Influence of Decoction Duration of FDY2004 on Its Physicochemical Components and Antioxidant and Antiproliferative Activities

In-Hee Lee1, Ho-Sung Lee1,2, Kyungrae Kang2, Sang-In Park3, Tae-wook Kwon2, Seung-Joon Moon2, Chol Hee Lee2, and Dae Yeon Lee1,2

Abstract
FDY2004 (Medicinal herbs: Rheum palmatum, Paeonia suffruticosa Andrews, and Prunus davidiana), an herbal drug composition with an antiproliferative effect, is prepared by boiling, which is the most common herbal extraction method in traditional Korean medicine. Several parameters are considered in the process, including herb-to-solvent ratio, extraction temperature and pressure, and total decoction time. The aim of this study was to examine the physicochemical changes, index compound analysis results, antioxidant activity, and antiproliferative activity of FDY2004 according to the decoction duration to establish the conditions that ensure efficacy while minimizing side effects. Different samples of FDY2004 were obtained by decocting for 30, 60, 90, 120, 180, and 240 minutes. Each sample was evaluated for hydrogen ion concentration (pH), total soluble solid content (TSSC), index compound profiles, and antioxidative and antiproliferative activity. pH was found to decrease, while TSSC increased with an increase in decoction duration. Index compound contents for FDY2004 (aloe emodin, emodin, rhein, chrysophanol, physcion, and sennoside A for R. palmatum, paeonol for P. suffruticosa Andrews, and amygdalin for P. davidiana) increased when the decoction duration was 120 minutes or more, while the content of sennoside A did not increase. The total d-glucose amount increased with an increase in boiling duration. Antioxidant activity of FDY2004 increased when the decoction duration was 120 minutes or more, and the antiproliferative activity of FDY2004 was concentration dependent. The decoction duration for FDY2004 needs to be carefully determined so as to maintain efficacy while reducing side effects related to digestive absorption.

Keywords
HPLC, anti-proliferative activity, antioxidative activity, boiling duration, starch

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Despite the development of the pharmaceutical industry and clinical medicine, in many countries, traditional medicine generally forms part of the culture and history of these countries.1 Traditional Chinese Medicine formulations usually contain 2 or more types of herbal medicines and are designed for relatively specific symptoms.2 In Korea, traditional medicine-based pills, decoctions, and pharmacopunctures are extensively used; however, the most commonly used formulations are decoctions that are taken orally. Because these are extracts, the active ingredients are already extracted and easy to receive and efficiently absorbed. The extraction methods employed may vary depending on various factors such as temperature, pH, extraction time, amount of solvent relative to solute, pressure, and the powder size of the herbal medicines.3,5 Depending on the extraction solvent, the extracted components show significant differences. Because decoctions are received orally, most of them are prepared using water.6 Furthermore, depending on the extraction time, many components may be extracted but may undergo decomposition and reduction.7 Thus, the extraction time may affect not only component bioactivity but also side effects. The longer the decoction time, the more the extraction of reactive substances such as starch. Starch can interfere with digestion and inhibit the absorption of the formulation’s active substances in the human body. The digestion rate has been reported to correlate negatively with starch crystallization,8 and starch granules have a crystalline chemical structure embedded in an amorphous matrix. A previous report linking naturally
occurring starch with a slow absorption rate suggested that many individuals are only partially capable of digesting plant starch, and high starch contents in herbal decoctions may adverse drug reactions such as bloating, indigestion, and acid reflux. FDY2004 is a processed formulation derived from Daehwangmokdan-pi-tang (named Dahuangmudani-tang in China), which comprises Rheum palmatum, Paeonia suffruticosa, and Prunus davidiana; it is known to be effective against pancreatitis and to have anti-inflammatory and antiproliferative effects in cancer cells through downregulation of matrix metalloproteinase 2 (a cancer cell metastasis factor), interleukin-1β, and tumor necrosis factor-α (an inflammatory gene). Therefore, it is expected to have an antiproliferative effect in other cancer cells. In this study, we aimed to confirm the antiproliferative efficacy in cancer cell lines of FDY2004, which is already in use in humans. Further, R. palmatum, a component of FDY2004, has side effects such as diarrhea, which is an inconvenience for patients, and rhein and sennoside A, components of R. palmatum, have been reported to affect digestion. Therefore, we tried to determine the appropriate decoction time for FDY2004 so as to minimize the amounts of starch, rhein, and sennoside A but maintain the antioxidant and antiproliferative efficacy of the formulation.

Materials and Methods

Preparation of FDY2004

All raw constituents of FDY2004 were purchased from Green Meong Pum Pharm Corp. (Namyangju, Korea). R. palmatum (6.67 g), P. suffruticosa (6.67 g), and P. davidiana (6.67 g) in ground form were added to 200 mL of distilled water and boiled for 30, 60, 90, 120, 180, or 240 minutes at 100 °C. The extract, after cooling, was passed through a 1-µm pore filter (Hyundai Micro, Seoul, South Korea). Partitions of each sample were analyzed for total soluble solid content (TSSC), pH, total starch, index compounds, and antioxidant activity. The remaining samples were freeze-dried at −80 °C for analyzing cytotoxicity against cancer cell lines and yield. All samples were extracted thrice separately under the same conditions and then freeze-dried. Before the assay, the freeze-dried samples were stored at −20 °C and dissolved in distilled water immediately.

TSSC and Hydrogen Ion Concentration (PH)

TSSC was measured with a PAL-1 pocket refractometer (ATAGO Co., Tokyo, Japan). After calibration, 200 µL of each sample was used for TSSC analysis. The pH of each sample was measured with an Orion Star A211 pH meter (Thermo Scientific, Waltham, USA). The pH meter was calibrated with premixed solutions of pH 4.0, 7.0, and 10.0 before running the assay. The mean values were obtained from triplicate experiments.

D-Glucose Measurement

The total starch content in each sample was measured with a Megazyme kit (K-TSTA, Chicago, USA). Eight milliliters of 95% ethanol was added to 2 mL of each FDY2004 sample. The samples were then vortexed, incubated at room temperature for 30 minutes, and centrifuged at 1800×g. Supernatants were removed, and the pellet was resuspended in 1 mL of distilled water. Thereafter, amyloglucosidase was added to each sample, and incubation was performed at 50 °C for 30 minutes. The samples were then subjected to a luminescence assay by adding glucose oxidase and 4-aminoantipyrine mixture solution. Absorbance was measured at 540 nm. A standard curve was obtained using multiple samples of the D-glucose standard at different concentrations (10, 50, 100, 500, and 1000 µg/mL), and the starch content in each sample was quantified based on the curve.

High-Performance Liquid Chromatography

The index compounds of FDY2004 were analyzed using high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan). HPLC-grade organic solvents (J.T. Baker, Center Valley, USA) and a C18 column (4.6 mm × 250 mm, 5 µm; Agilent, Santa Clara, USA) were used for the analysis. A standard curve was obtained using the standard control, and the index compound of each sample was determined by calculating the area under the corresponding slope.

Aloe emodin, emodin, rhein, chrysophanol, and physcion. The analysis of aloe emodin, emodin, rhein, chrysophanol, and physcion (Sigma-Aldrich, St. Louis, USA), which are the index compounds of R. palmatum, was performed at an ultraviolet (UV) wavelength of 254 nm. The mobile phase was composed of water and acetonitrile (15:85, v/v) with 0.1% phosphate, and the flow rate was 1 mL/min.

Sennoside A. Sennoside A (Sigma-Aldrich, St. Louis, USA), an index compound of R. palmatum, could not be analyzed simultaneously at a UV wavelength of 340 nm. A mobile phase composed of water and acetonitrile (29:71, v/v) with 1% acetic acid was used for its analysis. The flow rate was maintained at 1 mL/min.

Paeonol. Paeonol (Sigma-Aldrich, St. Louis, USA), the index compound of P. suffruticosa, was analyzed at a UV wavelength of 274 nm. The mobile phase was composed of water and acetonitrile (35:65, v/v) with 2% acetic acid, and the flow rate was 1 mL/min.

Amygdalin. The analysis of amygdalin (Sigma-Aldrich, St. Louis, USA), the index compound of P. davidiana, was conducted at a UV wavelength of 210 nm according to the Korean Pharmacopeia method. The mobile phase was composed of
water (A) and methanol (B). The gradient profile was applied with 5% B at the start and 100% B from 5 to 30 minutes. The flow rate was maintained at 1 mL/min.

Antioxidant Activity

1,1-Diphenyl-2-picrylhydrazyl assay. The methodology of Brand-Williams et al. was used for the 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich, St. Louis, USA) assay. Each sample of FDY2004 and DPPH solution was mixed in equal amounts. After incubation for 30 minutes at 37 °C, the reduction of the DPPH radical was measured by reading the absorbance at 517 nm using a microplate reader (TECAN, Chapel Hill, USA). A standard curve was plotted with multiple concentrations (250, 125, 62.5, 31.25, 16.63, and 8.21 µg/mL) of Trolox (Sigma-Aldrich, St. Louis, USA) as the standard.

2,2’-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid). For the 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, the procedure described by Arnao et al. was followed, whereby 7.4 mM ABTS and 2.6 mM potassium persulfate were mixed and then the mixture was left in complete darkness for 12 hours. FDY2004 sample (10 µL) was added to 190 µL of the mixture solution. Absorbance was then measured at 750 nm using a microplate reader. The results were expressed as milligrams of Trolox equivalents.

Ferric reducing/antioxidant power. The ferric reducing/antioxidant power (FRAP) assay was performed as described by Benzie and Strain. Acetate buffer (300 mM), 10 mM 2,4,6-tripyridyl-s-triazine with 40 mM hydrogen chloride, and 20 mM ferric chloride hexahydrate were mixed at a ratio of 10:1:1. Thereafter, 10 µL of the FDY2004 samples was added to 190 µL of the mixture solution and left in the dark for 30 minutes. Absorbance was read at 593 nm by using a microplate reader. The results were expressed as milligrams of Trolox equivalents.

Total phenolic compounds. The content of total phenols was determined according to the methodology of Singleton et al. FDY2004 sample (50 µL) was mixed with 1 mL of 2% sodium carbonate, after which 50 µL of 50% Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, USA) was added to the mixture, which was then left standing for 30 minutes. Absorbance was read at a wavelength of 750 nm using a microplate reader. The results were expressed as milligrams of gallic acid equivalents.

Total flavonoids. Total flavonoids content was determined as described by Chae et al. The FDY2004 sample solution (0.1 mL) was mixed with 1 mL of diethyleneglycol, after which 0.1 mL of 1 N sodium hydroxide was added to the mixture. After vortexing well, the absorbance of the mixture was measured at a UV wavelength of 415 nm. The results were expressed as milligrams of quercetin equivalents.

Antiproliferative Activity

The antiproliferative activity of FDY2004 was assessed using Mosmann’s method. The lung cancer cell line A549 and breast cancer cell line MDA-MB231 were purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea). Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (Gibco, Waltham, USA) and 1% antibiotic-antimycotic agent (Gibco, Waltham, USA) in a 5% carbon dioxide incubator maintained at 37 °C. The cells were seeded into 48-well plates at a density of 5.0 × 10^5 cells per well and then treated with various concentrations (1, 10, 100, and 1000 µg/mL) of FDY2004 extracts boiled for 120 minutes. The positive control group was treated with 1 µM paclitaxel. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by treating the cells with FDY2004 and paclitaxel for 48 hours, followed by incubation with 250 µL of MTT at 37 °C for 2 hours. The mitochondria-dependent reduction of MTT to formazan was followed by dissolution in dimethyl sulfoxide. The absorbance was then measured at a wavelength of 550 nm using a microplate reader (Epoch 2, BioTek, Winooski, USA).

Statistical Analysis

One-way analysis of variance was performed to determine the influence of decoction time on the biological activity and physicochemical changes of FDY2004; a paired t-test was performed to assess the length of time and concentration. Duncan’s multiple range test was conducted for post hoc analysis. All statistical analyses, including computation of P values, were performed using SPSS 18.0 statistical software packages (IBM, Armonk, USA).

Results

Yield

FDY2004 was boiled for different lengths of time (30, 60, 90, 120, 180, and 240 minutes) in a reflux extraction apparatus, cooled, and supplemented with distilled water to achieve a volume of 200 mL. Twenty milliliters of the extract was freeze-dried for additional experiments. Extraction for a longer duration with boiling increased the yield in a time-dependent manner. The yield was measured as the weight of the lyophilizate relative to the weight of the first herbal medicine. Decoction for 120 minutes instead of 30 minutes significantly increased the yield (Figure 1(A)).

TSSC and pH

TSSC significantly increased with a decoction duration of 120 minutes or more and decreased when the decoction duration was 90 minutes, although the decrease was not significant, and the overall TSSC increased. The pH of each sample ranged between 4.95 and 5.27; it showed a tendency to decrease with
an increase in boiling duration ($P < 0.0001$; Figure 1(B) and (C)). However, the pH did not decrease upon boiling for 180-240 minutes.

**Total Starch**

In order to measure whether the amount of starch changes depending on the decoction duration, we used a megazyme kit that measures starch through the amount of d-glucose. In the control group, measurements were performed without α-amylase treatment; the concentration of d-glucose did not significantly change depending on the decoction duration. In the experimental group, the d-glucose contents of the decoction extracts of 30, 60, 90, 120, 180, and 240 minutes were 0.29 ± 0.04, 0.32 ± 0.01, 0.33 ± 0.02, 0.44 ± 0.02, 0.58 ± 0.06, and 0.69 ± 0.04 mg/mL, respectively. When hydrolyzed, the d-glucose content increased significantly with a decoction duration of 120 minutes or more ($P < 0.0001$; Figure 1(D)).

**Analyses of Index Compounds**

The contents of the various index compounds were determined in different FDY2004 samples. The contents of aloe emodin, rhein, emodin, chrysophanol, and physcion, the index compounds of *R. palmatum*, showed a tendency to increase in proportion to the decoction duration (Figure 2(A)). On the other hand, the content of sennoside A tended to decrease (Figure 2(B)). The concentration of paenol, the index compound of *P. suffruticosa*, ranged from 42.50 ± 0.14 µg/mL to 82.37 ± 0.30 µg/mL among the samples and tended to increase with a decoction duration of 120 minutes or more (Figure 2(C)). The concentration of amygdalin, the index compound of *P. davidiana*, was found to be between 75.21 ± 0.41 µg/mL and 163.03 ± 0.72 µg/mL and tended to increase with a decoction duration of 120 minutes or more (Figure 2(D)). The concentrations of some index compounds increased with an increase in the duration of boiling; however, the content of sennoside A decreased when the decoction duration was 90 minutes.
Antioxidant Activity

The radical scavenging potential of FDY2004 was investigated in the DPPH, FRAP, ABTS, total flavonoid, and total phenolic compound assays. The antioxidative activity of each FDY2004 sample was quantified based on the standard curve. In all the assays, the antioxidant activity of FDY2004 tended to increase with a decoction duration of 120 minutes or more. Furthermore, the antioxidant activity increased significantly with a decoction duration of 180 minutes or more compared with that when the decoction duration was 30 minutes. The antioxidant activity of FDY2004 tended to decrease when the decoction duration was 90 minutes, as shown by HPLC, although the decrease was not significant (Figure 3(A–E)).

Antiproliferative Activity

The antiproliferative activity of FDY2004 (boiled for 120 minutes) was investigated at multiple concentrations (from 1 μg/mL to 1000 μg/mL) via the MTT assay. In the lung cancer cell line A549 and breast cancer cell line MDA-MB 231, FDY2004 displayed antiproliferative activity in a dose-dependent manner, which was expressed as a decrease in cell viability, at higher concentrations of FDY2004 (P < 0.0001; Figure 4(A) and (B)).

Discussion

In this study, changes in the amounts of index compounds and antioxidant and antiproliferative activities of FDY2004 were investigated in relation to the extraction duration. FDY2004 was chosen as the test product for this study because it has already been prescribed in Korean medicine hospitals as an anticancer drug and has been effective in many patients. We attempted to determine the appropriate decoction time for FDY2004 that would result in fewer side effects. *Rheum palmatum,* Paeonia suffruticosa, and Prunus davidiana, the constituents of FDY2004, are known for their antiproliferative efficacy. Formulations containing combinations of these species may differ in efficacy and side effects depending on the extraction method used. Previous studies on decoction methodologies, including the duration of boiling, have mainly focused on the differences in the yields of marker compounds and active constituents and the chemical denaturation of these compounds. The present study was aimed at identifying the main components of FDY2004 and its activities as well as the components that may cause side effects by measuring the total amount of starch in the formulation. In this study, pH decreased, while TSSC increased with an increase in the
duration of boiling (Figure 1(B) and (C)). It is natural for non-soluble, high-molecular-weight compounds to break down into soluble, low-molecular-weight compounds through the boiling process. Our results are in agreement with those reported previously.9 Prolonged application of physical stimuli such as heat can lead to protonation. In previous studies, the amount of d-glucose following enzyme hydrolysis was investigated to quantify the total starch amount.39,40 The results showed that boiling for an extended duration led to an increase in total starch (Figure 1(D)). Since the control group without enzyme treatment did not show a significant increase associated with the duration of decoction, the compounds whose quantities increased with a longer decoction time were more likely dietary fiber and resistant starch. Since FDY2004 is an herbal formulation, a certain amount of starch is obtained when it is extracted with water. A large amount of starch is not beneficial to the human body. The rate of digestion is negatively correlated with starch crystallization,8 and starch granules have a crystalline chemical structure embedded in an amorphous matrix. A previous report linking naturally occurring starch with a slow absorption rate39 indicated that many individuals are only partially capable of digesting plant starch, and excessive starch in herbal decoctions may lead to adverse drug reactions such as bloating, indigestion, and acid reflux. HPLC analyses of aloe

**Figure 3.** Difference in the antioxidant activity of FDY2004 according to varying decoction times. The antioxidative activity of FDY2004 samples was measured. A 2-sided analysis of variance test revealed a significant increase in antioxidant activity with an increase in concentration ($P < 0.05$). Statistically significant values were compared with values obtained with 30 minutes of decoction time by using the Student’s t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (A) 2,2-Diphenyl-1-picrylhydrazyl assay, (B) 2,2’-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt assay, (C) ferric reducing antioxidant power assay, (D) total phenolic compounds, and (E) total flavonoids.

**Figure 4.** Antiproliferative activity of FDY2004 in cancer cell lines. A 2-sided analysis of variance test revealed a significant increase in antiproliferative activity with an increase in concentration ($P < 0.05$). Student’s t-test revealed that the antiproliferative efficacy was higher in the A549 cell line than in the MDA-MB 231 cell line (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (A) Viability of FDY2004-treated A549 cells and (B) Viability of FDY2004-treated MDA-MB 231 cells.
emodin, rhein, emodin, chrysophanol, physcion, paeonol, and amygdalin showed that the amounts of these compounds tended to increase with an increase in boiling duration. However, the reverse trend was noted for sennoside A (Figure 2), probably because rhein and aloes are metabolites and derivatives of sennoside A.\textsuperscript{41,42} Rhein\textsuperscript{43} and sennoside A\textsuperscript{44} may cause side effects such as diarrhea and hence should not be used in high doses only for efficacy. The results of the physcion analysis differed from the trends noted for the other index compounds. This may be because physcion has the lowest solubility in the water among the compounds analyzed.

In terms of antioxidant activity, all the results showed a similar tendency according to the decoction time, and the antioxidant efficacy gradually increased with a decoction duration of 120 minutes or more (Figure 3). These results were in agreement with the analytical results of the contents of the index compounds.

In this study, we attempted to determine the optimal decoction time for FDY2004 to minimize digestive absorption-related side effects of its constituents such as total starch, rhein, and sennoside A while maintaining efficacy. The results of our analyses revealed that a decoction time of 120 minutes was the most appropriate for FDY2004 and confirmed the antiproliferative efficacy of FDY2004 in lung and breast cancer cell lines. The results showed significant concentration-dependent antiproliferative activity of FDY2004 in A549 and MDA-MB 231 cell lines. However, as the appropriate extraction method or extraction time for herbal drugs varies depending on constituent ingredients, further studies are needed to establish the optimal methods of extraction for other commonly used prescriptions.

Conclusion
In FDY2004, more active ingredients such as aloe emodin, emodin, chrysophanol, physcion, paeonol, and amygdalin are extracted as the decoction time increases. At the same time, more starch and ingredients such as sennoside A and rhein, which interfere with digestion and absorption, were also extracted. Therefore, it is difficult to find the proper decoction time for FDY2004, so it was determined by measuring the amount of physiological activity and extracting components. Rhein and starch, one of the side effects-causing substances, rapidly increased from 180 minutes of decoction time, and antioxidant activity increased from 120 minutes, and so the proper decoction time of FDY2004 was set to 120 minutes. FDY2004 with a 120-minute decoction time showed antiproliferative activities in lung cancer and breast cancer cell lines. However, additional research is needed because factors affecting the efficacy and side effects include not only the decoction time but also various factors.

Declaration of Conflicting Interests
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ORCID ID
Dae Yeon Lee https://orcid.org/0000-0002-3198-9881

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