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Original research article

Falcarindiol and dichloromethane fraction are bioactive components in *Oplopanax elatus*: Colorectal cancer chemoprevention via induction of apoptosis and G2/M cell cycle arrest mediated by cyclin A upregulation

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Abstract

Oplopanax elatus (Nakai) Nakai has a long history of use as an ethnomedicine by the people living in eastern Asia. However, its bioactive constituents and cancer chemopreventive mechanisms are largely unknown. The aim of this study was to prepare *O. elatus* extracts, fractions, and single compounds and to investigate the herb's antiproliferative effects on colon cancer cells and the involved mechanisms of action. Two polyyne compounds were isolated from *O. elatus*, falcarindiol and oplopandiol. Based on our HPLC analysis, falcarindiol and oplopandiol are major constituents in the dichloromethane (CH_2Cl_2) fraction. For the HCT-116 cell line, the dichloromethane fraction showed significant effects. Furthermore, the IC50 for falcarindiol and oplopandiol was 1.7 µM and 15.5 µM, respectively. In the mechanistic study, after treatment with 5 µg/ml for 48 h, dichloromethane fraction induced cancer cell apoptosis by 36.5% ($p < 0.01\%$ vs. control of 3.9%). Under the same treatment condition, dichloromethane fraction caused cell cycle arrest at the G2/M phase by 32.6% ($p < 0.01\%$ vs. control of 23.4%), supported by upregulation of key cell cycle regulator cyclin A to 21.6% ($p < 0.01\%$ vs. control of 8.6%). Similar trends were observed by using cell line HT-29. Data from this study filled the gap between phytochemical components and the cancer chemoprevention of *O. elatus*. The dichloromethane fraction is a bioactive fraction, and falcarindiol is identified as an active constituent. The mechanisms involved in cancer chemoprevention by *O. elatus* were apoptosis induction and G2/M cell cycle arrest mediated by a key cell cycle regulator cyclin A.

Keywords: Apoptosis; Cell cycle; Colorectal cancer; Cyclin A; Dichloromethane fraction; Falcarindiol; *Oplopanax elatus*

Highlights:
- Falcarindiol and oplopandiol are isolated from *Oplopanax elatus* CH_2Cl_2 fraction.
- *O. elatus* CH_2Cl_2 fraction is an active fraction on colon cancer cells.
- *O. elatus* CH_2Cl_2 fraction induces colon cancer cell apoptosis.
- *O. elatus* CH_2Cl_2 fraction induces G2/M cell cycle arrest via cyclin A upregulation.
- Falcarindiol is an active constituent and significantly inhibits cancer cell growth.

Abbreviations:

O. – Oplopanax; CRC – colorectal cancer; FITC – fluorescein isothiocyanate; CH_2Cl_2 – dichloromethane; BuOH – n-butanol; H_2O – water; DMSO – dimethylsulfoxide; PI – propidium iodide; NMR – nuclear magnetic resonance; HPLC – high performance liquid chromatography; FBS – fetal bovine serum; MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ANOVA – analysis of variance; NIH/NCCAM – National Institutes of Health/National Center for Complementary and Alternative Medicine.

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Introduction

Oplopanax is a small genus, which includes three species: *O. elatus*, *O. horridus*, and *O. japonicus*. *O. horridus* and *O. japonicus* are distributed throughout the Pacific Northwest of North America and Japan, respectively (Huang et al., 2014). *O. elatus* is distributed in eastern Asia, including northeastern China, Fareast Russia, and Korea (Huang et al., 2010). Although these three species are very close in taxonomy, the chemical composition and pharmacological activities are different (Calway et al., 2012; Huang et al., 2014). The main bioactive components from the root of *O. elatus* were polyynes, lignans, and phenylpropanoids (Huang et al., 2014). Phytochemical and pharmacological studies on another species in this genus, i.e., *O. horridus*, are more frequently reported than those of *O. elatus* (Calway et al., 2012; Jang et al., 2017; Wang et al., 2013). Thus, systematic bioactivity and the corresponding bioactive component characterization are critically needed for the effective use of *O. elatus*.

Oplopanax elatus (Nakai) Nakai has a long history of use as an ethnomedicine by the people living in eastern Asia, which was introduced in the *Jilin Chinese Herbal Medicine* (Changchun College of Traditional Chinese Medicine, 1970), and the claimed indication for *O. elatus* includes neurasthenia, schizophrenia, rheumatism, and cardiovascular diseases (Shikov et al., 2014). Data from human subjects suggest that *O. elatus* showed positive effects for the management of diabetes (Klimakova and Kazman, 1962), depression (Turova, 1974), rheumatic arthritis (Tian et al., 1985), and bacterial and fungal infections (Fu et al., 1997; Vereshchagin et al., 1982), with antioxidant and anti-inflammatory potentials (Kwon et al., 2020; Tian et al., 2019).

According to the book *Chinese Materia Medica*, *O. elatus* has characters of supplement qi, reinforces yang, frees network vessels and gets through impediments (Editorial Committee for Chinese Materia Medica 1999). Based on traditional Chinese medicine theory, cancer is caused by the obstruction of channels and network vessels. Due to *O. elatus* having the ability to free and break through the channels and network vessels, this herb may have the potential to prevent and/or treat cancer. Therefore, in recent years, *O. elatus* has been elected to investigate its cancer chemopreventive effects (Huang et al., 2014; Qiao et al., 2017; Wang et al., 2019a).

Colorectal cancer (CRC) is one of the most common cancers in the Western world. In the United States, it is estimated that there will be 104,270 newly diagnosed CRC cases and 52,980 CRC-related deaths in 2021, indicating the inadequacy of the currently available therapeutic approaches (Siegel et al., 2021). Botanicals are an attractive source of new therapeutic candidate compounds, as a tremendous chemical diversity is found in millions of species of plants (Lee et al., 2019; Liu et al., 2019). The treatment of human cancer owes much to the important drugs that have been derived from plants (Cragg et al., 2009). With the advent of phytochemical and pharmacological technologies, medicinal plants are likely to provide many of the lead structures for the construction of novel compounds with enhanced anticancer properties (Huang et al., 2019; Wu et al., 2020).

The genus *Oplopanax* is a member of the family Araliaceae, where a well-known plant ginseng (genus *Panax*) belongs. Over the past twenty years, over ten thousand research papers on ginseng have been published; however, for the genus *Oplopanax*, only less than one hundred studies have been published, and most of them were linked to *O. horridus*. An-

ticancer pharmacological investigations on *Oplopanax* genus plants have been reported, and the related literature can be traced back to 2006 (Tai et al., 2006). Thus, anticancer studies on *Oplopanax* herbs, especially *O. elatus*, are rare, although *O. elatus* has a wide region of distribution and in large quantity. Although clinical studies of its use for cancer are lacking, recent pre-clinical investigations revealed that bioactive components from *O. elatus* showed anti-proliferative and chemopreventive activities on different cancer cell lines (Qiao et al., 2017; Wang et al., 2019a). We conducted pharmacodynamics studies on this herb and observed that *O. elatus* components can enhance a chemotherapeutic agent's anticancer potential (Wang et al., 2019a, b). Reported *O. elatus* studies only used its extract or one fraction; systematic screening for different fractions and active constituent identification have not been conducted. Thus, the bioactive fractions and constituents of *O. elatus* and their cancer chemopreventive mechanisms are largely unknown.

In this study, we first prepared *O. elatus* extract, and three fractions were obtained. Then, two major constituents were isolated and identified from one fraction. The contents of representative compounds in the extract and fractions were analyzed. Anticancer effects of *O. elatus* extract and fractions were carefully evaluated and systematically compared, and the active fraction and compound were identified. The cancer cell growth inhibition involved in apoptosis induction and cell cycle arrest, including the key cell cycle regulator, were determined.

Materials and methods

Chemicals and reagents

All solvents were of high-performance liquid chromatography grade. Cell culture plasticware was obtained from Falcon Labware (Franklin Lakes, NJ) and Techno Plastic Products (Trasadingen, Switzerland). Glutamine, insulin trypsin, McCoy's 5A medium, and phosphate buffered saline were obtained from Mediatech, Inc. (Herndon, VA, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). The CellTiter 96 Aqueous Solution Cell Proliferation Assay, an MTS assay kit, was obtained from Promega (Madison, WI). PI/RNase staining buffer was obtained from BD Biosciences Pharmingen (San Diego, CA). Annexin V-FITC apoptosis detection kit and FITC-conjugated cyclin A were obtained from BD Biosciences (San Jose, CA).

Plant materials

The dried roots of *Oplopanax elatus* (Nakai) Nakai were collected from Benxi (Liaoning, China), in the northeastern area of China, and authenticated by Prof. De-Qiang Dou at Liaoning University of Traditional Chinese Medicine. A voucher specimen (number OES-20150820) is deposited in the Institute of Clinical Pharmacology, Central South University, Hunan Province, China.

Preparation of extract, fractions and single compounds

Air-dried, powdered root bark of *O. elatus* was extracted with methanol. The extraction method was boiling under reflux. The filtrate was collected, and the extraction procedure was repeated one more time on the residue. The combined filtrate was condensed under vacuum and lyophilized to yield dried *O. elatus* extract. The extract was suspended in water, then extracted with dichloromethane and n-butanol successively to give dichloromethane fraction (CH$_2$Cl$_2$) and n-butanol frac-

tion (BuOH). Water phase was condensed under vacuum to give water fraction (H$_2$O). The CH$_2$Cl$_2$ fraction was separated by preparative HPLC to afford compounds 1 and 2. Structures of isolated compounds were determined by a combination of

spectroscopic analyses, including 1H and 13C NMR and mass spectroscopic data. The compounds are identified as (1) falcarindiol and (2) oplopandiol (Fig. 1).
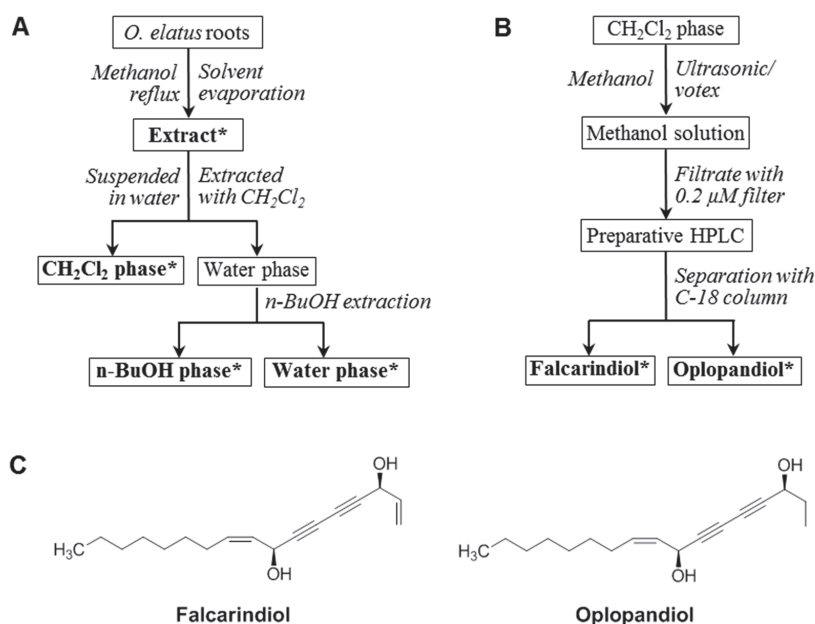

Fig. 1. Preparation of *O. elatus* fractions and single compounds. (**A**) Preparation flow chart for *O. elatus* extract and three fractions. (**B**) Isolation of representative single compounds from CH$_2$Cl$_2$ fraction. (**C**) Chemical structures of falcarindiol and oplopandiol; * those extract/fractions/compounds were used for biological evaluations.

HPLC analysis

Chromatographic analysis was performed on a Waters 2960 HPLC system (Milford, MA, USA) with a quaternary pump, an automatic injector, a 996 photodiode array detector, and a Waters Empower software for peak identification. A gradient mobile phase system of acetonitriol (eluent A) and water (eluent B) was applied as follows: 36% A and 64% B (0 min), 36% A and 64% B (10 min), 50% A and 50% B (20 min), 65% A and 35% B (35 min), 75% A and 25% B (40 min), 100% A (45 min), 100% A (50 min), 36% A and 64% B (53 min), and 36% A and 64% B (60 min). The flow rate was set at 1 ml/min. The sample volume injected was set at 10 μl, and the detection wavelength was set at 202 nm.

Cell culture

The research protocol was approved by the University of Chicago IACUC committee. The human colorectal cancer cell lines HCT-116 and HT-29 were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in McCoy's 5A medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% CO$_2$ at 37 °C.

MTS assay

O. elatus water fraction was dissolved in water. The extract, other fractions, and single compounds were dissolved in DMSO. HCT-116 and HT-29 cells were seeded in 96-well plates. After 1 day, various concentrations of extract/fractions/compounds were added to the wells. The final concentration of DMSO was 0.5%. Controls were exposed to a culture medium containing 0.5% DMSO without drugs. Cell proliferation was evaluated using an MTS assay according to the manufacturer's instructions. Briefly, after 24–72 h treatment, the medium was re-

placed with 100 μl of fresh medium and 20 μl of MTS reagent (CellTiter 96 Aqueous Solution) in each well, after which the plate was returned to the incubator for 1–4 h. A 60-μl aliquot of medium from each well was transferred to an ELISA 96-well plate, and its absorbance at 490 nm was recorded. Results are expressed as percent of control (0.5% DMSO control was set at 100%).

Apoptotic assay

Cancer cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed, and tested botanical components were added. After treatment for 48 h, the cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then, the culture medium containing FBS (and floating cells) was added to inactivate the trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 g. The supernatant was removed, and the cells were stained with annexin V-FITC and PI according to the manufacturer's instructions. Cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo 10.6.1 software (Tree Star, Ashland, OR). For each measurement, at least 20,000 cells were counted.

Cell cycle assay

Cancer cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed, and the cells were treated with different concentrations of tested samples. The cells were incubated for 48 h before harvesting. Next, the cells were gently fixed with 80% ethanol in a freezer for 2 h and then treated with 0.25% Triton X-100 for 5 min in an ice bath. The cells were resuspended in 300 μl of PBS containing

40 µg/ml PI and 0.1 mg/ml RNase, incubated in a dark room for 20 min at room temperature, and analyzed using a FACS-can flow cytometer. For each measurement, at least 10,000 cells were counted.

Cyclin A assay
After 48 h treatment with the tested botanical components, cancer cells were harvested in a similar manner to that used in the cell cycle assay. The cells were stained with 300 µl of PBS containing 40 µg/ml PI and 0.1 mg/ml RNase, and 20 µl of cyclin A-FITC. Then, the cells were analyzed with a FACScan flow cytometer. For each measurement, at least 10,000 cells were counted.

Statistical analysis
Data are presented as mean ± standard error (SE). A one-way ANOVA was employed to determine statistical significance of the results. In some cases, Student's t-test was used for comparing two groups. The level of statistical significance was set at $p < 0.05$.

Results

HPLC analysis of O. elatus extract and three fractions
Based on our pilot study, we observed two major constituents in CH_2Cl_2 fraction. Using preparative HPLC, we prepared these two compounds from CH_2Cl_2 fraction, and they were identified as falcarindiol and oplopandiol, both belonging to polyynes. These two compounds were employed as standards for further HPLC analysis. The HPLC chromatograms of $O.$ $elatus$ extract and three fractions are shown in Fig. 2A. It indicates that the main constituents in extract were falcarindiol and oplopandiol. In dichloromethane fraction (CH_2Cl_2), falcarindiol and oplopandiol were the major constituents, and peak areas of the two compounds are larger than that in the extract. On the other hand, the main constituents in n-butanol fraction (BuOH) were still falcarindiol and oplopandiol; however, peak areas for the two compounds are much smaller than that in the extract and CH_2Cl_2 fraction. While in water fraction (H_2O), only trace peaks were detected (Fig. 2A). As shown in
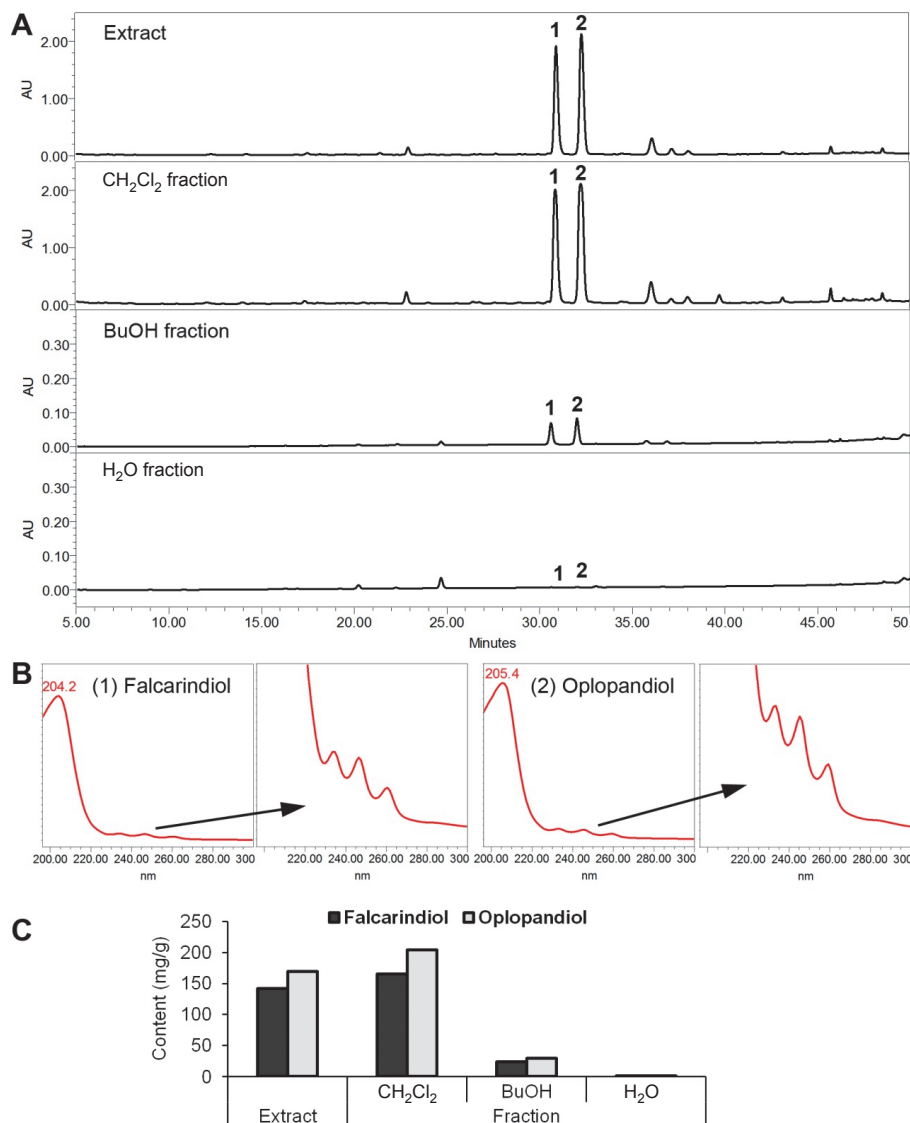

Fig. 2. HPLC analysis of *O. elatus* extract and fractions. (**A**) HPLC chromatograms recorded at 202 nm. (**B**) UV spectra of falcarindiol and oplopandiol. Polyyne specific fingerprint between 220 nm and 270 nm has been shown. (**C**) Contents of falcarindiol and oplopandiol in extract and different fractions. CH_2Cl_2, dichloromethane fraction; BuOH, n-butanol fraction; H_2O, water fraction.

Fig. 2B, the UV spectra of the two compounds displayed the typical fingerprint of polyyne structure, and three peaks were observed between 220 nm and 270 nm. The contents of the two determined polyynes in the extract and its three fractions are shown in Fig. 2C. In the extract, the content of falcarindiol and oplopandiol were 141.6 and 169.7 mg/g, respectively. The content of falcarindiol and oplopandiol in CH_2Cl_2 fraction were 165.7 and 204.4 mg/g, 23.3 and 29.0 mg/g in BuOH fraction, and 0.37 and 0.41 mg/g in H_2O fraction, respectively (Fig. 2C). Falcarindiol and oplopandiol have the highest contents in CH_2Cl_2 fraction.

Antiproliferative effects of O. elatus extract and fractions

Two steps were applied to evaluate antiproliferative effects. In the first step, we investigated antiproliferative effects at one time point with relatively large intervals of concentrations for extract and all three fractions. As shown in Fig. 3A, for the cell line HCT-116, cell growth was absolutely inhibited by the treatment of extract and CH_2Cl_2 with a concentration of 25 µg/ml. H_2O fraction did not show any antiproliferative

effects in the treatment concentration range. BuOH showed some cell growth inhibitory effects at 50 µg/ml. Extract and CH_2Cl_2 showed potent effects. At 5 and 12.5 µg/ml, the extract inhibited cell proliferation by 29.6% and 64.1%, respectively, while at the same concentrations, CH_2Cl_2 fraction inhibited cell growth by 52.5% and 93.1%, respectively. A similar trend was observed in HT-29 cells (Fig. 3A). Among the extract and three fractions, CH_2Cl_2 showed the strongest antiproliferative effects.

To further investigate antiproliferative potential, in the second step, time-, and dose-dependent effects were obtained. Cell proliferation was observed at 24, 48, and 72 h. More precise concentration ranges were employed. For the HCT-116 cell group, treatment concentrations for extract and CH_2Cl_2 fraction were 1–20 µg/ml for BuOH fraction at 5–75 µg/ml. For the HT-29 cell group, treatment concentrations for extract and CH_2Cl_2 fraction were 10–100 µg/ml for BuOH fraction at 50-400 µg/ml. As shown in Fig. 3B, for the cell line HCT-116, after treatment with CH_2Cl_2 fraction, the cell proliferation decreased in a dose- and time-dependent manner by doses greater than 2.5 µg/ml ($p < 0.01$ vs. control). The extract also
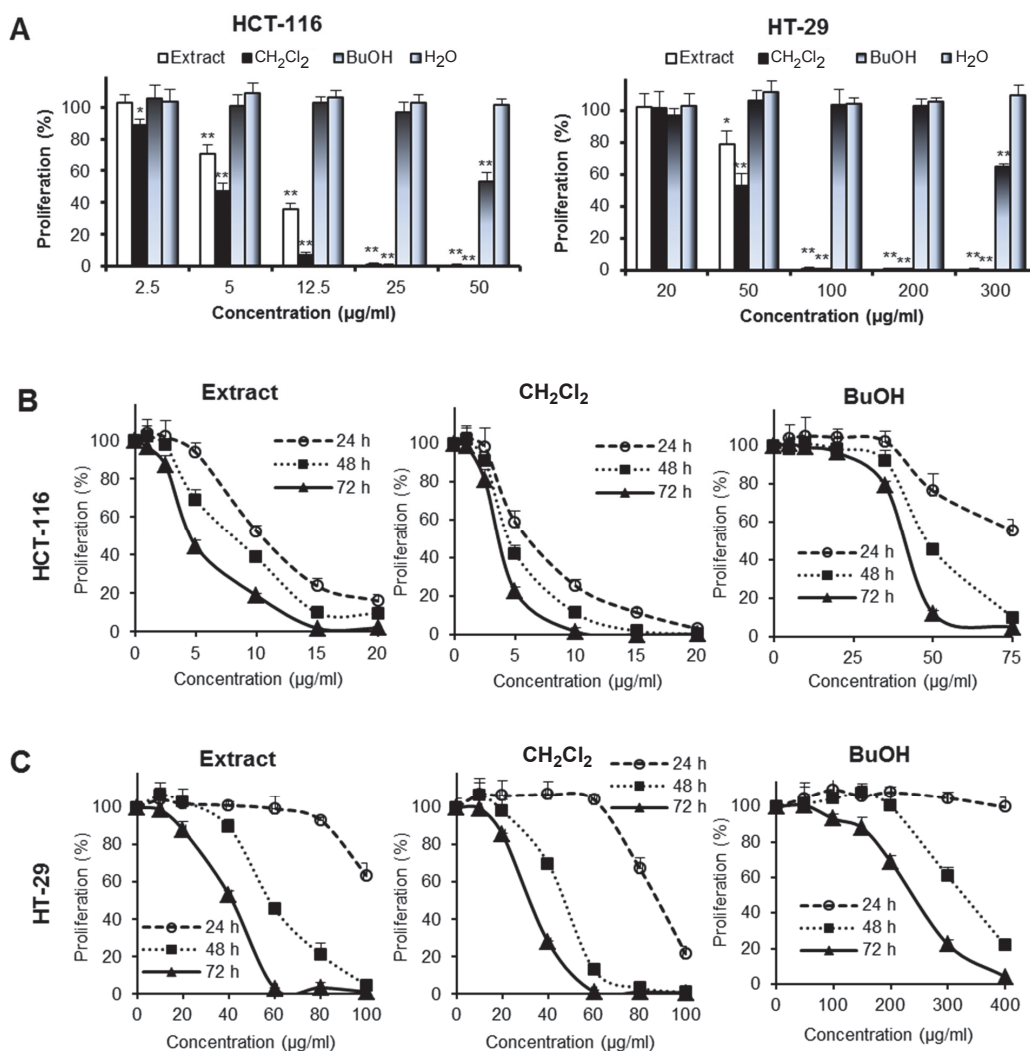

Fig. 3. Antiproliferative effects of *O. elatus* extract and fractions on human colorectal cancer cells. (**A**) HCT-116 and HT-29 cells were treated with extract/fractions at concentrations of 2.5–300 µg/ml for 48 h. (**B** + **C**) Time- and dose-dependent growth inhibitory effects of extract/fractions on colorectal cancer cell lines HCT-116 (upper panel) and HT-29 (lower panel). Cell proliferation was determined by MTS assay. Results were normalized to each control in percentage and expressed as mean ± standard error of triplicate experiments (solvent vehicle set at 100%). * $p < 0.05$, ** $p < 0.01$ vs. control. CH_2Cl_2, dichloromethane fraction; BuOH, n-butanol fraction; H_2O, water fraction.

showed potent antiproliferative effects with slightly weaker potential than that of CH_2Cl_2 fraction. Meanwhile, BuOH fraction showed dose- and time-dependent antiproliferative effects, however, the active concentrations were higher than 35 μg/ml (Fig. 3B). A similar trend for dose- and time-dependent effects were observed in HT-29 cells (Fig. 3C), but the active concentration ranges are higher than that of HCT-116 cells. Therefore, CH_2Cl_2 is an active fraction which is extracted from *O. elatus* extract.

Apoptotic effect of O. elatus *extract and fractions on* colorectal cancer cells

To explore the potential mechanism through which *O. elatus* induces cell death, we carried out an apoptotic assay by flow cytometry after staining with annexin V and PI. Based on the MTS assay data, using HCT-116 cells, an antiproliferative effect was observed by extract and CH_2Cl_2 treatment at >2.5 μg/ml. Thus, we used the treatment concentration range of 1-5 μg/ml to evaluate their effects on HCT-116 cell apoptosis. Annexin V can be detected in both the early and late stages of apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both annexin V and PI (lower left quadrant); early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic or necrotic cells displayed both positive annexin V and PI (upper right quadrant); non-viable cells which underwent necrosis were positive for PI and negative for annexin V (upper left quadrant) – Fig. 4A (Wang et al., 2013).
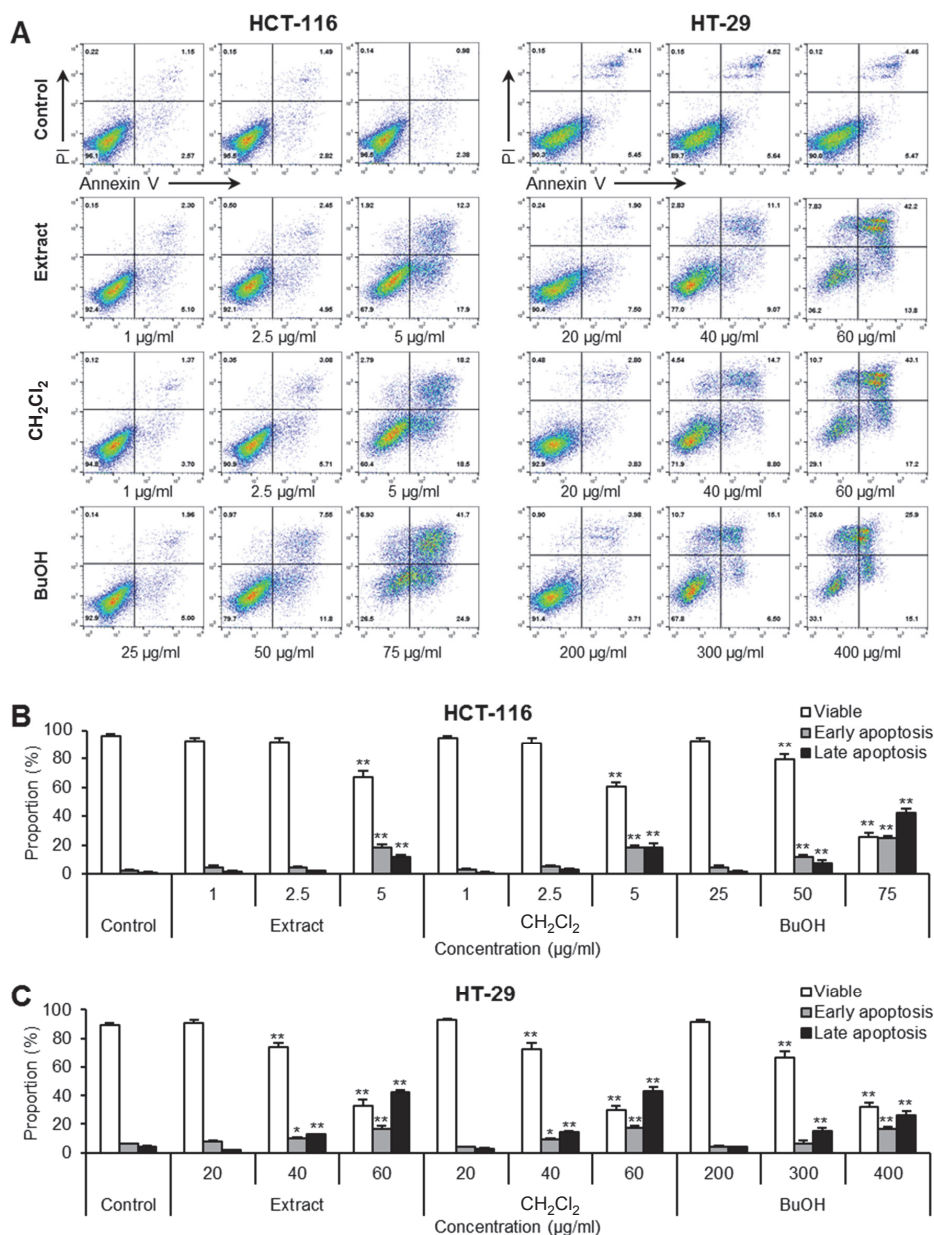

Fig. 4. Apoptotic analysis of HCT-116 and HT-29 cells treated with *O. elatus* extract and fractions. Cells were treated with extract/fractions for 48 h, then stained with annexin V/propidium iodide (PI) before the extent of apoptosis was determined by flow cytometry. (**A**) Representative scatter plots of PI (y-axis) versus annexin V (x-axis). (**B** + **C**) Percentage of viable, early apoptotic and late apoptotic cells. Data are presented as the mean ± standard error of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs. control. CH_2Cl_2, dichloromethane fraction; BuOH, n-butanol fraction.

Due to more experiments being conducted, the horizontal and vertical axis labels for the flow cytometry density plots were removed to display the original data and reduce space size. The bar graphs summarized the effects of different groups with statistical analytical results. For the HCT-116 cell group, after treatment for 48 h, cell percentage of the summary of early and late apoptosis for control was 3.9%; for 5 μg/ml of extract and CH_2Cl_2 fraction was 30.6% and 36.5%, respectively (Fig. 4B). BuOH also induced cell apoptosis, but active concentration was up to 50 μg/ml. Similar results were observed in HT-29 cells, although the active concentrations are higher than that for HCT-116 cells (Fig. 4). Thus, *O. elatus* extract and fractions clearly induced apoptosis in both cell lines, and CH_2Cl_2 showed more potent activity than the extract. This re-

sult suggested that the antiproliferative effect of *O. elatus* extract and active fraction (CH_2Cl_2) was in part mediated by the induction of apoptosis.

Effects of O. elatus *extract and fractions on colorectal cancer cell cycle*

The cell proliferation assay results suggested that after treatment with *O. elatus* extract and fractions, cell growth was inhibited in a dose- and time-dependent manner (Fig. 3). In the apoptotic assay, we also observed that viable cells were decreased with increasing treatment concentration. To further characterize the potential mechanism of *O. elatus* anticancer activity, the cell cycle profile was assayed by flow cytometry after staining with propidium iodide (PI). As shown in Fig. 5A,
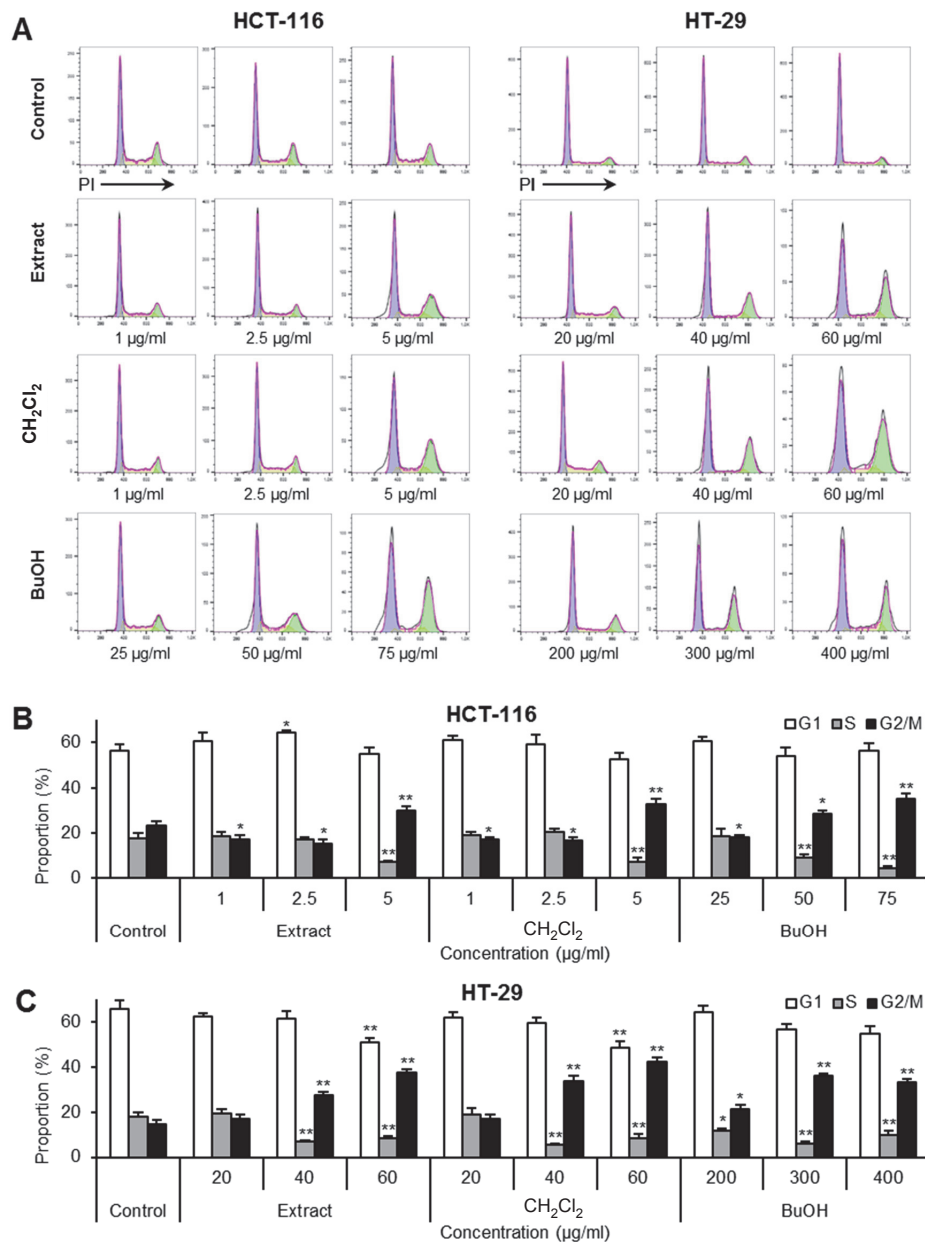

Fig. 5. Cell cycle analysis of HCT-116 and HT-29 cells treated with *O. elatus* extract and fractions. Cells were treated with extract/fractions for 48 h, then fixed in ethanol and stained with propidium iodide. DNA content was determined by flow cytometry. (**A**) Representative histograms of the DNA content in each experimental group. (**B** + **C**) Percentage of each cell cycle phase with various treatments or with control. Data are presented as the mean ± standard error of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs. control. CH_2Cl_2, dichloromethane fraction; BuOH, n-butanol fraction.

compared to the control, treatment with 1 and 2.5 μg/ml for 48 h did not obviously change the cell cycle profile. However, after treatment with 5 μg/ml, although the percentage of cells in G1-phase was slightly changed, extract and CH_2Cl_2 fraction obviously reduced the percentage of S-phase and increased G2/M-phase cells. Compared to the control (56.6% of G1-phase, 17.9% of S-phase, and 23.4% of G2/M-phase), treatment with 5 μg/ml of extract, 54.9% of cells were in G1-phase, 7.1% of cells were in S-phase, and 30.1% of cells were in G2/M-phase; treatment with CH_2Cl_2 fraction, 52.6% of G1-phase, 7.2% of S-phase, and 32.6% of G2/M-phase (Fig. 5B). Extract and CH_2Cl_2 fraction treatment obviously decreased S-phase cells, while increasing G2/M-phase cells. In addition, treatment with BuOH fraction also decreased S-phase and increased G2/M-phase cells; however, the active concentration is as high as 25 μg/ml.

A similar trend was also observed in HT-29 cells but with higher treatment concentrations. For example, compared to control (14.7% of G2/M), after treatment with 40 and 60 μg/ml of extract for 48 h, cells in G2/M phase were 27.6% and 37.5, respectively; while for CH_2Cl_2 fraction treatment, 33.7% and 42.4% of cells were arrested in G2/M phase, respectively. Although BuOH showed G2/M phase arrest effects in much higher concentrations (300-400 μg/ml), among extract and fractions, CH_2Cl_2 showed the strongest cell cycle arrest potential.

Effects of O. elatus extract and fractions on expression of cyclin A

Since cell cycle progression is regulated by the cyclins, and cyclin A is a key regulator for the passage through G2/M-phase (Zhao et al., 2016), to observe the regulation proteins involved in the cell cycle arrest, the expression of cyclin A in HCT-116 and HT-29 cancer cells was evaluated (Fig. 6A). For the HCT-116 cells, at 48 h, the percentage of cyclin A positive cells in the untreated control was 8.6%. With treatment concentrations of 2.5 and 5 μg/ml, for the extract, the proportion of cyclin A positive cells increased to 14.6% and 16.5%,
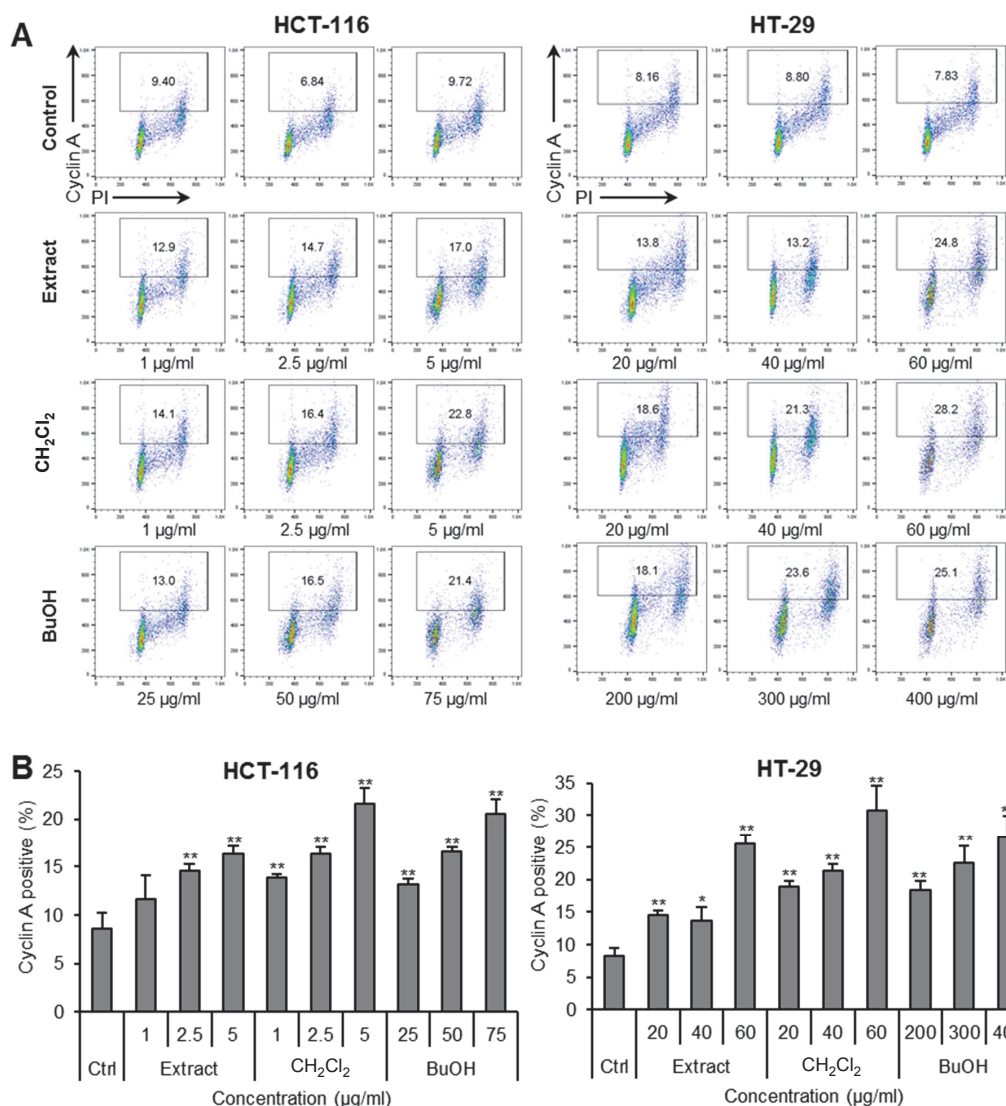

Fig. 6. Expression of cyclin A analysis of HCT-116 and HT-29 cells treated with *O. elatus* extract and fractions. Cells were treated with extract/fractions for 48 h, then fixed in ethanol and stained with propidium iodide and cyclin A. (**A**) Representative scatter plots of cyclin A (y-axis) versus PI (x-axis), and the percentage of cyclin A positive cells is shown in the gate. (**B**) Percentage of cyclin A positive cells with various treatments or with control. Data are presented as the mean ± standard error of triplicate experiments. * $p < 0.05$, ** $p < 0.01$ vs. control. CH_2Cl_2, dichloromethane fraction; BuOH, n-butanol fraction.

respectively; for the CH$_2$Cl$_2$ fraction, they were increased to 16.4% and 21.6%, respectively (Fig. 6B). Similar results were also observed in HT-29 cells. Compared to control (8.3%), 48 h treatment with 40 and 60 µg/ml of extract and CH$_2$Cl$_2$ fraction increased cyclin A positive cell proportions to 25.5% and 30.7%, respectively. BuOH fraction also arrested cell cycle in G2/M phase in both cell lines, but active concentrations are relatively high. Therefore, treatment with *O. elatus* extract and fractions caused a marked increase in the expression of cyclin A on human colorectal cancer cells, of which CH$_2$Cl$_2$ showed more potent activity.

Identification of active compound from O. elatus

To evaluate the contribution of representative single compounds to the antiproliferative activities of the extract and different fractions, two isolated polyynes were used to test their effects on HCT-116 and HT-29 cells. HPLC analysis showed that the major constituents in the *O. elatus* extract are fal-

carindiol and oplopandiol. They are also the major components in CH$_2$Cl$_2$ and BuOH fractions. As shown in Fig. 7A, for the HCT-116 cell line, treatment with 1–5 µM of oplopandiol for 48 h yielded no observable cell inhibitory effect, while at 2 and 5 µM, falcarindiol inhibited cell growth by 68.8% and 97.4% (both $p < 0.01$ vs. control), respectively. The IC50 for falcarindiol and oplopandiol was 1.7 µM and 15.5 µM, respectively. For the HT-29 cell line, oplopandiol did not show obvious antiproliferative effects up to 40 µM. Falcarindiol showed significant antiproliferative effects at 10 and 20 µM ($p < 0.01\%$), and IC50 of falcarindiol was 13.2 µM, in comparison to oplopandiol of >60 µM (Fig. 7B). These two compounds were found to have the highest contents in the extract and the active CH$_2$Cl$_2$ fraction, which represents the biological effects of the extract and fraction. Because oplopandiol showed low to moderate activity, while falcarindiol showed very potent antiproliferative effects, falcarindiol has been identified as an active constituent in the *O. elatus*.
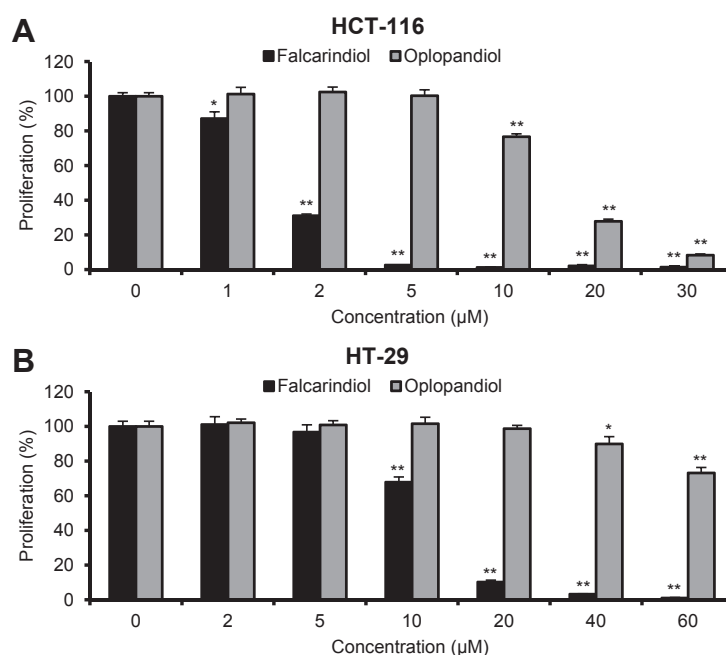

Fig. 7. Antiproliferative effects of falcarindiol and oplopandiol on human colorectal cancer cell lines HCT-116 (**A**) and HT-29 (**B**) Cancer cells were exposed to compounds (1–60 µM) for 48 h and cell proliferation was determined by the MTS assay. The results are expressed as the mean ± standard error of triplicate experiments. * $p < 0.05$; ** $p < 0.01$ vs. control.

Discussion

Human colorectal cancer is a leading cause of cancer-related death in the United States and worldwide. Half of all patients diagnosed with colorectal cancer eventually die from the disease; only less than 10% of patients with metastatic colorectal cancer will survive more than five years after diagnosis (Siegel et al., 2021). A number of recent controlled clinical trials supported a multimodal and multidisciplinary approach (including a combination of treatments and the schedule in which they are administered) to treating both early and advanced stages of colorectal cancer (Hurwitz et al., 2004; Redondo-Blanco et al., 2017). Studies also showed that patients with cancer often resort to complementary and alternative medical means to treat cancer, cancer-related symptoms, and/or

to reduce the side effects of chemotherapy (Cragg et al., 2009; Wang et al., 2012a). An estimated 30–50% of cancer patients use complementary and alternative medicines, including herbal medicines, along with their conventional medication (Buckner et al., 2018; Ernst 2000). While some beneficial effects of selected botanicals are documented, there are limited data to indicate whether herbs are effective in cancer therapeutics, and their mechanisms of action are unexplained as of now (Masui et al., 2013).

Plants from *Oplopanax* genus have been used as herbal medicines, and *O. horridus* has mainly been used by the Pacific indigenous peoples. In fact, *O. horridus* has reportedly been used by people from over 38 linguistic groups for the treatment of upwards of 34 different medical conditions (Lantz et al., 2004). In comparison to the long history ethnic medical use of *Oplopanax* plants, modern pharmacological studies on

this group of herbs are relatively fewer. *O. horridus* is a north American native botanical. Regarding *Oplopanax* species, most phytochemical and pharmacological reports are focused on this species (Calway et al., 2012; Huang et al., 2014). Several reports, including ours, indicated that *O. horridus* extract and dichloromethane fraction possess significant anticancer potential, and active constituents in this herb are identified as a group of polyynes (Sun et al., 2010; Wang et al., 2013). However, because many polyynes were identified from *O. horridus* and the contents of polyynes in the herbal extract are very low, it is difficult to standardize the herbal extract or fractions with polyynes (Huang et al., 2010).

O. elatus is another species in the genus *Oplopanax*. Recent studies have shown that *O. elatus* has significant anticancer activity (Wang et al., 2019a, b). Moreover, because there are only two major polyynes in the extract, the constituents in the herb are not as complicated as *O. horridus*, which makes it much easier than *O. horridus* to standardize the extract or active fraction. In this study, phytochemical and biological investigations were conducted on this herbal medicine.

We prepared three fractions by using dichloromethane and n-butanol extraction. Our pilot data showed that only two major constituents were found in the *O. elatus* extract and fractions. Thus, single compound isolation was focused on these compounds. With efficient preparative HPLC, we isolated these two compounds and identified them as falcarindiol and oplopandiol. HPLC analysis showed that these two compounds have relatively high content in the extract (72.7%). After dichloromethane extraction, the content of these two compounds was increased to 81.2%. Thus, polyyne content in the *O. elatus* extract and dichloromethane fraction is much higher than that in *O. horridus* extract and fraction (Huang et al., 2010; Sun et al., 2010). Analytical data encouraged us that *O. elatus* components may have stronger anticancer effects than *O. horridus*.

Using MTS assay, the antiproliferative effects of *O. elatus* and three fractions were determined. The whole extract inhibited colon cancer cell growth. The dichloromethane fraction (CH_2Cl_2) showed more potent antiproliferative effects than the extract. In contrast, the water fraction (H_2O) did not show any antiproliferative effect. In addition, the n-butanol fraction (BuOH) showed weak antiproliferative effects due to low content of polyynes. We recognized that the CH_2Cl_2 fraction is an active antiproliferative fraction.

Apoptosis is programmed cell death, a highly regulated process used to eliminate unwanted or defective cells (Wang et al., 2012b). Many chemotherapeutic agents, natural compounds, radiation, immunotherapy, and cytokines induce cancer cell death via the apoptotic pathway (Friedman, 2002). We observed *O. elatus* extract and fractions on the apoptotic induction of HCT-116 and HT-29 cells. The total extract and two fractions obviously induced cell apoptosis, while the active concentration for BuOH is relatively high. The CH_2Cl_2 fraction, which possessed the most potent anti-proliferative activity, has been identified as an active fraction. The CH_2Cl_2 fraction showed the strongest apoptotic induction activity (Fig. 4). This result suggests that antiproliferation of *O. elatus* extract and active fraction on human colorectal cancer cells is mediated in part by the induction of apoptosis.

Cancer cells lack normal growth controls, exhibit loss of cell cycle control, have unlimited reproductive potential, and have growth-signal self-sufficiency. Any compound aimed at controlling these processes would be beneficial in suppressing cancer progression (Lin et al., 2017). We investigated the effects of *O. elatus* extract and fractions on the cancer cell cycle.

In low concentrations, the extract and CH_2Cl_2 fraction decreases the S-phase and mainly arrests cells in the G2/M-phase but does not influence the proportion of the G1-phase (Fig. 5). G2/M phase arrest was also observed in BuOH fraction, but only in high treatment concentration. Both cell lines showed a similar trend, while active concentration on HT-29 cells is higher than on HCT-116 cells.

Since cyclins and cyclin-dependent kinases regulate cell cycle progression, and cyclin A is an important regulator of G2/M events (Zhao et al., 2016), we determined the expression of cyclin A in relation to the extract and fractions' G2/M-phase arrest. After treatment with CH_2Cl_2 fraction for 48 h (5 µg/ml for HCT-116, 60 µg/ml for HT-29), the fraction of cyclin A positive cells increased to 21.7% for HCT-116 and 30.7% for HT-29 cells; in untreated cells, the fraction was only 8.7% and 8.3%, respectively (Fig. 6). Because the accumulation of cyclin A was critical to promote cell cycle arrest in the G2/M-phases, our data proved that the active CH_2Cl_2 fraction-induced G2/M-phase arrest was regulated by the key cell cycle regulator cyclin A.

The biological activity of *Oplopanax* plants is the result of polyynes, which is believed to be the main active constituents in the herbs (Huang et al., 2014). While several but low content polyynes were detected in *O. horridus* extract (Huang et al., 2010), in this study, we demonstrated that in *O. elatus*, only two major polyynes were determined. These two polyynes were used to test for antiproliferative effects on colorectal cancer cells. From the results shown in Fig. 7, oplopandiol did not show a potent antiproliferative effect up to 10 µM on HCT-116 cells and 40 µM on HT-19 cells. In contrast, at much lower concentrations (2 µM on HCT-116, and 10 µM on HT-29), falcarindiol showed very potent antiproliferative effects. Falcarindiol's IC50 for HCT-116 cells was 1.7 µM (vs. oplopandiol of 15.5 µM) and, for HT-29 cells, was 13.2 µM, in comparison to oplopandiol of >60 µM. Thus, falcarindiol has been identified as an active constituent in the extract and active fraction. The chemopreventive mechanisms of falcarindiol in cancer cells and related illness will be investigated in our future studies. Because falcarindiol occupied a proportion of approximately 16% in the extract and active fraction, the cancer cell growth inhibition of *O. elatus* is a major contributor by falcarindiol. Due to the relatively simple composition in the herb and active fraction, in future studies, falcarindiol is a reasonable selection in standardizing herbal extract or active fraction.

To investigate responses of herbal components in different types of cancer cells, we employed two human colorectal cancer cell lines varied in p53 expression. HCT-116 is p53 wild type, while HT-29 cells contain a p53 mutation (Watson et al., 2010). Cancer cells with p53 mutations are resistant to many chemotherapeutic agents (Fan et al., 2014). Exposure to cellular stress triggered the p53 tumor suppressor and induced cell cycle arrest and apoptosis (Yogosawa and Yoshida, 2018). Our data showed that HCT-116 cells were more sensitive than HT-29 cells. Compared to HT-29, lower concentration of extract/fraction treatment inhibited HCT-116 cell growth, indicating that p53 plays a role in *O. elatus*-mediated colon cancer cell growth inhibition.

Conclusions

In summary, due to the limited reports available, the chemical composition and biological functions of *O. elatus* are still unknown. Our study filled the gap between the phytochemical basis and its potential in colorectal cancer chemoprevention.

HPLC analysis supplied original information of constituents in *O. elatus* extract and fractions, including the essential structural information of detected constituents. Pharmacological data obtained from this study suggested that *O. elatus* possesses significant chemopreventive potential on different types of human colorectal cancer cells. The dichloromethane fraction is an active fraction, and an active constituent has been identified as falcarindiol. The mechanisms involved in cancer chemoprevention by *O. elatus* were induction of apoptosis and cell cycle arrest at the G2/M phase mediated by a key cell cycle regulator cyclin A.

Author contributions
CZW, WD, MX and CSY designed the study, set up the experiments, participated in data collection, analyzed and interpreted the results, and drafted the manuscript. YL, WHH, JZ, CFZ and LC carried out some experiments and participated in the related data interpretations. All authors have read and approved the final manuscript.

Conflict of interests
The authors declare that they have no conflict of interests.

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