Pharmacogn. Mag.
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ORIGINAL ARTICLE

Peroxisome Proliferator-Activated Receptor-γ Agonistic Effect of *Chrysanthemum indicum* Capitulum and Its Active Ingredients

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Submitted: 20-12-2017 Revised: 10-01-2018 Published: 14-08-2018

ABSTRACT

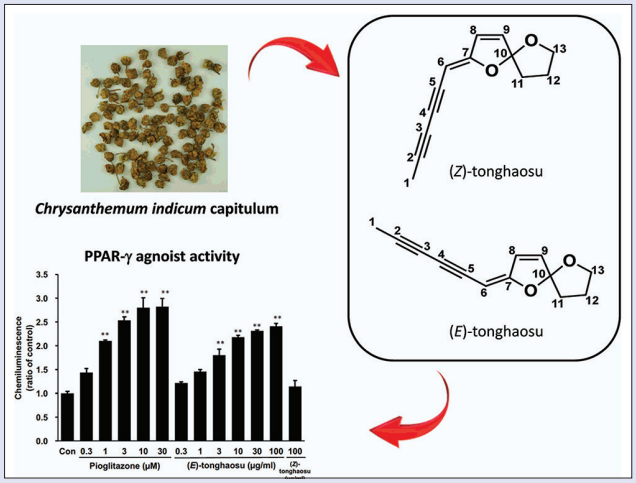
Objective: The capitulum of *Chrysanthemum indicum* (Compositae) is the source of a crude drug used in Japanese traditional Kampo and traditional Chinese medicine. Previous research showed that *C. indicum* flowers promote adipocyte differentiation through the activation of peroxisome proliferator-activated receptor (PPAR)-γ that may be an important mechanism for controlling systemic insulin resistance or other biological functions. **Materials and Methods:** The capitulum of *C. indicum* was extracted with methanol, and its PPAR-γ agonistic activity was measured using luciferase assay. The active ingredients were isolated by the activity-guided fractionations. **Results:** We isolated (*Z*)-tonghaosu and (*E*)-tonghaosu, and (*E*)-tonghaosu had PPAR-γ agonistic constituents by the activity-guided fractionation from the capitulum of *C. indicum*. The isomer (*Z*)-tonghaosu did not show PPAR-γ-agonistic activity. **Conclusion:** (*E*)-tonghaosu is one of the active ingredients as PPAR-γ agonist in the capitulum of *C. indicum*.

Key words: (E)-tonghaosu, (Z)-tonghaosu, activity-guided fractionation, *Chrysanthemum indicum*, diabetes, proliferator-activated receptor-γ

SUMMARY

- We found (*E*)-tonghaosu from the capitulum of *Chrysanthemum indicum* as PPAR-γ-agonist.

Abbreviations used: ANOVA: One-way analysis of variance; DMEM: Dulbecco's modified minimum essential medium; FBS: Fetal bovine serum; HPLC: High-performance liquid chromatography; MCI: Methanol extract of the capitulum of *Chrysanthemum indicum*; NMR: Nuclear magnetic resonance; PPAR: Peroxisome proliferator-activated receptor.



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DOI: 10.4103/pm.pm_607_17

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INTRODUCTION

Chrysanthemi Flos is a crude drug used in Japanese traditional Kampo medicine and traditional Chinese medicine. Japanese Pharmacopoeia 17th Edition (JPXVII)[1] defines that Chrysanthemi Flos is the capitulum of *Chrysanthemum morifolium* Ramatulle or *C. indicum* Linné (Compositae). On the other hand, Chinese Pharmacopoeia 2015 Edition[2] describes that the capitulum of these two plant species are recognized as different crude drug: the capitulum of the former plant species is defined as Chrysanthemi Flos, that is the same term as Japanese Pharmacopoeia, and that of the latter plant species is defined as Chrysanthemi Indici Flos. In traditional Chinese medicine, the capitulum of *C. indicum* is used to resolve *fire toxicity*,[3] and in Japanese traditional Kampo medicine, this crude drug is used as antipyretics, analgesics, and anti-inflammatory agents.[4]

The capitulum of *C. indicum* has been used as a tea beverage in the East Asian countries and as an edible garnish with raw fish served in Japan. *C. indicum* flowers are reported to have biological activities, including anti-inflammatory,[5,6] hepatoprotective,[7] analgesic,[8] and prophylactic for photoaging,[9] atopic dermatitis,[10] cadmium-induced toxicity,[11] and cisplatin-induced nephrotoxicity.[12] The capitulum of *C. morifolium*, a crude drug with the same genus origin, is reported to have the ability to promote adipocyte differentiation through peroxisome proliferator-activated receptor (PPAR)-γ activation.[13] In the present study, we evaluated the agonistic effect of methanol extract of the capitulum of *C. indicum* and isolated its active ingredients by the activity-guided fractionation.

Cite this article as: Zhang F, Iwaki A, Ishiuchi K, Sugiyama A, Ohsawa M, Makino T. Peroxisome proliferator-activated receptor-γ agonistic effect of *Chrysanthemum indicum* capitulum and its active ingredients. Phcog Mag 2018;14:461-4.

MATERIALS AND METHODS

Materials and its fractionation

The dried capitulum of *C. indicum* collected in Zhejian, China, with the grade of Japanese Pharmacopoeia seventeenth edition[1] (Lot number 8G08) is purchased from Daiko Shoyaku (Nagoya, Japan). Voucher specimens are deposited in the Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, and Nagoya City University. The dried capitulum of *C. indicum* (148 g) was powdered and mixed with 1.5 l of methanol and stirred overnight at room temperature. The solution was filtered with paper. The procedure was repeated twice with the resulting residue. The extracted solution was evaporated at 42°C under reduced pressure and then lyophilized to yield 36.5 g total methanol extract (methanol extract of the capitulum of *Chrysanthemum indicum* [MCI]). This extract (36 g) was suspended in water and serially partitioned with n-hexane, ethyl acetate, and water-saturated butanol to yield a hexane layer (5.7 g), an ethyl acetate layer (8.6 g), a butanol layer (8.0 g), and a water layer (14.0 g).

The hexane layer (100 mg) was spotted onto silica gel 60G F_{254} chromatography glass plate (2 mm, 20 cm × 20 cm, Merck Millipore, Billerica, MA, USA) and expanded with hexane/ethyl acetate (9:1) to yield fractions A–P. Because fraction F (*Rf* = 0.33) was the highest activity, hexane layer (5.5 g) was applied onto a silica gel open column chromatography (35 mm × 350 mm) and eluted with hexane/ethyl acetate (9:1) to yield 0.24 g of fraction F. Fraction F was further analyzed using silica gel open column chromatography (20 mm × 400 mm) eluted with chloroform to yield fractions F1–F14. Fraction F3 (27.3 mg) showed that the highest activity was analyzed with high-performance liquid chromatography (HPLC) (Cosmosil 5C18-AR-II, 10 mm × 150 mm, Nacalai Tesque, Kyoto, Japan), eluted with water/acetonitrile 1:1, and analyzed with ultraviolet absorbance at 238 nm to yield peak 1 (retention time, 15 min), 2 (21 min), and 3 (27 min). From 10 mg of F3, 1.0 mg of peak 1 and 2 and 1.4 mg of peak 3 were collected. The spectra of ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) of peaks 2 and 3 were analyzed, and their purities were 95% and 99%, respectively. Then, they were named compound 2 and 3, respectively, and NMR, electron impact ionization mass spectrometry (EI-MS) spectrum, and optical rotations data were measured as follows:

Compound 2: colorless amorphous solid; $[\alpha]_D^{20}$-1.4 (*c* 0.4, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 500MHz) 6.23 (1H, d, J = 6.0 Hz, H-8), 6.15 (1H, d, J = 6.0 Hz, H-9), 4.60 (1H, brs, H-6), 4.24 (1H, ddd, J = 8.0, 8.0, 4.0 Hz, H-13a), 3.99 (1H, ddd, J = 8.0, 8.0, 8.0 Hz, H-13b), 2.33 (1H, m, H-12a), 2.23 (1H, m, H-11a), 2.06 (1H, m, H-12b), 2.05 (1H, m, H-11b), and 1.99 (3H, brs, H-1); and ^{13}C-NMR ($CDCl_3$, 125 MHz) 167.1 (C-7), 135.2 (C-9), 127.5 (C-8), 121.0 (C-10), 80.6 (C-2), 78.9 (C-4), 78.8 (C-6), 70.7 (C-5), 69.7 (C-13), 65.2 (C-3), 35.6 (C-11), 24.5 (C-12), and 4.8 (C-1). EIMS m/z 200 $[M]^+$; HREIMS *m/z* 200.0828 (M; calcd for $C_{13}H_{12}O_2$, 200.0837).

Compound 3: colorless amorphous solid; $[\alpha]_D^{22}$ 0.1 (*c* 0.6, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 500MHz) 6.69 (1H, d, J = 6.0 Hz, H-8), 6.20 (1H, dd, J = 6.0, 2.0 Hz, H-9), 4.93 (1H, brs, H-6), 4.18 (1H, ddd, J = 8.0, 8.0, 3.0 Hz, H-13a), 3.99 (1H, ddd, J = 8.0, 8.0, 8.0 Hz, H-13b), 2.24 (1H, m, H-12a), 2.15 (1H, m, H-11a), 2.07 (1H, m, H-12b), 2.05 (1H, m, H-11b), and 1.98 (3H, d, J = 1.0 Hz, H-1); and ^{13}C-NMR ($CDCl_3$, 125 MHz) 168.8 (C-7), 135.7 (C-9), 126.0 (C-8), 120.8 (C-10), 79.9 (C-6), 79.7 (C-2), 76.3 (C-4), 71.5 (C-5), 69.7 (C-13), 64.9 (C-3), 35.5 (C-11), 24.5 (C-12), and 4.7 (C-1). EIMS m/z 200 $[M]^+$; HREIMS *m/z* 200.0844 (M; calcd for $C_{13}H_{12}O_2$, 200.0837).

Based on these spectra, compounds 2 and 3 were identified as (*Z*)- and (*E*)-tonghaosu, respectively.

Measurement of (*Z*)- and (*E*)-tonghaosu in the samples of chrysanthemi flos

Aliquot of the dried capitulum of *C. indicum* (5.0 g) was extracted using 100 ml of methanol with sonication for 30 min at room temperature, and the solution was filtered with paper. The procedure was repeated twice with the resulting residue. The solution was then evaporated at 42°C under reduced pressure and lyophilized to yield the extract. Three replicate extracts were prepared from one sample. Each methanol extract (50 μg) or (*E*)-tonghaosu (20 ng, 0.10, 0.50, and 1.0 μg) was injected to HPLC column (Cosmosil $5C_{18}$-AR-II, 4.6 × 150 mm, Nacalai) and eluted with water/acetonitrile 1:1 (1.0 ml/min) to measure the peak areas of (*Z*)- and (*E*)-tonghaosu (retention times, 8.8 and 11.3 min, respectively) detected with ultraviolet absorbance at 238 nm. A linear regression line with r^2 value > 0.999 was plotted within the range from 20 ng to 1.0 μg of (*Z*)- and (*E*)-tonghaosu, and the detection limit was approximately 0.5 ng.

Assay for peroxisome proliferator-activated receptor-γ agonistic activity

HEK293 (human embryonic kidney) cells were maintained in Dulbecco's modified minimum essential medium (DMEM, Nacalai) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai) under 5% CO_2 atmosphere at 37°C. To prepare defatted FBS, 2 g of Cab-O-sil® M-5 (Sigma) was added in 25 ml of FBS and stirred at 4°C overnight. The FBS was then centrifuged (1.5×10^3 ×g) at 4°C for 1 h and sterilized using membrane filtration. The cells (1.5×10^5 cells/well) were seeded in 24-well culture plates and incubated in DMEM containing 10% defatted FBS at 37°C overnight. The cells were dual transfected with 0.5 μg each of pCMV-SPORT-PPAR-γ vector (Open Biosystems of Thermo Scientific, Waltham, MA, USA) and pGL4.26 PPRE5 × 48 vector, that was constructed by inserting five repeats of PPAR-γ response sequence into HindIII site of pGL4.26 luciferase reporter vector (Promega, Madison, WI, USA), by lipofection using X-tremeGENE transfection reagents (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer's protocol. After transfection, the medium was exchanged with fresh medium containing 10% defatted FBS with or without test samples. After 24 h, the cells were harvested to determine the luciferase activities. Pioglitazone (Wako Pure Chemicals, Osaka) was used as a positive control. Luciferase activity was determined as the ratio of the sample to the chemiluminescence of control group. Experiments were conducted in triplicate or quadruplicate.

Statistics

To compare results among groups, we used a one-way analysis of variance (ANOVA), followed by Bonferroni/Dunnett's multiple *t*-test (PASQ Statistics version 18, SPSS, IBM, Armonk, NY, USA). Differences were considered statistically significant at values of $P < 0.05$.

RESULTS

MCI showed concentration-dependent PPAR-γ agonistic activity [Figure 1]. We partitioned n-hexane, ethyl acetate, and water-saturated butanol, and water layer from MCI, and both n-hexane and water layers showed a significant PPAR-γ agonistic activity at the concentration related to 100 μg/ml of the original CFM [Figure 2a]. Further, chromatography showed that fraction F from n-hexane layer exhibited a significant PPAR-γ agonistic activity at 20 μg/ml [Figure 2b]. Fraction F was further separated into 14 fractions, and one of these (F3) exhibited a significant PPAR-γ agonistic activity at 20 μg/ml (data not shown).

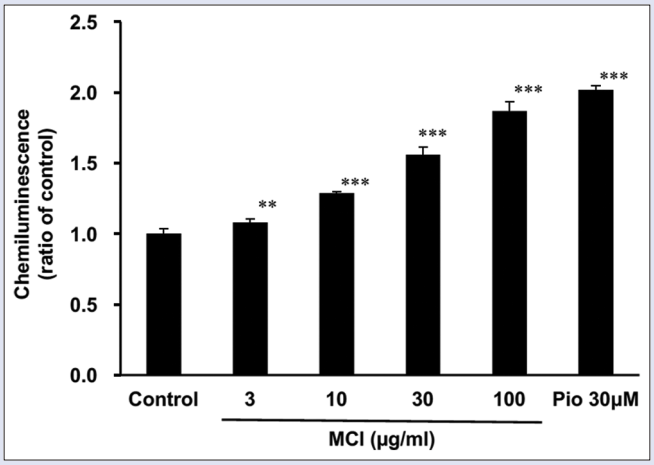

Figure 1: Peroxisome proliferator-activated receptor-γ agonistic activity of methanol extract of the capitulum of *Chrysanthemum indicum*. HEK293 cells were transiently transfected with pGL PPRE-luciferase reporter plasmid and pCMV-peroxisome proliferator-activated receptor-γ plasmid and incubated with the medium containing defatted fetal bovine serum for 24 h. methanol extract of the capitulum of *Chrysanthemum indicum* or pioglitazone (Pio) (30 μM) was dissolved in the media and further incubated for 24 h. Then, cells were lysed and the substrates for luciferase were added, and chemiluminescences were measured. Data are shown as the ratio of the control (without any treatment with methanol extract of the capitulum of *Chrysanthemum indicum* or pioglitazone) and expressed as mean ± standard error ($n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group evaluated by Bonferroni–Dunnett's multiple *t*-test

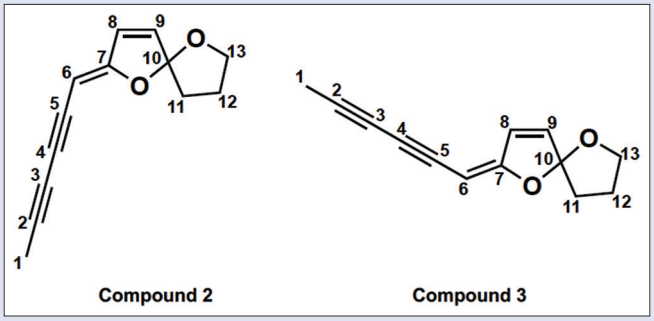

Figure 3: Chemical structures of compound 2 and 3

Analysis of F3 by HPLC revealed three distinct peaks. Peak 2 and Peak 3 were designated as compounds 2 and 3, respectively. On the basis of MS, NMR, optical rotation analysis, and comparison with previous studies,[14] compounds **2** and **3** were identified as (*Z*)-tonghaosu and (*E*)-tonghaosu, respectively [Figure 3]. The contents of (*Z*)- and (*E*)-tonghaosu in MCI were 0.063 ± 0.019 (w/w) % and 0.12 ± 0.04 (w/w) %, respectively. (*E*)-tonghaosu showed a significant concentration-dependent PPAR-γ agonistic activity, but (*Z*)-tonghaosu did not at a concentration of 100 μg/ml [Figure 4].

DISCUSSION

PPAR-γ agonists have a wide spectrum of biological functions, including regulating metabolism, reducing inflammation, influencing the balance of immune cells, inhibiting apoptosis and oxidative stress, and improving endothelial function; moreover, they are widely prescribed to type II diabetes mellitus in Japan.[15]

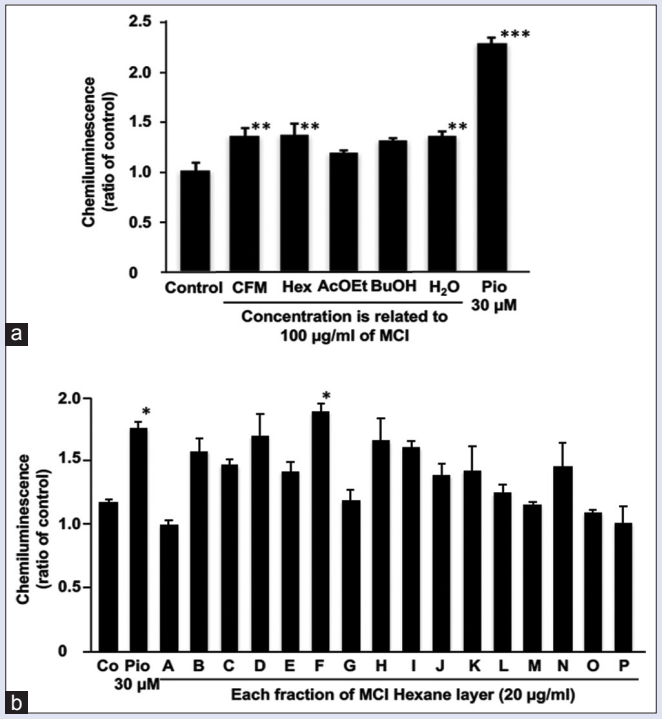

Figure 2: Peroxisome proliferator-activated receptor -γ agonistic activity of the fractions of methanol extract of the capitulum of *Chrysanthemum indicum*. (a) methanol extract of the capitulum of *Chrysanthemum indicum* was partitioned according to the descriptions of Materials and Methods, and the cells was treated with the medium containing each layer (Hex, hexane layer; AcOEt, ethyl acetate layer; BuOH, buthanol layer, H_2O, water layer; Pio, pioglitazone) at the concentration related to the original methanol extract of the capitulum of *Chrysanthemum indicum* (100 μg/ml). (b) Fractionated hexane layer of methanol extract of the capitulum of *Chrysanthemum indicum* shown as Fr. A– P. The cells were treated with the medium containing each fraction (20 μg/ml). Co, Control; Pio, pioglitazone. Data are shown as the ratio of the control and expressed as mean ± standard error ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group evaluated by Bonferroni–Dunnett's multiple *t*-test

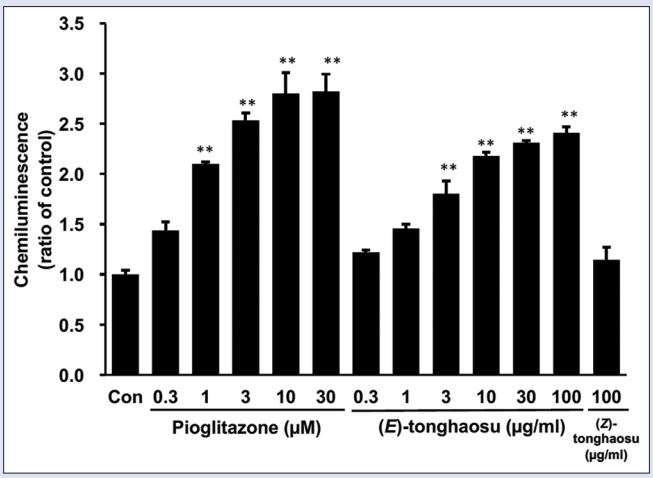

Figure 4: Peroxisome proliferator-activated receptor -γ agonistic activity of (*Z*)- and (*E*)-tonghaosu. (*Z*)-tonghaosu, (*E*)-tonghaosu, or pioglitazone was dissolved in the media and further incubated for 24 h. Then, cells were lysed and the substrates for luciferase were added, and chemiluminescence was measured. Data are shown as the ratio to the control and expressed as mean ± standard error ($n = 4$). $^{**}P < 0.01$ versus control group evaluated by Bonferroni–Dunnett's multiple *t*-test

In the present study, MCI at 100 μg/ml showed PPAR-γ agonistic activity approximately double of an experimental control. Previous study indicated that the hot water extract of *C. morifolium* edible flower (100 μg/ml) caused doubling of PPAR-γ mRNA expression in 3T3-L1 adipocyte cells.[16] Although *Chrysanthemum* species and the extracting methods are different from our study, the results are consistent with the previous report.[16]

We isolated the active constituents from CFM by activity-guided fractionation. Results show that PPAR-γ agonistic activities were distributed among several fractions of the extract, suggesting that Chrysanthemi Flos contained several active constituents. We isolated three peaks from the fraction exhibiting the highest activity and found that two of these (compounds **2** and **3**) had PPAR-γ agonistic activity. We further analyzed the chemical structures of compounds **2** and **3**. Both compounds were geometric isomers of tonghaosu, and only (*E*)-isomer had PPAR-γ agonistic activity. The polyacetylene motif binding to carbon 6 of tonghaosu with different geometry would affect the binding of tonghaosu to the target site of PPAR-γ. Although conjugated linoleic acids have different anti-inflammatory activities among geometric isomers,[17] the geometric character of tonghaosu could affect the PPAR-γ agonistic activity. (*E*)-tonghaosu showed significant PPAR-γ agonistic activity at 3 μg/ml = about 15 μM, suggesting that (*E*)-tonghaosu have about one-tenth activity of pioglitazone.

CONCLUSION

We identified (*E*)-tonghaosu as PPAR-γ agonistic constituents in the capitulum of *C. indicum*. Considerable chemical modulation would be needed to derive a new PPAR-γ agonist using (*E*)-tonghaosu as the seed.

Acknowledgements

This study was partially supported by the A-STEP Program from Japan Science and Technology Agency, JST (2013).

Financial support and sponsorship

This study was partially supported by the A-STEP Program from Japan. Science and Technology Agency, JST (2013).

Conflicts of interest

There are no conflicts of interest.

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