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Effect of Pre-soaking Mustard Green Seeds Prior to Cold Plasma Treatment on Bioactive Aspects of Microgreens

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ABSTRACT

Microgreens have been reported to contain higher phytochemicals than mature plants. Mustard green (MG) is rich in glucosinolates that produce isothiocyanates (ITCs) upon degradation. ITCs have well-known chemopreventive benefits. Previously, non-thermal plasma treatment on MG seeds could enhance bioactive content; However, seed pretreatment before non-thermal plasma treatment was yet to be studied. The aim of this work was to evaluate the impact of pre-soaking MG seeds overnight before non-thermal plasma treatment at 21 kV for 5 min on phytochemicals and activities of MG microgreens. Plasma-treated pre-soaked (PTPS) seeds and control seeds (no plasma treatment) received deionized water for 7 days. No difference in germination percentage, stem height or fresh/dried weight of MG between PTPS seeds and the control was observed. Total ITC content in MG microgreens from PTPS seeds (1.62 mmol/100 g DW) was significantly higher than control seeds (1.00 mmol/100 g DW). Antioxidant capacity and total phenolic/flavonoid compounds were similar in both MG microgreens. Lower IC_{50} values were recorded for MG microgreens from PTPS seeds at most time intervals, indicating higher cytotoxicity against MCF-7 and HepG2, and greater effectiveness in cancer proliferation inhibition than the control. No differences in *Bax*, *Caspase-3* and *Bcl-2* mRNA expressions were detected for all samples; however, *MMP-9* mRNA expression was reduced in MG microgreens from PTPS seeds for HepG2. Pre-soaking MG seeds before plasma treatment at 21 kV for 5 min increased ITC levels and increased anticancer activities of MG microgreens without adverse effects compared to MG microgreens from control seeds.

Keywords: Plasma, HepG2, MCF-7, Mustard green, Soaking.

Introduction

Mustard green (*Brassica juncea* (L.) Czern and Coss) is a glucosinolate (GSL)-rich local vegetable of the Brassicaceae family grown in Northeast Thailand. GSL is a secondary metabolite produced in the vacuoles of plant cells by the reaction of glucose and amino acids.[1] MYR stands for myrosinase enzyme, which is present in plant cells. GSLs are digested with MYR and transformed into isothiocyanates (ITCs), thiocyanates, nitriles and epithionitriles as tissues and cells are weakened.[2] ITCs can suppress a wide range of pathogens at low concentrations and also have antibacterial, anti-mold and anti-cancer effects.[3] Interestingly, multiple studies have shown that cold plasma technology improved seed surface, germination percentage and plant growth rate.[4,5]

Recently, mustard green (MG) microgreens from plasma-treated seeds at 23 kV showed increased ITC level, total phenolic content (TPC) and total flavonoid content (TFC). MG plasma-treated seeds at 21 kV demonstrated the greatest antioxidant capacity from 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Allyl isothiocyanate and 3-butenyl isothiocyanate were the dominant ITCs in MG.[6]

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The previous study concluded that plasma treatment positively influenced the bioactivities of MG and recommended further research on the impact of seed pretreatment before plasma treatment. Thus, the goal of this study was to examine whether pre-soaking MG seeds before cold plasma application influenced the phytochemical content and biological capacity of microgreens.

Materials and Methods

Plasma treatment on MG seeds

MG seeds were purchased from Punthawee mall shop (online) on 13th July 2019. The authentication of the plant sample was done by checking mature plant characteristics (broad, wavy frilled leaves with longitudinal veins and a deep green color) and distinct pungent flavor by Asst. Prof. Dr. Vijitra Luang-In, Mahasarakham University, Thailand. The plant sample was deposited (voucher number 78210) in the Queen Sirikit Botanical Garden Herbarium, Thailand. The test group of PTPS seeds (100 seeds/treatment with triplicate) were pre-soaked overnight prior to plasma treatment for 5 min at 21 kV at the Faculty of Engineering Srinakharinwirot University, Nakhon Nayok, Thailand. [7] The control group was MG seeds without soaking and without plasma treatment. Both PTPS and control seeds were cultivated as previously done till harvest (7 days).[7] After a gentle cut 1 cm above vermiculite, the physiology of MG microgreen was measured and they were stored at -80°C until the next experiment.

ITC extraction and quantification

ITCs were extracted and quantified as follows.[6] MG microgreens were freeze-dried, and the dried plants (250 mg) were mixed in 4 mL of

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0.1 M citrate-phosphate buffer pH 7.0 and macerated at 250 rpm for 1 h at 37°C. Dichloromethane (DCM) was used in a 1:1 ratio to extract the ITCs from the samples. The moisture in DCM layer was removed by adding 0.5 g $MgSO_4$ into it prior to centrifugation. The clear supernatants were diluted 1:4 in methanol, and the diluted sample (10 µL) was pipetted to each well of the 96-well plate containing 90 µL of methanol. The phosphate buffer (0.1 M, pH 8.0, 90 µL) and benzene 1,2 dithiol (0.08 M, 10 µL) were then added to the plates and incubated for 2 h at 60°C. The absorbance at 365 nm was measured using M965+ microplate reader (Metertech, Taipei, Taiwan). Benzyl isothiocyanate (BITC) was used as an authentic standard.

Determination of antioxidant activity and bioactive compounds

MG microgreens (100 mg) were freeze-dried and homogenized with the addition of 80% methanol (5 mL), and macerated for 24 h at 250 rpm at 37°C. The samples were spun down at 10,000 g for 15 min, and filtered. The plant extract supernatants were used for bioactivity and bioactive content assays. The ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods were assessed according to the previous work.[7] The FRAP reagent (180 µL) was mixed with MG extract solution (20 µL) and incubated for 30 min. The absorbance at 593 nm was observed and iron (II) sulfate was used as a standard. The DPPH reagent (180 µL) was mixed with MG extract (20 µL) and incubated for 30 min. The absorbance at 515 nm was recorded and the Trolox was used as a standard.

TPC and TFC were determined using previous methods.[7] The TPC was evaluated using a Folin-Ciocalteu colorimetric method. The reagent Folin-Ciocalteu (100 µL) was mixed with the MG microgreen extract (20 µL) and left for 1 min. The reaction mixture was added to a solution of 7.5 % (w/v) sodium bicarbonate (80 µL) and incubated for 30 min at room temperature. The measurement at 765 nm was then recorded, and the gallic acid was used as an authentic standard. For TFC, Deionized water (60 µL), 5% $NaNO_3$ (10 µL), 10% $AlCl_3.6H_2O$ (10 µL) and MG microgreen extract (20 µL) were mixed together and allowed to stand for 1 min. After that, 100 µL of 1 M NaOH was added, and incubated for 30 min before being measured at 500 nm using rutin (Sigma, St. Louis, MO, USA) as an authentic standard.

Cancer cell lines and cultivation conditions

The American Type Culture Collection (ATCC; Manassas, VA, USA) provided MCF-7 human breast cancer cell line (ATCC®HTB-22TM) and HepG2 human liver cancer cell line (ATCC®HB-8065TM). Both were cultured in Dulbecco's Modified Eagle's Medium (DMEM) added with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.).[8] They were held at 37°C in an incubator of 5% CO_2. The culture media were changed every 2 days until 80% confluency was obtained. Cells were washed with phosphate-buffered saline (PBS) pH 7.2 and then were trypsinized with 0.25% trypsin-EDTA. Cancer cells were put in a new multi-well microplate for the next experiment.

ITC extraction from microgreens for cell culture assays

ITCs were extracted for cell culture assays as follows. [6] Microgreens in fresh weight (50 g) were crushed in 50 mL citrate phosphate buffer (0.1 M, pH 7.0) and left shaking at 250 rpm for 2 h at 37°C. For extracting ITCs, DCM was applied to the mixed samples in a 1:1 ratio and stood for 30 min. The mixture samples were spun down for 15 min at 10,000 g. The upper DCM phases were transferred and combined. Next, 0.5 g magnesium sulfate was added to the samples to absorb moisture and the then they were centrifuged (10,000 g, 15 min). The supernatants were evaporated and then dried in a freeze dryer. The concentrated extracts were dissolved in dimethyl sulfoxide (DMSO) before use.

Cytotoxicity assay

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) method was conducted to assess cytotoxicity. [9] HepG2 or MCF-7 (5×10^3 cells/well) cells were transferred into 96-well plates and left for 24 h at 37°C under 5% CO_2. Wells were filled with MG extracts (0-250 µg/mL) and incubated for 72 h with monitoring the effect every 24 h. After that, MTT (0.5 mg/mL) dissolved in PBS buffer (pH 7.2) was transferred into wells and incubated for 4 h. MTT solution was discarded, and 200 µL of DMSO was added for formazan crystal dissolution, resulting in a purple hue that indicated that the cells were alive. A microplate reader was used to record the absorbance at 590 nm. The IC_{50} value was used to determine the cytotoxicity of MG extracts against cancer cells. A non-cytotoxic effect was expressed by a percent cytotoxicity less than 50%, and a cytotoxic effect was represented by a percent cytotoxicity greater than 50%.

Clonogenic assay

To assess colony formation in order to research viability and cell division in unhealthy conditions caused by factors that may cause genetic changes or interfere with the cell cycle after being extracted according to the protocol.[8] Cancer cells (800 cells/well) were transferred into 6-well plates and left for 24 h at 37°C. After removing the medium from the culture plates, MG extracts (0-100 µg/mL) were pipetted to cells for 24 h. Next, the cells were washed with PBS and cells were reared in fresh media for 14 days, and old media was replaced with fresh one every 3 days. Cells were fixed with cold methanol at -20°C for 30 min. Colonies were stained for 30 min using 0.5% Coomassie brilliant blue G-250 dissolved in methanol, and the excessive stain was removed under running tap water and dried at room temperature. Colony formation in triplicate was calculated as a percentage relative to untreated cells.

Wound healing assay

This assay was for the measurement of cell migration.[9] Cancer cells (2×10^5 cells) were transferred to each well of 24-well plates and allowed to attach overnight to approximately 90% confluency. MG extracts (25 µg/mL) were added to plates after a scratch wound was created using a 200 µL pipette tip and incubated for 24 h and 48 h. MG extract (25 µg/mL) was used in the wound healing assay because a lower concentration of the extract enabled a clearer observation of the gradual changes in cell migration over a time course of 0 h, 24 h and 48 h than using a higher extract concentration which may reach maximum effect at 24 h and no difference at 48 h. After that, cells were fixed using 4% formaldehyde and stained for 30 min with 0.5% crystal violet. Next, excessive stains were washed off and cells were captured. Percent relative closure of the scratch was determined in comparison with the untreated cells.

DNA fragmentation analysis

Gel electrophoresis was used to assess apoptosis as indicated by DNA fragmentation. [6] HepG2 and MCF-7 (2×10^5 cells) were transferred to each of 6-well plates and left for 24 h at 37ºC. MG extract (50 µg/mL) was applied to cells for 24 h. Genomic DNA from cancer cell pellet was extracted using DNA Extraction Kit (Vivantis, Malaysia) and examined on gel electrophoresis. Here, 50 µg/ml extract (higher concentration than that used in the wound healing assay) was used to ensure the effect on DNA fragmentation was observed at a fixed time point of 24 h (not a time-course manner as in the wound healing assay) using a sufficient extract concentration.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to investigate gene expressions in cancer cells. [6] Cancer cells (2×10^5 cells/well) were grown for 24 h at 37°C in 6-well plates before being treated to MG extracts (25 µg/mL) for another 24 h. TRIZol Reagent® was used for RNA isolation (Life Technologies, Carlsbad, CA, USA). The iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) was used to synthesize cDNAs from RNAs. The PCR reaction was done using 2× Master Mix (OnePCR, GeneDirex, Taiwan) with added primers to five genes (*Bax*, *Bcl-2*, *Caspase-3*, *MMP-9* and *Beta-actin* as reference gene).[7] The PCR products were analyzed using agarose gel electrophoresis with gel documentation and intensity measurements done with ImageJ software.

Statistical analysis of data

The data was gathered in triplicate. All measurements were expressed as means of standard deviations (SD). The program SPSS was used to do statistical research, which included a one-way analysis of variance (ANOVA) and Duncan multiple ranges analyses (demo version). If $p < 0.05$, statistically significant variations were considered.

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Results and Discussion

Insignificant differences in seed germination percentage (78-80%), length of microgreen stems (4.8 cm/microgreen), fresh weight (17-18 mg/microgreen) and dried weight (4.9-5.0 mg/microgreen) were recorded among MG microgreens from PTPS and control seeds (Figure 1A-D). Results suggested that pre-soaking seeds before plasma treatment had no influence on growth or development. By contrast, previously research found that the combined effects of a short life (less than 1 min) and a long life (minimum lifetime of one to two minutes) for reactive species, charged particles and electric fields in plasma wet treatments treated directly with plasma, led to changes in seed germination.[10-12] Enhanced seed germination resulted from chemical and/or biological germination impacts of chemical reactants occurring during plasma treatment.[11-12] Direct plasma wet treatment slowed mustard seed germination. This result was linked to longer-lasting chemicals created by plasma treatment as well as to other characteristics such as short lifespan species, electrical fields and UV radiation.

Similarly, no effect on TPC (3.9-4.0 mg GAE/g DW), TFC (0.136-0.140 mg RE/g DW) and antioxidant capacity by DPPH (2.3-2.7 mg TE/g DW) and FRAP (13-15 mg Fe (II)/g DW) was detected (Figure 1F-I). However, the ITC level from PTPS seeds (1.62 mmol/100 g DW) was significantly higher than control seeds (1.00 mmol/100 g DW) (Figure 1E). Plasma treatment on MG seeds enhanced ITC content by almost 2 folds.[6] Pre-soaking seeds before plasma treatment positively influenced ITC production by enhancing the GSL synthetic pathway.

Cytotoxicity results from the MTT assay showed that IC_{50} values of PTPS seeds for MCF-7 and HepG2 were lower than the control seeds at 24, 48 and 72 h and at 24 and 48 h, respectively suggesting that cytotoxicity of MG increased when seeds were pre-soaked before plasma treatment (Figure 2A-D). Similarly, MG from PTPS seeds had lower IC_{50} values (36 µg/mL and 54 µg/mL) than the control seeds (37 µg/mL and 61 µg/mL) in inhibition of colony formation of both MCF-7 and HepG2 cells, respectively (Figure 2E-H). MG was more effective toward MCF-7 than HepG2 due to the lower IC_{50} values. Wound healing assay results showed that the effectiveness of MG from PTPS and control seeds in inhibiting cell migration was similar in both cancer cells (Figure 2I-J).

The electrophoretic DNA fragmentation analysis for detection of apoptosis using agarose gel electrophoresis demonstrated that both MG extracts from PTPS and control seeds resulted in a typical ladder pattern of internucleosomal fragmentation, as indicated by the formation of ladder DNA in agarose gel from MCF-7 (Figure 3A) and HepG2 (Figure 3B) compared to unbroken DNA from untreated cells. This DNA fragmentation was a hallmark of apoptosis. Results indicated that the PTPS step induced apoptosis.

To determined gene expression related to the apoptotic pathway, RT-PCR assay was conducted. Similar gene expressions of *Bax*, *Caspase-3* and *Bcl-2* in MCF-7 were found in PTPS and control seeds (Figure 4A); however, *MMP-9* expression in HepG2 was significantly inhibited in MG from PTPS seeds than the control seeds (Figure 4B), suggesting that PTPS seeds were more effective in preventing HepG2 cancer migration.

Pre-soaking seeds before plasma treatment is an alternative means to increase bioactive compounds of MG microgreens, with anti-cancer effects from ITC toward MCF-7 and HepG2, with no adverse effects compared to control seeds. In a previous study by our group, plasma treatment alone without the pre-soaking seed step enhanced bioactive compounds and cytotoxicity of mustard green and Thai rat-tailed radish microgreens.[6,7] Soaking seeds before cold plasma treatment had positive effects as well as no seed pretreatment. Thus, hard-shelled seeds could also be pre-soaked before cold plasma treatment to aid germination and yield positive effects on bioactive compounds. Result supported the potential application of pre-soaking seeds before plasma treatment to benefit the microgreen industry and agriculture.

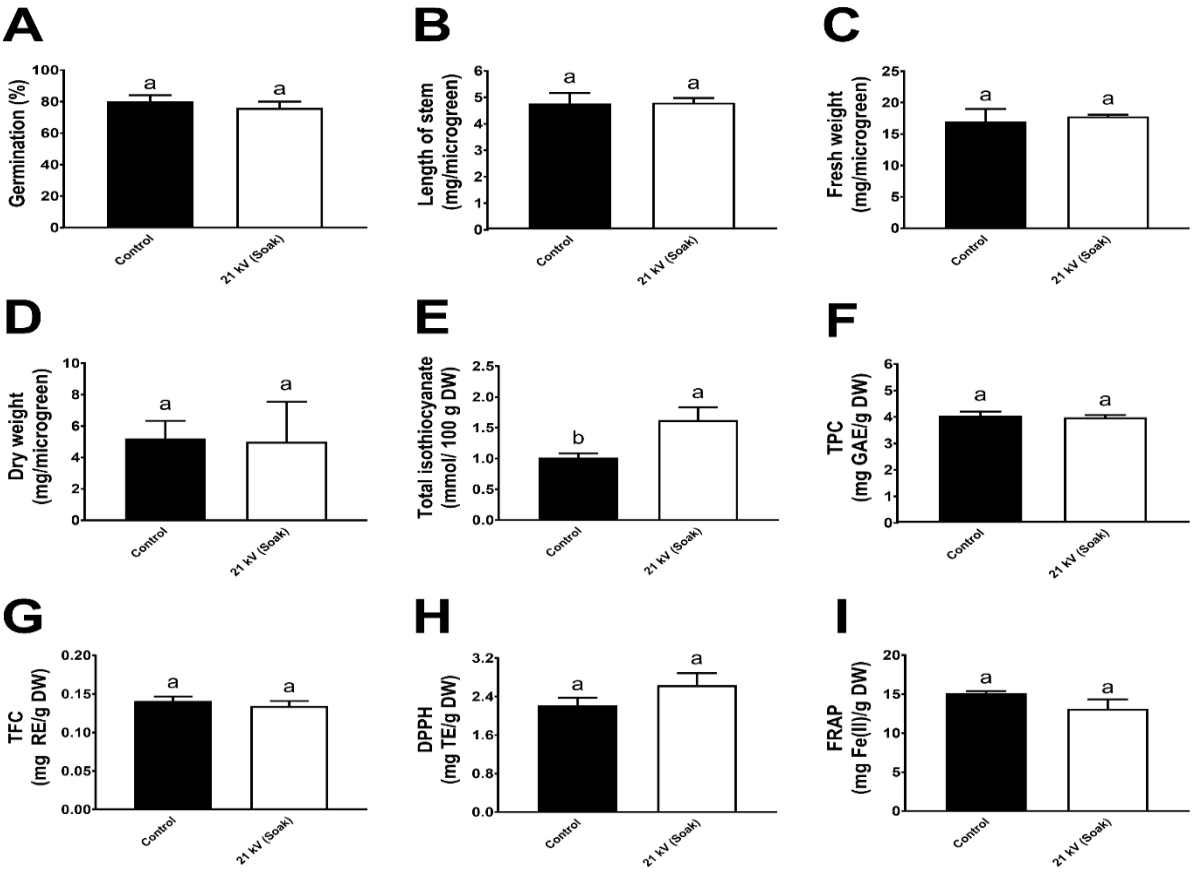

Figure 1: Physical changes, bioactive contents and antioxidant activities of MG. (A) Percentage germination. (B) Stem length. (C) Fresh weight. (D) Dry weight. (E) Total isothiocyanate. (F) TPC. (G) TFC. (H) DPPH scavenging capacity. (I) FRAP value. Different lowercase letters correspond to statistical differences ($p < 0.05$).

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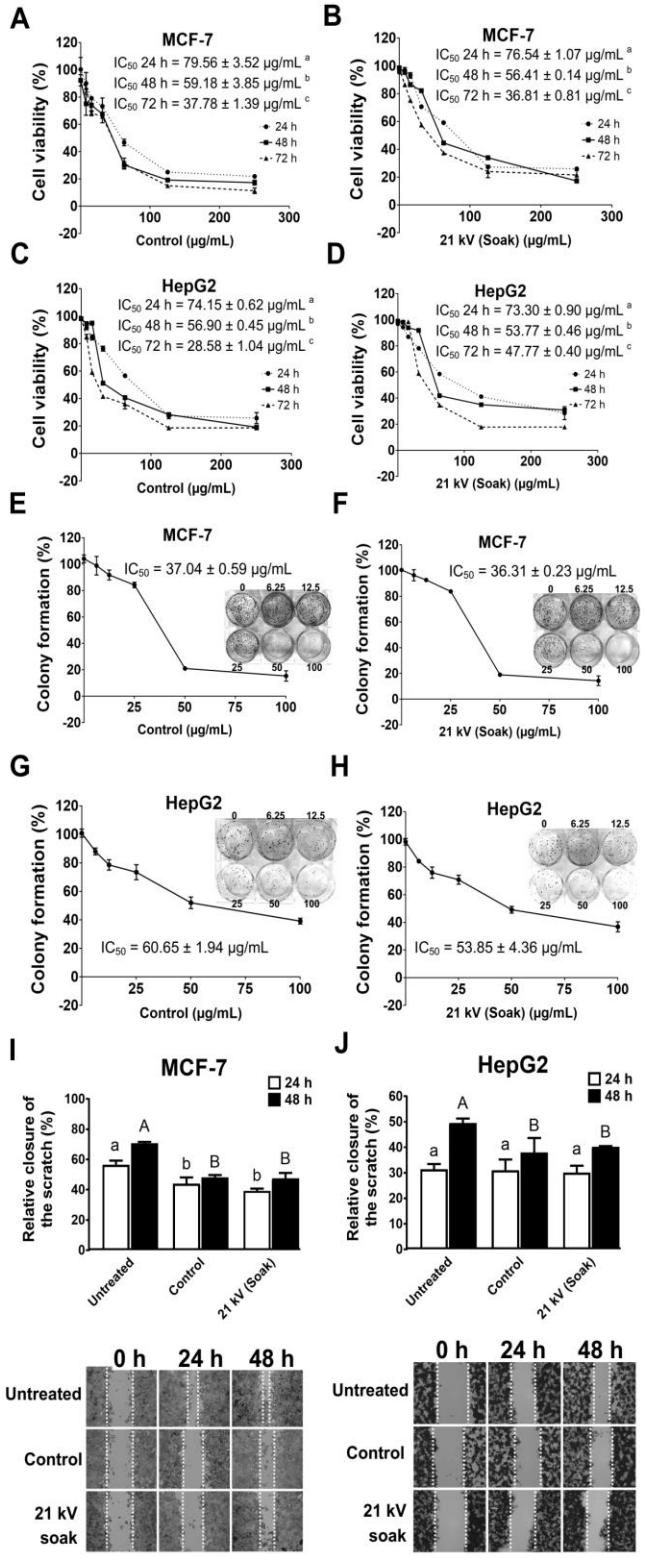

Figure 2: Cytotoxicity, colony formation and wound healing assay. (A-B) MCF-7 viability from control MG and soaked MG. (C-D) HepG2 viability from control MG and soaked MG. (E-F) Colony formation of MCF-7 from control MG and soaked MG. (G-H) Colony formation of HepG2 from control MG and soaked MG. (I-J) Distance of a wound and relative closure of the scratch (%) in MCF-7 and HepG2 from control MG and soaked MG. Different lowercase and uppercase letters correspond to significant differences ($p < 0.05$) at 24 h and 48 h, respectively.

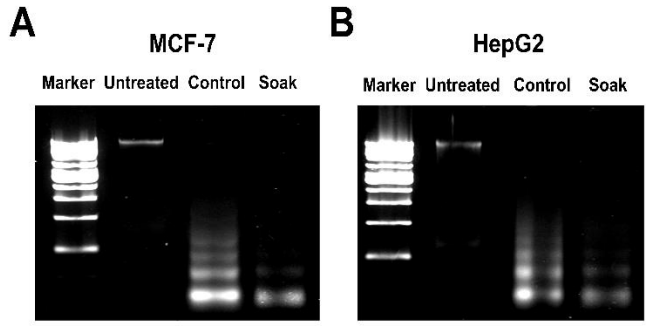

Figure 3: Agarose gel analysis of DNA fragmentation. (A) MCF-7. (B) HepG2. Marker = DNA marker; Untreated = cells only; Control = MG without plasma treatment and without pre-soaking; Soak = MG from pre-soaked seeds treated with plasma at 21 kV.

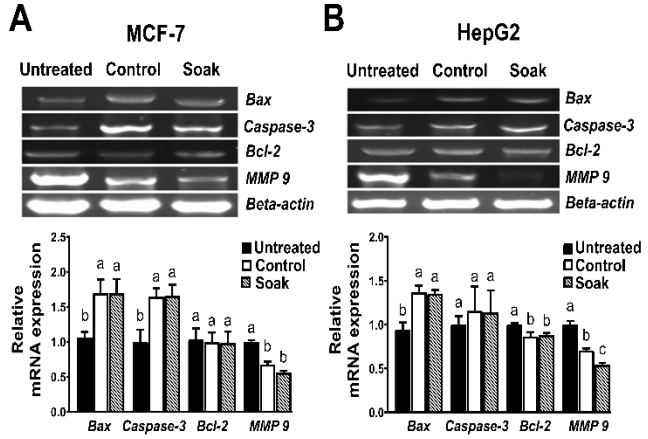

Figure 4: Gene expressions in cancer cells using RT-PCR. (A) MCF-7. (B) HepG2. Untreated = cells only; Control = MG without plasma treatment and without pre-soaking; Soak = MG from pre-soaked seeds treated with plasma at 21 kV. Different lowercase letters correspond to significant differences ($p < 0.05$).

Conclusion

Pre-soaking seeds before plasma treatment enhanced ITC content and certain anticancer aspects of microgreens, with no adverse effects compared to the control seeds with no pre-soaking or plasma treatment. Pre-soaking is an economical, environmentally friendly and effective way to produce microgreen products with improved health benefits.

Conflict of Interest

The authors declare no conflict of interests.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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