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Cedrol, a Sesquiterpene Isolated from *Juniperus chinensis*, Inhibits Human Colorectal Tumor Growth associated through Downregulation of Minichromosome Maintenance Proteins

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Cedrol, a sesquiterpene alcohol, isolated from *Juniperus chinensis* has been reported to inhibit minichromosome maintenance (MCM) proteins as cancer biomarkers in human lung cancer in vitro. In the present study, we investigated the anti-cancer activity of cedrol in vitro and in vivo using human colorectal cancer HT29 cells and a human colorectal tumor xenograft model. Cedrol inhibited MCM protein expression and cell growth in HT29 cells, which are associated with G1 arrest and the induction of apoptosis. We demonstrated that cedrol effectively reduced HT29 tumor growth without apparent weight loss in a human tumor xenograft model. Compared with vehicle- and adriamycin-treated tumor tissues, cedrol induced changes in the tumor tissue structure, resulting in a reduced cell density within the tumor parenchyma and reduced vascularization. Moreover, the expression of MCM7, an important subunit of MCM helicase, was significantly suppressed by cedrol in tumor tissue. Collectively, these results suggest that cedrol may act as a potential anti-cancer agent for colorectal cancer by inhibiting MCM protein expression and tumor growth.

Key Words Apoptosis, Cedrol, Colorectal cancer, G1 arrest, Minichromosome maintenance proteins

INTRODUCTION

Colorectal cancer is the third most common type of cancer worldwide, affecting all parts of the colorectal such as cecum, colon, rectum, anus and showing a high mortality rate due to difficulties in early detection and treatment [1,2]. Treatment strategies for colorectal cancer to improve the probability of survival include surgical treatments, such as resection, chemotherapy using cytotoxic drugs, and radiation therapies [3,4]. However, these treatment methods have been associated with various problems, mainly including side effects due to high toxicity, increased drug resistance, and metastasis [5]. Thus, in recent years, there has been an increasing emphasis on developing natural substance-derived materials that yield fewer side effects, along with safe and excellent anti-cancer activity and various studies on the molecular mechanisms of compounds contained in natural products are actively underway [6]. These natural compounds have various physiological activities such as anti-oxidant, anti-inflammatory, and anti-cancer effects and act selectively on cancer cells to inhibit proliferation and metastasis and to induce apoptosis. Apparently, many plant-derived drugs are known to exhibit anti-cancer activity by inhibiting cancer initiation, development, and progression [7]. Among these phytochemicals, substances that are actually used as anti-cancer agents include vinca alkaloids, taxanes, epipodophyllotoxin, and camptothecins

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are used to treat various types of cancers, including breast, ovarian, and lung cancers, and Kaposi's sarcoma [8-11].

Genomic instability is the hallmark of various cancers or cancer-related diseases with the increasing accumulation of DNA mutations or damage [12,13]. Thus, replication-related proteins or factors related to DNA repair can be targets for developing anti-cancer drugs [14]. The minichromosome maintenance (MCM) protein is considered as an important first step of formation of pre-replication complex in eukaryotes, with six subunits (MCM2–MCM7) forming a heterohexameric complex [15,16]. Several MCM proteins are closely related to cancer formation, and its overexpression has been identified in many cancer tissues or cancer cell lines [17-22]. Among them, particularly MCM7 has a crucial role in tumor formation and progression based on its involvement in cellular proliferation by interacting with numerous proteins and a high expression in multiple malignancies [20]. Therefore, the MCM protein is gaining attention as a biomarker for diagnosing cancers, as well as a target for cancer treatment [23]. Our purpose is to explore substances that regulate the expression of MCM proteins for developing novel anti-cancer agents.

Juniperus chinensis, an evergreen conifer belonging to the cypress family Cupressaceae, has been reported to show anti-tumor, anti-fungal and diuretic activities. Cedrol, a sesquiterpene alcohol, is a biologically active compound found in *J. chinensis*, and commonly used as a fine fragrance component of cosmetics. We previously reported that cedrol, a crystalline substance isolated from *J. chinensis*, inhibits the expression of several cell-specific markers available for cancer diagnosis, including MCM protein in the A549 lung cancer cell line [24]. Here we demonstrate that cedrol inhibits the expression of MCM proteins in human colorectal cancer model HT29 in vitro and in vivo. Furthermore, we assessed that cedrol inhibited cancer cell proliferation accompanied by cell cycle arrest and apoptosis induction, and cedrol suppressed oncogenucity of Human colorectal adenocarcinoma HT29 cells in vivo.

MATERIALS AND METHODS

Chemicals

Cedrol used in this study was isolated from *J. chinensis* and dissolved in dimethylsulfoxide (DMSO). Cedrol was stored at –20°C before the experiments and dilutions were performed in culture medium.

Cell line and culture

HT29 cells were purchased from American Type Culture Collection (Manassas, VA, USA). HT29 cells were cultured in RPMI medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin under standard culture conditions at 37°C and 5% CO_2.

Water-soluble tetrazolium salt (WST) assay

Cell viability was determined by the WST assay using the EZ-Cytox Cell Viability Assay Kit (Daeil Lab., Seoul, Korea). HT29 cells were treated with different concentrations of cedrol for 24 hours or 48 hours. The EZ-Cytox assay reagent (10 μL) was added to each cell culture well, and the mixture was incubated for 30 minutes at 37°C. The absorbance of each well was measured by a plate reader (Paradigm; Beckman Coulter, Brea, CA, USA) at the wavelength of 450 nm.

Flow vytometric analysis of cell cycle

The effects of cedrol on the cell cycle progression were examined using the MUSE™ Cell Cycle kit (Luminex, Austin, TX, USA) according to the manufacturer's instructions. Briefly, HT29 cells were treated with cedrol for 48 hours. The cells were then harvested and fixed in cold 70% ethanol for 3 hours at –20°C, followed by resuspending in PBS. After addition of an equal volume of Cell Cycle reagent, cells were incubated for 30 minutes at room temperature in the dark. Finally, flow cytometry was conducted (Muse™ Cell Analyzer; Luminex) at the Core Facility Center for Tissue Regeneration (Dong-eui University, Busan, Korea) and the Muse analysis software (version 1.9) (https://www.luminexcorp.com/muse-cell-analyzer/#documentation) was used to determine the relative DNA content.

Apoptosis analysis

Cell apoptosis was detected using an Annexin V & Dead Cell Kit (Merck Millipore, Darmstadt, Germany). Briefly, cells were treated with cedrol for 48 hours, harvested and then resuspended in PBS containing 1% FBS. Cells were stained with Annexin-V & Dead Cell reagent for 20 minutes under darkness at room temperature. The quantitative analysis of apoptosis was measured on a Muse™ Cell Analyzer (Merck Millipore).

4′,6-diamidino-2-phenylindole (DAPI) staining

HT29 cells were plated in 8-chamber slides and treated with various concentration of cedrol for 48 hours. The cells were fixed in 4% formaldehyde for 10 min at room temperature and then stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. After mounting, the stained cells were visualized under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at the Core Facility Center for Tissue Regeneration (Dong-eui University).

Western blot analysis

Western blot analysis was performed according to our previous protocol [25]. Cell lysates in Cytoskeletal buffer [10 mM Pipes (pH6.8), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid, 1mM dithiothreitol, 1 mM phenylmethane-sulfonyl fluoride] containing 0.1% Triton X-100, 1 mM ATP and protease inhibitor were incubated on ice for 15 minutes, disrupted by sonication. For preparation of cytosolic

proteins, the sonication process of the above was omitted. After centrifugation at 20,000 *g* for 30 min, the protein concentration within the supernatant was measured by using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). For Western blot analysis, 30–50 μg of proteins was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS containing 0.1% Triton X-100 for 1 hour and incubated with specific primary antibodies at 4°C overnight followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized by an enhanced chemiluminescence (ECL) detection system (FluorChem FC2; Alpha Innotech, San Leandro, CA, USA) at the Core Facility Center for Tissue Regeneration (Dong-eui University). MCMs, cyclin E, Cyclin-dependent kinase 2 (CDK2), caspase 3/8/9, poly ADP ribose polymerase (PARP), actin primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against Proliferating cell nuclear antigen (PCNA), p53, p21, retinoblastoma protein (pRb), phosphorylated pRb (p-pRb) and cytochrome *c* were purchased from Cell Signaling Technology (Danvers, MA, USA).

In vivo tumor xenograft study

All animal experiments were performed under an experimental protocol approved by the Ethics Review Committee for Animal Experimentation of the Done-eui University (A2015-018). This study was conducted in accordance with the ARRIVE guidelines. Five-week-old athymic BALB/c-nu female mice (SLC, Hamamatsu, Japan) were maintained under specific pathogen-free conditions with a temperature of 22–24°C, humidity of 50%–60%, and lighting regimen of 12 hours light and 12 hours dark. HT29 cells (5×10^6 cells) were subcutaneously injected into the lateral flank of mice. When the tumor mass was palpable, 50 mg/kg of cedrol or 3 mg/kg of Adriamycin (Sigma-Aldrich) was administered intravenously three times a week for 14 days to the mice (n = 5 each group). All the mice were weighed, and the tumor volume was measured and then calculated using the formula: tumor volume (mm^3) = [length × $(width)^2$] × π/6. After 14 days of injection, the mice were sacrificed, and all tumors were removed and weighed.

Histological study

To evaluate the histological changes, paraffin-embedded tumor blocks were sectioned at 5-μm thickness and stained with hematoxylin and eosin (H&E; Vector Laboratories Inc, CA, USA), followed by observation under a light microscope (Eclipse C; Nikon, Tokyo, Japan) at the Core Facility Center for Tissue Regeneration (Dong-eui University). For immunohistochemistry, sections were dewaxed, rehydrated, and boiled in antigen retrieving buffer (10 mM sodium citrate/0.05% Tween 20, pH 6.0) for 20 minutes. Sections were incubated with anti-MCM7 antibody (Santa Cruz Biotechnology) at 4°C overnight and further incubated with HRP-conjugated goat anti-mouse Immunoglobulin G (Santa Cruz Biotechnology) for 1 hour at room temperature, developed with diaminobenzidine substrate (Sigma-Aldrich). After counterstaining with hematoxylin, the sections were photographed under a light microscope (Eclipse C; Nikon).

Statistical analysis

The data were expressed as the mean ± standard deviation from at least three independent experiments. A student's *t*-test was used to compare the statistical difference within

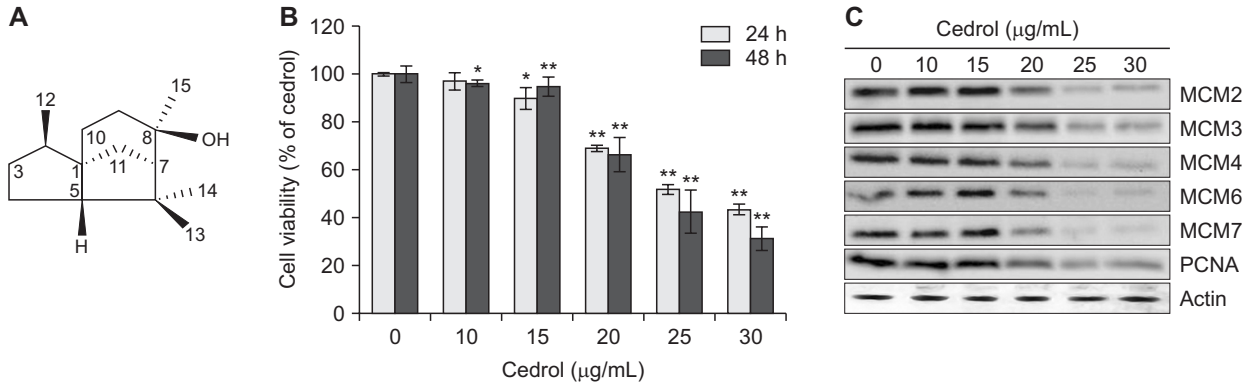

Figure 1. Cedrol inhibits cell growth and MCM protein expression in human adenocarcinoma HT29 cells. (A) Chemical of cedrol. (B) Inhibitory effects of cedrol on HT29 cell growth. Cells were treated with indicated concentrations of cedrol for 24 hours or 48 hours. Cell viability was measured by the water-soluble tetrazolium salt assay as described in Materials and Methods. Control cells were treated with 0.1% dimethylsulfoxide. All experiments were repeated three times, and the values and bars represented mean ± standard deviation, respectively. **P* < 0.05, ***P* < 0.01 vs. control. (C) Inhbition of minichromosome maintenance by cedrol treatment in HT29 cells. Cells were treated with various concentrations of cedrol for 48 hours. The cells were lysed, and the cellular proteins were then separated by sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were proved with the indicated antibodies and the proteins were visualized using the enhanced chemiluminescence detection system. Actin was used as an internal control. MCM, minichromosome maintenance; PCNA. proliferating cell nuclear antigen.

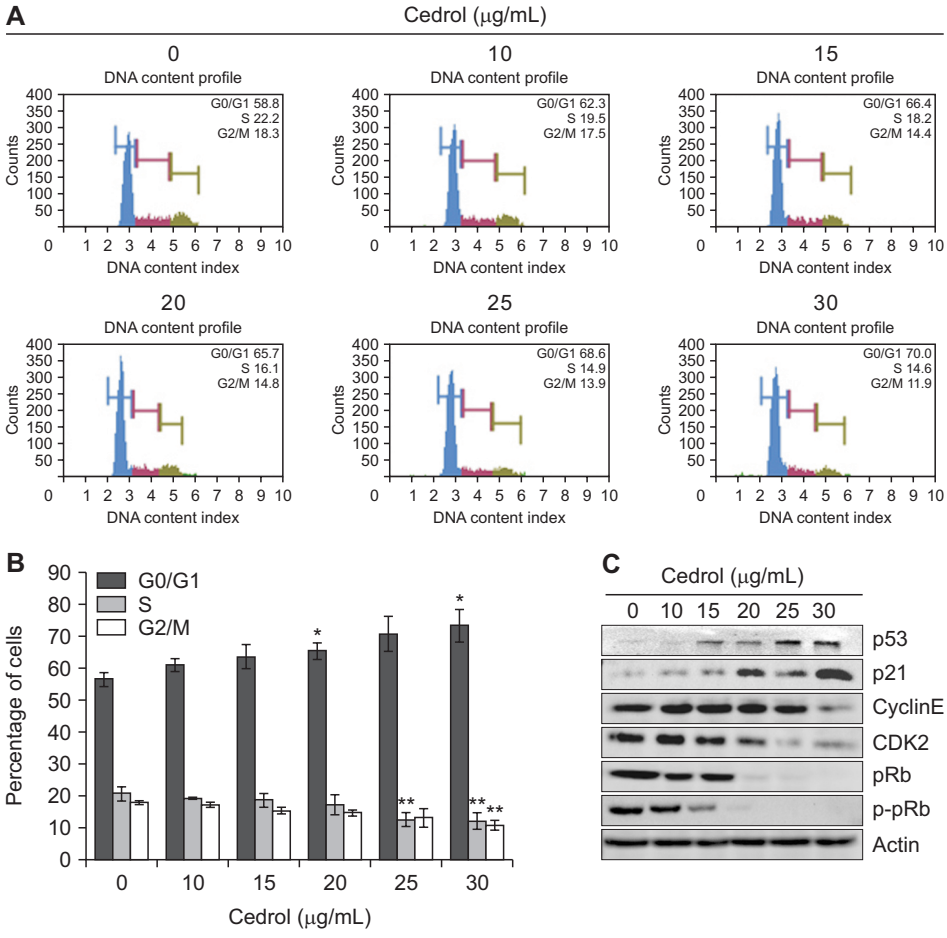

Figure 2. Cedrol induces G1 arrest and apoptosis in HT29 cells. (A) Cell cycle analysis of HT29 cells. Cells were treated with indicated concentrations of cedrol for 48 hours, stained with propidium iodide and analyzed by flow cytometry. DNA-fluorescence histogram of cell distribution is shown. (B) Cell cycle analysis data are presented as the mean ± standard deviation of triplicate experiments. **P* < 0.05, ***P* < 0.01 vs. control. (C) Effects of cedrol on the expression of cell cycle-related proteins. Western blotting was performed using indicated antibodies and actin was used as an internal control. CDK2, cyclin-dependent kinase 2; pRB, retinoblastoma protein; p-pRB, phosphorylated pRB.

two groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Cedrol shows the antiproliferative effect and the downregulation of MCM proteins in HT29 human colorectal cancer cells

To examine the inhibitory effect of cedrol on the HT29 cell proliferation, we performed a WST assay. Cells were treated with cedrol (Fig. 1A), incubated for 24 hours or 48 hours, and then the viable cells were measured. Compared to the control cells treated with vehicle, the cedrol-treated cell proliferation was significantly inhibited in a concentration- and time-dependent manner (Fig. 1B). When the concentration of cedrol was increased to 20, 25, and 30 μg/mL, the inhibitory rate of cell growth was increased to 33.64, 57.51 and 68.82%, respectively, after 48 hours of incubation.

Next, we investigated the effect of the cedrol on the expression of MCM proteins and PCNA, the proliferation markers, in HT29 cells based on our previous data (data not shown) that the methanol extract of *J. chinensis* inhibited the expression of MCM proteins in HeLa cells. As shown in Fig. 1C, MCM proteins (MCM2, MCM3, MCM4, MCM6, MCM7) and PCNA were downregulated in HT29 cells according to the increase of the cedrol concentration.

Cedrol induces the cell cycle arrest in HT29 cells

To demonstrate the molecular mechanism of suppression of HT29 cell proliferation by cedrol, we next examined the effects of cedrol on the cell cycle progression of HT29 cells. Figure 2A showed the increased distribution of cells in the G1 phases in a concentration dependent manner accompanied by a decrease in the S and G2/M phases, indicating that cedrol induces the G1 arrest. Compared to the untreated control, the treatment with 30 μg/mL of cedrol showed a significant increase in the G1 phase population from 56.36% to 73.26%, associated with the upregulation of p53 and p21, and downregulation of CDK2, cyclin E, pRb, and p-pRb (Fig. 2B and 2C).

Cedrol induces the apoptosis in HT29 cells

Next, cedrol-mediated apoptosis was examined by flow cytometry using Annexin V/7-Aminoactinomycin D (7-AAD). As shown in Fig. 3A and 3B, the apoptosis of HT29 cells was

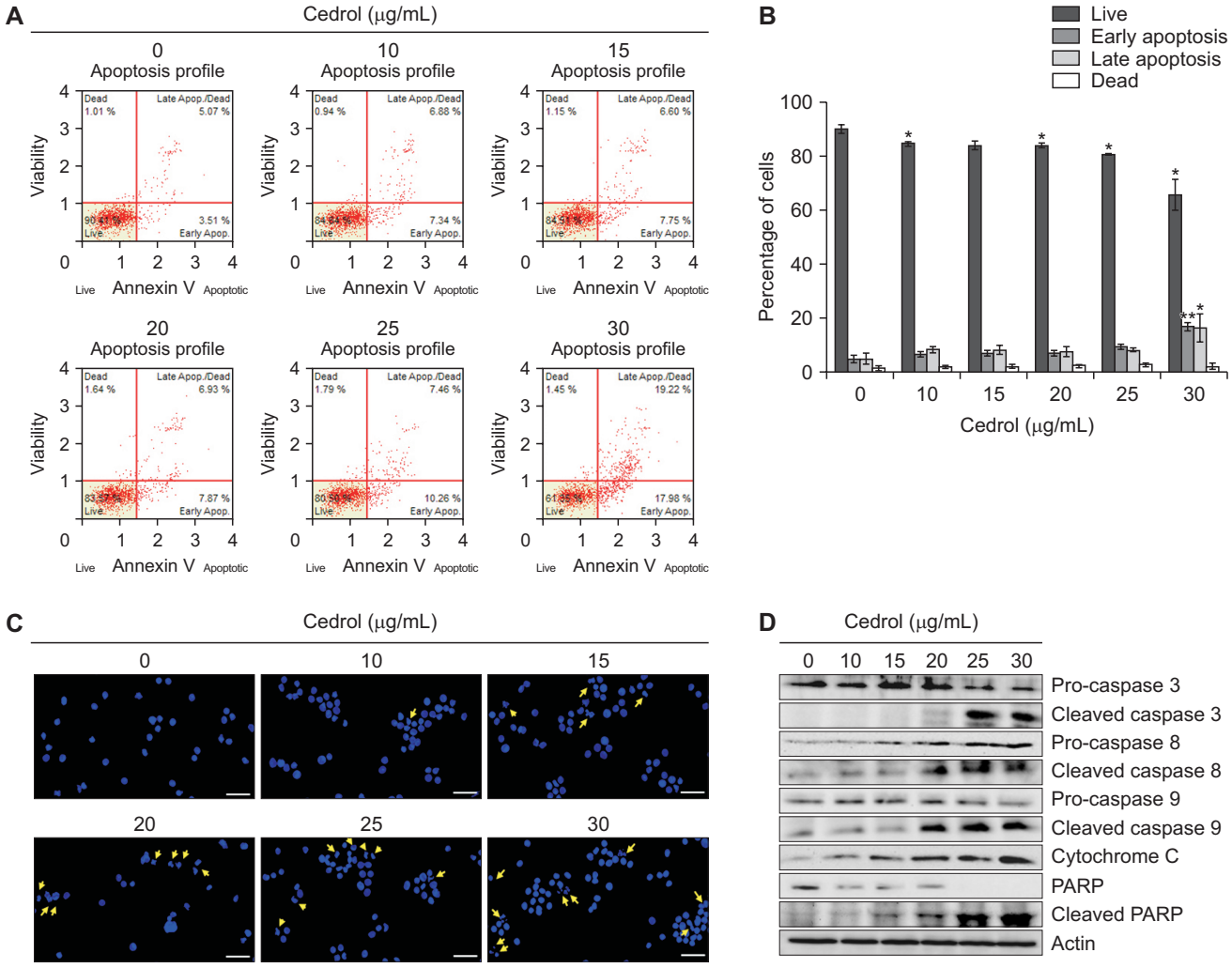

Figure 3. Cedrol induces apoptosis in HT29 cells. (A) Annexin V/7-Aminoactinomycin D (7-AAD) double staining. Cells were treated with cedrol for 48 hours and double stained with Annexin V and 7-AAD. Dot blot and the percentage of live, total apoptotic and dead cells are shown. Live cells (lower left): Annexin V$^-$/7-AAD$^-$, dead cells (upper left): Annexin V$^-$/7-AAD$^+$, early apoptotic cells (lower right): Annexin V$^+$/7-AAD$^-$, late apoptotic cells (upper right): Annexin V$^+$/7-AAD$^+$. (B) Distribution of cell population in each quadrant of the dot plots. Data are presented as the mean ± standard deviation of triplicate experiments. *P < 0.05, **P < 0.01 vs. control. (C) DAPI staining. Cedrol-treated cells were fixed and stained with DAPI. Arrows indicate the apoptotic bodies. Scale bars, 50 μm. (D) Modulation of apoptosis-related protein expression by cedrol in HT29 cells. Cells were treated with indicated concentrations of cedrol for 48 hours and western blotting was performed using apoptosis-related protein antibodies. Actin was used as an internal control. PARP, poly ADP ribose polymerase; DAPI, 4′,6-diamidino-2-phenylindol.

induced by cedrol in a concentration-dependent manner. Live cells (Annexin V$^-$/7AAD$^-$) decreased from 89.65% to 65.49%, and apoptotic cells (Annexin V$^+$) increased from 9.12% to 32.68%, after 48 hours incubation with 30 μg/mL of cedrol. Cedrol-mediated apoptosis was confirmed by observing the apoptotic bodies with chromatin condensation in a concentration-dependent manner using DAPI staining (Fig. 3C). Cedrol induced apoptosis of HT29 cells associated with cytosolic cytochrome-*c* release and casapase-3, -8, -9 activation, resulting in PARP cleavage (Fig. 3D). These results suggest that cedrol inhibits the proliferation of HT29 cells by the suppression of cell cycle progression and the induction of apoptosis.

Cedrol inhibits the tumor growth in a human xenograft model through MCM7 downregulation

To examine the antitumor effect of cedrol in a xenograft model tumor growth derived from HT29 cells was measured after treatment with cedrol or Adriamycin. As shown in Fig. 4A-4C, compared to vehicle-treated control, cedrol significantly inhibited tumor growth, showing the reduction of tumor volume and weight. Treatment with cedrol (50 mg/kg) reduced tumor volume more efficiently than Adriamycin (3 mg/kg) used as a positive control. Moreover, mice in the cedrol-treated group showed normal body weight whereas those in the Adriamycin-treated group exhibited body weight loss, suggesting that cedrol has an anti-cancer activity in vivo without any obvious

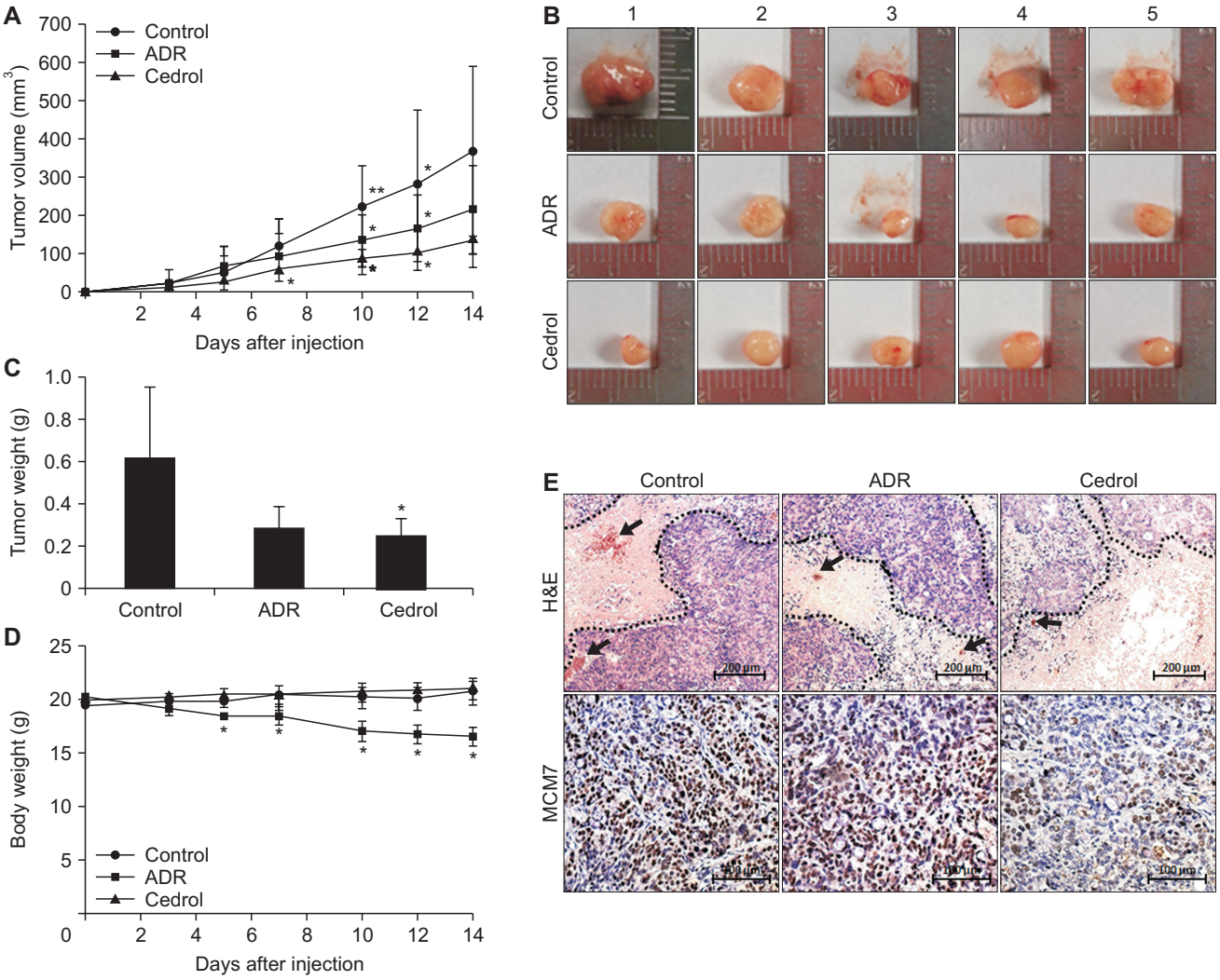

Figure 4. Cedrol suppresses the tumor growth and the expression of MCM7 in colorectal tumor xenograft mice. BALB/c nude mice (n = 5/group) were inoculated subcutaneously with HT29 cells. After the tumor formation, the mice were treated intravenously with vehicle (●), 3 mg/kg of ADR as a positive control (■), or 50 mg/kg of cedrol (▲). (A) The tumor volume was measured and calculated as described in Materials and Methods. (B, C) The tumor tissues were removed at 14 days after the first injection and weighed. (D) Mouse body weight was measured. Data are presented as mean ± standard deviation. *P < 0.05 and **P < 0.01 vs. control group. (E) Histological analysis. Tumor tissues were fixed with 10% formaldehyde and embedded in paraffin. Sectioned tissues were stained with H&E and with antibody against MCM7 for immunohistochemistry. Histological analysis was performed under a light microscope and the blood vessels are shown (arrow). Scale bars, 200 μm for H&E staining. 100 μm for MCM7 staining. ADR, Adriamycin; H&E, hematoxylin and eosin; MCM, minichromosome maintenance.

toxicity (Fig. 4D).

To confirm the anti-cancer activity of cedrol in vivo, the histological and molecular changes were assessed in tumor tissue. After H&E staining, the cedrol-treated tumor showed remarkably reduced cell density both in parenchyma and stroma, and the blood vessels (arrows) in stroma were not easily detected compared with those in vehicle- or Adriamycin-treated tumor (Fig. 4E). Since MCM proteins were downregulated in HT29 cells by cedrol treatment, we next performed immunohistochemical staining with specific antibody against MCM7. As shown in Fig. 4E, although MCM7 expression was detected in vehicle- and Adriamycin-treated tumor tissue, the proportion of MCM7-positive cells was significantly decreased in cedrol-treated tumor tissue.

DISCUSSION

In the present study, anti-cancer efficacy of cedrol, an aromatic component of *J. chinensis,* and its mechanism were analyzed in colorectal cancer in vitro and in vivo based on our previous study with human lung carcinoma A549 cells [24]. Natural products such as cedrol have been widely used to treat diseases and are still an important source for the development of new therapies [26]. In addition, compounds

derived from natural products or synthesized from natural substances play an important role in the development of new drugs, including anti-cancer drugs. The disadvantage of existing anti-cancer drugs are fatal side effects and low cure rates, so it is very important to develop alternative anti-cancer drugs to overcome such limitations. Treating cancer with natural products is becoming a new method of reducing side effects and increasing the effectiveness of concurrent treatment with anti-cancer drugs [27].

MCM protein is a major replication protein involved in the early stages of DNA replication along with topoisomerase due to its helicase activity [28]. It is attracting attention as a new target protein for anti-cancer drug development because of the recent characteristic overexpression in cancer cells. MCM proteins are essential for cell cycle progression, and they are overexpressed in various types of cancer cell lines [29]. Cedrol exhibited a potent anti-proliferative effect through reduction of MCM proteins in HT29 colorectal cancer cells, resulting in cell cycle arrest and apoptosis. Cedrol-mediated G1 phase arrest is likely to be caused by a decrease in CDK2 and Cyclin E as a result of p53-dependent p21 upregulation. The Rb protein restricts the cell's ability to replicate DNA, preventing the cell cycle progression from G1 to S phase [30]. Phosphorylation of Rb, which is a signal downstream of the Cdk2/cyclin E complex, was inhibited by cedrol, which was associated with inhibition of MCM (MCM2–MCM7) protein expression [31]. Cedrol-mediated apoptosis of HT29 cells occurred both internally by increasing cytoplasmic cytochrome *c* and the activating caspase-9, and an externally by activating caspase-8 and caspase-3 [32].

Moreover, we found that cedrol treatment suppressed tumor growth derived from HT29 cells and induced a number of histological changes in a xenograft tumor model. Compared to tumors in vehicle-treated mice, cedrol-treated mice showed a decreased tumor size and an indistinct tumor tissue structure as well as the decreased size and number of vessels in the tumor tissue. Angiogenesis plays an important role in tumor growth and metastasis in colorectal cancers; therefore, anti-angiogenesis is a promising approach for colorectal cancer therapy [33,34]. Our data on xenotransplantation are similar to previously reported data on the anti-angiogenic effect of widdrol, another active compound isolated from *J. chinenesis*. We have previously reported that widdrol suppresses angiogenesis by acting on vascular endothelial growth factor receptor 2, resulting in depletion of blood vessels and MCM7 expression [35]. These results suggest that cedrol may also have an inhibitory effect on angiogenesis as one of the putative mechanisms for its anti-cancer function.

In conclusion, we found that cedrol, an aromatic sesquiterpene compound present in *J. chinensis*, successfully reduced expression of MCM proteins (MCM2–MCM7), suggesting that cedrol may have anti-cancer activity by regulating cancer cell replication. The results of cell cycle arrest and apoptosis by cedrol may be related to the reduction of MCM in HT29 cells. In mouse tumor xenografts, cedrol administration significantly suppressed colorectal tumor growth and reduced MCM7 expression in tumor tissue, indicating that cedrol is a potential anti-cancer agent of natural origin.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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