


The Hexane Fraction of *Bursera microphylla* A. Gray Induces p21-Mediated Anti-Proliferative and Pro-Apoptotic Effects in Human Cancer-Derived Cell Lines

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Abstract

Bursera microphylla (BM), one of the common elephant trees, is widely distributed in the Sonoran Desert in Mexico. The Seri ethnic group in the Sonoran Desert uses BM as an anti-inflammatory and painkiller drug for the treatment of sore throat, herpes labialis, abscessed tooth, and wound healing. Dried stems and leaves of BM are used in a tea to relieve painful urination and to stimulate bronchial secretion. Furthermore, BM is used for fighting venereal diseases. To investigate the effects of the hexane fraction of resin methanol extract (BM-H) on cell growth, the acute myeloid cell line (OCI-AML3) was treated with 250, 25, or 2.5 µg/mL of BM-H. The first 2 concentrations were able to significantly decrease OCI-AML3 cell number. This reduced cell number was associated with decreased S-phase, blockade of the G₂/M phase of the cell cycle, and increased cell death. Similar results were obtained on all tested tumor cell lines of different origins. We found that blockade of the cell cycle was due to upregulation of p21 protein in a p53-independent way. Increase of p21 was possibly due to upstream upregulation of p-ERK (which stabilizes p21 protein) and downregulation of p-38 (which promotes its degradation). Regarding cell death, activation of caspase-3, but not of caspase-8 or -9, was detectable after BM-H treatment. In conclusion, these data suggest that the BM's hexane fraction inhibited proliferation of cell lines mainly by a p21-dependent, p53-independent mechanism and promoted apoptosis through activation of caspase-3, but not caspase-8 or -9.

Keywords

Bursera microphylla A. Gray, p21, Mexican traditional medicine, anti-proliferative effect, pro-apoptotic effect, human cancer cell lines

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Introduction

Traditional medicine is a comprehensive term that refers to systems such as traditional Chinese medicine, Indian ayurveda, and Arabic Unani medicine, as well as various forms of indigenous medicine. Traditional medicine is often termed “complementary,” “alternative,” or “nonconventional” medicine¹ because it relies on the use of primarily natural products, although approximately 60% of commercially available drugs are derived from bioactive compounds extracted from natural sources.² Many of these compounds have resulted from scientific studies of remedies traditionally used by various cultures worldwide.^{2,3} For example, herbal medicine and acupuncture are the most widely used traditional medicine therapies, and Mexican traditional medicine (MTM) is based mainly on phytotherapy.³ In laboratory settings, plant extracts have demonstrated diverse pharmacological effects, including anti-inflammatory, vasodilatory, antimicrobial, anticonvulsant, sedative, and antipyretic benefits.⁴ Evaluation of traditional medicine product efficacy is difficult, and accurately identifying the plant and isolating its active ingredients is essential to herbal medicine. This isolation is complex because medicinal plant collection and the area of plant origin (including environmental conditions) can influence the secondary metabolites present. A single medicinal plant can produce hundreds of natural by-products, and determining which compound is responsible for a particular bioactivity can be prohibitively expensive. Yet given the worldwide popularity of herbal medicines, a widely applicable and cost-effective method for characterizing herbal medicines is urgently needed; among priority areas is research investigating mechanisms of action of individual compounds produced by the medicinal plant.¹

The plant genus *Bursera* is distributed throughout the southwestern United States of America, most of Mexico, and in Central American tropical forests reaching into northwestern South America.⁵ The genus is endemic to the tropical dry forest of Mexico, where 70 species are currently present,⁶ and it is characterized by medically relevant exudates that arise from a system of resin canals.⁷ For example, the essential oil and exudates of *Bursera* spp exhibit anti-inflammatory activity.⁸

Specifically, *Bursera microphylla* A. Gray (Burseraceae),⁹ commonly known as “elephant tree” or “torote blanco,” is largely distributed in the Sonoran Desert of Mexico. In folk medicine, *B. microphylla* is steeped in alcoholic beverages to make a tincture for treating gum sores, cold sores, and abscessed teeth. Its resin is used to treat venereal diseases, while its stems and leaves are used to prepare a tea that relieves painful urination and symptoms of bronchitis.¹⁰ The Seri ethnic group in the Sonoran Desert uses different parts of the plant for treating several conditions, such as sore throats, headaches, and certain wounds.¹⁰ Studies on *B. microphylla* leaf and twig essential oils showed they are composed mostly (89%) of terpenoids,¹¹ especially α - and β -phellandrenes. The

essential oil of its oleo-gum-resin is composed mainly of β -caryophyllene,¹² while burseran and deoxydopodophyllotoxin have been identified from its ethyl acetate resin extract.^{13,14}

The phytochemical composition of *B. microphylla* hexane extract led to the isolation of several terpenoids, among which 3 were new (malabaricatrienol, malabaricatrienone, and microphyllanin), and 4 known lignans, namely, burseran, ariensin, burseranin, and dihydroclusin acetate.¹⁵ In view of the known cytotoxic activity of the isolated lignans,¹⁴⁻¹⁶ we sought to test the anti-proliferative activity of *B. microphylla* resin extract on the acute myeloid leukemia (AML) cell line OCI,¹ since this cell line is positive for both p21 and p53, molecules critical for both proliferation and apoptosis. We also extended the study to other cancer-derived cell lines to determine the extract's possible mechanism of action.

One classical anti-proliferative pathway relies on p21, a cell cycle inhibitor important in DNA damage response and in many cellular processes during normal cell growth. The main function of p21 involves arrest of cell cycle progression by inhibiting the activity of cyclin-dependent kinases (Cdk); other roles include regulation of transcription, apoptosis, and cell motility.¹⁷ As a biomarker of cellular response to toxic stimuli, p21 expression and function have been the focus of numerous studies investigating the cytotoxic effects of certain chemicals.¹⁷

In this study, we tested the effect of the hexane fraction from a *B. microphylla* methanol extract on AML and other cancer cells.

Materials and Methods

Plant Material

The collection of botanically certified resin samples was performed by Ing. José Jesús Sánchez Escalante, head of the Herbarium of Universidad de Sonora, Hermosillo, México, in Bahía de Kino (28°55' N and 112°2.5' W) in January 2013. The resin was obtained with natural exudates present in the bark of the plant. It was stocked in 1.5 mL polyethylene tubes that were sterile and free of contaminants. Five different trees of *B. microphylla* were sampled. A voucher specimen of the species was deposited at Herbarium of Universidad de Sonora (No. 22039), and a voucher specimen of resin (No. BM-1) was deposited at the Department of Pharmaceutical Sciences, University of Perugia.

Preparation of the Hexane Fraction and Analysis of the Content

The ground resin (4.0 g) was extracted by maceration in MeOH (3 × 100 mL) at room temperature for 24 hours. After filtration, the organic solutions were concentrated at 40°C to give a crude MeOH extract that was diluted with H₂O (50 mL) and sequentially partitioned with *n*-hexane (3 × 50 mL) and CH₂Cl₂ (3 × 50 mL). The hexane fraction was

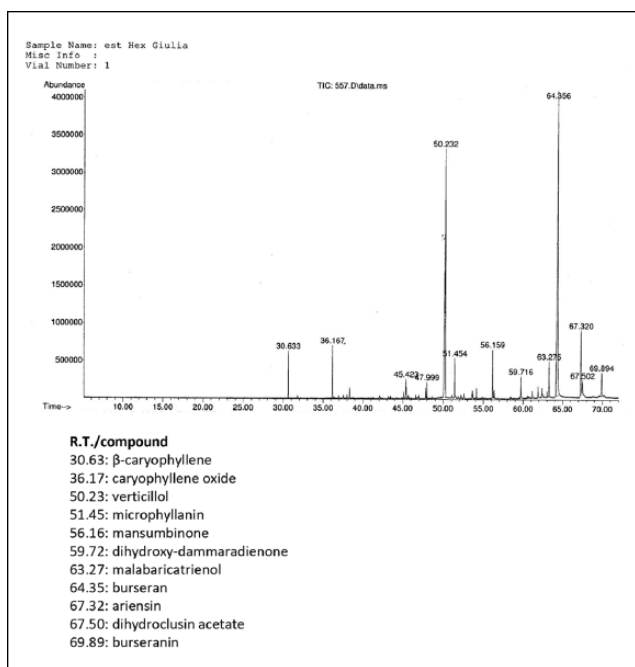


Figure 1. GC-MS fingerprint chromatogram of BM-H. Below the chromatogram, the list of compounds for each peak of the chromatogram is reported.

evaporated under a vacuum obtaining 0.90 g of extract. An amount of the hexane fraction was phytochemically studied and results were already reported.¹⁵

The extract was further characterized by a gas chromatography-mass spectrometry (GC-MS) fingerprint chromatogram: GC analyses were performed on an Agilent 6890N Network GC System, equipped with a flame ionization detector (FID) and a DB-35ms column (30 m \times 0.25 mm id, 0.25 μ m film thickness). The oven temperature was programmed from 40°C for 10 minutes, then ramped at 5°C/min to 300°C, and held for 10 minutes. Injector and detector temperatures were 250°C and 270°C, respectively. Sample was injected in the splitless mode using helium as carrier gas (1 mL/min); the samples were dissolved in dichloromethane to give 0.125 μ L/mL solution; the injection volume was 1 μ L (Figure 1).

Cell Line Culture and Characterization

OCI/AML3 (OCI), U937, HL-60, K562, and Jurkat cells (all lymphoma- or myeloma-derived) were maintained in RPMI medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C, 5% CO₂. C643 (thyroid carcinoma-derived) and MCF-7 (breast adenocarcinoma-derived) cells were maintained in high-glucose Dulbecco's Modified Eagle's medium with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C. HCT 116 cells, derived from human colon carcinoma, were maintained in McCoy's 5A medium with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C. All

cells were obtained from ATCC and were maintained in logarithmic growth and seeded in 24-well plates to evaluate their relative growth and morphologies. Cultures were maintained at 2×10^5 cells/mL and treated with varying concentrations of dimethyl sulfoxide or hexane fraction of resin methanol extract (BM-H; 250, 25, or 2.5 μ g/mL), were harvested after 24 hours, and counted with a hemacytometer.

Analysis of Cell Viability and Cell Cycle Progression

Cell viability and cell cycle progression were analyzed by flow cytometry to determine DNA content of cell nuclei stained with propidium iodide (PI). Briefly, cells were collected by centrifugation and washed in phosphate-buffered saline (PBS). DNA was stained by incubating the cells in PBS containing 50 μ g/mL PI and incubated for 30 minutes at 4°C. Fluorescence was measured and analyzed using a Becton Dickinson FACScan and Cell Fit software.

Western Blotting

Proteins were extracted by RIPA buffer, separated by SDS-PAGE, and analyzed by Western blotting. Primary antibodies (obtained from Cell Signaling, Danvers, MA, unless otherwise noted) included anti-caspase-3, anti-caspase-8 (Enzo Life Sciences, Farmingdale, NY), anti-p53, anti-p21, anti-phospho-p38, anti-p38, anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT, anti-BIM, anti-Puma, and anti-Bcl2 (Santa Cruz Biotechnology, Dallas, TX). Anti-actin and anti-laminin antibodies (Sigma-Aldrich, St Louis, MO) were used as controls. Secondary antibodies were labeled with horseradish peroxidase (Pierce/Thermo-Fisher Scientific, Waltham, MA). Antigen-antibody complexes were revealed by enhanced chemiluminescence by following the manufacturer's instructions (Millipore, Billerica, MA). Western blotting films were scanned, and band signal intensities were determined using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Statistical significance was determined using the Holm-Sidak method. Individual group means were compared using the Student's unpaired *t* test. Differences were considered statistically significant according to the following criteria: **P* < .05; ***P* < .01; ****P* < .001.

Results

Dose-Dependent Effect of BM-H on OCI-AML3 Cells

Because previous studies have shown that different compounds isolated from *B microphylla* have anti-proliferative

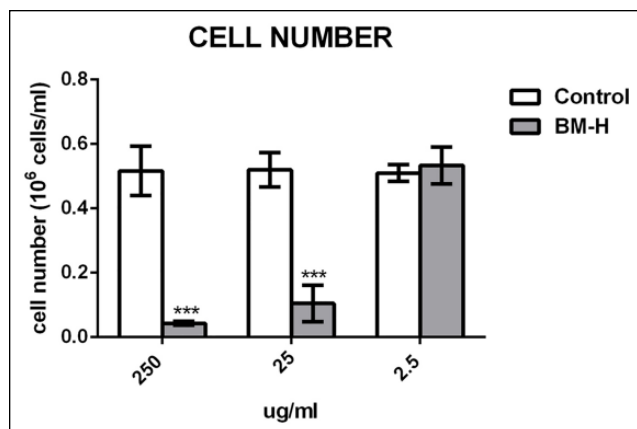


Figure 2. Effect of BM-H on OCI cell number. Bars represent the number of viable cells counted after 24 hours of treatment with either vehicle (white: control) or with BM-H (gray: BM-H) at the concentrations reported on the x-axis. Data from 3 independent experiments are reported as mean \pm SEM. *** $P < .001$.

effects,¹⁸ the possible impact of BM-H on p21/p53 positive OCI cells was tested. Figure 2 shows that after 24 hours of treatment with BM-H, the number of OCI cells was significantly decreased compared to the vehicle (control) at concentrations of 250 or 25 μ g/mL. Thus, 25 μ g/mL BM-H and a 24-hour incubation time were used in further experiments unless otherwise noted.

Effect of BM-H on Cell Death and Cell Cycle Progression

The BM-H-induced decrease in cell number could have been due to increased cell death, decreased proliferation, or both. Staining of nuclei with PI and subsequent flow cytometry analysis were applied to investigate both the cell cycle (indicator of the proliferation status of the cells) and frequency of cell death in BM-H-treated and nontreated cells. As shown in Figure 3A, we saw a significant decrease of cells in the S phase and an accumulation of cells in the G₂/M phase. The BM-H-dependent decrease in OCI cell number was due, at least partly, to blockage of DNA synthesis and mitosis. Figure 3B shows that, when cell death was analyzed under the same conditions, BM-H treatment also significantly increased occurrence of cell death. Thus, the decrease of OCI cell number was caused by BM-H-induced cell cycle arrest and cell death.

Effect of BM-H on Hematologic and Solid Tumors

We further investigated BM-H's effects on additional cancer cell lines. Specifically, Figure 4A shows that BM-H significantly decreased numbers of OCI, acute myeloid leukemia cells (U937, HL-60, and K562), and T-cell lymphoma Jurkat cells. Similar results were observed with

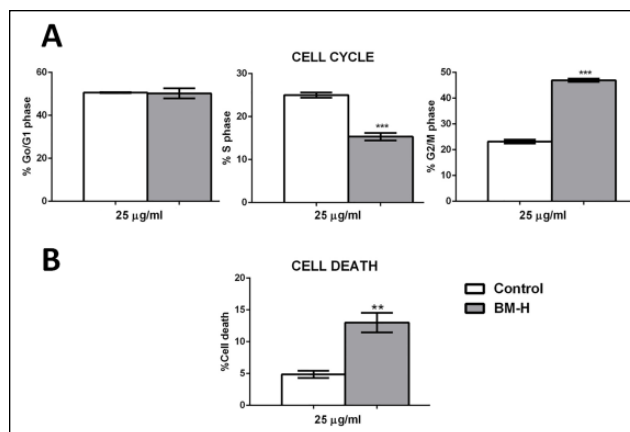


Figure 3. Effect of BM-H on OCI cell cycle progression and cell death. (A) Bars represent percentage of cells in G0/G1 (left panel), S (middle panel), or G2/M (right panel) phase after 24 hours of treatment with either vehicle (white: control) or with BM-H (gray: BM-H) at the concentrations reported on the x-axis. (B) Bars represent frequency of cell death under conditions described above. Data from 3 independent experiments are reported as mean \pm SEM. ** $P < .01$, *** $P < .001$.

respect to the solid tumors-derived cell lines C643, HCT 116, and MCF-7 (Figure 4B). These results indicate BM-H exerts a growth inhibitory effect on acute myeloid leukemia cells, other hematologic malignancies, and solid tumor-derived cells.

Effect of BM-H on Cell Proliferation Pathways

Because BM-H blocked cells in the G₂/M phase, we analyzed p21's possible role in BM-H-induced cell cycle arrest, as it regulates mitotic progression and promotes cellular stress response.¹⁹ We used Western blotting to measure expression of p21 in vehicle- and BM-H-treated cells. As seen in Figure 5, BM-H induced a strong time-dependent upregulation of p21 evident after 4 hours of treatment. Because p21 is regulated through either p53-dependent or p53-independent pathways,^{20,21} we also measured expression of p53, which did not change after treatment with BM-H. These expression data suggest BM-H induces activation of a p21-dependent, p53-independent pathway in OCI cells that could be responsible for their cell cycle arrest.

Effects of BM-H on the Cell Proliferation Pathways of Hematologic and Solid Tumors

To determine if the mechanism described above is also applicable to the other tested cancer cell lines, both hematologic and solid tumor cell lines were probed for the expression of p21 and p53 in Western blot experiments. Figure 6A shows that p21 is upregulated both in hematologic (U937, HL-60, and K562) and solid tumor (C643, HCT 116, and MCF-7) cell lines. The experiments

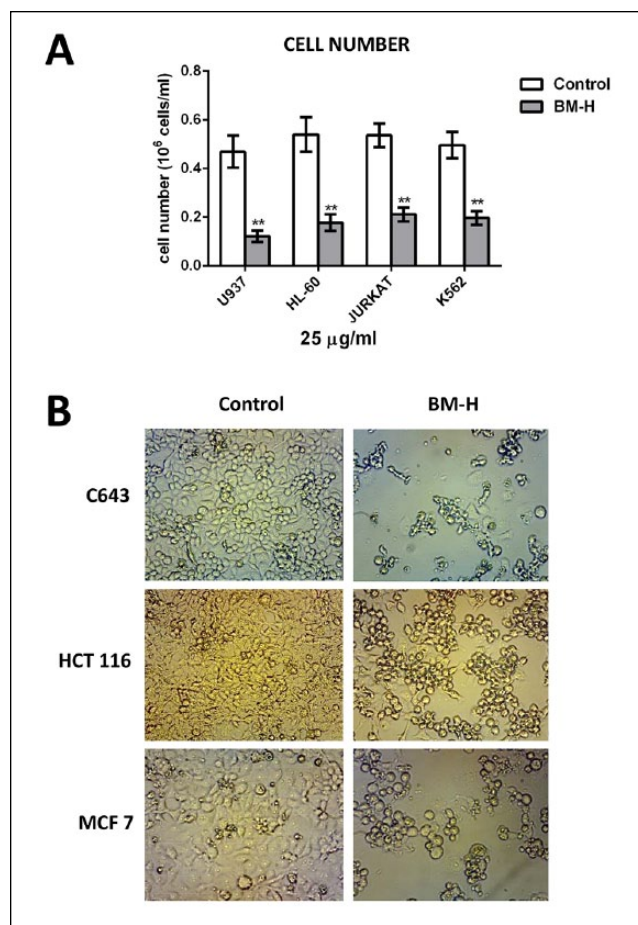


Figure 4. Effect of BM-H on hematologic- and solid tumor-derived cell numbers. (A) Bars represent number of viable cells counted after 24 hours of treatment with either vehicle (white: control) or with BM-H (gray: BM-H) at the concentrations reported on the x-axis. Data from 3 independent experiments are reported as mean \pm SEM. $**P < .01$. (B) Micrographs of cultured cells from different solid cancer cell lines treated either with vehicle (control) or with BM-H. Images are representative of 3 independent experiments.

confirmed that p53 is not expressed in U937, HL-60, and K562, whereas it is expressed at a low level in Jurkat cells. In the latter cell line (see the quantitation of protein bands in Figure 6B) along with the solid tumor cell lines, the expression of p53 did not increase on stimulation with BM-H, thus confirming the p21-dependent, p53-independent mechanism of BM-H extract on all tested cell lines. Since the mechanism was shown to be the same in all tested cell lines, only the OCI cell line was utilized in further experiments to elucidate the BM-H activity in depth.

Effect of BM-H on the Functional Regulation of p21

The functional regulation of p21 relies on 2 posttranslational modifications, phosphorylation and ubiquitylation.

Reversible phosphorylation is an indicator of p21 posttranslational regulation, as it controls p21's activity, localization, stability, and degradation.¹⁹ Because MAPK molecules significantly regulate p21,²²⁻²⁵ we also investigated the possible role of ERK, p38, and Akt on BM-H-induced p21 expression by analyzing via Western blot. Figure 7 reveals that levels of active phosphorylated ERK significantly decrease after 4 hours of BM-H treatment compared to controls. Levels of p-ERK continued to decline over time, reaching their minimum after 24 hours of BM-H treatment. Conversely, phosphorylation of p38 increased with BM-H treatment over time, whereas phosphorylated Akt levels remained unchanged.

Effect of BM-H on the Cyclin Pathway

p21 has been shown to inhibit cyclin E.¹⁷ During the G₁/S transition, cyclin E binds and activates CDK2, which is required for the phosphorylation of retinoblastoma protein (pRb). Phosphorylated pRb triggers cell proliferation.¹⁷ To determine if BM-H-induced p21 activation affects this cyclin pathway, we experimentally evaluated the expression of cyclin E and the subsequent phosphorylation of pRb. As shown in Figure 8, BM-H decreased the expression of cyclin E and inhibited the phosphorylation of pRb. Thus, BM-H decreases the expression of cyclin and inhibits the phosphorylation and activation of pRb.

Effect of BM-H on Apoptotic Pathways

Increased BM-H-mediated death of OCI cells prompted us to investigate the role of the caspase cascade, the hallmark of apoptotic cell death. We first examined caspase-3, one of the terminal caspases involved in apoptosis. We cultured OCI cells for 4, 8, 14, or 24 hours before extracting proteins for Western blotting. Figure 9 shows that activated cleaved caspase-3 was present after 4 hours of treatment with BM-H. The activated band of caspase-3 was evident after 14 or 24 hours of BM-H treatment. Thus, the increase in BM-H-dependent OCI cell death was attributed to apoptosis as it was correlated with augmented caspase-3 activation.

The executioner caspase-3 is activated by at least 2 different pathways. The mitochondrial (intrinsic) pathway leads to sequential release of cytochrome c from mitochondria and activation of caspase-9, which directly cleaves and activates caspase-3. The second (extrinsic) pathway involves activation of caspase-8, which also directly cleaves and activates caspase-3.²⁶ Both pathways were analyzed to determine the mechanism of caspase-3 activation in BM-H-treated OCI cells. Figure 9 shows also that, unlike caspase-3, neither caspase-8 nor caspase-9 were activated at any tested time points. Interestingly, BM-H inhibited caspase-9 activation in control cells. Thus, neither the caspase-8 nor caspase-9-dependent pathway was involved in BM-H-dependent apoptosis of OCI cells.

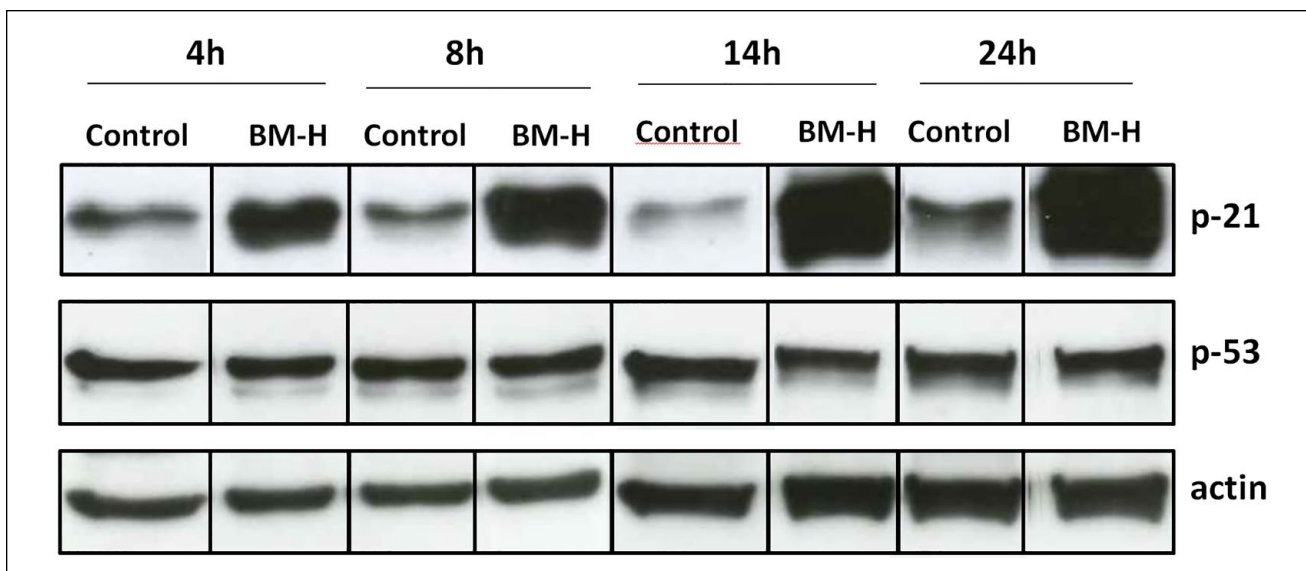


Figure 5. Time-course effects of BM-H on cell cycle protein expression. Western blot bands represent expression of p21 (upper lane), p53 (middle lane), or actin (lower lane) proteins extracted from OCI cells treated either with the vehicle (control) or with BM-H (BM-H) for 4, 8, 14, or 24 hours. Western blots are representative of 3 independent experiments.

Effect of BM-H on the Intrinsic Apoptotic Pathway

To confirm the lack of involvement of BM-H in the apoptotic intrinsic pathway, as suggested by the inhibition of caspase-9 activation, we evaluated the involvement of different molecules belonging to the intrinsic apoptotic pathway using Western blotting experiments. Figure 10 shows that when cells were treated with BM-H, the expressions of Bcl-2, Bcl-xL, and Puma were unchanged, and Bim was not cleaved.

Effect of BM-H on Nonneoplastic Spleen Cells

To determine the effect of BM-H on primary nonneoplastic cells, we tested the effect of BM-H extract on the cell numbers of normal murine splenocytes. Spleen cells isolated from mice were cultured after no treatment, treatment with 2.5 $\mu\text{g}/\text{mL}$ of BM-H, treatment with Concanavalin A (ConA) alone, and treatment with ConA plus BM-H. After 24 hours, the cells were harvested and counted using a hemocytometer. As shown in Figure 11, no significant differences were detected between the groups treated with BM-H and their controls. Thus, BM-H at a concentration of 2.5 $\mu\text{g}/\text{mL}$, both quiescent and stimulated with ConA, can be considered as safe to nonneoplastic murine spleen cells.

Discussion and Conclusions

Interest in traditional and complementary and alternative medicine practice is increasing in developed countries. In the United States, 38.3% of adults and 11.8% of children

have used some form of complementary and alternative medicine, and many physicians in Europe and North America have referred patients for acupuncture (43%), chiropractic (40%), and/or massage (21%).²⁷ Alternatively, MTM is based mainly on phytotherapy.³

Although *B. microphylla* is used for treating many conditions, obstacles to its use in modern medical practice involve validating its clinical efficacy and determining its safety (both biologically and commercially) regarding adverse effects. For example, herbs of the *Aconitum* genera are popular in Asia for their medicinal benefits, but they have a narrow therapeutic range, highlighting the potential risk associated with traditional herbal remedies and their potentially limited applications.²⁸

In this study, we investigated the anti-proliferative activity of the hexane fraction of *B. microphylla* in both hematologic- and solid tumor-derived cell lines. We found that BM-H significantly decreased cell growth of all cell lines tested and demonstrated that its growth inhibitory effect relied on both a significant arrest of cells in G_1/S transition and in G_2/M phase and a significant induction of caspase-3-mediated cell death. We investigated anti-proliferative and pro-apoptotic proteins modulated by BM-H to gain insight into its inhibitory mechanism(s) and to identify potential novel drug targets and/or biomarkers of the fraction's reported effect. We pursued these questions by analyzing classical anti-proliferative pathways linked to G_1/S transition and G_2/M blockage and expression of p21. During G_1/S transition and G_2 phase, p21 inhibits Cdk-activating kinase (CAK) and its consequent Thr116 phosphorylation.²⁹ The protein can also interact with the cyclin B1-Cdk1 complex in response to genotoxic stress, thus blocking activation by Cdc25 and CAK,³⁰ and sustain G_2

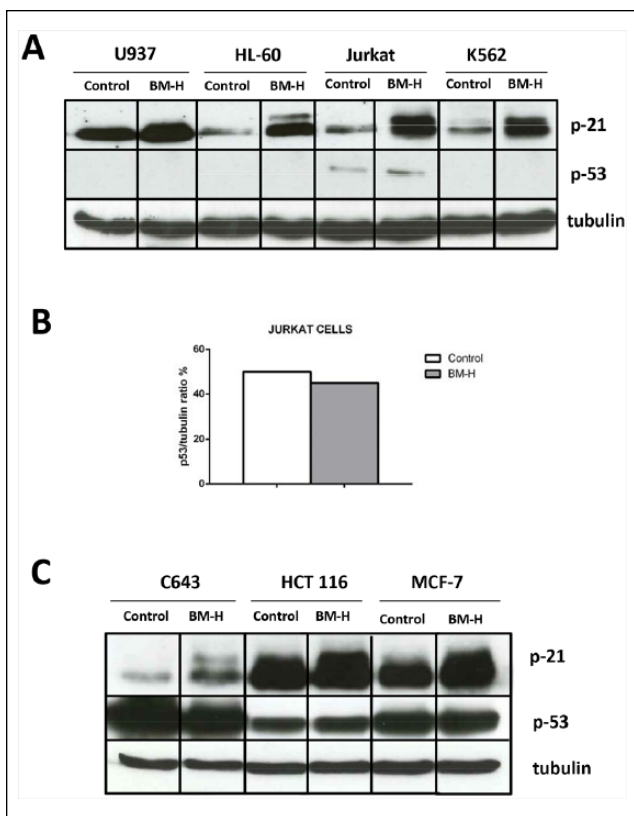


Figure 6. Effects of BM-H on cell cycle protein expression. Western blot bands represent expression of p21 (upper lane), p53 (middle lane), and tubulin (lower lane) proteins extracted from U937, HL-60, Jurkat, K562 (A) and C643, HCT 116, and MCF-7 (C) cells treated with the vehicle (control) or with BM-H (BM-H) for 24 hours. Western blots are representative of 3 independent experiments. (B) The quantitation of bands from untreated Jurkat cells (white bar) and Jurkat cells treated with BM-H (gray bar) from the experiment depicted in A.

arrest by mediating cyclin B1 degradation in the presence of DNA lesions.³¹ Furthermore, downregulation of early mitotic inhibitor 1 (Emi1) by p21 results in anaphase-promoting complex activation and degradation of cyclins A2 and B1, thus preventing G₂-arrested cells from proceeding to mitosis.³² These previous findings prompted us to investigate p21 as a possible target of BM-H action, which we confirmed via Western blotting, as p21 expression was significantly upregulated by BM-H at all times tested.

p21 also plays an important role in cell metabolism, and its expression and activity are finely regulated by multiple mechanisms, such as regulation of transcription, posttranscriptional modifications (eg, phosphorylation and ubiquitylation). In particular, transcriptional regulation mediated by p21 can occur through either p53-dependent or p53-independent pathways,³³ so we examined the mechanism of p21 upregulation and found that BM-H increases p21 expression in a p53-independent manner.

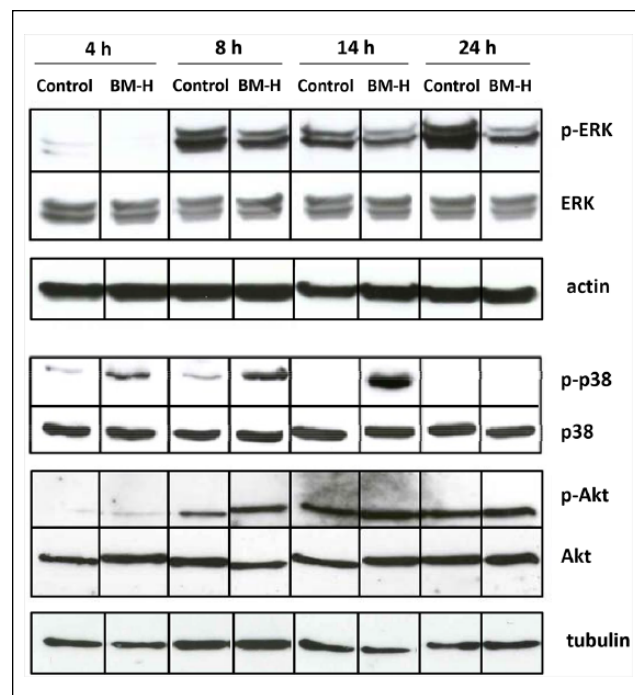


Figure 7. Time-course effects of BM-H on MAPK pathway. Western blot bands represent phosphorylated ERK (p-ERK), total ERK (ERK), actin (as a housekeeping gene for ERK), phosphorylated p38 (p-p38), total p38 (p38), phosphorylated Akt (p-Akt), total Akt (Akt), and the housekeeping gene tubulin (to control both p-38 and Akt). Proteins were extracted from OCI cells treated with the vehicle (control) or with BM-H (BM-H) after 4, 8, 14, or 24 hours. Western blots are representative of 3 independent experiments.

Next, we wanted to identify molecules involved in the BM-H-induced overexpression of p21. Several serine and threonine residues in p21 are phosphorylated by various protein kinases, especially MAPKs and p38, which phosphorylates Ser130 and increases p21's stability.²³ In contrast, phosphorylation at Ser130 by ERK2 decreases protein stability by promoting p21's degradation.²² Thus, to determine if upregulation of p21 was mediated by one or more of these posttranslational mechanisms, we explored expression of ERK2 and p38 in BM-H-treated cells. Our results showed that ERK2 phosphorylation and its consequent activation was enhanced by BM-H, whereas p38 phosphorylation decreased by BM-H exposure. Therefore, upregulation of p21 was likely due to increased protein stability promoted by ERK2 phosphorylation with a concomitant decrease in p21 degradation resulting from lower levels of p38 activity. In parallel, expression of Akt, which phosphorylates p21 at Thr145 and Ser146 and mediates its translocation from the nucleus to the cytoplasm in breast cancer cells,²⁵ was not affected by BM-H. These results are in agreement with literature data showing that Akt phosphorylates p21 in the p21/p53-dependent pathway,³⁴ unlike in the p21-dependent,

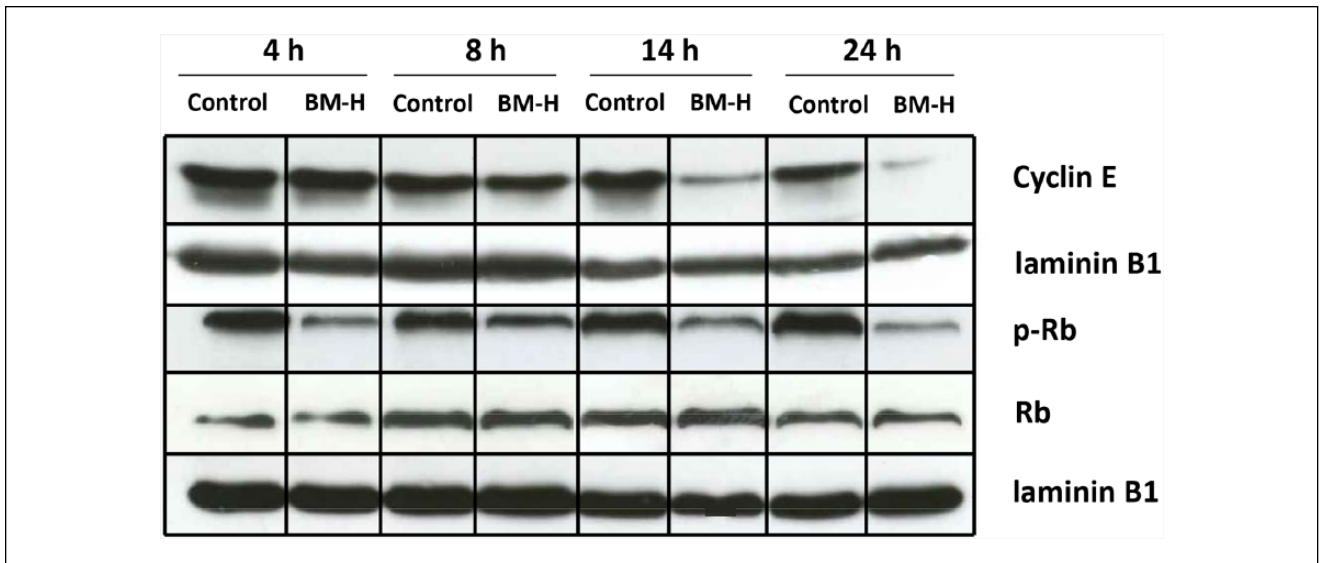


Figure 8. Time-course effects of BM-H on cyclin E pathway. Western blot bands represent the expressions of cyclin E, laminin B1 (housekeeping gene for cyclin E), phosphorylated Rb (p-Rb), total Rb (Rb), and laminin B1 (as a housekeeping gene for Rb). Proteins were extracted from OCI cells treated with the vehicle (control) or with BM-H (BM-H) after 4, 8, 14, or 24 hours. Western blots are representative of 3 independent experiments.

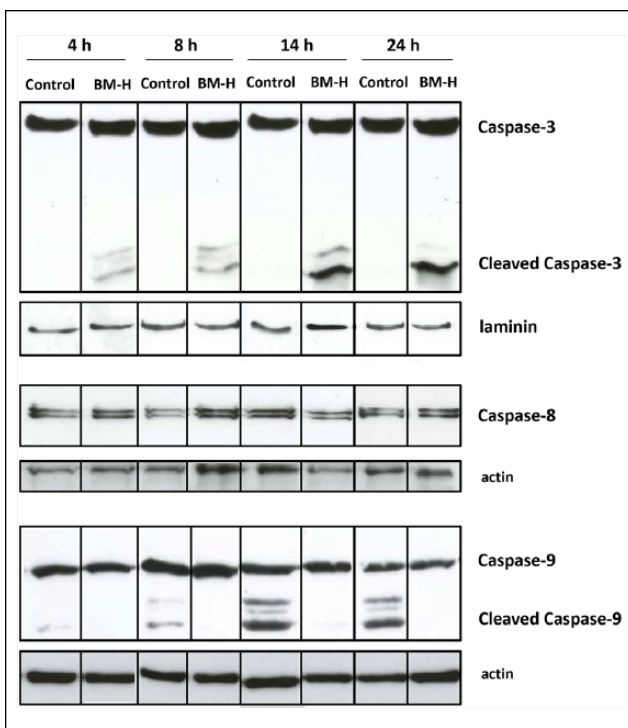


Figure 9. Time-course effects of BM-H on caspase-mediated apoptosis. Western blot bands represent pro-caspase-3 (caspase-3), activated caspase-3 (cleaved caspase-3), laminin (housekeeping gene for caspase-3), pro-caspase-8 (caspase-8), actin (housekeeping gene for caspase-8), pro-caspase-9 (caspase-9), activated caspase-9 (cleaved caspase-9), and actin (housekeeping gene for caspase-9). Proteins were extracted from OCI cells treated either with the vehicle (control) or with BM-H (BM-H) after 4, 8, 14, or 24 hours. Western blots are representative of 3 independent experiments.

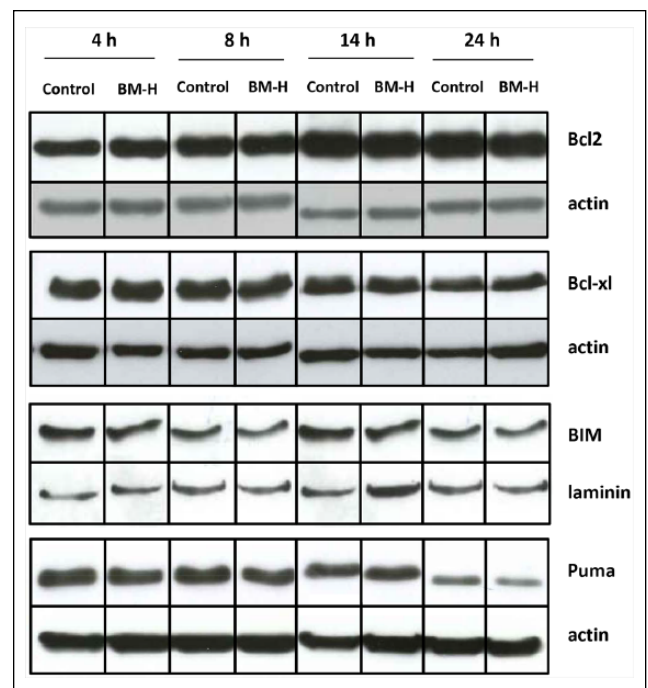


Figure 10. Time-course effects of BM-H on expression of intrinsic apoptotic pathway molecules. From upper to lower, Western blot bands represent the expressions of Bcl2, actin (housekeeping for Bcl-2), Bcl-xL, actin (housekeeping gene for Bcl-xL), BIM, laminin (housekeeping gene for BIM), Puma, and actin (housekeeping gene for Puma). Proteins were extracted from OCI cells treated either with the vehicle (control) or with BM-H (BM-H) for 4, 8, 14, or 24 hours. Western blots are representative of 3 independent experiments.

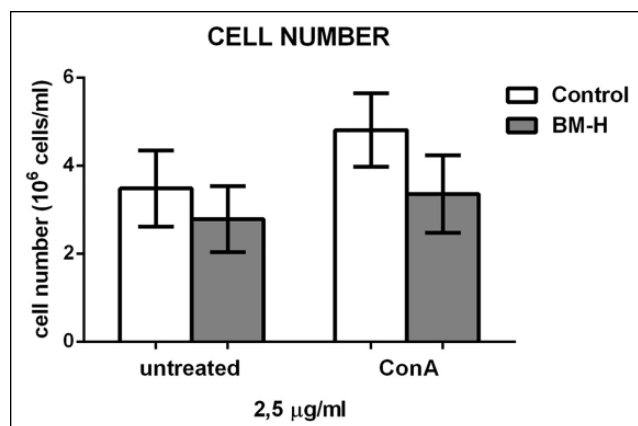


Figure 11. Effect of BM-H on primary murine spleen cell number. Bars represent the number of viable cells counted after 24 hours of treatment with vehicle (white: control) or BM-H (gray: BM-H) in the presence or absence of Concanavalin A (ConA) for 24 hours at the concentration reported on the x-axis. Data from 3 independent experiments are reported as mean \pm standard error from the mean.

p53-independent pathway triggered by BM-H. In addition, the phosphorylation and activation of Akt determines the translocation of p21 from the nucleus to the cytoplasm.¹⁷ This translocation of p21 is associated with its anti-apoptotic effect¹⁷; in contrast, in our system, the upregulation of p21 is associated with an increase in apoptosis.

We observed BM-H treatment induced caspase-3-mediated apoptosis, but surprisingly, this process was not a consequence of either caspase-8 or caspase-9 activation, the 2 classical pro-apoptotic extrinsic and intrinsic pathways. The lack of involvement of caspase-9 is especially interesting because it is usually involved in p21-mediated apoptosis. In our study, p21 inhibited caspase-9, a finding confirmed by unchanged levels of Bcl-2, Bcl-xL, Bim, and Puma, molecules often involved in apoptotic pathways. A certain degree of caspase-9 activation occurs in unstimulated OCI cells as a result of the spontaneous apoptosis of cultured cells: Since BM-H switched cells from caspase-9-dependent apoptosis to a different apoptotic pathway, this may explain the inhibition of caspase-9 activation operated by the addition of BM-H. However, additional studies are required to determine which apoptotic pathway is triggered by BM-H or if a novel apoptotic pathway is involved considering that in addition to caspase-8 and -9, approximately 170 molecules can bind caspase-3, a few of which may cleave and activate caspase-3 to regulate apoptosis (eg, caspase-2, -4, -6, -7, and -10; <http://visant.bu.edu/>).

The main question raised by our experiments is if the anti-proliferative effect of BM-H can be exploited for anticancer therapeutic purposes or if this effect is a marker of toxicity. To address this issue, additional experiments

should test BM-H's selectivity by utilizing primary cells and confirm its putative anti-proliferative effects in vivo with an appropriate animal model for cancer. Because BM-H's anti-proliferative effect was exerted on all cancer cell lines tested, the question remains if BM-H is cytotoxic and may lead to adverse effects during potential therapeutic uses.

Phytochemicals produced by medicinal herbs, like *B microphylla*, are investigated because they may influence human health, especially with respect to their anticancer properties. Such chemicals of interest include flavonoids, carotenoids, and allyl sulfides, which may act as estrogen-like compounds or may exert antioxidant effects. Although the role of phytochemicals in influencing p21 expression, thereby affecting cell cycle progression and/or apoptosis, has been explored in numerous studies, it is still difficult to accurately weigh their putative therapeutic benefits against their toxic effects.^{17,35}

In conclusion, we have shown that BM-H from the medicinal plant *B microphylla* exerted anti-proliferative effects on tumor-derived cells due to MAPK-dependent upregulation of p21 expression. Ideally, additional studies will clarify if this interesting effect could affect MTM due to BM-H's potential therapeutic benefits

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