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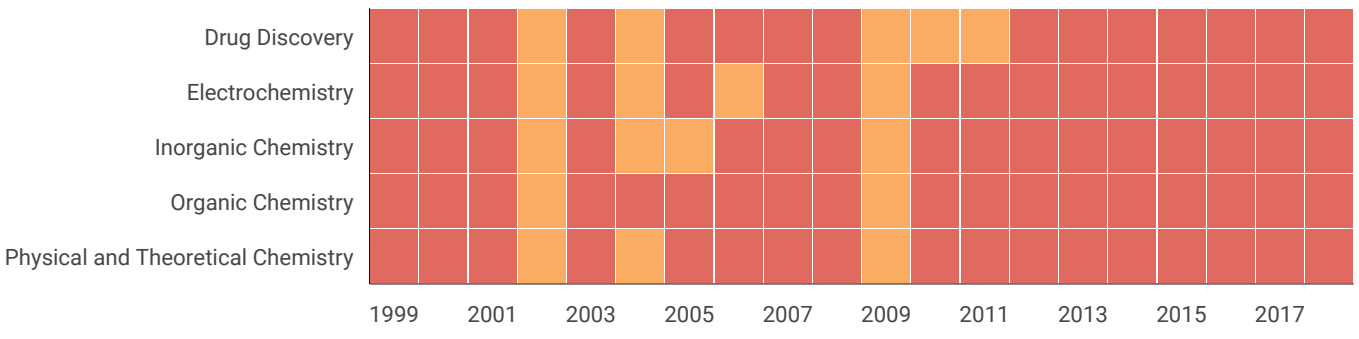

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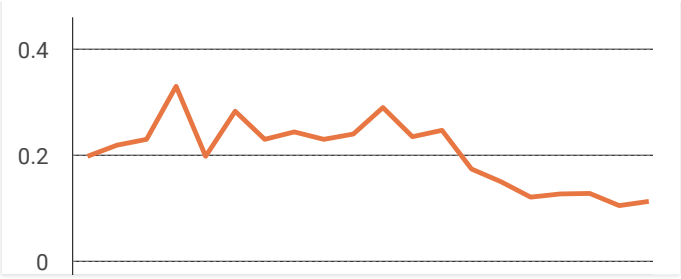

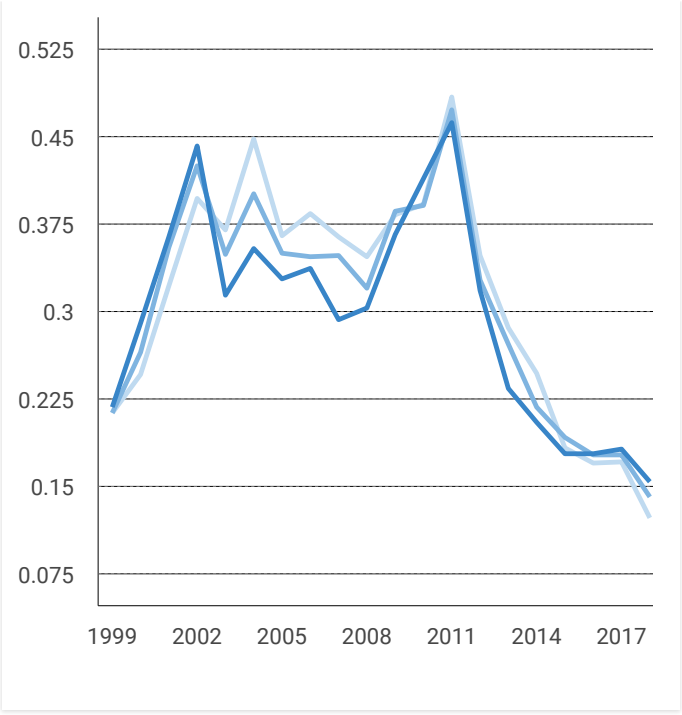

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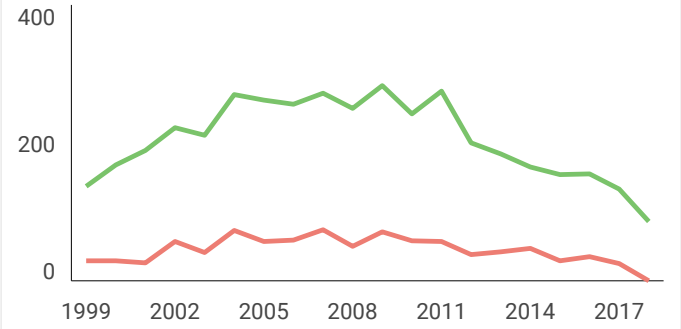

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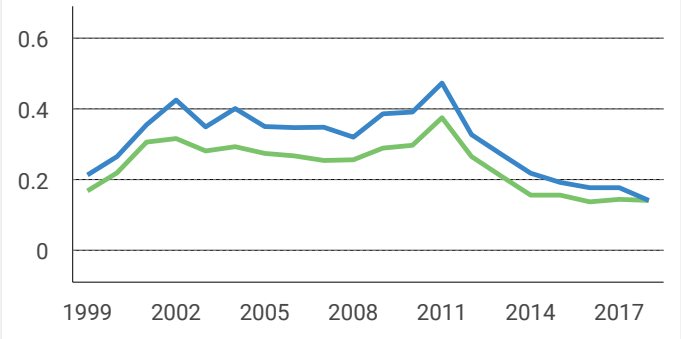

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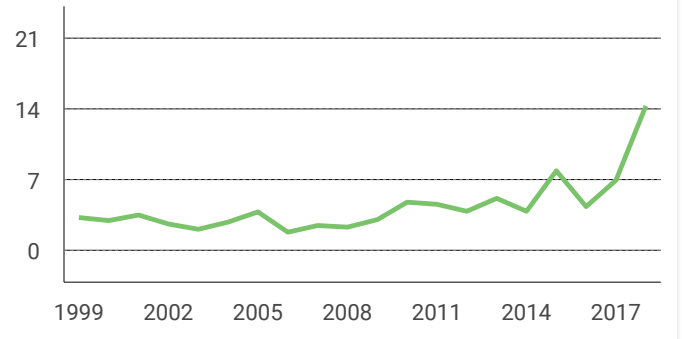

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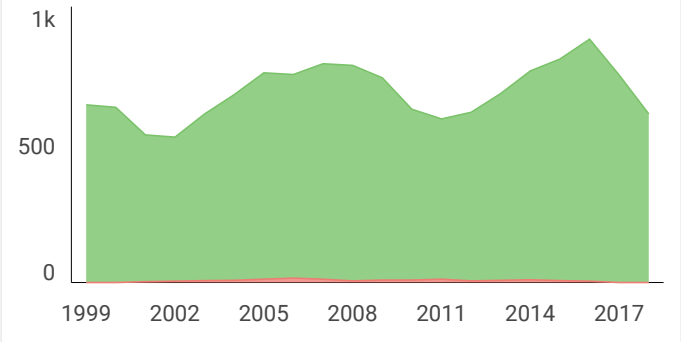

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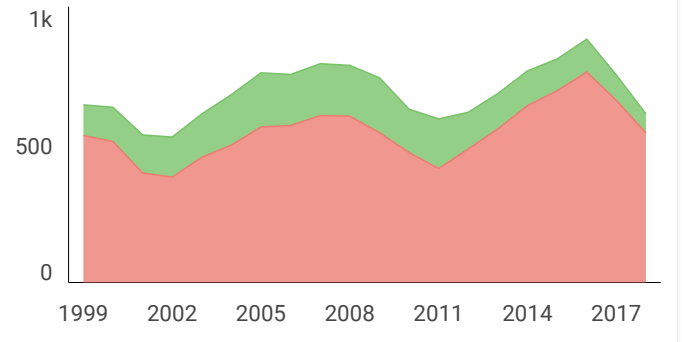

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Isolation of cytotoxic sesquiterpenes from *Curcuma comosa* and characterization of their structures

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Three quaiane-type sesquiterpenes named curcumenol (**1**), zedoarondiol (**2**), and (1*S*,4*S*,5*S*,10*R*)-isozedoarondiol (**3**) were isolated from the rhizomes of *Curcuma comosa*. Their structures were elucidated on the basis of extensive spectroscopic analysis. The cytotoxic activities of all isolated compounds were tested by MTT assay. Compounds **2** and **3** showed the most potent activities against T47D cell line with IC_{50} values 12.13 and 10.93 μg/mL, respectively.

Keywords: *Curcuma comosa*, Zingiberaceae, quaiane-type sesquiterpenes, MTT assay.

Introduction

Curcuma comosa (Zingiberaceae), widely grown in tropical and subtropical area of Asia, including Thailand, Indonesia, Malaysia and Taunggyi[1–3] (Shan State of Myanmar). Taunggyi is the fifth largest city of Myanmar. Taunggyi has a humid subtropical climate. The climate usually comprises three seasons: hot summer, rainy monsoon, and cold winter.

In Taunggyi, the rhizome of *Curcuma comosa* is called **Sa-nwin-ga** and local people have used it as a traditional medicine for stomach ache, diabetes mellitus and hypertension. In Thailand, the rhizome of *C. comosa* is called **Waam chak mod luuk** and it has been used for the treatment of reproductive disorders in women, and for relief of unpleasant menopausal symptoms among postmenopausal women, and it is also widely used as an aromatic stomachic and anti-inflammatory agent. Several compounds have been isolated from the rhizomes of *C. comosa*. Three major groups of structures reported include sesquiterpenes, diarylheptanoids, and flavonoids glycosides. The structure of sesquiterpenes can be classified into five sub-groups: (i) Germacrane type sesquiterpene, (ii) Guaiane type sesquiterpene, (iii) Bisaborane type sesquiterpene, (iv) Carabrane type sesquiterpene, and (v) Eudesmane type sesquiterpene. Pharmacological investigations on diarylheptanoids have displayed significant biological activities, including estrogenic, anti-bacteria, anti-inflammatory, and anti-osteoclastogenic properties[3–10].

This research focused on chemical investigation of a methanolic extract of *C. comosa* resulting in isolation and structure elucidation of three guaiane-type sesquiterpenes. Moreover, cytotoxic activity of all the isolated compounds also evaluated.

Experimental

General:

All chemical solvents used were of analytical grade and were purchased in Surabaya, Indonesia. The solvents used for extraction and chromatography were distilled at their boiling points. Column chromatography (CC) was carried out on silica gel 60. TLC was carried out on silica gel $60GF_{254}$ precoated plates (Merck). Melting points were determined using appropriate apparatus. The ^1H NMR and ^{13}C NMR spectra were recorded at 600 and 151 MHz, in $CDCl_3$, methanol-

d_4 and the residual solvent peaks were used as internal standard. Chemical shifts are reported in parts per million (δ) and coupling constants in Hertz. NMR assignments were obtained from investigation of 1D and 2D experiment (^{1}H NMR, ^{13}C NMR, DQF-COSY, NOESY, HMBC, HSQC, and DEPT). JEOL JMS HX-110 mass spectrometer was used to obtain HRFAB-MS spectra. The infrared spectra (IR) were obtained on FT IR-8400S (Shimadzu) using KBr. A Buichi Rotary Evaporator with a high vacuum pump was used for evaporation of solvents under reduced pressure at 40°C.

Table 1. ^{1}H NMR data of compounds **1**, **2** and **3**

H	**1**[a]	**2**[b]	**3**[c]
1	1.95 (m)	1.98 (m)	2.79 (m)
2	1.96 (m)	1.66 (m)	1.63 (m)
3	1.90 (m)	1.71 (m)	1.73 (m)
4	1.93 (m)	–	–
5		1.37 (d, 12.9 Hz)	2.02 (d, 12.9 Hz)
6	2.11 (d, 16.9 Hz), 2.66 (d, 16.9 Hz)	2.02 (m), 2.86 (d, 15.0 Hz)	2.52 (d, 13.9 Hz), 1.91 (d, 13.9 Hz)
7	–	–	–
8	–	–	–
9	5.77 (s)	2.51 (d, 12.7 Hz)	2.30 (dd, 16.1 Hz, 1.2 Hz)
	–	2.98 (d, 12.7 Hz)	3.34 (d, 16.1 Hz)
10	–	–	–
11	–	–	–
12	1.60 (s)	1.90 (s)	1.97 (s)
13	1.82 (s)	1.86 (s)	1.88 (s)
14	1.03 (d, 6.4 Hz)	1.17 (s)	1.39 (s)
15	1.67 (s)	1.11 (s)	1.19 (s)

[a]Spectra recorded at 600 MHz ($CDCl_3$), [b,c]Spectra recorded at 600 MHz (methanol-d_4).

Plant material:

The rhizomes of *Curcuma comosa* were collected in September 2016 from Taunggyi, Shan State, Myanmar.

Extraction and isolation:

The air-dried rhizomes of *Curcuma comosa* (1 kg) were successively extracted with methanol (3000 mL) at room temperature for three weeks. After concentration of the solvent under reduced pressure, the methanol extract (41.16 g) was partitioned with hexane/MeOH (100 mL×3, v/v). MeOH extract (15 g) was separated by vacuum liquid chromatography (VLC), eluting with hexane/EtOAc (100:0, 95:5, 80:20, 70:30, 0:100) step gradient as eluents to afford five fractions (CT-1 to CT-5). Fraction CT-2 (438 mg) was purified over silica gel by column chromatography, eluting with solvent mixtures of hexane/EtOAc (100:0, 90:10, 80:20, 0:100) to yield compound **1** (250.6 mg). Fraction CT-4 (1 g) was subjected to column chromatography using hexane/EtOAc (90:10, 80:20, 70:30, 0:100) step gradient solvent mixtures as eluents to give compounds **2** (150.2 mg) and **3** (10 mg).

MTT assay:

Cytotoxicity of the isolated compounds on HeLa and T47D cells were carried out, using MTT assay *in vitro*. The isolated compounds were dissolved in DMSO (100 μL) to obtain various concentrations. Doxorubicin was used as a positive control. The media control solution consists of media culture and cell control solutions consist of culture and cell media. Sufficient amount of HeLa cells and T47D cells (213.4×10^4 cells/well and 167×10^4 cell/well) solution were prepared and 100 μL were placed in each well in a-96-well plates. The plates containing the cancer cells were treated with compound and incubated for 24 h. The cells were washed and treated by the 100 μL MTT per well. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 4 h, and 0.1 mL of the extraction buffer (10% sodium dodecyl sulfate in 0.01% HCl) was added. After an overnight incubation at 37°C, the absorbance was measured at 595 nm using an ELISA reader and the results were compared with the control cultures without compound. To determine cell viability, percent viability was calculated by the formula

$$\% \text{ Viability} = \frac{A_t - A_{mc}}{A_{ng} - A_{mc}} \times 100$$

where, A_t = absorbance of treatment, A_{mc} = absorbance of media control and A_{nc} = absorbance of negative control.

Results and discussion

Chromatographic isolation work on the extracts of *Curcuma comosa* successfully yielded three guaiane-type sesquiterpenes, curcumenol (**1**), zedoarondiol (**2**), and (1*S*,4*S*,5*R*,10*R*)-isozedoarondiol (**3**). Their structures were elucidated by extensive spectroscopic techniques and by comparison with the data reported in the literature.

Compound (**1**) was obtained as colorless crystals. Its melting point was 98–100°C. The IR spectrum showed the absorption band for O-H stretching vibration at 3371 and 3321 cm^{-1}, C=C stretching vibration at 1695 and 1658 cm^{-1}, C-C-

O stretching vibration of alcohol group at 1274 cm^{-1}. The proton NMR spectrum of compound (**1**) revealed the presence of four methyl singlet signal peaks including a doublet peaks at δ_H 1.03 (6.4 Hz) and three singlet peaks at δ_H 1.60, 1.67 and 1.82. Three methylene signal peaks including two multiplet peaks at δ_H 1.97 and 1.90, and a doublet peak at 2.66/2.11 (16.9 Hz), and two methine multiplet signals at 1.91 and 1.95 were also observed. In addition, there were an olefinic singlet signal peak at δ_H 5.77 and a hydroxyl broad singlet signal peak at δ_H 6.06. ^{13}C NMR along with DEPT 135 and 90 experiments allowed the identification of four sp^3 methyl carbon, three sp^3 methylene carbons, two sp^3 methine carbons, one sp^2 methine carbon and a carbonyl carbon, respectively. Detailed structure of compound (**1**) was established by ^1H-^1H COSY and ^1H-^{13}C HMBC experiments (Figs. 1a and 1b). The two methyl signals showed HMBC correlation of H-12 (δ_H 1.60) with C-13/C-7/C-11, H-13 (δ_H 1.82) with C-12/C-7/C-11. An olefinic proton at δ_H 5.77 also showed long-range HMBC correlation to C-1 (δ_C 51.3), C-8 (δ_C 101.5) and C-15 (δ_C 20.9). Furthermore, the HMBC correlation showed one methyl doublet signal at δ_H 1.03 (d, 6.4 Hz) with C-1 (δ_C 51.3), C-9 (δ_C 125.6) and C-10 (δ_C 139.2), and another singlet methyl at δ_H 1.67 with C-1 (δ_H 51.3), C-9 (125.6) and C-10 (139.2). The configuration of compound (**1**) was considered the same with the previous data based on NOE correlations (Fig. 1c), as well as 1D, 2D NMR, and MS spectral data. Thus, based on above data, compound (**1**) was identified as curcumenol[11–14].

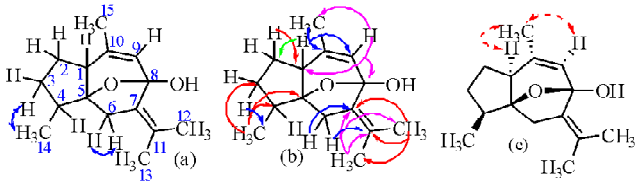

Fig. 1. DQF-COSY (a), HMBC (b), and NOESY (c) correlation in compound (**1**).

Compound (**2**) was obtained as yellow oil. The IR spectrum showed the presence of hydroxyl (3387 cm^{-1}), conjugated ketone (1708 and 1664 cm^{-1}), and olefinic (1694 cm^{-1}) groups. The proton NMR spectrum of compound (**2**) revealed the presence of four tertiary methyl signals at δ_H 1.11, 1.17, 1.86 and 1.90 (each 1H, s), four set of methlylene protons at δ_H 1.66 (2H, m) 1.71 (2H, m), 2.02 (1H, m)/2.86 (1H, d, 15 Hz), 2.51 (1H, d, 12.7)/2.98 (1H, d, 12.7), two methine proton at δ_H 1.37 (1H, d, 12.9 Hz), 1.98 (1H, m). The ^{13}C NMR and DEPT 135 and 90 spectra revealed the presence of four methyl carbon signals (δ_C 18.7, 20.9, 21.1, 21.7), four methylene carbon signals (δ_C 21.5, 28.1, 38.7, 59.5), two oxygenated carbon signals (δ_C 71.8, 79.1), two methine carbons signals (δ_C 51.5, 55.7), two olefinic quaternary carbon signals (δ_C 135.1, 141.8) and a carbonyl signal (δ_C 204.3), respectively. Based on the above spectra data, compound (**2**) might be a guaiane-type sesquiterpenes. This assumption was further determined through a variety of 2D NMR spectroscopic techniques. In the ^1H-^1H COSY correlations (Fig. 2a), the proton signal at δ_H 2.02 was coupled with the proton signal at δ_H 2.86; the proton signal at δ_H 2.51 was coupled with the proton signal at δ_H 2.98; the proton signal at δ_H 1.37 was coupled with the proton signal at δ_H 1.98; and the proton signal at δ_H 1.66 was coupled with the proton signal at δ_H 1.71, respectively. Furthermore, the ^1H-^{13}C HMBC long range correlation (Fig. 2b) of H-12 with C-7/C-8/C-11, H-13 (d_H 1.86) with C-7/C-8/C-11, H-14 with C-3/C-4/C-5, H-15 with C-1/C-9/C-10, H-5 with C-1/C-4/C-7/C-10, H-6a with C-1/C-4/C-5/C-7/C-8/C-11, H-9a with C-1/C-7/C-8/C-10, H-9b with C-1/C-8/C-10/C-15, respectively. The relative configuration of compound (**2**) was considered to be the same with previous data[1,15,16], based on the NOE correlations (Fig. 2c) between H-5 and H-15, H-1 and H-14. Based on these spectra data and comparison with the previous report, compound (**2**) was identified as zedoarondiol[1,15,16].

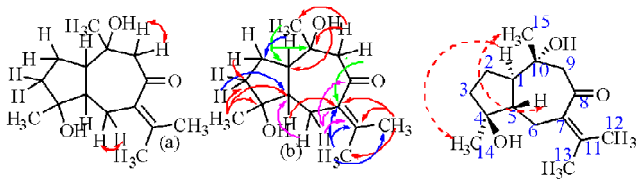

Fig. 2. DQF-COSY (a), HMBC (b), and NOESY (c) correlation in compound (**2**).

Compound (**3**) was also obtained as yellow oil. The IR spectrum showed absorption band due to conjugated ketone (1701 and 1665 cm^{-1}), a double bond (1612 cm^{-1}) and a hydroxyl group (3394 cm^{-1}). The ^1H NMR spectrum of the compound (**3**) exhibited characteristic signals for guaiane-type sesquiterpenes skeleton, including four methyl protons at δ_H 1.19, 1.39, 1.88, 1.97 (each 3H, s), two methine pro-

Table 2. ^{13}C NMR of compounds **1**, **2** and **3**

C	**1**[a]	**2**[b]	**3**[c]
1	51.3	55.7	51.2
2	27.6	21.5	24.6
3	31.2	38.7	36.0
4	40.4	79.1	81.7
5	85.7	51.5	52.6
6	37.3	28.1	27.0
7	137.4	135.1	134.0
8	101.5	204.3	204.7
9	125.6	59.5	49.9
10	139.2	71.8	72.5
11	122.2	141.8	143.0
12	22.3	21.7	20.9
13	18.9	20.9	21.8
14	11.8	21.1	23.4
15	20.9	18.7	31.3

[a]Spectra recorded at 151 MHz ($CDCl_3$), [b,c]Spectra recorded at 151 MHz (methanol-d_4).

tons at δ_H 2.02 (1H, d, 12.9 Hz) and 2.79 (1H, m), four methylene protons at δ_H 1.63 (2H, m), 1.73 (2H, m), 2.52 (1H, d, 13.9 Hz)/1.91 (1H, d, 13.9 Hz), 3.34 (1H, d, 16.1 Hz/2.30 (1H, dd, 16.1, 1.2 Hz). The ^{13}C NMR and HSQC experiment revealed the presence of 15 carbon signals due to one carbonyl function (δ_C 204.7), two sp^2 quaternaty carbons (δ_C 134.0, 143.0), two sp^3 quaternary carbony (δ_C 72.5, 81.7), two sp^3 methine carbon (δ_C 51.2, 52.6), four sp^3 methylene (δ_C 27.0, 24.6, 36.0, 49.9) and four sp^3 methyl carbons (δ_C 20.9, 21.8, 23.4, 31.3). HMBC analysis exhibited correlation of H-15 with C-5 and C-10; H-14 with C-1, C-3, C-4 and C-5; H-13 with C-7, C-8, C-11 and C-12; H-12, C-7, C-8, C-11, C-13; H-9a with C-1, C-8 and C-10; H-9b with C-1, C-7, C-8 and C-10; H-6a with C-1, C-4, C-7 and C-11; H-6b with C-5, C-7 and C-11; H-5 with C-2, C-3 and C-4; H-1 with C-2 and C-10. In the ^{1}H-^{1}H COSY spectrum, the following cross peak correlations were observed (Fig. 3a): the proton signal at δ_H 3.34 was correlated with the proton signal at δ_H 2.30; the proton signal at δ_H 2.52 was correlated with the proton signal at δ_H 1.91; the proton signal at δ_H 2.02 was correlated with the proton signal at δ_H 1.63; the proton signal at δ_H 3.34 was correlated with the proton signal at δ_H 1.73, respectively. In nuclear overhauser effect spectroscopy (NOESY) spectrum, the signal at δ 2.79 (H-1) had NOE enhancements with the signals at δ 1.19 (Me-15) and δ 2.02 (H-5). And NOE effects of Me-14 (δ 1.39) with H-1 (δ 2.79) and H-6 (δ 2.51) were also observed (Fig. 3b). With the aid of above data and previous report compound (**3**) was named as (1*S*,4*S*,5*S*,10*R*)-isozedoarondiol[16,17].

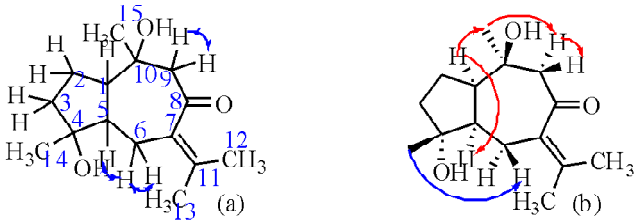

Fig. 3. DQF-COSY (a) and NOE (b) correlation in compound (**3**).

All isolated compounds (**1-3**) were examined for their cytotoxic activities against HeLa and T47D cell lines. Compound **2** and **3** showed the most potent activities against T47D cell line with IC_{50} values of 12.13 and 10.93 mg/mL.

Table 3. Cytotoxic activity of compounds (**1**-**3**) against HeLa and T47D

Compd.	IC_{50} (μg/mL)	
	HeLa	T47D
1	142.67	213.55
2	99.83	12.13
3	170.83	10.93
Doxorubicin	2.69	0.04

Biosynthesis pathway to compound (**1**)-(**3**) have been reported earlier[18].

Conclusions

In summary, this paper described the isolation and structure elucidation of three quaiane-type sesquiterpenes, namely curcumenol (**1**), zedoarondiol (**2**), and (1*S*,4*S*,5*S*,10*R*)-isozedoarondiol (**3**). The cytotoxic activity of all isolated compounds against two cancer cell lines was also reported. Compounds **2** and **3** showed the most potent activities against T47D cell lines with IC_{50} values 12.13 and 10.93 mg/mL.

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Supporting information

Supporting information accompanies this paper are described.

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