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Scientific Analysis of the Formulation Theory of Chungpesagan-tang; *In Vitro* Cytotoxicity of Chungpesagan-tang

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Abstract – To analyze scientifically the fundamental formulation theory and drug interaction of Chungpesagan-tang, the extraction level of puerarin and daidzin, the transforming activity of puerarin and daidzin to daidzein by human intestinal bacteria and *in vitro* cytotoxicity against tumor cell lines of Chungpesagan-tang were investigated. When Puerariae Radix was extracted with Chungpesagan-tang composing herbal medicines, the puerarin extraction level from these polyprescriptions was decreased by the extraction with Raphani Semen or Cimicifugae Rhizoma, but the other herbal medicines increased it. The activity transforming puerarin and daidzin to daidzein by human intestinal bacteria was increased by Raphani Semen, Cimicifugae Rhizoma and Angelicae Tenuissimae Radix, but decreased by Scutellariae Radix and Rhei Rhizoma. Puerariae Radix did not showed *in vitro* cytotoxicity against tumor cell lines. However, by its anaerobic incubation with human intestinal bacteria, it showed a potent cytotoxicity. When the main components, puerarin and daidzin, of Puerariae Radix were incubated with human intestinal bacteria, the main metabolites were daidzein and calycosin. These metabolites had the most potent cytotoxicity, compared to those of puerarin and daidzin. Raphani Semen, Rhei Rhizoma and Chungpesagan-tang had also the potent cytotoxicity against tumor cell lines by the anaerobic incubation with human intestinal bacteria.

Key words – Chungpesagan-tang, fundamental formulation theory, cytotoxicity, Puerariae Radix, human intestinal bacteria

The herbal medicinal polyprescription is composed of several herbal medicines. In Korea, this herbal medicinal polyprescription has been formulated according to the four regular components theory of Oriental medicines, which are consisted of King, Minister, Assistant and Laborer. However, this theory has not been elucidated by the scientific research method until now. If this theory could be understood through the experiment, new polyprescritpion could be developed. To scientifically understand the fundamental formulation theory of traditional herbal medicinal polyprescription, we studied herbal medicinal interaction on the purgative action of Chungpesagan-tang (Lee, 1996), which has been used in Oriental Clinic (Bae, 1987; Kwon, 1996): Chungpesagan-tang had better purgative action than Rhei Rhizoma (Jeon, 1996). The purgative action of Rhei Rhizoma was induced by Raphani Semen in Chunpesagan-tang.

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Here we investigated the effects of Chungpesagan-tang-composing herbal medicines on *in vitro* cytotoxicity of Puerariae Radix against tumor cell lines and their relation to the metabolism of puerarin and daidzin of Puerariae Radix by human intestinal bacteria in order to scientifically understand the fundamental formulation theory of Chungpesagan-tang.

Experimental

Materials – MTT, trypsin and RPMI1640 were purchased from Sigma Co., (U.S.A.). Antibiotics-antimycotics and FBS were from Gibco Co., (USA). GAM was from Nissui Pharm. Co., ltd., (Japan). Silica gel was from Merck Co., (USA). The other chemicals were of analytical reagent grade.

SNU-C4 (Human colon cancer cell line), A-549 (Human lung cancer cell line), P-388 (Mouse lymphoid neoplasma cell line) and L-1210 (Mouse lymphocytic leukemia cell line) were from Seoul University Cell Bank (Korea).

Rhei Rhizoma, Puerariae Radix, Scutellariae Radix, Angelicae Tenussimae Radix, Planticodi Raidx, Rhapani Semen, Cimicifugae Rhizoma and Angelciae Dahuricae Radix were purchased from Kyung-Dong traditional herbal medicinal market (Korea).

Extraction and Isolation of Puerarin and Daidzin from Puerariae Radix – The dried Puerariae Radix (500 g) was cut into small pieces and extracted with water at boiling water and evaporated. The evaporated extract (80 g) was extracted with ethylacetate. The ethylacetate fraction (20 g) was applied to silica-gel column chromatography (5×70 cm) and eluted with chloroform: MeOH (10:1 → 4:10). The fractions of puerarin and daidzin were again applied to silica-gel column chromatography (3×40 cm) and eluted with same solvents. The isolated puerarin and daidzin were identified by comparing to instrumental analysis of authentic compounds.

Puerarin Determination of Polyprescriptions – Fifty grams of each Chungpesagan-tang-composing herbal medicine, were extracted with 450 ml of water in a boiling water bath, filtrated, concentrated and dried with a freezing dryer. The combinations of each herbal medicine composing Chungpesagan-tang with Puerariae Radix (1:1, 1:0.5, 1:0.25) were also extracted with water. Chungpesagan-tang was also extracted (Table 1). All extracts were dried with a freezing dryer and used if necessary. One hundred milligrams of each dried extract of all polyprescriptions were dissolved and made to 10ml with methanol. The quantity of puerarin assayed with a HPLC. HPLC (Younglin system, Korea) was carried out as follows: column, μ-Bondapak C18 3.8×300 mm; solvent, 30% MeOH; wavelength of detector, 254 nm.

Thin layer chromatography – TLC for puerarin, daidzin, daidzein and calycosin was performed on silica gel plates (Merck, silica gel 60F-254) as follows; developing solvents system, $CHCl_3$/methanol (4:1). The quantity of these compounds were assayed with a TLC scanner (Shimadzu CS-920).

Table 1. Composition of Chungpesagantang

Herbal Medicine	Weight (g)
	Chungpesagantang
Pueraria thunbergiana (root)	15
Scutellaria baicalensis (root)	7.5
Angelica tenussima (root)	7.5
Platycodon gradiflorum (root)	3.75
Raphanus satius (seed)	3.75
Cimicifuga heracleifolia (root)	3.75
Angelica dahurica (root)	3.75
Rheum palmatum (root)	3.75

Metabolites of puerarin and daidzin by human intestinal bacteria – To isolate the metabolites of puerarin or daidzin isolated from Puerariae Radix by human intestinal bacteria, reaction mixture contained 0.1 mM puerarin (or daidzin) and 0.1 g fresh human fecal bacteria in a final volume of 50 ml of an anaerobic dilution medium was incubated at 37°C for 24 h. The reaction mixture was extracted three times with 500 ml of ethylacetate. The ethylacetate extract was applied to silica gel column chromatography (1.5×20 cm) with $CHCl_3$/MeOH (9:1). The isolated metabolites were crystallyzed with MeOH.

Daidzein : mp, 316-318°C. EI-MS, 254[M^+]. IR, 3215(OH), 1615(conjugated C=O), 1518 (aromatic C=C). ^{1}H-NMR (500MHz, CD_3OD) δ : 8.13(1H, s, H-2), 8.04(1H, d, J=8.9Hz, H-5), 6.92(1H, dd, J=8.9, 2.0Hz, H-6), 6.84 (1H, d, J=2.0Hz, H-8), 7.35 (1H, d, J=9.0Hz, H-2'), 6.81 (1H, d, 2.0Hz, H-3'), 6.84 (1H, d, J=9.0Hz, H-5'), 7.35 (1H, d, J=9.0Hz, H-6'). ^{13}C-NMR (125MHz, CD_3OD) δ : 155.0 (C-2), 126.25 (C-3), 178.10 (C-4), 128.31 (C-5), 116.27 (C-6), 162.98 (C-7), 105.00 (C-8), 158.79 (C-9), 117.05 (C-10), 124.11 (C-1'), 131.41 (C-2'), 115.85 (C-3'), 159.25 (C-4'), 115.80 (C-5'), 131.41 (C-6)

Calycosin : mp, 250-252°C. EI-MS, 282[M^+]. IR, 3241(OH), 1628 (conjugated C=O), 1510 (aromatic C=C). ^{1}H-NMR (500MHz, CD_3OD) : 8.16(1H, s, H-2), 8.05(1H, d, J=8.9Hz, H-5), 6.93 (1H, dd, J=8.9, 2.3Hz, H-6, 6.85 (1H, d, J=2.3Hz, H-8), 7.15(1H, d, J=2.0Hz, H-6), 6.48(1H, d, J=8.2Hz, H-5'), 6.95(1H, dd, J=8.9, 2.3Hz, H-6'), 3.89(3H, s, OCH_3). ^{13}C-NMR (125MHz, CD_3OD) δ : 147.9 (C-2), 124.8 (C-3), 178.2 (C-4), 126.0 (C-5), 116.2 (C-6), 164.8 (C-7), 103.2 (C-8), 159.8 (C-9), 116.5 (C-10), 122.9 (C-1'), 118.1 (C-2'), 154.7 (C-3'), 148.8 (C-4'), 114.1 (C-5), 128.5 (C-6'), 56.41 (OCH_3)

Time courses of metabolism of puerarin and daidzin by intestinal bacteria – The activities metabolizing puerarin and daidzin of Puerariae Radix and its containing polyprescriptions were measured as follows. The assay mixture contained puerarin (0.1 mM) and daidzin (0.02 mM) and fresh human fecal bacteria of human (0.1 g) in a final volume of 50 ml of an anaerobic dilution medium. The mixture was incubated at 37°C for 24 h and an aliquot (2 ml) of

the reaction mixture was periodically extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

***In Vitro* Cytotoxicity Assay** – The *in vitro* cytotoxicity was tested against SNU C4, A549, P388, L1210, MA104 by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Park, 1996). Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μl volumne) into 96-well microtiter plates. After cells were exposed to the test compounds for 2 d at 37°C, 50 μl MTT solution (2 mg/ml in PBS) was added to each well and the plates was incubated for 4 h. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plate was read on a microplate reader on a 540 nm. The IC_{50} (50% inhibitory concentration) of tumor cell growth was defined compared with the control cell culture.

Results and Discussion

Metabolism of puerarin and daidzin from Puerariae Radix by human intestinal bacteria and its relation to *in vitro* cytotoxicity against tumor cell lines – Two compounds, puerarin and daidzin were isolated from Puerariae Radix by silica-gel column chromatography. When the content of puerarin of Puerariae Radix was measured by HPLC, it was 3.07%. To investigate the metabolites of puerarin and daidzin by human intestinal bacteria, puerarin or daidzin were anaerobically incubated for 24 h with a bacterial mixture from human feces. And then the metabolites were extracted with ethylacetate and analyzed by TLC and ^{13}C- and ^{1}H-NMR and EI-MS after separating the metabolites by silica gel column chromatography. Two metabolites, Rf 0.59 and Rf 0.62, were observed by TLC ($CHCl_3$:MeOH). Compared with the authentic compounds, they were daidzein and calycosin, respectively.

The time course of transformation of Puerariae Radix containing puerarin and daidzin by human intestinal bacteria was shown in Fig. 1. Daidzin started to be converted to daidzein 3 h after incubation with the fecal mixture. It were transformed to calycosin as well as daidzein 6 h after incubation with the fecal mixture. Puerarin nearly was not transformed within 6 h after incubation. Puerarin started to be transformed 9 h after incubation with the fecal

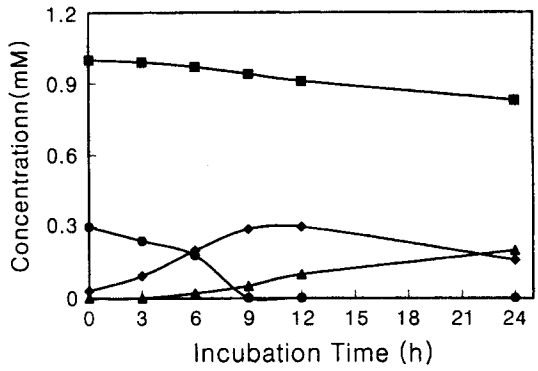

Fig. 1. Time course of the metabolism of puerarin and daidzin in Puerariae Radix extracts by human intestinal microflora. Symbols indicate as follows: ■, puerarin; ●, daidzin; ◆, daidzein; ▲, calycosin.

mixiture and 17% of puerarin was transformed. The biotransformation of daidzin to daidzein proceeded more easily than that of puerarin. These reults suggested that the enzyme activity hydrolyzing daidzin to daidzein, O-glycosidase, was more potent than what hydrolyzing puerarin to daidzein, C-glycosidase, in human intestinal microflora.

We also investigated *in vitro* cytotoxic activity of puerarin, daidzin and their metabolites against tumor cell lines (Table 2). Puerarin and daidzin did not show *in vitro* cytotoxicity. However, their metabolites, daidzein and calycosin showed potent cytotoxicity against tumor cell lines. We found that the cytotoxicity was increased when puerarin and daidzin were metabolized to daidzein and calycosin by human intestinal microflora. These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal microflora.

Effect of herbal medicines on puerarin content in water extract from Puerariae Radix containing polyprescriptions – When Puerariae Radix was

Table 2. Cytotoxicity of Puerarin and Daidzin and Their Metabolites against Tumor Cell Lines

Compound	IC_{50} (mM)				
	P-388	SNU C4	A 549	L 1210	MA 104
Puerarin	>2	>2	>2	>2	>2
Daidzin	>2	>2	>2	>2	>2
Daidzein	0.82	0.97	1.72	>2	>2
Calycosin	0.46	0.50	0.64	–[a]	>2
Adriamycin	0.003	0.05	0.1	–	>2

[a]not tested.

Table 3. Contents of puerarin per weight of Puerariae Radix on each polyprescriptions combined with Puerariae Radix

Herbal medicine[a]	Content (%)			
	1:1[b]	1:0.5	1:0.25	1:0
Pu				3.07±0.04
Pu:Sc	5.11±0.14[c]	4.20±0.72	3.20±0.46	
Pu:At	3.83±0.12[1)]	3.65±0.34	3.84±0.92	
Pu:Ra	2.82±0.66	2.59±0.24	2.78±0.51	
Pu:Pl	5.45±6.1	4.75±0.94	3.90±0.95	
Pu:Ci	1.93±0.26	2.88±0.68	3.15±0.94	
Pu:Ad	4.72±0.02	4.67±0.66	3.67±0.18	
Pu:Ph	3.19±0.56	3.36±0.94	3.95±0.54	
Chung				4.10±0.06

[a]Pu, Puerariae Radix; Sc, Scutellariae Radix; At, Angelicae Tenussimae Radix; Ra, Raphani Semen; Pl, Planticodi Rhizoma; Ci, Cimicifugae Radix; Ad, Angelicae Dahuricae Rhizoma; Rh, Rhei Rhizoma; Chung, Chungpesagantang.
[b]Ration of Puerariae Radix to the other herbal medicine composing Chungpesagan-tang.
[c](Mean±S.D)

extracted with water, the effect of Chungpesagan-tang composing herbal medicines on the puerarin content in water extract was investigated (Table 3). The content of puerarin in water extract of Puerariae Radix was 3.07%. However, the puerarin contents in water extract of Rhapani Semen or Cimicifugae Rhizoma with Puerariae Radix were decreased. The other Chungpesagan-tang composing herbal medicines increased the puerarin extraction level from Puerariae Radix. These actions, synergism and antagonism of puerarin extraction from Puerariae Radix by herbal medicines, could affect the biological action of Chungpesagan-tang. By the addition and subtraction of Chungpesagan-tang-composing herbal medicines, we thought, the puerarin level was controlled and the potency of the biological action could be also controlled.

Effect of herbal medicines on the metabolism of puerarin and daidzin in polyprescriptions by human intestinal bacteria – Puerarin and daidzin are the prodrug which can be activated to daidzein or calycosin, by human intestinal bacteria. Therefore, to evaluate herbal drug interaction and the *in vitro* cytotoxicity of Puerariae Radix in Chungpesagan-tang against tumor cell lines, the activities transforming puerarin and daidzin in each polyprescription to daidzein and calycosin were measured (Fig. 2). When Puerariae Radix was anaerobically incubated with human intestinal bacteria, more than 40% daidzin was transformed within 6 h. However, 3% puerarin only was transformed. This transforming activity of daidzin and puerarin was inhibited by Scutellariae Radix and Rhei Rhizoma. However, Raphani Semen, Cimicifugae Rhizoma and Angelicae Tenussimae Radix increased the transforming activity. These results suggested that the biological activities of Chungpesagan-tang could be controlled by the addition and subtraction of Chungpesagan-tang-composing herbal medicines.

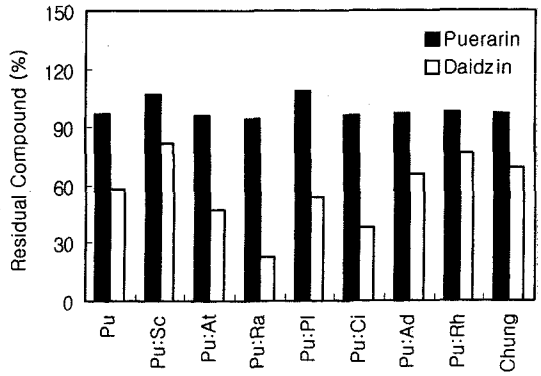

Fig. 2. Effect of herbal medicines on the metabolism of puerarin and daidzin in polyprescriptions by human intestinal bacteria. The reaction mixture contained water extract of Puerariae Radix (1 mM puerarin and 0.3 mM daidzin) only or with Chungpesagan-tang composing herbal medicines, and fecal suspension and was anaerobically incubated for 6 h at 37°C Symbols indicate as follows: ■, puerarin; □, daidzin. Abbreviation used: Pu, Puerariae Radix; Sc, Scutellariae Radix; At, Angelicae Tenussimae Radix; Ra, Raphani Semen; PI, Platycodi Rhizoma; Ci, Cimicifugae Radix; Ad, Angelicae Dahuricae Rhizoma; Rh, Rhei Rhizoma; Chung, Chungpesagan-tang.

***In vitro* Cytotoxicity of Chungpesagan-tang** – To investigate drug interaction on the *in vitro* cyto-

Table 4. Cytotoxicity of Constituted Herbs of Chungpesagan-tang and Chungpesagan-tang aganist tumor cell lines

Herbal medicine[a]	IC_{50} (mg/ml)				
	L1210	P388	SNU C4	A549	MA104
Pu	>1	>1	>1	>1	>1
Sc	0.15	0.05	0.3	>1	>1
Pu:Sc (1:1)	0.25	0.15	0.3	>1	>1
At	>1	>1	>1	>1	>1
Pu:At (1:1)	>1	>1	>1	>1	>1
Ra	>1	>1	>1	>1	>1
Ra:Pu (1:1)	>1	>1	>1	>1	>1
Pl	>1	>1	>1	>1	>1
Pu:Pl (1:1)	>1	>1	>1	>1	>1
Ci	0.78	0.69	>1	>1	>1
Pu:Ci (1:1)	>1	>1	>1	>1	>1
Ad	>1	>1	>1	>1	>1
Pu:Ad (1:1)	>1	>1	>1	>1	>1
Rh	0.25	0.05	0.28	0.95	>1
Pu:Rh (1:1)	0.5	0.15	0.75	>1	>1
Chung	0.97	0.78	0.32	>1	>1

[a]Pu, Puerariae Radix; Sc, Scutellariae Radix; At, Angelicae Tenussimae Radix; Ra, Raphani Semen; Pl, Plantricodi Rhizoma; Ci, Cimicifugae Radix; Ad, Angelicae Dahuricae Rhizoma; Rh, Rhei Rhizoma; Chung, Chungpesagan-tang.

toxic activity of Chungpesagan-tang, the *in vitro* cytotoxic activities of water extract of herbal medicines with Puerariae Radix against tumor cell lines were measured (Table 4). Most herbal medicines did not affect the *in vitro* cytotoxicity against MA104, normal cell lines, in the concentration of 1 mg per ml. However, Scutellariae Radix, Cimicifugae Radix and Rhei Rhizoma showed more potent *in vitro* cytotoxicity against tumor cell lines, particularly L1210, P388 and SNU C4, than the other herbal drugs. The best cytotoxic herbal medicines was Scutellariae Radix, followed by Rhei Rhizoma and Cimicifugae Radix. Chungpesagan-tang showed the weak *in vitro* cytotoxic activity against tumor cell lines.

Effect of human intestinal bacteria on the *in vitro* cytotoxic activity of Chungpesagan-tang – Water extract of herbal medicines were incubated at 37°C for 24 h with or without human intestinal bacteria and extracted with ethylacetate. We investigated *in vitro* cytotoxic activity of their extracts against SNU C4 tumor cell lines (Table 5). The *in vitro* cytotoxicity of Scutellariae Radix was not affected by human intestinal bacteria. However, the *in vitro* cytotoxicity of Raphani Semen and Rhei

Table 5. Cytotoxicity of Constituted Herbs of Chungpesagan-tang and Chungpesagan-tang aganist SNU-C4 Tumor Cell Lines

Herbal Medicine[a]	IC_{50} (mg/ml)	
	Untreated[b]	Treated
Pu	>0.5	0.05
Sc	0.05	0.04
Pu:Sc (1:1)	0.11	0.05
At	>0.5	>0.5
Pu:At (1:1)	>0.5	0.16
Ra	>0.5	0.01
Ra:Pu (1:1)	>0.5	0.11
Pl	>0.5	>0.5
Pu:Pl (1:1)	>0.5	0.12
Ci	>0.5	>0.5
Pu:Ci (1:1)	>0.5	0.01
Ad	>0.5	>0.5
Pu:Ad (1:1)	>0.5	0.02
Rh	0.03	0.01
Pu:Rh (1:1)	0.04	0.01
Chung	0.15	0.09

[a]Pu, Puerariae Radix; Sc, Scutellariae Radix; At, Angelicae Tenussimae Radix; Ra, Raphani Semen; Pl, Plantricodi Rhizoma; Ci, Cimicifugae Radix; Ad, Angelicae Dahuricae Rhizoma; Rh, Rhei Rhizoma; Chung, Chungpesagan-tang.
[b]Untreated, each herbal medicine or polyprescription was incubated at 37°C for 20 h without human intestinal bacteria and extracted with ethylacetate; Treated, each herbal medicine or polyprescription was incubated at 37°C for 20 h with human intestinal bacteria and extracted with ethylacetate.

Rhizoma was increased by human intestinal bacteria. These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal microflora.

In this point of view, the biological activities of Puerariae Radix, a Monarch of Chungpesagan-tang, could be controlled by the addition and substraction of herbal medicines. With a better understanding of these results, new polyprescritpions could be scientifically developed.

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