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Identification of hydroxychavicol and its dimers, the
lipase inhibitors contained in the Indonesian spice,
Eugenia polyantha

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Abstract

Inhibition of pancreatic lipase is effective for a prevention of obesity. *Eugenia polyantha* is a tropical tree whose leaves are known as a spice and also as an ingredient for Jamu, the traditional medicine of Indonesia. We found inhibitory activity against pancreatic lipase in the extract of *E. polyantha* leaves. Purification of the active principals resulted in isolation of hydroxychavicol, and two structurally new dimers. All of the isolated compounds showed inhibitory activity against the porcine pancreatic lipase and high content of hydroxychavicol (1.83 wt%) indicated this compound to be responsible for the majority of inhibitory activity of *E. polyantha* extract. Furthermore, hydroxychavicol is reported to possess anti-carcinogenic, anti-oxidant, anti-microbial and anti-inflammatory activity which is related to traditional usage of this plant. These results offer this plant as an attractive material for treating various health problems including obesity.

Keywords: *Eugenia polyantha*; pancreatic lipase inhibitor; obesity; spice; jamu

1. Introduction

Obesity is caused by an excess in energy intake compared with energy expenditure which leads to accumulation of fat in the body. This excess energy source is frequently derived from the fat contained in the food itself. Thus, to reduce fat accumulation, the digestion and absorption of fat into the body should be prevented (Hanefeld & Sachse, 2002). Pancreatic lipase is the most widely used target enzyme to prevent fat digestion. Orlistat (Xenical), the best-known highly active synthetic pancreatic lipase inhibitor, is currently approved and used as an anti-obesity drug in several countries (Filippatos, Derdemezis, Gazi, Nakou, Mikhailidis, & Elisaf, 2008; Hanefeld & Sachse, 2002; Henness & Perry, 2006).

In addition to synthetic drugs, expanding population of obesity state patients required convenient and easily accessible agent for a treatment or a prevention of obesity. For this purpose, foods and food additives are accepted as a suitable form, and various plants including traditional medicinal plants that are known to be used against obesity, are widely studied as the target of research (Ahn, Liu, Lee, Ahn, Yoo, Hwang, et al., 2012; Birari & Bhutani, 2007; de la Garza, Milagro, Boque, Campion, & Martinez, 2011; Jang, Lee, Kim, Lee, Kim, Kim, et al., 2008; Lee, Lee, Chung, Cho, Lee, Ahn, et al., 2010).

Eugenia polyantha, or a synonym *Syzygium polyanthum*, is a deciduous tropical tree belonging to the Myrtaceae family. The dried leaves of this plant are known by the name of daun salam in Indonesia and are used as a spice from their aromatic smell and somewhat sour taste. The leaves are also utilized as an ingredient in the Indonesian traditional medicine “Jamu”, and is known to be effective for treatment of ulcer, diabetes, inflammation, and diarrhea (Agus & Agustin, 2008).

Despite its familiar characteristics in Indonesia, the chemical constituents responsible for the beneficial effects of *E. polyantha* have not much been studied.

In the course of study to search for effective anti-obesity plants, we identified *E. polyantha* as a promising material with high inhibitory potential against pancreatic lipase. In this paper, we report the isolation and activity of chemicals responsible for the pancreatic lipase inhibitory activity of *E. polyantha*.

2. Material and methods

2.1. General Experimental Procedures

Dried leaves of *E. polyantha* were obtained from Merapi Farma Traditional Herbs Distributor (Yogyakarta, Indonesia). Commercially available chemicals were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan) and used without further purification unless otherwise noted. Inertsustain C18 ($\phi 4.6 \times 250$ mm) was obtained from GL Science Inc. (Tokyo, Japan). Bruker AMX500 was used to obtain NMR spectra, and TMS or the residual solvent peak was used as an internal standard (^1H NMR: TMS 0.00 ppm; ^{13}C NMR: CDCl_3 77.0 ppm). Thermo Scientific Exactive (ESI-MS) was used to obtain mass spectra. Absorbance was measured by the SynergyTM MX (BioTek Instruments Inc., Winooski, United States) microplate reader.

2.2. Extraction and Isolation of *E. polyantha*

Isolation procedure is detailed in §3.1. and Fig. 1. Activity guided fractionation was performed using the unit **n** as a concentration. 1**n** is a concentration when each fraction is dissolved in [g of extracted material $\times$ 10] mL of solvent representing the concentration in the material. The activity of each fraction was as followings. Aqueous layer, 26% at 0.1**n**; Ethyl Acetate layer,

83% at 0.1n; Fr. 1, 100% at 1n; Fr. 2, 41% at 1n; Fr. 3, 26% at 1n; Fr. 4, 50% at 1n; Fr. 1-1, 40% at 1n; Fr. 1-2, 52% at 1n; Fr. 1-3, 40% at 1n; Fr. 1-4, 97% at 1n.

Hydroxychavicol (1): ESI-MS (negative): m/z 149 [M-H]⁻; ¹H NMR (500 MHz, CDCl₃, rt): 6.75 (1H, d, J = 8.1 Hz, H-1), 6.67 (1H, d, J = 1.7 Hz, H-3), 6.57 (1H, dd, J = 8.1, 1.7 Hz, H-2), 5.94-5.80 (1H, m, H-6), 5.05-5.00 (2H, m, H-7, 8), 3.20 (2H, d, J = 6.7 Hz, H-4, 5).

4-Allyl-1-hydroxy-2-(2'-allyl-4'-hydroxy-5'-methoxyphenoxy)benzene (2): HRMS and NMR data, see main text and Table 1; IR (film) $\nu_{\max}$ 3449, 2927, 1638, 1604, 1443, 1361, 1267, 1226 cm⁻¹.

4-Allyl-2-hydroxy-1-(2'-allyl-4'-hydroxy-5'-methoxyphenoxy)benzene (3): HRMS and NMR data, see main text and Table 1; IR (film) $\nu_{\max}$ 3441, 2925, 1596, 1502, 1443, 1353, 1269 cm⁻¹.

2.3. Lipase Inhibitory Activity Assay

Lipase inhibitory activity was performed by Han's method with modifications (Han, Takaku, Li, Kimura, & Okuda, 1999). An emulsified solution of L- α -lecithin (Sigma-Aldrich; P4279, 10 mg), triolein (16 mg), and sodium taurocholate (5 mg) in Tris buffer (Tris 13 mM, NaCl 150 mM, CaCl₂ 1.3 mM, pH 8.0, 9.0 mL) was used as a substrate. Porcine pancreatic lipase (Sigma, L3126, 4.5 mg) was dissolved in Tris buffer and used as an enzyme solution. The substrate (100 μ L) and the sample (50 μ L, in 50% aq. DMSO) were preincubated at 37°C for 5 min. The reaction was started by the addition of an enzyme solution (50 μ L) and allowed to continue for 30 min at 37°C. The reaction was then stopped by the addition of orlistat (1 mg/mL, 50 μ L), and the liberated oleic acids were extracted by hexane (400 μ L). The hexane layer (200 μ L) was dried, dissolved in DMSO (100 μ L), and the oleic acid was quantitated by NEFA C-test Wako (Wako Pure Chem.

Ind. Ltd). Orlistat ($IC_{50} = 0.22 \mu M$) was used as a positive control. The assay was performed twice for each sample, and the mean value was used to evaluate IC_{50} .

3. Results

3.1. Isolation of lipase inhibitory activity principal from *E. polyantha*

The purification procedure of *E. polyantha* is summarized in Fig. 1. The 50% aq. methanol extract of dried *E. polyantha* leaves showed 73% inhibitory activity at 0.1n (2.75 mg/mL) against porcine pancreatic lipase. The crude extract was partitioned between ethyl acetate and water. Ethyl acetate layer showed higher activity, and was further purified by the silica-gel column with stepwise elution by 2:1 hexane/ethyl acetate, 1:1 hexane/ethyl acetate, ethyl acetate and methanol (1800 mL each). The most active fraction 1 eluted by 2:1 hexane/ethyl acetate was again separated by silica-gel column chromatography with chloroform/methanol (60:1, 30:1, and 4:1, 300 mL each) as an eluent. The eluate was fractionated, analyzed by TLC and combined as four fractions (Fr. 1-1 to 1-4) according to their TLC profiles. Fraction 1-2 and 1-4, which showed higher activity compared to other two, was selected for further purification and analysis of its component. Fr. 1-4 with highest activity was analyzed by thin layer chromatography (TLC) and NMR, and was confirmed to be consisted of a single compound (**1**, 730.3 mg). Fraction 1-2 was further purified by preparative TLC (2:1 hexane/EtOAc) and then by HPLC to obtain **2** (1.1 mg) and **3** (0.9 mg) as a brownish oil (Fig. 2).

3.2. Structure analysis of isolated compounds

The molecular weight of **1** was determined by ESI-MS to be 150 (negative, m/z 149 $[M-H]^-$). Analysis of the 1H NMR spectroscopic data revealed three signal (a doublet with $J = 1.7$ Hz, a

doublet with $J = 8.1$ Hz and a double doublet with $J = 1.7, 8.1$ Hz) in the aromatic region indicating 1,2,4-trisubstituted aromatic ring. The additional characteristic signal of multiplets at $\delta = 5.94$ - 5.80 and 5.05 - 5.00 and doublet at $\delta = 3.20$ indicated the presence of an allyl group. Further analysis using ^{13}C NMR spectroscopic data indicated **1** to be 4-allyl-1,2-dihydroxybenzene, commonly named hydroxychavicol, which was finally confirmed by comparing the data with the reported data (Fache, Suzan, & Piva, 2005).

The molecular formula of **2** was analyzed by HR-ESI-MS and determined to be $\text{C}_{19}\text{H}_{20}\text{O}_4$ (found m/z 311.1294 $[\text{M-H}]^-$, calcd. for $\text{C}_{19}\text{H}_{19}\text{O}_4$ 311.1289). Analysis of the $^1\text{H}/^{13}\text{C}$ NMR spectroscopic data of **2** indicated the presence of two sets of an aromatic ring and an allyl group which was presumed as the dimer of **1**. Coupling pattern of the ^1H NMR signal showed that one of the aromatic rings is 1,2,4-trisubstituted and the other is 1,2,4,5-tetrasubstituted. This also indicated that the two monomers are dimerized through the ether bond, using one of the hydroxyl groups of first monomer to connect with the second monomer at the aromatic carbon beside the allyl group. Also, an additional methyl group was seen from ^1H NMR spectra substituting one of the three free hydroxyl group. To determine the position of the ether bond between two monomers, the COSY, HMQC, and HMBC spectra of **2** were analyzed and all the signals were assigned (Table 1). Representative HMBC correlations are summarized in Fig. 3. The correlation from 1-OH ($\delta = 5.60$) to C-6 ($\delta = 115.4$) indicated 1-OH is free and, alternatively, 2-OH is used to form the ether bond between C-1'. The position of methylation was also determined from HMBC correlations. Correlation from the methyl group ($\delta = 3.78$) to C-5' ($\delta = 145.5$), and from 4'-OH ($\delta = 5.42$) to C-3' ($\delta = 115.83$) indicated the methylation of 5'-OH. From the overall results, the structure of **2** was determined as 4-Allyl-1-hydroxy-2-(2'-allyl-4'-hydroxy-5'-methoxyphenoxy)benzene which was a new compound to be reported.

The molecular formula of **3** was analyzed by HR-ESI-MS and determined to be C₁₉H₂₀O₄ (found m/z 311.1292 [M-H]⁻, calcd. for C₁₉H₁₉O₄ 311.1289). Analysis of the ¹H/¹³C NMR spectroscopic data of **3** indicated the presence of 1,2,4-trisubstituted and 1,2,4,5-tetrasubstituted aromatic ring, which was similar to **2**. All the signals of **3** assigned from the COSY, HMQC, and HMBC spectra are listed in Table 1, and HMBC correlations are summarized in Fig. 3. The correlation from 2-OH (δ = 5.68) to C-3 (δ = 115.79) indicated that compound **3** is the regioisomer of **2**, with a difference in the ether bond position. All the other HMBC correlations indicated this compound to be 4-allyl-2-hydroxy-1-(2'-allyl-4'-hydroxy-5'-methoxyphenoxy)benzene which was also a new compound.

3.3. Pancreatic lipase inhibitory activity of isolated compounds

The lipase inhibitory activity of the isolated compounds was tested against porcine pancreatic lipase (Fig.4). The inhibition curve of **1** showed linearity between the activity and the concentration, but for **2** and **3**, inhibitory activity did not correlate with the concentration at high concentration. The inhibitory activity of **2** and **3** reached maximum at concentrations higher than 1 mM for **2** and 0.5 mM for **3**. The IC₅₀ values of these two compounds were not determined owing to their low isolated yields and non-linear inhibition curve. Also, since eugenol (4-allyl-1-hydroxy-2-methoxybenzene) is reported as the constituent of *E. polyantha*, we tested the inhibitory activity of this compound together with 4-allyl-1,2-dimethoxybenzene. However, these two showed a scarce inhibitory activity (eugenol: IC₅₀ = 3.2 mM; 4-allyl-1,2-dimethoxybenzene: IC₅₀ = >5.0 mM).

4. Discussion

We found high inhibitory potential against pancreatic lipase in the extract of *E. polyantha*, the spice used in Indonesia. Activity guided fractionation resulted in isolation of three aromatic lipase inhibitors, hydroxychavicol (**1**) and new dimers **2** and **3**. Considering the high content of hydroxychavicol (**1**) in *E. polyantha* (1.83 wt% in dry leaf), this compound should be contributing to the majority of lipase inhibitory activity of the plant extract. Dimers **2** and **3** also showed inhibitory activity against pancreatic lipase, but unlike **1**, these compounds did not show linearity between concentration and inhibitory activity. The constant activity at high concentration is presumed to be due to low solubility in testing solution which reflects the effect of masked hydroxyl group by methylation and dimerization. This was supported by the results of eugenol and 4-allyl-1,2-dimethoxybenzene whose activities are limited by the additional methyl group which induces low solubility to the compounds.

Isolation of **1** is reported from *Piper betle* and *Dracaena cambodiana* (Luo, Wang, Xu, Mei, & Dai, 2010; Murata, Nakao, Hirata, Namba, Nomi, Kitamura, et al., 2009). *P. betle* is a plant used as a part of Indian traditional medicine, Ayurveda. The extract of this plant is traditionally used for disorders of respiratory tract like inflammation, infection, cough and dyspnea as well as for indigestion, diphtheria and hysteria (Nagori, Singh, Alexander, Kumar, Dewangan, Badwaik, et al., 2011). *D. cambodiana* is a material for the production of Dragon's blood that is known as a traditional medicine in the ancient Arab culture. Dragon's blood has been used for the treatment of wounds, leucorrhea, fractures, diarrhea, and intestinal/stomach ulcers (Luo, Wang, Xu, Mei, & Dai, 2010). *P. betle* is widely studied and modern studies of this plant revealed various positive effects in the extract including anti-carcinogenic, anti-oxidant, anti-microbial and anti-inflammatory effect (Chakraborty, Mahato, Joshi, Shinde, Rakshit, Biswas, et al., 2012; Murata, et al., 2009; Pandey & Bani, 2010; Saifudin, Kadota, & Tezuka, 2012; Sharma, Khan, Ali, Ali,

Kumar, Kumar, et al., 2009). And in these studies, hydroxychavicol (**1**) is identified as an active component. Therefore, the traditional use of *E. polyantha* as the ingredient in Jamu against ulcer, inflammation, and diarrhea can be explained by the activity of **1** in this plant. In addition, the finding of **1** as a pancreatic lipase inhibitor in this report may connects this compound with the anti-diabetic use of *E. polyantha*, since obesity have a high influence on diabetes development (Ashcroft & Rorsman, 2012).

In conclusion, three phenolic pancreatic lipase inhibitors **1-3** were isolated from *E. polyantha*. Although the activities of the isolated compounds were mild, the abundant content of hydroxychavicol (**1**) in this spice makes it quite attractive as a food additive for the treatment and prevention of obesity. Furthermore, various studies reported the beneficial effect of **1**. These reports and the results obtained here indicate that the traditional use of *E. polyantha* against ulcer, inflammation, diabetes and diarrhea largely attributes to the presence of **1** in this plant.

Supplementary data

^1H and ^{13}C NMR, COSY, HMQC, and HMBC spectra of **2** and **3**.

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Figures and Tables

Table 1. ^1H (500 MHz, rt) and ^{13}C (125 MHz, rt) NMR Spectroscopic Data for **2** and **3** in CDCl_3

	2		3	
position	δ_{H} (mult., J in Hz)	δ_{C}	δ_{H} (mult., J in Hz)	δ_{C}
1		144.5		143.5
2		144.9		146.1
3	6.45 (d, 1.7)	115.89	6.86 (d, 2.0)	115.79
4		132.2		135.4
5	6.77 (dd, 1.7, 8.2)	123.2	6.58 (dd, 2.0, 8.3)	120.2
6	6.94 (d, 8.2)	115.4	6.54 (d, 8.3)	115.6
7	3.21 (d, 6.5)	39.5	3.31 (d, 6.8)	39.6
8	5.93–5.82 (m)	137.6	5.99–5.89 (m)	137.4
9	5.04–4.96 (m)	115.82	5.11–5.04 (m)	115.79
1-OH	5.60 (s)			
2-OH			5.68 (s)	
1'		145.6		145.8
2'		124.1		124.1
3'	6.84 (s)	115.86	6.83 (s)	115.83
4'		142.4		142.4
5'		145.5		145.5
6'	6.50 (s)	103.5	6.49 (s)	103.5
7'	3.25 (d, 6.5)	33.7	3.25 (d, 6.5)	33.6
8'	5.93–5.82 (m)	136.6	5.95–5.85 (m)	136.7
9'	5.04–4.96 (m)	115.5	5.07–5.00 (m)	115.83
OMe	3.78 (s)	56.2	3.78 (s)	56.2
4'-OH	5.42 (s)		5.41 (s)	

Fig. 3. HMBC correlations of compound 2 and 3.

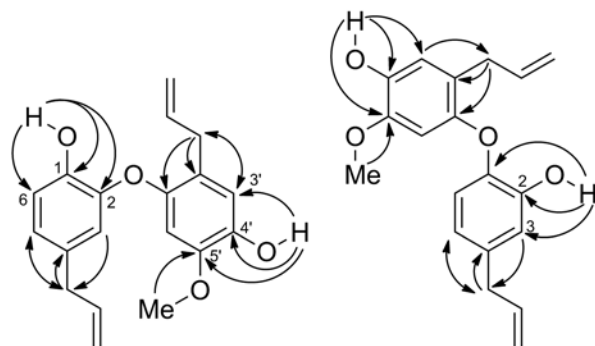


Fig. 4. Inhibitory activity of the isolated compounds.

