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Screening of Bioconversion Components from Gumiganghwal-tang on Fermentation by *Lactobacillus* Strains

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Abstract – Gumiganghwal-tang (GMT) is a traditional herbal prescription used for treatment of the common cold, pain, and inflammatory diseases. Variations in the amounts of bioactive components of GMT and GMT fermented with 10 *Lactobacillus* strains were investigated by high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Simultaneous qualitative and quantitative analyses of eleven bioactive compounds (prim-*O*-glucosylcimifugin, liquiritin, cimifugin, baicalin, liquiritigenin, wogonoside, baicalein, wogonin, butylphthalide, imperatorin, and isoimperatorin) were performed, with comparison of their retention times (t_R) and UV spectra with those of standard compounds. The amounts of baicalin (8.71 mg/g), liquiritigenin (5.28 mg/g) and butylphthalide (5.10 mg/g) were the major compounds in GMT. We found that *L. fermentum* KFRI 145 fermented wogonoside and baicalin to their aglycones, wogonin and baicalein, respectively. These results indicated that *L. fermentum* KFRI 145 has potential as a functional starter culture for manufacturing fermented GMT.
Keywords – Gumiganghwal-tang, Bioconversion, Identification, HPLC

Introduction

Oriental herbal medicines have gained increasing attention for the treatment of chronic diseases because of their effectiveness and small side effects.[1] Recently, the interest in fermented oriental herbal medicine has greatly increased the realm of oriental medicine. Fermented oriental herbal medicine, which is manufactured by fermenting traditional medicinal herbs using microorganisms in the air or selected microorganisms (such as lactic acid bacteria), has attracted considerable attention as a method to maximize intestinal absorption rates and the bioavailability of the active components of oriental herbal medicine.[2,3] And research on fermented oriental herbal medicine to enhance their biological activity is increasingly active. In one study, hepatoprotective effects of Ssanghwa-tang increased by fermentation with *L. fermentum* as demonstrated by decreases in serum transaminases levels and histological changes compared with the CCl_4 model group.[2] Other studies have suggested that Sipjeondaebo-tang fermented by *Lactobacillus* remarkably increases anti-inflammatory activity of Sipjeondaebo-tang without affecting cytotoxicity in LPS-stimulated RAW 264.7 macrophage cells.[4] However, very few studies have examined the changes of active components amount in before and after fermented oriental herbal medicine.

Gumiganghwal-tang (GMT) is a herbal prescription used for the clinical treatment of the common cold, pain, and inflammatory diseases,[5] and is one of the most commonly used herbal prescriptions in Asia.[6,7] Previous studies have shown that GMT exhibits anti-inflammatory effects on macrophage, possibly by inhibition of NF-κB.[5] GMT is representative cold medicine from 'Dongui Bogam'[8] that composed by nine herbs: *Ostericum koreanum*, *Saposhnikovia divaricate*, *Cnidium officinale*, *Angelica dahurica*, *Atractylodes lancea*, *Scutellaria baicalensis*, *Rehmannia glutinosa*, *Asarum heterotropoides*, *Gylycyrrhiza glabra*. This study, for enhance the biological activity of GMT we fermented the GMT by ten *Lactobacillus* strans. To evaluate GMT as the value of fermented oriental herbal medicine, we analyzed that variation in the amount of bioactive components of GMT and its fermentation GMT (FGMT) with ten *Lactobacillus* strains were investigated via high-performance liquid chromatography coupled with

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diode array detection (HPLC-DAD).

Experimental

General experimental procedures – GMT samples consisting of nine herbal medicines, *Ostericum koreanum*, *Saposhnikovia divaricate*, *Cnidium officinale*, *Angelica dahurica*, *Atractylodes lancea*, *Scutellaria baicalensis*, *Rehmannia glutinosa*, *Asarum heterotropoides* and *Gylycyrrhiza glabra* were purchase from the Korea Medicinal Herbs Association (yeongcheon, Korea). The origin of the samples was confirmed taxonomically by Prof. Ki Hwan Bae, College of Pharmacy, Chungnam National University. All voucher specimens were deposited in the herbal bank of the Korean Medicine (KM)-Based Herbal Drug Research Group, Korea Institute of Oriental Medicine. The reference components prim-O-glucosylcimifugin, cimifugin, baicalin, liquiritigenin, wogono-side, butylphthalide and imperatorin were purchase from Faces Biochemical Co., Ltd. (Wuhan, China). Liquiritin and isoimperatorin were purchased from Wako (Osaka, Japan) and Chengdu Biopurify Phytochemicals Ltd (Chengdu, China), respectively. Baicalein and wogonin were purchased from the Korea Food & Drug Administration. The purity of all reference standards was > 98.0%.

HPLC apparatus and chromatographic conditions – The HPLC system was an Elite Lachrom HPLC system (Hitachi High-Technologies Co., Tokyo, Japan) equipped with a pump (L-2130), an auto sampler (L-2200), a column oven (L-2350) and a diode array UV/VIS detector (L-2455). System control and data analyses were executed by EZchrom Elite software (version 3.3.1a) system. The analysis of compounds in the GMT and Fermented GMT samples was conducted using a RS-tech C_{18} column (5 μm, 4.60 mm I.D. × 250 mm) at 40 ºC. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid in water (A) and acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was a gradient of solvent A and solvent B as follows; 0 - 60 min, 10 - 40% B; 60 - 70 min, 40% B; 70 - 75 min, 40 - 100% B; 75 - 85 min, 100% B. The DAD detector UV wavelength was set at 254 nm according to the suitability UV absorption of eleven compounds. The sample injection volume was 10 μL.

Preparation of standard solutions – Each standard solution was prepared by dissolving standard compounds (prim-O-glucosylcimifugin, cimifugin, baicalin, liquiritigenin, wogonoside, butylphthalide, imperatorin, liquiritin, isoimperatorin, baicalein, and wogonin) in methanol at a concentration of 200 ppm. To prepare the analytical samples, the GMT and FGMT powder was weighed and dissolved in methanol at a concentration of 100 mg/mL. Prior to analysis, the sample preparation was filtered through a 0.45 μm membrane filter before HPLC analysis and all samples and standards solutions were stored at 4 ºC.

Sample preparation – A mixture of medicinal herbs (2000 g) consisting of *Ostericum koreanum*, *Saposhnikovia divaricate*, *Cnidium officinale*, *Angelica dahurica*, *Atractylodes lancea*, *Scutellaria baicalensis*, *Rehmannia glutinosa*, *Asarum heterotropoides* and *Gylycyrrhiza glabra* was boiled in 20 L of distilled water for 3 h using a COSMOS-660 extractor (Kyungseo Machine Co., Incheon, Korea), and the extract was filtered using standard testing sieves (150 μm) to yield 16 L of the decoction. The ten bacterial strains (*Lactobacillus casei* KFRI 127, *Lactobacillus acidophilus* KFRI 128, *Lactobacillus plantarum* KFRI 144, *Lactobacillus fermentum* KFRI 145, *Lactobacillus amylophillus* KFRI 161, *Lactobacillus acidophilus* KFRI 162, *Lactobacillus curvatus* KFRI 166, *Lactobacillus confuses* KFRI 227, *Lactobacillus amylophilus* KFRI 238 and *Lactobacillus thermophilum* KFRI 748) used in this study were obtained from the Korea Food Research Institute (KFRI, Korea). After successful transfer of the test organisms in MRS broth for *Lactobacillus sp*. at 37 ºC for 24 h, the activated culture was again inoculated into each broth under the same conditions. It was properly diluted to obtain an initial population of $1 - 5 \times 10^9$ CFU/mL and served as the inoculum. The GMT water extract was used as the culture media for fermentation after adjusting the pH to 7.0 using 1M NaOH and autoclaving for 15 min at 121 ºC. After cooling, 750 mL of GMT was inoculated with 7.5 mL inoculums as described above. This was incubated at 37 ºC for a period of 48 h. The fermented GMT was then filtered through a 60 μm nylon net filter (Millipore, Billerica, Mass, USA) and freeze-dried to give the dried fermented extract GMT which was stored in desiccators at 4 ºC until use.

Results and Discussion

Eleven active compounds, prim-O-glucosylcimifugin (**1**), liquiritin (**2**), cimifugin (**3**), baicalin (**4**), liquiritigenin (**5**), wogonoside (**6**), baicalein (**7**), wogonin (**8**), butylphthalide (**9**), imperatorin (**10**), and isoimperatorin (**11**), were analyzed in GMT and FGMT. The structures of eleven active compounds were shown in Fig. 1. Based on the absorption maxima in UV spectra with three-dimensional HPLC-DAD detection the monitoring wavelength was detected in 254 nm. Identification of the

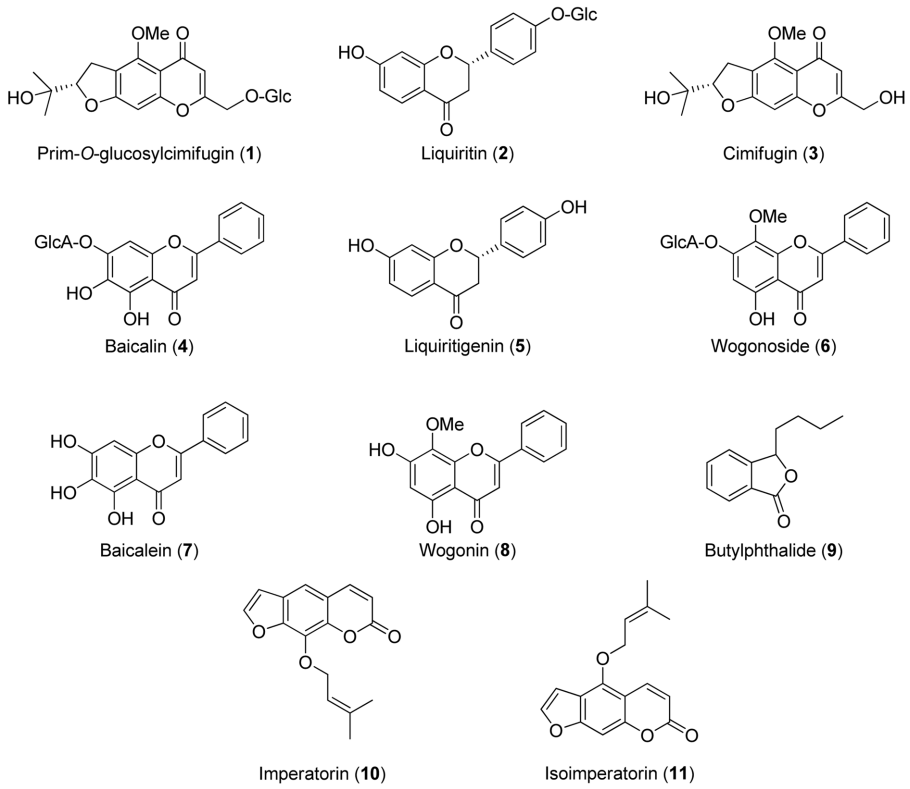

Fig. 1. Chemical structures of eleven active components.

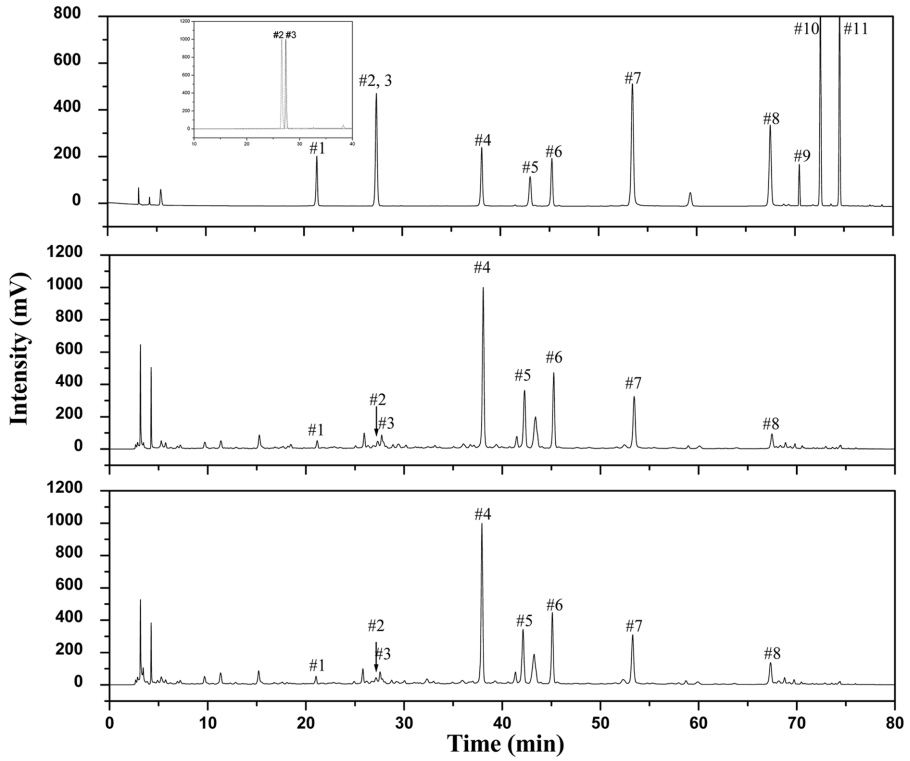

Fig. 2. Chromatograms of standard compounds (A), original Gumiganghwal-tang (B), and Gumiganghwal-tang fermented by *L. fermentum* KFRI 145 (C). (**1:** prim-*O* glucosylc-imifugin **2:** liquiritin **3:** cimifugin **4:** baicalin **5:** liquiritigenin **6:** wogonoside **7:** baica-lein **8:** wogonin **9:** butylphthalide **10:** imperatorin **11:** isoimperatorin).

Table 1. Amounts of active components in GMT and FGMT

Sample name	Amounts of active components [a] (mg/g) [b]										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Oreginal GMT	0.23 ± 0.02	0.28 ± 0.00	0.19 ± 0.00	8.71 ± 0.11	5.28 ± 0.09	2.73 ± 0.03	1.98 ± 0.01	0.45 ± 0.00	5.10 ± 0.16	0.37 ± 0.02	0.13 ± 0.00
Auto cleaved GMT	0.24 ± 0.04	0.15 ± 0.02	0.15 ± 0.01	7.56 ± 0.05	4.81 ± 0.02	2.60 ± 0.01	1.00 ± 0.01	0.43 ± 0.01	4.62 ± 0.40	0.27 ± 0.02	0.11 ± 0.02
L. casei KFRI 127	0.28 ± 0.00	0.16 ± 0.01	0.19 ± 0.00	9.29 ± 0.04	5.60 ± 0.00	2.80 ± 0.04	2.06 ± 0.01	0.80 ± 0.01	5.37 ± 0.00	0.32 ± 0.00	0.12 ± 0.01
L. acidophilus KFRI 128	0.26 ± 0.00	0.18 ± 0.01	0.17 ± 0.00	8.31 ± 0.03	5.28 ± 0.01	2.50 ± 0.02	1.78 ± 0.02	0.65 ± 0.01	5.14 ± 0.14	0.31 ± 0.00	0.11 ± 0.02
L.plantarum KFRI 144	0.27 ± 0.02	0.21 ± 0.00	0.20 ± 0.00	10.07 ± 0.03	5.45 ± 0.05	3.05 ± 0.01	1.70 ± 0.01	0.51 ± 0.00	5.43 ± 0.04	0.29 ± 0.03	0.12 ± 0.04
L. fermentum KFRI 145	N. D. [c]	0.24 ± 0.05	0.19 ± 0.01	8.43 ± 0.02	5.37 ± 0.03	2.41 ± 0.01	2.16 ± 0.01	0.89 ± 0.01	5.21 ± 0.10	0.30 ± 0.01	0.13 ± 0.03
L. amylophillus KFRI 161	0.26 ± 0.01	0.20 ± 0.01	0.20 ± 0.00	9.76 ± 0.04	5.35 ± 0.11	3.15 ± 0.02	1.68 ± 0.00	0.47 ± 0.07	5.21 ± 0.29	0.29 ± 0.00	0.14 ± 0.01
L. acidophilus KFRI 162	0.27 ± 0.00	0.18 ± 0.01	0.19 ± 0.00	9.06 ± 0.11	5.26 ± 0.08	2.84 ± 0.04	1.62 ± 0.06	0.49 ± 0.01	5.25 ± 0.01	0.29 ± 0.01	0.13 ± 0.00
L. curvatus KFRI 166	0.25 ± 0.03	0.20 ± 0.00	0.21 ± 0.00	9.86 ± 0.01	5.27 ± 0.00	3.14 ± 0.01	1.69 ± 0.01	0.51 ± 0.00	5.14 ± 0.01	0.27 ± 0.00	0.11 ± 0.02
L. confuses KFRI 227	0.29 ± 0.00	0.09 ± 0.01	0.20 ± 0.00	9.53 ± 0.14	5.41 ± 0.05	3.02 ± 0.04	1.66 ± 0.01	0.51 ± 0.02	5.40 ± 0.03	0.28 ± 0.04	0.12 ± 0.01
L. amylophilus KFRI 238	0.28 ± 0.00	0.15 ± 0.02	0.19 ± 0.00	9.53 ± 0.14	5.33 ± 0.03	3.15 ± 0.01	1.69 ± 0.02	0.51 ± 0.02	5.39 ± 0.12	0.31 ± 0.02	0.15 ± 0.00
L. thermophilum KFRI 748	0.27 ± 0.00	0.17 ± 0.00	0.20 ± 0.00	9.72 ± 0.02	5.23 ± 0.01	3.09 ± 0.01	1.58 ± 0.02	0.48 ± 0.00	5.25 ± 0.30	0.30 ± 0.02	0.15 ± 0.00

[a]Components: **1:** prim-O-glucosylcimifugin **2:** liquiritin **3:** cimifugin **4:** baicalin **5:** liquiritigenin **6:** wogonoside **7:** baicalein **8:** wogonin **9:** butylphthalide **10:** imperatorin **11:** isoimperatorin
[b] Data expressed mean ± SD (n = 2)
[c]N.D.: not detected

eleven active compounds was performed by comparing HPLC retention times (t_R) and UV absorption of target peaks in GMT and FGMT with standard compounds. The HPLC chromatograms are shown in Fig. 2. The profiles of each compound were identified in the GMT and FGMT samples: prim-O-glucosylcimifugin (**1**, t_R: 20.69 min), liquiritin (**2**, t_R: 26.64 min), cimifugin (**3**, t_R: 27.11 min), baicalin (**4**, t_R: 37.57 min), liquiritigenin (**5**, t_R: 41.76 min), wogonoside (**6**, t_R: 44.74 min), baicalein (**7**, t_R: 52.40 min), wogonin (**8**, t_R: 65.94 min), butylphthalide (**9**, t_R: 69.57 min), imperatorin (**10**, t_R: 71.97 min), and isoimperatorin (**11**, t_R: 74.04 min).

To analyze the amounts of each of the eleven active compounds in GMT and FGMT, linear regression analysis for each compound was performed by the external standard method. All marker substances showed good linearity ($r > 0.999$). The amounts of each compound based on the regression equation are shown in Table 1. The major components in GMT were found to be baicalin (8.71 mg/g), liquiritigenin (5.28 mg/g) and butylphthalide (5.10 mg/g). The contents of the other compounds were as follows: prim-O-glucosylcimifugin, 0.23 mg/g; liquiritin, 0.28 mg/g; cimifugin, 0.19 mg/g; wogonoside, 2.73 mg/g; baicalein, 1.98 mg/g; wogonin, 0.45 mg/g; imperatorin, 0.37 mg/g; and isoimperatorin, 0.13 mg/g.

For the analysis of the change of components content by *Lactobacillus* strains before and after GMT fermentation, the changes in each eleven activity compounds were compared and analyzed using HPLC. The amounts of wogonin in GMT fermented with *L. fermentum* KFRI 145 (0.89 mg/g) and *L. acidophilus* KFRI 128 (0.65 mg/g) were increased by 97.8% and 44.4%, respectively, compared to GMT (0.45 mg/g). Therefore its glycoside wogonoside in GMT, decreased by 8.4% and 11.7%, respectively, by *L. fermentum* KFRI 145 (2.41 mg/g) and *L. acidophilus* KFRI 128 (2.50 mg/g), compared to GMT (2.73 mg/g). The amounts of baicalein in GMT fermented with *L. fermentum* KFRI 145 (2.16 mg/g) increased by 9.0%, and the amounts of baicalin in GMT with *L. fermentum* KFRI 145 (8.43 mg/g) decreased by 3%, compared to GMT (1.98 mg/g and 8.71 mg/g). These results demonstrate that the *L. fermentum* KFRI 145 would be transformation of

baicalin and wogonoside to their aglycons baicalein and wogonin. Wogonoside and baicalin were main active compounds from *Scutellaria baicalensis* have been reported to possess anti-inflammatory[9,10], antiviral[11-13], antioxidant[14-16], and anticancer[17-19] properties. The β-glucuronidase gene from *Lactobacillus brevis* was cloned, overexpressed in *E. coli*, and used for the biotransformation of baicalin and wogonoside[20]. Ku et al. reported that *Lactobacillus delbrueckii* Rh2 used for biotransformation of baicalin and wogonoside to their aglycon baicalein and wogonin[21]. In this study, we found that *L. fermentum* KFRI 145 also transformation the wogonoside and baicalin to their aglycon wogonin and baicalein in GMT. In addition, most strains was increased the amounts of buthlphthalide, wogonin, baicalin, wogonoside and liquiritigenin, in fermented GMT, except strain *L. fermentum* KFRI 145. However further research is required to determine the mechanisms by which the levels of these components were increased by *Lactobacillus* strains.

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