# Microbial Metabolism of Yangonin, a Styryl Lactone from *Piper methysticum* (Kava)

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**Abstract** – Microbial metabolism studies of yangonin (**1**), a major styryl lactone from *Piper methysticum*, have resulted in the production of three hydroxylated metabolites (**2-4**). The chemical structures of these compounds were elucidated to be 4-methoxy-6-(12-hydroxystyryl)-2-pyrone (**2**), 4-methoxy-6-(11,12-dihydroxystyryl)-2-pyrone (**3**), and 4,12-dimethoxy-6-(7,8-dihydroxy-7,8-dihydrostyryl)-2-pyrone (**4**) on the basis of the chemical and spectroscopic analyses. The compounds **3** and **4** are reported herein as microbial metabolites of yangonin for the first time.
**Keywords** – Microbial metabolism, Yangonin, Styryl lactone, *Piper methysticum*, Kava

## Introduction

The roots of the kava shrub (*Piper methysticum* G. Forst, Piperaceae) have been used to prepare an intoxicating beverage for South Pacific Islanders' social occasions (Whitton, *et al.*, 2003). In Western countries, kava extracts have been widely used as an alternative medicine for the treatment of mental disorders, nervous anxiety, tension, and restlessness (Volz and Kieser, 1997). The kavalactones (styryl lactones of kava) were identified from kava roots and have been recognized as the major constituents responsible for the pharmacological effects of kava (Singh and Blumenthal, 1997; Singh and Singh, 2002; Bilia, *et al.*, 2002). Among the several kavalactones identified, yangonin (**1**) (4-methoxy-6-[(*E*)-2-(4-methoxyphenyl)ethylenyl] pyran-2-one) is one of the major constituents of kava extracts and it was shown to exhibit a weak melanogenesis stimulation activity (Matsuda, *et al.*, 2006). It has also shown a promising TNF-α release inhibitory activity (Hashimoto, *et al.*, 2003).

In the evaluation of safety and efficacy of kava, it is important to understand how its active constituents are metabolized. Metabolism studies have been carried out in attempts to identify the metabolic fate of kavalactones, and previous *in vivo* (Tarbah, *et al.*, 2003) and *in vitro* (Rasmussen, *et al.*, 1979; Duffield, *et al.*, 1989; Johnson,

*et al.*, 2003) studies provided several derivatives or metabolites including glucuronylated, hydroxylated, demethylated, and dehydrated metabolites. Microbial metabolism studies on kavalactones have been reported for kawain, methysticin (Abourashed and Khan, 2000), and dihydrokawain (Herath, *et al.*, 2004). Previous studies have reported metabolites of yangonin in rat and human urine. In both cases, however, unmetabolized kavalactones were detected in higher concentrations than any of their metabolites. In rats, the metabolism of yangonin occurred at its methoxyl group to produce two hydroxylated metabolites, namely, *p*-hydroxy-5,6-dehydrokawain and dihydroxy-5,6-dehydro-7,8-dihydrokawain as evidenced by mass spectrometry (Rasmussen, *et al.*, 1979). In humans, the yangonin metabolite was identified as a demethylated product at 4-methoxyl group (Duffield, *et al.*, 1989).

Microbial metabolism studies using microbes are well known as one of the useful tools to generate selective conversions of compounds to derivatives which are difficult to produce synthetically, as well as to mimic and predict mammalian metabolism for xenobiotic metabolism (Clark, *et al.*, 1985; Venisetty and Cidii, 2003). Preparative-scale microbial transformation of yangonin (**1**) by *Cunninghamella elegans* var. *elegans* KCTC 6992 and *Absidia coerulea* KCTC 6936 afforded three metabolites (**2-4**) (Fig. 1). Production and structure elucidation of these metabolites by microorganisms are described.

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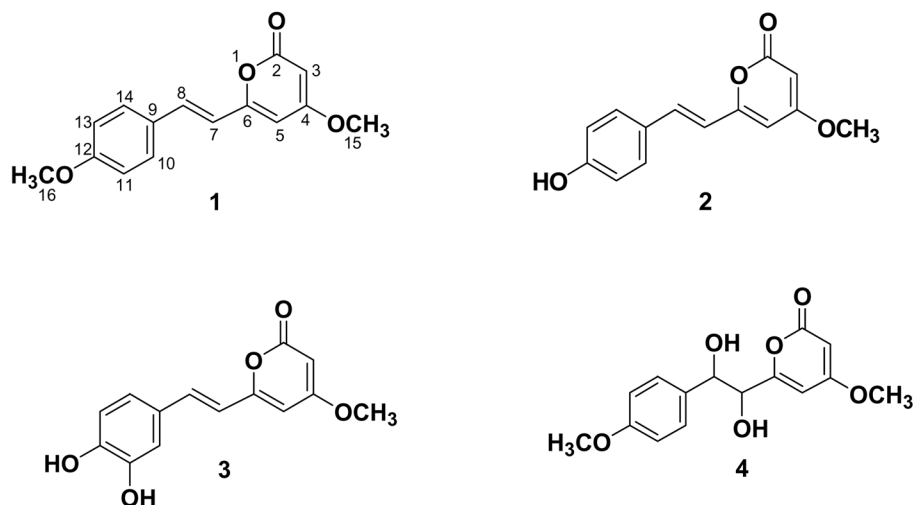Tel: +82-62-530-2932; E-mail: islee@chonnam.ac.kr

**Fig. 1.** Chemical structures of yangonin (**1**) and its metabolites (**2 - 4**).

## Experimental

**General experimental procedures** – UV spectra were recorded on a JASCO V-530 spectrophotometer, and IR spectra were obtained on a JASCO FT/IR 300-E spectrometer. NMR experiments were recorded using a Varian Unity INOVA 500 spectrometer. $^1H$ and $^{13}C$ NMR spectra were recorded at 500 and 125 MHz, respectively, and TMS was used as the internal standard. ESIMS and HRESIMS were determined on a Micromass QTQF2 mass and Waters Synapt HDMS LC-MS spectrometers, respectively. TLC was carried out on Merck silica gel 60 $F_{254}$-precoated glass plates and RP-18 $F_{254S}$ plates. Medium pressure liquid chromatography (MPLC) was carried out with Merck Lobar$^{TM}$ column (10 × 240 mm, 40 - 63 μm) using UV Detector at 254 and 280 nm. HPLC was performed on a Waters 600E multisolvent delivery system connected to a UV detector using Beckman Ultrasphere ODS column (4.6 × 250 mm, 5 μm) with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) at a flow rate of 1.0 mL/min.

**Chemicals** – The roots of *P. methysticum* were imported from Fiji Islands. *P. methysticum* (kava) root powder (3 kg) was extracted three times with methylene chloride at room temperature for 1 h under sonication to obtain kavalactones, which are lipophilic compounds. The combined methylene chloride extract was evaporated to give a dark green viscous semisolid. This semisolid was dissolved in methanol, diluted with diethyl ether and kept at −10 °C for 12 h to give 90 g of a crystalline material. Recrystallization of this material from methanol yielded yangonin and the mother liquor was subjected to silica gel (70 - 230 mesh, Merck) column chromatography. Elution

of the column with increasing amounts of acetone in hexane as the solvent system gave fractions with desmethoxyyangonin, yangonin, dihydroxykawain, kawain, dihydroxymethysticin, and methysticin as major compounds, and they were further purified by crystallization (Dharmaratne, *et al.*, 2002). The NMR data of yangonin were in agreement with data in the literature (Shao, *et al.*, 1998; Dharmaratne, *et al.*, 2002).

**Yangonin (1)** – $^1H$-NMR (CDCl$_3$) δ 7.46 (1H, d, $J$ = 15.9 Hz, H-8), 7.45 (2H, d, $J$ = 8.7 Hz, H-10, 14), 6.90 (2H, d, $J$ = 8.7 Hz, H-11, 13), 6.45 (1H, d, $J$ = 15.9 Hz, H-7), 5.90 (1H, d, $J$ = 2.1 Hz, H-5), 5.47 (1H, d, $J$ = 2.1 Hz, H-3), 3.84 (3H, s, 4-OCH$_3$), 3.82 (3H, s, 12-OCH$_3$); $^{13}C$-NMR (CDCl$_3$) δ 171.2 (C-4), 164.2 (C-2), 160.8 (C-12), 159.1 (C-6), 135.5 (C-8), 129.0 (C-10, 14), 128.0 (C-9), 116.4 (C-7), 114.4 (C-11, 13), 100.4 (C-5), 88.4 (C-3), 55.9 (4-OCH$_3$), 55.3 (12-OCH$_3$).

All the ingredients for microbial media including D-glucose, peptone, malt extract, yeast extract, and potato dextrose medium were purchased from Becton, Dickinson and Co., and sucrose was purchased from Sigma-Aldrich Co.

**Microorganisms and fermentation** – All the microorganisms were obtained from the Korean Collection for Type Cultures (KCTC). The cultures used for preliminary screening were as follows: *Absidia coerulea* KCTC 6936, *Aspergillus fumigatus* 6145, *Benisingtonia intermedia* 7207, *Candida albicans* 7965, *Candida famata* 7000, *Candida solani* 7689, *Cunninghamella elegans* var. *elegans* 6992, *Curvularia lunata* var. *lunata* 6919, *Debaryomyces occidentalis* var. *occidentalis* 7194, *Debaryomyces robertsiae* 7299, *Filobasidium capsuligenum* 7102, *Fusarium oxysporum* f.sp. *lini* 16325, *Gliocladium deliquescens* 6173, *Glomerella cingulata* 6104, *Hormoconis resinae* 6966,

*Kluyveromyces marxianus* 7155, *Lactobacillus brevis* 3102, *Leuconostoc inhae* 3774, *Metschnikowia pulcherrima* 7605, *Monascus rubber* 6122, *Mortierella ramanniana* var. *angulispora* 6137, *Mucor hiemalis* 6165, *Penicillium chrysogenum* 6933, *Pichia membranifaciens* 7006, *Pichia pastoris* 7190, *Rhizopus oryzae* 6399, *Rhizopus oryzae* 6946, *Saccharomyces cerevisiae* 7904, *Saccharomycodes ludwigii* 7126, *Torulaspora delbrueckii* 7116, *Trichoderma koningii* 6042, *Trigonopsis variabilis* 7263, *Weissella soli* 3789.

Fermentation experiments were performed in five types of media; *F. oxysporum* f.sp. *lini*, *C. lunata* var. *lunata*, *R. oryzae* (KCTC 6399) and *C. elegans* var. *elegans* were cultured on potato dextrose medium (24 g/L). *M. hiemalis* was incubated on potato sucrose medium (potato dextrose 24 g/L and sucrose 20 g/L). *P. membranifaciens* was incubated on malt medium (malt extract 20 g/L and peptone 5 g/L). Other microorganisms were cultured on yeast-malt medium (D-glucose 10 g/L, peptone 5 g/L, malt extract 3 g/L, and yeast extract 3 g/L).

**Screening procedure** – Microbial metabolism studies were performed according to the standard two-stage procedure (Clark *et al.*, 1985). In the screening studies, the actively growing microbial cultures were inoculated in 100 mL flasks containing 20 mL of media, and incubated with gentle agitation (200 rpm) at 25 °C in a temperature-controlled shaking incubator for 72 hours. Microbial culture (1 mL) was transferred to the fresh medium and incubated with gentle agitation (200 rpm) at 25 °C in a temperature-controlled shaking incubator for 24 hours. The DMSO solution (1 mg/100 μL) of yangonin (**1**) was added to each flask at a concentration of 40 μg/mL 24 h after inoculation, and incubated further at the same condition for 5 days. Substrate controls consisted of **1** and sterile YM medium incubated without microorganisms. Culture controls consisted of fermentation cultures in which the microorganisms were grown without addition of **1**. General sampling and TLC monitoring were performed on RP-18 $F_{254s}$ (65% MeOH) at 24 h intervals. UV light (254 and 355 nm) and anisaldehyde-sulfuric acid reagent were used for identification of the metabolites.

**Microbial metabolism of yangonin by *C. elegans* var. *elegans* KCTC 6992** – Scale-up fermentations were carried out under the same temperature-controlled shaking condition with sixteen 1 L flasks each containing 250 mL of medium and 10 mg of yangonin. After incubation for 5 days, the microbial cultures were extracted with the same volume of EtOAc (4 L) three times, and the organic layers were combined and concentrated at reduced pressure. The EtOAc extract (1 g) was subjected to silica gel (70 - 230

mesh, Merck) column chromatography with a $CHCl_3$-MeOH (500 : 1 → 1 : 1) gradient solvent system to give ten fractions. Fraction 4 (38 mg) was chromatographed on reversed-phase HPLC, eluting with 25% $CH_3CN$ (flow rate: 1 mL/min, UV detection: 254 nm) to afford the yangonin metabolite YM1 (**2**, 2.0 mg). Fraction 8 (25 mg) was chromatographed on reversed-phase HPLC, eluting with a 2-propanol : $CH_3CN$ : $H_2O$ : acetic acid isocratic eluent (16 : 16 : 84 : 0.1, flow rate: 1 mL/min, UV detection: 254 nm) to afford the metabolite YM2 (**3**, 2.0 mg).

**4-Methoxy-6-(12-hydroxystyryl)-2-pyrone (2)** – Yellow amorphous powder, UV $\lambda_{max}$ (MeOH) 205, 209, 211, 359 nm; IR (KBr) $\nu_{max}$ 3383, 2925, 2859, 1957, 1731, 1600, 1454, 1382, 1178, 1029 cm$^{-1}$; $^1$H-NMR (pyridine-$d_5$) δ 7.56 (1H, m, H-10, 14), 7.55 (1H, m, H-8), 7.19 (1H, m, H-11, 13), 6.74 (1H, d, $J = 15.5$Hz, H-7), 6.09 (1H, d, $J = 2.3$ Hz, H-5), 5.72 (1H, d, $J = 2.3$ Hz, H-3), 3.82 (3H, s, 4-OCH$_3$); $^{13}$C-NMR (pyridine-$d_5$) δ 171.7 (C-4), 163.1 (C-2), 161.1 (C-12), 160.1 (C-6), 131.8 (C-8), 130.3 (C-10, 14), 127.5 (C-5), 117.2 (C-11, 14), 116.8 (C-7), 100.8 (C-5), 89.1 (C-3), 56.3 (4-OCH$_3$); ESIMS $m/z$ 245 [M+H]$^+$.

**4-Methoxy-6-(11,12-dihydroxystyryl)-2-pyrone (3)** – Brown amorphous powder, UV $\lambda_{max}$ (MeOH) 202 (sh), 203, 265, 359 nm; IR (KBr) $\nu_{max}$ 3343, 2920, 1966, 1680, 1603, 1549, 1452, 1404, 1252, 1153, 1031 cm$^{-1}$; $^1$H-NMR (MeOH-$d_4$) δ 7.29 (1H, d, $J = 16.0$ Hz, H-8), 7.04 (1H, d, $J = 2.0$ Hz, H-10), 6.94 (1H, dd, $J = 2.0$, 8.5 Hz, H-14), 6.79 (1H, d, $J = 8.5$ Hz, H-13), 6.59 (1H, d, $J = 16.0$ Hz, H-7), 6.15 (1H, d, $J = 2.3$ Hz, H-5), 5.57 (1H, d, $J = 2.3$ Hz, H-3), 3.87 (3H, s, 4-OCH$_3$); $^{13}$C-NMR (MeOH-$d_4$) δ 174.2 (C-4), 167.3 (C-2), 161.1 (C-6), 148.8 (C-12), 146.7 (C-11), 137.4 (C-8), 129.0 (C-9), 122.2 (C-14), 116.8 (C-7), 116.7 (C-13), 115.0 (C-10), 101.6 (C-5), 88.7 (C-3), 57.1 (4-OCH$_3$); ESIMS $m/z$ 261 [M+H]$^+$.

**Microbial metabolism of yangonin by *A. coerulea* KCTC 6936** – The microbes were cultured in a shaking incubator with 250 mL of medium each in sixteen 1 L flasks under the same condition for scale-up fermentation. Yangonin was added evenly (10 mg/flask) and further incubated for 7 days, and then extracted with 4 L EtOAc. The concentrated EtOAc extract (1 g) was subjected to silica gel (70 - 230 mesh, Merck) chromatography using $CHCl_3$-MeOH (50 : 1 → 1 : 1) gradient solvent system. Fraction 4 (42 mg) was chromatographed on reversed-phase HPLC, eluting with a 2-propanol : $CH_3CN$ : $H_2O$ isocratic eluent (5 : 5 : 90, flow rate: 1 mL/min, UV detection: 254 nm) to afford the metabolite YM3 (**4**, 13.0 mg).

**4,12-Dimethoxy-6-(7,8-dihydroxy-7,8-dihydrostyryl)-2-pyrone (4)** – White amorphous powder; UV $\lambda_{max}$

(MeOH) 204, 209, 225, 281 nm; IR (KBr) $\nu_{max}$ 3368, 2924, 2854, 1967, 1696, 1565, 1247, 1030 cm$^{-1}$; $^1$H-NMR (MeOH-$d_4$) δ 7.28 (1H, d, $J$ = 8.4 Hz, H-10, 14), 6.85 (1H, d, $J$ = 8.7 Hz, H-11, 13), 6.12 (1H, d, $J$ = 2.4 Hz, H-5), 5.50 (1H, d, $J$ = 2.4 Hz, H-3), 4.90 (1H, d, $J$ = 5.1 Hz, H-8), 4.38 (1H, d, $J$ = 5.1 Hz, H-7), 3.81 (3H, s, 4-OCH$_3$), 3.76 (3H, s, 12-OCH$_3$); $^{13}$C-NMR (MeOH-$d_4$) δ 173.7 (C-4), 167.1 (C-2), 166.4 (C-6), 160.9 (C-12), 134.4 (C-9), 129.1 (C-10, 14), 114.7 (C-11, 13), 102.1 (C-5), 88.9 (C-3), 76.5 (C-7), 75.8 (C-8), 57.1 (4-OCH$_3$), 55.8 (12-OCH$_3$); ESIMS $m/z$ 315 [M+Na]$^+$; HRESIMS $m/z$ 315.0872 [M+Na]$^+$ (calcd. for C$_{15}$H$_{16}$O$_6$, 315.0845).

## Results and Discussion

Yangonin (C$_{15}$H$_{14}$O$_4$, MW 258) (**1**) was obtained as yellow crystal by column chromatography from *P. methysticum* (Dharmaratne *et al.*, 2002). Of thirty three microbial cultures screened, *C. elegans* var. *elegans* KCTC 6992 and *A. coerulea* KCTC 6936 were selected for scale-up fermentation studies since they showed the ability to metabolize yangonin based on the TLC profile analyses. The R$_f$ values of the metabolites YM1 (**2**: R$_f$ 0.5) and YM2 (**3**: R$_f$ 0.7) were higher than that of yangonin (**1**: R$_f$ 0.19) on RP-C$_{18}$ TLC using 60% CH$_3$CN as an eluent, and R$_f$ value of the metabolite YM3 (**4**: R$_f$ 0.4) was lower than that of yangonin on NP-TLC using CHCl$_3$ : MeOH = 9 : 1. These results suggested that more polar metabolites were produced. Substrate- and culture-controls showed that the metabolites were produced by enzymatic action of the microorganisms, but not as a consequence of chemical degradation or non-metabolic changes.

Metabolite YM1 (**2**) was produced by *C. elegans* var. *elegans* and obtained as yellow amorphous powder. ESIMS of the YM1 showed the [M+H]$^+$ peak at $m/z$ 245, which established a molecular formula of C$_{14}$H$_{12}$O$_4$. Its UV spectrum in MeOH showed absorption maxima at $\lambda_{max}$ 359, 211, 209, 205 nm, which is similar to that of yangonin. IR absorption due to a hydroxyl group was observed at $\nu_{max}$ 3383 cm$^{-1}$, which is different from that of yangonin, together with a conjugated carbonyl group at 1731 cm$^{-1}$, and aromatic rings at 1454-1382 cm$^{-1}$. The $^1$H-NMR spectrum was similar to that of yangonin except for aromatic proton signals displayed at δ 7.56 (2H, m, H-10, 14) and δ 7.19 (2H, m, H-11, 13) in the downfield region. A methoxyl proton signal at δ 3.84 (3H, s) disappeared, whereas the two doublets (in pyridine-$d_5$) in AB spin system characteristic of a 1,4-disubstituted benzene remained unchanged suggesting the *p*-hydroxylation of

the aromatic ring. Moreover, the $^{13}$C-NMR spectrum of **2** showed a singlet carbon signal at δ 161.1 corresponding to C-12. On the basis of the spectroscopic analyses and comparison with previous literature (Dong *et al.*, 1998; Rasmussen *et al.*, 1979; Tarbah *et al.*, 2003), structure of the metabolite YM1 was assigned 4-methoxy-6-(12-hydroxystyryl)-2-pyrone.

Metabolite YM2 (**3**) was obtained as brown amorphous powder that was positive to the FeCl$_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. ESIMS of metabolite YM2 showed the [M+H]$^+$ peak at $m/z$ 261, which established a molecular formula of C$_{14}$H$_{12}$O$_5$ indicating that it was a mono-oxygenated derivative of metabolite YM1. This suggested that YM2 is an analogue of YM1 having an additional hydroxyl group. Its UV spectrum in MeOH showed absorption maxima at $\lambda_{max}$ 359, 265, 203, and 202 (sh) nm, which was similar to those of yangonin and YM1. The IR spectrum showed a strong hydroxyl band at $\nu_{max}$ 3343 cm$^{-1}$, a conjugated carbonyl group at 1680 cm$^{-1}$, and aromatic rings at 1452-1252 cm$^{-1}$. The $^1$H NMR spectroscopic data of YM2 were similar to those of YM1 except that the AA'BB' spin system of the *para*-hydroxystyryl moiety of YM1 was replaced by an AMX spin system at δ 6.79 (1H, d, $J$ = 8.5 Hz, H-13), δ 6.94 (1H, dd, $J$ = 2.0, 8.5 Hz, H-14) and δ 7.04 (1H, d, $J$ = 2.0 Hz, H-10), suggesting the presence of 11,12-dihydroxystyryl moiety in YM2 instead of the 12-hydroxystyryl moiety present in YM1. Moreover, the $^{13}$C-NMR spectrum of YM2 showed carbon signals at δ 146.7 and δ 148.8 corresponding to C-11, C-12, respectively. The structure of YM2 was further confirmed on the basis of HSQC and HMBC NMR experiments. In the HMBC spectrum, the correlations from H-10 to C-11, H-13 to C-12, and H-14 to C-13 revealed the presence of the *ortho*-dihydroxystyryl moiety. Three bond correlations from H-10 to C-12 and C-14, H-13 to C-12 and C-14, and H-14 to C-10 and C-12 confirmed that the *ortho*-dihydroxystyryl moiety was located at C-11 and C-12. Based on the spectroscopic analyses and comparison with previous literature (Adam *et al.*, 1994; Edwards *et al.*, 1961; Edwards and Mir, 1967), structure of the metabolite YM2 was assigned 4-methoxy-6-(11,12-dihydroxystyryl)-2-pyrone.

Metabolite YM3 (**4**) was produced by *A. coerulea* and obtained as white amorphous powder. ESIMS of YM3 showed the [M+Na]$^+$ peak at $m/z$ 315, which established a molecular formula of C$_{15}$H$_{16}$O$_6$. Its UV spectrum in MeOH showed maximal absorption at $\lambda_{max}$ 281, 225, 209 and 204 nm, and IR absorption due to the two hydroxyl groups was observed at $\nu_{max}$ 3368 cm$^{-1}$, which are different

from those of yangonin. The $^1$H-NMR spectrum was similar to that of yangonin except for the two olefinic protons present in yangonin, and also displayed were two new oxymethine proton signals at $\delta$ 4.38 (1H, d, $J = 5.1$ Hz, H-7) and $\delta$ 4.90 (1H, d, $J = 5.1$ Hz, H-8). The $^{13}$C-NMR spectrum indicated the disappearance of the C-7, C-8 methylene carbon signals at $\delta$ 116.4 and $\delta$ 135.5 in yangonin, and the presence of new oxymethine signals at $\delta$ 75.6 and $\delta$ 75.8. These findings were confirmed by HMBC and HSQC correlations. Based on these data, the gross structure of the metabolite YM3 was assigned 4,12-dimethoxy-6-(7,8-dihydroxy-7,8-dihydrostyryl)-2-pyrone.

Metabolic fate of yangonin (**1**) to YM1 (**2**) was previously reported in rat urine using GC-MS, and the metabolites YM2 (**3**) and YM3 (**4**) are reported herein as microbial metabolites of yangonin for the first time.

## Acknowledgements

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