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Analysis of Anticancer Activity and Chemical Sensitization Effects of *Dendropanax morbifera* and *Commersonia bartramia* Extracts

SEONGMIN KIM[1*], SUNG GYU PARK[2,3*], YOON JAE SONG[2], JEONG KOO PARK[3], CHEOL HEE CHOI[4], SANGHOON LEE[1] and ROBERT M. HOFFMAN[5,6]

[1]*Department of Obstetrics and Gynecology, Korea University College of Medicine, Seoul, Republic of Korea;*
[2]*Department of Life Science, Gachon University, Seongnam, Republic of Korea;*
[3]*Metabio Inc., Seoul, Republic of Korea;*
[4]*Department of Pharmacology, Chosun University Medical School, Gwangju, Republic of Korea;*
[5]*AntiCancer Inc., San Diego, CA, U.S.A.;*
[6]*Department of Surgery, University of California, San Diego, CA, U.S.A.*

Abstract. *Background/Aim: Dendropanax morbifera (DM) and Commersonia bartramia (CB) are possible candidates for immunotherapy. In this study, the cytotoxicity and chemical sensitization of DM and CB extracts on gynecologic and colon cancers were evaluated. Materials and Methods: The malignant cell lines were cultured and analyzed for cytotoxicity and chemical sensitization. A mouse model was also constructed to make the condition similar to in vivo. Reverse transcription-polymerase chain reaction was conducted to determine alterations in drug-resistant genes. Results: The extracts from DM and CB showed specific cytotoxicity to malignant cell lines. DM increased chemical sensitivity to cervical and ovarian cancer, while CB showed improved sensitization to endometrial cancer. The effects of the extracts were confirmed using a mouse model. The extracts induced differences in the expression levels of a number of genes related to drug resistance. Conclusion: DM and CB extracts could be novel agents for immunotherapy and chemical sensitization in gynecologic and colon cancers.*

Gynecologic and colorectal cancers represent an important global health concern (1). Colon cancer is the third most common malignancy in both men and women in the United States (2), and is associated with both genetic and environmental factors. Genetically, it is correlated with familial adenomatous polyposis and hereditary non-polyposis colorectal cancers (3, 4). It has also been linked to environmental factors such as obesity, alcohol, and smoking, among others (5). Gynecologic cancers are one of the main causes of death in women (6). In Europe, endometrial cancer is the most common, followed by ovarian and cervical cancers. In the field of endometrial carcinoma, there is no standard screening test but a diagnosis is often made at the early stages because frequent vaginal bleeding is a common symptom. In contrast, ovarian cancer is often only diagnosed at an advanced stage due to lack of screening and asymptomatic disease progression (7). For cervical cancer, the Papanicolaou test has dramatically improved the rates of early diagnosis, and extensive human papillomavirus vaccination programs have markedly decreased the incidence of cervical cancer (8). Despite the different molecular profiles associated with each malignancy, these cancers have poor prognosis with traditional chemotherapy treatments in the more advanced stages of disease (9-11).

Immunotherapy is defined as the treatment of a disease by inducing, enhancing, or suppressing an immune response (12). It is the newest frontier of anticancer therapy and is based on initiating an endogenous immune response against cancer (6). In colon cancer, the combination of immunotherapy and chemotherapy has resulted in increased patient survival and an enhancement of immunologic responses (13). Many clinical trials for immunotherapy are showing promising results with positive antitumor immune responses. Likewise, vaccines have proven to be effective in preventing ovarian cancer (7). A number of preclinical and

*These Authors contributed equally to this study.

Correspondence to: Sanghoon Lee, Department of Obstetrics and Gynecology, Korea University Anam Hospital, 73 Inchon-ro, Seongbuk-gu, Seoul 02841, Republic of Korea. Tel: +82 29206773, Fax: +82 29215357, e-mail: mdleesh@gmail.com

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phase I trials are being assessed for the application of immunotherapy in endometrial cancer (14), while several immunotherapies targeting human papilloma virus are under investigation for cervical cancer (15).

Dendropanax morbifera (DM) belongs to the *Araliaceae* family and has been used as a traditional medicinal plant in the Republic of Korea (16). In a previous study, it was found to reduce the lipid profiles in mice serum in a dose-dependent manner (17), and also showed antioxidant and anticancer activities (18). *Commersonia bartramia* (CB) belongs to the *Malvaceae* family and is usually found in Australia and Southeast Asia (19). In a previous study, it was found to have a cytotoxic effect on leukemia cell lines (20).

In this study, we evaluated the cytotoxic effect and chemical sensitization properties of DM and CB extracts was evaluated on gynecologic and colon cancers.

Materials and Methods

Extraction from plants. A total of 1 kg of dried DM leaves was divided into two five-liter Erlenmeyer flasks, followed by the addition of 3.5 l of methanol in each flask. After left at room temperature for 24 h, they were filtered using paper filters to obtain their concentrates. This was followed by the addition of 1 l of normal saline and 1 l of hexane. After another 24 h, the hexane layer was removed and another layer of 1 l of hexane was added for repeat extraction. This process was repeated twice. The CM extract was produced using a similar method. After dividing 1 kg of dried CM leaves into two flasks, the same amount of methanol as in the above extraction was added. The flasks were then stored at 50°C for 3 days, then filtered using paper filters.

Cell culture. Cell lines of endometrial cancer (SUN-1077), ovarian cancer (SK-OV-3, SNU-840, SNU-8, OVCAR3, Caov3), and colon cancer (HT-29, SNU-C5, HCT 116, SW-480, HCT-15) were cultured using RPMI-1640 medium (Roswell Park Memorial Institute medium; Welgene, Kyungbook, Republic of Korea). The HeLa cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium; Welgene). MEM (Medical Research Council cell strain 5) was used for the culture of MRC-5 (Medical Research Council cell strain 5). All cultures were supplemented with 10% fetal bovine serum (Welgene) and 1% antibiotics (100×) including penicillin and streptomycin, at 37˚C in humidified air and 5% CO_2.

MTT assay. A. Chemical sensitivity profile: The chemical sensitization and cytotoxicity were analyzed using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay. The detailed methodology of this assay has been described previously (21, 22). Briefly, 5×10^4 cells of each HeLa, SUN-1077, SK-OV-3, and HT-29 cell line were seeded in 96-well plates in 100 μl culture medium. The cells were incubated at 37˚C, 5% CO_2 overnight for attachment. As a preliminary study, 20 μl of several drugs used for chemotherapy in endometrial cancer, ovarian cancer, cervical cancer, and colon cancer were added, and the mixture was cultured for 72 h. Thereafter, the cells were treated with MTT. After that, all of the medium was aspirated from the wells. The remaining formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the inhibition rate (IR) was measured at 540/630 nm using a 96-well microplate reader. As a result of this preliminary study, 2 drugs with good sensitivity and 2 drugs with low sensitivity were chosen for the experiment. For the next stage of our study, 20 μl of each of the 4 drugs was added to 5×10^4 cells in 96-well plates after resolution in 1X phosphate buffered saline (PBS) and analyzed using an MTT assay. All experiments were performed more than twice before carrying out a statistical analysis.

B. Cytotoxicity: Vero and MRC-5 cell-lines with same cell count as in chemical sensitivity analysis were seeded in 96 well plates in 100 μL culture medium. The cells were incubated at 37˚C, 5% CO_2 for 24 h. The extracts from DM and CB were resolved with 1X PBS and added. After 72 h of culture, the supernatant was removed, and the cells were treated with MTT for cytotoxic analysis. All experiments were performed more than twice before statistical analysis.

C. Chemical sensitization effect of extracts: Here, 5×10^4 cells of each HeLa, SUN-1077, SK-OV-3, and HT-29 cell line were seeded in 96-well plates in 100 μl of culture medium and incubated. After removal of the medium, the DM and CB extracts were dissolved in 1X PBS at various concentrations (1500 μg/ml, 1000 /ml, 500 μg/ml, 200 μg/ml 150 μg/ml, 100 μg/ml, 50 μg/ml). After 72 h, the supernatant was removed and the cells were treated with MTT for analysis. All experiments were performed more than twice.

D. Animal model: Several ovarian cancer (SK-OV-3, SNU-840, SNU-9, OVCAR3, Caov3) and colon cancer (SNU-C5, HT-29, HCT 116, SW-480, HCT-15) cell lines were cultured in RPMI-1640 medium. Each cell line was cultured in medium including 10% FBS, then separated from the culture dish using trypsin-EDTA and diluted with PBS at 6×10^6 cells in 0.2 ml. These cells were injected subcutaneously to 5-week-old female nude mice adapted for one week at 23±3˚C with a humidity of 55±15%. They were raised in the same sterile conditions. The tumors were removed when the size reached 250 mm^3.

3-Dimensional histoculture drug response assay (3D-HDRA). The cancerous portions of the specimens were minced into pieces of approximately 1 mm in diameter. Cancer tissues were further cut into 10-mg pieces, weighed on a chemical balance, and placed onto collagen gels, immersed in 1 ml Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma). After incubation for 72 h at 37˚C with 5% CO_2, MTT was added to each well. Plates were incubated for another 2 h, the media were removed, and 0.5 ml dimethyl sulfoxide was added to each well to extract MTT formazan. The absorbance was measured using an ELISA reader. The inhibition rate of tumor growth (IR) was calculated using the following equation: IR (%)=(1 − mean absorbance of treated wells per gram of tumor/mean absorbance of control wells per gram of tumor) ×100. In this study, the IR cut-off value for a positive response was previously determined to be ≥30% (23, 24).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). RNA was separated from 4 types of cancer cells using Trizol Reagent (RNAiso Plus, Thermo Fisher, MA, USA). 1 μg of RNA was used for cDNA synthesis using a superscript first-strand system (Invitrogen, CA, USA). cDNA was diluted for use in RT-PCR. The reaction solution contained 4 μl of 10× PCR buffer, 0.2 mmol/l of each dNTP, 10 pmol of each primer, 2.5U of Taq DNA polymerase (Takara; Japan) and 4 μl of cDNA. The real-time quantitative analysis was performed using a Light cycler 2.0 instrument (Roche, Mannheim, Germany).

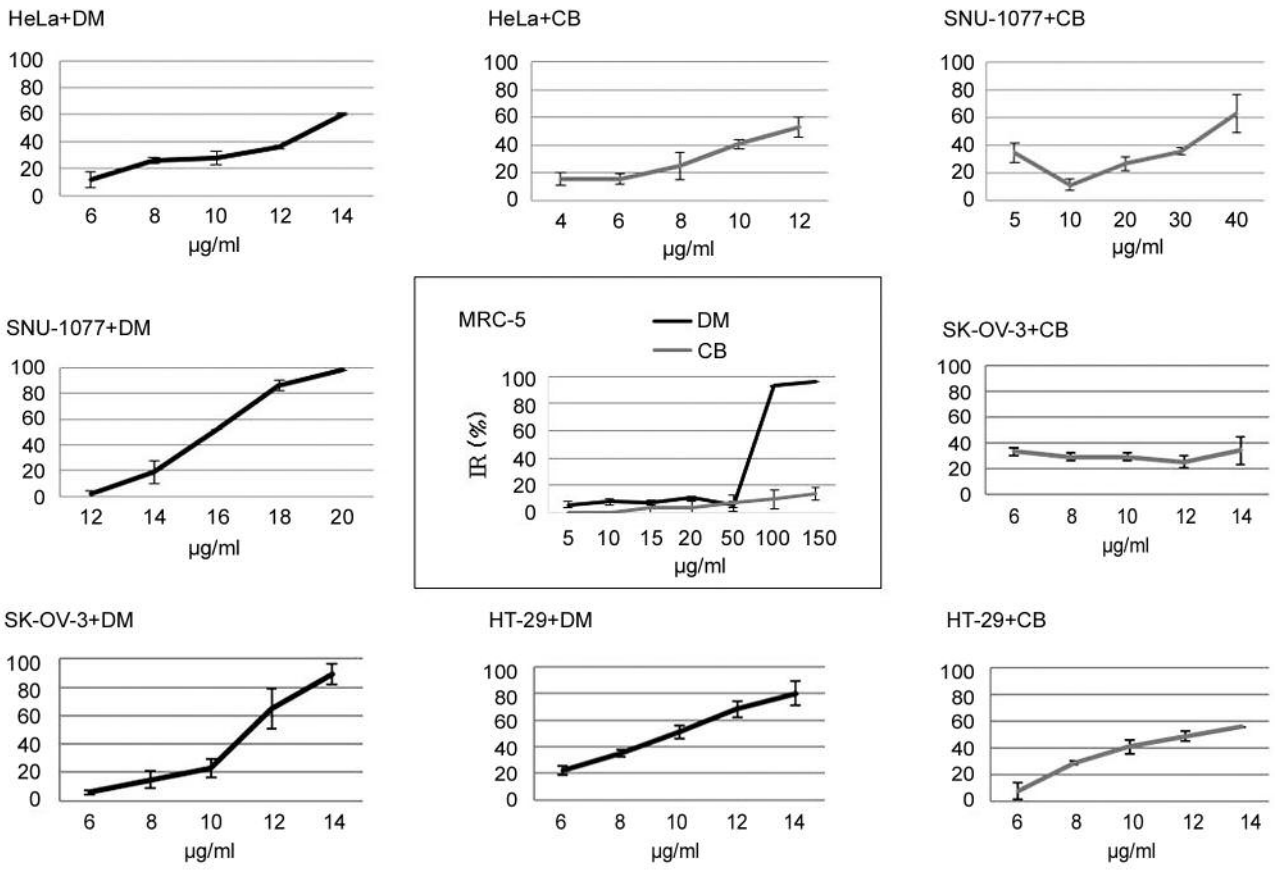

Figure 1. *Cytotoxic effects of extracts of Dendropanax morbifera and Commersonia bartramia. The extracts showed a more cytotoxic effect to cancer cell lines than MRC-5.*

Statistical analysis. Statistical analysis was performed using a Kruskal-Wallis rank test, and a Mann-Whitney *U*-test was used to calculate the *p*-value and to compare the immuno-histochemistry differences of the groups. Statistical SPSS software package version 20.0 (SPSS Inc, Chicago, IL, USA) was used to analyze the data. Differences were considered statistically significant at $p<0.05$.

Results

Chemical sensitivity profiles. For the chemical sensitivity profiles of the chemical agents, docetaxel and paclitaxel showed an IR over 80% for SK-OV-3 and HeLa cell-lines. Carboplatin and cisplatin showed an IR of less than 25%. On the other hand, docetaxel and paclitaxel had an IR over 30%; carboplatin and cisplatin had an IR of less than 20% for SNU-1077. Otherwise, fluorouracil, oxaliplatin, and irinotecan showed an IR of 56%, 55%, and 28% IR for HT-29, respectively.

Cytotoxicity of extracts of Dendropanax morbifera and Commersonia bartramia. The cytotoxic activity of the DM and CB extracts was studied against cultured MRC-5 (normal), SK-OV-3, HeLa, SNU-1077, and HT-29 cell lines using MTT assays. The MRC-5 cell line was subjected to increasing doses of extracts ranging from 5 to 150 µg/ml (Figure 1). DM showed cytotoxicity of more than 93% at doses >100 µg/ml, and 6~11% at doses lower than 50 µg/ml. CB extracts showed 10% cytotoxicity at doses >100 µg/ml, and 4~6% at doses ranging from 15~50 µg/ml. The lower doses of DM and CB extracts caused toxicity to cancer cells compared to the doses of MRC-5, indicating a degree of specificity for malignant cell lines.

Chemical sensitization effect on cancer cell lines. To explore the role of DM/CB in the regulation of chemosensitivity of human cancer cells, the drug sensitivity of cancer cells was compared with that of control cells, using an MTT assay. Each of the cytotoxic agents at various concentrations (reference dose from previous chemosensitivity test) were mixed in a 1:1 ratio with 5 or 10 µg/ml DM/CB extracts before treating the HeLa cell line (cervical cancer). The IR was found to increase more than 1.5 times when the DM extracts were added to carboplatin (Figure 2). Cisplatin, paclitaxel, and docetaxel showed a similar chemical sensitization effect as DM and no

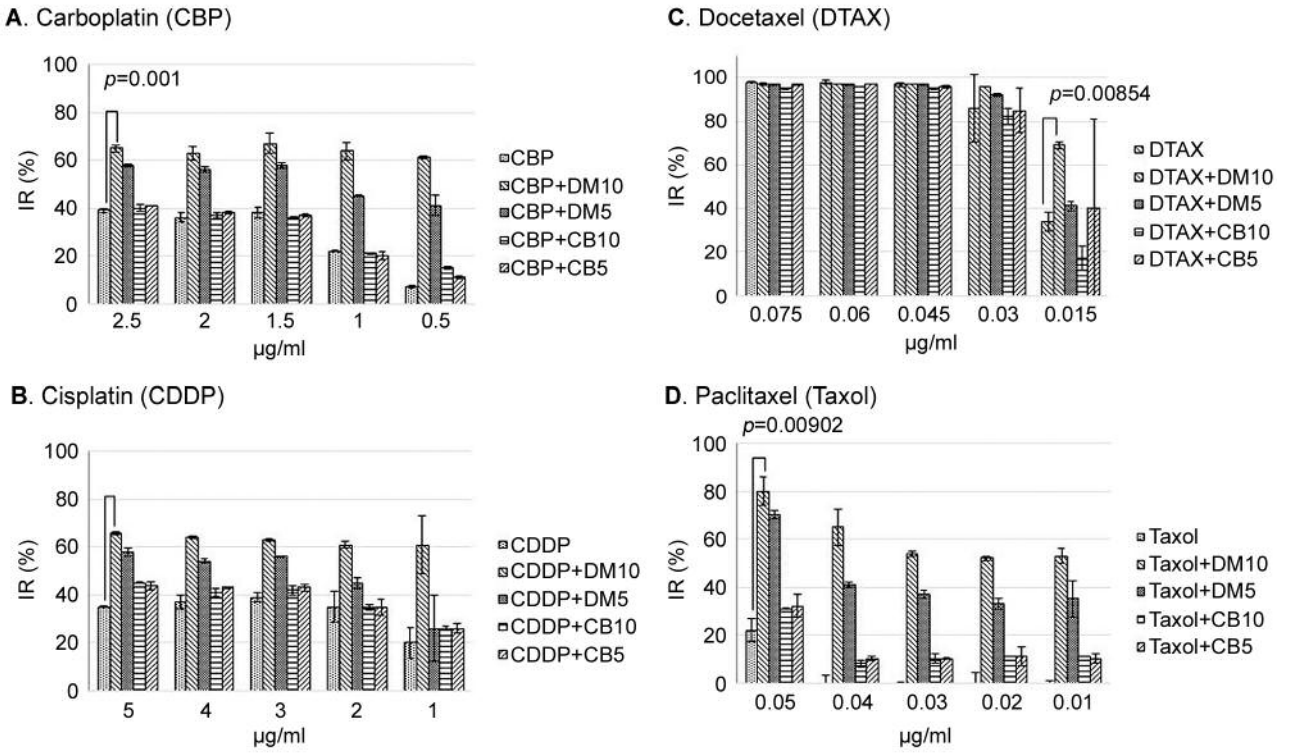

Figure 2. *Chemical sensitization effect of Dendropanax morbifera and Commersonia bartramia on HeLa cell line. IR was increased by Dendropanax morbifera, but not by Commersonia bartramia.*

effect was confirmed with CB. With the SK-OV-3 cell line of ovarian cancer, carboplatin, docetaxel, paclitaxel, and cisplatin also showed increased sensitivity to DM, but not to CB (Figure 3). However, the drugs showed the opposite result when they were added to the SNU-1077 cell line (endometrial cancer). None of the agents were sensitized with DM but a remarkable change in IR was observed when they were mixed with CB (Figure 4). Finally, HT-29 cells (colon cancer) were treated with three different drugs. 5-fluorouracil and oxaliplatin showed moderate sensitization only with CB, but irinotecan showed dramatic changes in its IR when it was mixed with DM and CB (Figure 5).

In vivo evaluation using 3D-HDRA. 3D-HDRA was used to evaluate the cytotoxicity and chemosensitivity of DM/CB in *in vivo* conditions. Several types of ovarian cancer and colon cancer cell lines were cultured and implanted in mice, and the resected tumors were analyzed by 3D-HDRA. CB showed chemical sensitization effects with irinotecan on most types of colon cancer cell lines, and DM increased the sensitivity of ovarian cancer cell lines to paclitaxel (Figure 6).

Analysis of drug-resistant gene expression by DM/CB. The DM/CB extracts were added to various cancer cell lines using a multidrug resistance diagnostic kit (Drugsporter; Chosun University, Republic of Korea) (Table I). In HeLa cells, DM caused a reduction in the expression of 11 genes related to drug resistance and upregulation of the expression of 1 gene, while CB decreased the expression of 8 genes and increased the expression of 2 genes. There were also several alterations of drug resistant genes in other cell lines (SK-OV-3, SNU-1077, HT-29).

Discussion

Most chemotherapeutic agents act by inducing apoptosis in cancer cells, which affects their sensitivity to anticancer drugs (25). In a previous study, DM extracts showed a cytotoxicity of up to 26% on normal hepatocytes, and over 50% on various cancer cell lines (26). These extracts are considered to have selective toxic effects on malignant cells. In another study, DM inhibited cell proliferation of the U937 cell line (leukemia) and induced apoptosis *via* intrinsic and extrinsic pathways (27).

In this study, DM and CB showed a greater cytotoxic effect on four types of cancer cell lines compared to normal cell lines. The specific molecular sensitization effects of the DM and CB extracts on diverse chemotherapeutic agents was

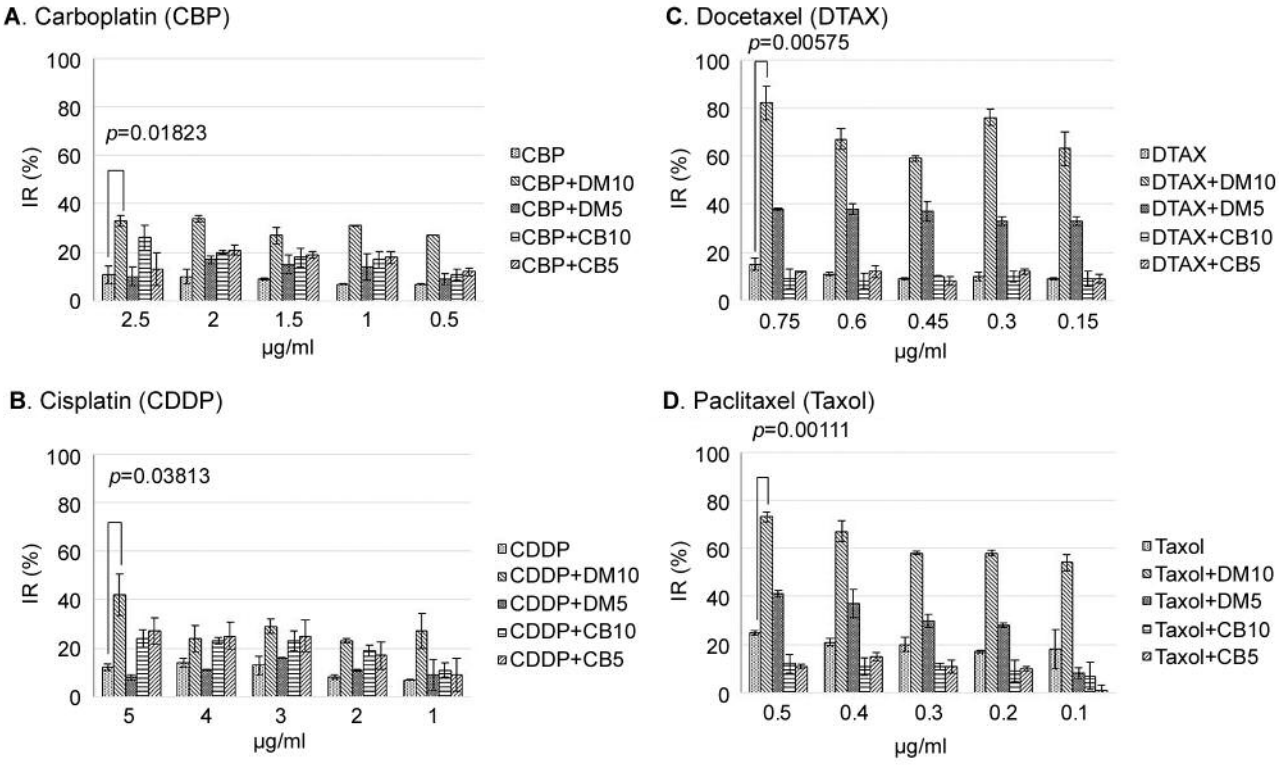

Figure 3. *Chemical sensitization effect of Dendropanax morbifera and Commersonia bartramia on SK-OV-3 cell line. Dendropanax raised IR, but Commersonia did not.*

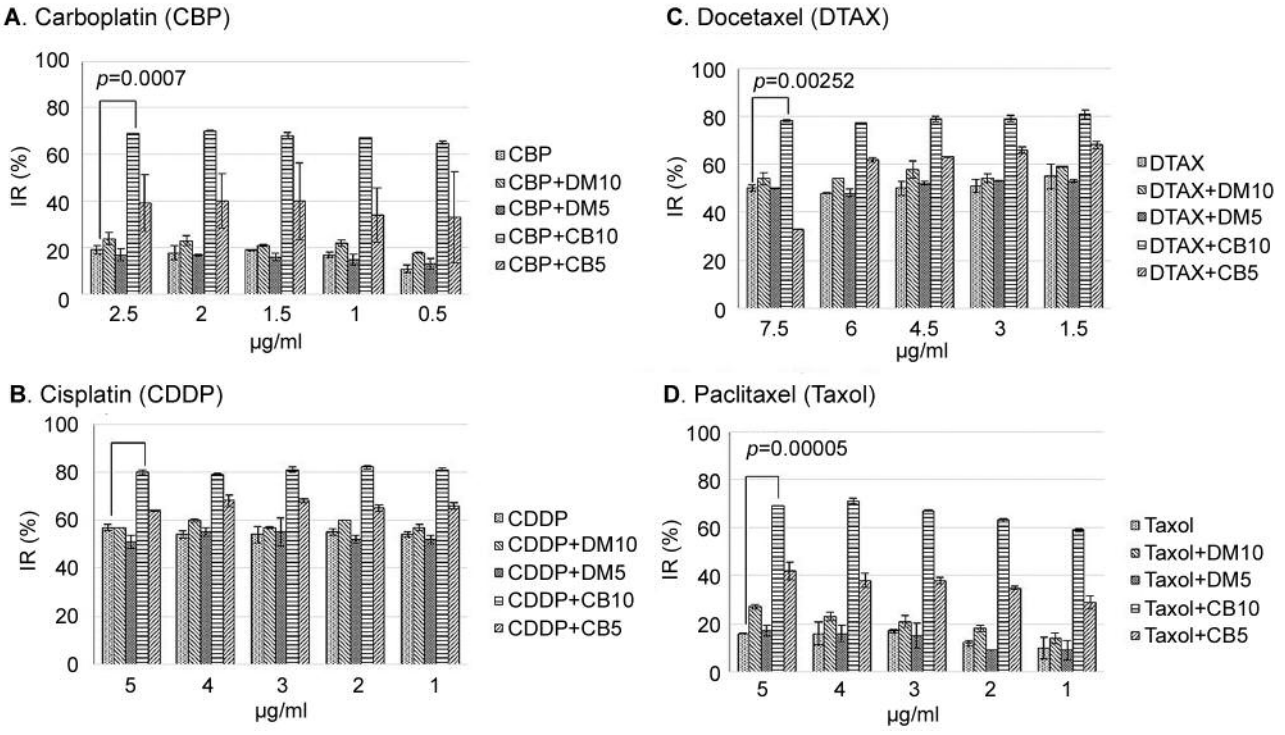

Figure 4. *Chemical sensitization effect of Dendropanax morbifera and Commersonia bartramia on SNU-1077 cell line. Inhibition rate was remarkably increased only by Commersonia bartramia.*

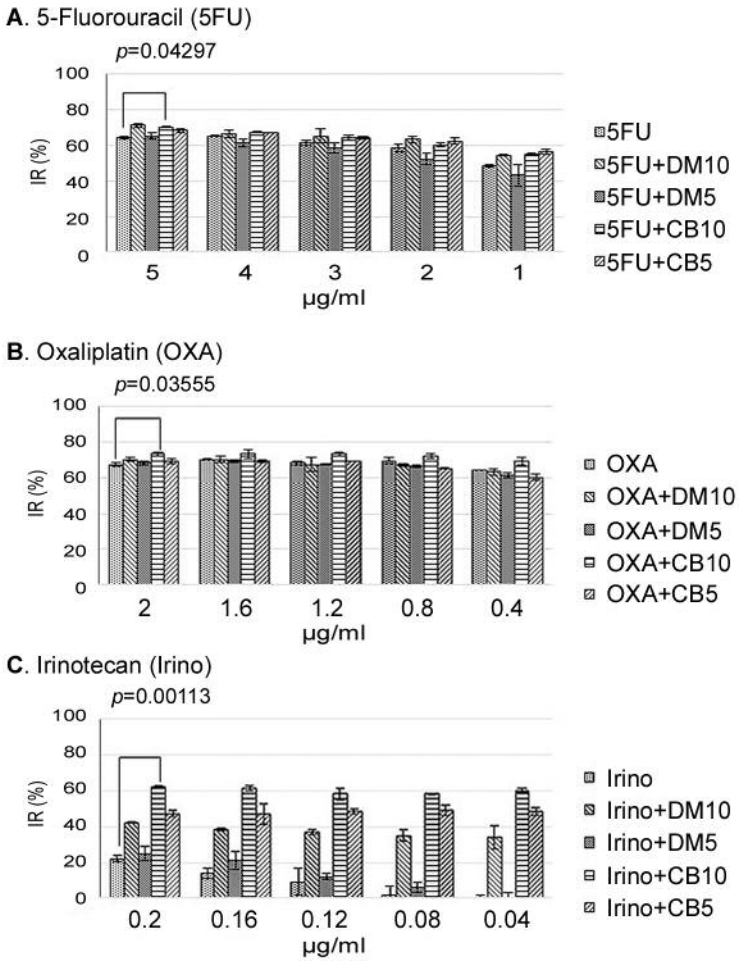

Figure 5. *Chemical sensitization effect of Dendropanax morbifera and Commersonia bartramia on HT-29 cell line. Commersonia showed significant changes in its inhibition rate.*

confirmed. DM showed a dose-dependent sensitization to all agents tested on HeLa, while CB had no effect. SNU-1077 was susceptible to CB but not to DM. In the SK-OV-3 cell line, DM showed a high inhibitory function when it was added to docetaxel and paclitaxel. However, the sensitization decreased with lower doses (5 μg/ml) of DM, suggesting that the chemical sensitization is dependent on the concentration of anticancer drugs. The IR was diminished when CB was mixed with paclitaxel, suggesting that CB suppresses the reaction of paclitaxel in SK-OV-3. In colon cancer cell lines, fluorouracil and oxaliplatin were not affected by DM and CB, while irinotecan showed increased sensitivity to both DM and CB.

Paclitaxel is used for the treatment of carcinomas in the ovaries, breasts, lungs, head, and neck, and induces polymerization of microtubules irreversibly, which is also a target of vinca alkaloids (28). The action of irinotecan occurs *via* the topoisomerase I inhibitor, which is then inactivated by glucuronidation by uridine diphosphate glucuronosyltransferase 1A1, and leads to the inhibition of DNA replication and transcription (29). The mechanism of action of DM and CB is not well understood, but the resulting apoptosis is considered to be the main stream of action as with most agents.

Since the characteristics and genetic manifestations of cancer cells are affected by the method and condition of the cell culture, the interactions between cancer cells and normal cells are important. A 3D culture is more similar to the *in vivo* environment than a 2D culture, and therefore can yield results that are closer to the drug responses seen in clinical trials (30). 3D-HDRA is a type of 3D culture that is being used to determine the efficacy of chemotherapeutic agents in individuals by identifying their chemical sensitization profiles (31). Here, a mouse model was constructed using ovarian and colon cancer cell lines to obtain cancer tissues

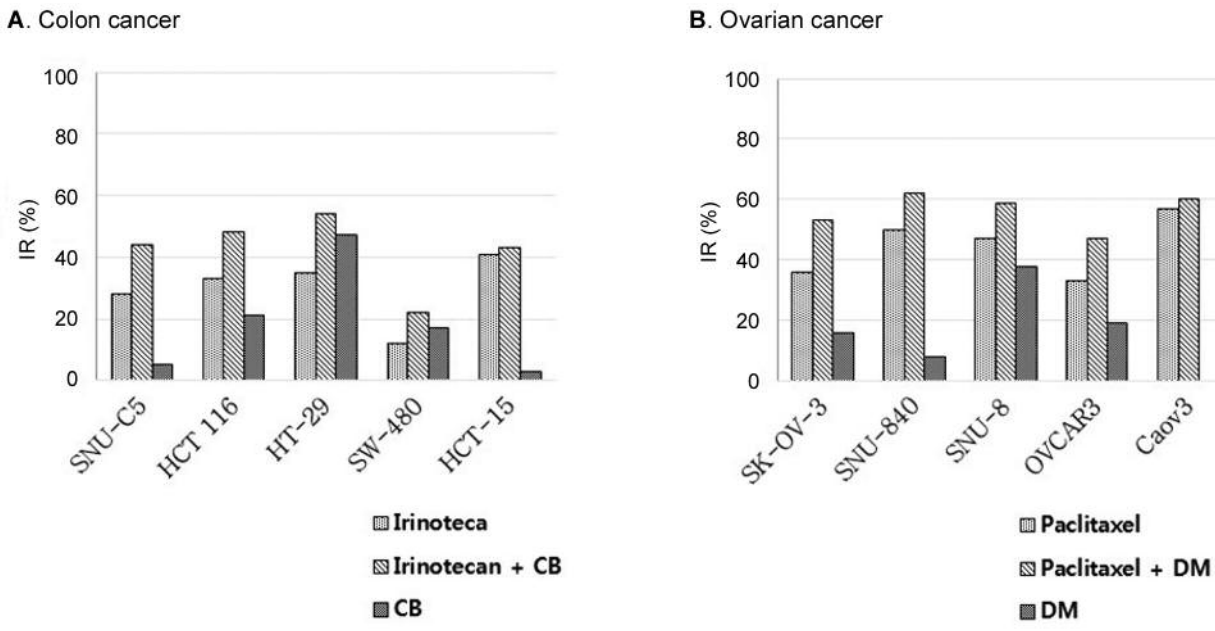

Figure 6. *Chemosensitization evaluation of extracts using 3D-HDRA.*

Table I. *Analysis of drug-resistant gene expression by DM/CB.*

		Endometrial cancer			Ovarian cancer			Cervical cancer			Colon cancer		
		SNU-1077	SNU-1077 +DM	SNU-1077 +CB	SK-OV-3	SK-OV-3 +DM	SK-OV-3 +CB	HeLa	HeLa +DM	HeLa +CB	HT-29	HT-29 +DM	HT-29 +CB
GAPDH		0.160	0.160	0.160	0.200	0.200	0.200	0.200	0.200	0.200	0.115	0.115	0.115
BCRP	(ABCG2)							0.275	0.045		0.130	0.347	0.123
MRP1	(ABCC1)	3.844	3.405	0.615	1.800	1.862	1.616	3.775	3.240	0.948	0.696	1.190	0.985
LRP	(MVP)	3.313	3.321	3.380	4.525	1.923	5.216	2.800	2.071	1.354	1.478	1.303	1.464
WND	(ATP7B)											0.008	
CFTR	(ABCC7)	0.313	0.553								0.435	0.440	0.479
MRP8	(ABCC11)	0.125					0.257						0.178
ENT1	(SLC29A1)	0.063											
CNT1	(SLC25A1)	0.031	0.027		0.175	0.285	0.147	0.025	0.015	0.037		0.274	0.082
SUR2	(ABCC9)	0.188	0.056	0.088	0.100	0.062	0.074	0.050	0.059	0.037	0.261	0.020	0.027
CTR1	(SLC31A1)	0.469	0.083		0.100	0.285	0.036	0.150	0.029			0.085	0.150
MRP4	(ABCC4)	1.406	0.498	0.044	0.625	0.869	0.184	0.475	0.104	0.013	0.261	0.161	0.780
MDR3	(ABCB4)	0.063											
ABC8	(ABCG1)	0.156			0.025	0.062		1.400	0.622	0.493	0.391	0.702	0.670
MRP6	(ABCC6)	0.188											
MRP3	(ABCC3)	1.094	0.138	0.044	0.350	0.577	0.331	0.375	0.266	0.086	0.348	0.638	0.493
ABCB5	(ABCB5)	0.250				0.015							
MRP7	(ABCC10)	0.719	0.305	0.175	0.225	0.223	0.293	0.050	0.029			0.044	0.123
MRP5	(ABCC5)	4.781	4.180	0.966	1.650	1.985	0.221	0.600	0.429	0.086	0.087	0.157	0.164
MRP2	(ABCC2)	0.406	0.083		0.050	0.146		0.025				0.020	
ABC3	(ABCA3)							0.375					
MDR1	(ABCB1)	0.344	0.166	0.088	1.050	1.154	1.616	0.050	0.192	0.123	0.043	0.065	
ENT2	(SLC29A2)	0.375				0.031							
ABCA2	(ABCA2)	0.563	0.249	0.088	0.325	0.338	0.367	0.300	0.178	0.086	0.130	0.573	0.548
CNT2	(SLC28A2)	0.281	0.111									0.016	0.013

with which to evaluate cytotoxicity and chemical sensitization. All ovarian cancer cells except Caov3 showed an increased IR with DM in combination with paclitaxel, and most colon cancer cells were more sensitive to CB combined with irinotecan. This suggests that sensitivity can vary according to the type of cell line. Although the experiment was repeated 5 times, the number of samples was not enough to deduce the exact effect of the extracts, and therefore would require further analysis using 3D-HDRA.

Membrane transporters maintain cellular homeostasis by importing nutrients and exporting toxins. Transporters also have roles in drug response, serving as drug targets and setting drug levels (32). In this study, the gene expression of specific transporters was analyzed by normalizing with glyceraldehyde 3-phosphate dehydrogenase. When DM was applied to HeLa, the expression of genes resistant to cisplatin and carboplatin (ABCC1, SLC31A1, ABCC3) decreased, which is concordant to the increased IR by DM to these drugs. The genes expressing resistance to paclitaxel and docetaxel (ABCC10 and ABCB1) also decreased, and the increased IR with improved sensitivity could be an explanation. Januchowski *et al*. reported that the ATP-binding cassette family protein is related to the expression of the ABCB1 gene in more than half of drug-resistant cancers (33). This is because DM suppresses the expression of specific transporter genes. In SNU-1077, CB was related with impairment of carboplatin resistance genes (SLC31A1), paclitaxel resistance genes (ABCC1, ABCC10, and ABCB1), and cisplatin resistance genes (SLC31A1, ABCC3). This is reflected by the increased IR in SNU-1077 after the application of the CB extract.

Nakayama *et al*. reported an increased expression of ABCB1, ABCC1, ABCC2, MVP, ABCG2, and ATP7B genes in patients with untreated ovarian cancer, and found a specific relation between ATP7B and cisplatin resistance (34). Treatment of SK-OV-3 cell line with DM extracts resulted in inhibition of MVP gene expression. On the other hand, the genes expressing resistance to docetaxel did not show any change with DM, but this does not correspond to the increases in IR. As such, there could be another mechanism, such as a synergistic effect, between DM and docetaxel. The sensitization of CB with irinotecan in HT-29 cells also showed a discordance with the gene analysis. The ABCC2 gene was not altered by CB. According to a previous study, some errors could occur in evaluating the therapeutic effect in colon cancer due to resistant genes including ABCB1, ABCC1, and ABCG2 (35), so further studies are necessary to confirm the mechanism and regulation of drug-resistant gene expression. The above results suggest that DM and CB extracts could play important roles in reducing the expression of transporter genes that exist on the cellular membrane and therefore to improve sensitivity to specific anticancer drugs.

A limitation of this study is that the results were deducted mainly from in vitro experiments, so the exact efficacy of the extracts should be evaluated using fresh tissues to identify actual functions. Therefore, an animal model was constructed to resemble *in vivo* condition. A significant increase in chemosensitivity was also observed *in vivo*. Additional work using different agents is ongoing in our institution.

Our study is significant for two reasons. Above all, this is the first study evaluating the cytotoxic effect of DM and CB in gynecologic and colorectal cancers. It could lead to the identification of a new target therapy for these malignancies or other cancers. Secondly, the experimental design of the study was well established and conducted deliberately. The cytotoxicity and sensitization of materials were evaluated step by step with various concentrations, and this could determine the dose dependent activity of the materials.

In conclusion, DM and CB extracts could be used as anticancer drugs and chemical sensitization agents for gynecologic and colon cancers. Drug resistance in these cancers is still an important obstacle in the success of chemotherapy to which this study might provide a novel therapeutic strategy. Further studies are needed in order to identify their exact mechanism of action and to determine their appropriate clinical application.

Acknowledgements

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