

1 Running Title: Phenolic Compounds Showed Adipogenesis Activity

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3 Phenolic Compounds from Leaves of *Casimiroa edulis* Showed Adipogenesis Activity

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19
20 *Abbreviations:* LC/MS, liquid chromatography mass spectrometry; PPAR, peroxisome
21 proliferator-activated receptor; FA, fatty acid

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23 *Casimiroa edulis* is known as *cochitzapotl*, and it belongs a species of tropical
24 fruiting tree in the family Rutaceae, native to eastern Mexico and Central America south
25 to Costa Rica. In this study, we isolated two furocoumarins and two
26 polymethoxyflavones from leaves of *Casimiroa edulis* and evaluated the functions of
27 glucose and lipid metabolism activity with 3T3-L1 adipocytes. We discovered that the
28 addition of furocoumarins increased glucose uptake and lipid accumulation in 3T3-L1

1 adipocyte. These results suggest that furocoumarin compounds can be used as
2 functional food-derived compounds, to regulate adipocyte functioning for the
3 management of metabolic syndrome, which is associated with dysfunctions of glucose
4 and lipid metabolism.

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6 **Key words:** liquid chromatography/mass spectrometry (LC-MS); adipocyte;
7 peroxisome proliferator-activated receptor (PPAR); fatty acid (FA) syntheses; phenolic
8 compounds

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14 *Casimiroa edulis* is a species of tropical fruiting tree. Zapotin in *Casimiroa edulis*
15 has been found to induce both cell differentiation and apoptosis in cultured human
16 promyelocytic leukemia HL-60 cells.¹⁾ This compound inhibits
17 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC)
18 activity in human bladder carcinoma cells (T24 cells) and TPA-induced nuclear
19 factor-kappa B (NF-κB) activity in human hepatocellular liver carcinoma cells (HepG2
20 cells).²⁾ The data suggest that zapotin deserves further investigation as a potential cancer
21 suppressive agent. Crude plant extracts of seeds and of leaves of *Casimiroa edulis* have
22 been found to affect blood pressure, cardiac activity,³⁻⁵⁾ and aortic muscular tone,⁶⁾ and
23 to possess anticonvulsant activity,^{7,8)} but previous study has focused mainly on seed
24 components and leaf components that were not studied well.

25 Adipocytes play a major role in the maintenance of energy balance. The amount
26 of glucose uptake into the adipocytes is significant in the maintenance of serum glucose
27 levels, because adipocyte-specific knock-out of an insulin-dependent glucose
28 transporter (glut4) results in diabetic symptoms.⁹⁾ Lipid synthesis (lipogenesis) is also a

1 vital function of adipocytes. *In vivo* studies have suggested that adipose tissue is
2 responsible for more than 50% of fatty acid synthesis in mammals.¹⁰⁾ Fatty acid (FA)
3 synthesis is a cytosolic process utilizing acetyl CoA as precursor. It is generally
4 accepted that fatty acid is synthesized from various substrates (glucose, pyruvate, lactate
5 and acetate).¹¹⁾ It is indispensable for the management of adipocyte functions to
6 evaluate complicated metabolisms, including lipogenesis and glucose uptake.

7 In this study, we tried to isolate phenolic compounds from *Casimiroa edulis*
8 leaves, and investigated other effect, adipogenesis, including glucose uptake, fatty acid
9 synthesis activity, and lipid accumulation with 3T3-L1 adipocytes. PPAR γ synthesized
10 ligand, pioglitazone, was used as positive control, since PPAR γ is abundantly expressed
11 in 3T3-L1 adipocytes and plays a central role in metabolism and differentiation.¹²⁾
12 Pioglitazone, a major insulin sensitizer, promotes preadipocyte metabolism including
13 glucose uptake and FA synthesis through PPAR γ activation ¹³⁾ and is widely used in the
14 treatment of type 2 diabetes.

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20 **Materials and Methods**

21 Leaves of *Casimiroa edulis* were collected at the Botanical Garden of Nippon
22 Shinyaku Co., Kyoto, Japan. The voucher specimen was deposited at Gifu
23 Pharmaceutical University. ¹³C₆ glucose and pioglitazone was purchased from Sigma
24 (St. Louis, MO). [1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16-¹³C] palmitic acid was from
25 Watari (Tokyo). Unless otherwise indicated, all chemicals were purchased from Nacalai
26 Tesque (Kyoto, Japan) or Wako Pure Chemicals (Osaka, Japan) and were of guaranteed
27 reagent grade or tissue culture grade.

1 *General experimental procedures.* ¹H- and ¹³C-NMR spectra were recorded on an
2 EX400 (¹H at 400 MHz and ¹³C at 100 MHz, JEOL) spectrometer. Chemical shifts
3 appeared as δ values with trimethylsilane (TMS) as internal reference. Peak
4 multiplicities were quoted in Hz. EI-MS was recorded on a JMSDX-300 (JOEL)
5 spectrometer. Kiesel gel 60 (60-230 mesh, Merck, Japan) and Fuji Silysia Chemical
6 Chromatorex ODS (100—200 mesh) were used for column chromatography. Kiesel-gel
7 60 F₂₅₆ (Merck, Tokyo) at 0.25 mm was used for analytical and 0.5 mm for preparative
8 TLC.

9

10 *Extraction and isolation of compounds.* Dried leaves (550 g) extracted with
11 MeOH (1 L x 5 times) at room temperature and was evaporated in *vacuo*. The MeOH
12 extract (52 g) obtained was chromatographed on Silica gel (250 mm × 30 mm) with a
13 mixture of chloroform –MeOH by increasing the polarity from 0% to 10 % methanol.
14 The chloroform-MeOH (10:1) (3.5g) was further chromatographed on an ODS column
15 (250 mm × 20 mm) eluted with H₂O, followed by increasing the concentration of
16 MeOH to give four fractions. Fraction 2 (263 mg, 70% methanol) was repeatedly
17 purified by preparative TLC (*n*-hexane-EtOAc 1:4) to give **1** (5 mg), **2** (7 mg), **3** (12
18 mg), and **4** (10 mg).

19

20 *8-(3'-Hydroxymethyl-but-2-enyloxy)-psoralen acetate (compound 1).* White
21 amorphous solid. Negative ion FABMS [M-H]⁻ *m/z* 327. ¹H NMR(CDCl₃) δ: 1.81 (3H,
22 br s, H-5'), 2.04 (3H, s, acetyl-Me), 4.66 (2H, br s, H-4'), 5.09 (2H, br d, *J* = 7.0 Hz,
23 H-1'), 5.86 (1H, br t, *J* = 7.0 Hz, H-3'), 6.38 (1H, d, *J* = 10.0 Hz, H-4), 6.82 (1H, d, *J* =
24 2.5 Hz, H-9), 7.37 (1H, s, H-5), 7.69 (1H, d, *J* = 2.5 Hz, H-10), 7.77 (1H, d, *J* = 10.0 Hz,
25 H-3): ¹³C NMR (CDCl₃) δ: 160.43 (C-2), 114.83 (C-4), 144.27 (C-4), 116.54 (C-4a),
26 113.81 (C-5), 125.95 (C-6), 148.32 (C-7), 131.39 (C-8), 143.67 (C-8a), 106.76 (C-9),
27 146.76 (C-10), 69.12 (C-1'), 125.13 (C-2'), 136.57 (C-3'), 62.79 (C-4'), 21.42 (C-5'),
28 20.83 (acetyl-Me), 170.85 (acetyl C=O).

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2 *8-(3'-Hydroxymethyl-but-2-enyloxy)-5-methoxyorsolen acetate (compound 2).*

3 White amorphous solid. Negative ion FABMS $[M-H]^-$ m/z 357. 1H NMR($CDCl_3$) δ :
4 1.80 (3H, s, H-5'), 2.03 (3H, s, acetyl Me), 4.18 (3H, s, C-5 OMe), 4.66 (2H, br s, H-4'),
5 4.91 (2H, br d, $J = 7.0$ Hz, H-1'), 5.86 (1H, br t, $J = 7.0$ Hz, H-2'), 6.28 (1H, d, $J = 10.1$
6 Hz, H-3), 6.99 (1H, d, $J = 2.5$ Hz, H-9), 7.64 (1H, d, $J = 2.5$ Hz, H-10), 8.12 (1H, d, $J =$
7 10.1 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ : 160.42 (C-2), 112.91 (C-3), 139.55 (C-4), 107.61
8 (C-4a), 144.26 (C-5), 114.61 (C-6), 150.57 (C-7), 136.51 (C-8), 125.63 (C-8a), 105.12
9 (C-9), 145.16 (C-10), 69.36 (C-1'), 125.27 (C-2'), 136.51 (C-3'), 62.78 (C-4'), 21.41
10 (C-5'), 20.84 (acetyl Me), 170.84 (acetyl C=O), 60.79 (3H, s, C-5 OMe).

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12 *5,6,2',3',5',6'-Hexamethoxyflavone (compound 3).* White amorphous solid:
13 exhibited spectral data (1H NMR and EIMS) comparable to published data.¹⁴⁾

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15 *5.6.2'-Trimethoxyflavone (compound 4).* White amorphous solid: 1H NMR data
16 was comparable to published data;⁶⁾ ^{13}C NMR ($CDCl_3$) δ : 159.05, 157.90 (C-2, C-2'),
17 111.68 (C-2), 178.41 (C-4), 119.07, 119.02 (C-4a, 8), 147.82 (C-5), 149.65 (C-6),
18 113.33 (C-7), 151.82 (C-8a), 120.65 (C-1'), 112.96 (C-3'), 132.13 (C-4'), 120.62 (C-5'),
19 129.07 (C-6')

20

21 *LC/MS system.* The Agilent Series 1100 LC system (Agilent Technologies,
22 Waldbronn, Germany) consisting of a G1323B control module, a G1312A quaternary
23 pump, a G1322 degasser, a G1329A, a G1330A autosampler, a G1316A column oven,
24 and a G1314A detector, was used. The column used was an XbridgeTM (C18 3.5 μm ,
25 2.1 \times 150 mm, Waters, Milfold, MA). The LC pump gradient was as follows: 85%
26 mobile phase A (water in 5 mM ammonium acetate) and 15% mobile B (methanol in 5
27 mM ammonium acetate) from 0 to 20 min, 99% mobile phase A and 1% mobile phase B
28 from 20 to 20.1 min, and 85% mobile phase A and 15% mobile B from 20.1 to 35 min.

1 The injection volume, flow rate, and column temperature were 5 μ L, 0.2 mL, and 40°C,
2 respectively. MS conditions were selected ion monitoring (SIM); polarity negative; 10
3 psi nebulizer gas, 150 V fragment ion voltage; 3,000 V Capillary voltage, 350°C; dwell
4 time 50 msec. Unless otherwise specified, all reagents used were of analytical grade,
5 and the water was deionized. Solvent: LC/MS grade methanol. [1,2,3,4,5,6-¹³C] glucose
6 and [1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16-¹³C] palmitic acid were detected at 185 and
7 271, m/z respectively.¹⁵⁾

8 Spectra of furocoumarins were determined in LC/MS scan mode, polarity
9 negative (50- 400 m/z).

10

11 *GC/MS system.* Gas-chromatographic analyses were performed using an Agilent
12 6890 quadrupole gas chromatograph (GC) equipped with an Agilent 5973B mass
13 spectrometer (MS). The GC column was a DB-5MS+DG capillary type (30m \times 0.25
14 mm i.d., 0.25 μ m film thickness; J&W Scientific, Folsom, CA). A 1- μ L extract was
15 injected in splitless mode at an injection temperature of 250°C. The oven temperature
16 was programmed to increase from an initial 60°C (held for 1 min) to 300°C (held for 2
17 min) at a rate of 20°C/min. The temperatures of the quadrupole and ion source were 150
18 and 230°C. The GC-MS system was operated in scan mode (50-500 m/z) with the
19 electron multiplier tune value.

20

21 *Cell culture.* 3T3-L1 preadipocytes, purchased from the American Type Culture
22 Collection (Rockville, MD), were grown in DMEM-high glucose medium
23 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μ g/mL
24 streptomycin at 37°C in 5% CO₂ using a 12-well plate (5×10^4 cell/well).
25 Post-confluent preadipocytes were differentiated into adipocytes by adding 10 μ g/mL
26 insulin, 0.1 μ g/mL dexamethasone, and 112 μ g/mL isobutylmethylxanthine to the
27 medium with 5 μ M pioglitazone, and 5 μ M each test compound in DMSO, as previously
28 described.¹²⁾ The differentiation medium was removed after 2 days and replaced with

1 DMEM-high glucose medium containing 5 µg/mL insulin with TZD or the test
2 compound every 2 days. Ten days after the addition of the differentiation medium, the
3 cells were plated in culture dishes for a given experiment. After differentiation, they
4 were washed with warm PBS, and the medium was replaced with glucose-free DMEM
5 containing 4.5 mg/mL [1,2,3,4,5,6-¹³C] glucose. They were incubated for a further 48 h.
6 This was repeated twice.

7
8 *Extract and saponification.* The media were collected after incubation for specific
9 durations and diluted with methanol. Diluents were filtered with a 0.22 µm filter
10 membrane, and were analyzed by the LC/MS system. The cells were dissolved in 5 mL
11 KOH 20% (ethanol:water 4:6; v/v), and hydrolyzed for 60 min at 80°C, followed by
12 acidification with 6 mL 5 N HCl. The FAs were extracted twice with 4 ml diethylether,
13 dried under N₂ at 40°C, dissolved in 5 ml methanol, and analyzed by the LC/MS
14 system.

15
16 *Oil Red O staining.* Cells were fixed with 10% formaldehyde/PBS and stained
17 with Oil Red O solution (0.5% Oil Red O-isopropyl alcohol/H₂O (3:2, v/v)).

18
19 *Statistical analysis.* Results are expressed as mean and standard error of mean
20 (SEM). Statistical significance of differences among groups was evaluated by
21 Student's *t*-test. Differences were considered significant at $p < 0.05$.

22 23 24 25 26 27 **Results and Discussion**

28 *NMR analysis.* The ¹H NMR of compound **1** showed the presence of two sets of

1 coupled protons that δ 6.38 and 7.77 ($J = 10.0$ Hz) and δ 6.82 and 7.69 ($J = 2.5$ Hz).
2 The data indicated compound **1** was a furocoumarin. An aromatic singlet (δ 7.37) was
3 also observed that correlated with the C-4 signal of the coumarin skeleton in the HMBC
4 spectrum. Other HMBC correlations indicated that the furan ring was attached to C-6, 7,
5 like psoralen. ^1H and ^{13}C NMR showed the presence of two oxymethylene (δ_{H} 4.66 and
6 5.09, and δ_{C} 62.79 and 69.12), a tri-substituted double bond (δ_{H} 5.09; δ_{C} 125.13 and
7 136.57), a methyl attached to a double bond (δ_{H} 1.81; δ_{C} 21.41), and an acetyl group (δ_{H}
8 2.04; δ_{C} 20.83 and 170.85). In the HMBC spectrum, an oxymethylene proton was
9 correlated with C-8 of a coumarin. These data are characteristic of a prenyloxy group, in
10 which one of methyl groups had been oxidized to a hydroxyl methyl and acetylated. The
11 hydroxymethyl group was of the *cis* form with respect to the oxymethylene group (H-1')
12 of the prenyl substituent on the basis of the NOESY spectrum (H-1'/H-4' and H-2'/H-5').
13 Therefore, the structure was determined to be as described in Fig. 1. This compound
14 was first isolated from leaves of *Casimiroa pringlei*.^{16, 17)}

15 The ^1H NMR spectrum of compound **2** was very similar to that of **1**, but an
16 aromatic proton disappeared and a methoxy group (δ 4.18) was observed. An NOE was
17 observed between the methoxy group and H-4 of the coumarin skeleton. By detailed
18 analysis of NMR by 2D methods, the structure of **2** was determined to 5-methoxylated
19 compound of **1**. A deacetylated coumarin of **2** was isolated from *Casimiroa tetrameria*.
20 To the best of our knowledge, compound **2** was a new compound.

21 The structures of compounds **3** and **4** were determined and identified by
22 comparison to previous data as 5,6,2',3',5',6'-hexamethoxyflavone (**3**)¹⁸⁾ and
23 5,6,2'-trimethoxyflavone (**4**)^{14,18)} respectively.

24 Figure 2 shows spectra of furocoumarins and polymethoxyflavones. Parent ions
25 of them as determined by NMR were obtained by LC/MS and GC/MS analysis (327,
26 357, 402, 312 m/z). This result confirms the NMR analysis data.

27

28 *Glucose uptake and glycerol output activity.* To elucidate the effects of

1 compounds **1-4** on adipocyte functioning, we examined the insulin-dependent glucose
2 uptake and glycerol output of differentiated 3T3-L1 adipocytes. As shown in Fig. 3A,
3 the addition of 5 μM compounds **1** and **2** enhanced the insulin-dependent uptake of
4 glucose (1.2-1.3 fold increases as compared to control). The addition of the other
5 compounds did not enhance insulin-dependent uptake of glucose. On the other hand, the
6 addition of 5 μM compounds **1-4** did not enhance glycerol output or lipolysis (Fig. 3B).
7 These compounds at the concentrations used in this study did not affect cell viability
8 (data not shown).

9

10 *De novo FA synthesis activity and lipid accumulation of adipocytes.* To assess the
11 effects of compounds **1-4** on lipid metabolism in 3T3-L1 adipocytes, we analyzed *de*
12 *novo* FA syntheses activity with compounds **1-4**. Figure 3C shows new synthesis of
13 palmitic acid in 3T3-L1 adipocytes treated with compounds **1-4** for 48 h. The addition
14 of pioglitazone at (5.0 μM) increased the amounts of synthesized FA, by approximately
15 2.2-fold in the 3T3-L1 adipocytes as compared with control. The addition of 5.0 μM
16 compounds **1** and **2** enhanced FA syntheses by approximately 1.5-fold as compared with
17 control. Finally, to investigate the dose-dependent effects of furocoumarins on the lipid
18 accumulation of adipocytes, we performed Oil Red O staining. The addition of
19 furocoumarins increased lipid accumulation dose-dependently (Fig. 4).

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21 Obesity and associated disorders are the major noncommunicable public health
22 problems of the 21st century. Studies indicate that high levels of body fat are associated
23 with an increased risk of developing numerous adverse health conditions.^{19,20)} Hence,
24 effective therapeutic approaches to obesity and obesity-induced metabolic syndrome are
25 of wide interest. In this study, we isolated two furocoumarins and two
26 polymethoxyflavones from leaves of *Casimiroa edulis* fruit and evaluated the functions
27 of glucose and lipids metabolism activation. We found that the addition of
28 furocoumarins (compounds **1** and **2**) caused increased glucose uptake, FA synthesis, and

1 lipid accumulation in 3T3-L1 adipocytes. Furanocoumarins are known to have
2 biological effects in humans. For example, bergamottin and dihydroxybergamottin are
3 attributable to the grapefruit juice effect, in which these furanocoumarins affect the
4 metabolism of certain drugs,²¹⁾ but the concentration of furanocoumarins used in our
5 assays had no influence on cell viability. They might lower blood glucose levels by
6 orally ingested in a meal or an ordinary drink, and this calls for further evaluation *in*
7 *vivo*.

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13 **References**

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11 **Figure legends**

12 **Fig.1.** Structures of Compounds Isolated from Leaves of *Casimiroa edulis*.

13 Furocoumarins (compounds **1** and **2**), polymethoxyflavones (compounds **3** and **4**)

14

15 **Fig.2.** Mass Spectra of the Isolated Compounds.

16 Mass patterns of compounds **1-4** are shown. Compounds **1-2** and Compound **3-4**
17 were analyzed by GC/MS and LC/MS respectively.

18

19 **Fig.3.** $^{13}\text{C}_6$ -Glucose Uptake (A), Glycerol Output (B), and *De Novo* ^{13}C -FA Synthesis
20 (C) in 3T3-L1 Adipocytes after the Addition of Compounds As Determined by LC/MS
21 Analysis of the Medium at 48 h. Incubation.

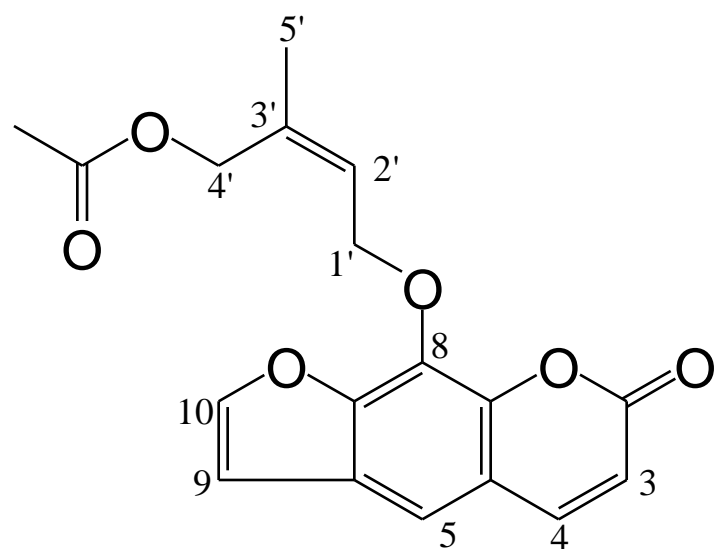
22 $^{13}\text{C}_6$ -Glucose uptake and glycerol output of 3T3-L1 adipocytes after addition of
23 coumarins and polymethoxyflavones were determined by LC/MS. The concentrations of
24 $^{13}\text{C}_6$ -glucose and glycerol were determined by the LC/MS system at 185 m/z
25 ($^{13}\text{C}_6$ -glucose) and 91 m/z (glycerol) respectively. *De novo* synthesis of palmitic acid
26 was monitored by analyzing 271 (m/z). Lane 1, control; lane 2, pioglitazone; lanes 3-4,
27 compounds **1** and **2**; lanes 5 and 6, compounds **3** and **4** respectively. Lane 1, control;
28 lane 2, pioglitazone; lanes 3 and 4, compounds **1** and **2**; lanes 5 and 6, compounds **3** and

1 **4** respectively. Concentrations (in μM) at all data points is 5.0 $**p < 0.05$ compared to
2 control.

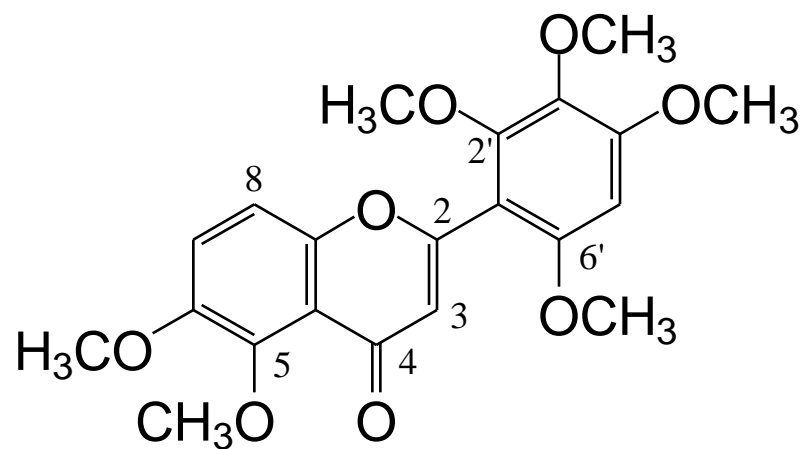
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4 **Fig. 4.** *De Novo* ^{13}C -FA Synthesis and Lipid Accumulation of 3T3-L1 Adipocytes.

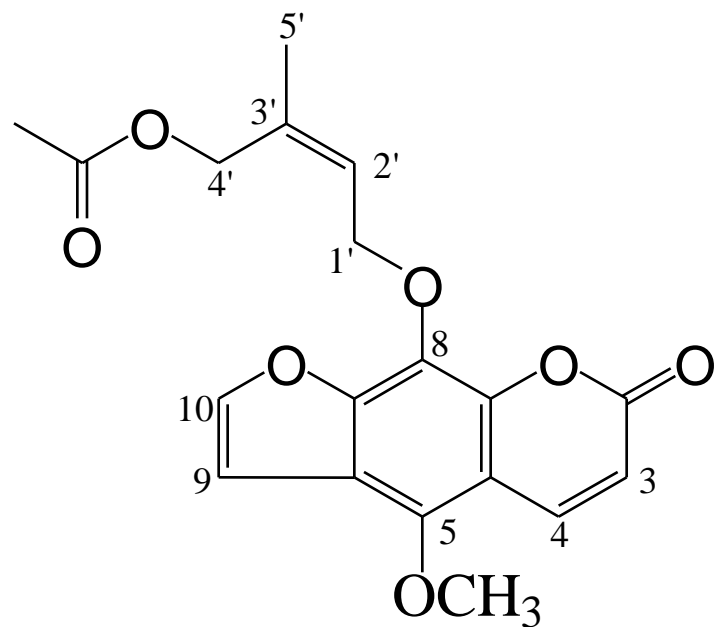
5 Adipocytes treated with pioglitazone or compounds **1,2** (furocoumarins) (1-100
6 μM) were fixed with 10% formaldehyde/PBS and stained with Oil Red O solution (0.5%
7 Oil Red O-isopropyl alcohol/ H_2O 3:2, v/v). Microscopy views of representative 3T3-L1
8 cells (original magnification, x 100).



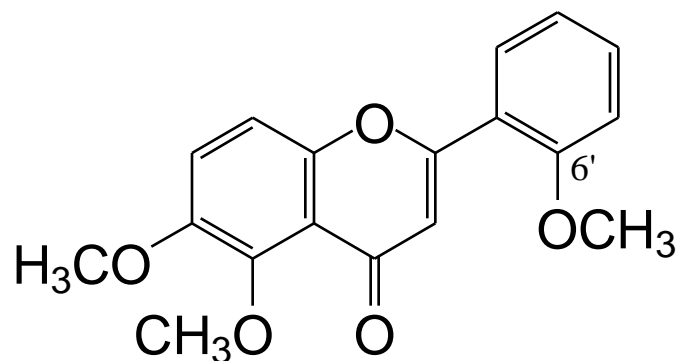
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Figure 1, Nagai

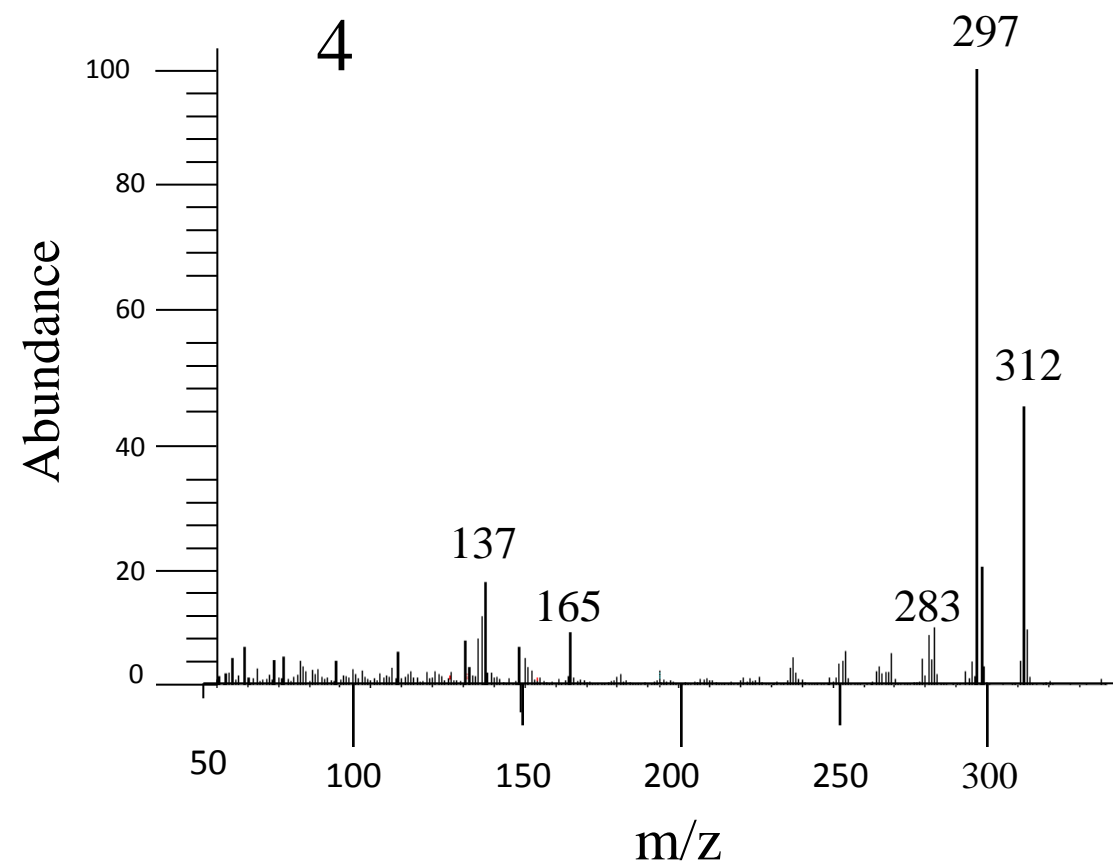
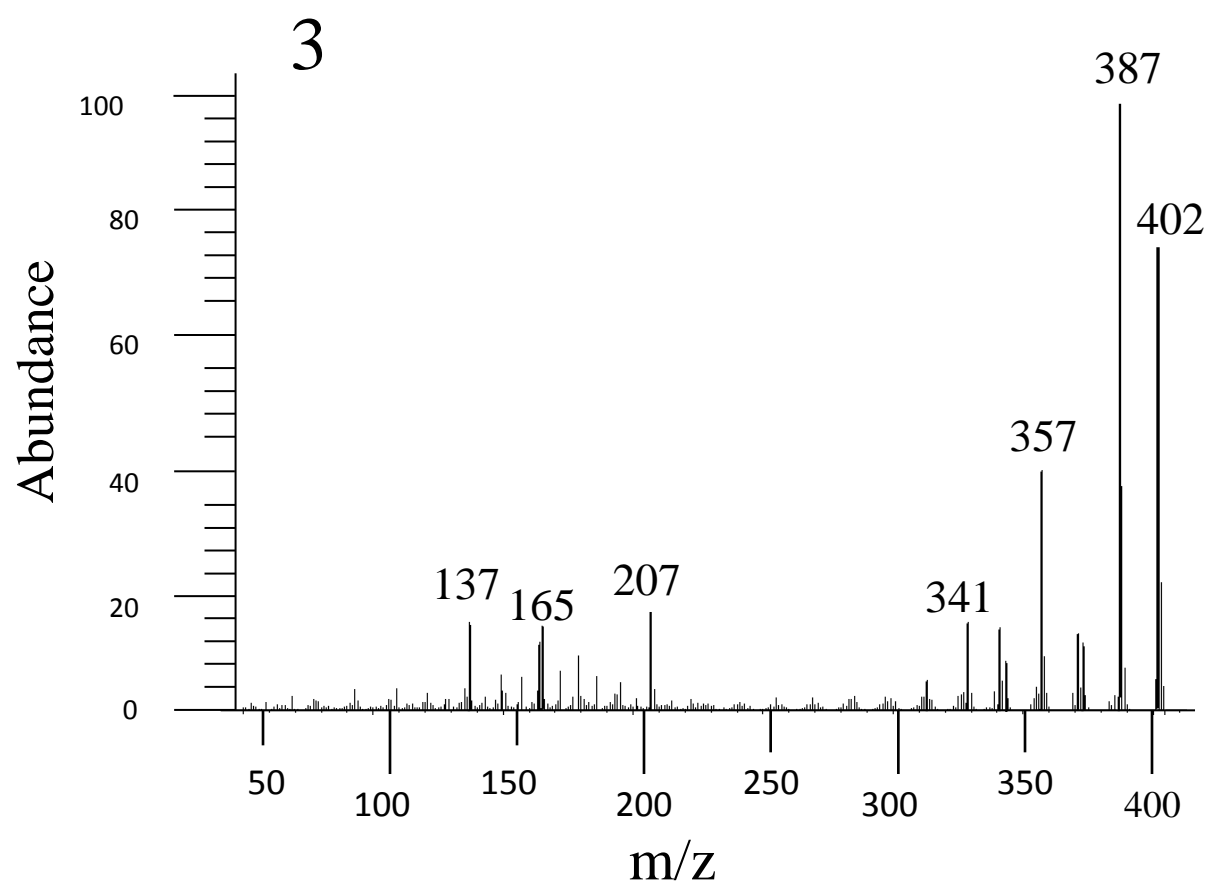
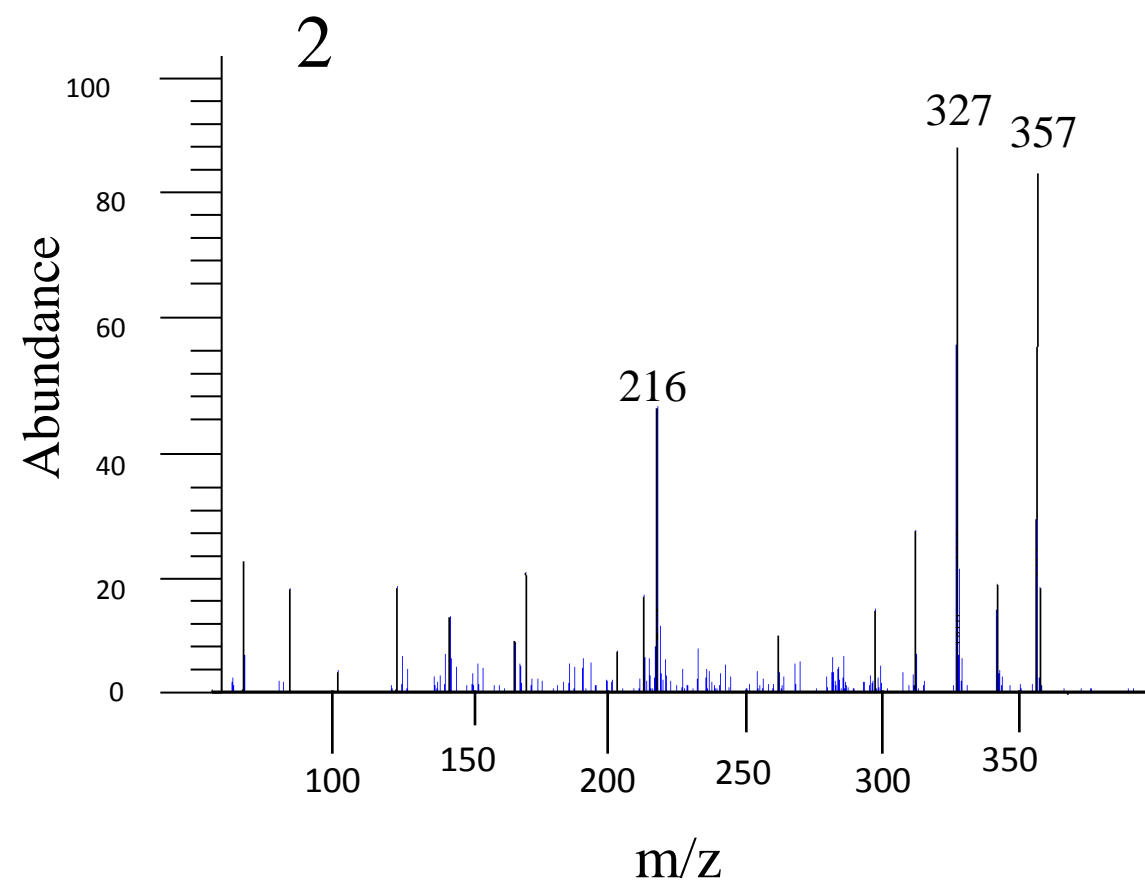
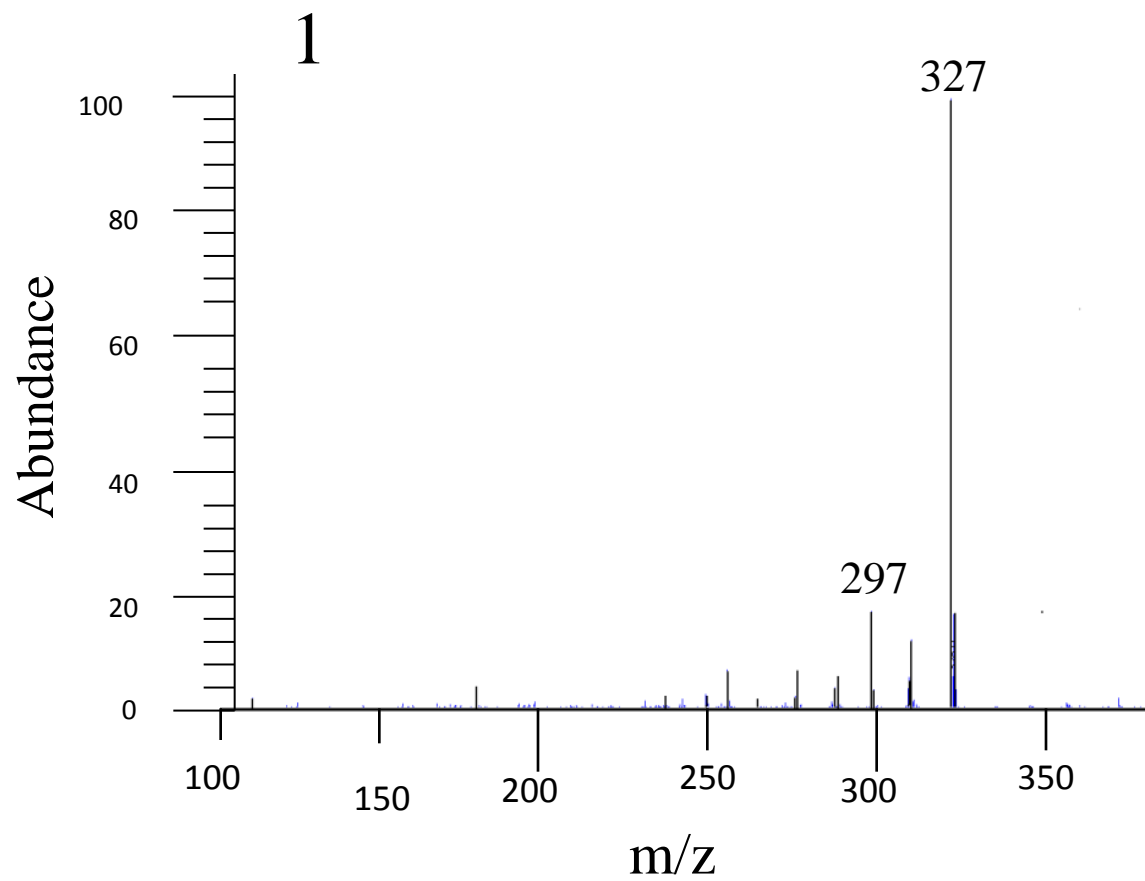
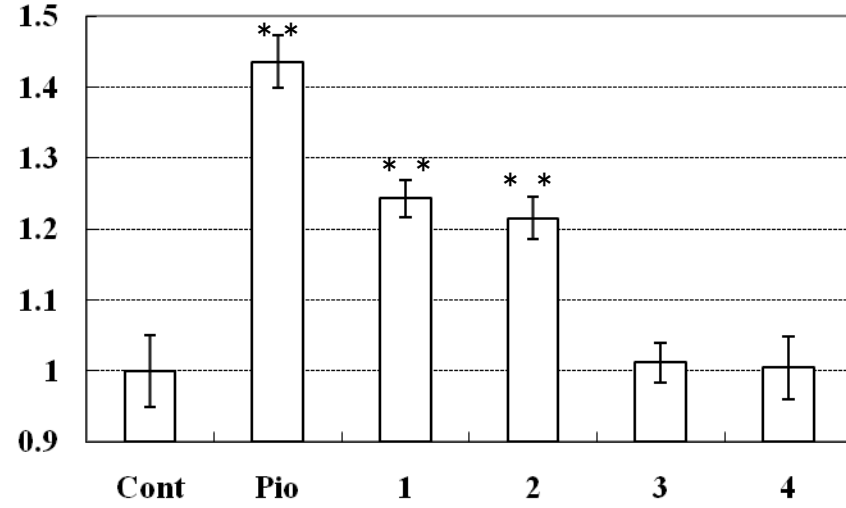


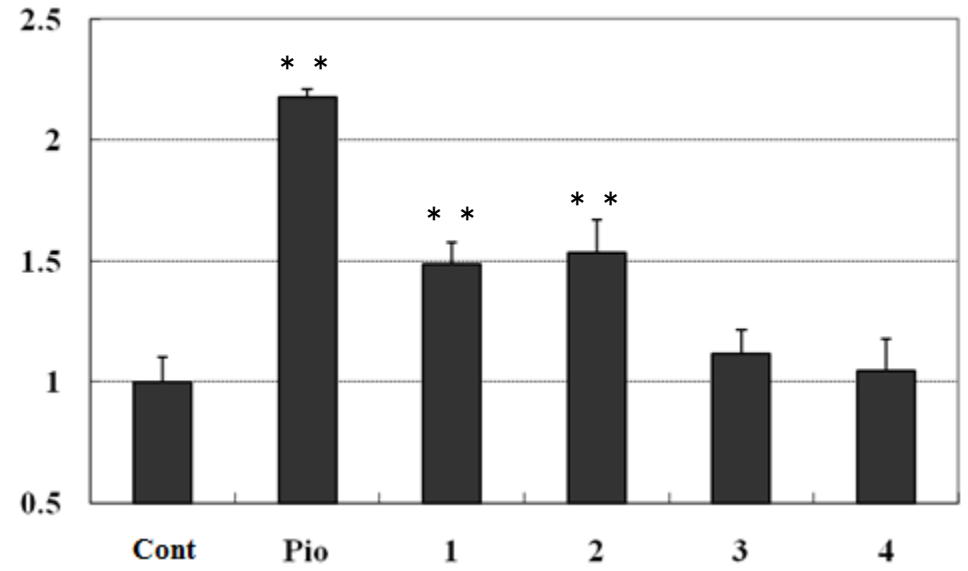
Figure 2, Nagai

Relative glucose uptake
(%/control)



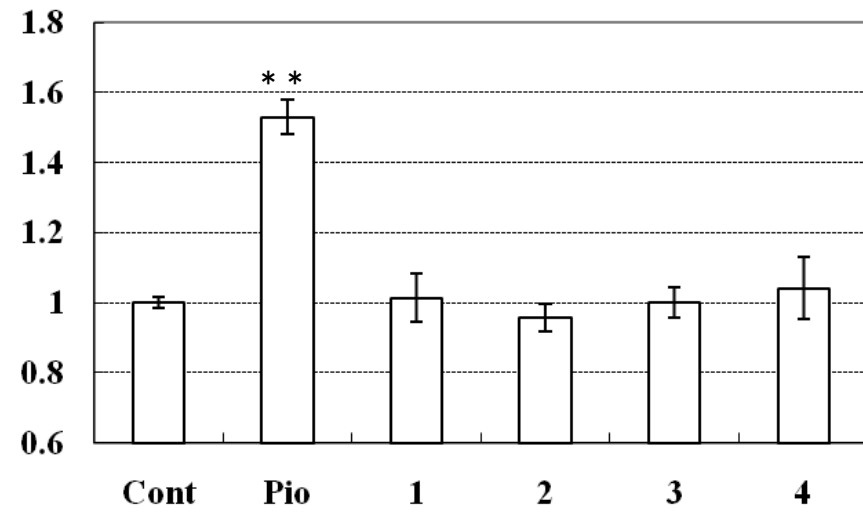
A

Relative Activity of fatty acid
synthesis (%/control)



C

Relative activity of lipolysis
(%/control)



B

Figure 3, Nagai

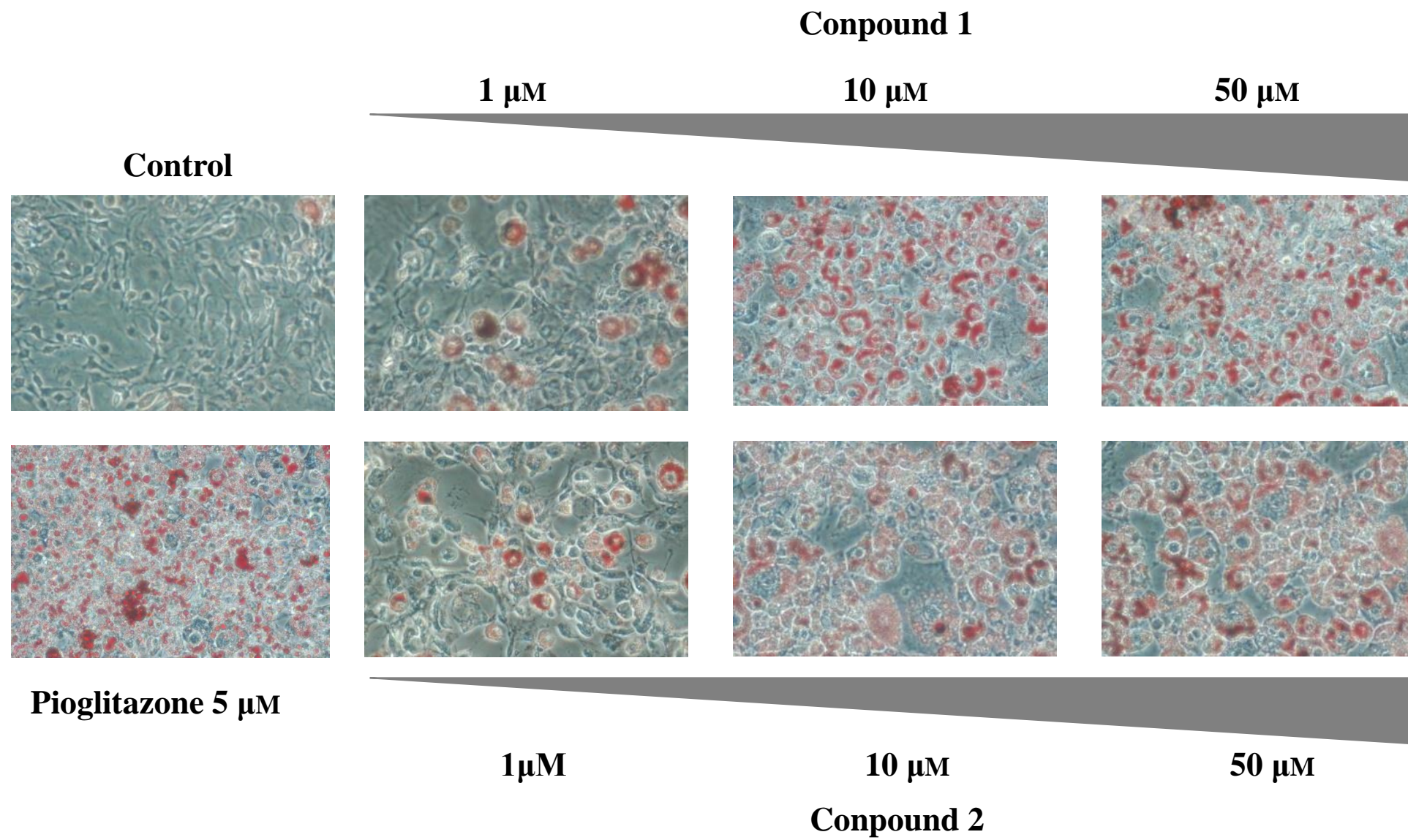


Figure 4, Nagai