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Bromelain Enhances Radiosensitivity in HT-29 Colorectal Cancer Cells: Increased Chromosomal Damage and Reduced Clonogenic Survival

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Abstract

Background: Bromelain, a proteolytic enzyme extracted from pineapple stems, has demonstrated anticancer activity in several experimental models. However, its radiosensitizing effect in colorectal cancer remains insufficiently understood. The present study aimed to investigate the radiosensitizing effect of bromelain in HT-29 human colorectal adenocarcinoma cells.

Method: In this in-vitro experimental study, HT-29 cells were pretreated with bromelain (70 µg/mL) for 3 h before exposure to 2 or 4 Gy X-ray irradiation. Radiosensitivity was evaluated using the cytokinesis-block micronucleus assay, clonogenic survival assay, and catalase (CAT) and superoxide dismutase (SOD) activities. Statistical analyses were performed using GraphPad Prism version 8.0, with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; A $P < 0.05$ was considered to be statistically significant.

Results: Bromelain alone produced minimal effects. Bromelain pretreatment significantly increased micronucleus frequency from 39.22 ± 2.03 to 46.00 ± 2.00 after 2 Gy and from 43.11 ± 2.00 to 53.00 ± 1.00 after 4 Gy irradiation ($P < 0.01$). It also reduced clonogenic survival from 57.33 ± 2.04% to 49.00 ± 1.00% after 2 Gy and from 46.24 ± 1.67% to 29.10 ± 2.01% after 4 Gy ($P < 0.001$). Irradiation reduced CAT and SOD activities, whereas bromelain produced an additional significant decrease only in SOD activity.

Conclusion: Bromelain enhanced radiosensitivity in HT-29 cells by increasing radiation-induced chromosomal damage, reducing clonogenic survival, and further suppressing SOD activity. These

findings support further investigation of bromelain as a naturally derived radiosensitizer for colorectal cancer.

Keywords: Bromelain, Colorectal neoplasms, Radiosensitization, Micronucleus assay, Clonogenic survival

Introduction

Colorectal cancer (CRC) remains one of the most significant global health challenges and is currently among the most frequently diagnosed malignancies, worldwide. According to recent global cancer statistics, CRC ranks as the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality, accounting for approximately 1.9 million new cases and more than 900,000 deaths annually. Despite substantial advances in screening programs, surgical techniques, systemic therapies, and personalized treatment approaches, CRC continues to impose a considerable clinical and socioeconomic burden worldwide, particularly in patients with advanced-stage disease.[1-3]

Radiotherapy constitutes an integral component of multimodal treatment strategies for colorectal malignancies, especially rectal cancer. Preoperative and postoperative radiotherapy have significantly improved local tumor control, reduced recurrence rates, and enhanced surgical outcomes. Nevertheless, the therapeutic effectiveness of ionizing radiation is frequently limited by intrinsic or acquired radioresistance of tumor cells. Furthermore, the escalation of radiation dose is often constrained by unavoidable toxicity to surrounding normal tissues. Consequently, identifying approaches capable of selectively enhancing tumor radiosensitivity while preserving normal tissue integrity, remains a major objective in contemporary radiation oncology.[4-6]

The biological effects of ionizing radiation are mediated predominantly through the generation of reactive oxygen species (ROS), resulting in oxidative stress and damage to cellular macromolecules. DNA double-strand breaks represent a particularly lethal form of radiation-induced injury and play a critical role in determining cellular fate after irradiation. However, antioxidant defense systems and DNA repair pathways may enable tumor cells to survive radiation exposure and contribute to treatment resistance.[7-9] Therefore, modulation of oxidative stress and DNA damage responses represents a potential strategy for improving the therapeutic efficacy of radiation treatment.

Naturally derived bioactive compounds have attracted increasing attention as potential radiosensitizers due to their ability to influence multiple biological pathways. Several phytochemicals, including curcumin, resveratrol, quercetin, epigallocatechin gallate, and genistein, have demonstrated radiosensitizing properties through modulation of oxidative stress, apoptosis, cell-cycle regulation, inflammation, and DNA repair mechanisms.[10, 11]

Bromelain is a complex proteolytic extract primarily obtained from the stem and fruit of *Ananas comosus* (pineapple). It contains a heterogeneous mixture of cysteine proteases and other bioactive components and has traditionally been investigated for its anti-inflammatory, anti-edematous, fibrinolytic, and wound-healing properties. Growing evidence has also demonstrated anticancer effects of bromelain in several experimental cancer models.[12]

Experimental studies have shown that bromelain can suppress tumor cell proliferation and promote apoptosis and autophagy, while also modulating cellular

redox balance and signaling pathways involved in tumor progression.[12, 13] Bromelain has additionally been reported to enhance the effects of conventional anticancer therapies in experimental models. These biological activities, together with the central role of oxidative stress in radiation-induced cell killing, provide a rationale for investigating bromelain as a potential natural radiosensitizer.[12-14]

CRC represents one of the malignancies in which novel radiosensitizing approaches may be particularly beneficial. Among available experimental models, the HT-29 human colorectal adenocarcinoma cell line is widely employed in radiobiological research due to its well-characterized molecular profile and relatively resistant phenotype. Evaluation of radiation-induced micronucleus formation provides a sensitive indicator of chromosomal instability and unrepaired DNA damage, whereas the clonogenic survival assay remains the gold standard for determining long-term reproductive cell death following irradiation. Furthermore, measurement of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) offers valuable mechanistic insight into radiation-induced alterations in cellular redox homeostasis.[15-18]

Although bromelain has been extensively investigated for its anticancer activities, its potential as a radiosensitizer remains insufficiently characterized. In particular, limited evidence is available regarding its effects on radiation-induced chromosomal damage, long-term clonogenic survival, and antioxidant defense in CRC cells.

Accordingly, the present study was designed to investigate the radiosensitizing potential of bromelain in HT-29 human CRC cells exposed to ionizing radiation by evaluating radiation-induced chromosomal damage using the cytokinesis-block micronucleus (CBMN) assay, reproductive cell survival using the clonogenic assay, and alterations in antioxidant defense through the measurement of SOD and CAT activities.

Materials and Methods

Ethical considerations

The experimental protocol was approved by the Ethics Committee of Guilan University of Medical Sciences, Rasht, Iran (Approval No. IR.GUMS.REC.1402.248). All experimental procedures were conducted in accordance with the institutional guidelines for biomedical research.

Chemicals and reagents

Bromelain (2500 GDU/g), extracted from the stem of Ananas comosus, was purchased as a bulk powder from DarouSazi Salamat Parmon Amin Co. (Iran). Cytochalasin-B was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 culture medium, penicillin, and streptomycin were obtained from Dacell Co. (Iran). Fetal bovine serum and 0.25% trypsin-EDTA were obtained from Gibco-Invitrogen (Thermo Fisher Scientific, UK). Commercial SOD and CAT assay kits were purchased from Navand Salamat Co. (Urmia, Iran). Giemsa stain, methanol, glacial acetic acid, and phosphate-buffered saline were obtained from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade.

Bromelain preparation and irradiation conditions

Bromelain was freshly prepared immediately before each experiment by dissolving the powder in sterile distilled water under aseptic conditions and sterilizing the solution through a 0.22-μm syringe filter. Working solutions were prepared by dilution with complete culture medium to obtain a final concentration of 70 μg/mL. Since bromelain is a proteolytic enzyme complex rather than a single purified compound, concentrations are expressed as μg/mL throughout the study.

For irradiation, culture plates were transported to the radiotherapy unit and exposed to either 2 Gy or 4 Gy using a 6 MV

medical linear accelerator (Shinva, China). Irradiation was delivered at a dose rate of 1.96 Gy/min, with a source-to-surface distance of 100 cm and a treatment depth of 2 cm. Sham-irradiated control cultures were handled under identical transportation and environmental conditions but were not exposed to ionizing radiation.

Cell line and cell culture

The human colorectal adenocarcinoma cell line HT-29 was obtained from the National Center for Genetic and Biological Resources of Iran (IBRC), Tehran, Iran. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2. The culture medium was renewed every 2–3 days, and cells were routinely subcultured upon reaching approximately 80% confluence using 0.25% trypsin-EDTA.

Study design

The present in-vitro study was designed to investigate the potential radiosensitizing effect of bromelain on the human colorectal adenocarcinoma HT-29 cell line. The experimental design consisted of six experimental groups: untreated control, bromelain alone (70 µg/mL), 2 Gy irradiation, 4 Gy irradiation, bromelain + 2 Gy irradiation, and bromelain + 4 Gy irradiation. Bromelain was administered as a pretreatment 3 h before irradiation to evaluate its ability to enhance radiation-induced cellular responses.

To comprehensively assess the radiosensitizing potential of bromelain, four complementary biological endpoints were evaluated. Radiation-induced chromosomal damage was assessed using the CBMN assay, reproductive cell survival was determined by the clonogenic assay, and oxidative stress responses were evaluated by measuring the activities of the antioxidant enzymes SOD and CAT. Each experiment was performed independently in triplicate under identical experimental conditions.

CBMN assay

HT-29 cells were seeded into 12-well culture plates at a density of 1×10^5 cells/well and incubated for 24 h to allow cell attachment. Cells were subsequently treated with bromelain at a final concentration of 70 µg/mL for 3 h before irradiation. Irradiation was then performed with either 2 Gy or 4 Gy under the conditions described above.

Immediately after irradiation, the bromelain-containing medium was removed and replaced with fresh complete RPMI-1640 medium, and the cells were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Cytokinesis was then blocked by adding cytochalasin B to achieve a final concentration of 6 µg/mL. The cultures were further incubated for an additional 28 h before cell harvesting.

The harvested cells were fixed with cold methanol: glacial acetic acid (6:1, v/v). Three microscope slides were prepared from each sample, air-dried at room temperature for 24 h, stained with 10% Giemsa solution for 3 min, rinsed with distilled water, and examined under a light microscope.[16] For each sample, at least 1000 binucleated cells were scored, and the frequency of micronucleated binucleated cells was recorded for statistical analysis.

Clonogenic survival assay

HT-29 cells were seeded into 6-well culture plates at a density of 2×10^3 cells/well in triplicate and incubated for 24 h to allow cell attachment. The cells were subsequently treated with bromelain at a final concentration of 70 µg/mL for 3 h before irradiation. Irradiation was performed with either 2 Gy or 4 Gy under the conditions described above.

Immediately after irradiation, the bromelain-containing medium was replaced with fresh complete RPMI-1640 medium, and the cells were incubated for 14 days under standard

culture conditions to allow colony formation. The culture medium was renewed every 2–3 days throughout the incubation period.[15]

After 14 days, the colonies were gently washed with phosphate-buffered saline, fixed with cold methanol:glacial acetic acid (6:1, v/v), and stained with 10% Giemsa solution. Colonies containing 50 or more cells were considered viable and counted under a light microscope.[15]

The plating efficiency (PE) and surviving fraction (SF) were calculated using the following equations:

PE = (Number of colonies formed / Number of cells seeded) × 100%

SF = Number of colonies formed / (Number of cells seeded × PE)

where PE represents the PE of untreated control cells and SF represents the SF following each treatment condition.

Determination of CAT activity

HT-29 cells were seeded into 12-well culture plates and incubated for 24 h before treatment. The cells were subsequently treated with bromelain at a final concentration of 70 µg/mL for 3 h before irradiation. The culture plates were then transferred to the radiotherapy center and exposed to either 2 Gy or 4 Gy X-ray irradiation under the conditions described above. Immediately after irradiation, the plates were returned to the laboratory, and CAT activity was determined.[17]

CAT activity was measured using a commercially available Nasdox™ CAT Activity Assay Kit (Navand Salamat Co., Iran) according to the manufacturer's instructions. Cell lysates were prepared using the lysis buffer provided with the kit, and the resulting supernatants were used for enzyme activity measurement. Absorbance was recorded at 550 nm using a microplate reader, and CAT activity was calculated according to the manufacturer's protocol.

Determination of SOD activity

HT-29 cells were treated under the same experimental conditions described for the CAT assay. Immediately after irradiation, SOD activity was determined.

SOD activity was assessed using a commercially available Nactaz™ SOD Assay Kit (Navand Salamat Co., Iran) according to the manufacturer's instructions. Cell lysates were prepared using the lysis buffer supplied with the kit. The lysates were centrifuged, and the resulting supernatants were collected for SOD activity measurement.[18] Absorbance was measured at 405 nm using a microplate reader, and SOD activity was calculated according to the manufacturer's protocol.

Statistical analysis

All experiments were performed in three independent biological replicates (n = 3). Data are presented as mean ± standard deviation (SD) values. Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software Inc., San Diego, CA, USA). Differences among experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. A P-value < 0.05 was considered statistically significant.

Results

Bromelain enhanced radiation-induced micronucleus formation in HT-29 cells

The CBMN assay was performed to evaluate chromosomal damage induced by bromelain, ionizing radiation, and their combination in HT-29 cells (Figure 1).

As expected, exposure to X-ray irradiation significantly increased micronucleus formation compared with untreated control cells. The mean micronucleus frequency increased from 12.21 ± 2.00 in the control group to 39.22 ± 2.03 and 43.11 ± 2.00 following exposure to 2 Gy and 4 Gy, respectively (both P < 0.0001 vs. control). In

contrast, treatment with bromelain alone (70 μg/mL) produced only a slight increase in micronucleus frequency (16.36 ± 0.62), which was not statistically different from the control group ($P = 0.0966$).

Pretreatment with bromelain significantly potentiated radiation-induced chromosomal damage. In irradiated cells receiving bromelain, the mean micronucleus frequency increased to 46.00 ± 2.00 following 2 Gy irradiation and to 53.00 ± 1.00 following 4 Gy irradiation. Compared with radiation alone, bromelain pretreatment significantly increased micronucleus formation after 2 Gy irradiation ($P = 0.0042$) and after 4 Gy irradiation ($P = 0.0001$).

No statistically significant difference was observed between the 2 Gy and 4 Gy irradiation groups ($P = 0.1305$), nor between the 4 Gy and bromelain + 2 Gy groups ($P = 0.3640$). However, micronucleus formation in the bromelain + 4 Gy group was significantly higher than that observed in all other irradiated treatment groups, indicating the greatest extent of radiation-induced chromosomal damage under combined treatment conditions ($P < 0.01$).

Overall, the CBMN assay demonstrated that bromelain alone exerted minimal genotoxicity, whereas pretreatment with bromelain significantly enhanced radiation-induced chromosomal damage at both radiation doses, consistent with a radiosensitizing effect in HT-29 CRC cells.

Bromelain potentiated the inhibitory effect of ionizing radiation on clonogenic survival of HT-29 cells

The clonogenic survival assay was performed to determine whether bromelain pretreatment enhanced the inhibitory effect of ionizing radiation on the long-term proliferative capacity of HT-29 cells (Figure 2).

Exposure to ionizing radiation significantly reduced clonogenic survival compared with untreated control cells. SF decreased from 100.00 ± 0.00 in the control group to 57.33 ± 2.04 following 2 Gy irradiation and 46.24 ± 1.67 following 4 Gy irradiation (both $P < 0.0001$ vs. control). In contrast, treatment with bromelain alone resulted in only a slight reduction in clonogenic survival (96.43 ± 0.77), which was not statistically different from the untreated control ($P = 0.1165$).

Pretreatment with bromelain further enhanced the inhibitory effect of radiation on colony formation. SF decreased to 49.00 ± 1.00 in the bromelain + 2 Gy group and 29.10 ± 2.01 in the bromelain + 4 Gy group. Compared with irradiation alone, bromelain pretreatment significantly reduced clonogenic survival following 2 Gy irradiation ($P = 0.0003$) and 4 Gy irradiation ($P < 0.0001$).

A significant difference was also observed between the 2 Gy and 4 Gy irradiation groups ($P < 0.0001$), indicating a dose-dependent reduction in clonogenic survival following radiation exposure. No statistically significant difference was found between the 4 Gy and bromelain + 2 Gy groups ($P = 0.3049$). The bromelain + 4 Gy group exhibited the lowest SF among all experimental groups, demonstrating the greatest inhibition of clonogenic growth.

Overall, these findings indicate that bromelain alone had minimal effects on clonogenic survival, whereas pretreatment with bromelain significantly potentiated the inhibitory effect of ionizing radiation on the reproductive survival of HT-29 CRC cells.

Effect of bromelain pretreatment on CAT activity in irradiated HT-29 cells

CAT activity was measured to evaluate whether bromelain pretreatment influenced the antioxidant response of HT-29 cells following ionizing radiation (Figure 3).

Treatment with bromelain alone did not significantly alter CAT activity compared with untreated control cells (2.260 ± 0.093 vs. 2.285 ± 0.013, $P = 0.9821$). In contrast, exposure to ionizing radiation significantly reduced CAT activity. The mean CAT

activity decreased to 1.973 ± 0.005 following 2 Gy irradiation and to 1.931 ± 0.008 following 4 Gy irradiation, both of which were significantly lower than the untreated control ($P < 0.0001$).

Pretreatment with bromelain before irradiation resulted in CAT activities of 1.950 ± 0.057 in the bromelain + 2 Gy group and 1.857 ± 0.025 in the bromelain + 4 Gy group. However, these values were not significantly different from their corresponding irradiation-only groups ($P = 0.9883$ for 2 Gy vs. bromelain + 2 Gy and $P = 0.4171$ for 4 Gy vs. bromelain + 4 Gy). Likewise, no significant difference was observed between the 2 Gy and 4 Gy irradiation groups ($P = 0.8680$).

Overall, these findings indicate that ionizing radiation significantly decreased CAT activity in HT-29 cells, whereas bromelain pretreatment did not produce an additional statistically significant effect on CAT activity beyond that induced by irradiation.

Effect of bromelain pretreatment on SOD activity in irradiated HT-29 cells

SOD activity was evaluated to determine whether bromelain pretreatment modulated the antioxidant response of HT-29 cells following ionizing radiation (Figure 4).

Treatment with bromelain alone did not significantly affect SOD activity compared with the untreated control group (1.201 ± 0.054 vs. 1.360 ± 0.010, $P = 0.0907$). In contrast, exposure to ionizing radiation significantly reduced SOD activity. The mean SOD activity decreased to 1.140 ± 0.034 following 2 Gy irradiation ($P = 0.0132$) and to 1.084 ± 0.034 following 4 Gy irradiation ($P = 0.0024$) compared with the control group.

Pretreatment with bromelain before irradiation produced a further significant reduction in SOD activity. The bromelain + 2 Gy group exhibited a mean SOD activity of 0.853 ± 0.140, which was significantly lower than that observed in the 2 Gy irradiation-only group ($P = 0.0017$). Likewise, the bromelain + 4 Gy group showed a mean SOD activity of 0.701 ± 0.024, representing a significant decrease compared with the 4 Gy irradiation-only group ($P = 0.0001$).

No significant differences were observed between the 2 Gy and 4 Gy irradiation groups ($P = 0.8948$) or between the bromelain + 2 Gy and bromelain + 4 Gy groups ($P = 0.1122$).

Overall, these findings demonstrate that bromelain significantly potentiated the radiation-induced reduction in SOD activity, indicating a greater impairment of the cellular antioxidant defense system following combined treatment. A summary of the effects of bromelain pretreatment on radiation-induced chromosomal damage, clonogenic survival, and antioxidant enzyme activities is presented in Table 1. Bromelain pretreatment significantly increased micronucleus frequency and reduced clonogenic survival at both radiation doses. It also further reduced SOD activity, whereas the additional reduction in CAT activity was not statistically significant.

Discussion

The present study demonstrates that bromelain enhances the radiosensitivity of HT-29 CRC cells when administered prior to ionizing radiation. Bromelain alone exerted minimal effects on chromosomal damage, clonogenic survival and antioxidant enzyme activity, whereas its combination with irradiation significantly increased micronucleus formation, reduced clonogenic survival and selectively altered the antioxidant defense system.

A major finding of the present study is the significant increase in micronucleus formation following bromelain pretreatment before irradiation. Micronuclei are well-established biomarkers of unrepaired DNA double-strand breaks and chromosome mis-segregation during mitosis and therefore

reflect genomic instability induced by ionizing radiation.[19, 20] Since bromelain alone did not significantly increase micronucleus frequency, the enhanced chromosomal damage observed in the combined treatment groups is unlikely to result from intrinsic genotoxicity. Instead, bromelain appears to increase the susceptibility of irradiated cells to DNA damage, thereby amplifying the biological consequences of radiation exposure.

The mechanism underlying this radiosensitizing effect is likely multifactorial. Previous studies have shown that bromelain suppresses CRC cell growth through induction of apoptosis and autophagy, increased intracellular ROS generation, and modulation of signaling pathways involved in cell survival, including PI3K/Akt and ERK.[21, 22] These effects may interfere with cellular recovery following radiation-induced damage and thereby contribute to the increased chromosomal damage observed in the present study. In addition, modulation of oxidative stress may be particularly relevant because ROS are important mediators of radiation-induced DNA damage. However, the present study did not directly assess DNA repair pathways, apoptosis, autophagy, or intracellular ROS levels; therefore, these mechanisms should be considered potential explanations rather than directly demonstrated mechanisms.

Similar radiosensitizing mechanisms have been described for several naturally derived compounds, including curcumin, quercetin, resveratrol and thymoquinone, which can enhance radiation-induced DNA damage through increased oxidative stress, inhibition of DNA repair and activation of apoptotic signaling pathways.[23] Our previous study using T47D breast cancer cells similarly demonstrated that pretreatment with quercetin increased radiation-induced micronucleus formation, reduced clonogenic survival and further suppressed antioxidant defenses.[24] Although bromelain has not previously been investigated as a radiosensitizer in HT-29 CRC cells, these findings support the broader concept that naturally derived compounds may enhance the response of tumor cells to ionizing radiation through modulation of multiple biological pathways.

The clonogenic assay further confirmed the radiosensitizing activity of bromelain. Although bromelain alone had little effect on colony formation, pretreatment significantly reduced the SF after irradiation, indicating that irradiated HT-29 cells lost their capacity for sustained proliferation. Because clonogenic survival reflects the long-term reproductive integrity of cancer cells, it is considered the gold standard for assessing radiosensitivity.[15] The parallel increase in micronucleus frequency and decrease in clonogenic survival observed in the present study suggests that the additional DNA damage induced by bromelain is biologically relevant and ultimately translated into irreversible loss of proliferative potential. Our findings are also consistent with our recent study in MCF-7 breast cancer cells, in which bromelain significantly enhanced radiation-induced micronucleus formation, reduced clonogenic survival, and potentiated oxidative stress–related responses. Although the tumour models differed, the comparable biological effects observed in both breast and CRC cells suggest that bromelain-mediated radiosensitization may not be limited to a single cancer type and may indicate that bromelain-mediated radiosensitization is not restricted to a single tumour model.[25]

These findings are also consistent with previous studies demonstrating that radiosensitization in CRC cells can be accompanied by reduced clonogenic survival following inhibition of DNA repair and activation of apoptosis.[26, 27] In particular, suppression of the PI3K/Akt/mTOR signaling pathway has been shown to

enhance the radiosensitivity of HT-29 and HCT116 CRC cells by attenuating DNA double-strand break repair through inhibition of ATM and DNA-PKcs activation.[27] Recent evidence further indicates that agents capable of increasing oxidative stress while interfering with pro-survival signaling pathways may represent promising adjuncts to radiotherapy. Although the present study did not directly investigate these signaling pathways, the observed enhancement of radiation-induced chromosomal damage and loss of clonogenic survival is compatible with such mechanisms.

Another noteworthy observation was that bromelain enhanced the radiation response at both 2 Gy and 4 Gy. These doses were selected to represent clinically relevant irradiation conditions. A dose of 2 Gy corresponds to a conventional daily fraction used in external beam radiotherapy, whereas doses around 4 Gy are used in moderate hypofractionated treatment protocols for several solid tumors, including rectal cancer.[28, 29] Evaluating bromelain under both irradiation conditions therefore provides a broader assessment of its potential radiosensitizing activity. The absence of a statistically significant difference between the two radiation doses for some endpoints suggests that the magnitude of bromelain-mediated radiosensitization may not increase proportionally with radiation dose.

Interestingly, although increasing the radiation dose from 2 Gy to 4 Gy produced only a modest increase in micronucleus frequency, it resulted in a substantially greater reduction in clonogenic survival. This finding suggests that increasing radiation dose does not necessarily lead to a proportional increase in early chromosomal damage but may produce a greater loss of long-term reproductive capacity. Such observations are consistent with the radiobiological concept that clonogenic survival integrates the cumulative consequences of DNA damage, mitotic catastrophe and delayed cell death, whereas micronucleus formation reflects only one component of radiation-induced chromosomal injury.[15]

The antioxidant enzyme findings provide additional insight into the potential contribution of redox regulation to bromelain-mediated radiosensitization. Irradiation significantly reduced the activities of both CAT and SOD, confirming alterations in the antioxidant defense system following radiation exposure.[30-32] However, bromelain pretreatment produced an additional significant reduction only in SOD activity, whereas CAT activity did not differ significantly from the corresponding radiation-alone groups. This differential response suggests that bromelain may selectively influence components of the cellular antioxidant defense system rather than causing a generalized suppression of antioxidant enzymes.

The distinct behaviour of these enzymes is biologically plausible. SOD represents the first enzymatic barrier against radiation-induced oxidative stress by catalysing the conversion of superoxide anions into hydrogen peroxide, whereas CAT functions downstream by detoxifying hydrogen peroxide into water and oxygen. Consequently, impairment of SOD activity is expected to promote intracellular ROS accumulation at an earlier stage following irradiation, thereby facilitating oxidative DNA damage and enhancing radiosensitivity.[30, 33] The absence of a further reduction in CAT activity may reflect compensatory regulation of hydrogen peroxide metabolism or the involvement of alternative antioxidant pathways, indicating that bromelain primarily influences superoxide-mediated oxidative stress.

The present findings are consistent with the study of Huang et al., who demonstrated that bromelain increased intracellular ROS

generation and induced apoptosis and autophagy in HT-29 and HCT116 CRC cells through modulation of PI3K/Akt and ERK signalling pathways.[21] Although antioxidant enzyme activities were not measured in their study, their observations support the hypothesis that oxidative stress contributes to bromelain-mediated radiosensitization.

Similar observations have also been reported for other naturally derived radiosensitizers. Several phytochemicals, including quercetin, curcumin, resveratrol and thymoquinone, have been shown to enhance radiation-induced oxidative stress while simultaneously suppressing DNA repair and survival signalling pathways, thereby increasing tumour radiosensitivity.[23] The consistency of these findings across different tumour models suggests that modulation of oxidative stress represents a common mechanism through which phytochemicals potentiate the biological effects of ionizing radiation.

The findings of the present study further support the radiosensitizing potential of bromelain in HT-29 CRC cells. Bromelain pretreatment increased radiation-induced chromosomal damage, reduced long-term clonogenic survival and selectively modulated the cellular antioxidant defense system, particularly through suppression of SOD activity. These findings suggest that bromelain may facilitate radiation-induced oxidative stress and impair the capacity of tumour cells to recover from radiation-induced damage. Further studies investigating the underlying molecular mechanisms and validating these findings in appropriate in vivo CRC models are warranted.

This study has some limitations. First, the experiments were conducted using a single CRC cell line (HT-29), which may limit the generalizability of the findings to other cancer cell types. Second, the evaluation was limited to micronucleus formation, clonogenic survival, and SOD and CAT activities. Further studies using additional cell lines, a broader range of bromelain concentrations, and complementary molecular assays are needed to clarify the mechanisms underlying the observed effects and to determine the potential of bromelain as a radiosensitizing agent.

Conclusion

The present study demonstrated that bromelain pretreatment enhances the radiosensitivity of HT-29 CRC cells. Bromelain significantly increased radiation-induced chromosomal damage and reduced clonogenic survival, while selectively enhancing the radiation-induced reduction in SOD activity without producing a significant additional effect on CAT activity. These findings support the potential of bromelain as a naturally derived radiosensitizing agent and provide a basis for further investigation of its underlying molecular mechanisms and therapeutic potential in combination with radiotherapy.

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Authors' Contributions

Mona Haddad Zahmatkesh conceived and designed the study, performed the experiments, analyzed the data, interpreted the results, and drafted the manuscript. Amir Shojaeifar contributed to the experimental work and data collection. Hamid Saeidi Saedi supervised and performed the irradiation procedures. All authors read and approved the final manuscript.

Conflict of Interest

None declared.

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Table 1. Summary of the effects of bromelain pretreatment on radiation-induced responses in HT-29 cells

Endpoint	Dose	Radiation alone	Bromelain + radiation	*P*-value
Micronucleus frequency	2 Gy	39.22 ± 2.03	46.00 ± 2.00	0.0042
Micronucleus frequency	4 Gy	43.11 ± 2.00	53.00 ± 1.00	0.0001
Clonogenic survival (%)	2 Gy	57.33 ± 2.04	49.00 ± 1.00	0.0003
Clonogenic survival (%)	4 Gy	46.24 ± 1.67	29.10 ± 2.01	<0.0001
CAT activity (n mol/ml/min)	2 Gy	1.97 ± 0.01	1.95 ± 0.06	0.9883
CAT activity (n mol/ml/min)	4 Gy	1.93 ± 0.01	1.86 ± 0.03	0.4171
SOD activity (U/ml)	2 Gy	1.14 ± 0.03	0.85 ± 0.14	0.0017
SOD activity (U/ml)	4 Gy	1.08 ± 0.03	0.70 ± 0.02	0.0001

CAT: Catalase; SOD: Superoxide dismutase; SD: Standard deviation; Gy: Gray. Values are presented as mean ± standard deviation. *P*-values compare each bromelain-plus-radiation group with the corresponding radiation-alone group.

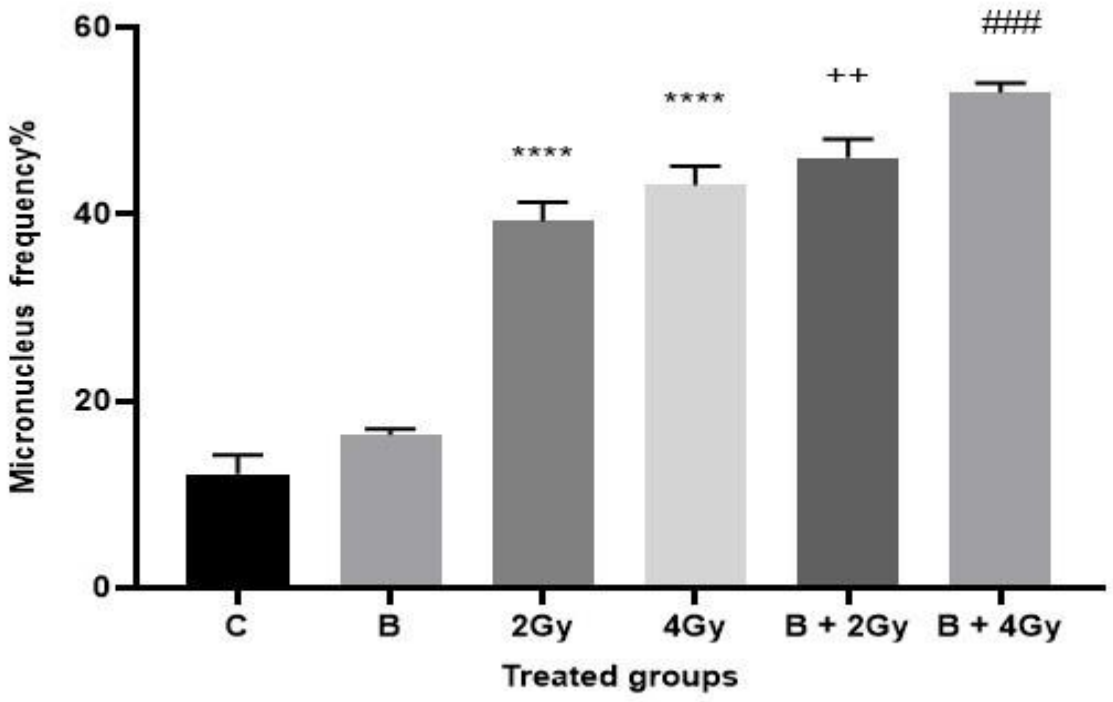


Figure 1. This figure shows the effect of bromelain pretreatment on radiation-induced micronucleus formation in HT-29 CRC cells; HT-29 cells were pretreated with bromelain (70 µg/mL) for 3 h before exposure to X-ray irradiation (2 or 4 Gy). Chromosomal damage was evaluated using the CBMN assay. Data are presented as mean ± SD from three independent experiments (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

$^{****}P < 0.0001$ versus untreated control; $^{++}P < 0.01$ versus the 2 Gy irradiation group; $^{###}P < 0.001$ versus the 4 Gy irradiation group. SD: Standard deviation; n: Number; CBMN: cytokinesis-block micronucleus; CRC: Colorectal cancer; Gy: Gray

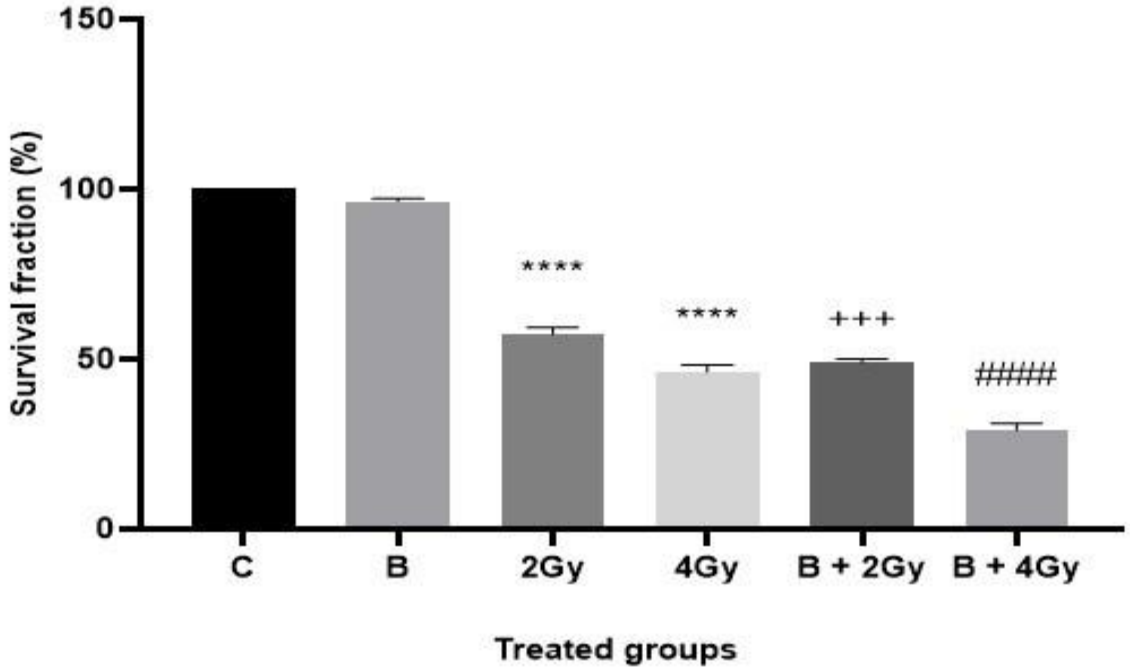


Figure 2. This figure shows the effect of bromelain pretreatment on clonogenic survival of HT-29 CRC cells following ionizing radiation; HT-29 cells were pretreated with bromelain (70 μg/mL) for 3 h before exposure to X-ray irradiation (2 or 4 Gy). Following treatment, cells were cultured for 14 days to allow colony formation. Colonies containing at least 50 cells were counted, and SF was calculated relative to the untreated control. Data are presented as mean ± SD values from three independent experiments (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

$^{****}P < 0.0001$ versus untreated control; $^{+++}P < 0.001$ versus the 2 Gy irradiation group; $^{\#\#\#\#}P < 0.0001$ versus the 4 Gy irradiation group. SD: Standard deviation; n: Number; CRC: Colorectal cancer; Gy: Gray; SF: Survival fraction

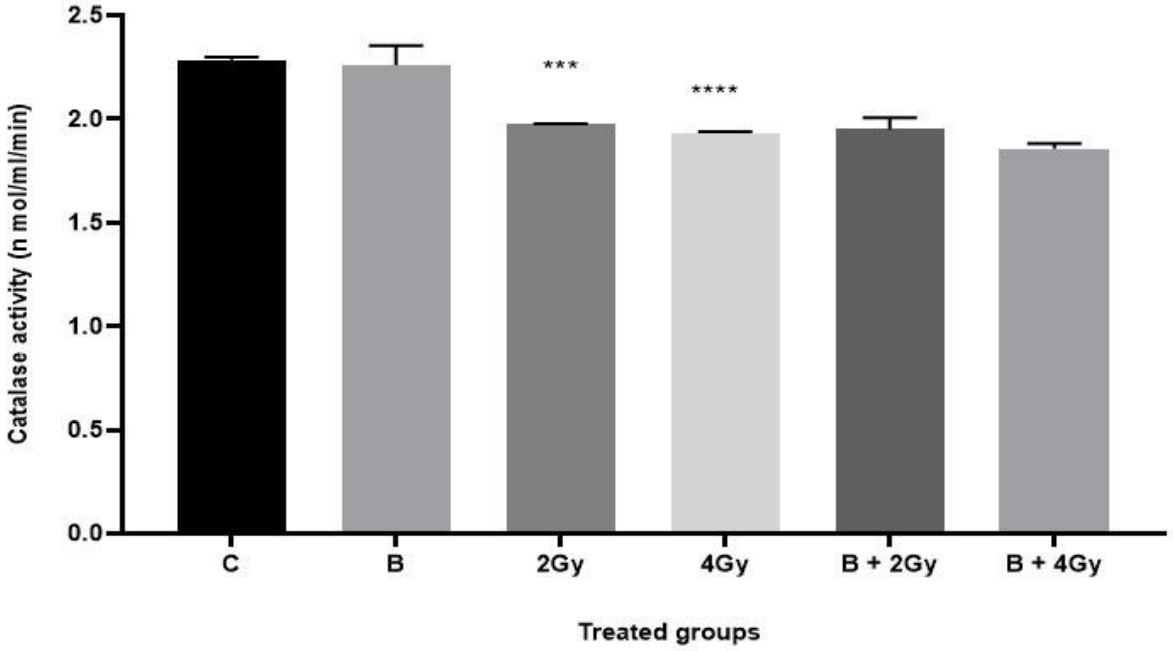


Figure 3. This figure shows the effect of bromelain pretreatment on CAT activity in HT-29 CRC cells following ionizing radiation; HT-29 cells were pretreated with bromelain (70 μg/mL) for 3 h before exposure to X-ray irradiation (2 or 4 Gy). CAT activity was measured immediately after irradiation using a commercial CAT activity assay kit according to the manufacturer's instructions. Data are presented as mean ± SD values from three independent experiments (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

$^{****}P < 0.0001$ versus untreated control. CAT: Catalase; CRC: Colorectal cancer; SD: Standard deviation; n: Number; Gy: Gray

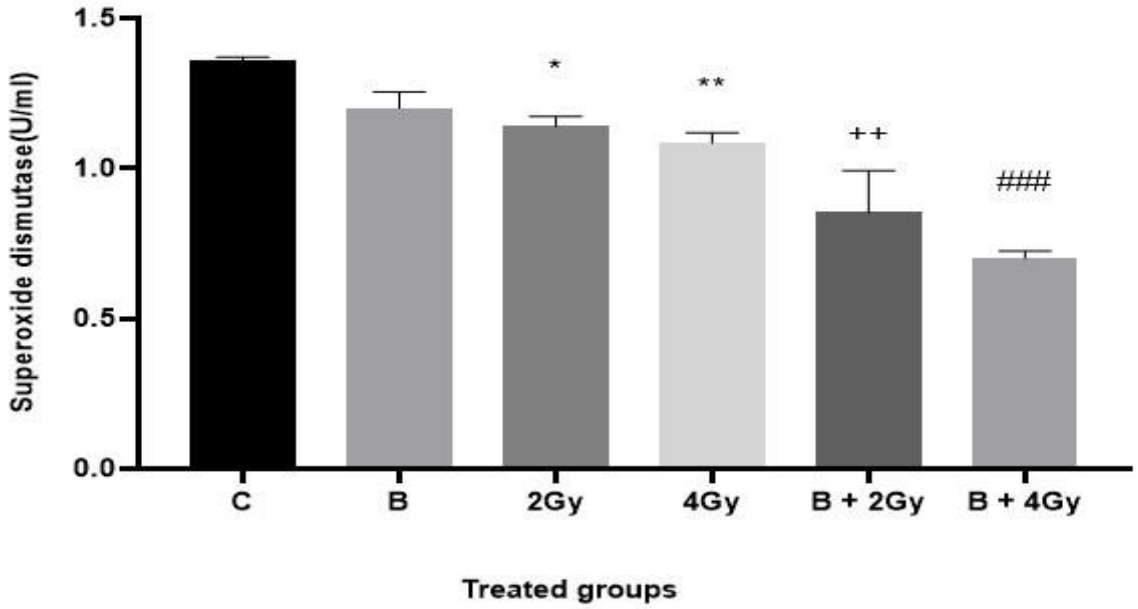


Figure 4. This figure shows the effect of bromelain pretreatment on SOD activity in HT-29 CRC cells following ionizing radiation; HT-29 cells were pretreated with bromelain (70 µg/mL) for 3 h before exposure to X-ray irradiation at doses of 2 or 4 Gy. SOD activity was measured immediately after irradiation using a commercial SOD activity assay kit according to the manufacturer's instructions. Data are presented as mean ± SD values from three independent experiments (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

$^{*}P < 0.05$ and $^{**}P < 0.01$ versus the untreated control group; $^{++}P < 0.01$ versus the 2 Gy group; $^{\#\#\#}P < 0.001$ versus the 4 Gy group.
SOD: Super oxide dismutase; CRC: Colorectal cancer; SD: Standard deviation; n: Number; Gy: Gray