Chemical constituents of *Zingiber ligulatum* Roxb.

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ABTRACT

The chemical constituents of ethanolic fresh rhizome extract from *Zingiber ligulatum* Roxb. were studied. Three pure compounds were isolated and the structures were elucidated and confirmed by spectroscopic data. They were identified as kaempferol 7,4'-dimethyl ether, quercetin 7,4'-dimethyl ether and *n*-propyl *p*-hydroxybenzoate. In addition, the volatile components were also analyzed by GC-MS. Six fatty acid ethyl esters including palmitic acid ethyl ester, magaric acid ethyl ester, linoleic acid ethyl ester, oleic acid ethyl ester and stearic acid ethyl ester together with elemol, β-eudesmol, α-eudesmol and β-sitosterol were found.

Keywords: Zingiber ligulatum, Khing Haeng

INTRODUCTION

Zingiber ligulatum Roxb. (Zingiberaceae) is in genus *Zingiber*. It is known as Khing Haeng. In Thailand, Khing Haeng is one of four species are used as spice and medicine i.e. *Z. ligulatum*, *Z. montanum*, *Z. officinale* and *Z. ottensii* (Triboun and Chantaranothai 2015). *Z. ligulatum* has been mainly used as carminative (Tappayuthpijarn et al. 2012) and showed antioxidant activity with EC_{50} value of 15.89 ± 2.92 µg/mL (Phuaklee, Sakpakdeejaroen, and Itharat 2010). *Z. ligulatum* together with four other herbs are known as a set of traditional medicine called Benchakoon. In addition, it is known and used among the healers in central, western and northern Thailand (Wongsuwan 2009). In Thailand, there are many ginger types that are called Khing Haeng. *Z. ligulatum* is often misunderstood and misused as dried *Z. officinale* because the powder of *Z. ligulatum* and *Z. officinale* have very similar characters (Tappayuthpijarn et al. 2012). However, there are many differences between *Z. ligulatum* and *Z. officinale*. For example, *Z. ligulatum* has cold taste and less pungent smell whereas *Z. officinale* has more spicy taste. The active compound, 6-gingerol, was found only in *Z. officinale* (Tappayuthpijarn et al. 2012). In addition, the flower and rhizome of *Z. ligulatum* (**Fig. 1**) is clearly disparate from *Z. officinale*. *Z. ligulatum* which is cultivated in Thailand nowadays may be imported from India or Myanmar for long time (Triboun and Boonkorkaew 2013).

In this work, the fresh rhizome of *Z. ligulatum* were extracted by 95% ethanol. The procedures for isolation and structural elucidation are reported. Then the pure compounds were structural elucidated and confirmed by spectroscopic data. The compound groups were investigated by GC-MS. To the best of our knowledge, the

chemical constituents of *Z. ligulatum* have never been previously reported, so this literature is the first report about these.

Fig. 1 The flower and rhizomes of *Z. ligulatum*

METHODOLOGY

General Experimental Procedures

The 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR spectra were obtained from Bruker Avance 400 spectrometer (USA). The HPLC (Agilent 1260 Infinity, USA) were used in separation with platinum^{TM} C18-EPS column (150×4.6 mm, 5 μm) from Grace davison discovery sciences. The GC-MS (6890N Network GC system, Agilent, USA) with wiley7n library was used in identification of compound groups. The molecular masses of pure compounds were confirmed by 6540 UHD Accurate Q-TOF LC/MS, Agilent (USA).

Plant material

Z. ligulatum rhizome (cultivated plant) was collected in 2014 at Asst.Prof.Dr.Kongsak Promtep's house area in Phitsanulok province and was identified by Asst.Prof.Dr.Pranee Nangngam. A voucher specimen (no. 003506) is kept at Department of Biology, Faculty of Science, Naresuan University, Phitsanulok.

Extraction, isolation and identification

Fresh rhizomes of *Z. ligulatum* (1.32 kg) were sliced into small pieces, extracted twice with 95% EtOH (2.2 L) for 15 days at room temperature of each. The solvent was removed under reduced pressure to give EtOH extract (16.02 g). The EtOH extract was further partitioned with EtOAc/H_2O to yield the EtOAc extract (2.43g). The EtOAc extract was subjected to silica gel (mesh 5-40 μm, Merck) column chromatography with air pump, eluted by a gradient of 5-40% MeOH/CH_2Cl_2 to afford 40 fractions. The 40 fractions were combined to 5 groups (G1-G5) by their characteristic, weight, TLC profiles and ^{1}H-NMR profiles. G1 (132 mg) was subjected

to silica gel (mesh 50-40 μm, Merck) column chromatography with air pump, eluted by a gradient of hexane/CH_2Cl_2. G1-2 (38 mg) was prepared in acetone for GC-MS analysis to afford **compound group A**. G2 (120 mg) was subjected to silica gel (mesh 5-40 μm, Merck) column chromatography with air pump, eluted by a gradient of 0.5-1% acetone/CH_2Cl_2. G2-2 (12 mg) was subjected to semi-preparative HPLC (flow rate 3 mL/min) to afford **compound 1** (5 mg). G3 (238 mg) was suspended in hexane to afford **compound 2** (10.22 mg). G4 (324 mg) was subjected to sephadex LH-20 column chromatography with air pump, with MeOH as an eluent to afford 42 fractions (G4-1–G4-42). After separation by sephadex LH-20, fraction G4-19 afforded **compound 3** (8.4 mg). G4-15 (46.5 mg) was prepared in CH_2Cl_2 for GC-MS analysis to afford **compound group B**. G4-16 (36.7 mg) was prepared in CH_2Cl_2 for GC-MS analysis to afford **compound group C**. The flow chart of column chromatography separation of G1 to G4 is shown in **Fig.2**

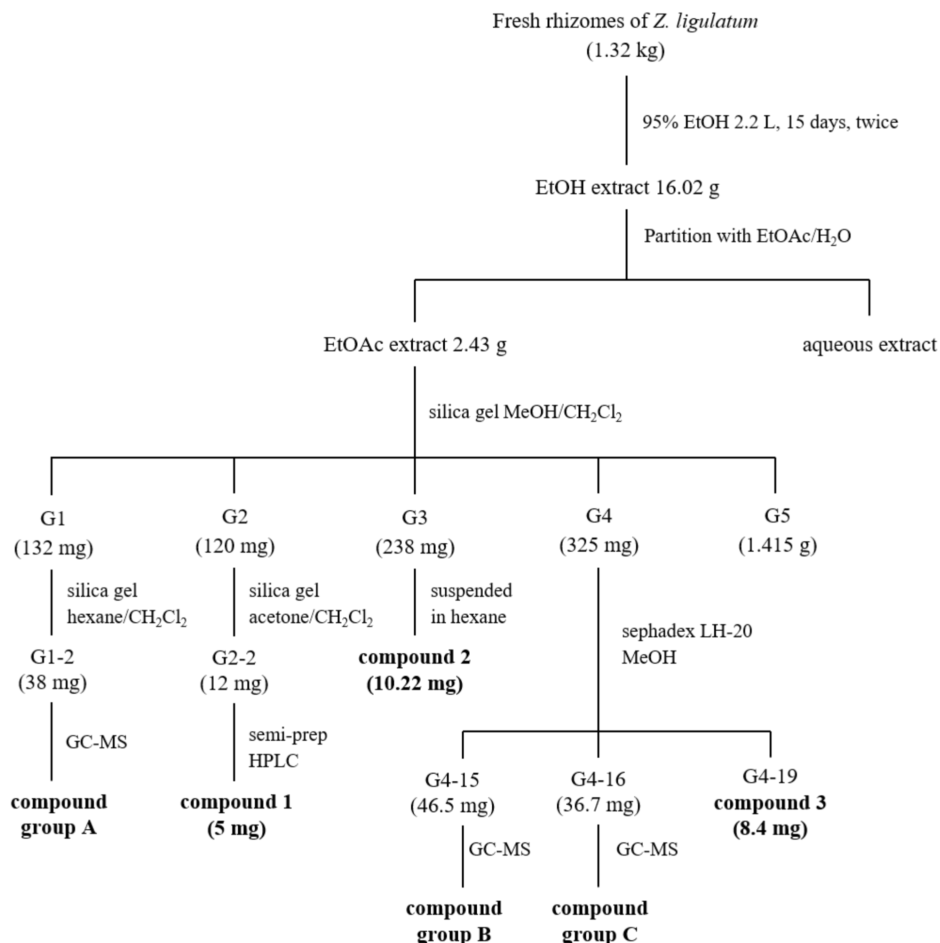

Fig. 2 The flow chart of column chromatography separation of G1 to G4

Gas chromatography-mass spectrometry conditions

The gas chromatography was fitted with a HP-5MS capillary column (0.25 mm × 30 m × 0.25 μm). Column temperature programs were operated using two methods.

Method 1: **compound group A** analysis; column oven temperature programmed at 60 °C for 3 min then the column oven was programmed at 10 °C/min to 280 °C and hold at 280 °C for 5 min.

Method 2: **compound groups B** and **C** analyses; column oven temperature programmed at 70 °C for 3 min then the column oven was programmed at 3 °C/min to 280 °C and hold at 280 °C for 10 min.

Operating conditions were set as following parameters: injection temperature, 250 °C; injected volume, 1 μL; carrier gas, UHP helium; flow rate, 1.0 mL/min; ion source temperature, 230 °C. Mass spectra were obtained by electron impact ionization mode at 70 eV and mass range detection was 50-550 amu. The mass spectra obtained were compared with those of Wiley7n library.

RESULTS AND DISCUSSION

Compound **1** exhibited two signals of methoxy groups and four signals of aromatic protons, all of those were doublet. The signals of two aromatic protons at δ 6.31 (1H, d, J = 2.1 Hz) and 6.70 (1H, d, J = 2.1 Hz) showed the same small J constant (2.1 Hz) which indicated that these protons were in M shape or W shape. This also suggested that these protons were on the same aromatic ring which contained four substituents. The two signals of aromatic protons at δ 7.10 (2H, d, J = 9.0 Hz) and 8.24 (2H, d, J = 8.9 Hz) indicated the presence of an aromatic A_2X_2 system. Coupling constant of these aromatic protons showed these protons were in *cis*- position of each other. Therefore, this aromatic ring was substituted by two groups in symmetry position. The ^{13}C-NMR spectrum showed signals of a carbonyl group (δ 177.2), two methoxy groups (δ 55.8, 56.4) and twelve signals of two aromatic rings. The link of two aromatic rings were partially supported by HMBC correlations. The correlated signals between H-2' to C-2 and H-6' to C-2 were observed. The structure and molecular formula were confirmed by MS with $[M+H]^+$ ion peak at *m/z* 315.0710 (calcd for $(C_{17}H_{14}O_6+H)^+$, 315.0863) and $[M+Na]^+$ ion peak at *m/z* 337.0683 (calcd for $(C_{17}H_{14}O_6+Na)^+$, 337.0513). The obtained spectroscopic data supported that the structure of compound **1** was kaempferol 7,4'-dimethyl ether.

Compound **2** had two aromatic rings which was similar to compound **1** except that there was an additional aromatic proton signal, while integration suggested five protons in total. The signals of aromatic protons at δ 7.08 (1H, d, J = 8.7 Hz), 7.68 (1H, dd, J = 8.6, 2.4 Hz) and 7.71 (1H, d, J = 2.2 Hz) in compound **2** was an ABX system. The J constant of a proton at δ 7.08 was 8.7 Hz indicating that this proton was in *cis*-position of each other. The splitting pattern of a proton at δ 7.68 was doublet of doublet suggesting that this proton was in between two protons. The small J constant between protons at δ 7.71 and δ 7.68 suggested that they were correlated to each other in M shape or W shape position. From ^{1}H-NMR spectrum, this aromatic ring was not symmetry, which suggested the present of three substituents. In addition, ^{1}H-NMR spectrum indicated the hydrogen-bonded hydroxyl group at δ 12.44 (1H, s) and hydroxyl group at δ 9.33 (1H, s). The skeleton of C_6-C_3-C_6 was supported by HMBC

correlations of H-2' to C-2, H-6' to C-2 and hydrogen-bonded between hydroxyl group (at C-5) together with carbonyl carbon (C-4). NMR spectra of compound **2** strongly indicated that compound **2** was quercetin 7,4'-dimethyl ether. This structure was confirmed by MS with $[M+H]^+$ ion peak at *m/z* 331.0648 (calcd for $(C_{17}H_{14}O_7+H)^+$, 331.0812), $[M+Na]^+$ ion peak at *m/z* 353.0455 (calcd for $(C_{17}H_{14}O_7+Na)^+$, 353.0632) and $[M-H]^-$ ion peak at *m/z* 329.0586 (calcd for $(C_{17}H_{14}O_7-H)^-$, 329.0667). The NMR data of compounds **1** and **2** are shown in **Table 1**.

Table 1 ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of kaempferol 7,4'-dimethyl ether (**1**) in acetone-d_6 and quercetin 7,4'-dimethyl ether (**2**) in DMSO-d_6

Positions	kaempferol 7,4'-dimethyl ether (**1**)		quercetin 7,4'-dimethyl ether (**2**)	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
2		147.2		146.8
3		137.5		136.5
4		177.2		176.1
5		161.6		160.4
6	6.31 (d, 2.1)	98.3	6.34 (d, 2.2)	97.6
7		166.6		165.0
8	6.70 (d, 2.1)	92.8	6.70 (d, 2.2)	91.9
9		157.7		156.2
10		104.9		104.1
1'		124.3		123.4
2'	8.24 (d, 8.9)	130.3	7.71 (d, 2.2)	114.8
3'	7.10 (d, 9.0)	114.8		146.2
4'		162.2		149.5
5'	7.10 (d, 9.0)	114.8	7.08 (d, 8.7)	111.8
6'	8.24 (d, 8.9)	130.3	7.68 (dd, 8.6, 2.4)	119.9
5-OH			12.44 (s)	
3'-OH			9.33 (s)	
7-OCH_3	3.90 (s)	55.8	3.85 (s)	55.7
4'-OCH_3	3.91 (s)	56.4	3.86 (s)	56.1

Compound **3** exhibited two signals of four aromatic protons and three signals of aliphatic protons. Two aromatic protons were A_2X_2 system, which suggested the present of an aromatic ring which was substituted with two groups in a symmetrical position. In addition, the terminal methyl protons at δ 1.02 (3H, t, $J = 7.4$ Hz), the two set of methylene protons at δ 1.78 (2H, sextet, $J = 7.2, 6.8$ Hz) and 4.25 (2H, t, $J = 6.6$ Hz) were observed in ^{1}H-NMR spectrum of compound **3**. The methylene protons at δ 4.25 was deshielded more than other, indicating that this proton was located near the electron withdrawing group. The ^{13}C-NMR showed signal of carbonyl group (δ 167.0). The ^{1}H-NMR, ^{13}C-NMR and HMBC data of compound **3** suggested that this compound was *n*-propyl *p*-hydroxybenzoate and it structure was confirmed by MS with $[M-H]^-$ ion peak at *m/z* 179.0653 (calcd for $(C_{10}H_{12}O_3-H)^-$, 179.0714). The NMR data of compound **3** is shown in **Table 2**. The structures of compounds **1**, **2** and **3** are shown in **Fig. 3**.

Table 2 ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectroscopic data of *n*-propyl *p*-hydroxybenzoate (**3**) in $CDCl_3$

Positions	δ_H (multiplicity. *J* in Hz)	δ_C
1		122.9
2, 6	7.96 (d, 8.8)	132.0
3, 5	6.87 (d, 8.7)	115.4
4		160.3
7		167.0
1'	4.25 (t, 6.6)	66.6
2'	1.78 (sextet, 7.2, 6.8)	22.3
3'	1.02 (t, 7.4)	10.6

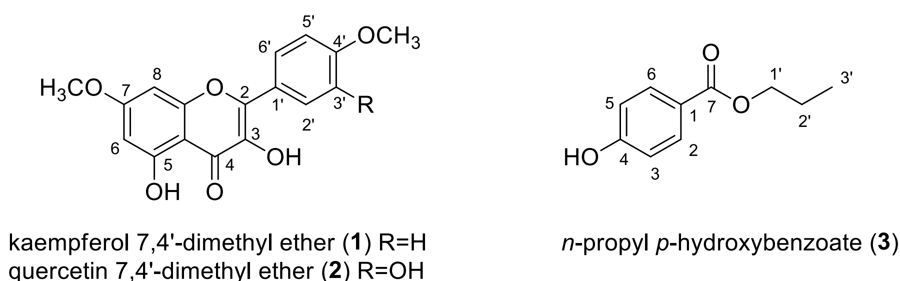

Fig. 3 The chemical structures of compounds **1**, **2** and **3**

^{1}H-NMR spectrum of compound group **A** (liquid) suggested a mixture of fatty acid ethyl esters and therefore it was subjected for GC-MS analysis. The GC chromatogram of compound group **A** is shown in **Fig. 4** and compounds in group **A** are listed in **Table 3**.

Table 3 Compound group **A** identified by GC-MS (*Method 1*)

Peak	RT (min)	Compounds	Molecular formula	$M^{+\cdot}$ (obs.)	Base peak (*m/z*)	Fragmentations (*m/z*)
a	22.16	palmitic acid ethyl ester	$C_{18}H_{36}O_2$	284	88	284, 101, 88
b	23.05	margaric acid ethyl ester	$C_{19}H_{38}O_2$	298	88	298, 101, 88
c	23.70	linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308	67	308, 263, 81, 67
d	23.77	oleic acid ethyl ester	$C_{20}H_{38}O_2$	310	55	310, 265, 69, 55
e	23.97	stearic acid ethyl ester	$C_{20}H_{40}O_2$	312	88	312, 101, 88

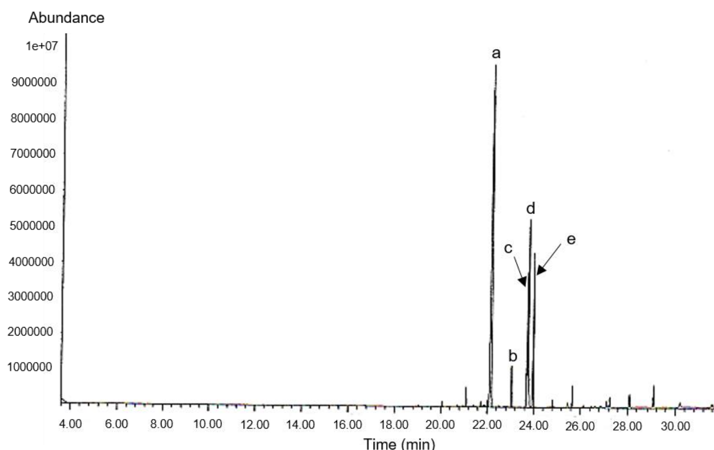

Fig. 4 GC chromatogram of compound group **A**

Normally, fatty acid ethyl esters are not found in nature. The unusual detection of fatty acid ethyl esters in compound group **A** could be explained by the conversion of fatty acid to fatty acid ethyl esters during the extraction with EtOH. However, assuming that the esterification was occurring in the same extent for each fatty acid, the percentage ratio of the esters would be related to the ratio of fatty acids in sample plant. Saturated fatty acid ethyl esters (palmitic, margaric and stearic acid ethyl esters) and unsaturated fatty acid ethyl esters (linoleic and oleic acid ethyl esters) were found in compound group **A**. Linoleic acid is known as an essential fatty acid (omega-6). In addition, the high content of margaric acid was found in dolphins' blood that made them had lower level of insulin and triglycerides in blood (Venn-Watson et al. 2015).

Compound groups **B** and **C** also obtained as liquid, whereas their ^{1}H-NMR spectra were observed to be not so complicated. Therefore, they were also analyzed with GC-MS. The GC chromatogram of compound groups **B** and **C** are shown in **Fig. 5** and compounds in groups **B** and **C** are listed in **Table 4**.

Table 4 Compound groups **B** and **C** identified by GC-MS (*Method 2*)

Peak	RT (min)	Compounds	Molecular formula	$M^{+\cdot}$ (obs.)	Base peak (*m/z*)	Fragmentations (*m/z*)
Compound group **B**						
f	29.56	elemol	$C_{15}H_{26}O$	222	161	161, 107
g	33.36	*β*-eudesmol	$C_{15}H_{26}O$	222	149	164, 149
i	78.48	*β*-sitosterol	$C_{29}H_{50}O$	414	414	396, 329, 213
Compound group **C**						
g	33.36	*β*-eudesmol	$C_{15}H_{26}O$	222	149	164, 149
h	33.42	*α*-eudesmol	$C_{15}H_{26}O$	222	149	161, 149
i	78.48	*β*-sitosterol	$C_{29}H_{50}O$	414	414	396, 329, 213

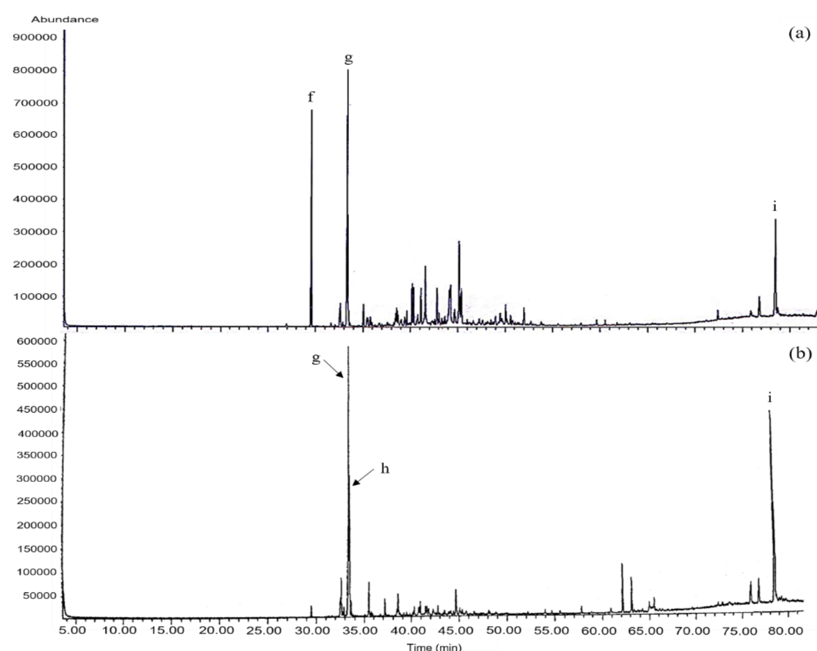

Fig. 5 GC chromatogram of compound groups **B** (a) and **C** (b)

Volatile components of compound groups **B** and **C** were analyzed using GC-MS. Compound group **B** consisted of elemol, β-eudesmol and β-sitosterol (**Fig. 5a**) while compound group **C** consisted β-eudesmol, α-eudesmol and β-sitosterol (**Fig. 5b**). The results indicated that two compounds; β-eudesmol and phytosterol (β-sitosterol) were observed on both groups. For the structural relationship, elemene-type (elemol) and eudesmane-type (β-eudesmol and α-eudesmol) sesquiterpenes were isomers. In addition, elemol can be changed to β-eudesmol by alder-ene reaction as shown in **Fig. 6**. Elemene- and eudesmane-type sesquiterpenes which found in *Z. ligulatum* have been previously reported to be the components in essential oil from *Zingiber* such as *Z. roseum* (Prakash et al. 2006) and Z. *mekongense* (Chareonkla et al. 2011).

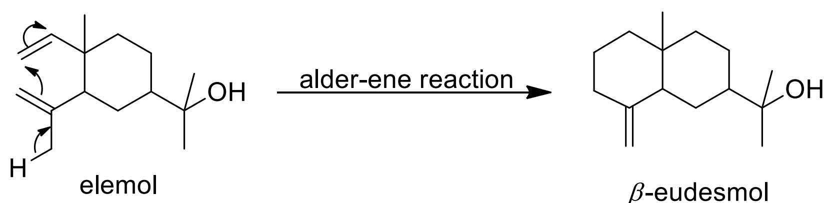

Fig. 6 Proposed structural relationship of elemol and β-eudesmol.

The finding in this study were found to be corresponded to the studies of Chareonkla and coworker (2011). Kaempferol 7,4'-dimethyl ether and quercetin 7,4'-dimethyl ether (Compounds **1** and **2**) have been isolated from *Z. mekongense*. Both of

compounds have anti-HIV1 activity (Chareonkla et al. 2011). Moreover, compound **2** isolated from *Miliusa balansae* showed cytotoxicity for cancer cells (Huong et al. 2005). Interestingly, many compounds isolated from *Z. ligulatum* are similar to those found in *Z. mekongense*. In *Z. ligulatum*, we found eudesmane-type sesquiterpene monohydric alcohols (eudemols) and in *Z. mekongense* they found eudesmane-type sesquiterpene dihydric alcohols; 5αH-eudesmane-4α,11-diol and 5αH-eudesmane-4β,11-diol. In addition, a benzoic acid derivative; *n*-propyl *p*-hydroxybenzoate; which is an antifungal compound that inhibited growth of *Cladosporium* sp. (Chirawut 2005) was found in *Z. ligulatum*. Also in *Z. mekongense* they found docosyl *trans*-ferulate which was a ferulic acid derivative. The results of this work suggested that Z. *ligulatum* and Z. *mekongense* which were in same genus have some structural relationships i.e. flavonols and eudesmane-type sesquiterpene skeletons.

CONCLUSION

Two flavonols (kaempferol 7,4'-dimethyl ether and quercetin 7,4'-dimethyl ether) and benzoic acid derivative (*n*-propyl *p*-hydroxybenzoate) were isolated from *Z. ligulatum* ethanolic fresh rhizome extract. Fatty acid ethyl esters, elemene-type (elemol), eudesmane-type (β-eudesmol and α-eudesmol) sesquiterpenes and phytosterol (β-sitosterol) were also found. This work is the first report on chemical constituents of *Z. ligulatum*.

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