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Original Article

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Anticancer and Antimutagenic Properties of *Pogonatherum paniceum* on Colorectal Cancer Cells

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Abstract

Background: *Pogonatherum paniceum* (*P. paniceum*) (Lam.) Hack. plays an important role in detoxification. However, its anticancer activity has not yet been elucidated. The aim of our study was to examine the suppressive proliferation, anti-migration and mutagenic/antimutagenic properties of *P. paniceum*. Moreover, we set out to determine the cellular mechanism underlying its antiproliferation.

Methods: To investigate *P. paniceum*'s anticancer ability, HCT116 and HT29 cell lines were treated with a water extract containing *P. paniceum*, and then the cell viability was examined using the trypan blue exclusion method which were compared to HEK293 (noncancerous cells). The anticancer effects were investigated by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and colony formation assay. Apoptosis induction, cell cycle distribution, and migration abilities were assessed by cell death detection enzyme-linked immunoassay (ELISA), flow cytometry, and wound healing assay. Finally, the mutagenicity and antimutagenicity were evaluated using the micronucleus assay.

Results: Treatment with *P. paniceum* caused a loss of cell viability in HCT116 and HT29 cells (not found in HEK293), which had an IC_{50} (half-maximal inhibitory concentration) of 1,156.2 and 1,207.0 µg/mL, respectively. We found that *P. paniceum* significantly inhibited the proliferative function of HCT116 and HT29 cells. To find the mechanism that exerts a suppressive proliferation effect on *P. paniceum*, we determined the DNA fragmentation and cell cycle distribution. We also found that *P. paniceum* treatment increased apoptosis and arrested of the cell cycle at G0/G1 remarkably when compared with the control group. Moreover, *P. paniceum* could decrease the migration of HCT116 and HT29 cancer cells. Finally, the treatment of *P. paniceum* did not induce micronucleus formation but did decrease the micronucleus frequency against mutagen-mitomycin C.

Conclusions: *P. paniceum* did not possess any toxicity (cytotoxic and mutagenic) but has the potential for anticancer activity against human colorectal cells by increasing apoptosis, which leads to the suppression of cell proliferation. *P. paniceum* also inhibits cell migration and exerts antimutagenicity, thereby suggesting that *P. paniceum* might be useful for colorectal cancer treatment.

Keywords: Colorectal cancer cells; Anticancer; Antimutagenicity; *Pogonatherum paniceum*

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Introduction

Cancer continues to be a major cause of death worldwide [1]. The greatest number of cancer cases relate to the detection of cancer in the breast, lungs, colon, and rectum. Among incidences of cancer, colorectal cancer (CRC) is the most commonly occurring cancer, accounting for approximately one-third of all cancer cases worldwide and is the second leading cause of cancer-related mortality in the world [2-4]. A diverse amount of stimuli contributes to genomic alterations that accumulate over time, which then drive the transformation of normal colonic epithelium cells into a colorectal carcinoma. CRC cells have a frequency characterized by excessive cell proliferation, most of which are able to evade apoptosis. These are important hallmarks of cancer [5, 6]. Apoptosis is one mechanism leading to cell death and is characterized by specific morphological and biochemical changes to cells, including cell blebbing, cytoplasmic condensation, loss of cell contact and cleavage of

chromosomal DNA. Therefore, the evasion of apoptosis is a major cause of cancer, but it is also providing opportunities for anticancer drug design [7-9].

Moreover, most cancer patients develop metastasis leading to poor prognosis due to limited therapeutic options [10, 11]. Tumor metastasis is a crucial mechanism consisting of a series of biological processes by which cancer cells move from the primary neoplasm to distant locations. Migration is a key cellular mechanism for cancer cells moving out, thereby disturbance in this process eliminates cancer progression [12, 13].

Various research studies have demonstrated that natural products, in particular plants, play a role as potential cancer agents. The World Health Organization (WHO) has estimated that approximately 75-80% of the world's population depends mainly on traditional medicines for their health care [14, 15]. In addition, there are several natural products and their analogs that are currently in the preclinical and clinical stages of development. In cancer treatment, natural products can be more efficient with fewer side effects, which makes them a promising substitute for chemotherapy, which has deleterious side effects and does not meet clinical needs [16, 17].

Pogonatherum paniceum (*P. paniceum*) is a perennial grass that belongs to the family of Poaceae. This plant is widespread in tropical and subtropical regions of Asia, Africa, and Oceania. A previous study has shown that *P. paniceum* can differentially respond to a number of environmental stress factors by the activation of various abiotic stress-specific proteins through alternative transcript splicing [18, 19]. Another study has shown that expression of GDP-D-mannose pyrophosphorylase (GMPase) was upregulated in the leaves of *P. paniceum* under stressed conditions (salinity and drought), and this contributes to increasing the content of L-ascorbic, which plays a crucial role in the detoxification of reactive oxygen species (ROS) [20]. *P. paniceum* might be a good candidate for the treatment of cancer due to its exertion of biological functions (e.g., detoxification). However, it has not yet been studied in the field of medical research. Therefore, the aim of our study was to assess suppressive cell proliferation and any possible mechanism responsible for anticancer activity on CRC cells. We selected human colorectal HCT116 and HT29 cancer cells as a research object, and a preliminary cytotoxicity test revealed that water extract containing *P. paniceum* exerts selected cytotoxic activity on those CRC cell lines and significantly inhibits the proliferative function of those cells, suggesting that it might be of potential as an anticancer agent. Interestingly, to further highlight the mechanisms of its proliferative suppression, we first evaluated its effects on programmed cell death or apoptosis and on the cell cycle progression of both CRC cells. Finally, we also explored, for the first time, mutagenicity and antimutagenicity of *P. paniceum.*

Materials and Methods

Sample collection and polyphenolic analysis

P. paniceum plants were collected in May 2020 from a local medicinal herb store in Kamphaengphet Province, Thailand. Leaves from the *P. paniceum* plants were extracted using aqueous according to previously reported with some modification [21]. A stock solution for our experiments was prepared in deionized water. The solution was then filtered and stored in a sterilized container and stored in the refrigerator (2 - 4 °C). Due to its average polyphenolic concentration, the *P. paniceum* extract was further tested using high performance liquid chromatography (HPLC) from the Central Laboratory Co., Ltd. Service Center (Chiangmai, Thailand). The HPLC analysis used 25 g of *P. paniceum* extract. The final solvent was filtered through 0.22-µm sterile membrane filters, and 20 µL was injected for HPLC analysis. Analytical HPLC analyses were performed on a binary solvent system (absolute methanol + 1% acetic acid). The column used was a 3.5-mm Kromasil reversed-phase column (150 × 4 mm) protected by a Kromasil C 18 (10 mm pre-column) (Scantec Lab, Savedalen, Sweden). The ultraviolet (UV) detection was monitored at 280, 360 and 530 nm. The concentration of each component was quantified with reference to samples of the commercial standards.

Cell culture and *in vitro* experiments

Human colorectal HCT116 (*p53*^{wt} tumor suppressor gene) (CCL-247™) and HT29 (p53^{R273H} tumor suppressor gene) (HTB-38™) cancer cells were supplied from American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (p/s) in a CO_2 incubator at 37 °C. A non-cancer HEK293 (human kidney epithelium) cell line was purchased from ATCC (CRL-1573™) and grown in completed medium consisting of Eagle's minimum essential medium (EMEM), 10% FBS, 1% p/s (all from Gibco). For subculturing, the media were discarded, and the cells were washed twice with phosphate buffered saline (PBS). The cells were treated with 0.25% trypsin-ethylenediaminetetraacetic acid solution (0.25% trypsin/EDTA) and centrifuged at 1,500 rpm for 5 min. The cells were collected and transferred to a new dish. The cells were sub-cultured every 2 to 3 days or at 80-90% confluency.

To assess the anticancer effect of *P. paniceum,* the HCT116 and HT29 cells were exposed to serial concentrations of *P. paniceum* for 24 - 48 h. Cell viability was evaluated using the trypan blue exclusion method. Then, a 50% reduction in cell survival (half-maximal inhibitory concentration (IC_{50}) value) was indicated. To analyze the effect of *P. paniceum* on antiproliferative ability, the HCT116 and HT29 cells were exposed to noncytotoxic doses of *P. paniceum* in DMEM containing 10% FBS and then subsequently incubated for 48 h. Furthermore, a study of the mechanism responsible for proliferative suppression was conducted. In this study, we investigated the effect of *P. paniceum* on apoptosis induction and cell cycle distribution through the evaluation of DNA fragmentation and cell cycle analysis, respectively. Inhibition of the migration of cancer cells was also evaluated using the scratch wound healing method. Finally, we performed mutagenicity and antimutagenicity using the *in vitro* cytokinesis-block micronucleus (CBMN) assay.

Cell viability assessment

The cytotoxicity of *P. paniceum* was assessed using the trypan blue exclusion method. The HCT116 and HT29 cell suspensions were seeded at a density of 8×10^4 cells/mL in a 12-well plate and allowed to attach for 24 - 48 h. The cells were exposed to *P. paniceum* at concentrations of 0, 500, 1,000, 1,250, and 1,500 µg/mL for 24 and 48 h in the condition mentioned above. After the treatment period, the chemical-containing medium was removed and washed several times with PBS. Then, the treated cells were collected using trypsinization and centrifugation at 1,500 rpm, for 5 min. The cell suspensions were analyzed after being mixed with 0.4% trypan blue dye, and the cells were counted using a hemocytometer under an inverted microscope. The percentage of viable cells was calculated and the cytotoxicity of the *P. paniceum* on those cell lines was indicated as an IC_{50} value, which was calculated by a linear equation of the relative viability rate versus the concentrations of *P. paniceum*.

The cytotoxicity in non-cancerous cells

The selective cytotoxicity of *P. paniceum* for CRC cells was evaluated by screening the IC_{50} value of *P. paniceum* against the non-cancerous HEK293 cell line. After 24 - 48 h of treatment periods, cell viability was assessed by cell counting using trypan blue dye. Then, the IC_{50} value was determined as described above.

Antiproliferative ability evaluation

Cell viability was investigated using the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction assay (Promega, Madison, WI). Briefly, 2,000 cells of HCT116 and HT29 cell suspensions were seeded into a 96-well plate with 100 µL seeded into each well. Subsequently, *P. paniceum* was added to the culture at final concentrations of 0, 600, 800, and 1,000 µg/mL, and it was then further incubated for 48 h. At the end of the incubation period, the medium containing *P. paniceum* was replaced with a new medium, and 20 µL of MTS solution was added to each well. The solution was incubated further for 1 h at 37 °C, after which the plate was shaken, and the absorbance at a wavelength of 492 nm was detected using a microplate reader (Synergy H1, Biotek, USA).

Colony formation assay

Cell lines at a density of 6×10^2 cells/well were plated in a six-well plate, and then treated with varying concentrations of *P. paniceum* extract (600, 800, and 1,000 µg/mL). The control cells were incubated without a test sample. After the cells were incubated for 48 h at 37 °C, the treated cells were replaced with fresh medium and incubated for another 7 - 9 days. Next, the medium was discarded, and the treated cells were washed with cold PBS before being fixed with ice-cold methanol. Then, the treated cells were stained with crystal violet (1%) and the cellular colonies attained in each well plate were photographed with a phase-contrast microscope. The number of colonies was counted.

Cell cycle analysis

HCT116 and HT29 cells were grown in six-well plates (2×10^5 cells/well) and then incubated with or without various concentrations of *P. paniceum* extract (0 - 800 µg/mL) for 48 h. The cell suspension was then pelleted, centrifuged, washed twice with cold PBS, and fixed with 70% ethanol at -20 °C for 24 h. Following this, the cells were stained with 50 µg/mL of propidium iodide (Biolegend, California) containing RNaseA (Geneaid, Taiwan) solution (20 µg/mL) for 30 min in the dark. The DNA contents at different phases of the cell cycle were measured by flow cytometry using a FACScan apparatus (Becton Dickinson, USA).

Apoptosis induction determination by cell death detection enzyme-linked immunoassay (ELISA)

Apoptosis was quantified using a cell death detection ELISA PLUS kit (Sigma-Aldrich, Mannheim, Germany) in accordance with previous study [22]. Briefly, the cells were seeded at a density of 6×10^4 cells/well in a 12-well plate and led to attach overnight. The cells were treated with *P. paniceum* at concentrations of 600 and 800 µg/mL and incubated for 72 h. Next, the cells were lysed with lysis solution and incubated at room temperature for 30 min. The lysate cells were transferred to the ELISA plate and incubated with anti-histone-biotin and anti-DNA-peroxidase before analysis, according to the manufacturer's protocol. The absorbance level was measured at 405 nm using the microplate reader (Synergy H1, Biotek, USA).

Wound-healing anti-migratory assay

The *in vitro* cell migration ability was determined by the wound healing assay. HCT116 and HT29 cells (1×10^6 cells/mL) were seeded in each 12-well plate. After incubation in a 5% CO_2 incubator at 37 °C to complete confluency, the old medium was replaced by a serum-free culture medium, which was then further incubated overnight. Then, the cultures were scratched to produce a wound using sterile 200 µL pipette tips. After removing the cell debris, the cells were exposed to *P. paniceum* (600, 800, and 1,000 µg/mL) and the untreated control was included for 24 - 48 h. The gap width was measured at 0, 24, or 48 h under an inverted microscope at magnification × 10. The percentage of wound closure was measured by using the following formula:

$$\%\ \text{Wound closure} = \frac{\text{Area of wound closure of cells at each time point}}{\text{Area of wound at 0 h}} \times 100\%.$$

Mutagenicity and antimutagenicity using *in vitro* CBMN test

The CBMN test was carried out to evaluate the mutagenicity and anti-mutagenicity of the *P. paniceum* extract using the protocol described by Fenech [23]. Chinese hamster lung fibroblast cells (V79 cells) were cultured at a density of 1×10^5 cells/mL. Following overnight incubation, the cells were treated with *P. paniceum* extract at various concentrations (600 and 1,000 µg/mL) for 24 h. A negative (untreated) and positive (mitomycin C (MMC): 1.25 µg/mL) controls were also evaluated. Eighteen hours before harvesting, cytochalasin B at a final concentration of 6 µg/mL was added to the cells to block the cytokinesis, which led to the cells being produced at the binucleated stage. After being washed by centrifugation at 800 rpm for 10 min at 4 °C, the cell pellets were resuspended in hypotonic solution (0.56% KCl) for 10 min at room temperature. Then, the pellets were incubated with fixing solution 1 (methanol: acetic acid: 0.9% NaCl) and fixing solution 2 (methanol: acetic acid) for 10 min at 4 °C, followed by centrifugation. The cell pellets were washed twice with the fixative, and then the cell suspension was dropped onto a clean microscope slide. After drying, the slides were stained with 4',6-diamidino-2-phenylindole (DAPI).

For micronucleus (MN) scoring, all slides were coded and analyzed blindly using a fluorescence microscope (Metafer, Germany) following the criteria for MN scoring in binucleated cells (BNC) as described by Fenech [23]. MN frequency was expressed as the number of micronucleated cells in 2,000 BNC scored. The cell cycle alterations, including cytotoxic and cytostatic effects, were concurrently expressed as a cytokinesis-blocked proliferation index (CBPI). Five hundred cells per concentrations were examined to determine the CBPI value, which was calculated from the following formula:

$$\mathrm{CBPI} = \frac{(1\times \mathrm{M1}) + (2\times \mathrm{M2}) + (3\times \mathrm{M3}) + (4\times \mathrm{M4})}{\mathrm{N}}$$

M1 - M4 represent the number of cells with one to four nuclei and N as a total number of cells count.

Statistical analysis

Data are presented as the mean ± standard deviation. Differences between the mean values for individual groups were assessed using a one-way analysis of variance (ANOVA) with Tukey's test applied to compare the mean frequencies of treatment groups with the control. Statistical analysis was conducted using SPSS version 25 (IBM). $P < 0.05$ was identified as indicating a statistically significant difference.

Results

P. paniceum possess cytotoxic effect on CRC cells

In order to study the effect of *P. paniceum* on proliferative inhibition activity in CRC cells, we first evaluated cell cytotoxicity. This allowed us to establish the IC_{50} value for *P. paniceum* on HCT116 and HT29 human CRC cell lines. When those cells were exposed to *P. paniceum* extract for 24 h, it had no effect on reducing the cell viability of the cell lines tested (Fig. 1a), as evidenced by there being no difference in cell morphology and cell population between the treatment groups and the control. After 48 h of *P. paniceum* treatment, we observed that the cells became damaged, starting from 500 to 1,500 µg/mL, and that the cell cultures exhibited a decrease in cell population after *P. paniceum* treatment compared to the control (Fig. 1a). Moreover, the treated cells became round in shape and detached from the surface leading to loss of cell viability. We further evaluated the cell viability using the trypan blue exclusion method. The results showed that *P. paniceum* significantly decreased the viability of both cells in a dose-dependent manner ($P < 0.05$, $P < 0.01$) (500 - 1,500 µg/mL), as shown in Figure 1b, demonstrating that *P. paniceum* treatment induces a cytotoxic effect on the cell line tested. Then, the IC_{50} value of each cell was determined to be 1,156.2 µg/mL for the HCT116 and 1,207.0 µg/mL for the HT29 cells after 48 h of treatment. According to these IC_{50} values, non-cytotoxic doses were used for subsequent experiments. Moreover, the viability of normal human epithelium (HEK293) with *P. paniceum* was similar to the control group at all selected concentrations for 24 and 48 h (excepted for the highest doses at 48 h, IC_{50} = 2,691.589 µg/mL), as evidenced by the non-neoplastic cell lines remaining above 70% even with the extract dose of 1,250 µg/mL at 48 h (Fig. 1b).

Polyphenolic content from the leaves of *P. paniceum*

The HPLC analysis of *P. paniceum* revealed the presence of several polyphenolic constituents and data are presented in Table 1.

P. paniceum exerts proliferative suppression on CRC cells

To explore the anticancer potential of *P. paniceum* in CRC cells, the effect of *P. paniceum*, at concentrations that do not cause massive cell death, was evaluated for cell proliferation in HCT116 and HT29 cells using MTS assay. We treated those cells with different concentrations of *P. paniceum* (600, 800, and 1,000 µg/mL) for 48 h. The results revealed that the proliferative abilities were different when comparing the control and treatment groups (Fig. 2a). Treatment with *P. paniceum* led to a very evident decrease in cell proliferation on both cells in a dose-dependent manner. To confirm the proliferative suppression ability, a colony-forming assay was conducted in both cancer cells. The results revealed that the number of colonies of HCT116 and HT29 cells in the *P. paniceum* treatment groups was significantly less than in the control group ($P < 0.05$) (Fig. 2b). Collectively, *P. paniceum* exerts the antiproliferation of HCT116 and HT29 cells. However, the mechanism responsible for proliferative suppression remains unclear.

P. paniceum induced cell cycle arrest on CRC cells

To elucidate the mechanism underlying cellular suppression, we carried out a cell cycle phase analysis of HCT116 and

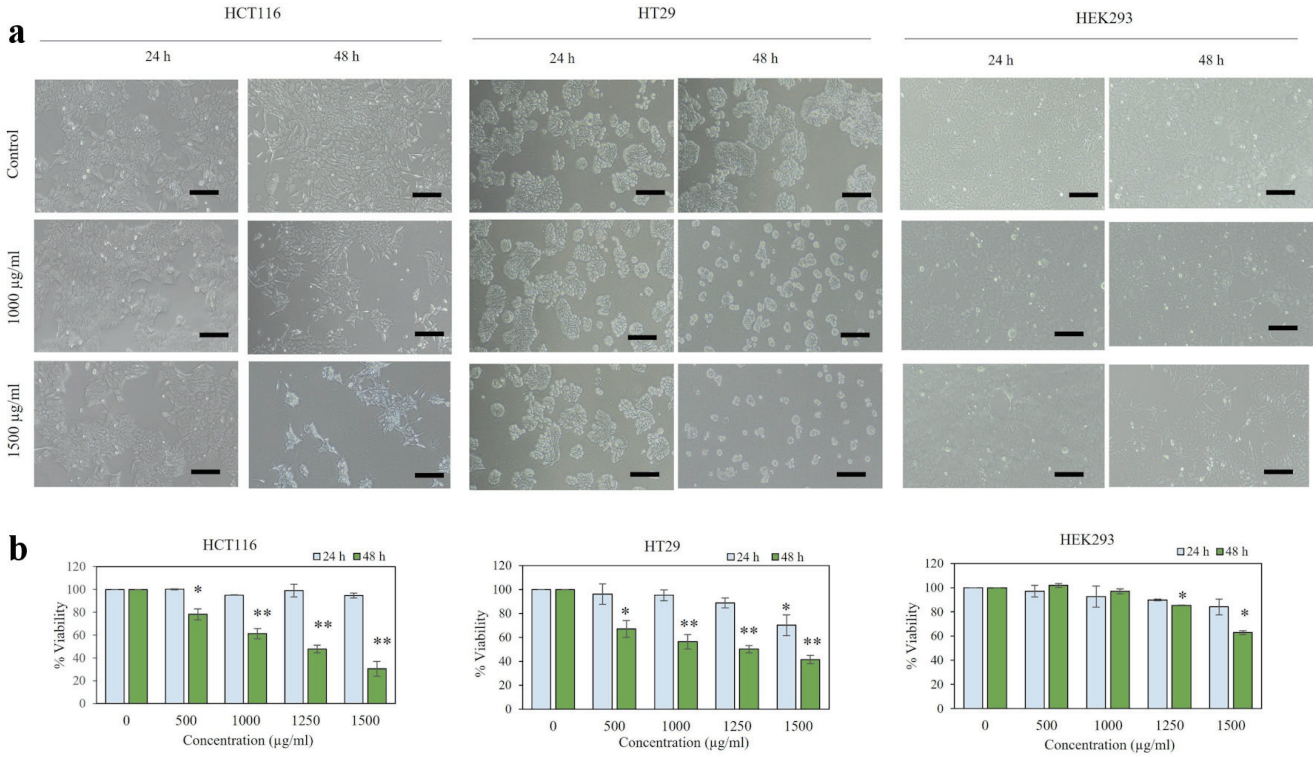

Figure 1. Viability of *P. paniceum* in HCT116, HT29, and HEK293 cells. (a) Representative images (scale bar = 50 µm) are shown. (b) Cells were treated for 24 and 48 h with *P. paniceum*, and cell viability was measured using trypan blue exclusion method. Data shown are mean ± SD. *P < 0.05, **P < 0.01, significantly different from control (untreated group). *P. paniceum*: *Pogonatherum paniceum*; SD: standard deviation.

HT29 cells following treatment with *P. paniceum* (600 and 800 µg/mL). The flow cytometry results showed that *P. paniceum* caused a G0/G1 phase arrest in both HCT116 and HT29 cells compared to the control group ($P < 0.05$) (Fig. 3). In the G0/G1 phase after treatment with *P. paniceum*, it was found that the highest accumulations of HCT116 cells and HT29 cells was 75.35% for 800 µg/mL and 82.40% for 600 µg/mL, respectively. Concomitantly, a significant decrease in the number of cells in the S phase was also observed ($P < 0.05$).

P. paniceum induces apoptosis on CRC cells

We conducted a further experiment to investigate the effects on apoptosis induction. This experiment was carried out to clarify whether apoptosis induction upon proliferative suppression on CRC cells is caused by *P. paniceum*. We determined this on the basis of DNA fragmentation, which is a biochemical hallmark of apoptosis, using a cell death detection ELISA assay. This assay is used for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments after induced cell death. The results revealed that the treatment of non-cytotoxic concentrations of *P. paniceum* on HCT116 and HT29 cells led to morphological changes, which were observed using inverted microscopy. Representative results are shown in Figure 4a. We found that there were more cell shrinkages and a tendency for them to float in the medium, which was seen more in the *P. paniceum* treatment groups when compared to the control. Interestingly, the apoptosis analysis results show that as the concentrations of *P. paniceum* increased (600 and 800 µg/mL), the apoptotic induction in both cells increased compared to the untreated control ($P < 0.05$) (Fig. 4b).

Table 1. HPLC Analysis Revealed the Presence of Phenolic Components in Aqueous Extract of *P. paniceum*

Polyphenolic compound of *P. paniceum* (mg/kg)	
Tannic acid	2,014
Gallic acid	1,101
Rutin	414.77
Catechin	308.29
Quercetin	195.50

HPLC: high performance liquid chromatography; *P. paniceum*: *Pogonatherum paniceum*.

P. paniceum abolishes migration activity of CRC cells

Next, we evaluated the effect of *P. paniceum* on the migration of CRC cells, which was investigated by scratch assays. The migration ability of HCT116 and HT29 cells was observed following treatment with *P. paniceum* at 600, 800, and 1,000 µg/mL for 24 and 44 h, respectively. The differences

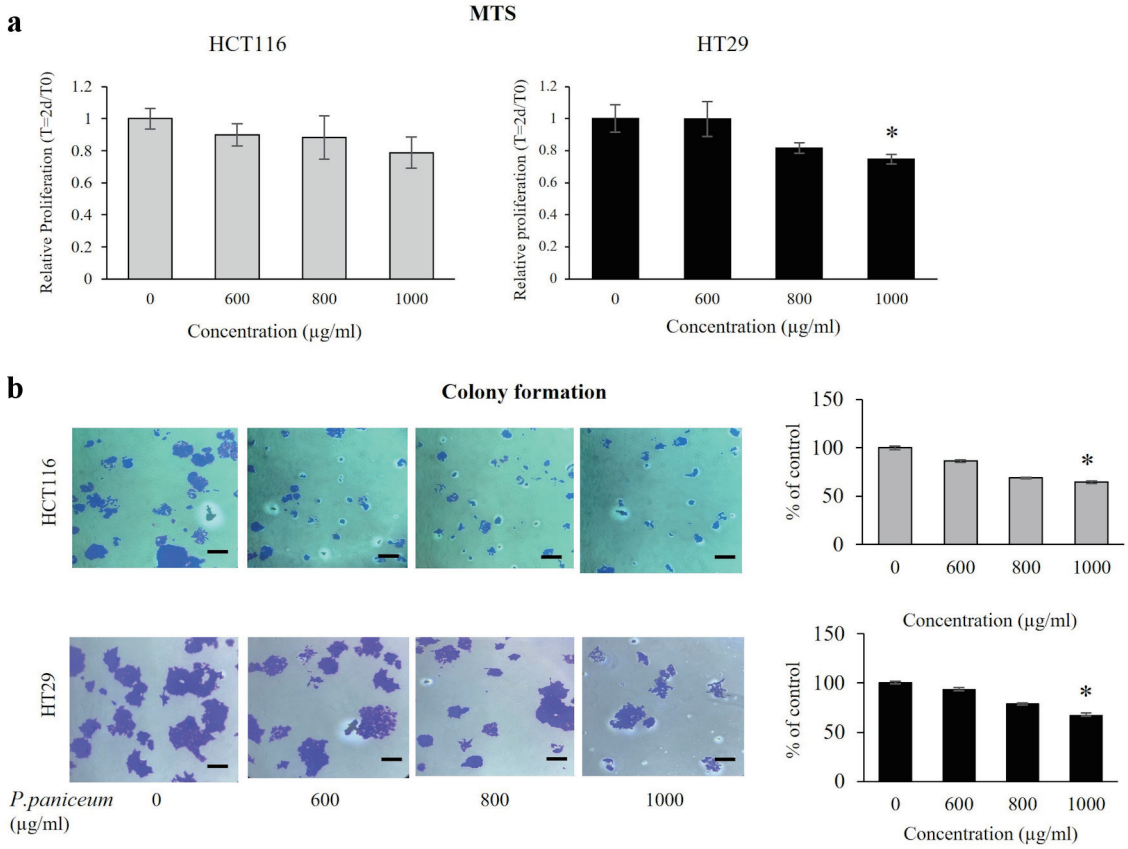

Figure 2. Proliferation and clonogenic property of HCT116 and HT29 cancer cells. (a) Cells were treated with varying doses of *P. paniceum* for 48 h, and proliferation was measured using MTS assay. (b) The anticolony formation activity of *P. paniceum* (48 h). The scale bar is 50 µm with magnification (× 20). The data shown are mean value ± SD. *P < 0.05, significantly different from control (untreated group). MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; *P. paniceum*: *Pogonatherum paniceum*; SD: standard deviation.

in the wideness of the scratch area were measured under an inverted microscope. While the untreated control increased the migration, treatment with *P. paniceum* resulted in a remarkable reduction in the migration of both HCT116 and HT29 cells (Fig. 5a). The treatment of HCT116 with 600, 800 and 1,000 µg/mL of *P. paniceum* for 24 h suppressed wound healing by 41.98%, 29.66%, and 29.17%, respectively, whereas in HT29 after 44 h, the width was 44.14%, 34.45%, and 25.32%, respectively. This result suggests that *P. paniceum* inhibits the *in vitro* migration of HCT116 and HT29 cells (Fig. 5b).

Mutagenicity and antimutagenic properties of *P. paniceum*

The mutagenicity and antimutagenicity of *P. paniceum* on V79 cells were evaluated using an *in vitro* CBMN assay. Before the antimutagenicity evaluation, we performed the mutagenicity test of *P. paniceum.* The analysis of MN frequency revealed that the extract concentrations at 600 and 800 µg/mL did not induce a dramatic increase in the MN formation compared to the negative and positive control groups (Fig. 6a, b). In the untreated negative control, the baseline of MN frequency was 30.00 ± 1.78 MN/2,000 BNC. Similar to the negative control, the MN formation was found at 26.00 ± 1.08 and 32.50 ± 1.04 for *P. paniceum* doses of 600 and 800 µg/mL, whereas the MMC-treated positive control showed a significant increase of MN of up to 144.50 ± 6.76 compared to the negative control ($P < 0.05$). These results indicate that *P. paniceum* did not induce mutagenicity in the *in vitro* MN assay.

The same concentrations used for mutagenicity testing were also used to evaluate the antimutagenic potential of the combination of *P. paniceum* and MMC (Fig. 6c). The frequency of MN in the negative control group was 30.75 ± 1.55 MN/2,000 BNC. In contrast, the MMC-treated group induced significant increase in MN frequency by 158.0 ± 6.76 ($P < 0.05$). For the combination of the extract with MMC, the data demonstrated that the combination treatment elicited a significant reduce in the MN frequency ($P < 0.05$). The MN frequencies were 100.0 ± 4.04 and 92.5 ± 2.31 MN/2,000 BNC when the cells were treated with doses of 600 and 800 µg/mL of *P. paniceum.*

The effect of cytostatic for all treated groups, including negative and positive controls and sample cultures, were measured and determined as CBPI values in accordance with OECD 487 guidelines. The results show that there were no sig-

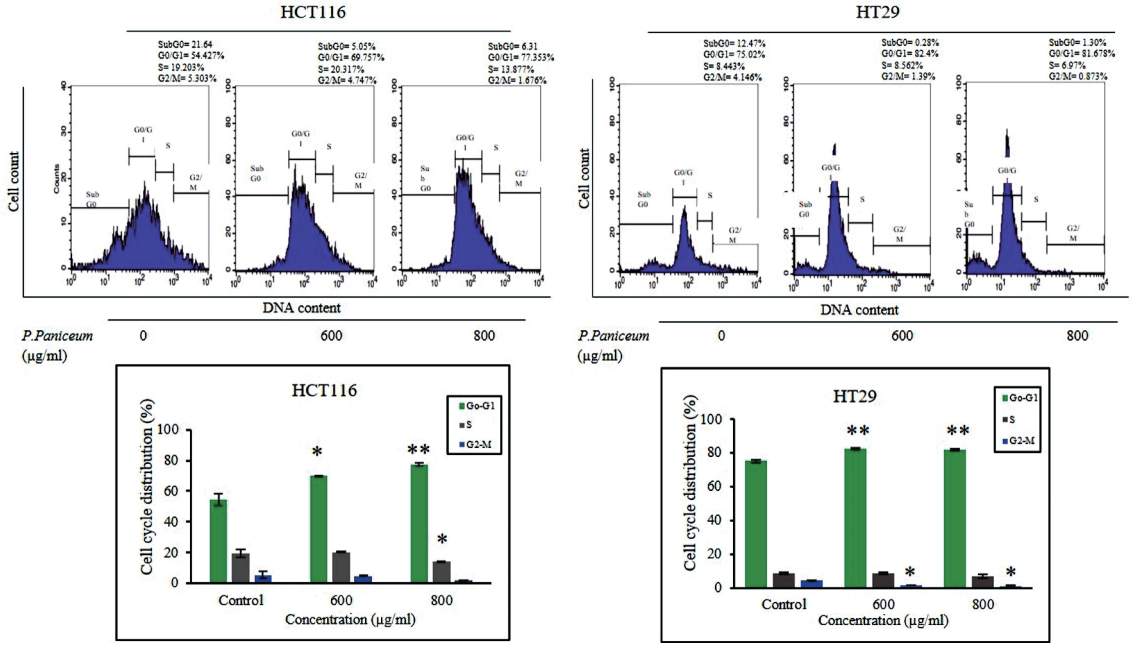

Figure 3. *P. paniceum* induced G0 - G1 cell cycle arrest of HCT116 and HT29 cancer cells. Cells were treated for 48 h with *P. paniceum*, and flow cytometry was used to determine the cell cycle distribution. All data are expressed as the mean ± SD. *P < 0.05, **P < 0.01 compared to the control. *P. paniceum*: *Pogonatherum paniceum*; SD: standard deviation.

nificant differences between the CBPI values of all treatment and untreated control groups (P > 0.05) (Table 2).

Discussion

Colon cancer is a common form of cancer that occurs in the digestive system and is a leading cause of cancer-related death. Moreover, the incidence rate of colon cancer is still increasing year by year. The courses used for therapy generally include surgical intervention and chemotherapy. However, these therapies can cause unexpected outcomes due to serious toxic side effects occurring, such as leading to damage of normal cells/tissue, and most patients with CRC die as a result of this disease. As a consequence, the screening of new anticancer drugs has become an increasing challenge.

In this study, we present the effects of *P. paniceum* on CRC cells. We have shown that *P. paniceum* extract, at non-cytotoxic concentrations, exerts a sign of damage on cancer cells and contributes to proliferative suppression while also

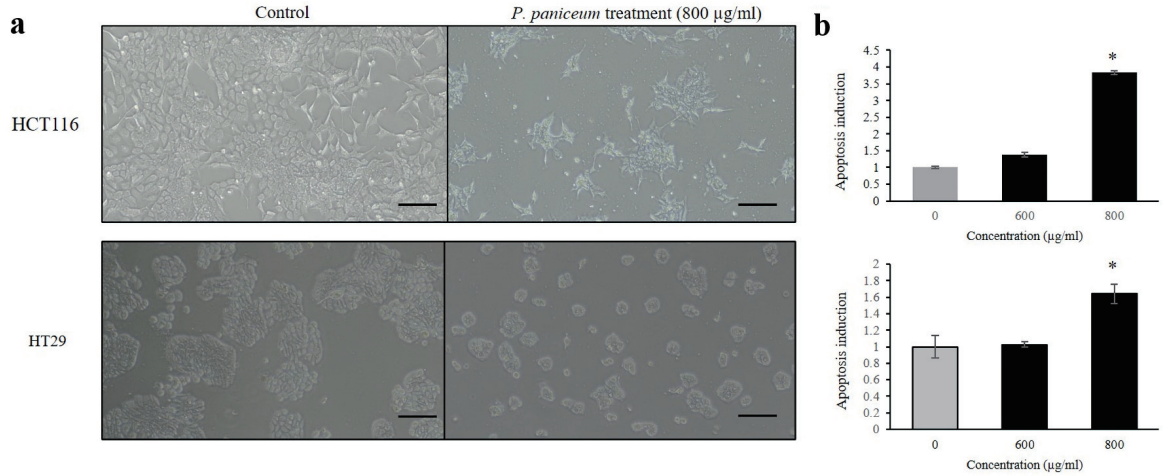

Figure 4. Apoptosis induction of HCT116 and HT29 cancer cells. (a) Cells were treated for 48 h with *P. paniceum*, and (b) apoptosis induction was measured using cell death detection ELISA (scale bar = 50 µm). The data shown are the mean value ± SD. *P < 0.05, significantly different from control (untreated group). *P. paniceum*: *Pogonatherum paniceum*; SD: standard deviation; ELISA: enzyme-linked immunoassay.

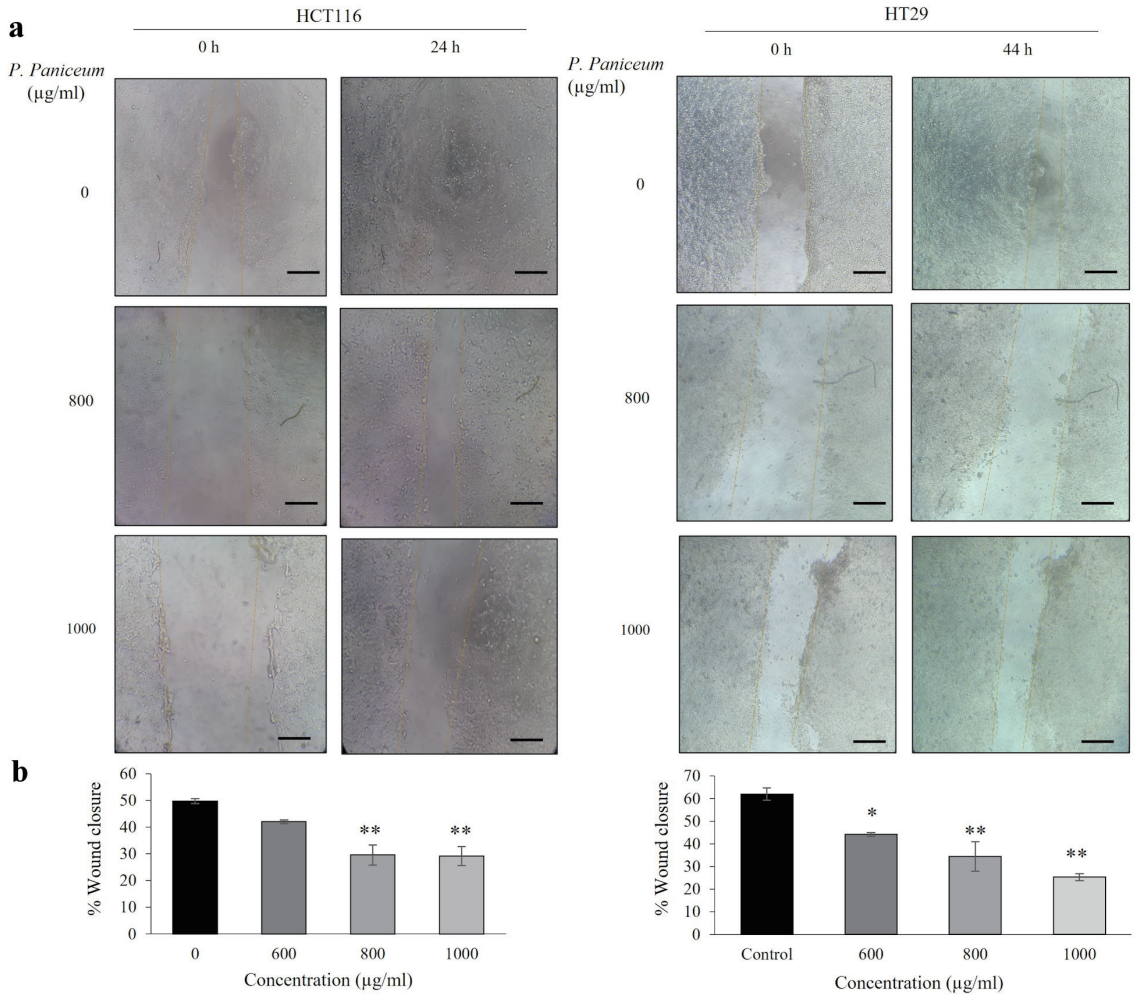

Figure 5. Migration of HCT116 and HT29 cancer cells following *P. paniceum* incubation for 24 and 44 h. (a) The photograph of migrating cells shown at magnification × 20 (scale bar = 50 µm). (b) The migration ability indicated as the percentage of wound healing. *P < 0.05, **P < 0.01 compared to the control. *P. paniceum*: *Pogonatherum paniceum*.

not being toxic to non-cancerous cells. To elucidate the compound association of the biological effect, we first explored the biochemical compound in the extract sample. Interestingly, we found that a variety of polyphenolic compounds are most abundant in *P. paniceum.* These include tannic acid, gallic acid, catechin, and rutin. In cancer research, it has been suggested that the bioactive component may also have anticancer properties. This is in agreement with previous studies which reported that phytochemicals in plants can induce cytotoxic activity in various types of cancer cells, when compared with known chemotherapy (e.g., doxorubicin), which is reliant on a rich source of active ingredients such as flavonoids, carotenoids, and phenolics [24, 25]. In addition, related research has indicated that active compounds and their antioxidants might have potential anticancer activities due to their inhibitory effect on free radicals [26]. Tannic acid is also known for its inhibitory action against breast cancer stem cells [27]. Another study demonstrated that tannic acid could attenuate the formation of cancer stem cells by inhibiting NF-κB activation and thereby preventing the phenotype transition of breast cancer cells [28]. Similarly, catechin has been reported to inhibit oxidative stress and to possess an anticancer effect on a variety of cancer cells, including breast and colon cancer cells [29, 30].

We first explored the relevance of *P. paniceum* for its anticancer effects by looking at its ability to help with proliferative suppression and its possibility for use in the induction of apoptosis in CRC cells. Excessive cell proliferation and evasion of apoptosis are characteristic hallmarks of cancer [31]. Morphological changes in apoptosis begin primarily with decreased cell volume and the condensation and subsequent fragmentation of its nucleus [32, 33]. Several studies have reported that targeting apoptosis can be used as a criterion for developing anticancer agents [32, 34]. Therefore, therapeutic approaches to the prevention or suppression of the proliferation and targeting of apoptosis can possibly be used as anticancer agents, as well as enhancing patient survival rates. The mechanism of apoptosis occurs due to several factors involving intrinsic and extrinsic pathways. These would lead to the conveyance of signals to initiate the execution process, resulting in the degradation of cytoskeletal and nuclear pro-

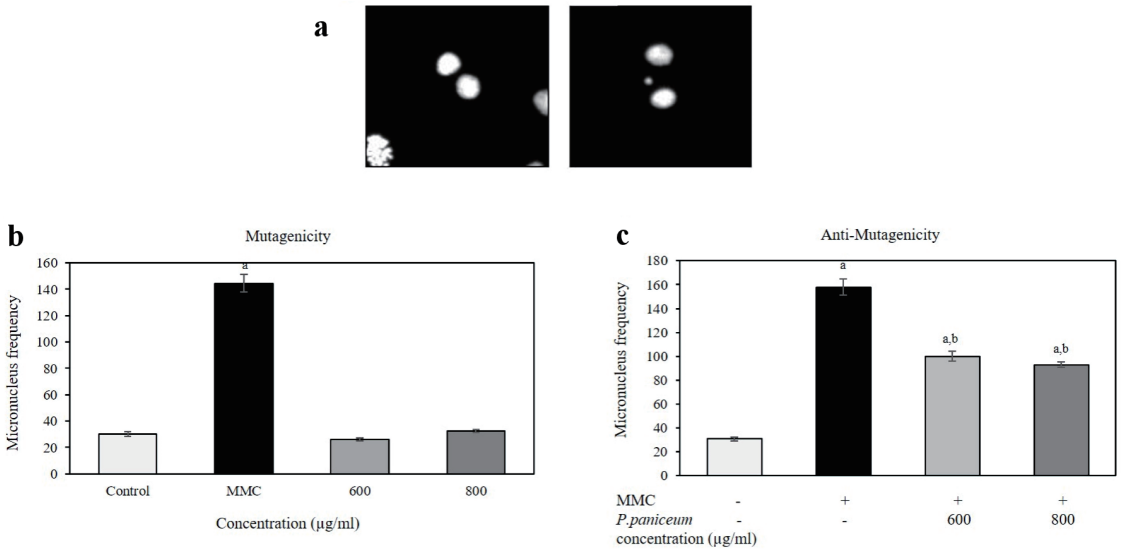

Figure 6. The frequency of micronuclei (MN) in V79 cells after the treatment of *P. paniceum* for 24 h. (a) Representative images shown binucleated cell (left) and MN in binucleated cell (right). (b) Cells were treated for 24 h with *P. paniceum*, and MN frequency was scored in 2,000 binucleated cells. (c) Cells were treated for 24 h with *P. paniceum* combined with mitomycin C ((MMC), 1.25 µg/mL). (a) and (b) significantly different from control (untreated group) and MMC, respectively. *P. paniceum*: *Pogonatherum paniceum*.

teins and the gradual loss of cell size, leading to the formation of apoptotic bodies [7, 35, 36]. Consistent with previous research, the findings of our study revealed a marked increase in the morphological hallmarks of apoptosis with regard to cell size loss. Some previous reports have shown an inducement of apoptosis in several types of cancer cells. In those studies, it was found that at high concentrations (2 mg/mL), water extract from *Momordica cochinchinensis* causes apoptosis of breast and melanoma cancer cells in a dose- and time-dependent manner [37].

Additionally, the cell cycle arrest of CRC cells in *P. paniceum* may also indicate its potential anticancer activity. However, there is currently no data on the cell cycle distribution of *P. paniceum*. In this study, the extract of *P. paniceum* at the concentration used showed a slight inhibition in the G0/G1 phase of the cell cycle. In this regard, previous studies have demonstrated that inducing the cell cycle arrest of cancer cells reflects an accumulation of responses to DNA damage that sequentially affect cell growth and could contribute to inhibiting cancer cell proliferation [38]. However, there may also be some activation of unique processes following the treatment of *P. paniceum.*

Table 2. The Cytokinesis-Block Proliferation Index (CBPI) in V79 Cells After the Treatments With *P. paniceum* and *P. paniceum* Combination With MMC

Treatments (µg/mL)	CBPI (mean ± SD)	
	P. paniceum	*P. paniceum* + MMC
Negative control	1.13 ± 0.02	1.13 ± 0.02
600	1.15 ± 0.03[a]	1.15 ± 0.03[a]
800	1.13 ± 0.01[a]	1.13 ± 0.01[a]
Positive control	1.07 ± 0.04[a]	1.07 ± 0.03[a]

[a]Not significant compared to negative control (P > 0.05). *P. paniceum*: *Pogonatherum paniceum*; MMC: mitomycin C.

Cell migration is a crucial process of spreading cancer into the surrounding area. It is involved in many processes, such as metastasis, which is the main cause of mortality. Therefore, disturbance of the metastasis pathway is clinically promising, and inhibition in this process eliminates cancer progression [12, 39]. However, this important property of *P. paniceum* has not been extensively investigated. We demonstrated the capability of *P. paniceum* to inhibit the migration of HCT116 and HT29 cells in a dose-dependent manner. Our findings reveal that *P. paniceum* has an antimigration effect against those human CRC cells.

It is important to knowledge the properties of *P. paniceum* regarding its effect on human genetic material. In our current study, we used the *in vitro* CBMN which is the widely used method to evaluate genetic damage. In this work, we followed the regulatory guidelines to evaluate the genetic potential according to OECD guidelines [40], and some modifications of Fenech [23]. The MN are small chromatin bodies surrounded by a nuclear envelope, which are not incorporated into the nucleus of the daughter cell after nuclear division, thereby indicating DNA damage as clastogenic or aneugenic activities [41-43]. In the present study, the extract of *P. paniceum* did not possess significant mutagenicity up to a dose level of 800 µg/mL in the MN test. Interestingly, *P. paniceum* exhibited antimutagenic activity against mitomycin C. However, there was no previous evidence of the mutagenic/antimutagenic potentials of *P. paniceum* detected in any *in vitro* and *in vivo* studies, and the MN test alone does not provide direct information on the mutagenic and antimutagenic potency.

Moreover, there are some limitations in this study that

should be noted. The *in vivo* studies were not completely understood. Future study studies may be conducted to find out the antiproliferative and its toxicity effects *in vivo* of *P. paniceum*. The information from these future tests will be beneficial for the usage of *P. paniceum* as an anticancer agent.

Conclusions

In conclusion, we reported for the first time that *P. paniceum* extract exhibits antiproliferative and antimigration activities against CRC cells. The antiproliferative effect was shown to be mediated through the induction of apoptosis and cell cycle arrest. Our results also reveal that *P. paniceum* caused an absence of cytotoxic or mutagenic potential at all concentrations tested but exerts antimutagenicity effects under the condition of the micronucleus assay. These results provide a scientific basis for the traditional use of *P. paniceum* as a potential anticancer agent.

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Conflict of Interest

The authors declare no conflict of interest.

Informed Consent

Not applicable.

Author Contributions

RP and WP designed and performed the study. RP drafted the manuscript and did critical editing. ST, WK, PJ, PK, and SL did the experiments. RT supported sample collection and extraction. PS provided cell lines. AR, PS, WP, and PCS have carefully supervised this manuscript.

Data Availability

The authors declare that data supporting the findings of this study are available with the article.

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