ORIGINAL ARTICLE

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Rec. Nat. Prod. 10:2 (2016) 148-153

records of natural products

Pancreatic Lipase Inhibitory Phthalide Derivatives from the Rhizome of *Cnidium officinale*

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(Received December 04, 2014; Revised June 12, 2015; Accepted June 15, 2015)

Abstract: Pancreatic lipase plays an important role in the digestion and absorption of fats; it has become a target of interest in the treatment of obesity. Investigations of pancreatic lipase inhibitory compounds from *Cnidium officinale* rhizomes have resulted in the isolation of a new phthalide derivative (1) together with ten known phthalides (2-11). Phthalide derivatives from *C. officinale* showed mild inhibition against pancreatic lipase with 13 - 56% inhibition at 100 μM. Structure activity relationship suggested that the double bond in the side chain of phthalide increased its inhibitory activity, whereas the addition of hydroxyl moiety to side chain reduced activity. Lineweaver-Burk plot analysis also demonstrated that compound 2 was a noncompetitive inhibitor with an IC_{50} of 86.4 μM. Taken together, *C. officinale* and its phthalide constituents might be beneficial for the regulation of obesity through pancreatic lipase inhibition.

Keywords: *Cnidium officinale*; pancreatic lipase; phthalide; obesity.

1. Introduction

Obesity is currently one of the major threats to global health. It is a multifactorial disease and is associated with several pathological disorders, including diabetes, hypertension, atherosclerosis and cancer [1-2]. It is characterized by excessive weight due to a prolonged imbalance between the levels of energy intake and expenditure. Dietary fats account for a major source of energy intake, with triacylglycerol being the main component. Energy intake starts from fat absorption through the digestion of fat into monoglycerides and fatty acids, mainly by pancreatic lipase. Therefore, pancreatic lipase has become a target of interest in the treatment of obesity [3-4]. Orlistat, a specific pancreatic lipase inhibitor, reduces the hydrolysis of triacylglycerol and has been clinically used for the prevention of obesity [5-6].

Cnidium officinale Makino is a perennial plant of the Umbelliferae family, which is widely distributed throughout Asia, including Korea. In traditional medicine, the rhizomes of *C. officinale* have been used for sedative, hematic, and anti-fungal activities [7]. A number of phthalide derivatives

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 08/01/2015 EISSN:1307-6167

from *C. officinale* with anti-inflammatory, anticancer and antioxidant activities have been reported [8-11]. In the course of screening, the total methanolic extract of *C. officinale* rhizomes significantly inhibited pancreatic lipase activity (49.6% inhibition at 100 μg/mL). The methanolic extract was further fractionated into *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH fractions. Among them, CH_2Cl_2 and EtOAc fractions inhibited pancreatic lipase activity (77.7% and 70.4% inhibition, respectively, at 100 μg/mL). Thus, we attempted to isolate and characterize active compounds from *C. officinale*.

2. Materials and Methods

2.1. Plant Material

The rhizomes of *C. officinale* were purchased from the local herbal market in Chungbuk, Korea in November 2013. They were identified by the herbarium of the College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU201311-CO).

2.2. Extraction and Isolation

The rhizomes of *C. officinalis* (1.0 kg) were extracted 3 times with 80% MeOH using a sonic apparatus, which yielded the methanolic extract. The methanolic extract was then suspended in H_2O and partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The CH_2Cl_2 and EtOAc fractions, which showed inhibition against pancreatic lipase, were subjected to further chromatographic separation.

The CH_2Cl_2 fraction (COM) was subjected to silica gel column chromatography with a mixture of *n*-hexane- EtOAc to produce 8 fractions (COM1-COM8). Compounds **5**, **11**, and **3**, **8** were isolated from COM2 and COM3, respectively, by semi-preparative HPLC eluted with acetonitrile-H_2O (45:55). Compounds **2**, **4**, and **6** were isolated from COM4 by semi-preparative HPLC eluted with acetonitrile-H_2O (45:55). COM6 was subjected to column chromatography over Sephadex LH-20 eluted with CH_2Cl_2-MeOH (1:1) to produce 6 subfractions (COM6A-COM6F). COM6D was further divided into 5 fractions (COM6D1-COM6D5) by column chromatography over silica gel eluted with the mixture of CH_2Cl_2-MeOH. Semi-preparative analysis of COM6D4 resulted in the isolation of compound **7**. The EtOAc fraction (COE) was subjected to silica gel column chromatography with a mixture of CH_2Cl_2-MeOH to produce 8 fractions (COE1-COE8). COE4 was subjected to column chromatography over Sephadex LH-20 eluted with MeOH to produce 4 subfractions (COE4A-COE4D). Compounds **9**, **10**, and **1** were isolated from COE4A and COE4B, respectively, by semi-preparative HPLC eluted with acetonitrile-H_2O (30:70).

Compound (**1**): (*Z*)-5-hydroxy-3-(2-hydroxybutylidene)isobenzofuran-1(3*H*)-one, coloress syrup; $[\alpha]_D^{25}$-7.0 (c 0.2, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.73 (1H, d, *J* = 8.5 Hz, H-7), 7.13 (1H, d, *J* = 1.5 Hz, H-4), 7.02 (1H, dd, *J* = 8.5, 2.0 Hz, H-6), 5.64 (1H, d, *J* = 9.0 Hz, H-8), 4.73 (1H, m, H-9), 1.76 (1H, m, H-10b), 1.64 (1H, m, H-10a), 0.99 (3H, t, *J* = 7.5 Hz, H-11). ^{13}C NMR (125 MHz, CD_3OD) δ 166.8 (C-1), 164.5 (C-5), 145.3 (C-3), 142.1 (C-3a), 126.5 (C-7), 118.7 (C-6), 115.3 (C-7a), 110.0 (C-8), 105.3 (C-4), 67.2 (C-9), 29.8 (C-10), 8.6 (C-11). IR_{max} cm^{-1} 3324, 1646. UV λ_{max} (MeOH) nm 254. HRESIMS *m/z*: 243.0627 (Calcd for $C_{12}H_{12}O_4Na$: 243.0633). ESI-MS *m/z*: 219 $(M-H)^-$.

2.3. Assessment of Pancreatic Lipase Activity

Pancreatic lipase inhibitory activity was evaluated as previously reported [12]. Briefly, the test sample was mixed with enzyme buffer and incubated for 15 min at 37°C. After incubation, 10mM of *p*-nitrophenylbutyrate (*p*-NPB) was added and the enzyme reaction was allowed to proceed for 15 min at 37°C. Pancreatic lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm using a microplate reader. Pancreatic lipase inhibition (%) was calculated as follows: 100 – [(absorbance of reaction mixture with compound and substrate – absorbance of reaction

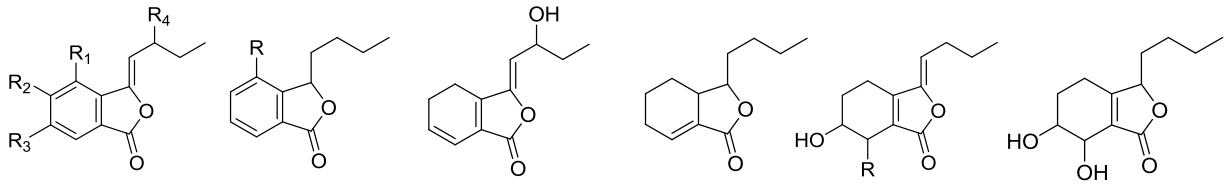
mixture with compound without substrate) / (absorbance of reaction mixture with substrate without compound)x 100]. The evaluation of statistical significance was determined using a one-way ANOVA test with a value of $p<0.05$ considered statistically significant.

3. Results and Discussion

3.1. Structure elucidation

Activity-guided fractionation of *C. officinale* was carried out for the isolation of pancreatic lipase inhibitory constituents. The fractionation and separation of CH_2Cl_2 and EtOAc fractions by several chromatographic methods yielded a new phthalide (**1**) and 10 known compounds (**2-11**) (Figure 1).

Compound **1** was purified as a colorless syrup, and a molecular formula of $C_{12}H_{12}O_4$ was determined on the basis of the ^{13}C NMR spectroscopic data and an HREIMS ion at 243.0627 ($[M+Na]^+$, calcd 243.0633). The IR spectrum showed the presence of hydroxy (3324 cm^{-1}) and carbonyl (1646 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra of **1** showed the resonances for a 1, 3, 4-trisubstituted aromatic ring at [δ_H 7.13 (1H, d, J = 1.5 Hz, H-4), 7.02 (1H, dd, J = 8.5, 2.0 Hz, H-6), and 7.73 (1H, d, J = 8.5 Hz, H-7); δ_C 142.1 (C-3a), 105.3 (C-4), 164.5 (C-5), 118.7 (C-6), 126.5 (C-7) and 115.3 (C-7a)], which was confirmed by the HSQC spectrum. In addition, the resonances were observed for a methine at [δ_H 5.64 (1H, d, J = 9.0 Hz); δ_C 110.0], a hydroxymethine at [δ_H 4.73 (1H, m); δ_C 67.2)], a methylene at [δ_H 1.64 (1H, m), 1.76 (1H, m); δ_C 29.8)], and a methyl at [δ_H 0.99 (3H, d, t, J = 7.5 Hz); δ_C 8.6] in the ^1H and ^{13}C NMR spectra. The ^{13}C NMR spectrum also showed additional resonances at δ_C 166.8 and 145.3. In the HMBC spectrum, correlations between CH_3-11 and C-9, 10, between H-10 and C-9, between H-8, 9 and C-3 suggested the presence of C=CH-CH(OH)-CH_2-CH_3 moiety (Figure 2). These data suggested that compound **1** was a phthalide derivative [13]. The position of hydroxy group in the aromatic ring was determined to be C-5 by the correlation between H-4 and H-8 in NOESY spectrum. The NOESY correlation between H-6 and Me-13 also suggested a *Z*-configuration of the 3, 8 double bond. On the basis of the obtained data, the structure of compound **1** was elucidated as (*Z*)-5-hydroxy-3-(2-hydroxybutylidene)isobenzofuran-1(3*H*)-one and the compound was named senkyunolide Z.



1 R_2=OH, R_4=OH
2 R_1=OH
3 R_1=OCH_3
4 R_3=OH

5 R=H
6 R=OH

7

8

9 R=OH
10 R=OCH_3

11

Figure 1. Structures of compounds **1-11** isolated from *C. officinale*.

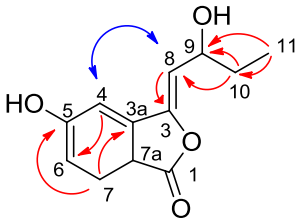


Figure 2. Key HMBC (→) and NOESY (↔) correlation of compound **1.**

The structure of 10 known phathalide derivatives were identified as senkyunolide B (**2**), 3-butylidene-4-methoxy-isobenzofuranone (**3**), 3-butylidene-6-hydroxy-isobenzofuranone (**4**), butylphthalide (**5**), 3-butyl-4-hydroxy- 1 (3*H*)-isobenzofuranone (**6**), senkyunolide F (**7**), neocnidilide (**8**), ligusilidiol (**9**), senkyunolide J (**10**), and *Z*-6-hydroxy-7-methoxy-dihydroligustilide (**11**) by direct comparison of their physicochemical and spectroscopic data with those previously reported [11, 14-19].

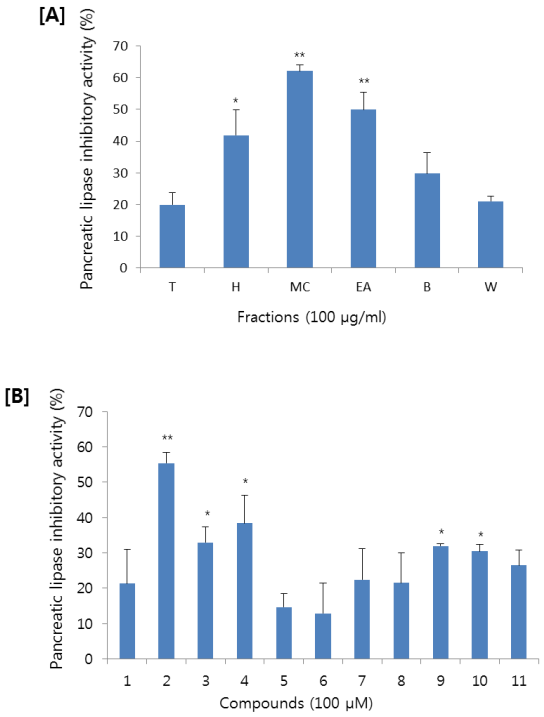


Figure 3. Effects of [A] fractions of *C. officinale* and [B] compounds **1**-**11** on pancreatic lipase inhibition. Pancreatic lipase activity was measured using porcine pancreatic lipase *in vitro*. Results are expressed as mean ± SD of three independent experiments, each performed using triplicate wells. $^{*}p<0.05$; $^{**}p<0.01$ compared with control.

3.2. Pancreatic lipase inhibitory activity

All isolates were evaluated for their inhibitory effects on pancreatic lipase activity using porcine pancreatic lipase *in vitro* (Figure 3). Among the isolated phthalide derivatives, compound **2** inhibited pancreatic lipase with an IC_{50} of 86.7 μM. Compound **6** has the same structure as compound **2,** except for the absence of the double bond in the side chain showed less inhibition (12.9% inhibition) than compound **2** (55.3% inhibition), which suggests the importance of the double bond. Compound **4** is also identical to compound **2**, except for the presence of a hydroxy group at C-6 instead of a hydroxy group at C-4 of compound **2**, but less inhibition (38.4% inhibition) than compound **2**. Therefore, these results suggested that the number and position of the hydroxy group, and the double bond in the side chain are important for the pancreatic lipase inhibitory activity of phthalide derivatives. Consistent with these postulations, compounds **2**, **3**, **4**, **9**, and **10** having double bonds but no hydroxy moiety in the side chain exerted 30.4- 55.3% pancreatic lipase activity at 100 μM whereas compounds **1**, **5**-**8**, and **11** exerted < 30% pancreatic lipase activity inhibition (Figure 3).

For further characterization of the mechanism of compound **2**'s inhibitory effect on pancreatic lipase, Lineweaver-Burk analysis was performed. As the compound **2** concentration increased, the value for the *y*-intercept in the equation for each curve increased, whereas the *x*-intercept remained at a

fixed point (Figure 4). These results suggest that compound **2** exerted an inhibitory effect on pancreatic lipase in a noncompetitive manner.

The present study showed that the total extract of *C. officinale* and its phthalide derivatives inhibited pancreatic lipase activity. Natural products contain diverse constituents, which allow multiple activities [20]. Therefore, phthalide derivatives may contribute to the inhibitory activity of total extract of *C. officinale* together with other constituents. Moreover, therapeutics targets for obesity can be developed in combinatorial ways, such as through lipase inhibition, the suppression of food intake, the stimulation of energy expenditure, the inhibition of adipocyte differentiation, and the regulation of lipid metabolism [21]. Therefore, further investigation of other anti-obesity mechanisms is still needed to obtain a better understanding. Conclusively, the present study will provide further insight into the design of new approaches for anti-obesity therapeutics.

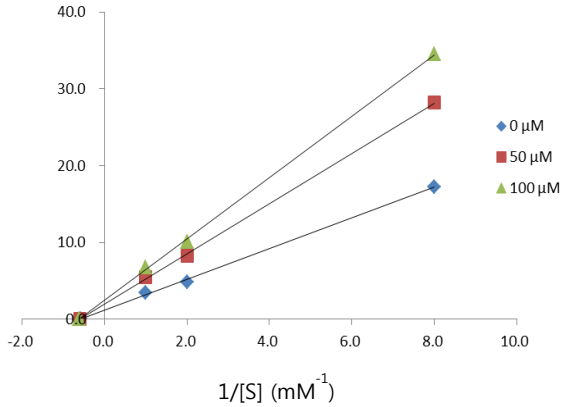


Figure 4. Lineweaver-Burk plots of the inhibitory activity of compound **2** in the presence of various concentrations of compound **2**

Acknowledgments

This work was supported by the Basic Science Research Program (2012-0008023) and the Medical Research Center program (2010-0029480) through the National Research Foundation of Korea.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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