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ORIGINAL ARTICLE

FDY003 Inhibits Colon Cancer in a Colo205 Xenograft Mouse Model by Decreasing Oxidative Stress

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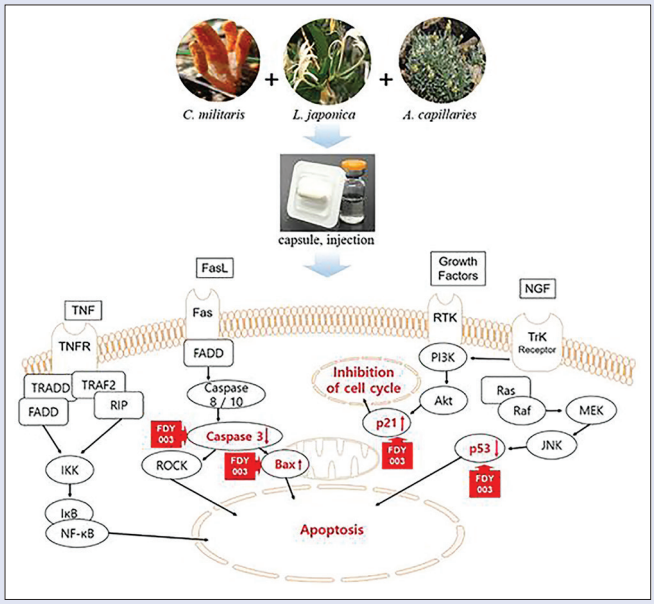
ABSTRACT

Background: FDY003 is a traditional Korean medicine that has been developed as a complementary therapy for cancer. FDY003 contains various herbs such as *Lonicera japonica, Artemisia capillaris* Thunb., and *Cordyceps militaris* known to exhibit antioxidant and anticancer activities. **Objective:** The objective of this is to determine whether FDY003 represents a complementary therapy for colon cancer when it is injected using a syringe. **Materials and Methods:** High-performance liquid chromatography (HPLC) analysis was performed to determine the active ingredients of FDY003. The effect of FDY003 on the proliferation of Colo205 cells was investigated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The antioxidant effects of FDY003 on Colo205 cells were ascertained using oxidative markers such as lipid peroxidation and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Markers of apoptosis in Colo205 cells after treatment with FDY003 were also measured. Based on the *in vitro* results, *in vivo* experiments were performed using Colo205 cell-induced xenograft mouse cancer model treated with FDY003. **Results:** HPLC analysis revealed that FDY003 contained various active ingredients known to possess antioxidant activities. The viability of Colo205 cells was decreased by FDY003 in a concentration-dependent manner. Cancer size and weight were significantly decreased in the group treated with FDY003, similar to those in the group treated with anticancer drug irinotecan. The expression of Bcl-2-associated X protein and caspase-3 was increased in cancer tissues derived from the FDY003-treated group. Serum levels of lipid peroxidation and DPPH were also significantly increased in the FDY003-treated group. **Conclusion:** FDY003 represents a potential complementary therapy for cancer due to its antioxidative effects and anticancer activity.

Key words: Colo205 cells, colon cancer, FDY003, oxidative stress, xenograft

SUMMARY

- FDY003, containing various antioxidants, showed antitumor activity mediated via antioxidative effects, reducing blood lipid peroxide levels and inducing apoptosis of cancer cells *in vivo* and *in vitro*.



Abbreviations used: DPPH: 2,2-diphenyl-1-picrylhydrazyl; Bax: Bcl-2-associated X protein; KCLB: Korean Cell Line Bank; RPMI: Roswell Park Memorial Institute; MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide; EDTA: Ethylenediaminetetraacetic acid; MDA: Malondialdehyde; TBARS: Thiobarbituric acid reactive substances; PBS: Phosphate-buffered saline; RIPA: Radioimmunoprecipitation assay; PVDF: Polyvinylidene fluoride; ANOVA: Analysis of variance; SEM: Standard error of the mean; SPSS: Statistical Package for the Social Sciences; ROS: Reactive oxygen species; RSA %: Radical scavenging activity percentage.

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INTRODUCTION

In the last few decades, colon cancer has become the third most common cancer worldwide, with increasing incidence in many countries.[1,2] Strategies for the screening of colon cancer have not reduced the incidence or mortality.[3] Current drug treatments for colon cancer are associated with toxic side effects and fail to inhibit the metastatic progression of the disease.[4] Therefore, many drugs and treatments targeting the nutritional and genetic risk factors of colon cancer are being investigated.[5] Furthermore, complementary therapies containing safe phytochemical agents along with acupuncture have been proposed as novel therapeutic strategies for cancer.[6,7]

We are interested in developing an effective therapeutic intervention for colon cancer based on complementary medicine. Our ultimate goal is

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to develop an herbal extract based on Korean traditional medicine with antioxidative effects and anticancer activity, for administration as an injection clinically. We blended herbal extracts obtained from medicinal herbs such as *Lonicera japonica, Artemisia capillaris* Thunb., and *Cordyceps militaris*. *L. japonica* is a broad-leaved tree belonging to family Caprifoliaceae.[8] It is known to possess antioxidant, anti-inflammatory, and antibacterial activities due to the presence of various active ingredients such as saponin, luteolin, tannin, triterpenoid glycoside, and loniceran.[9] *A. capillaris* Thunb. is a deciduous shrub belonging to family Compositae. It is traditionally used as a medicinal herb.[10] *A. capillaris* Thunb. is known to possess antioxidant and anticancer activities.[11]

Both of these medicines carry a variety of active ingredients, especially chlorogenic and caffeic acids, which exhibit strong anticancer activities.[12-16] *C. militaris* is a parasite living on insects.[17] Cordycepin, the main ingredient occurring in *C. militaris*, has been reported to exhibit high anticancer efficacy.[18,19] The prescription based on FDY003 combined with these three plants has already been used in Korea as an injection or capsule in cancer treatment. However, it is necessary to establish the pharmacological basis of the intervention supported by an appropriate level of evidence.

We hypothesized that our blended herbal medicine known as FDY003 could be used as an anticancer therapy for administration by injection. To determine the anticancer activity of our blended herbal medicine administered as an injection, we performed *in vitro* experiments using Colo205 human colon cancer cells and *in vivo* experiments using a Colo205 xenograft mouse model.

MATERIALS AND METHODS

Antibodies, cell line, and chemicals

The human colorectal carcinoma cell line Colo205 was obtained from the Korean Cell Line Bank (Seoul, Korea). These cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo, USA) in a humidified incubator (37°C, 5% CO_2). Anti-Bcl-2-associated X protein (Bax), beta-actin, caspase-3, cleaved caspase-3 (Asp175), phospho-p53 (Ser15), and p53 (1C12) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). P21 was purchased from Abcam (Cambridge, UK). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard markers required for analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade organic solvents used for analysis were obtained from J. T. Baker (Center Valley, PA, USA).

FDY003 preparation

All raw constituents of FDY003 were purchased from Green Meong Pum Pharm Corp. (Namyangju, Korea). Dried plant materials of *L. japonica* (4.16 g), *A. capillaris* Thunb. (6.25 g), and *C. militaris* (6.25 g) were ground and added to 500 mL of 70% ethanol, followed by reflux extraction at 80°C for 180 min. After filtration using a 1-μm pore filter (Hyundai Micro, Seoul, South Korea), the extract was purified by treatment with 80% and 90% ethanol. Finally, the solution was freeze-dried at −80°C. Freeze-dried samples were stored at −20°C before they were dissolved in distilled water for experimental analysis.

High-performance liquid chromatography analysis

Components of FDY003 were analyzed using a Shimadzu LC-20A HPLC system consisting of a LC-20AT solvent delivery system, a SIL-20AC auto-injector, a CTO-20AC column oven, and a SaPD-M20A photodiode array. FDY003 was added to the Agilent TC-C_{18} (2) column (5 m, 250 mm × 4.6 mm, Agilent Co., Ltd., USA) and detected at a wavelength of 254 nm. The column temperature was set to 35°C and the flow rate at 1 mL/min. The mobile phase was prepared using 100% acetonitrile for 180 min mixed with water containing 1% formic acid.

2,2-Diphenyl-1-picrylhydrazyl assay

To determine the antioxidant activity of FDY003, the DPPH assay was performed as reported previously[20] by monitoring the absorbance at 492 nm with a microplate reader (Epoch 2, BioTek, USA). A standard curve was plotted with multiple concentrations (0.1, 1, 10, 100 and 1000 μg/mL) of gallic acid (Sigma-Aldrich, St. Louis, MO, USA). The scavenging activity of FDY003 for DPPH radicals was measured by comparing the absorbance values to the standard curve.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay

Colo205 cells were seeded into 48-well plates at a density of 5.0×10^4 cells per well. The MTT assay was conducted by treating the cells with FDY003 (0.1–500 μg/mL) for 24 h, followed by incubation at 37°C with 250 μL MTT for 2 h. The mitochondrial-dependent reduction of MTT to formazan was followed by dissolution in dimethyl sulfoxide (DMSO). The absorbance was then measured at a wavelength of 550 nm using a microplate reader (Epoch 2, BioTek, Winooski, VT, USA).

Animal study

Athymic nude-Foxn1nu mice (5 weeks old, Harlan Co, USA) weighing 18–22 g were used after 1 week of acclimation (temperature: 23°C ± 3°C, humidity: 55 ± 15%, free access to water and standard food). The Colo205 xenograft model was developed by culturing Colo205 cells as a monolayer and harvesting them after treatment with 0.25% trypsin-ethylenediaminetetraacetic acid. Next, 5×10^6 cells were implanted subcutaneously into the right flanks of 6-week-old athymic nude mice using a 26-gauge needle (0.1 mL/head). Once the tumor size reached 100–150 mm^3, mice were randomly assigned to a negative control group, a positive control group (irinotecan 20 mg/kg i. p.), and an FDY003-treated group (0.2 mg/kg intravenous [i. v.]). Irinotecan and FDY003 were used for treatment twice a week for 4 weeks. The body weight of each mouse was measured once a week on the day of separation or administration and the autopsy day. On the day of separation or treatment initiation, the tumor size was measured using a caliper. It was then measured twice a week. All the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of KNOTUS Co. Ltd., Korea (approval number: KNOTUS IACUC 18-KE-043).

Free radical scavenging activity

Free radical scavenging activity was determined by measuring the ability of cells to reduce DPPH based on the principle of decreased reaction of DPPH radicals in the presence of hydrogen-donating antioxidants.[21,22] The DPPH solution (0.2 mM) was prepared in 95% ethanol. FDY003 only, FDY003-treated Colo205 cell supernatant, and the serum sample from experimental mouse model were mixed with DPPH solution and incubated at 37°C for 30 min. DMSO was used as a control. The absorbance was then measured at a wavelength of 492 nm using a spectrophotometer (Epoch 2, BioTek, USA). The scavenging activity was determined using the published formula:[23] Scavenging activity% = $100 - [(Abs_{sample} - Abs_{blank}) \times 100/Abs_{control}]$.

Determination of malondialdehyde level

Levels of malondialdehyde (MDA), a lipid peroxidation product, in the Colo205 cells and the serum obtained from the experimental mouse model were determined by quantifying thiobarbituric acid reactive substances (TBARS). Briefly, the Colo205 cells were treated with FDY003 (0.5, 1, or 5 μg/mL) for 24 h. After treatment, the cells were harvested and washed with PBS twice. The MDA levels were then measured using commercially available kits (Bio-Vision, USA) in accordance with the manufacturer's instructions.

Protein extraction and immunoblotting

Immunoblotting was performed for both *in vitro* and *in vivo* samples. Briefly, Colo205 cells were treated with FDY003 (0.5, 1, or 5 μg/mL) for 24 h. After incubation, cells were harvested and washed twice with PBS. Tumor tissues were obtained from mice at a specified endpoint. These cells and tissues were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA) containing Protease inhibitor cocktail and phosphatase inhibitors (GenDepot, USA). Total protein concentration was measured using a bicinchoninic acid assay (Thermo, USA). Proteins in the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 8%, 10%, and 14% gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). These membranes were blocked with 5% skim milk in PBS and individually incubated with each primary antibody such as Bax, caspase-3, cleaved caspase-3, p21, p53, and p-p53 (diluted 1:1,000 in 1% skim milk in PBS) at 4°C overnight. Blots were further incubated with each secondary antibody (diluted at 1:10,000) at room temperature for 1 h. These immunoreactions were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, San Jose, CA, USA) and analyzed with a ChemiImager system (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Data were analyzed using Student's *t*-test (for two groups), one-way analysis of variance, and Tukey's test (for more than two groups) and are presented as mean and standard deviation of the mean. The *P* values were indicated as $P < 0.05$, $P < 0.01$, and $P < 0.001$ in order to express the degree of significance. However, statistical significance was set at $P < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences version 13.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Component analysis of FDY003

The analysis of FDY003 components using HPLC revealed a large amount of caffeic acid derived from *L. japonica* and *A. capillaris* Thunb. Chlorogenic acid and 6,7- dimethylesculetin from *A. capillaris* Thunb. and cordycepin derived from *C. militaris* were also detected [Figure 1].

FDY003 inhibits proliferation of Colo205 cells

The antioxidant activity of FDY003 was measured in comparison with gallic acid as a positive control. Although FDY003 showed a lower antioxidative activity than gallic acid, its activity was concentration dependent in the range of 1–1000 μg/mL [Figure 2a].

Effect of FDY003 on cell proliferation was evaluated using Colo205 cells via MTT assay. FDY003 at concentrations ranging from 0.1 μg/mL to 500 μg/mL significantly inhibited the proliferation of Colo205 cells after 24 h of exposure [Figure 2b].

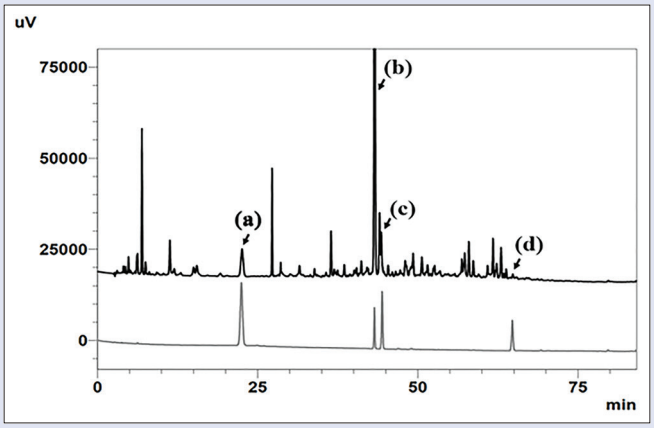

Figure 1: Component analysis of FDY003 by high-performance liquid chromatography**.** Gray line represents the standard chromatogram while black line represents the FDY003 chromatogram. (a) Cordycepin, (b) caffeic acid, (c) 6, 7-dimethylesculetin, (d) chlorogenic acid

FDY003 shows antioxidative effect in Colo205 cells

The antioxidative effect of FDY003 was determined by analyzing DPPH radical scavenging activity percentage (RSA %) and lipid peroxidation in Colo205 cells and cell supernatant. DMSO served as a negative control. The FDY003 treatment groups showed higher RSA % in cells and cell supernatants compared to DMSO control [Figure 3a and b]. TBARS values in Colo205 cells were significantly decreased in the FDY003-treated groups compared to the control group [Figure 3c and d].

FDY003 induces apoptosis signaling in Colo205 cells

To identify the impact of FDY003 on the induction of apoptosis in Colo205 cells, the expression of apoptotic signaling molecules such as Bax, caspase-3, cleaved forms of caspase-3 (C-caspase-3), p21, and phosphorylation forms of P53 (p-P53) was quantified. The expression of Bax in Colo205 cell was significantly increased (up to 1.2-fold) in the FDY003-treated groups as compared to the control group [Figure 4a]. The expression of C-caspase-3/caspase-3 and p-P53/P53 was also significantly increased (up to 1.6-fold) after treatment with FDY003 at concentrations ranging from 1 μg/mL to 5 μg/mL [Figure 4b and c]. The expression of P21 was also significantly increased in the group treated with FDY003 at 5 μg/mL [Figure 4d].

FDY003 reduces tumor size in Colo205 xenograft mouse model

To assess the antitumor effect of FDY003 on colorectal tumor, athymic nude mice were implanted with Colo205 cells to develop a xenograft mouse model. FDY003 was used to treat mice twice a week for 4 weeks (0.2 mg/kg, i. v.). The average tumor size in mice treated with FDY003 was significantly decreased compared to that of control mice at 18–25 days after FDY003 treatment [Figure 5b and c]. No significant difference was observed in weight between the FDY003-treated and control groups during the test period. The irinotecan-treated group showed a decrease in body weight compared to the control group [Figure 5a].

FDY003 exhibits antioxidative effect in Colo205 xenograft mouse model

The antioxidative effect of FDY003 was determined by analyzing DPPH radical scavenging activity and measuring lipid peroxidation in the serum

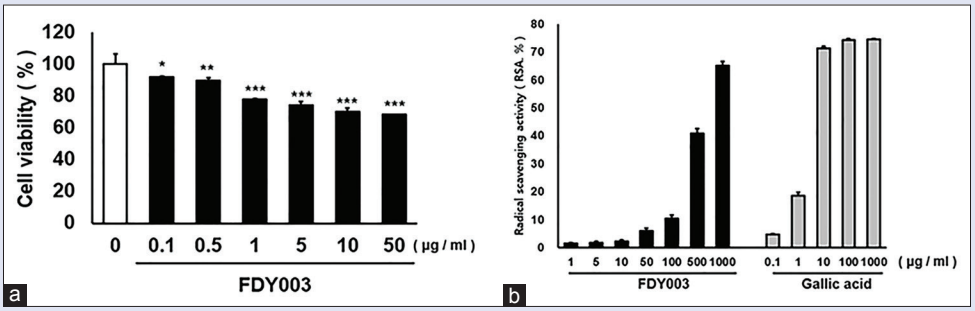

Figure 2: Antioxidant activity of FDY003 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay of the effect of FDY003 on Colo205 cell growth. (a) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of FDY003. FDY003 shows a concentration-dependent antioxidant activity. (b) Effect of FDY003 on Colo205 cell growth by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. Cells were incubated with various concentrations (0.1, 0.5, 1, 5, 10, and 50 μg/mL) of FDY003 for 24 h. Data are presented as mean ± standard deviation (n = 3). ***P < 0.001; **P < 0.01; *P < 0.05 compared to the control

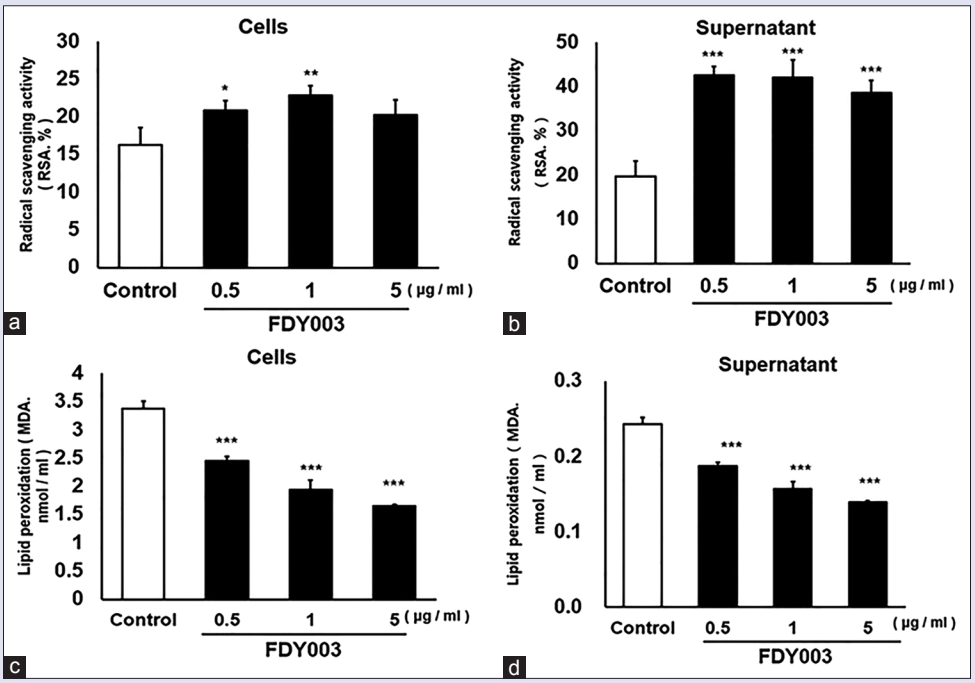

Figure 3: Antioxidant potential of FDY003-treated Colo205 cells and cell supernatant. Cells were incubated with various concentrations (0.5, 1, and 5 μg/mL) of FDY003 for 24 h. (a and b) 2,2-Diphenyl-1-picrylhydrazyl assay. (c and d) Thiobarbituric acid reactive substances assay. (a) 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity of FDY003-treated Colo205 cells; (b) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of FDY003-treated Colo205 cell supernatant; (c) lipid peroxidation in Colo205 cells; (d) lipid peroxidation in cell supernatant. Data are presented as mean ± standard deviation (n = 3). ***P < 0.001; **P < 0.01; *P < 0.05 compared to the control

of a mouse. The DPPH RSA % was significantly higher (up to 18-fold) in the FDY003-treated group than in the control group [Figure 6a]. Serum TBARS values of the FDY003-treated group were significantly (P < 0.001) lower than those in the control group [Figure 6b].

Apoptosis marker is increased in tumor tissues of FDY003-treated Colo205 xenograft mouse model

To evaluate the effect of FDY003 on tumor apoptosis, immunoblotting was performed using the mouse tumor tissue. The levels of Bax and cleaved caspase-3/caspase-3 were examined [Figure 7]. As a result, the expression of Bax was significantly higher (up to 1.6-fold) in the FDY003-treated group than in the control group [Figure 7a]. The ratio of cleaved caspase-3 to caspase-3 expression was significantly increased (up to 1.4-fold) in the FDY003-treated group compared to the control group [Figure 7b].

DISCUSSION

The interest in developing cancer therapies and preventive agents derived from natural compounds has been increasing considerably. Extensive research studies have been performed over the past few decades focusing on traditional medicinal herbs and derivatives.[24] Moreover, complementary medicines using herbal extracts along with acupuncture administration are being presented as a new therapeutic strategy called pharmacopuncture.[6,7] Common acupuncture treatment consists of physical stimulation of associated meridians and acupoints while pharmacopuncture adds chemical ingredients derived from medicinal

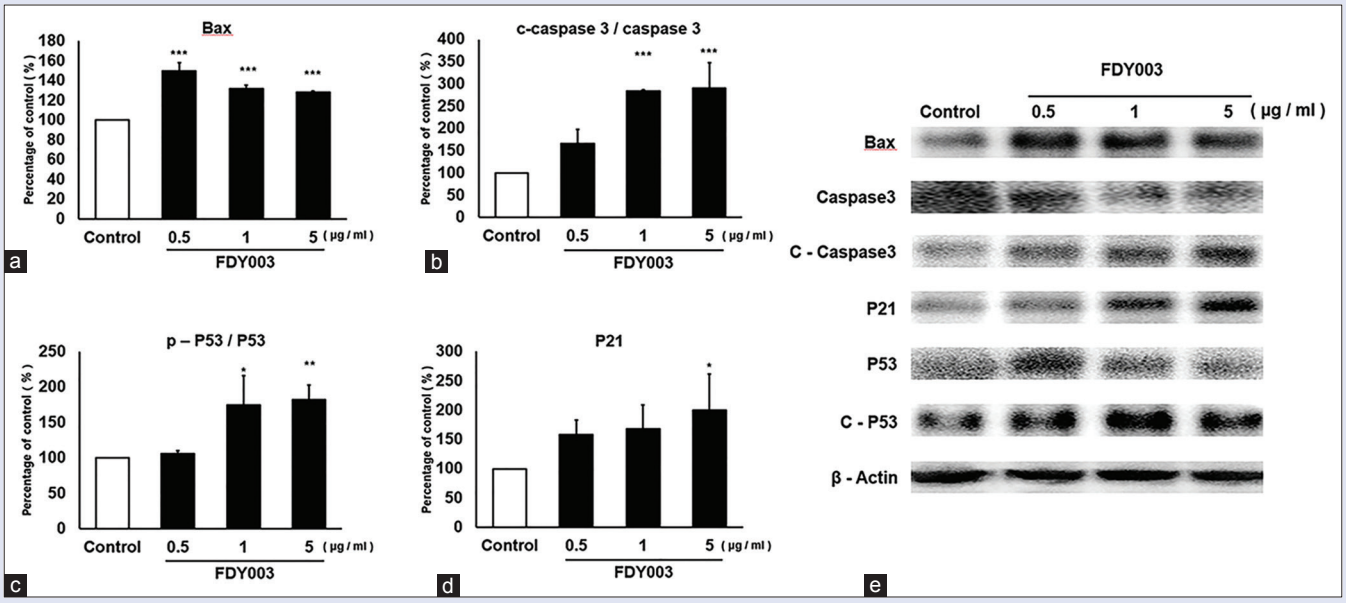

Figure 4: Effect of FDY003 on the expression of tumor apoptosis-related molecules in Colo205 cells. Colo205 cells were treated with various concentrations (0.5, 1, and 5 μg/mL) of FDY003 for 24 h and protein expression was evaluated by Western blot. Each bar chart represents the results of Western blotting analysis involving the expression of Bax (a), caspase-3 (b), p53 (c), and p21 (d). Data are presented as mean ± standard deviation (n = 3). ***P < 0.001; **P < 0.01; *P < 0.05 compared to the control. (e) Immunoblotting image

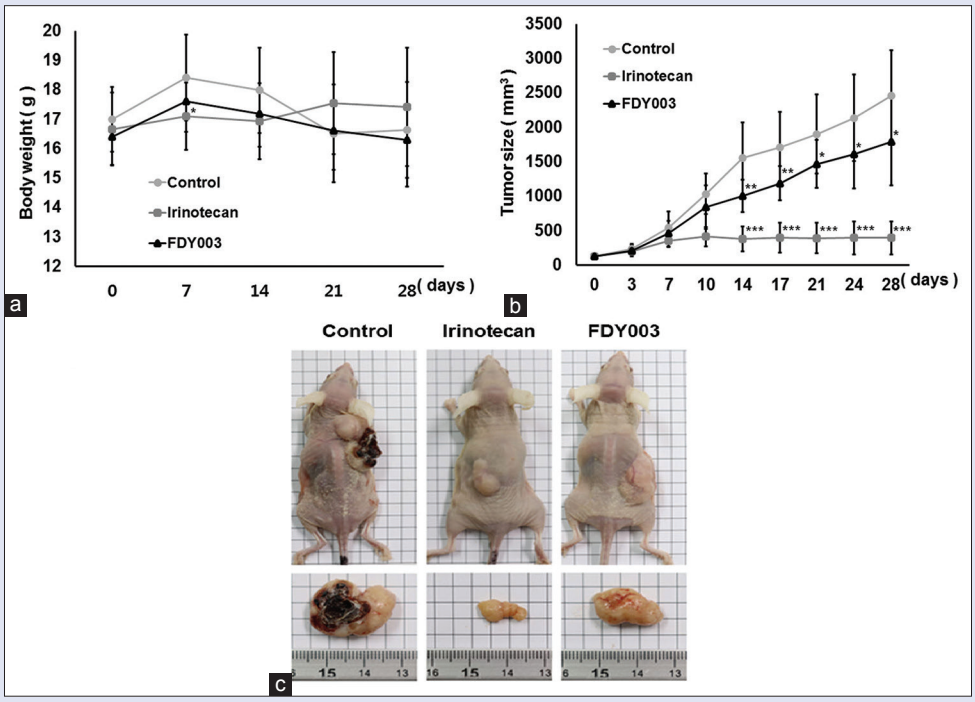

Figure 5: Effect of FDY003 on tumor growth in Colo205 xenograft mouse model. (a) Corresponding body weight changes after FDY003 treatment. (b) Time course of tumor growth in mice. (c) Autopsied tumors at 28 days after FDY003 treatment. Data are presented as means ± standard deviation (n = 10). ***P < 0.001; **P < 0.01; *P < 0.05 compared to the control

herbs with pharmacological effects.[25] The effect of pharmacopuncture is better than the routine acupuncture treatment because herbal extracts administered via acupuncture are immediately and directly absorbed without passing through the gastrointestinal tract. Acupuncture and herbal extracts can yield synergistic effects. Accordingly, patients with swallowing difficulty and those reluctant to take herbal medicine might benefit from pharmacopuncture.[25] Despite these advantages of pharmacopuncture over acupuncture, few studies have been published due to the difficulty involved in performing biological experiments in pharmacopuncture. Thus, the present study investigated whether FDY003 exhibited anticancer effects.

We also evaluated the role of oxidative markers in FDY003-treated cells. Although many factors trigger cancer, oxidative stress is

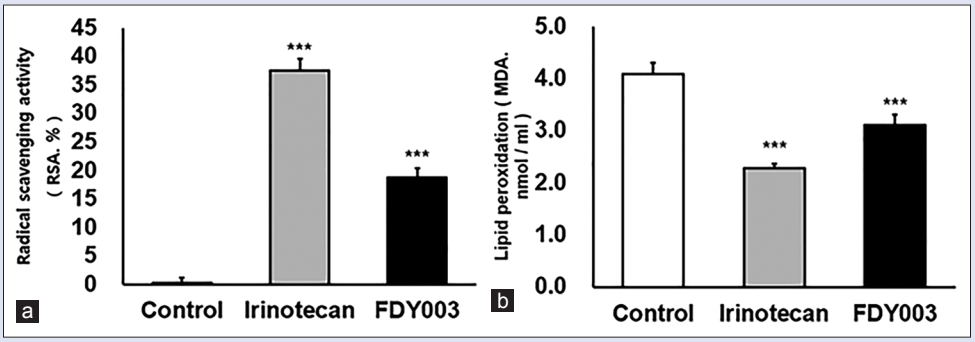

Figure 6: Antioxidant ability of FYD003 in mouse serum. (a) 2,2-Diphenyl-1-picrylhydrazyl and (b) thiobarbituric acid reactive substances in mouse sera compared to the control. Data are presented as means ± standard deviation (n = 10). ***P < 0.001 compared to the control

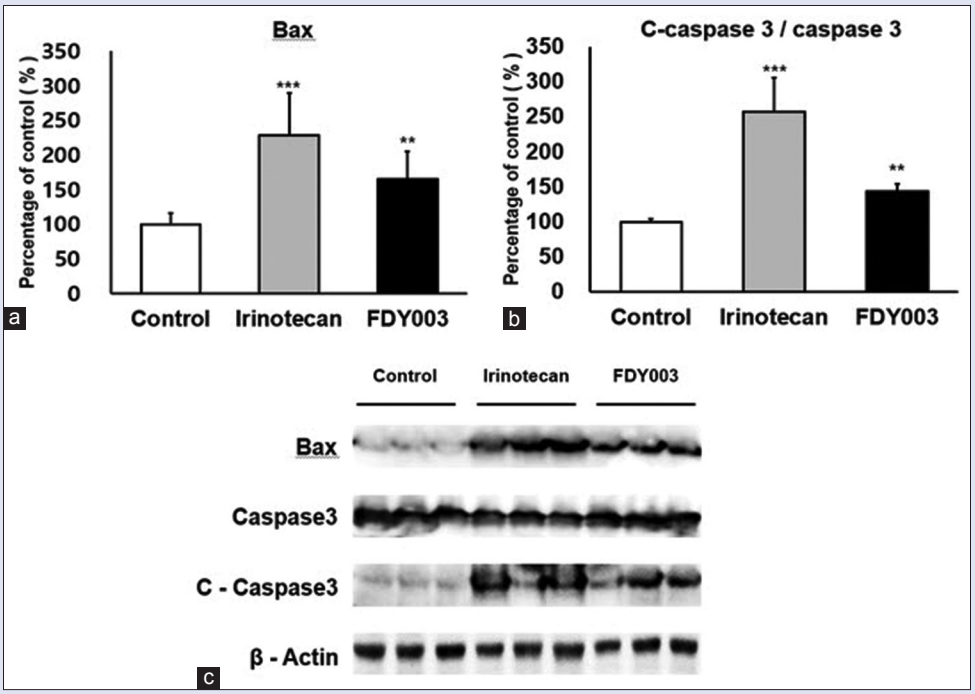

Figure 7: Immunoblotting analysis of apoptosis-related molecules in mouse tumor tissues following FDY003 treatment. The bar charts show results of Western blot analysis for the expression of Bax (a) and caspase-3 (b). Data are presented as means ± standard deviation (n = 10). ***P < 0.001; **P < 0.01 compared to the control. (c) Immunoblotting image

a major one.[26] FDY003 treatment increased the RSA % while reducing the accumulation of lipid peroxidation products caused by oxidative stress [Figure 3]. To determine the impact of FDY003 on the induction of apoptosis in Colo205 cells, the expression of apoptotic signaling molecules such as Bax, caspase-3, cleaved forms of caspase-3 (C-caspase-3), p21, and phosphorylated forms of P53 (p-P53) was quantified using immunoblotting.[27] The expression of all apoptotic signaling molecules was increased by FDY003 treatment [Figure 4]. Based on these *in vitro* results, we evaluated the therapeutic efficacy of FDY003 in a Colo205 cell xenograft mouse model using the same experimental parameters. The average tumor size in mice treated with FDY003 was significantly decreased compared to that in controls on days 18–25 after injection. Interestingly, the anticancer agent irinotecan reduced the body weights of treated mice whereas the FDY003-treated group did not show any change in body weight compared to the control group [Figure 5]. The levels of RSA % and lipid peroxidation products in sera of mice were similar to *in vitro* results. The FDY003-treated group showed a significantly higher RSA % compared to the control group whereas the lipid peroxidation products were lower in the FDY003-treated group than in the control group [Figure 6]. Finally, to evaluate the effect of FDY003 on tumor apoptosis, immunoblotting was performed using the mouse tumor tissue to determine the levels of Bax and cleaved caspase-3/caspase-3 [Figure 7]. As expected, the expression of Bax was significantly higher in the FDY003-treated group than in the control group. The ratio of cleaved caspase-3 to caspase-3 expression was also significantly increased in the FDY003-treated group as compared to the control group. Although the present experimental findings can be partially extrapolated to the clinical setting, the FDY003 showed a beneficial effect due to its anticancer activity. The progression of colon cancer is affected by oxidative stress.[28] We confirmed the antioxidative effects of FDY003 based on the reduction of oxidative stress in Colo205 cells and the Colo205 cell xenograft mouse model. Moreover, FDY003 induced apoptotic signaling molecules to inhibit cancer development.

Taken together, our results suggest that FDY003, a blended herbal medicine, exerts anticancer activity due to its antioxidative effect.

CONCLUSION

FDY003 is a blended herbal medicine with anticancer activity mediated via decreased lipid peroxidation and induction of apoptosis in Colo205 cells. In addition, it decreases the tumor size without affecting the body weight, in contrast to the anticancer agent irinotecan. Further studies are needed to determine whether FDY003 can be used therapeutically against colon cancer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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