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# Scientific Analysis of Formulation Theory of Chungpesagan-tang; *In vitro* Cytotoxicity of Cisplatin Combined with Chungpesagan-tang

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**Abstract** – *In vitro* cytotoxic activities of cisplatin combined with Chungpesagan-tang or puerarin, which were treated with or without human intestinal bacteria, were measured. When cisplatin was combined with Chungpesagan-tang and its ingredient treated without intestinal bacteria, they did not affect the *in vitro* cytotoxicity of cisplatin against tumor cell lines. However, when cisplatin was combined with intestinal bacteria-treated Chungpesagan-tang and its ingredients, the cytotoxicities against SNU C4, L1210, A549 and P388 tumor cell lines were synergistically increased. Puerarin, which was isolated from Puerariae Radix, did not show *in vitro* cytotoxicity. However, its metabolite, daidzein, showed potent cytotoxicity against tumor cell lines and was synergistic by the combined usage of cisplatin. These results suggest that natural glycosides are not only prodrugs which can be transformed to active compounds by intestinal microflora, but the combined usage of cisplatin with natural components, such as daidzein, and herbal medicinal polyprescriptions, such as Chungpesagan-tang, may be a new method for prevention and minimization of the toxicity of cisplatin.

**Key words** – Chungpesagan-tang, combined usage, cisplatin, 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 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 *et al.*, 1987; Kown *et al.*, 1996). Thus, Chungpesagan-tang had better purgative action than Rhei Rhizoma (Jeon *et al.*, 1999). The purgative action of Rhei Rhizoma was induced by Raphani Semen in Chunpesagan-tang. In addition, we also reported that *in vitro* cytotoxicities of Chungpesagan-tang and Purariae Radix against tumor cell lines were activated by human intestinal bacteria and the cytotoxicity of Puerariae Radix was affected by the addition and subtraction of some herbal medicines (Kim *et al.*, 2000). Most tumor patients use herbal medicines as well as anti-cancer drugs in Korea. Therefore, we here investigated the cytotoxic effects of the combined usage of Chungpesagan-tang and its ingredients with cisplatin and the cytotoxic effect of cisplatin combined with puerarin, which is a main component of Puerariae Radix, in order to scientifically understand the fundamental formulation theory of Chungpesagan-tang.

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## Experimental

**Materials** – MTT, trypsin and RPMI1640 were purchase from Sigma Co., (USA). 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 lymphoma cell line) and L-1210 (Mouse lymphocytic leukemia cell line) were from Seoul University Cell Bank (Korea).

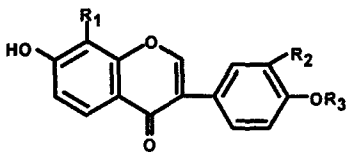
Rhei Rhizoma, Puerariae Radix, Scutellariae Radix, Angelicae Tenuissimae Radix, Platycodi Radix, Raphani

Semen, Cimicifugae Rhizoma and Angelicae Dahuricae Radix were purchased from Kyung-Dong traditional herbal medicinal market (Seoul, Korea).

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

**Thin layer chromatography** – TLC for puerarin and daidzein were performed on silica gel plates (Merk, 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).

**Metabolites of puerarin and daidzin by human intestinal bacteria** – To isolate the metabolites of puerarin isolated from Puerariae Radix by human intestinal bacteria, reaction mixture contained 0.1 mM puerarin 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 ethyl acetate. The ethyl acetate extract was applied to silica gel column chromatography (1.5×20 cm) with $CHCl_3$/MeOH (9:1). The isolated metabolites were crystallized with MeOH. These isolated metabolites, daidzein and calycosin, were identified by comparing to instrumental analysis of authentic compounds.




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| puerarin | Glc | H | H |
| daidzein | H | H | H |
| calycosin | H | OH | $CH_3$ |

**Table 1.** Composition of Chungpesagan-tang

| Herbal Medicine | Weight (g) |
|---|---|
| *Pueraria thunbergiana* (root) | 15 |
| *Scutellaria baicalensis* (root) | 7.5 |
| *Angelica tenuissima* (root) | 7.5 |
| *Platycodon gradiflorum* (root) | 3.75 |
| *Raphanus sativus* (seed) | 3.75 |
| *Cimicifuga heracleifolia* (root) | 3.75 |
| *Angelica dahurica* (root) | 3.75 |
| *Rheum palmatum* (root) | 3.75 |

***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 according to the method of Carmichael *et al.* (1987). Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μl volume) into 96-well microtiter plates. After cells were exposed to the test compounds for 2 day 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. After 30 min at room temperature, 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

***In vitro* Cytotoxicity of cisplatin combined with Chungpesagan-tang and its ingredients** – To investigate drug interaction on the *in vitro* cytotoxic activity of cisplatin combined with Chungpesagan-tang, the *in vitro* cytotoxic activities of cisplatin combined with water extract of herbal medicines against tumor cell lines were measured (Table 2). Compared to the *in vitro* cytotoxicity of cisplatin alone against tumor cell lines, most herbal medicines (0.05 mg/ml) did not affect the *in vitro* cytotoxicity of cisplatin against tumor cell lines. However, Scutellariae Radix and Rhei Rhizoma, which showed the potent *in vitro* cytotoxicity against L1210, P388 and A549 tumor cell line, synergistically increased the cytotoxicity of cisplatin against L1210, P388 and A549. The best synergistic herbal medicines was Scutellariae Radix, followed by Rhei Rhizoma. Chungpesagan-tang also synergistically increased the *in vitro* cytotoxic activity of cisplatin against L1210 tumor cell line only.

**Table 2.** Cytotoxicity of Chungpesagan-tang and its ingredients against tumor cell lines

| Herbal medicine[a] | $EC_{50}$ (mM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | L1210 | | P388 | | SNU C4 | | A549 | |
| | – | +cisplatin | – | +cisplatin | – | +cisplatin | – | +cisplatin |
| None | – | 0.01 | – | 0.020 | – | 0.06 | – | 0.09 |
| Pu | >1 | 0.009 | >1 | 0.018 | >1 | 0.06 | >1 | 0.10 |
| Sc | 0.15 | 0.003 | 0.05 | 0.018 | 0.3 | 0.06 | >1 | 0.05 |
| Pu:Sc (1:1) | 0.25 | 0.004 | 0.15 | 0.018 | 0.3 | 0.06 | >1 | 0.08 |
| At | >1 | 0.010 | >1 | 0.020 | >1 | 0.06 | >1 | 0.09 |
| Pu:At (1:1) | >1 | 0.014 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Ra | >1 | 0.009 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Ra:Pu (1:1) | >1 | 0.015 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Pl | >1 | 0.014 | >1 | 0.020 | >1 | 0.06 | >1 | 0.09 |
| Pu:Pl (1:1) | >1 | 0.015 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Ci | 0.78 | 0.017 | 0.69 | 0.018 | >1 | 0.06 | >1 | 0.07 |
| Pu:Ci (1:1) | >1 | 0.005 | >1 | 0.014 | >1 | 0.06 | >1 | 0.09 |
| Ad | >1 | 0.012 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Pu:Ad (1:1) | >1 | 0.009 | >1 | 0.020 | >1 | 0.06 | >1 | 0.10 |
| Rh | 0.25 | 0.005 | 0.05 | 0.020 | 0.28 | 0.05 | 0.95 | 0.08 |
| Pu:Rh (1:1) | 0.5 | 0.004 | 0.15 | 0.020 | 0.75 | 0.06 | >1 | 0.10 |
| Chung | 0.97 | 0.003 | 0.78 | 0.020 | 0.32 | 0.06 | >1 | 0.10 |

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

To investigate the drug interaction of Chungpesagan -tang on cytotoxicity of cisplatin against tumor cell lines, Puerariae Radix, which is a monarch drug of Chungpesagan-tang, was extracted with ingredients of Chungpesagan-tang and their cytotoxicities were measured. Among the cytotoxic herbal medicines, the *in vitro* cytotoxicities of Scutellariae Radix and Rhei Rhizoma were increased by Puerariae Radix. These cytotoxicity was affected by the addition and subtraction of Puerariae Radix (Data not shown). The biological activities of Puerariae Radix, a Monarch of Chungpesagan-tang, could be controlled by the addition and subtraction of herbal medicines.

**Effect of human intestinal bacteria on the *in vitro* cytotoxic activity of cisplatin combined with Chungpesagan-tang and its ingredients** – We previously reported that some ingredients of Chungpesagan-tang were transformed to more cytotoxic ones against tumor cell lines by human intestinal bacteria. Therefore, we investigated effect of human intestinal bacteria on the *in vitro* cytotoxic activity of cisplatin combined with Chungpesagantang and its ingredients (Table 3). The *in vitro* cytotoxicities of cisplatin combined with Chungpesagan-tang and its ingredients against tumor cell lines were more potent than those of cisplatin alone. The *in vitro* cytotoxicities of cisplatin combined with intestinal bacteria-treated fractions of Chungpesagan-tang and its ingredients against tumor cell lines, particularly L1210 and P388, were more potent than those of cisplatin combined with intestinal bacteria-untreated fractions of Chungpesagan-tang and its ingredients. For example, the components of Chungpesagan-tang, Puerariae Radix and Scutellariae Radix were transformed to potent cytotoxic ones by human intestinal bacteria. These transformed components synergistically increased *in vitro* cytotoxicity of cisplatin alone.

To investigate the drug interaction of Chungpesagan -tang treated with human intestinal bacteria on cytotoxicity of cisplatin against tumor cell lines, Puerariae Radix was extracted with ingredients of Chungpesagan-tang and treated with human intestinal bacteria. The cytotoxicities of cisplatin combined with the treated fractions were measured. Among the cytotoxic herbal medicines, the *in vitro* cytotoxicities of Scutellariae Radix and Rhei Rhizoma against L1210 were increased by Puerariae Radix. These cytotoxicities were affected by the addition and subtraction of Puerariae Radix (Data not shown). These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal microflora. With a better understanding of these results, new polyprescriptions could be scientifically developed.

**Metabolism of puerarin and daidzin from Puerariae Radix by human intestinal bacteria**

**Table 3.** Cytotoxicity of Cisplatin Combined with Constituent Herbs of Chungpesagan-tang (0.05 mg/ml) against tumor cell lines

| Herbal medicine[a] | $EC_{50}$ (mM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | L1210 | | P388 | | SNU C4 | | A549 | |
| | untreated[b] | treated | untreated | treated | untreated | treated | untreated | treated |
| Cisplatin | 0.01 | | 0.02 | | 0.06 | | 0.09 | |
| Pu | 0.006 | 0.004 | 0.016 | 0.008 | 0.063 | 0.052 | 0.09 | 0.08 |
| Sc | 0.004 | 0.003 | 0.010 | 0.008 | 0.063 | 0.040 | 0.05 | 0.06 |
| Pu:Sc (1:1) | 0.005 | 0.003 | 0.012 | 0.010 | 0.062 | 0.050 | 0.09 | 0.08 |
| At | 0.004 | 0.003 | 0.006 | 0.005 | 0.063 | 0.063 | 0.09 | 0.10 |
| Pu:At (1:1) | 0.004 | 0.004 | 0.015 | 0.014 | 0.063 | 0.061 | 0.09 | 0.06 |
| Ra | 0.005 | 0.003 | 0.010 | 0.010 | 0.063 | 0.061 | 0.10 | 0.06 |
| Ra:Pu (1:1) | 0.005 | 0.004 | 0.006 | 0.013 | 0.060 | 0.060 | 0.10 | 0.05 |
| Pl | 0.006 | 0.004 | 0.017 | 0.015 | 0.060 | 0.060 | 0.10 | 0.10 |
| Pu:Pl (1:1) | 0.009 | 0.003 | 0.016 | 0.010 | 0.060 | 0.060 | 0.10 | 0.07 |
| Ci | 0.004 | 0.002 | 0.007 | 0.080 | 0.063 | 0.062 | 0.08 | 0.09 |
| Pu:Ci (1:1) | 0.004 | 0.003 | 0.005 | 0.004 | 0.063 | 0.058 | 0.10 | 0.09 |
| Ad | 0.006 | 0.004 | 0.014 | 0.008 | 0.063 | 0.060 | 0.01 | 0.10 |
| Pu:Ad (1:1) | 0.004 | 0.004 | 0.010 | 0.006 | 0.038 | 0.027 | 0.10 | 0.09 |
| Rh | 0.006 | 0.005 | 0.018 | 0.015 | 0.063 | 0.020 | 0.04 | 0.04 |
| Pu:Rh (1:1) | 0.004 | 0.004 | 0.018 | 0.013 | 0.050 | 0.037 | 0.06 | 0.02 |
| Chung | 0.005 | 0.004 | 0.016 | 0.008 | 0.059 | 0.041 | 0.10 | 0.08 |

[a]Pu, Puerariae Radix; Sc, Scutellariae Radix; At, Angelicae Tenussimae Radix; Ra, Raphani Semen; Pl, Platycodi 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 ethyl acetate; Treated, each herbal medicine or polyprescription was incubated at 37°C for 20 h with human intestinal bacteria and extracted with ethyl acetate.

**and its relation to *in vitro* cytotoxicity against tumor cell lines** – We previously reported that two compounds, puerarin and daidzin were isolated from Puerariae Radix by silica gel column chromatography, and their main metabolites, daidzein and calycosin, by human intestinal bacteria were isolated. Therefore, we here investigated *in vitro* cytotoxic activity of puerarin and its metabolite, daidzein, against tumor cell lines (Table 4). Puerarin did not show *in vitro* cytotoxicity. However, its metabolite, daidzein, showed potent cytotoxicity against tumor cell lines. We found that the cytotoxicity was increased when puerarin was metabolized to daidzein by human intestinal microflora. These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal microflora.

When cisplatin was combined with these compounds, puerarin or daidzein, its *in vitro* cytotoxicity was measured (Fig. 1). When cisplatin was combined with 0.5 mM puerarin, its in vitro cytotoxicity was similar to that of cisplatin only. When cisplatin combined with 1.0 mM puerarin, its cytotoxicity was weakly increased. However, when cisplatin was combined with daidzein, its *in vitro* cytotoxicity was synergistically increased.

**Table 4.** Cytotoxicity of Puerarin, Daidzein and Cisplatin against Tumor Cell Lines

| Compound | $ED_{50}$ (mM) | | | |
|---|---|---|---|---|
| | P-388 | SNU C4 | A 549 | L 1210 |
| Puerarin | >2 | >2 | >2 | >2 |
| Daidzein | 0.82 | 0.97 | 1.72 | >2 |
| Cisplatin | 0.02 | 0.06 | 0.09 | 0.01 |

When cisplatin only was treated into tumor cell lines, SNU C4, L1210, A549 and P388 tumor cell lines, its cytotoxic $EC_{50}$ was 0.06, 0.01, 0.09 and 0.02 mM respectively. However, when cisplatin combined with daidzein was treated into SNU C4, L1210 and P388 tumor cell lines, its cytotoxicity was synergistically increased. When cisplatin combined with 0.5 mM daidzein, its cytotoxic $EC_{50}$ was 0.002, 0.0003, 0.0004 mM, respectively. These results suggest that natural glycosides are not only prodrugs which can be transformed to active compounds by intestinal microflora, but the combined usage of cisplatin with natural components, such as daidzein, and herbal medicinal polyprescriptions, such as Chungpesagan-tang, may be a new method for prevention and minimization of the toxicity of cisplatin.

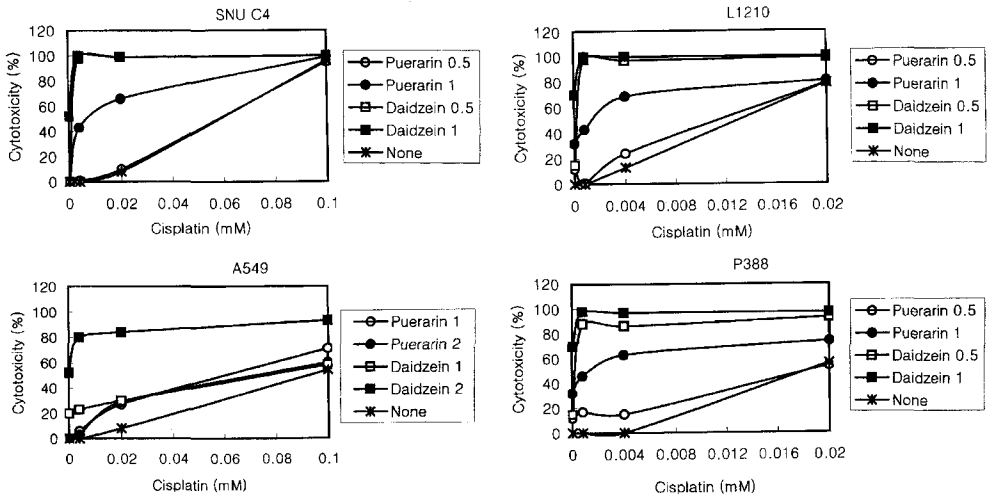



**Fig. 1.** Cytotoxicity of Cisplatin Combined with Puerarin or Daidzein against Tumor Cell Lines.

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