being partitioned with 1-butanol generating 17.6 g extract. The obtained extracts were examined its activity against parasite *L. major*. From figure 2, it showed that ethanol and ethyl acetate extracts had the highest inhibitory activity, while 1-butanol extract exhibited no activity. Based on bioassay guided fractionation procedure, ethyl acetate extract was selected to be proceeded furthermore.

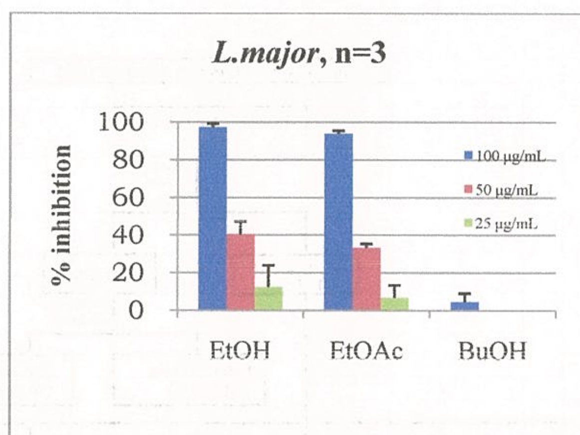

Figure 2. Inhibitory activity of *C. heyneana* extracts

About 50.0 g ethyl acetate extract was separated on a silica gel open column chromatography (CC) and eluting with increasing amount of MeOH in $CHCl_3$ (20:1, 10:1, 7:1, 5:1, 3:1, 1:1, and 100% MeOH, 500 mL each), generating nine fractions. Fraction II (20.9 g) was subjected to octadesyl silica gel (ODS) open column chromatography (CC) with a linear gradient of 10% aq. MeOH, from 20%MeOH to 100% MeOH, (1.2 L each), leading to nine fractions (f.1-f.9).

The fraction 1 (1.7 g) was purified by HPLC using 20% aq. acetone to give **2** (660 mg), **3** (340 mg), and **4** (80 mg). The fraction 3 (1.7 mg) was purified by HPLC using 70% aq. acetone to give **1** (357 mg) and **5** (700 mg). The fraction 4 (1.3 mg) was purified by HPLC using 50% aq. acetone to give **5** (560 mg), **6** (10.0 mg) and **10** (34.7 mg). The fraction 5 (0.7 mg) was purified by HPLC using 60% aq. acetone to give **6** (77.1 mg) and **11** (27.3 mg). The fraction 6 (0.5 mg) was purified by HPLC using 70% aq. acetone to give **9** (70 mg) and **25** (91.9 mg). The fraction 7 (1.0 mg) was purified by HPLC using 80% aq. acetone to give **7** (10.0 mg), **8** (45.9 mg), **12** (100 mg), and **24** (73 mg).

Fraction VI (1.7 g) was subjected to ODS open CC with a linear gradient of 10% aq. MeOH, from 20%MeOH to 100% MeOH, (300 ml each), leading to nine fractions (f.1-f.9). The fraction 1 (0.2 g) was purified by HPLC using 20% aq. MeOH to give **13** (3.6 mg). The chart of extraction and isolation of chemical constituents of *C. heyneana* extract as described in fig. 2.

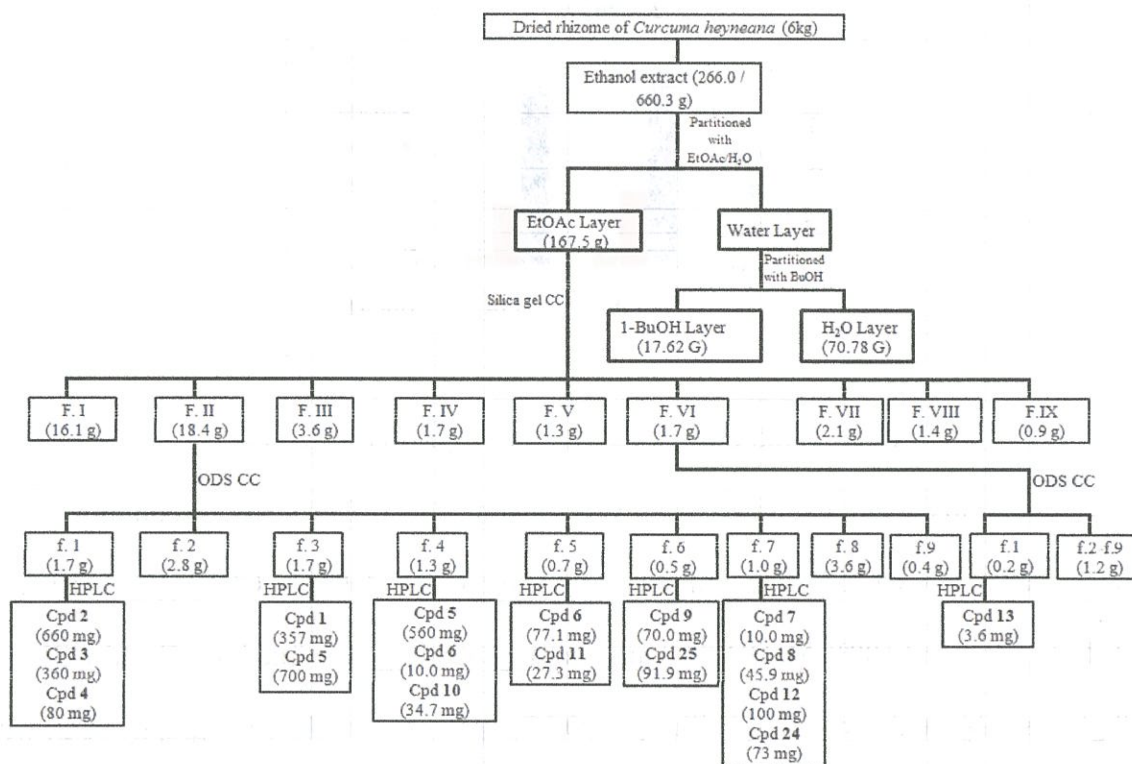

Figure 3. Extraction and isolation of *C. heyneana*

1.3 Structural Elucidation of Isolated Compounds

The ethyl acetate extract from ethanolic extract of *C. heyneana* rhizomes was fractionated by Silica gel and ODS open CC, then further purified by HPLC to provide 15 known compounds (fig. 4). The compounds were identified as procurcumenol (**1**), aerugidiol (**2**), zedoarondiol (**3**), isozedoarondiol (**4**), curcumenol (**5**), 4-epi-curcumenol (**6**), octadecatrienoic acid (**7**), eicosadienoic acid (**8**), curcumin (**9**), gingerol (**10**), curcuzedoalide (**11**), zerumin A (**12**), adenosine (**13**), 14,15,16-trinorlabda-diene-13-oic acid (**24**) and labdan-8 (17)-dien-14,15,16-triol (**25**).

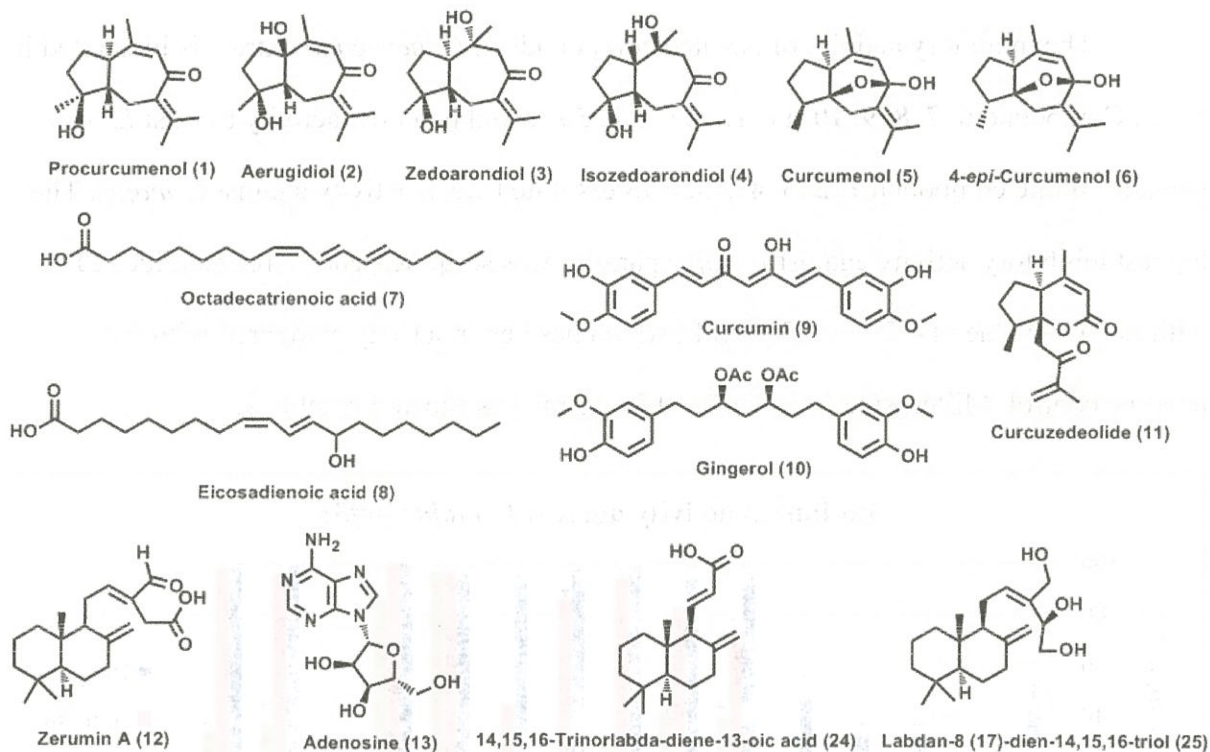

Figure 4. Isolated compounds of *C. heyneana*

1.4 Bioactivity examination of Isolated Compounds

Leishmaniasis is one of neglected-tropical diseases (NTD) categorized by WHO, which is instigated by the protozoan *Leishmania* parasite, through transmission by the bite of certain species of sandflies. As per October 2018, about 60% endemic countries have been reported by World Health Organization (WHO). The most common leishmaniasis is cutaneous leishmaniases (CL), and *Leishmania major* is one of species which caused that disease. It is estimated up to 1 million new cases and about 26.000 to 65.000 mortality cases arise, per annum. The available medicines to treat this disease are limited and have many side effect such as hepatotoxic, cardiotoxic, ototoxic and nephotoxic [6,7].

The inhibitory activity of isolated compounds of *C. heyneana* extract is illustrated in fig. 5. Compound **6, 7, 8, 9, 10, 11, 12, 24** and **25** showed potential activity against *L. major* parasite. While compound **1, 2, 3, 4, 5** and **13** exhibited weak activity against *L. major*. The highest inhibitory activity against *L major* parasite was sesquiterpene Curcuzeoalide (**11**) with the IC_{50} value of 4.53 ± 2.63 µg/mL, which has better activity compared with the positive control, Miltefosine (IC_{50} 7.47 ± 0.30 µg/mL) as showed in table 1.

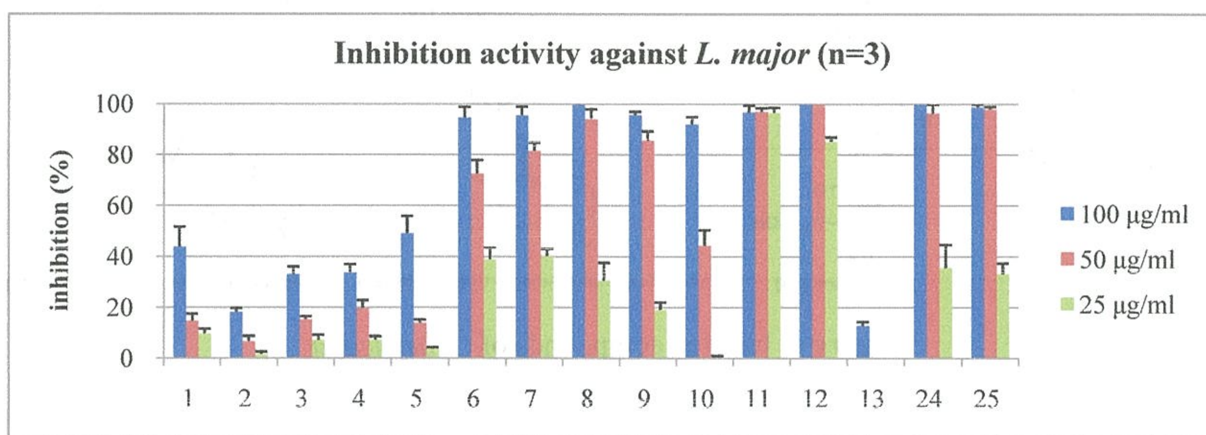

Figure 5. *L. major* inhibitory activity of isolated compound

In this study, we were also evaluating the cytotoxicity of isolated compounds against human lung cancer cell, A549. The growth inhibitory activity (IC_{50}) of isolated compounds of *C. heyneana* extract against as summarized in table 1.

Table 1. Anti-leishmania activity of isolated compounds from *C. heyneana* extract.

Isolated Compound	***L. major*** **IC_{50} (µg/ml)**	**A549 IC_{50} (µg/mL)**	**SI**
6	33.23 ± 4.11	>100	3.01
7	30.96 ± 3.46	15.70 ± 3.33	0.51
8	32.65± 1.45	62.96± 3.88	1.93
9	36.60 ± 1.35	15.33 ± 3.65	0.42
10	55.93 ± 2.85	22.45 ± 6.03	0.40
11	4.53 ± 2.63	27.95 ± 1.45	6.17
12	14.98 ± 1.88	32.22 ± 1.68	2.15
24	30.91 ± 1.90	42.14 ± 3.51	1.36
25	31.47 ± 1.23	16.01 ± 1.00	0.51
Etoposide	n.d	7.58±0.06	n.d
Miltefosine	7.47 ± 0.30	n.d	n.d

The selectivity index (SI) is a relative amount that assesses the chance between citotoxicity and anti leishmania activity. Supposedly, the higher SI ratio, the safer and more effective a medicine would be during medication for treating leishmaniasis. From table 1, the sesquiterpene Curcuzeoalide (**11**) has the highest SI value (6.17), means that this compound is potential to be developed furthermore as anti leishmania candidate.

1.5 Experimental Section

1.5.1. General Methods

Optical rotations were determined on a JASCO P-1030 digital polarimeter. NMR measurements were established on a Bruker Ultrashield 600 spectrometer. HR-ESI-MS data were performed with a LTQ Orbitrap XL mass spectrometer. Column chromatography (CC) was carried out on silica gel 60 (E. Merck, Darmstadt, Germany), and octadecyl silica (ODS) gel (Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan; Φ= 35 mm, L = 350 mm). HPLC was performed on ODS gel (Inertsil ODS-3, GL-science, Φ=6 mm, 250 mm) and the solvent eluation was observed by refractive index detector with a Jasco RI-930 intelligent detector and a Jasco PU-1580 intelligent pump. All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and TCI (Tokyo, Japan).

1.5.2. Isolated compounds

Procurcumenol (**1**)

Colorless oil, $[\alpha]_D^{24.4}$ +206.0° (c = 0.61, $CHCl_3$); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 1.22 (3H, s), 1.73 (3H, s), 1.78 (3H, s), 1.86 – 1.88 (1H, overlapped), 1.85-1.90 (2H, overlapped), 1.92 (3H, s), 1.98-2.03 (2H, m), 2.21-2.28 (2H, m), 2.44-2.48 (1H, m), 5.85 (1H, q, J= 1.3 Hz). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 21.3, 22.6, 23.6, 24.3, 28.0, 29.6, 40.5, 51.7, 55.2, 80.8, 129.5, 137.7, 137.7, 4, 159.2, 201.6. HRESIMS (positive ion) m/z: 257.1512 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_2Na$: 257.1512).The obtained data were similar with the references [8,9].

Aerugidiol (**2**)

Colorless oil, $[\alpha]_D^{24.4}$ -19.3° (c = 1.55, MeOH); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 5.89 (1H, d, J= 1.2 Hz), 4.58 (2H, s), 2.75 (1H, dd, J= 14.0, 2.5 Hz), 2.21 – 2.10 (2H, m), 2.02 (3H, s), 2.01 (3H, s), 1.94-2.00 (3H, m), 1.93 (3H, s), 1.72 (1H, dd, J= 10.5, 6.9 Hz), 1.22 (3H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 22.6, 23.3, 23.5, 23.8, 28.4, 38.3, 38.7, 63.4, 82.0, 84.9, 130.8, 133. 6, 145.9, 155.6, 197.5. HRESIMS (positive ion) m/z: 273.1465 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_3Na$: 273.1461). The obtained data were similar with the literature [10].

Zedoarondiol (**3**)

Colorless oil, $[\alpha]_D^{24.4}$ -39.4° (c = 7.86, MeOH); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 5.30 (2H, br.s), 2.99 (1H, d, J= 12.7 Hz), 2.86 (1H, d, J= 14.9 Hz), 2.51 (1H, d, J= 12.7 Hz), 2.05-1.95 (2H, m), 1.91 (3H, s), 1.86 (3H, s), 1.75-1.65 (4H, m), 1.40 – 1.33 (1H, t, J=12 Hz), 1.17 (3H, s), 1.11 (3H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 205.5, 143.2, 136.3, 80.5, 73.1, 60.8, 57.0, 52.8, 40.0, 29.4, 23.2, 22.9, 22.6, 22.4, 20.2.

HRESIMS (positive ion) *m/z*: 273.1465 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_3Na$: 273.1461). The obtained data are similar with the reference [11].

Isozedoarondiol (**4**)

Colorless oil, $[\alpha]_D^{24.4}$ -125° (*c* = 1.15, MeOH); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 4.57 (2H, br.s), 3.33 (1H, br. s), 2.82 – 2.76 (1H, m), 2.52 (1H, d, *J* = 13.9 Hz), 2.31 (1H, d, *J* = 16.1 Hz), 2.04 (1H d, s), 2.03 – 1.99 (1H, m), 1.97 (3H, s), 1.93 – 1.89 (1H, m), 1.88 (3H, s), 1.83 – 1.76 (1H, s), 1.72 (1H, ddd, *J* = 14.2, 9.7, 4.5 Hz), 1.62 (1H, ddd, *J* = 24.2, 11.8, 4.6 Hz), 1.39 (3H, s), 1.19 (3H, s). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 206.2, 144.6, 135.4, 83.12, 73.9, 53.9, 52.6, 51.2, 49.8, 37.4, 32.7, 28.4, 26.0, 24.8, 23.2, 22.4. HRESIMS (positive ion) *m/z*: 275.1620 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$: 275.1618). The obtained data are similar with the literature [11].

Curcumenol (**5**)

Colorless needles, $[\alpha]_D^{24.4}$ -367.1° (*c* = 0.7, MeOH); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 5.70 (1H, br.s), 2.69 – 2.62 (1H, m), 2.14 (1H, dd, *J* = 15.6, 1.1 Hz), 1.96 (3H, m), 1.85 (2H, s), 1.79 (3H, s), 1.64 (3H, s), 1.59 (3H, s), 1.53 – 1.46 (1H, m), 1.01 (3H, d, *J* = 6.6 Hz). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 139.4, 138.1, 127.5, 122.4, 102.6, 86.6, 52.4, 41.4, 38.2, 32.3, 28.7, 22.5, 21.1, 19.2, 12.3. HRESIMS (positive ion) *m/z*: 257.1510 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_2Na$: 257.1512). The obtained data are similar with the references [4, 12].

4-Epi-curcumenol (**6**)

yellowish amorphous solid, $[\alpha]_D^{17.9}$ +20.8° (*c* = 0.76, MeOH); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 5.90 – 5.58 (1H, m), 2.71 – 2.63 (1H, m), 2.23 (1H, d, *J* = 15.5 Hz, 1H), 2.17 – 2.12 (1H, m), 2.09 – 2.01 (3H, m), 1.78 (3H, s), 1.66 (3H, s), 1.60 (s, 3H), 1.50 (1H, m), 1.38 – 1.32 (m, 1H), 0.98 (3H, d, J = 7.3 Hz). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 139.0, 137.9, 127.8, 122.6, 102.3, 88.7, 50.0, 42.9, 38.6, 32.7, 29.5, 22.4, 21.2, 19.3, 17.2. HRESIMS (positive ion) *m/z*: 257.1509 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_2Na$: 257.1512). The obtained data are similar with the references [13].

9-11-13-Octadecatrienoic acid (*α-eleostearic acid*) (**7**)

white amorphous solid, ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 5.40 – 5.37 (1H, m), 5.36 (1H, dd, *J* = 8.4, 1.5 Hz), 5.35 (1H, *d*, J = 8.1 Hz), 5.34 (1H, d, *J* = 15.4 Hz), 5.32 (1H, d, *J* = 15.4 Hz), 5.31 – 5.28 (1H, m), 2.81 (3H, t, *J* = 5.7 Hz), 2.28 (3H, t, *J* = 7.4 Hz), 2.15 – 2.03 (2H, m), 1.60 (d, *J* = 1.4 Hz, 4H), 1.35 (dd, J = 49.1, 27.5 Hz, 10H), 0.97 (3H, t, *J* = 7.5 Hz). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 177.9, 132.7, 131.1, 129.2, 129.1, 128.8, 128.2, 35.0, 30.6, 30.2, 30.1, 30.0, 28.1, 26.5, 26.4, 26.0, 21.5, 14.6. HRESIMS (positive ion) *m/z*: 279.1588 $[M+H]^+$ (calcd for $C_{18}H_{31}O_2$: 279.2319). The obtained data are similar with the references [14,15].

11-hydroxy-12,14-Eicosadienoic acid (*coriolic acid*) (**8**)

white amorphous solid, ^{1}H-NMR (600 MHz, methanol-d_4, δ): 6.48 (1H, dd, J = 15.2, 11.1 Hz), 5.97 (1H, t, J = 11.0 Hz), 5.61 (1H, dd, J = 15.2, 6.8 Hz), 5.44 – 5.37 (1H, m), 4.08 (1H, dd, J = 13.1, 6.5 Hz), 3.94 – 3.77 (3H, m), 2.65 (1H, s), 2.27 (5H, t, J = 7.4 Hz), 2.18 (3H, dd, J = 14.2, 7.3 Hz), 1.35-1.25 (11H, m), 0.93 – 0.87 (3H, m). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 177.8, 137.2, 133.0, 129.3, 126.5, 73.3, 38.3, 34.9, 32.5, 30.4, 30.3, 30.2, 30.1, 28.5, 26.4, 26.0, 23.6, 14.4. HRESIMS (positive ion) *m/z*: 319.2237 [M+Na]$^+$ (calcd for $C_{18}H_{33}O_3Na$: 319.2244). The obtained data are similar with the references [14,16].

Curcumin (**9**)

yellow crystalline, ^{1}H-NMR (600 MHz, methanol-d_4, δ): 7.57 (1H, d, J = 15.9 Hz), 7.49 (1H, d, J = 15.7 Hz), 7.22 (1H, d, J = 15.2 Hz, 3H), 7.10 (1H, d, J = 15.5 Hz), 6.98 (1H, d, J = 16.2 Hz), 6.84 – 6.81 (4H, m), 6.77 (1H, br.s), 6.68 (2H, d, J = 8.2 Hz), 4.58 (2H, br. s), 3.91 (6H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 184.9, 184.7, 150.4, 149.4, 142.1, 141.9, 131.2, 130.4, 128.6, 128.0, 124.1, 122.3, 122.0, 116.9, 116.9, 116.6, 111.8, 109.6, 56.5, 56.5. HRESIMS (positive ion) *m/z*: 369.1318 [M+H]$^+$ (calcd for $C_{21}H_{21}O_6$: 369.1333). The obtained data are similar with the literature [17].

3,5-Diacetoxy-1,7-bis-(4'-hydroxy-3'-methoxyphenyl)-heptane (Gingerol) (**10**)

Colorless oil, ^{1}H-NMR (600 MHz, methanol-d_4, δ): 6.95 (2H, d, J = 8.4 Hz), 6.70 (2H, dd, J = 4.9, 2.0 Hz), 6.66 (1H, d, J = 8.0 Hz), 6.60 – 6.54 (2H, m), 6.45 (1H, dd, J = 8.0, 2.2 Hz), 6.28 (2H, s), 3.82 (6H, s), 2.58 – 2.39 (4H, m), 1.98 (1H, br.s), 1.97 (4H, q, J = 7.4 Hz), 1.95 (6H, s), 1.87 (1H, m). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 172.7, 172.7, 148.8, 146.1, 145.5, 144.3, 134.2, 134.1, 121.8, 120.7, 116.4, 116.2, 116.2, 113.2, 72.4, 72.4, 56.4, 56.4, 39.4, 37.0, 36.9, 31.8, 31.8, 21.1, 21.1. HRESIMS (positive ion) *m/z*: 461.2130 [M+H]$^+$ (calcd for $C_{25}H_{33}O_8$: 461.2170). The obtained data are similar with the references [18, 19].

Curcuzedoalide (**11**)

white amorphous solid, $[\alpha]_D^{18.6}$ +24.8° (c = 1.25, $CHCl_3$); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 6.10 (1H, s), 5.92 (1H, d, J = 1.4 Hz), 5.58 (1H, d, J = 1.3 Hz), 3.93 – 3.75 (2H, m), 3.34 (1H, d, J = 6.2 Hz), 2.88 (1H, dd, J = 11.2, 6.5 Hz), 2.84 (1H, d, J = 14.0 Hz), 2.30 – 2.20 (1H, m), 2.20 – 2.10 (1H, m), 1.91 (3H, d, J = 1.3 Hz), 1.78 (3H, s), 1.49 – 1.44 (1H, m), 1.09 (3H, d, J = 6.8 Hz). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 201.5, 166.5, 164.5, 146.4, 128.3, 122.3, 114.4, 92.6, 46.3, 46.2, 43.3, 32.2, 31.4, 22.7, 17.6, 12.2. HRESIMS (positive ion) *m/z*: 271.1322 [M+Na]$^+$ (calcd for $C_{15}H_{20}O_3Na$: 257.1305). The obtained data are similar with the references [20].

Zerumin A (**12**)

yellowish oil, $[\alpha]_D^{17.0}$ +6.8° (c = 1.7, ethanol); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 9.32 (1H, s), 6.74 (1H, t, J = 6.4 Hz), 4.86 (1H, s), 4.48 (1H, s), 2.60 (2H, ddd, J = 16.8, 5.9, 2.9 Hz,), 2.49 – 2.39 (1H, m), 2.00 (1H, d, J = 10.7 Hz), 1.90 – 1.70 (4H, m), 1.70 – 1.48 (4H, m), 1.48 – 1.10 (5H, m), 0.90 (3H, s), 0.85 (3H, s), 0.77 (3H, s). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 195.5, 173.8, 160.7, 149.4, 137.9, 108.6, 57.5, 56.5, 43.1, 40.5, 40.2, 38.9, 34.4, 34.1, 30.2, 25.6, 25.3, 22.3, 20.3, 14.9. HRESIMS (positive ion) m/z: 341.2079 [M+Na]$^+$ (calcd for $C_{20}H_{30}O_3Na$: 341.2087). The obtained data are similar with the references [21].

Adenosine (**13**)

White amorphous solid, $[\alpha]_D^{17.0}$ -17.1° (c = 0.24, methanol); ^{1}H-NMR (600 MHz, pyridine-d$_5$, δ): 8.63 (1H, s), 8.38 (1H, s), 6.74 (1H, d, J = 5.9 Hz), 5.51 (1H, t, J = 5.4 Hz,), 5.09 (1H, m), 4.78 (1H, m), 4.33 (1H, dd, J = 12.3, 2.6 Hz), 4.16 (1H, dt, J = 12.3, 2.5 Hz). ^{13}C-NMR (151 MHz, pyridine-d$_5$, δ): 158, 2, 153.8, 150.4, 121.9, 141.0, 91.3, 88, 3, 76.0, 72.9, 63.5. HRESIMS (positive ion) m/z: 268.1041 [M+H]$^+$ (calcd for $C_{10}H_{14}O_4N_5$: 268.1040). The obtained data are similar with the references [22].

14,15,16-Trinorlabda-8(17), 11-diene-13-oic acid (**24**)

Colorless oil, $[\alpha]_D^{18.3}$ +16° (c = 4.0, $CHCl_3$); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 7.02 (1H, dd, J = 15.5, 10.5 Hz), 5.79 (1H, d, J = 15.5 Hz), 4.78 (1H, br.s), 4.41 (1H, br.s), 2.49 (1H, d, J = 12.0 Hz), 2.20 – 1.98 (2H, m), 1.82 – 1.70 (1H, m), 1.70 – 1.55 (5H, m), 1.51 – 1.24 (3H, m), 0.91 (3H, s), 0.90 (3H, s), 0.87 (3H, s). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 169.5, 149.9, 149.3, 125.0, 109.1, 61.6, 55.6, 43.2, 41.8, 40.1, 37.6, 34.4, 34.0, 24.4, 22.4, 20.0, 15.5. HRESIMS (negative ion) m/z: 261.1863 [M-H]$^-$ (calcd for $C_{17}H_{25}O_2$: 261.1849). The obtained data are similar with the references [23].

Labdan-8 (17)-dien-14,15,16-triol (**25**)

White amorphous solid, $[\alpha]_D^{24.4}$ +5.3° (c = 1.16, $CHCl_3$); ^{1}H-NMR (600 MHz, methanol-d$_4$, δ): 5.50 (1H, t, J = 6.5 Hz), 4.69 (1H, dd, J = 7.5, 4.6 Hz), 4.53 (1H, d, J = 11.0 Hz), 4.16 – 4.10 (1H, m), 4.02 – 3.96 (1H, m), 3.68 – 3.62 (1H, m), 3.59 – 3.53 (1H, m), 2.24 – 1.96 (4H, m), 1.93 – 1.47 (5H, m), 1.48 – 1.28 (3H, m), 1.31 – 1.04 (3H, m), 0.89 (3H, s), 0.84 (3H, s), 0.75 (3H, s). ^{13}C-NMR (151 MHz, methanol-d$_4$, δ): 149.5, 138.4, 132.5, 108.3, 72.0, 66.2, 64.3, 58.7, 56. 8, 43.3, 40.7, 40.3, 39.2, 34.5, 34.1, 25.4, 23.2, 22.2, 20.4, 15.0. HRESIMS (positive ion) m/z: 345.2400 [M+Na]$^+$ (calcd for $C_{20}H_{34}O_3Na$: 345.2400). The obtained data are similar with the references [24].

1.5.3. Bioassay examination of isolated compounds

1.5.3.1. Antileishmania assay

The leishmanicidal activities of isolated compounds were conducted using MTT assay. In a 96-well micro titration plate, 1 μL of sample solutions in DMSO (1% as final concentration) and *L. major* cells (1×10^5 cells/well) in 99 μL medium were added to each well. Then being incubated for 72 h in a CO_2 incubator at 25 °C. M-199 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/ml of kanamycin was used as the cell culture medium. After that, a 100 μL of MTT solution was added to each well and continued the incubation for another 8 hours. The absorbance of the DMSO dissolved-formazan products was recorded using a microplate reader at λ540 nm. IC_{50} was observed as the concentration of the compounds to inhibit 50% *L. major* parasite. The equation (1) was used to estimate % inhibition of the samples: [25].

$$\text{Inhibition } (\%) = [1 \times \frac{(Asample - Ablank)}{(Acontrol - Ablank)} \times 100 \qquad (1)$$

1.5.3.2. A549 Growth Inhibition Assay

This experiment was performed using A549 cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Procedure of this assay was referred to the reference without any modification. Doxorubicin was used as a positive control. All the tested compounds were prepared in DMSO solution. The cell growth inhibition was calculated using the following equation 1. IC_{50} was determined as the concentration of sample to inhibit 50% of the growth of cancer cell. The smaller the IC_{50} value of the compound compared to the standard compound, the higher activity of the compound to inhibit A549 cancer cell [25].

CHAPTER 2.

CHEMICAL CONVERSION STUDY OF *ALPINIA GALANGA* AND *CURCUMA HEYNEANA*

2.1 Introduction

Natural products have been acknowledged as a main resource in drug development due to their structural assortment and complexity. It's also frequently offering greatly definite biological activity. It has been informed that the employment of natural products and/or their novel structures, with the purpose of finding and extends the drug entity, is yet prosperous and satisfactory. For instance, from the 1940s up to 2014, of the 175 small molecules approved as anticancer, and about 49% is natural products or directly resulting thence. [26,27]. Therefore, natural products and its derivatives are even now beneficial for discovery of drug-lead compounds.

In the last decade or so, it has been introduced an approach to produce bioactive compounds via chemical modification of crude natural extracts. This approach is an obvious method for altering the chemical composition of crude herbal extracts, which caused changes in their biological activity in a negative or positive manner. Some methods such as sulfonylation with *p*-toluene sulfonyl chloride [28,29], ammonolysis with hydrazine monohydrate [29,30,31], bromination with bromine [29,32], oxidation with Dess-Martin periodinane (DMP), reduction with sodium borohydride, epoxidation with *m*-chloroperbenzoic acid (*m*CPBA) [33] and methyl(trifluoromethyl)dioxirane then followed by transannulation with a Lewis acid catalyst [34], ethanolysis with hydroxylamine hydrochloride [35] to natural extracts have been carried out to produce novel or bioactive compounds.

Alpinia galanga (L.) belongs to family Zingiberaceae, which extensively distributed in China, India and Southeast Asian countries include Indonesia. *A. galanga* is an is a tropical plant with an aromatic perennial growing up to 7 m tall. The leaves are lanceolate while the flowers are white with pink markings. The rhizome has an aromatic odor while the taste is pungent and spicy. The parts of this plant include rhizome, fruit and flowers have been used as a spice, flavoring agent, food additives and gastroprotective. The picture of *A. galanga* as portrayed in fig. 6. Traditionally it is being applied for treating dyspepsia and abdominal colic pain, motion sickness, and gastralgia [36,37]. Up to now, there are numerous articles about pharmacological activities of *A. galanga*, for instance antialergic [38], anticancer [39-41], anti-inflammatory [42], antimicrobial [43-45], antioxidant [45,46], antiamnesic [47], antiasthma [48], and also melanogenesis inhibitor [49]. Nevertheless, there is very rare report about its activity as anti-leishmanial agent. Thanh Binh Le, *et al.* screened 37 Vietnamese plants, including *A. galanga,* against *Leishmania Mexicana Mexicana (Lmm)*. They reported that *A. galanga* didn't' posses ability in inhibiting *Lmm* [50].

In this report, a chemical alteration method was conducted concurrently with bioactivity-guided isolation of an inactive ethyl acetate extract from *A. galanga* and 1-butanol extract from *C. heyneana* to generate some new phenylpropanoid analogues (**14-17**) and a new sesquiterpene (**30**), along with other known compounds (**18-23**, **26-29**). Furthermore, the isolated compounds were also being examined against parasite *L. major*.

Figure 6. *Alpinia galanga (*L*)*

(https://www.naturalmedicinefacts.info/plant/alpinia-galanga.html)

2.2 Extraction and Isolation of Chemically Converted Extract of *A. galanga*

The dried rhizomes of *A. galanga* (2.0 kg) were extracted with MeOH (3x 1.5 L) at room temperature to give MeOH extract. Afterward, this extract was partitioned by solvent fractionation with EtOAc/H_2O to give EtOAc extract. The ethyl acetate extract was used for being treated with dioxirane to produce the chemically converted extract (CCE). Dioxirane is powerful reagent for epoxidation, which generated *in situ*, by reacting acetone with potassium peroxomonosulfate [51]. After being quenched and evaporated, HPLC analyses of the CCE and original extract were carried out, and followed by biological assay. The antileishmania activity of both extracts (original extract and CCE) against promastigotes *of L. major* was conducted by the colorimetric method 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [52]. Figure 7 exhibited that the inhibitory activity of CCE is significantly increased after treating of dioxirane.

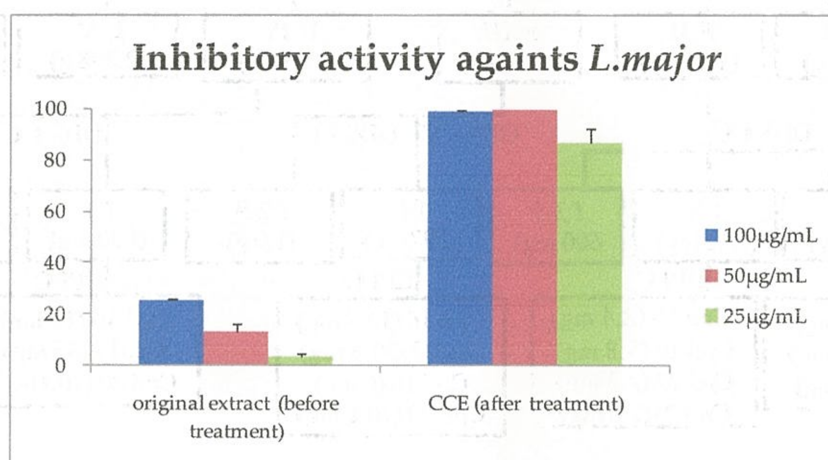

Figure 7. Inhibitory activity of *A. galanga* extracts.

The chart of extraction and isolation of chemical constituents from CCE of *A. galanga* as described in fig. 8.

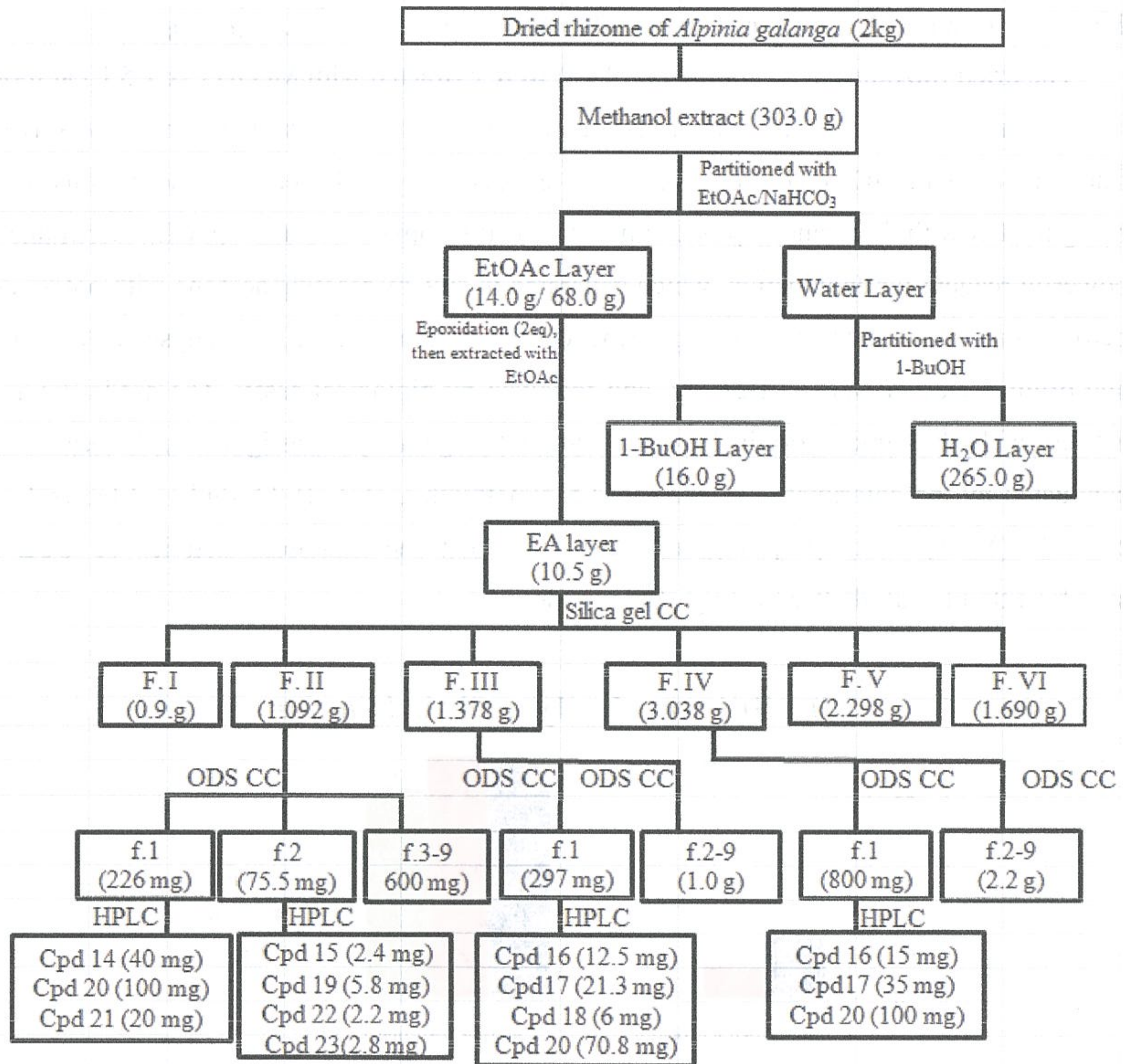

Figure 8. Extraction and isolation of CCE from *A.galanga*

2.3 Structural Elucidation of Isolated Compounds

By using bio-guided isolation procedure, the CCE of *A. galanga* was further separated by assorted column chromatographies and HPLC to provide compounds **A–F** **(14-19)**. In addition, by referring the NMR data from literatures, the structures of the known compounds were named as hydroquinone (**20**) [53], 4-hydroxy(4-hydroxyphenyl)methoxy)-benzaldehyde (**21**) [54], isocoumarin cis 4-hydroxymelein (**22**) [55] and (*2S, 3S, 6R, 7R, 9S, 10S)*-humulene triepoxide (**23**) [56].

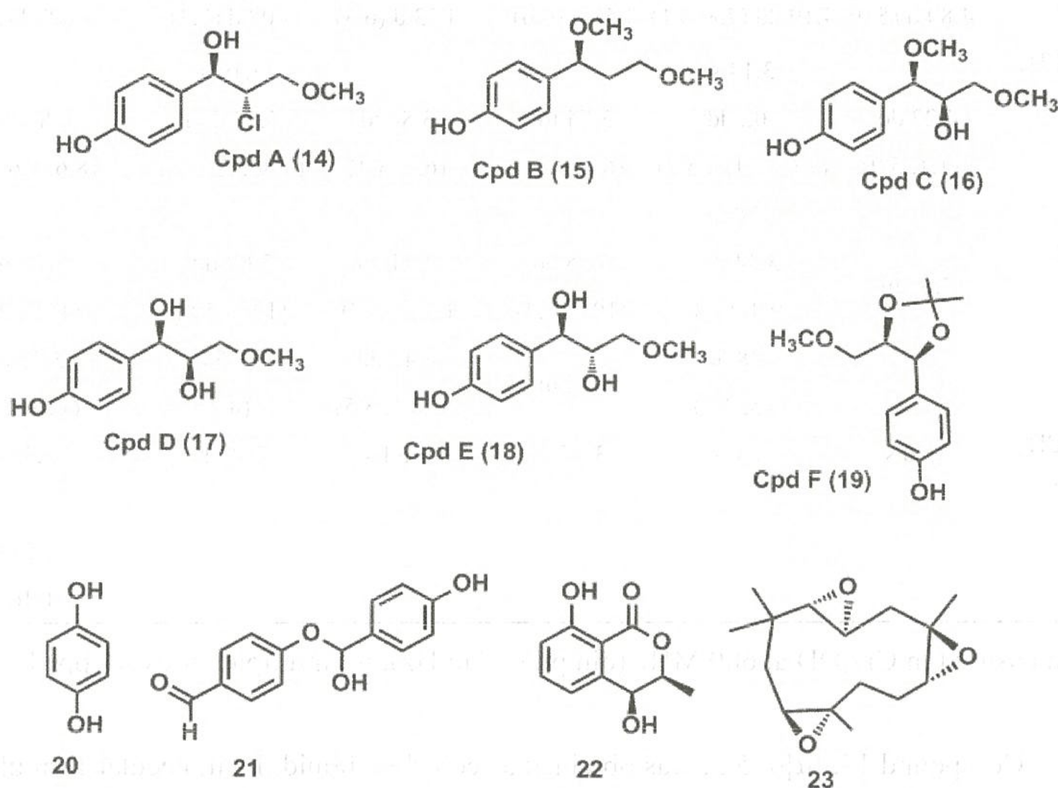

Figure 9. Isolated compounds of *A. galanga*

Compound **14** was attained as colorless liquid. Its molecular formula was revealed to be $C_{10}H_{13}O_3ClNa$ from an ion at *m/z* 239.0446, with characteristic pattern of halide ion (Cl-) fragmentation at [M+2] in the HRESIMS. The IR data exhibited bands for hydroxyl group (3350 cm-1) and C-Cl stretching bond (850 cm-1) absorption The ^{1}H NMR data (table 2) showed the presence of one methoxy group at 3.36 ppm, and *para*-substituted aromatic rings at 7.21 and 6.76 ppm as doublet *(J* = 8.6Hz) each in **14**. The ^{1}H and ^{13}C-NMR data were

nearly similar as those of (*anti*)-2-chloro-3-phenyl-1,3-propandiol. On the basis of the coupling constant of H-7 and H-8 (J=5.6Hz), **14** was assigned as *threo-* compound too, in accordance with a reference [57].

Table 2. ^{1}H-NMR spectroscopic data for compounds **14-19**[1]

Position	14	15	17	18	16	19
1						
2,6	7.21 d (8.6)	7.10 d (8.5)	7.19 d (8.4)	7.21 d (8.6)	7.13 d (8.5)	7.21 d (8.6)
3,5	6.76 d (8.6)	6.77 d (8.5)	6.76 d (8.6)	6.76 d (8.6)	6.78 d (8.5)	6.79 d (8.6)
4						
7	4.80 d (5.9)	4.19 dd (7.8, 6.1)	4.51 d (6.6)	4.53 d (6.3)	4.08 d (7.3)	4.69 d (8.8)
7-OCH$_3$		3.14 s			3.19 s	
8	4.22 dd (11.1, 5.9)	2.02 ddt (16.5, 11.9, 5.1)	3.74 td (6.6, 3.4)	3.84 td (6.5, 3.4)	3.74 ddd (7.4, 5.8, 3.2)	3.89 ddd (8.6, 5.6, 2.8)
		1.78 m				
9	3.61 m	3.44 dt (9.4, 6.5)	3.15 dd (10.1, 6.2)	3.44 dd (10.1, 6.7)	3.08 dd (10.1, 5.8)	3.52 dd (11.0, 2.8)
		3.28 dd (6.5, 2.3)	3.32 m	3.49 dd (10.1, 3.5)	3.26 t (8.4)	3.47 dd (11.0, 5.6)
9-OCH$_3$	3.36 s	3.30 s	3.27 s	3.34 s	3.24 s	3.35 s
1'						
2'						1.51 s
3'						1.46 s

[1] measured in CD_3OD at 600 MHz (δin ppm, J in Hz); m=multiplet or overlapped

Compound **15,** $[\alpha]_D$ -5.3, was obtained as colorless liquid. Its molecular formula was showed to be $C_{11}H_{16}O_3Na$ from an ion at *m/z* 219.0989 in the HRESIMS. The IR data showed bands for hydroxyl group (3377 cm-1) and the presence of aromatic ring (1603 and 1507 cm-1) absorption. The ^{1}H NMR data (table 2) showed the presence of two methoxy group at 3.14 and 3.30 ppm, and *para*-substituted aromatic rings at 7.10 and 6.77 ppm as doublet (J = 8.5 Hz) each in **5**. According to (heteronuclear multiple bond conectivity) HMBC correlation (Fig.2) and also similarity NMR data especially on position H-7 at 4.19 ppm, **15** was regarded as 4-(1,3-dimethoxypropyl)phenol [58]. Zhang, *et al.* revealed that the related

compounds (**3a** and **3b**) as *R*-type had $[\alpha]_D$ positive rotations [59]. While Hodgson, D.M and Man. S., reported that comparable compound as *S*-type had specific rotation as negative value [60]. These findings added confidence to conclude the structure of **15** as ***S***-type compound.

Table 3. ^{13}C-NMR spectroscopic data for compounds **14-19**[1]

Position	14	15	17	18	16	19
1	132.8	133.5	133.8	133.9	130.6	129.5
2,6	129.4	129.0	129.1	129.4	129.8	129.2
3,5	115.7	116.2	115.9	115.8	116.8	116.3
4	158.2	158.2	158.0	157.9	158.5	158.8
7	75.1	81.7	75.5	75.6	85.7	80.8
7-OCH_3		56.4			56.8	
8	64.9	38.9	76.0	75.0	75.5	83.7
9	74.6	70.4	74.9	75.1	74.6	72.6
9-OCH_3	59.1	58.8	59.2	59.3	59.2	59.6
1'						110.1
2'						27.4
3'						27.3

[1] measured in CD_3OD at150 MHz (δin ppm)

Compound 17 was isolated as colorless liquid with molecular formula $C_{10}H_{14}O_4Na$ as measured by HRESIMS at *m/z* 221.0785. The IR data indicated bands for hydroxyl group (3420 cm-1) and the presence of aromatic ring (1514 and 1455 cm-1) absorption. The ^{1}H NMR data (table 2) displayed signals caused by the presence of one methoxy group at 3.27 ppm, and an AA'BB' type coupling system at δ_H 6.76 ppm (2H, d, *J*= 8.6 Hz) and 7.19 (2H, d, *J*= 8.4 Hz). The C-NMR data (table 3) exhibited 10 carbon peaks that were classified by chemical shift values and heteronuclear single quantum coherence (HSQC) spectrum (Fig.2) as one methoxy carbon group (δ_C 59.2), two *sp2* methine carbons (δ_H 115.9 (2xC), 129.1(2xC)), five *sp2*quartery carbons (δ_C 74.9, 76.0, 75.5, 133.8, 158.0). Mc.Elhanon, J.R., *et al.* wrote asymmetric dihydroxylation of cinnamates yielded (*R*, *R*)-diols-**24** which had $[\alpha]_D$ -2.70 [61]. From ^{1}H-NMR spectrum, diol-**30**, **34**, **35** of reference data mentioned the

coupling constants between H-7 and H-8 are approximately 4.5 Hz for the *erythro* isomer while 7.2 and 7.7 Hz for the *threo* isomer. Based on the coupling constants of H-7 and H-8 (*J*= 6.6 Hz), compound 17 assigned to be the *threo* isomer [62].

Compound **18** was obtained as colorless liquid with molecular formula $C_{10}H_{14}O_4Na$ as measured by HRESIMS at *m/z* 221.0785. The IR data indicated bands for hydroxyl group (3416 cm-1) and the presence of aromatic ring (1514 and 1455 cm-1) absorption. Almost similar white those in diol-**17**, the ^{1}H NMR data (table 2) showed peaks caused by one methoxy group at 3.34 ppm, and an AA'BB' type coupling system at δ_H 6.76 ppm (2H, d, *J* = 8.6 Hz) and 7.21(2H, d, *J*= 8.6 Hz). The C-NMR data (table 3) exhibited 10 carbon peaks that were classified by chemical shift values and heteronuclear single quantum coherence (HSQC) spectrum (Fig.2) as one methoxy carbon group (δ_C 59.3), two *sp2* methine carbons (δ_H 115.8 (2xC), 129.4(2xC)), five *sp2*quartery carbons (δ_C 75.0, 75.1, 75.6, 133.9, 157.9). As mentioned above, from ^{1}H-NMR spectrum, diol-**30**, **34**, **35** of reference data described the coupling constants between H-7 and H-8 of the *erythro* isomer are smaller than *threo* isomer (*J* less than 7.2 Hz) and the coupling constants data of H-7 and H-8 (*J*= 6.3 Hz), therefore compound **18** supposed to be reported as *erythro* isomer [62].

Compound **16,** $[\alpha]_D$ -11.6, was yielded as colorless liquid with molecular formula $C_{11}H_{16}O_4Na$ as determined by HRESIMS at *m/z* 235.0941. The IR data indicated bands for hydroxyl group (3407 cm-1) and the presence of aromatic ring (1510 and 1455 cm-1) absorption. From the ^{1}H NMR data (table 2) showed peaks caused by two methoxy group at 3.19 and 3.24 ppm, and an AA'BB' type coupling system at δ_H 6.78 ppm (2H, d, *J*= 8.5 Hz) and 7.13(2H, d, *J*= 8.6 Hz). The ^{13}C-NMR data (table 3) exhibited 10 carbon peaks that were classified by chemical shift values and heteronuclear single quantum coherence (HSQC) spectrum (Fig.2) as two methoxy carbons group (δ_C 56.8, 59.2), two *sp2* methine carbons (δ_H 116.8 (2xC), 129.8(2xC)), five *sp2*quartery carbons (δ_C 74.6, 75.5, 85.7, 130.6, 158.5). It has been reported that in the case of glycerol derivatives, the coupling constants between H-7 and

H-8 are approximately 5 Hz for the *erythro* isomer and 7 Hz for the *threo* isomer. Phenyl propanoid **16**, $[\alpha]_D$ -8.2, has been assigned as *threo* isomer, based on the coupling constants of H-7 and H-8 are 6.6 Hz [63]. Another reference mentioned that *erythro* isomer had been assigned because of the coupling constants of H-7 and H-8 are 6.3 Hz, which smaller that its *threo* isomer (J = 7.2 Hz) [64]. According to those reports, compound **16** which has the coupling constants of H-7 and H-8 (J=7.3 Hz), can be concluded as *threo* isomer.

Compound **19,** $[\alpha]_D$ -2.4, was produced as colorless liquid with molecular formula $C_{13}H_{18}O_4Na$ as analyzed by HRESIMS at *m/z* 261.1099. The IR data indicated bands for hydroxyl group (3368 cm-1) and the presence of aromatic ring (1512 cm-1) absorption. From the ^{1}H NMR data (table 2) showed peaks caused by one methoxy group at 3.35ppm, two methyl group at 1.46 and 1.51 ppm, and an AA'BB' type coupling system at δ_H 6.79 ppm (2H, d, J= 8.6 Hz) and 7.21 (2H, d, J= 8.6 Hz). The ^{13}C-NMR data (table 3) exhibited 13 carbon peaks that were categorized by chemical shift values and heteronuclear single quantum coherence (HSQC) spectrum (Fig.2) as two methyl carbons group (δ_C 27.3, 27.4), one methoxy carbons group (δ_C 59.6), two *sp2* methine carbons (δ_H 116.3 (2xC), 129.2(2xC)), five *sp*^{2} quartery carbons (δ_C 72.6, 80.8, 83.7, 129.5, 158.8). It has been reported that acetonide protection of the 1,2-diol moiety, reduction of the ester group and hydrogenolysis of the benzyl ether protecting group of **24** to provide acetonide-**4** (***R***, ***R***), $[\alpha]_D$ +18.0, a *threo* isomer, has coupling constants of H-7 and H-8 are 8.1 Hz [61]**.** The other reference also described that acetonide-**10** **(*R*, *R*),** $[\alpha]_D$ +1.0, a *threo* isomer, has coupling constants of H-7 and H-8 are 8.4 Hz [63]**.** Morikawa, T., *et al.* explained acetonide-**2a** and acetonide-**3a** has similar NMR data, except for the coupling constants of H-7 and H-8 of acetonide-**2a** **(**J=2.8 Hz) as *erythro*-form, while acetonide-**3a** **(**J=10.1 Hz) as *threo*-form [65]. Therefore, based on those literatures and NMR data, the coupling constants of H-7 and H-8 (J=8.0 Hz), phenyl propanoid **19**, can be assigned as *threo* isomer. Since the specific rotation of **19** exhibited negative value, compound 19 must be (*S*,*S*)-isomer.

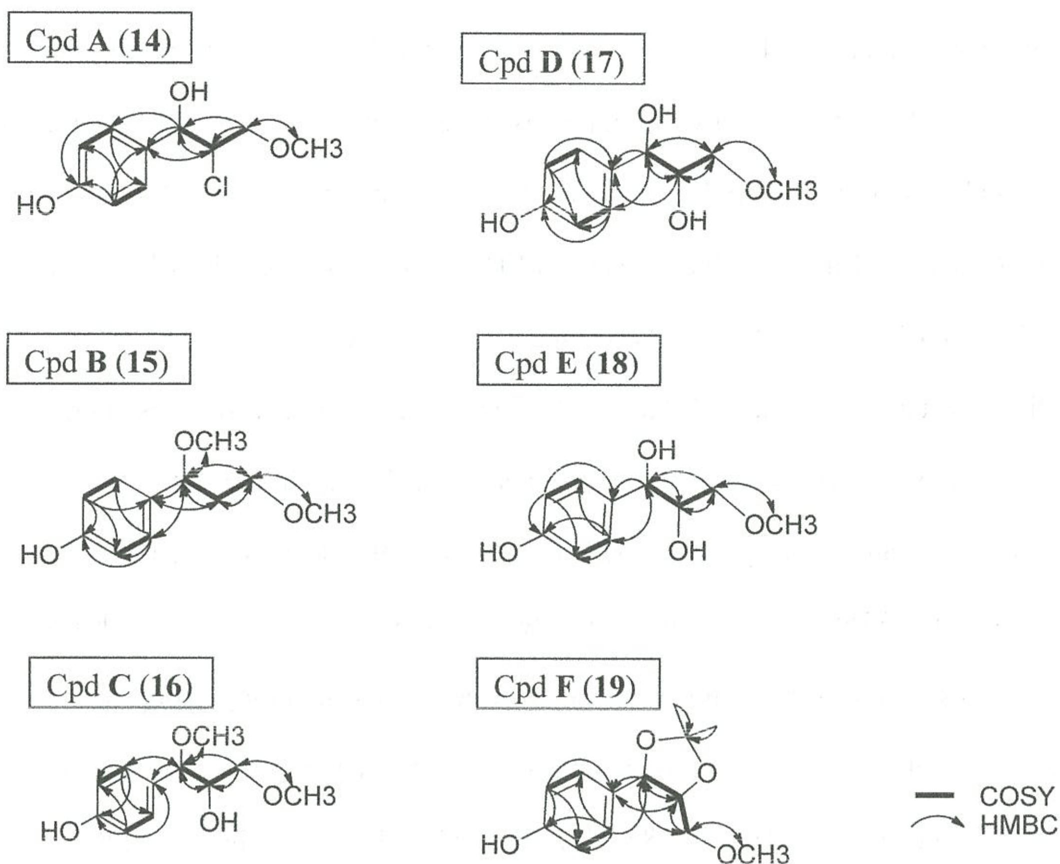

Figure 10. H-H COSY and H-C HMBC correlation of compound **14-19**.

2.4. Anti-parasite activity examination of Compounds 14-23.

All isolated compounds were being investigated their activity toward promastigotes of *L. major*. Table 4 showed that **20** as the most potent against parasite of *L. major*. Compound **15**, **21** and **22** also possessed substantial cytotoxicities against *L. major*.

Table 4. Inhibition Rate of compounds **14-23** against *L .major*

Compound	IC_{50} (μg/mL) [1]
Cpd 14	>100
Cpd 15	27.83 ± 0.34
Cpd 16	70.33 ± 0.10
Cpd 17	>100
Cpd 18	>100
Cpd 19	87.60 ± 1.50
Cpd 20	0. 37 ± 1.37
Cpd 21	51.10 ± 1.19
Cpd 22	40.55 ± 0.95
Cpd 23	>100
Miltefosine	7.47 ± 0.30

[1] each value represents mean ±SE (three independent experiments).

2.5. Experimental Section

2.5.1. General Methods

Optical rotations were determined on a JASCO P-1030 digital polarimeter. IR spectra were measure on a Horiba FT-710 Fourier transform infrared spectrophotometer (Horiba, Kyoto, Japan), while UV spectra were taken on a Jasco V-520 UV/Vis spectrophotometer. NMR measurements were established on a Bruker Ultrashield 600 spectrometer with tetramethylsilane (TMS) as an internal standard. Positive ion HR-ESI-MS data were performed with a LTQ Orbitrap XL mass spectrometer. Column chromatography (CC) was carried out on silica gel 60 (E. Merck, Darmstadt, Germany), and octadecyl silica (ODS) gel (Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan; Φ= 35 mm, L = 350 mm). HPLC was performed on ODS gel (Inertsil ODS-3, GL-science, Φ=6 mm, 250 mm) and the solvent eluation was observed by refractive index detector. with a Jasco RI-930 intelligent detector and a Jasco PU-1580 intelligent pump. All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and TCI (Tokyo, Japan).

2.5.2 Preparation of Chemically Converted Extract of *A. galanga* and Isolation of Compounds 14-23

The Rhizome of *A. galanga* (2.0 Kg) was extracted with MeOH (10 L) three times and then evaporated to give a methanolic extract (303.0 g). This residue was suspended in 1 L of saturated sodium carbonate solution and partitioned with ethyl acetate (1 L) three times producing ethyl acetate extract (68.0 g).

To a solution of EtOAc extract (14 g) in acetonitrile (360 mL) and water (360 mL) was added acetone 10.35 mL, sodium bicarbonate (19.6 g) and potassium peroxosulphate (28.2 g) sequentially at 0°C and being stirred for 3 hours. After 3 hours, add potassium peroxosulphate (28.2 g) to the solution at 0°C and being stirred for another 3 hours. The mixture continued to be stirred up to 24 hours. The following step was pouring into water and being extracted with ethyl acetate (three times). The combined organic layer was washed with water, brine and dried over sodium sulfate and evaporated to yield CCE of *A.galanga* (10.5 g).

The CCE was separated on a silica gel (50 g) CC and eluting with increasing amount of MeOH in $CHCl_3$ (20:1, 10:1, 7:1, 5:1, 3:1, 1:1, and 100% MeOH, 500 mL each), generating 9 fractions. Fraction CCE2 (1.1 g), CCE3 (1.4 g) and CCE4 (4.0 g) were subjected to ODS CC with a linear gradient of 10% aq. MeOH, from 30%MeOH to 100% MeOH, (400 mL each), leading to eight fractions. The residue of fraction CCE 2.1 (226 mg) was purified by HPLC using 25% aq. acetone to give **14** (40 mg), hydroquinone (**20**, 100.0 mg), and

4-hydroxy(4-hydroxyphenyl)methoxy)benzaldehyde **21** (20.0 mg). The residue of fraction CCE 2.2 (79.5 mg) was purified by HPLC using 30% aq. MeOH to give Isocoumarin cis 4-hydroxymelein (**22,** 2.2 mg), **15** (2.4 mg), **19** (5.8 mg), and *(2S, 3S, 6R, 7R, 9S, 10S)*-humulene triepoxide (**23,** 2.8 mg). The residue of fraction CCE 3.1 (297.5 mg) was purified by HPLC using 20% aq. acetone to give **20** (70.1 mg), 17 (20.1 mg), **18** (6.0 mg), and **16** (12.5 mg).

The chemical structures of the **14-19** and other know compounds (**20-23**) were analyzed and described through spectroscopic measurement based on IR, UV-visible, HR-ESI-MS data and 1D and 2D NMR, as follow.

(erythro)-2-chloro-1-phenylpropane-1,3-diol (**14**)

Colorless oil, $[\alpha]_D^{27}$ -7.5 (*c* 1.20, MeOH); IR υ_{max} (film) cm^{-1}: 3350, 2935, 1668, 1607, 1454, 1377, 1236, 1118, 1038, 850; UV λ_{max} (MeOH) nm (log ε): 276 (3.58); ^1H NMR (CD_3OD, 600 MHz) data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 239.0446 $[M+Na]^+$ (calcd for $C_{10}H_{13}O_3ClNa$: 239.0445).

(S)-4-(1,3-dimethoxypropyl)phenol (**15**)

Colorless oil, $[\alpha]_D^{27}$ -5.3 (*c* 0.13, MeOH); IR υ_{max} (film) cm^{-1}: 3377, 2927, 2857, 1701, 1603, 1507, 1442, 1233, 1104; UV λ_{max} (MeOH) nm (log ε): 259 (2.57); ^1H NMR (CD_3OD, 600 MHz) data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 219.0989 $[M+Na]^+$ (calcd for $C_{11}H_{16}O_3Na$: 219.0992).

(threo)-1-(4-hydroxyphenyl)-3-methoxypropane-1,2-diol (**17**)

Colorless oil, $[\alpha]_D^{27}$ -4.8 (*c* 1.34, MeOH); IR υ_{max} (film) cm^{-1}: 3420, 2923, 2821, 1677, 1606, 1514, 1455, 1240, 1119; UV λ_{max} (MeOH) nm (log ε): 276 (2.96); ^1H NMR (CD_3OD, 600 MHz) data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 221.0785 $[M+Na]^+$ (calcd for $C_{10}H_{14}O_4Na$: 221.0784).

(erythro)-1-(4-hydroxyphenyl)-3-methoxypropane-1,2-diol (**18**)

Colorless oil, $[\alpha]_D^{27}$ +6.7 (*c* 0.90, MeOH); IR υ_{max} (film) cm^{-1}: 3416, 2927, 2360, 1679, 1616, 1514, 1455, 1240; UV λ_{max} (MeOH) nm (log ε): 276 (2.33); ^1H NMR (CD_3OD, 600 MHz)

data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 221.0785 $[M+Na]^+$ (calcd for $C_{10}H_{14}O_4Na$: 221.0784).

(threo)- 4-(2-hydroxy-1,3-dimethoxypropyl)phenol (**16**)

Colorless oil, $[\alpha]_D^{27}$ -11.6 (*c* 0.86, MeOH); IR υ_{max} (film) cm^{-1}: 3407, 2929, 2360, 1603, 1500, 1455, 1375; UV λ_{max} (MeOH) nm (log ε): 279 (3.26); ^{1}H NMR (CD_3OD, 600 MHz) data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 235.0941 $[M+Na]^+$ (calcd for $C_{11}H_{16}O_4Na$: 235.0941).

4-((4*S*,5*S*)-5-(methoxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)phenol

(threo)- 4-(5-(methoxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)phenol (**19**)

Colorless oil, $[\alpha]_D^{27}$ -2.4 (*c* 0.38, MeOH); IR υ_{max} (film) cm^{-1}: 3368, 2986, 1732, 1607, 1512, 1375, 1230, 1165, 1078; UV λ_{max} (MeOH) nm (log ε): 276 (2.95); ^{1}H NMR (CD_3OD, 600 MHz) data, see Table 1 and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 2. HRESIMS (positive ion) *m/z*: 261.1099 $[M+Na]^+$ (calcd for $C_{11}H_{16}O_4Na$: 261.1097).

Hydroquinone (**20**)

White amorphous solid, ^{1}H-NMR (600 MHz, methanol-d_4, δ): 6.63 (4H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 151.1, 151.1, 116.8, 116.8, 116.8, 116.8. The obtained data are similar with the references [53].

4-hydroxy(4-hydroxyphenyl)methoxy)benzaldehyde (**21**)

Colorless oil, $[\alpha]_D^{27}$ -1.65 (*c* 1.33, MeOH); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 9.76 (1H, s), 7.78 (2H, d, *J* = 8.7 Hz), 7.73 (2H, m), 6.92 (2H, d, *J*= 8.6 Hz), 6.77 (2H, d, *J*= 8.6 Hz), 5.27 (1H, s).^{3}C-NMR (151 MHz, methanol-d_4, δ): 192.9, 166.2, 158.8, 133.4, 116.9, 130.3, 104.8, 130.5, 129.0, 115.8. HRESIMS (negative ion) *m/z*: 243.0661 $[M-H]^-$ (calcd for $C_{14}H_{11}O_4Na$: 243.0652). The obtained data are similar with the references [54].

Isocoumarin cis 4-hydroxymelein (**22**)

Colorless oil, $[\alpha]_D^{27}$ -6° (*c* = 0.15, methanol); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 7.57 (1H, dd, *J* = 8.4, 7.5 Hz), 7.08 (1H, d, *J* = 7.5 Hz), 6.94 (1H, dd, *J* = 8.4, 0.7 Hz), 4.56 (1H, m),

4.55 (1H, m), 1.47 (3H, m). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 170.2, 162.9, 144.1, 127.8, 117.8, 117.7, 108. 0, 81.64, 69.5, 18.2. HRESIMS (negative ion) *m/z*: 193.0507 $[M-H]^-$ (calcd for $C_{10}H_9O_4$: 193.0495). The obtained data are similar with the references [55].

humulene triepoxide (**23**)
White amorphous solid, $[\alpha]_D^{27}$ +0.5° (*c* = 0.19, methanol); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 2.89 (1H, dd, *J* = 9.8, 5.1 Hz), 1.40 (3H, s), 2.66 (1H, dd, *J* = 12.9, 2.8 Hz), 0.67 (1H, m), 2.52 (1H, d, *J* = 2.3 Hz), 0.87 (3H, s), 1.08 (3H, s), 1.76 (1H, dd, *J* = 15.2, 7.6 Hz), 1.61 (1H, d, *J* = 15.2 Hz), 2.76 (1H, d, *J* = 7.5 Hz), 1.38 (3H, s). 2.12 (1H, dd, *J* = 13.4, 5.5 Hz), 1.18 (1H, dd, *J* = 13.5, 5.7 Hz), 2.25 (1H, m), 1.15 (1H, dd, *J* = 13.5, 5.6 Hz). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 60.9, 59.5, 17.2, 44.7, 54.1, 66.7, 34.2, 18.3, 29.0, 38.8, 62.9, 61.7, 16.7, 36.0, 26.5. HRESIMS (positive ion) *m/z*: 252. 0890 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$: 252.1726). The obtained data are similar with the references [56].

2.5.3. Anti-leishmania assay of Isolated Compounds

The leishmanicidal activities of isolated compounds were conducted using MTT assay. In a 96-well micro titration plate, 1 μL of sample solutions in DMSO and *L. major* cells (2×10^5 cells/well) in 99 μL medium were added to each well. Then being incubated for 72 h in a CO_2 incubator at 25 °C. M-199 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/ml of kanamycin was used as the cell culture medium. After that, a 100 μL of MTT solution was added to each well and continued the incubation for another 8 hours. The absorbance of the DMSO dissolved-formazan products was recorded using a microplate reader at λ540 nm. The equation (1) was used to estimate % inhibition of the samples:

2.6 Extraction and Isolation of Chemically converted extract of *C. heyneana*

The 1-butanol extract of *C. heyneana* was dissolved in DMSO (10mg/ml) then being heated in oil bath at 160 °C for several hours. Later on, this extract was partitioned by EtOAc/H_2O to give the CCE of *C.heyneana*. The antileishmania activity of original extract and CCE against *L. major* parasite was performed by MTT assay. **Fig. 11** exhibited that in the 1-butanol extract there was some major peaks that vanished in the CCE, while in the CCE there was new peaks that appeared. It also showed that the inhibitory activity of CCE is majorly increased if compared with its original extract. The following step was bioactive compounds isolation using chromatographic techniques, including SiO_2, ODS CC, and also HPLC The elucidation structure of the isolated compounds was carried out by spectroscopy methods, including NMR, MS, UV, and IR spectroscopy. The chart of extraction and isolation of chemical constituents of *C.heyneana* extract as described in **fig. 12**:

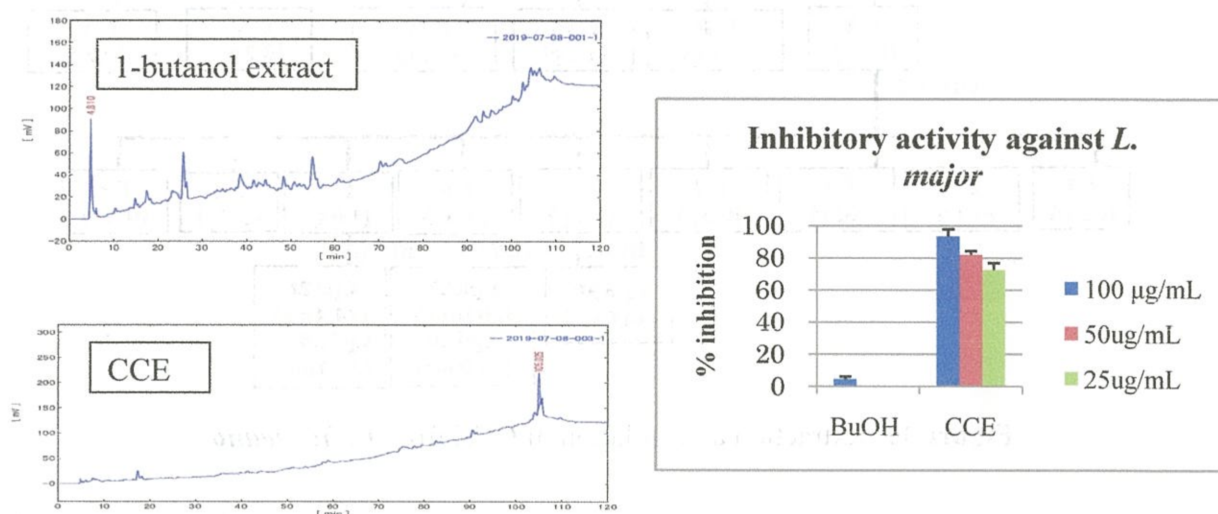

Figure 11. The Distinction of HPLC chart (left side) and Biological activity (right side) of *C. heynana* extracts before and after modification.

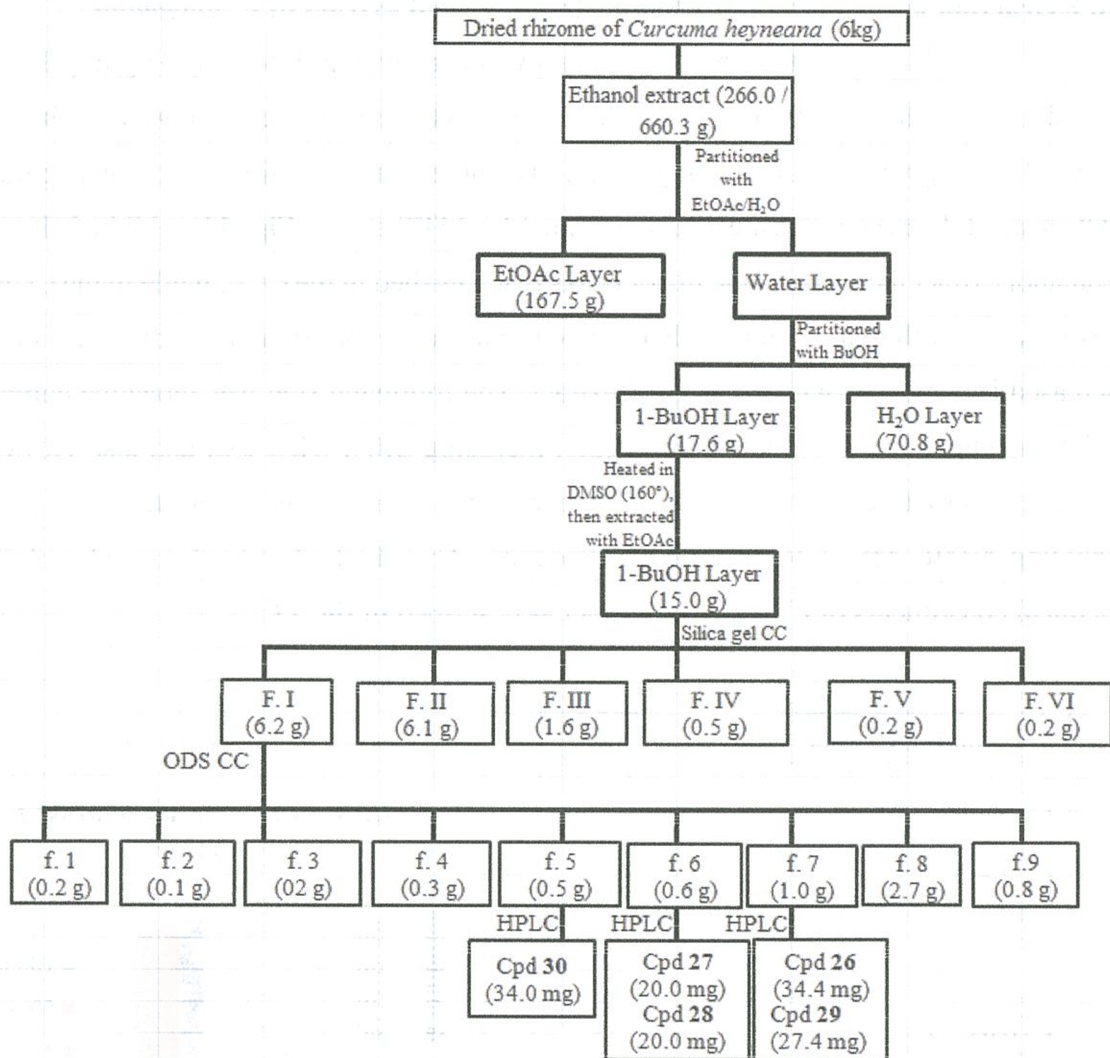

Figure 12. Extraction and isolation of CCE from ***C. heyneana***

2.7 Structural Elucidation of Isolated Compounds of CCE from *C. heyneana*

By using bio-guided isolation method, the CCE of ***C. heyneana*** was further isolated to generate a new sesquiterpene **30,** along with the known compounds: Longpene A (**26**) [66] Gajustulactone A (**27**) [13], Gajustulactone B (**28**) [13], and a norlabdane-type diterpene, 13,14,15,16 tetranorlabdan-8(17)-en-12-carboxylic acid (**29**) [67].

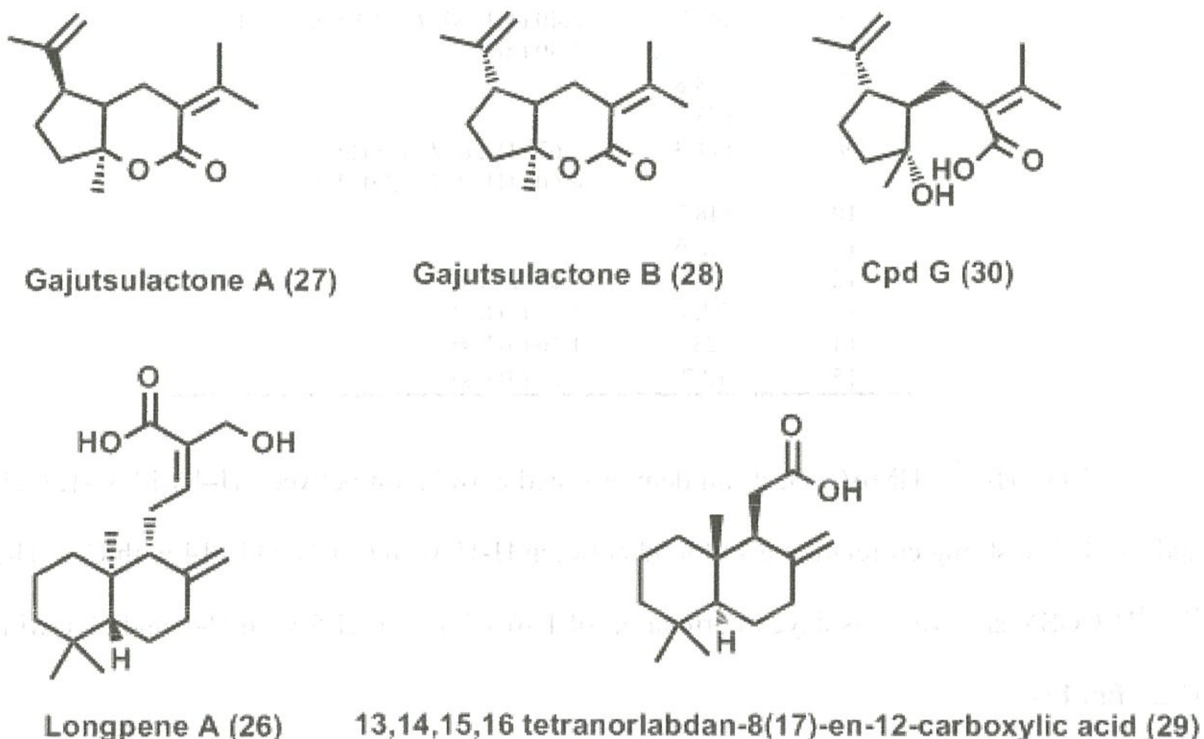

Figure 13. Isolated compounds of CCE from ***C. heyneana***

Compound **30** was attained as yellowish liquid. Its molecular formula was revealed to be $C_{15}H_{24}O_3Na$ from an ion at *m/z* 275.1612 in the HRESIMS. The IR data exhibited bands for hydroxyl group (3425 cm^{-1}) and the occurrence of aromatic ring (1646 cm^{-1}) absorption. The ^1H NMR data (table 5) showed the presence of four methyl group at 1.16, 1.64, 1.81 and 1.87 ppm. From ^{13}C NMR data exhibited the existence of carbonyl group of carboxylic acid at 173.8 ppm, hydroxyl group at 81.2 ppm, alkenyl group at 111.3, 128.8, 142.6 and 148.7 ppm subsequently and also methyl group at 18.7 22, 5, 23.0 and 23.3 ppm, respectively.

Table 5. ^{1}H-NMR data of compound **30**

^{13}C NMR and ^{1}H NMR data (150MHz and 600 MHz, MeOD)		
Position	**^{13}C**	**^{1}H**
1	52.2	2.30 (m)
2	28.3	1.69 (m)
		1.56 (m)
3	41.2	1.71 (m)
		1.61 (m)
4	81.2	
5	52.5	1.99 (1H, ddd, J = 10.5, 8.0, 6.2 Hz)
6	30.3	2.40 (1H, dd, J = 10.5, 6.2 Hz)
		2.30 (m)
7	128.8	
8	173.8	
9	111.3	4.67 (1H, d, J=1.9 Hz)
		4.60 (1H, d, J = 2.0 Hz)
10	148.7	
11	142.6	
12	23.3	1.87 (3H, s)
13	22.5	1.81 (3H, s)
14	23	1.16 (3H, s)
15	18.7	1.64 (3H, s)

The ^{1}H- ^{13}C HMBC spectrum demonstrated correlation between H-4 with C-1, C-4, and C-10. The strong correlation also found between H-15 with C-10 and H-14 with C-4. The ^{1}H- ^{1}H COSY spectrum displayed correlation of H-6 with H-5, H-5 with H-1 and H-1 with H-2. (**fig. 14**).

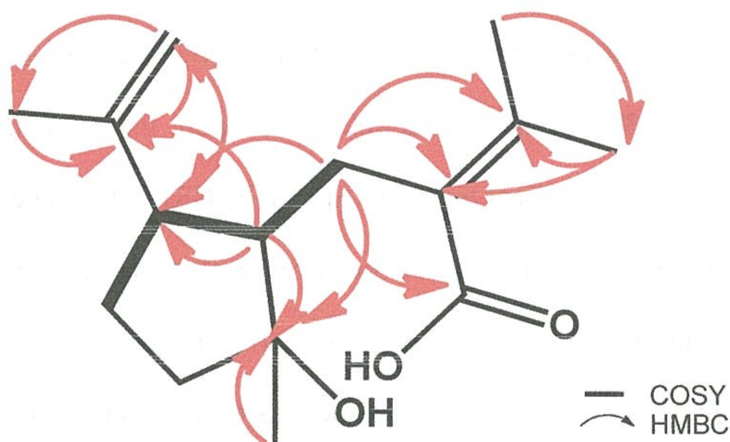

Figure 14. COSY and HMBC correlation of **30**

From **fig. 15**, it showed the NOESY spectrum which exhibited the correlation between H-1 with H-9, H-9 with H-15, H-14 with H-6, and H-5 with H-15.

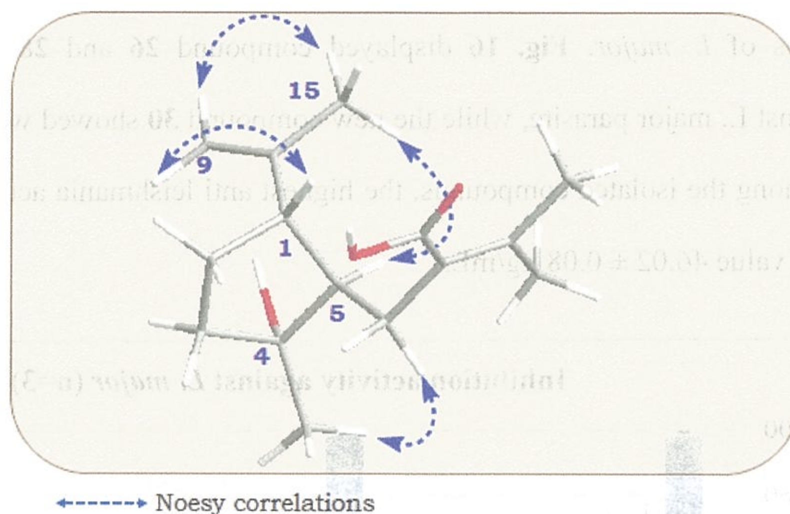

Figure 15. H-H NOESYcorrelation of compound **30**

2.8. Anti-leishmaniasis Activity Examination of Compounds 26-30

All isolated compounds were being evaluated their inhibitory activity toward promastigotes of *L. major.* **Fig. 16** displayed compound **26** and **28** possessed potential activity against L. major parasite, while the new compound **30** showed weak activity. Table 6 exhibited among the isolated compounds, the highest anti leishmania activity was compound **26** with IC_{50} value 46.02 ± 0.08 μg/mL.

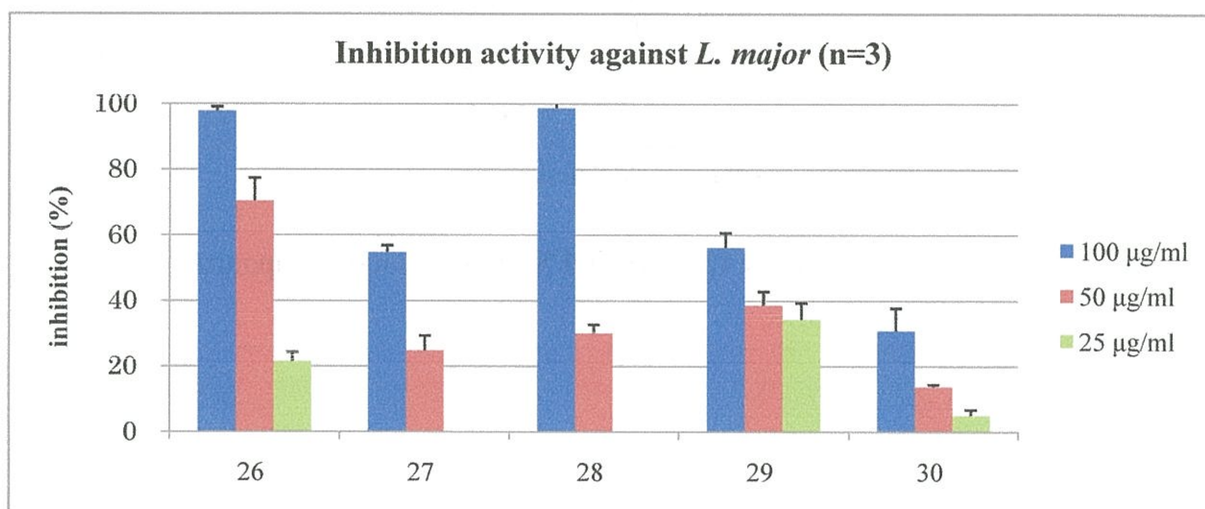

Figure 16. Biological activity of isolated compounds of CCE from *C. heynana*

Table 6. Inhibition Rate of compounds **26-30** against *L .major*

Isolated Compounds	*L. major* IC_{50} (μg/mL)
26	46.02 ± 0.08
27	91.94 ± 2.04
28	64.47 ± 2.25
29	82.08 ± 4.43
30	>100
Miltefosine	7.47 ± 0.30

[1] each value represents mean ±SE (three independent experiments).

2.9. Experimental Section

2.9.1 Preparation of Chemically Converted extract of *C. heyneana* and Isolation of Compounds 26-30

About 17.6 g of 1-butanol extract of *C. heyneana* was dissolved in DMSO (10mg/ml) then being heated in oil bath at 160 °C for 8 hours. This extract then was extracted with partitioned of ethyl acetate and water. The combined organic layer was washed with water, brine and dried over sodium sulfate and evaporated to give the CCE of *C. heyneana* (15.0 g).

The CCE was separated on a silica gel CC and eluting with MeOH/$CHCl_3$ solvent producing 6 fractions. Fraction CCE1 (6.2 g) was subjected to ODS CC with a linear gradient of 10% aq. MeOH, from 10%MeOH to 90% MeOH, (1.5 L each), leading to nine fractions. The residue of fraction CCE 1.5 (500 mg) was purified by HPLC using 65% aq. acetone to give **30**. The residue of fraction CCE 1.6 (600 mg) was purified by HPLC using 65% aq. acetone to yield **27** and **28**. The residue of fraction CCE 1.7 (1.0 g) was purified by HPLC using 65% aq. acetone to give **26** and **29**.

The chemical structures of the **26-30** were analyzed and described through spectroscopic measurement based on IR, UV-visible, HR-ESI-MS data and NMR, as follow.

Longpene A (26)

Colorless oil, $[\alpha]_D^{27}$ +1.6° (c = 1.8, dichloromethane); ^{1}H-NMR (600 MHz, methanol-d_4, δ): δ 6.87 (1H, t, J = 6.8 Hz), 4.96 (2H, s), 4.86 (1H, d, J = 1.1 Hz), 4.49 (1H, s), 2.58 (1H, ddd, J = 16.5, 6.3, 2.9 Hz), 2.46 – 2.39 (2H, m), 2.06 (1H, td, J = 12.8, 4.7 Hz), 1.90 (1H, d, J = 10.8 Hz), 1.80 – 1.75 (1H, m), 1.68 – 1.58 (1H, m), 1.57 – 1.48 (1H, m), 1.37 (1H, dd, J = 12.9, 4.2 Hz, 1H), 1.29 – 1.11 (1H, m), 0.90 (s, 3H), 0.85 (s, 3H), 0.77 (s, 3H). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 170.6, 149.6, 149.3, 132.1, 108.5, 58.2, 56.9, 56.6, 43.2, 40.6, 40.3, 39.0, 34.5, 34.1, 25.4, 24.6, 22.2, 20.4, 14.9. HRESIMS (positive ion) m/z: 329. 2113 $[M+Na]^+$ (calcd for $C_{19}H_{30}O_3Na$: 329.2087). The obtained data are similar with the references [66].

Gajutsulactone A (27)

Colorless oil, $[\alpha]_D^{17.4}$ -124.9° (c = 0. 9, methanol); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 4.80 (1H, d, J = 0.5 Hz), 4.77 (1H, br.s), 2.49 (1H, dd, J = 15.9, 5.9 Hz), 2.39 – 2.31 (1H, m), 2.23 – 2.18 (1H, m), 2.16 (3H, s), 2.08 – 2.02 (1H, m), 1.95 (1H, ddd, J = 24.3, 15.2, 7.9 Hz), 1.88 (3H, s), 1.86 – 1.84 (1H, m) 1.75 (3H, s), 1.74 – 1.70 (2H, m), 1.23 (3H, s).^{13}C-NMR (151 MHz, methanol-d_4, δ): 169.6, 154.4, 147.2, 120.8, 111.0, 86.8, 47.3, 46.9, 37.6, 27.7, 27.6, 23.7, 23.5,

19.6, 19.6. HRESIMS (positive ion) *m/z*: 235. 1695 $[M+H]^+$ (calcd for $C_{15}H_{23}O_2$: 235.1693). The obtained data are similar with the references [13].

*Gajutsulactone B(**28**)*

Colorless oil, $[\alpha]_D^{17.6}$ -41.3° (*c* = 0.89, methanol); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 5.03 (1H, s), 4.90 (1H, s), 2.98 – 2.93 (1H, m), 2.61 – 2.57 (1H, m), 2.32 (1H, dd, *J* = 10.6, 5.2 Hz), 2.30 – 2.25 (1H, m), 2.14 (3H, s), 2.09 – 2.03 (2H, m), 1.90 (2H, t, *J* = 1.7 Hz), 1.89 (3H, s), 1.81 (3H, s), 1.21 (3H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 169.8, 154.3, 146.7, 121.6, 112.4, 86.9, 46.9, 43.6, 39.0, 27.1, 26.8, 25.4, 23.7, 23.4, 20.1. HRESIMS (positive ion) *m/z*: 235. 1694 $[M+H]^+$ (calcd for $C_{15}H_{23}O_2$: 235.1693). The obtained data are similar with the references [13].

*Tetralabdane-carboxylic acid (**29**)*

Colorless oil, $[\alpha]_D^{16.1}$ +5.2° (*c* = 6.7,$CHCl_3$); ^{1}H-NMR (600 MHz, methanol-d_4, δ): 4.92 (1H, s), 4.76 (1H, d, *J* = 4.1 Hz), 4.55 (1H, d, *J* = 4.1 Hz), 2.49 (1H, d, *J* = 12.4 Hz), 2.43 – 2.38 (1H, m), 2.32 (1H, d, *J* = 3.1 Hz), 2.13 – 2.05 (1H, m), 1.80 – 1.74 (1H, m), 1.67 – 1.59 (2H, m), 1.54 – 1.47 (1H, m), 1.45 – 1.39 (1H, m), 1.36 (1H, dd, *J* = 12.9, 4.3 Hz), 1.24 – 1.21 (1H, m), 1.16 (1H, m), 0.91 (3H, s), 0.85 (3H, s), 0.73 (3H, s). ^{13}C-NMR (151 MHz, methanol-d_4, δ): 177.8, 150.4, 106.9, 56.5, 54.0, 43.2, 40.1, 39.9, 38.7, 34.4, 34.1, 31.6, 25.2, 22.2, 20.3, 14.9. HRESIMS (positive ion) *m/z*: 251. 2007 $[M+Na]^+$ (calcd for $C_{16}H_{27}O_2Na$: 251. 2006). The obtained data are similar with the references [67].

2-hydroxy-2-methyl-5-(prop-1-en-2-yl)cyclopentyl)methyl)-3-methylbut-2-enoic acid (**30**)

Yellowish oil, $[\alpha]_D^{17.9}$ +10.5 (*c* 1.5, methanol); IR υ_{max} (film) cm^{-1}3425, 2918, 1646, 1442, 1375, 1240, 1060; UV λ_{max} (MeOH) nm (log ε): 223 (4.02), 202 (4.16); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 151 MHz) data, see Table 5. HRESIMS (positive ion) *m/z*: 275.1612 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$: 275.1618).

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