eProceedings Chemistry 4 (2019) 70-76

eISSN 2550-1453 http://eproceedings.chemistry.utm.my/

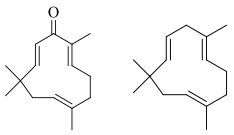
Phytochemicals and Biological Activities of Zingiber Zerumbet (L.) Smith.

Anamira Zakaria and Shajarahtunnur Jamil*

Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Malaysia
**Corresponding Author: shaja@kimia.fs.utm.my*

Article history:
Received 11 June 2019
Accepted 17 July 2019

GRAPHICAL ABSTRACT



Zerumbone α-Humulene

ABSTRACT

Zingiber zerumbet is one of the species belongs to the genus of Zingiber in the Zingiberaceace family. The phytochemicals of the crude extracts from the rhizome of Zingiber zerumbet were isolated and its biological activities were evaluated. Tyrosinase and acetylcholinesterase inhibition activities were screened on crude extracts and isolated compounds of the rhizome of Z. zerumbet. Phytochemical studies on the rhizome of Z. zerumbet have resulted in the isolation of two sesquiterpenoids. Their structures were determined as zerumbone (1) and α-humulene (2) by spectroscopic methods. All isolates were evaluated for the first time for antityrosinase and antiacetylcholinesterase activities. Antityrosinase activity of the samples was determined against mushroom tyrosinase using microplate reader. Zerumbone (1) and α-humulene (2) exhibited an excellent inhibitory activity against mushroom tyrosinase comparable to the standard kojic acid with 83% and 90% inhibition, respectively. Inhibition of acetylcholinesterase (AChE), tested by Ellman's method showed zerumbone (1) was active agents with percentage inhibition of 87%.

Keywords: Zingiber zerumbet, zerumbone, α-humulene. antityrosinase, antiacetylcholinesterase

1. INTRODUCTION

Natural products are typically secondary metabolites produced by the organisms in response to external stimuli such as nutritional changes, infection and competition. Natural products produced by plants have been extensively used in medical practices and have proved to be the major remedy in traditional system of medicine due to their biomedical benefits as well as place in cultural beliefs in many parts of world [1]. Plants of Zingiberaceae family are among the tropical plants that had shown interesting chemistry and biological activities. The family comprises about 52 genera and approximately 1400 species that form a significant element in the flora of the tropical region of Southeast Asia. The most investigated genera are Curcuma, Etlingera and Zingiber [2].

The genus Zingiber is a perennial plant comprises about 85 species of herbs found abundant mainly in Southeast Asia, the Pacific, Oceania and Malay Peninsula with medicinal, economic and horticulture significance. Plants in this genus are mostly sterile cultivars grown for the edible rhizomes and flowers are rarely seen. This genus is known worldwide for its economically important spice crops. The ginger species are ginger (Zingiber), galangal or Thai ginger (Alpinia galanga), myoga (Zingiber mioga), and turmeric (Curcuma).

Previous phytochemical studies of Zingiber species had revealed that this genus is rich source of terpenoids, flavonoids, aromatic compounds and kaempferol derivatives. Rhizomes of several Zingiber species have revealed the presence of bioactive compounds such as gingerols, shogaols, diarylheptanoids, phenylbutenoids, flavanoids, diterpenoids and sesquiterpenoids [3]. Many of the compounds have been reported to possess pharmacological potentials such as anti-inflammatory, antinociceptive, antiulcer and antioxidant [4]. Previous study on the Z. officinale had successfully isolated several monoterpene hydrocarbons. The components in ginger that are responsible for the antiemetic effect are thought to be the gingerol, shogaol and galanolactone [5]. Zingiber montanum rhizome contains volatile oil that contain the active compounds terpinen-4-ol and (E)-1-(3′,4′-dimethoxyphenyl)butadiene (DMPBD). This compound exhibit strong anti-inflammatory activity and are used to treat sprains, contusions, muscular pain, and inflammation related disorders [6]. 6-Gingerol isolated from Z. cassumunar has a notable inhibitory effect on oxidation and inflammatory activity [7].

On continuing research on Zingiber species, this study focused on the potential of the crude extracts and the isolated compounds from the rhizomes of Z. zerumbet for their tyrosinase and acetylcholinesterase inhibitory activities.

2. EXPERIMENTAL

2.1. Plant Material

The fresh rhizomes of *Zingiber zerumbet* were purchased from a wet market in Larkin, Johor Bahru. The rhizomes were cleaned and chopped into small pieces. The sample was kept in dried condition.

2.2. Extraction and Isolation

The fresh rhizomes (900 g) of *Z. zerumbet* were cleaned and chopped The chopped rhizomes of *Z. zerumbet* were macerated twice with different polarity of solvent starting from hexane and dichloromethane sequentially at room temperature for 48 hours. Evaporation of each solvent gave hexane crude extracts (ZZRH) and dichloromethane crude extract (ZZRD) which were then fractionated to afford different fractions based on solvent polarity by column chromatography. The hexane crude extract (0.85 g) gave three (ZZRH1 - ZZRH3) major fractions. Purification of ZZRH2 gave **(1)** (0.14 g, 16.5%) corresponding as reddish solid with melting point 61 – 63˚C. The dichloromethane crude extracts (ZZRD) (4.30 g) was fractionated to give five (ZZRD1 - ZZRD5) major fractions. Compound **(2)** (0.17 g, 4.0%) was isolated from ZZRD2 as colorless liquid.

Zerumbone **(1)**: R_f= 0.58 (PE: EtO_2=3:2). IR spectrum ν_{max} cm^{-1} 3026 (sp^2 CH), 2922 and 2964 (sp^3 CH), 1656 (conjugated C=O), 1620 (C=C). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.09 (3H, s, H-14), 1.23 (3H, s, H-15), 1.56 (3H, s, H-12), 1.82 (3H, s, H-13), 1.93 (1H, d, *J*=12.0 Hz, H-1), 2.26 (1H, m, H-1a, H-4a), 2.38 (2H, m, H-4b, H-5a), 2.47 (1H, m, H-5b), 5.28 (1H, d, *J*= 12.0 Hz, H-2), 5.88 (1H, d, *J*=16.4 Hz, H-10), 5.98 (1H, d, *J*=16.4 Hz, H-9), 6.04 (1H, brd. d, *J*= 12.4 Hz, H6). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 11.8 (CH_3, C-13), 15.2 (CH_3, C-12), 29.4 (CH_3, C-14), 24.2 (CH_3, C-15), 24.4 (CH_2, C-5), 37.9 (C, C-11), 39.5 (CH_2, C-4), 42.4 (CH_2, C-1), 125.0 (=CH, C-2), 127.2 (=CH, C-9), 136.3 (C=C, C-3), 138.0 (C=C, C-7), 148.7 (=CH, C-6), 160.7 (C=C, C-10), 204.3 (C=O, C-8).

α-Humulene **(2)**: R_f= 0.80 (PE: EtO_2=3:2). IR spectrum ν_{max} cm^{-1} 3020 (sp^2 CH), 2930 and 2960 (sp^3 CH), 1678 (C=C). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.12 (6H, s, H-13, H-14), 1.45 (3H, s, H-15), 1.65 (3H, s, H-11), 1.93 (2H, m, *J*=7.6 Hz, H-7), 2.14 (4H, m, H-10, H-11), 2.55 (2H, d, *J*= 8.0 Hz, H-3), 4.89 (1H, m, H-8), 4.99 (1H, m, H-1), 5.16 (1H, d, *J*= 16.0 Hz, H-5) and 5.59 (1H, m, H-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 14.8 (CH_3, C-15), 17.2 (CH_3, C-12), 17.9 (CH_3, C-13), 23.3 (CH_2, C-11), 27.1 (CH_3, C-14), 37.3 (C, C-6), 39.7 (CH_2, C-10), 40.4 (CH_2, C-3), 42.0 (CH_2, C-7), 125.0 (=CH, C-8), 125.9 (=CH, C-1), 127.7 (=CH, C-4), 133.1 (C=C, C-9), 139.2 (C=C, C-2), 141.0 (=CH, C-5).

2.3. Tyrosinase Inhibitory Activity

Tyrosinase inhibition assay with slight modifications was performed by using the dopachrome method by Likhitwitayawuid *et al* and Kamken *et al*, with minor modifications [8, 9]. A flat–bottomed 96 well plate was used and each well contained 0.1 M phosphate buffer solution (pH 6.8) and L-DOPA. Four wells labelled as well 1 and well 2 contained sample stock solution (40 µL). Kojic acid was used as the positive control in this assay while DMSO acted as negative controlled and it replaced the sample in well 3. The plate was incubated for 10 minutes at 25°C. Tyrosinase enzyme was then added to well 1 and well 4. The plate was the incubated again for 20 minutes at 25°C. Lastly, the absorbance of the tested samples was measured using ELISA microplate reader at 475 nm with 700 nm as reference. The activity was screened as the sample concentration that gave a 70% enzyme activity. The percentage of tyrosinase inihibition was calculated as follows:

$$\text{Percentage of Tyrosinase inhibitor} = ((4\text{-}3) - (1\text{-}2))/(4\text{-}3) \times 100\%$$

1: absorbance of sample solution with enzyme.
2: absorbance of sample solution without enzyme.
3: absorbance of blank solution without enzyme
4: absorbance of blank solution with enzyme.

2.4. Acetylcholinesterase Inhibitory Activity

Ellman's method was used for AChE inhibitory activity of the crude extract and isolated compound from rhizome of *Zingiber zerumbet* [10]. Concisely, 140 µl of 0.1 M sodium phosphate buffer, with pH 8 and 20 µl of 1 mg/ml tested sample solution mixed with enzyme (AChE). The Amount of AChE used was 15 µl with activity of 0.25 U/ml. The reaction was started by adding 10 µl of 0.01 M DTNB and they were pre incubated at 25°C for 15 min. The sample and blank were then added with 10 µl of 0.075 M of ATCI and incubated at 25°C for another 15 min. Galantamine was used as positive control in this experiment. Lastly, the absorbance of the tested samples was measured using ELISA microplate reader at 412 nm. The percentage of inhibitory activity was calculated by using formula:

$$\text{Percentage of AChE inhibitor} = (\text{Absorbance of sample}/\ \text{Absorbance of control}) \times 100\%$$

3. RESULTS AND DISCUSSION

3.1. Extraction and Isolation

The fresh rhizomes of *Zingiber zerumbet* (900.0 g) were extracted using cold extraction method to yield hexane and dichloromethane crude extracts. Purification and isolation of the hexane crude extract of the rhizomes yielded two sesquiterpenes identified as zerumbone **(1)** and α-humulene **(2)**. Zerumbone **(1)** was purified and isolated from the hexane crude extract while α-humulene **(2)** was isolated from the dichloromethane crude extract of the rhizomes. The structures of the isolated compounds were fully characterized on the basis of their spectroscopic data and direct comparison with published reports.

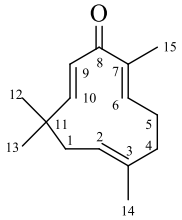


Figure 1. Structure of zerumbone (1)

The IR spectrum of compound **(1)** displayed the characteristic bands for sp^2 CH at 3026 cm^{-1}, two absorption bands of sp^3 CH at 2964 and 2922 cm^{-1}, and strong absorption of conjugated C=O at 1656 cm^{-1} for carbonyl group of α,β-unsaturated ketone. There is also absorption band at 1620 cm^{-1} corresponding to the presence of C=C. The ^{1}H NMR spectrum of compound **(1)** showed the presence of four methyl singlets at δ 1.09, 1.23, 1.56 and 1.82 representing H-14, H-15, H-12 H-13, respectively. Doublet signals observed at δ 5.28 (*J*=12.0 Hz) and 6.04 (*J*=12.4 Hz) exhibited the characteristic of olefenic protons assigned to H-2 and H-6 respectively. A doublet signal at δ 1.93 (*J*=12.0 Hz), 5.88 (*J*=16.4 Hz) and 5.98 (*J*=16.4 Hz) showed the presence of vinyl proton at H-1, H-9 and H-10. Three methylene protons were observed at δ 2.26, 2.38 and 2.47 indicating H-1, H-4 and H-5 with all having multiplet signals.

Table 1. ^{1}H NMR data of compound **(1)**

Carbon	δ ppm Compound (1)	δ ppm Zerumbone (1) [11]
1	1.93 (1H, d, *J*=12.0 Hz)	1.90 (lH, d, *J*=12.0 Hz)
	2.38 (2H, m)	2.34 (2H, m)
2	5.28 (1H, d, *J*=12.0 Hz)	5.25 (1H, dd, *J* = 3.0 Hz, *J*=12.0 Hz)
3	-	-
4	2.38 (1H, m)	2.34 (1H, m)
	2.26 (1H, m)	2.22 (1H, m)
5	2.47 (1H, m)	2.44 (lH, m)
	2.26 (1H, m)	2.22 (1H, m)
6	6.04 (1H, brd. d, *J*=12.4 Hz)	6.01 (1H, dd, *J* =2.0 Hz, *J* =11.5 Hz,)
7	-	-
8	-	-
9	5.98 (1H, d, *J*=16.4 Hz)	5.98 (IH, d, *J*=17.0 Hz)
10	5.88 (1H, d, *J*=16.4 Hz)	5.86 (lH, d, *J*=17.0 Hz)
11	-	-
12	1.56 (3H, s)	1.54 (3H, s)
13	1.82 (3H, s)	1.80 (3H, s)
14	1.09 (3H, s)	1.07 (3H, s)
15	1.23 (3H, s)	1.20 (3H, s)

The ^{13}C NMR spectrum of compound **(1)** showed 15 signals which corresponded to 15 carbons in the molecule. The NMR spectrum were obtained under condition of proton decoupling. Analysis of the spectrum showed four methyl groups C-12 (15.2), C-13 (11.8), C-14 (29.4), C-15 (24.2), four olefinic carbons C-2 (125.0), C-6 (148.7), C-9 (127.2) and C-10 (160.7), three secondary carbon C-1 (42.4), C-4 (39.5) and C-5 (24.4), one tertiary carbon C-6 (148.7), three quaternary carbon C-3 (136.3), C-7 (138.0) and C-11 (37.9) and also one carbonyl group C-8 (204.3). The IR and NMR spectra indicated a sesquiterpene backbone structure. Comparison of the spectrum with previously published data [33] showed that compound **(1)** is zerumbone.

Table 2. ^{13}C NMR data of compound **(1)**

Carbon	δ ppm Compound (1)	δ ppm Zerumbone (1) [33]
1	42.4	42.4
2	125.0	125.0
3	136.3	136.2
4	39.5	39.5
5	24.4	24.4
6	148.7	148.8
7	138.0	138.0
8	204.3	204.4
9	127.2	127.2
10	160.7	160.8
11	37.9	37.9
12	15.2	15.2
13	11.8	11.8
14	29.4	29.4
15	24.2	24.2

The IR spectrum of compound **(2)** displayed the characteristic bands for sp^2 CH at 3020 cm^{-1}, two absorption bands of sp^3 CH at 2930 and 2960 cm^{-1}. There is also absorption band at 1678 cm^{-1} corresponding to the presence of C=C. The ^{1}H NMR spectrum of compound (**2**) showed the presence of two singlet signals indicated the presence of geminal dimethyl group (C-13 and C-14) with δ 1.12 for both. A multiplet signal at δ 4.99 was attributed to methyl group on trisubtituted double bond at C-1, while a singlet signal at δ 4.89 demonstrate the presence of methyl group at C-8. Two doublet signals at δ 1.93 (2H, d, *J*=7.6 Hz) and 2.55 (2H, d, *J*=8.0 Hz) indicate the presence of vinyl proton at H-7 and H-3 respectively. A methylene group with multiplet signal at δ 2.14 was observed for H-10 and H-11. Olefinic proton was observed at signal δ 5.16 at C-5 (*J*=16.0 Hz). Other olefinic proton with multiplet signal were observed at δ 4.99 which assigned as H-1, δ 4.89 as H-3 and δ 5.59 as H-4.

Table 3. ^{1}H NMR data of compound **(2)**

Carbon	δ ppm Compound (2)	δ ppm α-humulene (2) [12]
1	4.99 (1H, m)	5.02 (1H, m)
2	-	-
3	2.55 (2H, d, *J*=8.0 Hz)	2.55 (2H, d, *J*=7.4 Hz)
4	5.59 (1H, m)	5.62−5.56 (1H, m)
5	5.16 (1H, d, *J*=16.0 Hz)	5.13 (1H, d, *J*=15.8 Hz)
6	-	-
7	1.93 (2H, d, *J*=7.6 Hz)	1.97 (2H, d, *J*=7.3 Hz)
8	4.89 (1H, m)	4.94 (1H, m)
9		
10	2.14 (2H, m)	2.18− 2.11 (4H, m)
11	2.14 (2H, m)	2.18− 2.11 (4H, m)
12	1.65 (3H, s)	1.69 (3H, s)
13	1.12 (3H, s)	1.11 (6H, s)
14	1.12 (3H, s)	1.11 (6H, s)
15	1.45 (3H, s)	1.50 (3H, s)

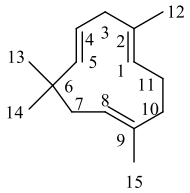


Figure 2. Structure of α-humulene (**2**)

The ^{13}C NMR spectrum of compound (**2**) was obtained under condition of proton decoupling and comparison with literature value [13]. Analysis of the spectrum showed four methyl groups C-12 (17.2), C-13 (17.9), C-14 (27.1), C-15 (14.80), two disubtituted carbons C-3 (40.4) and C-7 (42.0), four olefinic carbons C-1 (125.9), C-4 (127.7), C-5 (141.0) and C-8 (125.0) as well as three quarternary carbon C-2 (139.2), C-6 (37.3) and C-9 (133.1). Based on the IR, ^{1}H and ^{13}C NMR spectral data together with comparison with previous report, compound (**2**) was identified as α-humulene **(2)**.

Table 4. ^{13}C NMR data of compound **(2)**

Carbon	δ ppm Compound (2)	δ ppm α-humulene (2) [13]
1	125.9	125.8
2	139.2	139.1
3	40.4	40.4
4	127.7	127.7
5	141.0	140.9
6	37.3	37.2
7	42.0	41.9
8	125.0	124.9
9	133.1	133.0
10	39.7	39.7
11	23.3	23.3
12	17.2	17.6
13	17.9	17.8
14	27.1	26.8
15	14.8	14.8

3.2. Tyrosinase Inhibitory Activity

Table 5 presented the percentage inhibition of tyrosinase at 1000 µg/mL for the crude extracts and isolated compounds. The hexane (ZZRH) and dichloromethane (ZZRD) crude extracts showed strong tyrosinase inhibition with 81% and 97% inhibition respectively comparable to the positive control, kojic acid with the percentage inhibition 96%. The isolated compounds, zerumbone **(1)** and α-humulene **(2)** also displayed a high percentage of inhibition of 83% and 90%. Therefore, this plant is a potential source of active compounds for tyrosinase inhibitory activity.

Table 5. Percentage inhibition of Tyrosinase inhibitory activity of crude extracts and isolated compounds

Samples	Inhibition at 1000µg/mL (%)
Crude Extracts	
ZZRH	81
ZZRD	97
Isolated Compounds	
Zerumbone **(1)**	83
α-Humulene **(2)**	90
Positive Control	
Kojic Acid	75

Based on previous research, the tyrosinase inhibitory activity of compounds drives in part from hydrophobic interaction with the hydrophobic protein pocket surrounding the binuclear copper active site in the enzyme [14]. The hydrophobic group, which is exposed on the outer side of the molecule, may disrupt tyrosinase's quaternary structure. The low conformational stabilities of native proteins make them easily susceptible to denaturation by altering the balance of the weak nonbonding forces that maintain the native conformation. On close inspection of the inhibitory activity exerted by the compound, hydrophobicity of α-humulene **(2)** is higher, which led to significantly stronger inhibitory activity than that of zerumbone **(1).**

3.3. Acetylcholinesterase Inhibitory Activity

Table 6 presented the percentage inhibition of acetylcholinesterase at 1000 µg/mL for the crude extracts and isolated compounds. The hexane (ZZRH) and dichloromethane (ZZRD) crude extracts as well as α-humulene **(2)** gave low inhibition of acetylcholinesterase activity with 6%, 28% and 14% inhibition respectively, whereas zerumbone **(1)** displayed a significant antiacetylcholinesterase activity with percentage of inhibition 87%, making it a promising candidate to be used as cholinesterase inhibitors in clinical practice.

Table 6. Percentage of Acetylcholinesterase Inhibitory Activity of Crude Extracts and Isolated Compounds

Samples	**Inhibition at 1000µg/mL (%)**
Crude Extracts	
ZZRH	6
ZZRD	28
Isolated Compounds	
Zerumbone **(1)**	87
Humulene **(2)**	14
Positive Control	
5,5′-Dithiobis-(2-nitrobenzoic acid)	98

This study showed that the presence of ketone group that more closely resembling ethyl acetate, making it a good inhibitors and transition state analogues for acetylcholinesterase [15]. Thus explaining the significantly stronger inhibitory activity of zerumbone **(1)** compared than that of α-humulene **(2)** by the presence of ketone group on its structure.

4. CONCLUSION

The crude extracts and the two isolated sesquiterpene from rhizomes of *Z. zerumbet* were evaluated for their tyrosinase and cholinesterase inhibitory activity. The crude extracts, ZZRH and ZZRD gave positive potential on inhibition of tyrosinase activity as well as the isolated compounds, zerumbone **(1)** and humulene **(2)** by 90% and 83% respectively. While the cholinesterase inhibitory activity of the crude extracts (ZZRH and ZZRD) as well as humulene **(2)** gave low inhibition, whereas zerumbone **(1)** exhibit the potential as the cholinesterase inhibitor with 87% inhibition on the enzyme. The tyrosinase inhibitory activity of these compounds depends on the hydrophobicity groups of the molecules and influenced by configuration of other substituent. While the presence of ketone group that more closely resembling ethyl acetate make a compound to be a good inhibitors and transition state analogues for acetylcholinesterase.

.

REFERENCES

[1] Ali, B. H., Blunden, G., Tanira, M. O., and Nemmar, A. Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale* Roscoe): a review of recent research. *Food and chemical Toxicology*, 46(2) (2008) 409-420.

[2] Mascolo, N., Jain, R., Jain, S. C., and Capasso, F. Ethnopharmacologic investigation of ginger (*Zingiber officinale*). *Journal of ethnopharmacology*, 27(1) (1989) 129-140.

[3] Sharifi-Rad, M., Varoni, E., Salehi, B., Sharifi-Rad, J., Matthews, K., Ayatollahi, S. and Sharifi-Rad, M. Plants of the genus *Zingiber* as a source of bioactive phytochemicals: From tradition to pharmacy. *Molecules*, 22(12) (2017) 2145.

[4] Singh, G., Maurya, S., Catalan, C., and De Lampasona, M. P. Studies on essential oils, Part 42: chemical, antifungal, antioxidant and sprout suppressant studies on ginger essential oil and its oleoresin. *Flavour and Fragrance Journal*, 20(1) (2005) 1-6.

[5] Das, A., Kasoju, N., Bora, U., and Rangan, L. Chemico-biological investigation of rhizome essential oil of *Zingiber moran*—native to Northeast India. *Medicinal Chemistry Research*, 22(9) (2013) 4308-4315.

[6] Anasamy, T., Abdul, A.B, Sukari, M.A., Abdelwahab, S.I., Mohan, S., Kamalidehghan, B., Azid, M.Z. A Phenylbutenoid Dimer, cis-3-(30 ,40 -Dimethoxyphenyl)-4-[(E)-3000,4000-Dimethoxystyryl] Cyclohex-1-ene, Exhibits Apoptogenic Properties in T-Acute Lymphoblastic Leukemia Cells via Induction of p53-Independent Mitochondrial Signalling Pathway. *The Journal of Alternative and Complementary Medicine* (2013) 14-16.

[7] Agrawal, A. K., Rao, C. V., Sairam, K., and Joshi, V. K. Antipyretic and analgesic activities of *Zingiber zerumbet* extracts. *Indian Journal of Experimental Biology*, 38 (2000) 994-98.

[8] Kamkaen, N., Mulsri, N., Treesak, C. Screening of Some Tropical Vegetables for Anti-tyrosinase Activity. *Thai Pharmacy & Health Science Journal*. 2 (2007) 15–19.

[9] Likhitwitayawuid, K., Sritularak, B. A New Dimericstilbene with Tyrosinase Inhibitory Activity from *Artocarpus gomeizianus*. *Journal Natural Product*. 64 (2001) 1457–1459.

[10] Ellman, G. L., Courtney, K. D., Andres, Jr. V. and Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochemical Pharmacology*. 7 (1961) 88-95.

[11] Dai, J. R., Cardellina, J. H., Mahon, J. B. M., and Boyd, M. R. Zerumbone, an HIV-inhibitory and cytotoxic sesquiterpene of *Zingiber aromaticum* and *Z. zerumbet*. *Natural Product Letters*, 10(2) (1997) 115-118.

[12] Neuenschwander, U., Czarniecki, B., and Hermans, I. Origin of regioselectivity in α-humulene functionalization. *The Journal of organic chemistry*, 77(6) (2012) 2865-2869.

[13] Saewan, N., Koysomboon, S., and Chantrapromma, K. Antityrosinase and Anti-cancer Activities of Flavonoids from *Blumea balsamifera* DC. *Journal of Medicinal Plants Research*, 5(6) (2011) 1018-1025.

[14] Freeman, J. V., Dewey, F. E., Hadley, D. M., Myers, J., and Froelicher, V. F. Autonomic nervous system interaction with the cardiovascular system during exercise. *Progress in cardiovascular diseases*, 48(5) (2006) 342-362.

[15] Allen, K. N., and Abeles, R. H. Inhibition kinetics of acetylcholinesterase with fluoromethyl ketones. *Biochemistry*, 28(21) (1989) 8466-8473.