Extracts of *Musa basjoo* induce growth inhibition and changes in the protein expression of cell cycle control molecules in human colorectal cancer cell lines

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Abstract. *Musa basjoo* (MB) is a species of the banana plant belonging to the genus *Musa* that has been used as a folk medicine. However, evidence-based biological activities and the molecular mechanism of action of MB are unknown. Thus, the aim of the present study was to examine whether the crude dried leaf extracts of MB inhibit the growth of colorectal (HT29 and HCT116) and other types (HepG2, MCF-7 and PC-3) of human cancer cell lines. Crude extracts of MB inhibited the growth of cells with IC_{50} values of 136 μg/ml (acetone extract, HT29), 51 μg/ml (acetone extract, HCT116), 45 μg/ml (acetone extract, HepG2), 40 μg/ml (acetone extract, MCF-7), 29 μg/ml (acetone extract, PC-3), 175 μg/ml (methanol extract, HT29), 137 μg/ml (methanol extract, HCT116), 102 μg/ml (methanol extract, HepG2), 85 μg/ml (methanol extract, MCF-7), and 85 μg/ml (methanol extract, PC-3) in colony formation assays, and 126 μg/ml (acetone extract, HT29), 68 μg/ml (acetone extract, HCT116), 260 μg/ml (methanol extract, HT29), and 216 μg/ml (methanol extract, HCT116) in MTT assays. Thin layer chromatography analysis revealed the potential existence of aromatic compounds in the acetone extract of MB. Flow cytometric analysis indicated that the percentage of cells in G1 increased, and this was associated with a concomitant decrease of cells in the S and/or G2-M phases of the cell cycle. When colorectal cancer cells were treated with acetone extract of MB, there was a marked decrease in the levels of expression of the cyclin D1, cyclin E, cdk2 and cdk4 proteins and a marked increase in the levels of the expression of the $p21^{CIP1}$, $p27^{KIP1}$, and p53 proteins, but those of apoptosis-associated protein PARP did not change. There was a tendency for acetone extract of MB to inhibit xenograft tumor growth in mice. Collectively, the crude extracts of MB contain active components that exert growth inhibition of human cancer cells. This is the first systematic study of the anticancer activity of MB and may broaden insights into the possible clinical approach of specific herbal medicines.

Introduction

There is increasing interest in the use of herbal medicines for the treatment and/or prevention of a variety of diseases (1,2). *Musa basjoo* (MB) has been used as a traditional herb, grown for the treatment of inflammatory conditions accompanying high fever (3,4). MB is classified as one of the 50 species belonging to the genus *Musa* that includes edible banana species, such as *Musa paradisiaca* and *Musa sapientum* (5). In addition, the fiber of *Musa balbisiana* has been used as a material for clothing termed Bashofu from ancient times in Japan (3).

Previous findings showed that the compounds isolated from herbs have a broad range of biological activities including anticancer and/or cancer preventive properties (6,7). In fact, the dietary administration of powdered leaves of *Peucedanum japonicum* and *Terminalia catappa* reduce the occurrence of azoxymethane-induced aberrant crypt foci, preneoplastic lesions in rat colon carcinogenesis (8,9). In addition, growth inhibitory activities of crude extracts obtained from herbal plants in the Ryukyu Islands on several human colorectal cancer cell lines have been identified (10).

Although the above-mentioned findings obtained with herbal plants in experimental studies are promising, the molecular mechanisms by which the crude extracts of MB exert anticancer effects have yet to be clarified. Even if such herbal products are often perceived as being of natural origin

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and therefore harmless, investigation of their molecular mechanisms of action and their specific cellular targets is crucial to predict possible side effects. Furthermore, this information is useful for developing and designing new drugs for more effective treatment and prevention of cancer and other diseases. Based on the findings of a previous study (11), authors of the present study were interested in examining the crude extracts of MB in the p53 wild-type and p53 mutant human colorectal cancer cell lines. Thus, the aim of the present study was to determine whether crude extracts of MB contain compounds that may inhibit the growth of human colorectal and other types of cancer cell lines.

Materials and methods

Crude dried leaf extracts of MB. Samples of MB in the current study were collected from the Herbal Garden, Gifu Pharmaceutical University, Gifu, Japan. In brief, the dried leaf with blade and stalk was extracted with acetone or methanol, filtered and evaporated under reduced pressure to remove the acetone or methanol, as described in a previous study (10). Remaining crude extracts were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and stored at -20˚C until use. Each extract (acetone extract sample and methanol extract sample) was used for the biological assays described below.

Thin layer chromatography. Thin layer chromatography (TLC) was performed with silica gel 60 F_{254} (1.05715.0001; Merck KGaA) to analyze crude extracts of MB. The extracts were developed with ethyl acetate (AcOEt):*n*-hexane at 1:3 for 9 cm. Then, the resulting chromatogram was visualized with a handheld UV (254/365 nm) lamp (UVGL-58; Analytik Jena AG) equipped with Chromato-Vue Cabinet (C-10; Analytik Jena AG) followed by treatment with 5% phosphomolybdic acid hydrate in ethanol solution and then heating to enhance visualization [phosphomolybdic acid staining at 200˚C for enough time to distinguish coloration (~1 min)].

Cell lines and treatment with the crude extracts of MB. HT29 (HTB38) and HCT116 (CCL247) human colorectal cancer, HepG2 (HB8065) liver cancer/hepatoma, MCF-7 (HTB22) human breast cancer, and PC-3 (CRL1435) human prostate cancer cell lines [American Type Culture Collection (ATCC)] were maintained in Dulbecco's modified Eagle's medium (DMEM) (FUJIFILM Wako Pure Chemical Corp.) supplemented with 5% (v/v) fetal bovine serum (FBS) (Biowest) in an incubator with humidified air at 37˚C with 5% CO_2. Cells were plated in 10-cm culture dishes, treated with the indicated concentrations of the crude acetone or methanol extracts, and harvested at the indicated times. As an untreated solvent control, the cells were treated with DMSO at a final concentration <0.5%.

Cell proliferation assays. Cell proliferation was measured by both the colony formation assay (HT29, HCT116, HepG2, MCF-7 and PC-3 cell lines) and MTT assay (HT29 and HCT116 cell lines) as described elsewhere (12,13). The MTT assay was performed to confirm the results of the colony formation assay. Experiments were performed at an optimal timing point, at which cells were dividing and did not reach a plateau. Cells were plated into 6-well 35-mm diameter culture plates ($5x10^2$ cells per well) and treated with different concentrations (12.5-200 μg/ml) of crude acetone or methanol extracts of MB at 37˚C for 7 days in DMEM plus 5% FBS. After washing with phosphate-buffered saline (PBS), colonies were stained with Giemsa solution (Sigma-Aldrich) and then counted. The clonogenicity of cancer cells was tested using the colony formation assay. Results were expressed as the percentage of the control untreated culture. Each concentration of the extracts was tested at least in duplicate. The relative surviving fraction, when compared with cells treated with the vehicle, was plotted on the dose-response curve. A viability of 100% corresponded to the control cells.

Cells were plated onto 96-well plates ($5x10^3$ cells per well) and cultured overnight to allow for cell attachment. Subsequently, these cells were treated with increasing concentrations (25-400 μg/ml) of crude acetone or methanol extracts of MB, grown in DMEM containing 5% FBS at 37˚C for 96 h, and assayed using an MTT assay kit (Cytiva). The growth inhibitory activity of the extracts of MB was examined by the MTT assay. Each concentration of the extracts was tested in duplicate. Solubilization buffer (10% SDS in 0.01 M HCl) was used to dissolve the formazan. The quantity of formazan product was measured using a spectrophotometric microplate reader (Bio-Rad Laboratories Inc.) at 595 nm wavelength. A viability of 100% corresponded to the control cells.

Flow cytometry. Flow cytometric analysis was performed as described previously (13,14). HT29 and HCT116 cells were plated onto 10-cm dishes ($5x10^5$ cells per dish) in DMEM containing 5% FBS and grown at 37˚C overnight to allow for cell attachment. Cells were then treated with DMSO (<0.5%) or 100 μg/ml crude acetone extract of MB at 37˚C for 96 h, harvested, fixed with 70% ethanol, centrifuged (750 x g, at room temperature for 5 min), resuspended in 400 μl of PBS containing 2 mg/ml RNase (Nacalai Tesque Inc.), and stained with 400 μl of 0.1 mg/ml propidium iodide (Nacalai Tesque). The cell suspension was filtered through a 40 μm nylon filter (Ikemoto Scientific Technology Co. Ltd.). Samples of 20,000 cells suspended in BD FACSFlow™ Sheath Fluid at room temperature were then analyzed for DNA histograms (<1 min/run) and cell cycle phase distributions by flow cytometry using a FACSCalibur instrument (BD Biosciences), and the data were analyzed by a CELLQuest computer program (BD Biosciences), as previously described (13-15). Each assay was repeated in triplicate to confirm the results.

Western blot analysis. The assay was carried out as described previously (13,16). HT29 and HCT116 cells were treated with DMSO (<0.5%), 50 or 100 μg/ml acetone extract of MB at 37˚C for 96 h and harvested. Then, the cells were lysed with modified radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 0.5% deoxycholic acid, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, and 25% glycerol]. Cell lysates (40 μg per lane) were separated by SDS-PAGE (12.0-13.5% gel) and transferred onto an Immobilon-P transfer membrane (Merck KGaA). Immunoblots using monoclonal or polyclonal antibodies were then prepared by established methods (12). Primary antibodies used in this study included cyclin D1 (06-137; Merck KGaA; 1:1,000), cyclin E (sc-377100; Santa Cruz Biotechnology

Inc.; 1:100), $p21^{CIP1}$ (sc-817; Santa Cruz Biotechnology Inc.; 1:200), $p27^{KIP1}$ (610242; BD Biosciences; 1:2,500), p53 (sc-126; Santa Cruz Biotechnology Inc.; 1:200), cdk2 (sc-163; Santa Cruz Biotechnology Inc.; 1:200), cdk4 (sc-260; Santa Cruz Biotechnology Inc.; 1:200), poly (ADP-ribose) polymerase (PARP; 9542; Cell Signaling Technology, Inc.; 1:1,000), and β-actin (sc-1616-R; Santa Cruz Biotechnology Inc.; 1:200). Anti-mouse IgG, Horseradish peroxidase-linked species-specific $F(ab')_2$ fragment (NA9310; Cytiva; 1:4,000) or anti-rabbit IgG, horseradish peroxidase-linked species-specific whole antibody (NA934; Cytiva; 1:3,000) antibodies were used as the secondary antibodies. The membranes were then incubated at room temperature for 1 h. Each membrane was developed using an ImmunoStar Long Detection System (FUJIFILM Wako Pure Chemical Corp.) and visualized with Light-CaptureII imaging analyzer (ATTO Corp.). Each assay was repeated to confirm the results. Densitometry was performed to obtain relative band intensity using ImageJ computer program (Image J software, National Institute of Health) as described earlier (11,13,15).

Tumor xenograft assay. A total of 8 female BALB/cSlc-nu/nu mice aged 6 weeks and weighing 18.8±0.22 g were purchased from Japan SLC, Inc. Dose level (2 mg/kg) was determined based on the results of our preliminary experiments (unpublished data). All 8 mice were quarantined for 1 week and housed in plastic cages (4 mice/cage) with free access to tap water and basal diet (MF diet; Oriental Yeast Co., Ltd.) under controlled conditions of humidity (50±10%), temperature (23±2˚C) and lighting (12 h light/12 h dark cycle; 8 am light on, 8 pm light off). All the animal experiments were performed with the approval of the Animal Ethics Committee of the Nagoya City University (approval no. H28M-03) and according to the guidelines of the committee.

Viable HT29 human colorectal cancer cells ($2.5x10^6$ cells/200 µl DMEM without L-glutamine and phenol red) were subcutaneously injected into the flank of all 8 mice. After confirming the visible tumor mass, the mice were assigned into two experimental groups (4 mice in each group). Crude acetone extract was dissolved into saline containing 20% ethanol. As shown in Fig. 1, mice in group 1 (control) and group 2 (treatment) received intratumoral injections of the vehicle (saline containing 20% ethanol) and crude acetone extract of MB (2 mg/kg), respectively, every other day (a total of 7 times). The mice were observed on a daily basis for tumor growth, body weight, and symptomatic adverse side effects. Tumors were measured three times a week. The mean volume per tumor was calculated using the formula: $V\ (mm^3)=axb^2/2$ where V is the volume, and a is the maximum and b is the minimum diameter of the tumor (17). At the 4th experimental week, all 8 mice were euthanized by decapitation following anesthesia with 3% isoflurane and complete autopsy was performed. Tumors and main organs (heart, lung, liver, and kidney) were carefully removed, fixed with 10% buffered formalin and processed for histopathological examination and [hematoxylin and eosin (HE) staining]. Furthermore, we measured the necrosis area in the HE tumor sections using an imaging system (Digital microscope VHX-5000; Keyence Corp.). The experiment was terminated when a tumor reached 20 mm or 2 cm in dimension, or when the study period finished.

Statistical analysis. Comparisons between the vehicle-treated control group and the acetone extract-treated group were made using one-way ANOVA (western blot analysis) or two-way ANOVA (xenograft assay), and Tukey's multiple comparison test was applied to evaluate statistical significance. IBM SPSS Statistics version 24 was used to evaluate the data. Student's unpaired t-test was used to compare two groups with normal data distribution and homogenous variances. If variances were heterogenous, Welch's t-test was used. Mann-Whitney U test was used to compare two groups with non-normal data distribution. Results were expressed as means ± SEs. Differences between groups of $P<0.05$ were considered statistically significant.

Results

Crude extracts of MB exhibited the difference in migration of spots on TLC. To examine dissimilar contents of the possible active components contained in both acetone and methanol extracts, TLC was performed. Although both extracts had the same several fluorescent spots, visualized with 365 nm UV lamp irradiation, the acetone extract had specific spots migrated marginally from its origin [Retardation factor (Rf): 0.03] (Fig. 2). These unique spots were visualized with both 254 nm UV lamp irradiation and phosphomolybdic acid staining, suggesting that they are aromatic compounds.

Crude extracts of MB inhibited the proliferation of five human cancer cell lines. To examine the antiproliferative activity of acetone or methanol extracts in a variety of human cancer cell lines, colorectal (HT29 and HCT116) and other types (HepG2, MCF-7 and PC-3) of human cancer cell lines were examined. Exponentially dividing cells were treated with increasing doses of the MB extracts (12.5-200 µg/ml for colony assays, 25-400 µg/ml for MTT assays). In these five cell lines, both acetone and methanol extracts inhibited cell proliferation, in a dose-dependent manner, with IC_{50} values in the range of 29-136 µg/ml (acetone extract) and 85-175 µg/ml (methanol extract) (Table I and Fig. 3). Representative images of culture plates (HCT116 and MCF-7 cell lines) of colony formation assay are shown in Figure S1. Additional studies were performed with the HT29 and HCT116 cell lines to confirm the growth inhibition by crude extracts of MB using the MTT assays. In the HT29 cell line, both acetone and methanol extracts of MB showed marked inhibition of the growth of these cells, with IC_{50} values of ~126 and 260 µg/ml, respectively (Table II and Fig. 4). In the HCT116 cell line, both acetone and methanol extracts decreased the growth of cells in a dose-dependent manner, with IC_{50} values of about 68 and 216 µg/ml, respectively (Table II and Fig. 4). As a result of the colony formation assay and MTT assay, the acetone extract exhibited stronger antiproliferative activity than the methanol extract (Tables I and II).

Acetone extract of MB caused an increase in cells of G1-phase in the colorectal cancer cell line. In view of the above-mentioned growth inhibitory effects, it was of interest to examine the effects of the acetone extract on cell cycle progression in exponentially dividing cultures of the HT29 and HCT116 cell lines. Cells were treated with either DMSO (control) or 100 µg/ml acetone extract at 37˚C for 96 h. Since we were interested in simultaneously

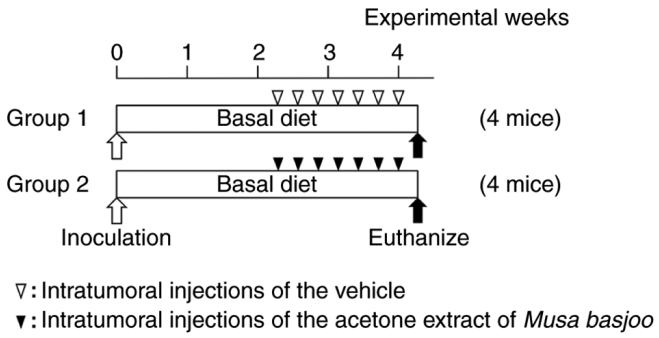

Figure 1. Experimental protocol of a mouse xenograft model.

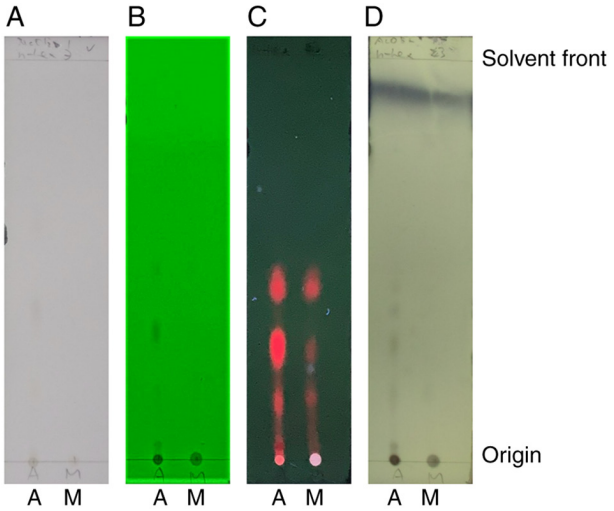

Figure 2. TLC of acetone extract and methanol extract of *Musa basjoo*. The extracts were developed with AcOEt:*n*-hexane at 1:3 for 9 cm. The resulting TLC plate was observed by (A) white light, (B) 254 nm UV light, (C) 365 nm UV light, and (D) phosphomolybdic acid staining, respectively. TLC, Thin-layer chromatogram; A, acetone extract; M, methanol extract.

demonstrating cell cycle arrest and changes in protein expression in each cell line, a single concentration of 100 μg/ml was selected for flow cytometry or two different concentrations of 50 and 100 μg/ml for western blot analysis. The dose 100 μg/ml is almost the IC_{50} value of these two cell lines. A representative histogram for the HT29 and HCT116 cell lines is shown in Fig. 5. Flow cytometric analysis indicated that when cells were treated with the indicated concentration of acetone extract of MB, the percentage of the HT29 and HCT116 cell lines in G1 significantly increased by 5 and 9%, respectively, after 96 h of treatment, and this was associated with a concomitant decrease of cells in the S and/or G2-M phases of the cell cycle. There was no evidence of apoptosis by an increase in the sub-G1 population of DNA when cancer cells were treated with 100 μg/ml of acetone extract of MB.

Acetone extract of MB causes an increase in the protein expression levels of p21^{CIP1}, p27^{KIP1}, and p53 and a decrease in those of cyclin D1, cyclin E, cdk2, and cdk4. As the acetone extract induced a G1 arrest in the cell cycle (Fig. 5), western blot analysis was performed to determine whether the treatment of HT29 and HCT116 cells with the acetone extract

Table I. Effects of the extracts of *Musa basjoo*.

	IC_{50} (μg/ml)	
Cell line	Acetone extract	Methanol extract
HT29	136±9.5	175±17.0
HCT116	51±10.8[a]	137±10.3
HepG2	45±16.2	102±17.0
MCF-7	40±12.7	85±12.3
PC-3	29±10.7	85±11.3

Values are presented as the mean ± SE. [a]P<0.05.

Table II. Effects of the extracts of *Musa basjoo*.

	IC_{50} (μg/ml)	
Cell line	Acetone extract	Methanol extract
HT29	126±4.3[a]	260±6.4
HCT116	68±17.4	216±20.3

Values are presented as the mean ± SE. [a]P<0.05.

of MB alters the cellular expression levels of the G1 cell cycle control proteins, cyclin D1/cdk4 (early G1 phase) and cyclin E/cdk2 (late G1 phase), and the cell cycle inhibitor proteins p21^{CIP1}, p27^{KIP1}, and p53. Investigation of the acetone extract of MB in the p53 wild-type HCT116 and p53 mutant HT29 cell lines was also conducted, as presented in a previous study (11). Thus, these two colorectal cancer cell lines were chosen for the analysis. The results revealed that when these two cell lines were treated with the indicated concentrations (50 and 100 μg/ml) of the acetone extract of MB at 37˚C for 96 h, there was a marked increase in the p21^{CIP1}, p27^{KIP1}, and p53 proteins (Fig. 6). There was also a marked inhibition in expression levels of the cyclin D1, cyclin E, cdk2, and cdk4 proteins (Fig. 6). The sub-G1 population of DNA was not detected in the flow cytometric analysis (Fig. 5). Next, the expression level of apoptosis-associated molecule PARP was examined. Western blot analysis demonstrated that the expression level of PARP did not change and cleaved PARP was not detected after HT29 and HCT116 cells were treated with 50 and 100 μg/ml MB extract.

Tendency for acetone extract of MB to inhibit xenograft tumor growth in BALB/cSlc-nu/nu mice. At the end of the experiment, the treatment of mice with acetone extract of MB caused a decrease by 42% in tumor volume, but this effect was not statistically significant (Fig. 7A). There was a tendency of an increase in the necrosis area (mm^2) of the MB treatment group when compared to that of the control group (Fig. 7B and C). At the end of the experiment, there was no statistically significant difference in body, liver, kidney, relative liver, and relative kidney weights between control and MB treatment groups (Table III). Additionally, there were no symptomatic side effects

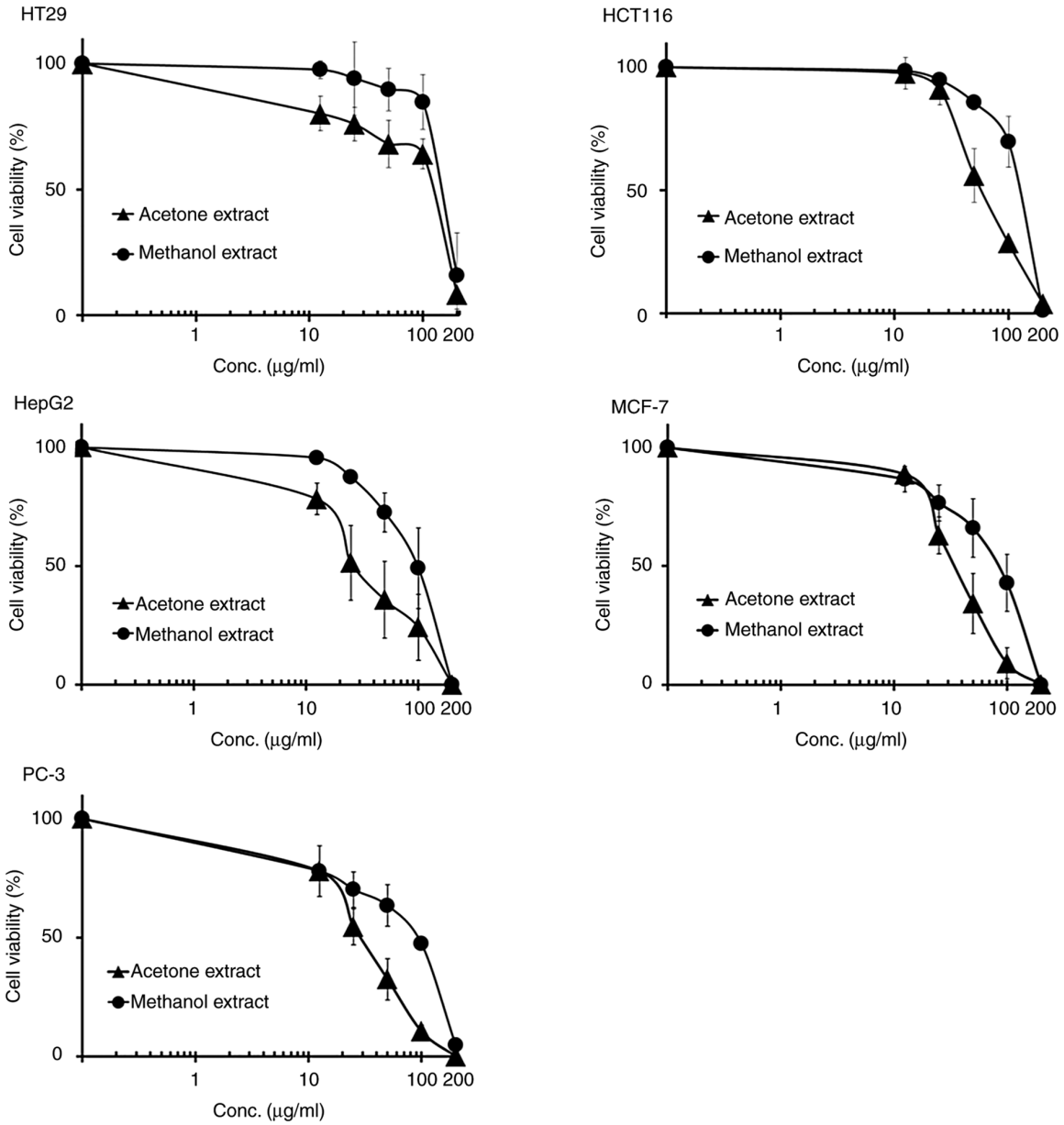

Figure 3. Growth inhibition by the crude extracts of MB in five cancer cell lines in colony formation assay. Exponentially dividing cells were treated with acetone/methanol extracts of MB at a dose range of 12.5-200 μg/ml for 7 days in DMEM/5% FBS. Results are the means ± SEs. Representative images of culture plates of the colony formation assay are shown in Fig. S1. The IC_{50} values are presented in Table I. MB, *Musa basjoo*.

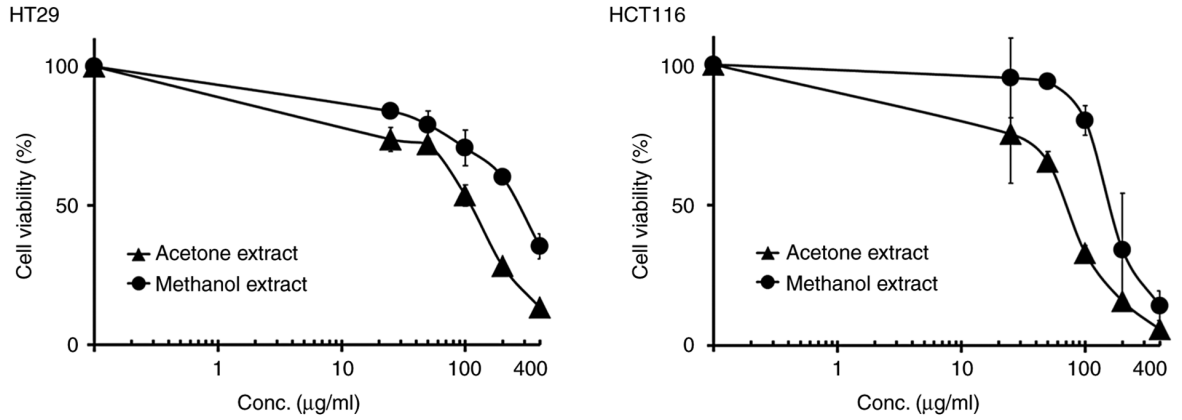

Figure 4. Growth inhibition by the crude extracts of MB in HT29 and HCT116 cell lines in MTT assay. Exponentially dividing cells were treated with acetone/methanol extracts of MB at a dose range of 25-400 μg/ml for 96 h in DMEM/5% FBS. Results are the means ± SEs. The IC_{50} values are presented in Table II. MB, *Musa basjoo*.

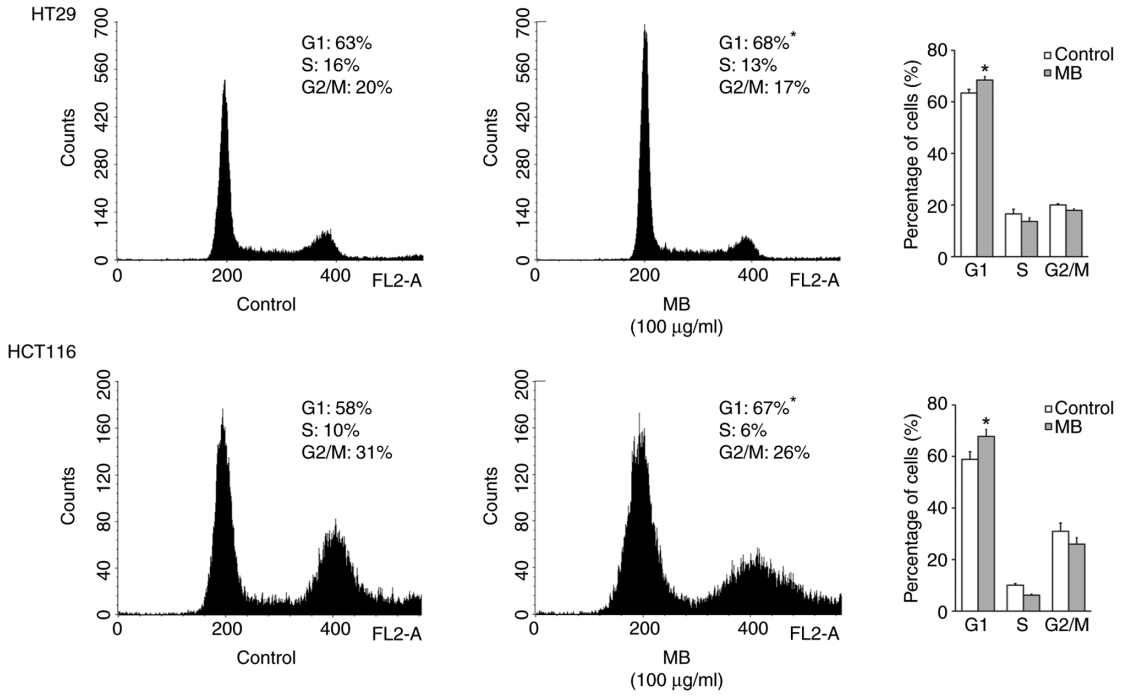

Figure 5. Representative results of flow cytometry analysis and graphs indicating the quantified results. HT29 and HCT116 cells were treated with DMSO (control) or 100 μg/ml acetone extract of MB for 96 h. The data indicate the percentage of cells in each phase of the cell cycle. Asterisk (*) indicates a statistically significant difference between cells treated with DMSO and those treated with the acetone extract (*P<0.05). Each assay was performed in triplicate. MB, *Musa basjoo*.

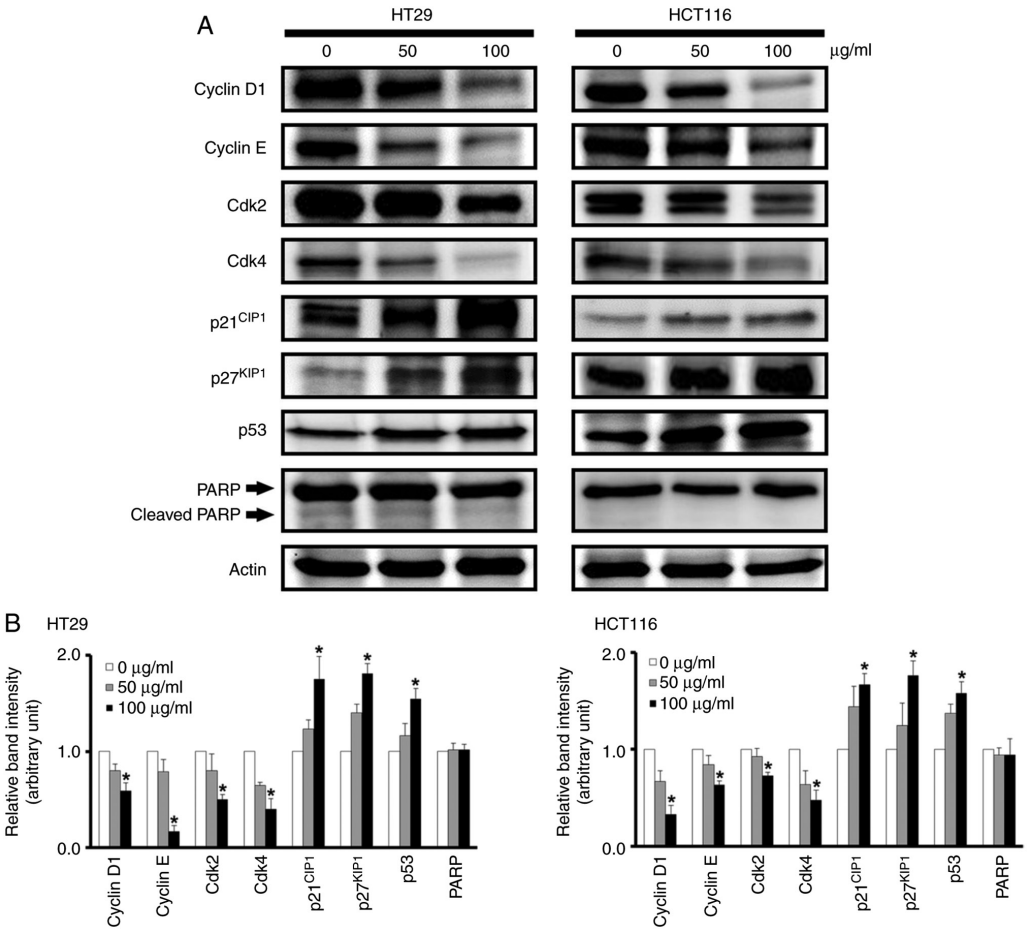

Figure 6. Representative results of western blot assays (A) and densitometry indicating relative band intensity (B). HT29 and HCT116 cells were treated with 0, 50 or 100 μg/ml acetone extract of MB for 96 h in DMEM/5% FBS. Asterisk (*) indicates a statistically significant difference between cells treated with DMSO and those treated with 100 μg/ml acetone extract of MB (*P<0.05). Each assay was repeated in triplicate to confirm the results. MB, *Musa basjoo*.

Table III. Body, organ, and relative organ weights of mice at the end of the experiment.

Group no.	Effective no. of mice	Treatment	Body weight (g)	Liver weight (g)	Relative liver weight[a]	Kidney weight (g)	Relative kidney weight[b]
1	4	None	19.15±0.56	0.95±0.04	0.05±0.0004	0.36±0.01	0.03±0.0004
2	4	Acetone extract of MB	19.33±0.33	1.00±0.04	0.06±0.001	0.35±0.01	0.02±0.001

Values are presented as the mean ± SE. [a]Liver weight (g)/body weight (g). [b]Kidney weight (g)/body weight (g). MB, *Musa basjoo*.

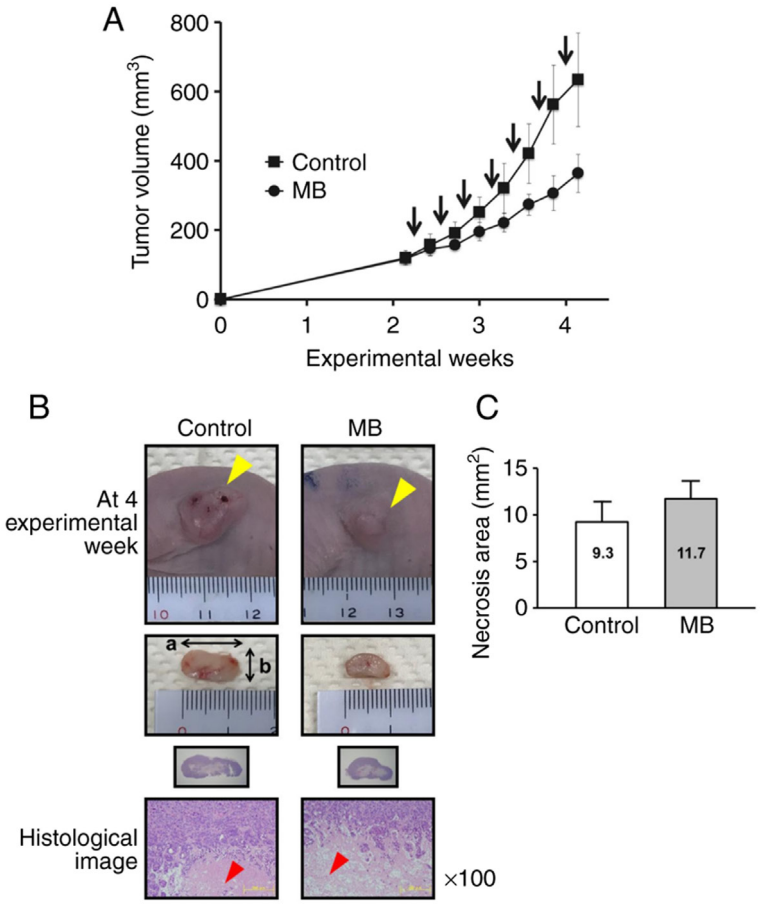

Figure 7. Tumor volume during experiment (A), representative images of animals with tumors (B) and necrosis area (mm^2) of the tumor (C). (A) Nude mice received intratumoral injections (arrows) of vehicle or the acetone extract of MB every other day (a total of 7 times). (B) Yellow arrowheads indicate tumors in the right flank of mice. The tumor was excised and cut in half. In the middle panel, a is the maximum and b is the minimum diameter of the tumor. Necrosis areas (red arrowheads) were observed in the inner part of the tumor in the HE image (lowest panel). (C) Y-axis indicates the necrosis area (mm^2). There was a tendency of an increase in the necrosis area of the MB treatment group (11.7 mm^2) when compared to that of the control group (9.3 mm^2). Results are the means ± SEs. MB, *Musa basjoo*.

evident between the control and MB treatment groups during the experimental period. No histologically significant adverse side effects were observed in heart, lung, liver, and kidney (Fig. S2).

Discussion

Although MB has previously been shown to have biological activities (3,4), there are no experimental data characterizing its anticancer properties during human consumption. Current studies have provided further evidence to support the empirical use of MB and identify a potential active component of MB for evaluating its *in vivo* treatment and/or prevention efficacy. The present study demonstrated the broad anticancer properties of MB in a wide variety of human cancer cell lines. Both the acetone and methanol extracts of MB caused a dose-dependent cancer cell growth inhibition, indicating that MB contains component(s) that have antiproliferative activity in these cell lines. In the human colorectal cancer cell lines, this antiproliferative activity appears to be due to its ability to induce G1-phase arrest by causing a decrease in the cellular levels of the cyclin D1, cyclin E, cdk2, and cdk4 proteins. In the current study, acetone and methanol extracts were

examined for their growth inhibitory activity using cell proliferation assays and the results showed that the acetone extract exerted stronger growth inhibition than the methanol extract in the cell lines tested. The differences observed between the two extracts are presumably due to the dissimilar contents of the active components contained in the extracts. The finding that the acetone extract may possibly contain components that inhibit the growth of cancer cells more effectively than the methanol extract requires further investigation.

In addition, HCT116 cells were found to be more sensitive to growth inhibition in the acetone/methanol extracts than HT29 cells. As similar results concerning different sensitivity in several human colorectal cancer cell lines were previously identified (10), the above-mentioned findings may provide clues as to why susceptibility to growth inhibition differs depending on the cell lines. $p21^{CIP1}$ is a tumor suppressor protein and acts as an inhibitor of cell cycle progression by negatively regulating the activity of cyclin D1-cyclin-dependent kinase (CDK) complex via a p53-dependent or -independent mechanism (18). In the current study, the levels of expression of $p21^{CIP1}$ and p53 proteins in the p53 wild-type HCT116 and p53 mutant HT29 cell lines were examined. The acetone extract of MB caused an increase in the cellular levels of the $p21^{CIP1}$ and p53 proteins. The results presumably indicate that p53 status, at least in part, is critical to the antiproliferative activity in HCT116 cells, as seen in a previous study (11). The reason for which the p53 mutant cell line HT29 is sensitive to the treatment of the MB extract requires further studies. It is known that the p53 family consists of three proteins, p53, p63 and p73 that are homologous at the amino acid level (19). Both p63 and p73 use two different promoters, resulting in the expression of two isoforms with different N-terminal domains, including transactivation domain (TA) and ΔN (amino-truncated) isoforms (20). Despite the high frequency of p53 mutations, p63 and p73 genes are rarely mutated in cancer (20). *In vivo* models indicated that the TA isoforms of p63 and p73 are tumor suppressors since the loss of these isoforms causes spontaneous tumor formation (21,22). In addition, p63 isoforms induced apoptosis in BHK cells transfected with wild-type p53, mutant p53 or p63 isoforms (23).

There are more than 50 species in genus *Musa* (3). The constituents of a plant may depend on the phytogeographical factors including plant sources, specific location of plant distribution, and seasonal time periods of the harvest (24-28). These factors may influence the biological activities and amount of active component present in the plant. Thus, it is of interest to investigate whether the chemical composition of various *Musa* species differs depending on the above-mentioned factors. Related to this fact, we had previously reported such discrepancy in the growth inhibition of cancer cells via investigation of the ethanol extracts between Brazilian and Chinese propolis (11). Furthermore, to evaluate the possibility of the clinical application of MB, it is necessary to identify the specific component present in the extracts of MB that cause growth inhibitory activity in human cancer cells, and to determine the precise chemical structure of this compound. As for active components obtained from rhizomes of MB, three known compounds 4-(4'-hydroxyphenyl)-2-methoxyphenalen-1-one, 2-phenyl-naphthalic anhydride, and 1,7-*bis*(4-hydroxyphenyl) hepta-4(*E*), 6(*E*)-dien-3-one exhibited significant cytotoxicities with IC_{50} values of 23-28, 6.5-18, and 3.7-11 μM against human cancer cell lines, respectively (29). In the present study, the dried leaf with a leaf blade and a stalk was extracted with acetone or methanol, a TLC-based simple identification method was performed, and the results showed that the acetone extract had specific spots visualized with UV lamp irradiation (254 and 365 nm) and phosphomolybdic acid staining. The results indicated that the crude extract of MB may contain aromatic compounds with a certain number of conjugated double bond (30-35) and/or an antioxidant compound with a hydroxy group (36,37), suggesting polyphenols and/or flavonoids as potential active components. The extract of Chinese propolis contains polyphenols and flavonoids (38-40). Previous findings demonstrated that the extract of Chinese propolis caused a marked growth inhibition on the HCT116 and HT29 human colorectal cancer cell lines but only a marginal growth inhibition on the FHC normal human colonic epithelial cell line (11). Therefore, cancer cell-specific effects may be caused by the MB treatment; however, this aspect should be further investigated. Limitations of the present study include the lack of data elucidating cell proliferation in the tumor tissue (e.g., Ki-68 immunohistochemical staining) and expression levels of apoptosis-associated proteins other than PARP (e.g., caspase-3). Fractionation followed by chromatographic analysis of the extract of MB is also needed to obtain information on structure-activity relationships (41-43).

There is limited information on the toxicity of the acetone or methanol extracts in experimental animal models, and there are, apparently, no clinical toxicity data on the use of the extracts of MB in humans. In the present study, in a mouse xenograft bioassay system no significant adverse side effects were caused by the administration of the acetone extract of MB into the tumor (Fig. S2), although a decrease in tumor volume was not statistically significant. The possible applications of the present study may depend on whether the acetone extract of MB can be given safely to humans at amounts appropriate enough to achieve pharmacologically active conditions. The important consideration is whether sufficient blood and tissue levels of the active component can be achieved and whether the extracts exert significant adverse side effects. To answer these questions, further investigations are in progress to identify the active component of the crude dried leaf extracts and its underlying mechanism of action.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HM, MI and MS designed the study. HM, SA, EY, TN, NS, MI, KO and MS performed the experiments and acquired the data. HM and MS confirm the authenticity of all the raw data. HM, KF, TN, MI, KO, KK and MS analyzed and interpreted the data. HM, KF, MI, KO, KK and MS wrote, reviewed and/or revised the manuscript. MI and MS contributed to the material management. MS supervised the study. All of the authors are fully aware of the contents of this paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed with the approval of the Animal Ethics Committee of the Nagoya City University (approval no. H28M-03) and according to the guidelines of the committee. Samples of *Musa basjoo* used in the current study were identified by MI, and located at the Herbal Garden, Gifu Pharmaceutical University, Japan. Voucher specimens can be made available for researchers, when requested via authentic certification.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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