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A Pancreatic Lipase Inhibitory Activity by Mango (*Mangifera indica*) Leaf Methanolic Extract

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Abstract

The objective of this study was to identify pancreatic lipase inhibitory active ingredients of mango leaves, and to examine a relationship between leaves maturation and pancreatic lipase inhibitory activity. A methanolic extract of old dark green mango leaves (OML-ext) showed a porcine pancreatic lipase inhibitory activity. The pancreatic lipase inhibitory activity of OML-ext was attributable to 3-C-*β*-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone (**2**) and mangiferin (**1**). The pancreatic lipase inhibitory activity of young mango leaf extract was superior to that of old leaf extract. It was suggested that the activity is correlated with the content of **2** in these extract. Considering the amounts of leaves obtained from pruning, old dark green leaves may be a reasonable natural resource for the preparation of ingredients with lipase inhibitory activity.

Keywords: 3-C-*β*-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone, lipase inhibitory activity, *Mangifera indica*, mangiferin, obesity

1. Introduction

Obesity is recognized as a major life style disorder especially in developing countries and it is prevailing at an alarming speed in the world due to fast food intake, industrialization, and reduction of physical activity (Cairns, 2005).

Pancreatic lipase is a key enzyme for lipid absorption by hydrolysis of total dietary fats (Seyedan et al., 2015). Two pancreatic lipase inhibitors, namely orlistat (Xenical®) (Ballinger & Peikin, 2002) in U.S.A. and cetilistat (Oblean®) (Gras, 2013) in Japan, have been approved for the treatment of obesity syndrome. In order to find new pancreatic lipase inhibitors from natural resources, screening of plant extracts has been considered as one of strategies. Hitherto, several extracts of the plants, such as chokeberry fruit (Sosnowska et al., 2015), *Nelumbo nucifera* leaves (Liu et al., 2013), *Coffea arabica* seeds (Patui et al., 2014) and *Panax japonicus* rhizomes (Han et al., 2005), have been reported to have lipase inhibitory activities. As a folk tradition in India and Thailand when the high fat diet was taken, young mango (*Mangifera indica* Linne) leaves have been eaten together for health (Iwasa, 1984). Lipid metabolic enzyme inhibitory (Moreno et al., 2006) and cholesterol esterase inhibitory activities (Gururaja et al., 2015) of mango leaves have been reported, however pancreatic lipase inhibitory activity of young mango leaves has never been examined.

Several types of C-glucosyl-polyphenols, such as **1** and **2**, were isolated from mango leaves (Severi et al., 2009). Moreno et al. (2006) reported that 95% ethanolic extracts of mango leaves and bark inhibited human pancreatic lipase inhibitory activity at a high concentration of 1 mg/ml, and they suggested that these extracts may provide botanical therapeutics useful in weight control. However, they did not identify any active constituents. Severi et al. (2009) reported that mango leaf extract showed antiulcerogenic activity and the activity was attributable to phenolic compounds such as **2**. Zhang et al. (2011, 2013) isolated several benzophenone C-glycosides namely foliamangiferosides, in addition to **1** and **2** from 70% ethanolic extract of mango leaves as active inhibitor of triglyceride accumulation in 3T3-L1 cells. Recently, Gururaja et al. (2015) reported that the extracts of mango

leaves exhibited cholesterol esterase inhibitory activity, and fractions containing 3-*β*-taraxanol or **2** showed a potent activity. Compound **1** has been reported as active constituent of lipolytic effect in rat epididymal fat-delivered cultured adipocytes, but **1** did not inhibit pancreatic lipase in the literature (Yoshikawa et al., 2002).

These reports prompt us to reexamine the pancreatic lipase inhibitory activity of mango leaf extracts and identify active constituents. Considering with the folk tradition described above, we also examined pancreatic lipase inhibitory effect of young mango leaf extract.

2. Materials and Methods

2.1 Plant Materials

Leaves of *M. indica* (cv. Irwin) were collected in the Experimental Farm, Kindai University (34° 2′ N, 135° 11′ E, 17 m ASL), located in Wakayama Prefecture, Japan. The *M. indica* trees planted in the ground are commercially grown in a plastic greenhouse {temperature: winter, min. 5ºC (room) and 13ºC (soil); summer, max. 39ºC (room) and 30ºC (soil)}. 'Irwin' mango is a representative cultivar especially in Japan and Taiwan because of its early ripening and relative good cold resistance. The leaves were collected from 250 mango trees which were propagated by grafting (the height of trees; 2 m, the age of trees; 18-26 years old, the life span of trees; 40-50 years). The collection date of the materials were as follows, old leaves; July 2013 and August 2015, young dark brown and yellow leaves; August 2015. These young and old leaves were collectable at the same time of pruning in late summer due to mango is an evergreen plant. The collected leaves were visually classified by the color of leaf into three groups, namely dark brown, yellow and dark green (Photo 1). The samples were identified by the Experimental Farm at Kindai University, air-dried at 50ºC for 72 h in an automatic air-drying apparatus (Vianove Inc., Tokyo, Japan), and powdered. Voucher specimens of leaves (Mango old Leaf: OML201307DG-S and OML201508DG-S, Mango young dark brown Leaf: YML201508DB-S, Mango young yellow leaf: YML201508Y-S) are deposited in the Experimental Farm, Kindai University.

1-a; Young dark brown leaves 1-b; Young yellow leaves 1-c; Old dark green leaves

Photo 1. Photographs of typical mango leaves at various stages of development

2.2 Extraction

The leaf powder (10 g) was extracted with methanol (MeOH, 200 ml) for 72 h at room temperature. Each extract solution was evaporated under reduced pressure to give each MeOH extract. The yields of MeOH extract of young dark brown leaves, young yellow leaves, and old dark green leaves (OML-ext) were 28%, 25%, and 23%, respectively.

2.3 Reagents

4-Metylumbeliiferyl oleate, lipase (type II, from porcine pancreas, Lot #: SLBN3801V), authentic **1** were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Orlistat was purchased from Tokyo Chemical Industry (Tokyo, Japan). Other chemical and biochemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and/or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise noted.

2.4 In Vitro Pancreatic Lipase Inhibition Assay

Porcine pancreatic lipase (type II, from porcine pancreas) activity was measured according to the method of Nakai et al. (2005) with minor modification. The test sample was dissolved with dimethyl sulfoxide (DMSO) and diluted with 13 mM Tris-HCl buffer containing 150 mM NaCl, 1.3 mM $CaCl_2$ (pH 8.0) to a final DMSO concentration of 2.5% v/v. 4-Methylumbelliferyl oleate (4-MU) was used as a substrate. The substrate and

enzyme were both diluted in above mentioned buffer immediately before use. An aliquot of 25 µl of the test solution and 50 µl of 0.1 mM 4-MU solution were mixed in back colored microtiter plates, 25 µl of 0.2 mg/ml enzyme solution was then added to each well to start the reaction. After incubation for 30 min at 37ºC, 100 µl of 0.1 M citrate buffer (pH 4.2) was added to stop the reaction. The fluorescence associated with enzymatically released 4-methylumbelliferone product was monitored at an excitation wavelength of 355 nm and emission of 460 nm using a multi-label counter (PerkinElmer 2030 ARVO X4，PerkinElmer Life and Analytical Sciences). Orlistat, a known inhibitor of pancreatic lipase, was used as reference compound. The activity of negative control was also evaluated by adding stop solution before enzymatic reaction. The inhibition activity was calculated using the following formula:

$$\% \text{ inhibition} = [(A-B)-(C-D)]/(A-B)\times 100$$

where A is the fluorescence with enzyme and substrate, but without test substance (adding stop solution after enzymatic reaction); B the fluorescence with enzyme and substrate, but without test substance (adding stop solution before enzymatic reaction); C the fluorescence with enzyme, substrate and test substance (adding stop solution after enzymatic reaction); and D the fluorescence with enzyme, substrate and test substance (adding stop solution before enzymatic reaction).

Each concentration of samples was confirmed in triplicate (P value < 0.01). IC_{50} value represents the concentration required to inhibit 50% of pancreatic lipase activity.

2.5 Spectroscopy

ESI mass spectra were recorded on a Triple TOF 5600+ mass spectrometer (SICEX) combined with an LC-20A HPLC system (Shimadzu, Kyoto, Japan). The samples were eluted with 0.1% formic acid aqueous solution and acetonitrile at a constant flow rate of 0.2 ml/min.

^{1}H NMR (800 MHz), ^{13}C NMR (200 MHz), and 2D NMR (COSY, HSQC, and HMBC) spectra were obtained with JNM-ECA 800 MHz NMR spectrometer (JOEL, Tokyo, Japan) using a 5 mm probe, DMSO-d_6 as solvents.

*2.6 Isolation of 3-C-β-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone (**2**) and Mangiferin (**1**)*

The OML-ext (5.44 g) was submitted to column chromatography over 125 g of silica gel (Wakogel C-100, Wako, Osaka, Japan, 36 mm × 205 mm). Elution with stepwise gradient of 1.2 l of $CHCl_3$/MeOH (10:0 v/v), (30:1 v/v), (10:1 v/v), (5:1 v/v) and (0:10 v/v) to give 30 fractions. The lipase inhibitory activity at 25 µg/ml of each fraction was assayed. Fractions (1-6) eluted with $CHCl_3$/MeOH 10:0 v/v were almost inactive. Active fractions (7-25) eluted with $CHCl_3$/MeOH 30:1 v/v, 5:1 v/v and 0:10 v/v were further purified by a preparative HPLC (column: Inertsil ODS-3 column, 20 mm × 50 mm, GL Sciences, Tokyo, Japan. elution; 15% acetonitrile aqueous solution, flow rate; 9.0 ml/min, detection; UV 280 nm) gave compound **A** and compound **B**. Identification of the isolated compound **A** as **1** were done by co-chromatography and comparison of spectrum analysis with authentic **1** under the same HPLC condition as described in the section of HPLC Analysis conditions. Compound **B** was identified as **2** by comparison of ^{1}H, ^{13}C-NMR and mass spectra data (Severi et al., 2009) as described in the section of Identification of 3-C-*β*-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone (**2**).

*2.7 Identification of 3-C-β-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone (**2**)*

^{1}H-NMR δ: 3.17 (1H, *dd*, *J*=2.3, 2.5 Hz, H-5"), 3.20 (2H, *m*, H-3", -4"), 3.49 (1H, *m*, H-6"a), 3.57 (1H, *dd*, *J*=8.9, 9.5 Hz, H-2"), 3.61 (1H, *m*, H-6"b), 4.58 (1H, *d*, *J* =9.5 Hz, H-1"), 5.93 (1H, *s*, H-5), 6.76 (2H, *d*, *J* =8.7 Hz, H-3', 5'), 7.55 (2H, *d*, *J* =8.7 Hz, H-2', 6'). ^{13}C-NMRδ: 60.7 (C-6"), 69.8 (C-4"), 72.1 (C-2"), 74.9 (C-1"), 78.5 (C-3"), 81.2 (C-5"), 103.9 (C-5), 107.1 (C-1), 114.8 (C-3', -5'), 131.0 (C-1'), 131.6 (C-2', -6'), 157.0 (C-2), 161.0 (C-3), 161.3 (C-4'), 194.8 (C-7). ESI-MS: $[M+H]^+$ = *m/z* 409.1.

2.8 HPLC Analysis Conditions

An HPLC system consisted of LC-20A pump and SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan), which were used for detection and UV spectrometry. The samples were analyzed by using an Inertsil ODS-3 reverse phase column (4.6 × 150 mm, GL Sciences, Tokyo, Japan) and gradient elution with acetonitrile aqueous solution at a constant flow rate of 0.8 ml/min. The elution was carried out using linear gradient condition as follows; initial condition was set at 5% acetonitrile and maintained for 5 min., followed by a linear gradient from 5% to 100% acetonitrile for 35 min. The column temperature was set at 40ºC, and eluted compounds were detected at a range of 200 to 700 nm. Under this condition, **1** and **2** were eluted at the retention time of 20 min and 17 min, respectively. Concentration of **1** and **2** were calculated from linear calibration curves made from external standards, authentic **1** and the isolated **2**. Linear calculation curves in the range of 0.5 to 5 µg were made from the peak areas analyzed at 280 nm, and the correlation coefficients of **1** and **2** were 0.967 and

0.996, respectively. Dried extracts were dissolved in MeOH, and an appropriate amount of sample solution was applied to HPLC analysis in triplicate. Values represent the mean ± standard deviation.

2.9 Statistical Analysis

The experimental data were evaluated for statistical significance using Bonferroni/Dunn's multiple-range test with GraphPad Prism for Windows, Ver. 5 (GraphPad Software Inc., 2007).

3. Results and Discussion

3.1 Identification of Pancreatic Lipase Inhibitory Active Ingredients of OML-ext

In the preliminary evaluation of mango leaf extract on porcine pancreatic lipase inhibitory activity using 4-metylumbeliiferyl oleate as a substrate, the methanolic old mango leaf extract (OML-ext) inhibited a pancreatic lipase activity with the IC_{50} value of 13.9 μg/ml. To identify the active constituents, we carried out activity-guided fractionation of OML-ext using lipase inhibitory assay. Silica gel column chromatographic fractionation of OML-ext gave active fractions eluted with $CHCl_3$/MeOH 30:1 v/v, 5:1 v/v and 0:10 v/v, and inactive fractions eluted with $CHCl_3$/MeOH 10:0 v/v. Further purification of active fractions using preparative HPLC led to isolation of **1** and **2** as active constituents. The IC_{50} values (Table 1) of **1** and **2** were 273 and 226 μM, respectively. As shown in Table 1, the IC_{50} value of orlistat as a reference compound was 0.1 μM (= 0.0495 μg/ml) in accordance with the reported IC_{50} value (0.05 μg/ml) (Ado et al., 2013). Thus, a part of the pancreatic lipase inhibitory activity of OML-ext is attributable to these two compounds. To the best of our knowledge, this is the first report on lipase inhibitory activity of **2**.

Table 1. Inhibitory activities of 3-C-*β*-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone (**2**) and mangiferin (**1**) on pancreatic lipase

Samples	**IC_{50} values [a] (μM)**
3-C-*β*-D-glucosyl-2,4,4',6-tetrahydroxybenzophenone **(2)**	226 μM
Mangiferin **(1)**	273 μM
Orlistat	0.1 μM

Orlistat was used as reference compound. a); IC_{50} value represents the concentration required to inhibit 50% of pancreatic lipase activity.

As described above, **1** is a xanthone found in various parts of mango and has several biological activities, *e.g.* antitumor (Yoshimi et al., 2001), antiviral (Zheng & Lu, 1990), antidiabetic (Miura et al., 2001), anti-inflammatory (Garrido et al., 2004) and potent antioxidant (Sato et al., 1992) activities.

Yoshikawa et al. (2002) described that **1** showed lipolytic effect in rat epididymal fat-delivered cultured adipocytes, but **1** did not inhibit pancreatic lipase. In addition, Koga et al. (2013) reported IC_{50} value of **1** as lipase inhibitor was above 2962 μM. In our experiments, **1** inhibited pancreatic lipase (IC_{50} value; 273 μM, Table 1) in contrast to the reports by Yoshikawa et al. (2002) and Koga et al. (2013). Because they did not use a reference compound such as orlistat, it may be difficult to discuss the reasons anymore. We assumed that a part of the discrepancy of the activity of **1** might be due to the some differences in experimental conditions, such as substrate and evaluation method.

3.2 A Relationship Between Leaves Maturation and Pancreatic Lipase Inhibitory Activity

Considering with the use of mango leaves and also the folk tradition in India and Thailand described above, we examined the inhibitory activity of young and old leaves on pancreatic lipase. The color of mango leaves turns from dark brown to dark green with an increase in area after unfolding. Since the mango is an evergreen plant, young dark brown, young yellow and old dark green leaves were collectable at the same time of pruning in late summer after fruit harvest. The collected leaves were visually classified by the color of leaf into three groups, namely dark brown, yellow and dark green.

As shown in Table 2, lipase inhibitory activity of young dark brown leaf extract showed most potent activity with the IC_{50} value of 2.9 μg/ml. In accordance with the leaves mature from yellow to dark green, activities of these extracts were slightly decreased. The IC_{50} values of young yellow leaves and old dark green leaves were 4.5 and 13.9 μg/ml, respectively (Table 2).

Table 2. Inhibitory activities of MeOH extracts of young dark brown and young yellow leaves and old dark green mango leaves on pancreatic lipase

Samples	IC_{50} values [a] (μg/ml or μM)
Young dark brown leaf extract	2.9 μg/ml
Young yellow leaf extract	4.5 μg/ml
Old dark green leaf extract (= OML-ext)	13.9 μg/ml
Orlistat	0.1 μM

Orlistat was used as reference compound. a); IC_{50} value represents the concentration required to inhibit 50% of pancreatic lipase activity.

The contents (mg/g extract) of **2** and **1** in these leaf extracts were determined by HPLC analysis. As a result, young dark brown leaf extract contained 400.0 ± 11.0 mg/g of **2** and 78.6 ± 1.2 mg/g of **1**. The corresponding content data for other two leaf extracts were as follows; young yellow leaf extract, 278.6 ± 15.5 and 79.4 ±1.0 mg/g, and OML-ext, 205.9 ± 7.6 and 85.1 ± 0.5 mg/g. The pancreatic lipase inhibitory activity of young dark brown and young yellow leaf extracts was superior to that of OML-ext. These results suggested that the inhibitory activity of extract was in accordance with the content of **2** in extracts.

High contents of **2** and **1** in the extracts indicated that these two compounds would be partly responsible to their inhibitory activities. On the other hand, the potent inhibitory activities of leaf extracts can't exclude a hypothesis that other ingredients may also contribute to the activity. To identify other active ingredients, further studies are required, and now undergoing.

In the cultivation process of mango fruit, pruning in summer after fruit harvest is important so as to obtain excellent flower buds in next spring and a rich harvest of superior fruit in next summer, and pruned leaves are usually discarded. As shown in Photo 1, anthocyanin content in young dark brown leaves is high (Photo 1-a), but during their enlargement, the anthocyanin content is decreased (Photo 1-b), while the chlorophylls content is increased. Concurrently, the leaves become thick and rigid (Photo 1-c) (Ali et al., 1999). The period of young dark brown and young yellow leaves were short, leaves rapidly turned to dark green with developing due to rapid decrease of anthocyanin content. Therefore pruned amounts of young dark brown and young yellow leaves are much less than that of old dark green leaves. Considering the amount of leaves obtained from pruning, old dark green leaves may be a reasonable natural resource for the preparation of ingredients with lipase inhibitory activity.

4. Conclusion

Young dark brown and young yellow mango leaf extracts and OML-ext exhibited pancreatic lipase inhibitory activities. The activities of these young mango leaf extracts was superior to that of old mango leaves, this is the first report to reveal a relationship between leaves maturation and pancreatic lipase inhibitory activity. These findings may support the folk tradition in India and Thailand. It was revealed that a part of the pancreatic lipase activity of leaf extract was attributable to **2** and **1**. This is the first report on lipase inhibitory activity of **2**. Hitherto, pruned mango leaves were unworthy and discarded during the cultivation process of mango fruit, this finding suggested that pruned mango leaves may be a useful resource for the preparation of ingredients for the treatment of obesity. However, further investigations are required to examine of administration safety and the mechanisms involved and to reveal other active constituents.

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