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Biological activity and alkaloid compounds of *Mahonia napaulensis* DC. extracts

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ABSTRACT: *Mahonia napaulensis* DC. has historically been used for traditional medicine in Vietnam. However, the biological mechanisms underlying its medicinal effects are not yet fully understood. In the present study, a crude extract derived from the *M. napaulensis* stem was fractionated with *n*-hexane, ethyl acetate (EtOAc), and water-saturated butanol (BuOH). The antioxidant activities of the crude extract and the fractionated samples were evaluated using a DPPH assay and a reducing power assay. Our results revealed that the EtOAc fraction showed the highest total phenolic content as well as the highest free radical scavenging activity and reducing power. The antiproliferative effects of *M. napaulensis* DC. extracts were detected using a MTT assay. The results showed a significant reduction in cell viability of over 70% in human colon carcinoma CoLo 205 cells at the highest concentration tested (200 μg/ml). We also used quantitative reverse transcription-polymerase chain reaction (RT-PCR) to measure the expression of mRNA transcripts involved in the regulation of cell proliferation such as survivin, p53, caspase-3, and caspase-9 in CoLo 205 cells treated with the crude extract and fractions to better understand the changes induced by the BuOH fraction. Two alkaloid compounds that possess strong antiproliferative activities in colon cancer cells, berberine and palmatine, were isolated from the BuOH fraction. The results from this study indicate that *M. napaulensis* may be considered a medicinal plant due to its anti-oxidative and antiproliferative properties.

KEYWORDS: antioxidant, anti-proliferative, *Mahonia nepalensis* DC, alkaloids compounds

INTRODUCTION

Cancer is the second leading cause of death in the Western world [1] associated with approximately 7.6 million deaths worldwide, which is predicted to increase to 13.1 million deaths by 2030 [2]. Free radicals are defined as atoms or molecules that have one or more unpaired electrons such as reactive oxygen species (ROS) [3]. Elevated ROS levels can lead to oxidative stress, causing DNA damage or mutations [4] and inducing the cellular processes underlying the development of cardiovascular disease, diabetes, and aging [5]. Antioxidants interact with ROS and other free radicals, donating an electron to the reactive molecules, leading to their stabilization and preventing the adverse effects and chronic complications associated with oxidative stress [6]. There are a number of synthetic phenolic antioxidants. Butylated hydroxyanisole (BHA) and butylated hydroxytoluence (BHT) are the two main synthesized antioxidants, but some reports indicated the possibility of carcinogenic effect and high dose toxicity to the cell. As a result of such restriction, natural antioxidants are now being given significant attention because of their minimal side effect and limited toxicity.

Various agents have been identified to show a wide range of anticancer effects, but these drugs often have unpleasant side effects and other complications [7]. Thus, alternative medicines are necessary, preferably herbal therapies. *Mahonia napaulensis* DC. is a fully hardy perennial evergreen shrub with yellow flowers widely distributed in the high mountainous areas at altitudes between 1700–1900 m in China, Nepal, and Bhutan [8]. The *M. napaulensis* DC. stem has been found to exhibit anti-inflammatory, anti-bacterial, and antifungal activities and is used particularly for the treatment of eczema, psoriasis, and other skin conditions [9]. However, the bioactive compounds underlying these effects have not been identified. Therefore, this study evaluated *M. napaulensis* crude extract and various derived fractions for their antioxidant activities and potential antiproliferative effects against cancer cells, in addition to determining the mechanisms driving these effects.

MATERIALS AND METHODS

Plant material and preparation of crude extract and fractions

The stem of *M. napaulensis* was collected in Hoa Binh province, a mountainous province of Vietnam. The plant was identified and authenticated by Dr. HeoK-

weon, a taxonomist in the department of Applied plant sciences of Kangwon National University. A voucher specimen is deposited in the herbarium of Department of Applied plant sciences, Kangwon National University. *M. napaulensis* stem pieces were dried and extracted three times with 100% methanol. Then, the methanol extract was filtered and then evaporated under reduced pressure using a vacuum rotary evaporator to produce a crude extract. After that, the crude extract was suspended in distilled water and then partitioned with *n*-hexane, ethyl-acetate (EtOAc), and butanol (water saturated BuOH). The extract and fractions were evaporated using a vacuum rotary evaporator. The dried samples were weighed and kept in a refrigerator at −20 °C for further analysis.

Analysis of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu reagent method [10]. Briefly, 0.1 ml of the diluted sample was reacted with 0.05 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, F9252, USA) for about 4 min, and then a 0.3 ml of 20% sodium carbonate was added into the reaction mixture and shaken immediately. Finally, 1 ml of distilled water was added after incubating at room temperature for 15 min. The absorbance reading was taken at 725 nm in triplicate, and the results were expressed as gallic acid equivalent per gram of extract by referencing the gallic acid standard calibration curve (mg GAE/g).

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of fraction, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) was assayed using a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). The reaction mixture contained 1 ml of 0.15 mM DPPH (59.148 mg/l in 100% methanol) and 4 ml of 100% methanol and the extract or fraction at various concentrations. The reaction mixture was allowed to stand for 30 min at room temperature, and the radical scavenging activity of each antioxidant was quantified by decolorization at 517 nm. The IC_{50} value (mg/ml) is the concentration at which the scavenging activity is 50%.

Reducing power assay

The reducing power of *M. napaulensis* was measured according to a method of Oyaizu [11] with some modifications. Predetermined concentrations of samples in 0.1 ml were mixed with 0.1 ml of sodium phosphate buffer (pH 6.6, 0.2 M) and 0.1 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 0.1 ml of 10% trichloroacetic acid (w/w), 0.4 ml of distilled water was then added, followed by 0.05 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm, which is directly related to the reducing power of intrinsic active substances. Ascorbic acid was used for comparison.

Cell culture

The 293 (Human Embryonic Kidney; p53-wild type), CoLo 205 (Human colo carcinoma cell; KRAS-WT, BRAFV600E), AGS (Human gastric carcinoma cell; p53-wild type), and PC-3 (human prostate cancer cell; p53-wild type) cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The 293 cells were cultured in Dulbecco's modified Eagle (DMEM) medium (supplemented with 10% fetal bovine serum (FBS)) and 1% of penicillin. CoLo 205, AGS, and PC-3 cells were grown in RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 10% FBS and 1% penicillin. Cells were incubated at 37 °C, 5% CO_2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The viability assay most commonly used throughout the world is the MTT assay. The cells were seeded at the density of 10^4 cells/well in a 96-well plate and incubated for 24 h, and then 100 µl of diluted samples made up in medium was treated to each well. The culture supernatants in the culture wells were collected after 24 h incubation, and 0.1 ml of MTT (0.5 mg/ml) solution was added. After an additional 4 h incubation, the formazan crystals were dissolved by adding DMSO (0.1 ml). The absorbance was read at 540 nm.

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR)

The CoLo 205 cells were incubated in a 6-well plate with experiment predetermined density. After 24 h incubation, cells were treated with desired concentrations of samples and then were harvested to use for further investigation. Total RNA from the CoLo 205 cells was extracted following the protocol of iNtRON Biotechnology Inc. (Seoul, Korea). Then, the first strand cDNA was synthesized from 2 µl of total RNA containing oligo (dT) primers and Moloney murine leukemia virus reserve transcriptase (M-MLV RT). The primer and probe sequences and concentrations were optimized according to the manufacturer's instructions in exTaq DNA polymerase and were as followed:

Survivin-F, 5′-GGACCACCGCATCTCTACAT-3′;
Survivin-R, 5′-GCACTTTCTTCGCAGTTTCC-3′;
p53-F, 5′-GCGCACAGAGGAAGAGAATC-3′;
p53-R, 5′-CTCTCGGAACATCTCGAAGC-3′;
Caspase-3-F, 5′-GAACTGGACTGTGGCATTGA-3′;
Caspase-3-R, 5′- TGTCGGCATACTCTTTCAGA-3′;
Caspase-9-F, 5′- TGGACGACATCTTTGAGCAG-3′;
Caspase-9-R, 5′- GCAAGATAAGGCAGGGTGAG-3′;
β-actin-F, 5′-GGATTCCTATGTGGGCGA-3′; and
β-actin-R, 5′-CGCTCGGTGAGGATCTTCATG-3′.

Table 1 Total phenolic content and DPPH radical scavenging activity of extract and fractions from *M. napaulensis*.

Fraction	DPPH radical scavenging activity, IC_{50} (μg/ml)	TPC† (mg GAE/g)
MeOH	92.36 ± 2.15^{b}	74.11 ± 1.16^{b}
n-Hexane	128.28 ± 0.14^{d}	57.81 ± 1.11^{c}
EtOAc	48.93 ± 0.59^{a}	122.94 ± 4.93^{a}
BuOH	104.64 ± 1.79^{c}	94.92 ± 0.28^{b}
Water	223.43 ± 4.02^{e}	4.95 ± 1.05^{d}
BHA	7.29 ± 0.06	
BHT	125.25 ± 0.80	
Ascorbic acid	2.40 ± 0.22	

† Total phenolic contents, [a–d] Means values followed by the same letter in the same column are not significantly different at $p < 0.05$ by Duncan's multiple range test.

Isolation of biological compounds

The BuOH fraction (20.8 g) was dissolved in sulfuric acid and filtered, after which the extract was suspended in ammonia water and rinsed with saturated sodium chloride solution to yield four fractions (Fr. 1–4). Fr. 3 (8.4 g) was chromatographed on alumina gel (90.4 g, 0.2–0.1 mm) by gradient elution with chloroform:methanol (50:0–0:50) and then separated by alumina gel by isocratic elution with ethyl alcohol:water (7:3) to produce compound 1 (98.2 mg) and compound 2 (119.8 mg). The two compounds were identified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses. ^{1}H and ^{13}C NMR profile were adapted as supplementary materials.

Statistical analysis

Data were statistically evaluated via Duncan's one-way analysis of variance (ANOVA). The level considered significant when $p < 0.05$. Data were presented as means ± standard errors.

RESULTS AND DISCUSSION

Total phenolic content

Phenolic compounds are constituents of many plants and have attracted a great deal of public and scientific interest because of their health-promoting effects as antioxidants [12, 13]. Thus, the TPCs (total phenolic contents) of *M. napaulensis* DC. crude extract and solvent fractions were analyzed in this study, and the results are displayed in Table 1. The TPCs differed among the crude extract and solvent fractions depending upon their distinct polarities. Briefly, the ethyl acetate (EtOAc) fraction showed the highest TPC (122.94±4.93 mg GAE/g), followed by the BuOH fraction (94.92±0.28 mg GAE/g). The aqueous fraction had the lowest TPC (4.95±1.05 mg GAE/g). These results align with the findings from a previous study on the antioxidant activity of *Zostera marina* L. extracts conducted by Gao et al [14].

DPPH radical scavenging activity

The free radical scavenging activities of *M. napaulensis* DC. crude extract and solvent fractions are displayed in Table 1. The results revealed that the EtOAc fraction of *M. napaulensis* DC. extract exhibited the strongest ability to reduce DPPH free radicals (IC_{50}, 48.93±0.59 μg/ml) compared with the other fractions and the control, 2,6-di-tert-Butyl-4-methylphenol (BHT; IC_{50}, 125.25±0.80 μg/ml). The EtOAc fraction was also reported to have the highest DPPH radical scavenging activity in a previous study by Yang et al [15], in which they evaluated the free radical scavenging activity and inhibition of platelet function of a polyphenol-rich fraction of *Salvia miltiorrhiza* Bunge extract. This prior study also reported that the EtOAc fraction possessed the highest TPC relative to other fractions, similar to our findings. This result was corroborated by Sahreen et al [16] in a report demonstrating a significant correlation between TPC and DPPH free radical scavenging activity.

Reducing power assay

In the present study, the reducing power of different concentrations of *M. napaulensis* DC. crude extract and solvent fractions were investigated. Our results indicate that the reducing power increased in a concentration-dependent manner and differed significantly among the crude extract and the solvent fractions. The greatest reducing activity was detected in the EtOAc fraction, for which we observed absorbance values of 0.578 at 100 μg/ml, 0.743 at 300 μg/ml, and 0.815 at 500 μg/ml. These values were similar to those observed for ascorbic acid (0.802 at 100 μg/ml, 0.849 at 300 μg/ml, and 0.946 at 500 μg/ml; Fig. 1). In a previous study examining the antioxidant activities of methanol fractions of *Polyalthia longifolia* var. Pendula leaf extract, Adaramola et al [17] also reported that the highest reducing power was found in the EtOAc fraction. They also observed correlations between reducing power, TPC, and DPPH radical scavenging activity, in which the EtOAc fraction possessed high TPC and strong antioxidant activity in the DPPH radical scavenging activity assay. Reductones inhibit lactoperoxidase (LPO) by donating a hydrogen atom, terminating the free radical chain reaction [18]; therefore, the antioxidant activity of *M. napaulensis* DC. may be related to its reducing power.

Cytotoxicity of *M. napaulensis* DC. extract in 293 cells, CoLo 205 cells, AGS, and PC-3 cells

The BuOH fraction did not exhibit significant cytotoxic effects in 293 cells at concentrations of 100 or 200 μg/ml. However, we observed cytotoxic effects when the *n*-hexane fraction was applied to 293 cells at a concentration of 200 μg/ml (cell viability < 80%; Fig. 2). In the MTT assay, all fractions inhibited the proliferation of CoLo 205 cells in a concentration-

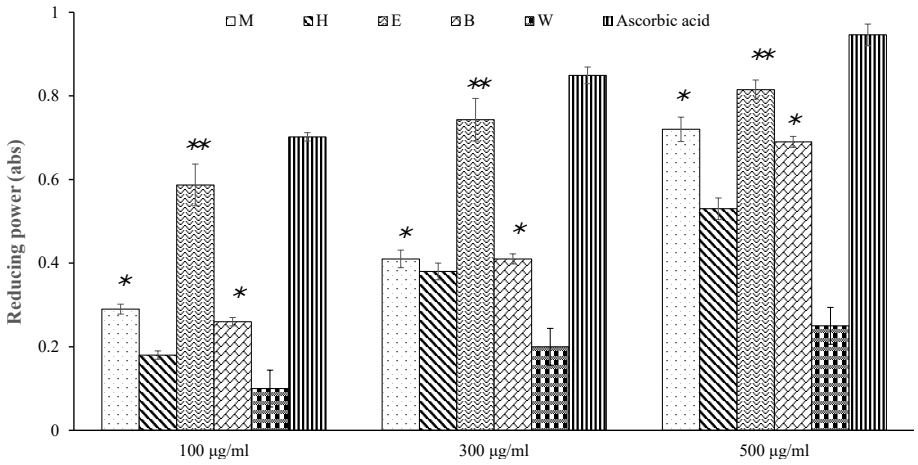

Fig. 1 Reducing power of extract and fractions from *M. napaulensis*. M: Methanol extract, H: *n*-hexane fraction, E: ethyl acetate fraction, B: butanol fraction, and W: water fraction; Data are means ± SD of three experiments performed in triplicate ($^{*}p < 0.05$, $^{***}p < 0.001$).

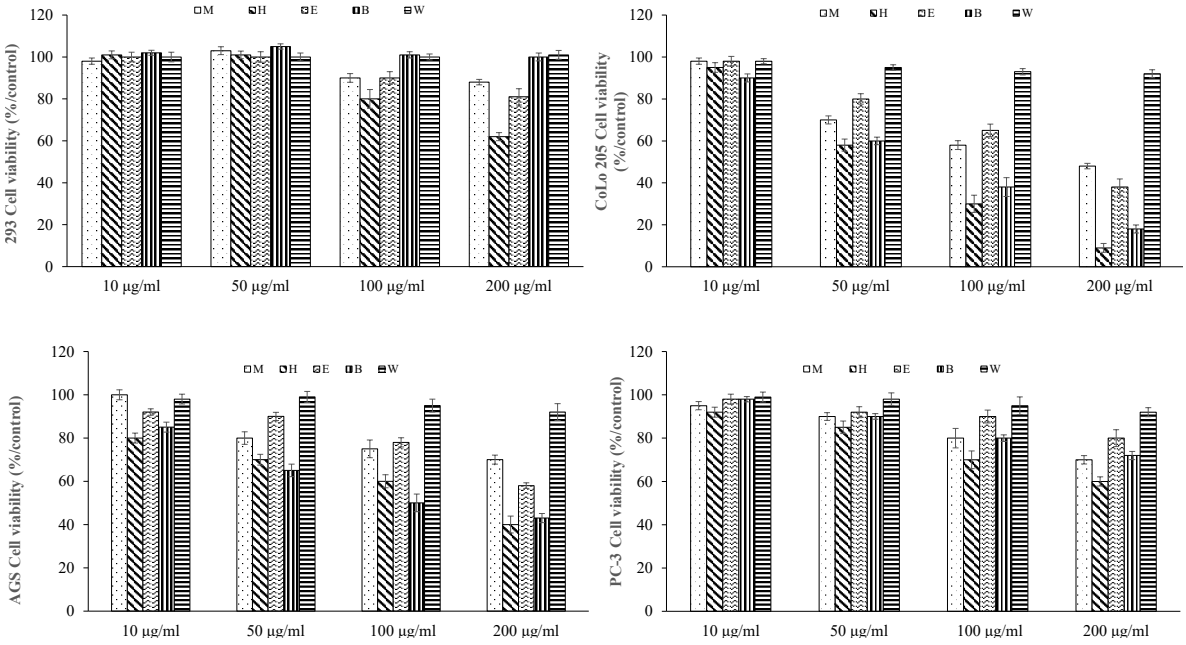

Fig. 2 Inhibitory effect of extract and fractions from *M. napaulensis* on proliferation of cells. M: methanol extract, H: *n*-hexane fraction, E: ethyl acetate fraction, B: butanol fraction, and W: water fraction.

dependent manner. At the highest concentration tested (200 µg/ml), cell viability ranged from 40.21% to 17.66% across the crude extract and solvent fractions. The *n*-hexane fraction exhibited the greatest inhibition of cell proliferation (cell viability, 17.66%), followed by the BuOH fraction (cell viability, 22.01%). However, because the *n*-hexane fraction exhibited cytotoxic effects at 200 µg/ml, the inhibition of cell proliferation may have been the result of cytotoxicity.

The BuOH fraction also exhibited the greatest antiproliferative effects in AGS and PC-3 cells at a concentration of 200 µg/ml. Treatment of AGS and PC-3 cells with the BuOH fraction resulted in cell viability values of 8.87% and 14.87%, respectively, suggesting that the BuOH fraction exhibited anticancer activity in CoLo 205, AGS, and PC-3 cells (Fig. 2). These results were similar to those reported in a previous study by Gao et al [15], in which the BuOH fraction of *Sauromatum giganteum* (Engl.) *Cusimano & Hett* extract was found to exhibit the strongest antitumor activity. The BuOH fraction was also found to effectively inhibit the proliferation of the breast cancer cell line MCF-7

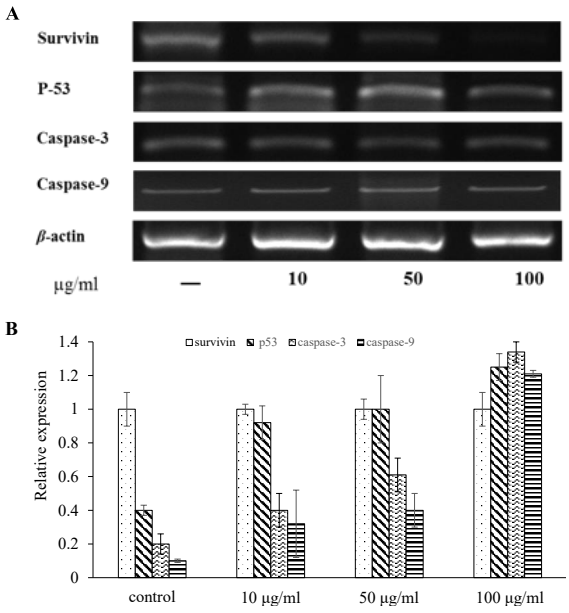

Fig. 3 The inhibition of gene expression of BuOH fraction from *M. napaulensis* in COLO 205 (Human colon cancer cell). A: Gel electrophoresis of amplified product of β-actin, survivin, p53, caspase-3, and caspase-9; B: Quantified bands of survivin, p53, caspase-3, and caspase-9 with β-actin as a reference.

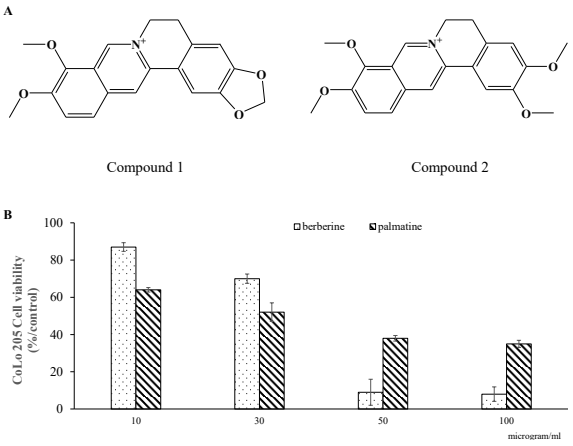

Fig. 4 Structures and antiproliferative activity of compounds. A: Structures of compounds 1 and 2 isolated from *M. napaulensis*; B: Inhibitory effect of compound 1 (berberine) and compound 2 (palmatine) on proliferation of COLO 205 cell.

[19]. In addition, Dhru et al [20] concluded that the *n*-butanol fraction of an extract of *Oroxylum indicum* root bark might be considered a potential source of anticancer compounds.

mRNA expression levels in CoLo 205 cells treated with the BuOH fraction from *M. napaulensis* DC. extract

Our results demonstrated that the BuOH fraction exhibited significant antiproliferative effects in the CoLo 205 cell line. These apoptotic effects could be mediated by several mechanisms. Anticancer agents are known to suppress the proliferation of cancer cells via enhancing apoptosis [21, 22]. Therefore, to further investigate the molecular mechanisms responsible for the effects of the BuOH fraction, the mRNA expression levels of several apoptosis-related genes were analyzed using RT-PCR. The expression of mRNA transcripts in CoLo 205 cells treated with the BuOH fraction is shown in Fig. 3. All concentrations of the BuOH fraction resulted in the decreased mRNA expression of survivin, p53, and caspase-3 compared with the unexposed control sample; however, caspase-9 mRNA expression increased in a concentration-dependent manner. Previous findings by Yamamoto et al [23] indicated that survivin is a highly conserved protein that is indispensable for cell survival during development. Downregulation of the survivin gene causes cell death through apoptosis or mitotic catastrophe. Survivin expression is induced together with p53 in response to DNA damage. Importantly, the level of p53 gene expression is largely cell type-dependent. The p53 gene is either non-functional or mutated in most human cancers, and p53 gene mutations are the most common mutations reported in human cancers. Moreover, the mutated form of p53 is commonly upregulated in tumors [24]. Thus, in this study, the downregulation of p53 suggests that p53 may be non-functional in CoLo 205 cells treated with the BuOH fraction. Gomyo et al [25] reported that the upregulation of caspase-9 induced apoptosis in human lung cancer cells. Therefore, the BuOH fraction likely induced apoptosis by both upregulating pro-apoptotic genes (caspase-9) and downregulating anti-apoptotic genes (survivin).

Identification of the chemical structures of the isolated compounds

The compounds derived from *M. napaulensis* DC. extract were identified by comparison of ^{1}H and ^{13}C NMR data with the literature. The structures of compounds 1 and 2, identified as berberine and palmatine, respectively, are presented in Fig. 4A.

Antiproliferative activities of palmatine and berberine

As shown in Fig. 4B, different concentrations of berberine and palmatine exhibited strong antiproliferative effects in CoLo 205 cells, resulting in dose-dependent reductions in cell viability. For example, berberine decreased CoLo 205 cell viability to 87% at 10 μg/ml, 68% at 30 μg/ml, and 8% at 100 μg/ml. We also observed similar antiproliferative effects for palmatine in cancer cells. Hu et al [26] analyzed the anticancer activity of berberine in HT-29 cells, a human colon

cancer cell line, which showed strong antiproliferative activity. Berberine has also been shown to exhibit strong antitumor effects in A549, Hep-G2, and MCF-7 cells [27] and amelioration of astrocyte activation and migration via NDUFC2 [28]. Further, in a report from Ma et al [29], ApcMin/+ mice, a genetically engineered model, were used to investigate the effects of palmatine on the initiation and progression of gut inflammation and tumorigenesis enhanced by a high fat diet. Palmatine from *Mahonia bealei* was shown to attenuate gut tumorigenesis in ApcMin/+ mice via inhibition of inflammatory cytokines. Palmatine also significantly inhibited the proliferation of colorectal cancer cells. These results suggest that berberine and palmatine may mediate the anticancer effects of the BuOH fraction from *M. napaulensis* DC. extract.

CONCLUSION

In summary, the results of the present study provide evidence for the antioxidant and antiproliferative activities of *M. napaulensis* DC. extract. In particular, we found that the EtOAc and BuOH solvent fractions derived from *M. napaulensis* DC. extract contained substantial phenolic contents and possessed robust free radical scavenging activities and reducing power. In addition, the BuOH fraction demonstrated the robust inhibition of human colon cancer cell proliferation. Furthermore, we identified the alkaloid compounds, berberine and palmatine, as the bioactive components most likely to be responsible for the antioxidant and antiproliferative activities of the BuOH fraction. Overall, our findings support the potential clinical utility of *M. napaulensis* DC. extract as a therapeutic agent for colon cancer.

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