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Full Length Article

Cold Plasma Treatment on Mustard Green Seeds and its Effect on Growth, Isothiocyanates, Antioxidant Activity and Anticancer Activity of Microgreens

Worachot Saengha[1], Thipphiya Karirat[1], Benjaporn Buranrat[2], Khanit Matra[3], Sirirat Deeseenthum[1], Teeraporn Katisart[4] and Vijitra Luang-In[1*]

[1]*Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand*
[2]*Faculty of Medicine, Mahasarakham University, Maha Sarakham 44000, Thailand*
[3]*Department of Electrical Engineering, Faculty of Engineering, Srinakharinwirot University, Nakhon Nayok 26120, Thailand*
[4]*Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand*
[*]For correspondence: vijitra.l@msu.ac.th; vijitra.luangin@gmail.com
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Abstract

The aims of this work were to study growth, isothiocyanate (ITC), bioactive content, antioxidant activity and anticancer activity of mustard green (MG) microgreens grown from seeds treated with cold plasma at 21 and 23 kV for 5 min. Microgreens from plasma-treated seeds at 23 kV showed almost 2-fold increased ITC content (1.57 ± 0.05 mmol/100 g DW) compared to MG from seeds without plasma (control), showed the highest total phenolic content (TPC) (6.76 ± 0.14 mg GAE/g DW) and total flavonoid content (TFC) (0.16±0.01 mg RE/g DW). However, MG plasma-treated seeds at 21 kV showed the highest antioxidant activity from 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (3.51 ± 0.38 mg TE/g DW). Allyl isothiocyanate and 3-butenyl isothiocyanate were the dominant ITCs in MG. The highest cytotoxicities using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against MCF-7 (IC_{50} of 32.44 ± 1.64 μg/mL) and HepG2 (IC_{50} of 28.58 ± 1.04 μg/mL) after 72 h exposure were found in MG from plasma-treated seeds at 23 kV and MG from control seeds, respectively. However, MG from plasma-treated seeds at 21 kV exhibited the highest antiproliferative effect against MCF-7 (IC_{50} of 23.23 ± 0.23 μg/mL) and HepG2 (IC_{50} of 20.44 ± 0.56 μg/mL) for 14 days and also the most potent antimigratory effect. MG from cold plasma inhibited MMP-9 protein expression in both cancers indicating antimigratory property. MG from cold plasma also significantly reduced *MMP-9* mRNA expression in both cancers when compared to the control and untreated cells. In conclusion, cold plasma treatment on seeds seemed to be an innovative tool to enhance ITC, TPC, TFC and anticancer properties of MG microgreens for better health implications.

Keywords: Allyl isothiocyanate; Chemoprevention; Cold plasma; Microgreen; Mustard green

Introduction

Microgreens are plants with a few cotyledons from vegetable seeds after planting for approximately 7–14 days and have gained momentum among healthy consumers in the past ten years (Kyriacou *et al*. 2016). Microgreens contain several phytochemicals and thus they are known for risks reduction of various diseases (Shahidi 2006), such as ischemic heart disease, stroke and cancers (Kennedy and Wightman 2011; Khanam *et al*. 2012; Alrifai *et al*. 2019).

In Thailand, approximately half of the cancer burden is due to five types of cancers: lung, liver, breast, colorectal, and gall bladder cancers (Bray *et al*. 2018). Several cancers are preventable by a nationwide campaign to encourage a regular consumption of local Thai vegetables, especially those in Cruciferae family. *Brassica juncea* (L.) Czern and Coss or mustard green (MG) is a local Cruciferous vegetable commonly grown in Northeast Thailand. Glucosinolates are secondary metabolites ubiquitous in vegetables of Cruciferae family (Fahey *et al*. 2001). Once come into contact with myrosinase enzyme (Van Eylen *et al*. 2006), glucosinolates are converted into isothiocyanates (ITCs), thiocyanates, nitriles and epithionitriles (Halkier and Gershenzon 2006). ITCs are bioactive compounds exhibiting antibacterial, antimould and anticancer effects (Singh and Singh 2012). These are able to induce Phase II enzyme through an expression of NF-E2-related factor-2 (Thimmulappa *et al*. 2002; Zhang and Hannink 2003), inhibit histone deacetylases (Wang *et al*. 2008), inhibit cell cycle and Bcl-2 protein expression (Xiao *et al*. 2006; Zhang

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and Tang 2007; Geng *et al*. 2011), stimulate caspases and activates the transcription factor 3 (Wu *et al*. 2005; Park *et al*. 2007). The MG contains sinigrin and allyl isothiocyanate (AITC) (Ishida *et al*. 2014) and also other phytochemicals including carotenoids and phenolic compounds (Frazie *et al*. 2017) exhibiting antioxidant capacity (Ishida *et al*. 2014). To date, only reports on MG mature plants have existed and the knowledge on the type and quantity of ITCs and bioactivities of MG microgreens is still scarce.

At present, non-thermal plasma (NTP) or cold plasma technology application in food and agriculture has gained momentum. The benefits of NTP lie with its non-thermal, economical, flexible and environmentally friendly nature (Pankaj *et al*. 2017). During the food processing stage, applications of NTP for improving functionality of food were recorded (Muhammad *et al*. 2018). The changes caused by NTP are primarily related to oxidative degradation and double bonds cleavage in plant-derived organic compounds. To date, the effects have been reported mostly on increases in polyphenol, vitamin C and antioxidant activity in a time-dependent manner (Muhammad *et al*. 2018) and also the extraction efficiency of polyphenols has been improved. However, the effect of NTP on food functionality of seeds or plants prior to food processing step has not yet been extensively examined. Very few studies have shown that NTP was used to improve seed surface, germination percentage and growth rate of certain plants (Dobrin *et al*. 2015; Butscher *et al*. 2016; Burnett *et al*. 2017). Therefore, this work aimed to test the hypothesis that cold plasma treatment on MG seeds was able to increase seed germination, ITC content, bioactive compounds and anticancer activity of MG microgreens when compared to those grown from the control seeds without plasma treatment.

Materials and Methods

MG microgreen cultivation and cold plasma treatment

Cold plasma treatment on MG seeds was carried out at Faculty of Engineering Srinakharinwirot University, Nakhon Nayok Province. The experimental set up design is shown in Fig. 1. MG seeds of Lanna cultivar (Sorndang brand) were purchased from Punthawee mall shop (https://www.pwcmallonline.com), Lot. no. 304333. MG seeds (100 seeds/replicate/treatment) in triplicate were treated with cold plasma at the supplied voltages of 21 or 23 kV for 5 min. Both supplied voltages were chosen for the test since our preliminary work showed the optimal voltage at 21 kV to increase ITC content and bioactivity of Thai rat-tailed radish microgreens from cold plasma-treated seeds (unpublished data). MG seeds were germinated on vermiculite in a tray at 25°C (12 h light/12 dark cycle, light intensity controlled at 42 μmol/s/m^2) and were sprayed with 20 mL of deionized water for 7 days' till harvested. Percent seed germination, stem length and dry weight of MG microgreens were measured after gentle cut 1 cm above the vermiculite surface. Fresh microgreens were harvested for further analyses.

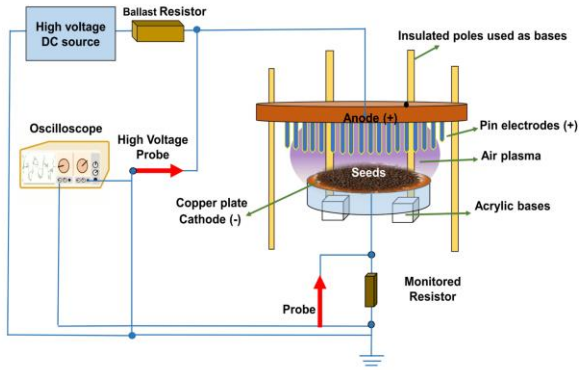

Fig. 1: Schematic drawing of cold plasma device setup
A DC high voltage power supply (Matsusada, AU-30P10) was connected to the 16Ø cm circular PCB BreadBoard anode, attached to 0.2Ø mm multi-pin electrodes, through a 6 MΩ ballast resistor. The 0.5 cm space was placed between two pin electrodes on the anode plate. A 12×12 cm^2 copper cathode plate as a tray for placing the plant seeds, was grounded. Between the multi-pin anodes and cathode tray with a space gap of 1.4 cm, air plasma was generated at room temperature when the high voltage was applied across both electrodes at 19 and 23 kV with the average supplied current of 0.53 mA

Extraction and determination of ITCs

ITCs of 7-day old MG microgreens were extracted as previously reported (Luang-In *et al*. 2018). Briefly, MG microgreens were freeze-dried and then dried samples (250 mg) were added with 0.1 *M* citrate-phosphate buffer pH 7.0 (4 mL) and incubated in a shaking incubator at 250 rpm (LSI-1005R, Lab Tech, Korea) for 1 h at 37°C. The extract was mixed with dichloromethane (DCM) (RCI Labscan, Thailand) in the ratio of 1:1 and was incubated for 30 min at 37°C with shaking at 250 rpm. The samples were centrifuged at 10,000 × g for 15 min and thereafter DCM phase (bottom layer) was obtained and mixed with 0.5 g of $MgSO_4$ for water removal. Extracted samples were centrifuged as previously and the supernatants were obtained for total ITC content determination (Amron and Konsue 2018). The supernatants were diluted in methanol in the ratio of 1:4 and the diluted sample (10 μL) was added to the 96-well plate and mixed with 90 μL of methanol. An aliquot (90 μL) of 0.1 *M* phosphate buffer pH 8.0 and 10 μL of 0.08 *M* benzene 1,2 dithiol (Sigma, St. Louis, MO, USA) were added and incubated at 60°C for 2 h. The A365 nm measurement was recorded using M965+ microplate reader (Metertech, Taipei, Taiwan). Benzyl isothiocyanate (BITC) was used to calibrate ITC standard curve. ITCs were identified as previously done (Luang-In *et al*. 2014). Gas chromatograph-mass spectrometry (GC-MS) (Agilent HP-5MS) with 5% phenylmethylsiloxane column (30 m × 0.25 mm, 0.25 μm) was used with helium as a carrier gas. The temperature of oven was started at 50°C for 5 min and ramped up to 150°C and 250°C (4°C /min). The amount of injection was 1 μL, the flow rate was 1 mL/min, the average

velocity was 36 cm/min, the pressure was 7.56 kPa and the overall runtime was 40 min. ITCs were identified using the mass spectral library in GC-MS and standard reference database. AITC and 3-butenyl ITC fingerprint fragment ions were 99, 72 and 55 (m/z) and 113, 72 and 55 (m/z), and113, 72 and 55 (M^+), respectively at the retention times of 7.25 and 9.78 min, respectively.

Microgreen extract preparation

Freeze-dried MG microgreens (100 mg) were added to 5 mL of 80% methanol, homogenized, incubated with shaking at 250 rpm at 37°C for 24 h. The samples were centrifuged at 10,000 × g for 15 min, and filtered. Supernatants were used for antioxidant activity and bioactive content assays.

Scavenging of DPPH free radical

The 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution (Sigma, St. Louis, MO, USA) (180 μL) was combined with the MG microgreen extract (20 μL) and incubated for 30 min in the dark as in the previous report (Zhang *et al*. 2016) with a slight modification. Sample absorption was estimated at 515 nm and the Trolox equivalent (TE) (Sigma, St. Louis, MO, USA) was used.

Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent was prepared in 300 m*M* acetate buffer pH 3.6, 10 m*M* 2,4,6-Tris(2-pyridyl)-s-triazine solution in 40 mM HCl and 20 m*M* iron(III) chloride solution in a 1:1:10 (v/v) ratio (Wei *et al*. 2011). Subsequently, the FRAP reagent (180 μL) was applied to the MG microgreen extract solution (20 μL), mixed well and incubated for 30 min in the dark. The sample absorbance was measured at 593 nm and the iron (II) sulfate standard (Sigma, St. Louis, MO, USA) was used.

Total phenolic content (TPC) and total flavonoid content (TFC)

A Folin-Ciocalteu colorimetric approach was used to evaluate the TPC (Radošević *et al.* 2017). The MG microgreen extract (20 μL) was combined with the reagent Folin-Ciocalteu (100 μL) and left for 1 min. The solution of 7.5% (w/v) sodium bicarbonate (80 μL) was added to the reaction mixture and incubated for 30 min at room temperature. After that, the measurement at 765 nm was recorded and the gallic acid equivalent (GAE) (Sigma, St. Louis, MO, USA) was used.

TFC was done according to the previous approach (Tian *et al.* 2016) with some modifications. Deionized water (60 μL), 5% $NaNO_3$ (10 μL), 10% $AlCl_3.6H_2O$ (10 μL) and MG microgreen extract (20 μL) were combined and stood for 1 min. Afterwards, 1 *M* NaOH (100 μL) was added, mixed and incubated for 30 min prior to measurement at 500 nm and rutin equivalent (RE) (Sigma, St. Louis, MO, USA) was used.

Cancer cell cultures

The human breast adenocarcinoma (MCF-7 ATCC ® HTB-22TM) and human hepatocellular carcinoma (HepG2 ATCC ® HB-8065TM) were received from the American Type Culture Collections (ATCC, Manassas, VA, USA). MCF-7 and HepG2 cells were cultured with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA). Cells were cultured under 5% CO_2 at 37°C. When 80% confluence was reached, DMEM media was refreshed every 2–3 days. Cultured cell lines were washed with phosphate-buffered saline (PBS), pH 7.2, and trypsinized with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA). Cancer cells were placed in a fresh DMEM medium before further tests.

Microgreen extraction for cell cultures

MG extraction for cell lines was performed accordingly (Pocasap *et al*. 2013). Fresh MG microgreens (50 g) were ground in 0.1 *M* citrate phosphate buffer (50 mL), pH 7.0 and mixed at 250 rpm for 2 h at 37°C. DCM was then added in the mixed samples in the ratio of 1:1 for ITC extraction for 30 min. The samples were centrifuged at 10,000 g for 15 min, filtered, evaporated and freeze-dried. Dried samples were mixed with 1% dimethyl sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK) and the MG microgreen extract solution was used in the next step.

Cytotoxicity assay

MCF-7 or HepG2 cells (5×10^3 cells/mL) were cultured in 96-well plates under 5% CO_2 for 24 h at 37°C. The MG microgreen extract (0–250 μg/mL) was exposed to cancer cells for 24, 48 and 72 h. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) was added to wells and incubated for 4 h and removed before DMSO (200 μL) was added. The appearance of purple color corresponded to alive cells.

The absorbance (A) at 590 nm was measured. *E*max (maximum cytotoxicity (%)) and half maximal inhibitory concentration (IC_{50}) values were calculated for the cytotoxicity of microgreen MG extracts against cancer cells.

$$Emax = \frac{(\text{Acontrol} - \text{Asample})}{(\text{Acontrol})} \times 100 .$$

Clonogenic assay

The colony forming test was used to determine the influence of MG microgreen extracts on cancer cell proliferation as previously done (Buranrat *et al*. 2017). The viable cancer cells (800 cells/well) were seeded in 6-well plates for 24 h prior to exposure to microgreen

extracts (0, 6.25, 12.5, 25, 50 and 100 μg/mL) for 24 h. Cells were then washed with PBS and replenished into a new DMEM for 14-day cultivation. The DMEM medium has subsequently been discarded and cells were washed, fixed and stained with 0.5% crystal violet for 1 h. After washing cells, the colonies were captured using a digital camera (Nikon D50).

Wound healing assay

Cell migration was measured with the previously mentioned wound healing assay (Buranrat *et al.* 2016). MCF-7 or HepG2 (2×10^5 cells/well) were seeded into 24-well plates overnight. After 90% confluency was reached, a sterile 0.2 mL pipette tip was used for scraping the cells to make a straight line. Cells were exposed to MG microgreen extracts (25 mg / mL). Pictures at 0, 24 h and 48 h were recorded.

$$\text{Relative closure of the scratch}(\%) = \frac{(\text{width of wound at T h})}{(\text{width of wound at0 h})} \times 100$$

Cell migration was observed using phase contrast microscopy (NIB-9000 inverted microscope, Xenon, China).

Gelatin zymography analysis

The influence of MG microgreen extracts on MMP-2 and MMP-9 protein expression was determined by gelatine zymography (Buranrat *et al.* 2017). MCF-7 and HepG2 (2×10^5 cells/well) were cultured in the 24-well plates and were exposed to MG microgreen extracts (25 μg/mL) for 24 h. Cells were collected and extracted for protein content which was later quantified using Bradford reagent (Bio-Rad, UK) with bovine serum albumin as a standard (Bradford 1976). The sample protein (30 μg) was studied on 10% SDS-polyacrylamide gel containing 0.1% gelatin as substrate under electrophoresis running conditions at 150 V for 50 min before staining with 0.25% Coomassie Brilliant Blue R250. Proteins were analyzed for the relative densities of the bands using ImageJ software (v. 1.46r; U.S. National Institutes of Health) *(*Rueden *et al*. 2017*).*

Reverse transcription-polymerase chain reaction (RT-PCR)

Gene expressions in cancer cells were analyzed using RT-PCR. MCF-7 and HepG2 (2×10^5 cells/well) were cultured into 6-well plates for 24 h at 37°C and then exposed to MG extracts (25 μg/mL) for 24 h in fresh media. Cells were collected for RNA isolation using a TRIZol Reagent® (Life Technologies, Carlsbad, CA, USA). The synthesis of cDNAs from RNAs was performed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). After that, PCR reaction using specific primers to genes of interest (Table 1) was conducted. The PCR reaction contained cDNA products (1 μL) and 2× Master Mix (OnePCR, GeneDirex, Taiwan). PCR thermocycler (Thermo Scientific Hybaid P×2) was programmed at initial denaturation for 3 min at 94ºC for 1 cycle, 40 cycles for denaturation at 94°C for 45 s, annealing at specific temperatures (Table 1) for 45 s, extension for 1 min at 72°C followed by final extension 1 cycle at 72°C for 7 min. Beta-actin was used as an internal standard. The PCR products were analyzed on a 1.5% agarose gel at 100 V for 30 min and were viewed using gel documentation and measured for intensity using ImageJ software.

Cell morphology

MCF-7 and HepG2 cells (5,000 cells/well) were cultured into 24-well plates for overnight and then were exposed to MG microgreen extracts (50 μg/mL) for 24 h. Cells were captured using an inverted light microscope (NIB-9000, Xenon, China) at 400× magnification.

DNA fragmentation

MCF-7 and HepG2 (2×10^5 cells/well) were cultured into 6-well plates at 37°C under 5% CO_2 for 24 h and afterwards were exposed to MG microgreen extracts (100 μg/mL) for 24 h. Cells were collected and the genomic DNA (1 μg/mL) was then extracted using DNA Extraction Kit (Vivantis, Malaysia), analyzed on gel electrophoresis in 1% agarose gel and viewed using gel documentation (Syngene Gene Flash, UK).

Statistical analysis

Data collection was done in triplicate and the results were presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) and Duncan multiple range test by the software S.P.S.S. (demo version) were used for statistical analyses. Statistically significant differences were considered if $P < 0.05$.

Results

Growth of MG microgreens

The results showed that the MG from cold plasma-treated seeds at 23 kV had the highest germination (88.66±3.05%); however, MG from control seeds (without cold plasma treatment) and those from cold plasma-treated seeds at 21 kV resulted in similar germination approximately 80% (Fig. 2A). All three groups of tested MG showed no significant differences in microgreen stem length (Fig. 2B). The highest dry weight of MG came from cold plasma-treated seeds at 23 kV (7.30 ± 1.83 mg/microgreen), followed by MG from cold plasma-treated seeds at 21 kV (7.00 ± 0.28 mg/microgreen) and control MG (5.20 ± 1.13 mg/microgreen) (Fig. 2C); however, these measurements were not significant.

Table 1: Primer sequences for RT-PCR

Gene	Product (bp)	Annealing temp (°C)	Forward and reverse primer sequences
Beta-actin	290	52	5′ – CTGTCTGGCGGCACCACCAT – 3′ 5′ – GCAACTAAGTCATAGTCCGC – 3
Bax	538	65	5′ – CAGCTCTGAGCAGATCATGAAGACA – 3′ 5′ – GCCCATCTTCTTCCAGATGGTGAGC – 3′
Bcl-2	459	65	5′ – GGTGCCACCTGTGGTCCACCTG – 3′ 5′ – CTTCACTTGTGGCCCAGATAGG – 3′
Caspase-3	419	55	5′ – CGGTCTGGTACAGATGTCGAT – 3′ 5′ – TAACCAGGTGCTGTGGAGTATG – 3
MMP-9	460	61	5′ – CGCTGGGCTTAGATCATTCC– 3′ 5′ – TTGTCGGCGATAAGGAA– 3′

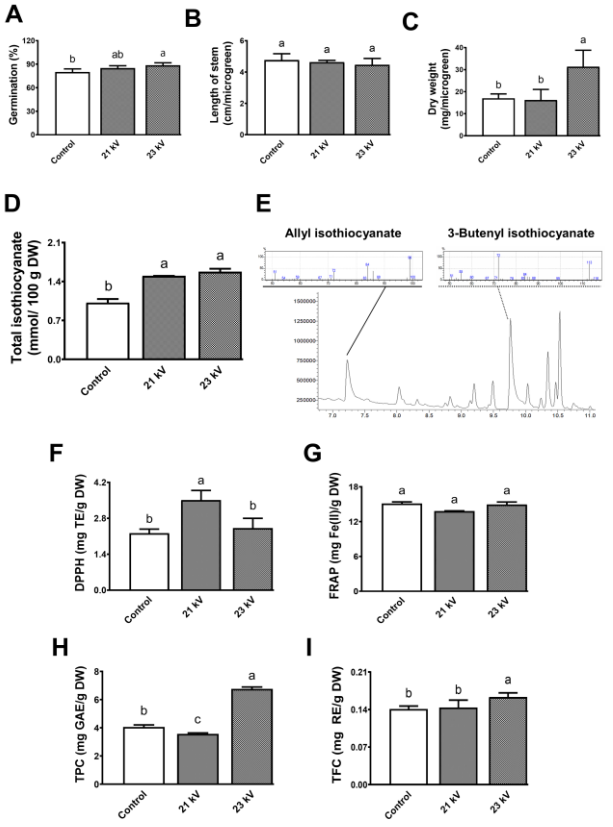

Fig. 2: Growth, bioactive content and antioxidant activities of 7-day old MG microgreens: **A**) Germination (%), **B**) Stem length, **C**) Dry weight, **D**) Total ITC content, **E**) GC-MS chromatogram and fingerprint of ITCs, **F**) DPPH scavenging activity, **G**) FRAP activity, **H**) Total phenolic compound (TPC) and **I**) Total flavonoid compound (TFC). Data represented as mean ± SE of three independent experiments. Different letters above columns indicate significant differences ($P < 0.05$)

Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

ITCs in MG microgreens

The total ITC content increased significantly in MG from cold plasma-treated seeds when compared to those from control seeds (Fig. 2D). The highest total ITC content was found in cold plasma treatment at 23 kV (1.57±0.05 mmol/100 g DW). This value was not significantly different from that of cold plasma treatment at 21 kV (1.50±0.00 mmol/100 g DW); however, it was almost 2-fold higher than that of the control (1.02±0.07 mmol/100 g DW) (Fig. 2D). This suggested that cold plasma treatment at 23 kV on MG seeds was optimal for increasing total ITC content in MG microgreens. Two types of ITCs; AITC and 3-butenyl ITC at 7.25 min and 9.78 min, respectively were detected in MG samples in three groups using GC-MS (Fig. 2E).

Antioxidant activity and bioactive compounds in MG microgreens

The highest DPPH antioxidant activity of MG microgreens was found from cold plasma-treated seeds at 21 kV (3.51 ± 0.38 mg TE/g DW) which was significantly different from those of the control and 23 kV treatment (Fig. 2F). However, no statistical difference in FRAP activity was found amongst three groups of microgreens (Fig. 2G). The TPC results were significantly different ($P < 0.05$) among all treatments (Fig. 2H). Cold plasma treatment at 23 kV on seeds led to the highest TPC (6.76 ± 0.14 mg GAE/g DW) which was significantly higher than that of the control and cold plasma treatment at 21 kV (Fig. 2H). Likewise, the highest TFC (0.16 ± 0.01 mg RE/g DW) was from cold plasma treatment at 23 kV and was significantly different from those of the control and cold plasma treatment at 21 kV (Fig. 2I).

Cytotoxicity and antiproliferation of MG microgreens

Cytotoxicity of MG microgreen extracts (0–250 μg/mL) against the MCF-7 and HepG2 after 24, 48, and 72 h incubation was determined using MTT assay (Fig. 3). The results showed that the cytotoxicity was time- and dose-dependent. MG microgreen extracts from cold plasma-treated seeds at 23 kV exhibited the highest cytotoxicity against MCF-7 after 72 h exposure with IC_{50} of 32.44 ± 1.64 μg/mL) (Fig. 3A). However, MG microgreen extracts from control seeds exhibited the highest cytotoxicity against HepG2 after 72 h exposure with IC_{50} of 28.58 ± 1.04 μg/mL) (Fig. 3B).

The anti-colony formation was performed to measure the effects of MG microgreen extracts (0–100 μg/mL) on long-term cancer cell viability and replicative potential.

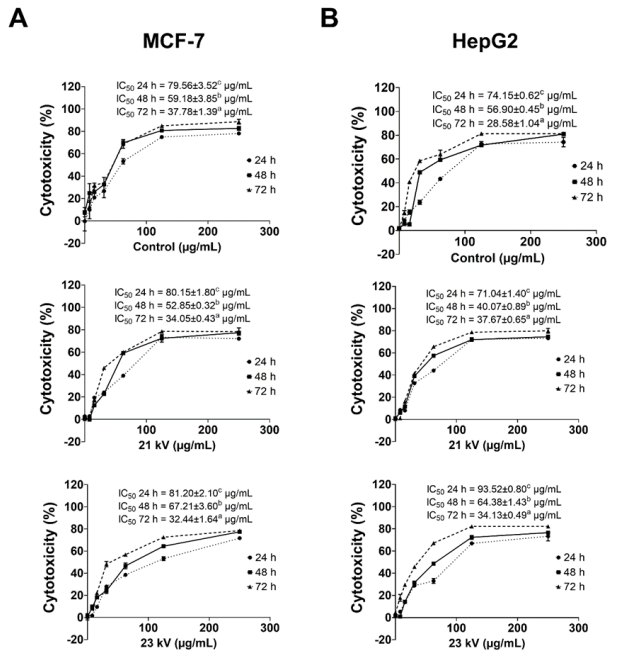

Fig. 3: Cytotoxicity of MG microgreen extracts on cancer cells: **A**) MCF-7 and **B**) HepG2

Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

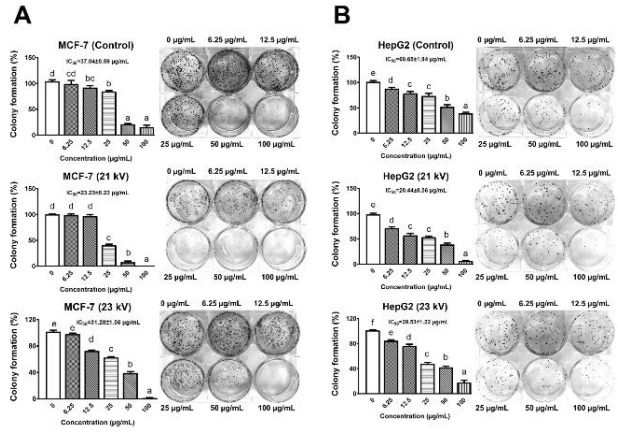

Fig. 4: Anticolony formation: **A**) MCF-7 and **B**) HepG2. Data represented as mean ± SE of three independent experiments. Different letters above columns indicate significant differences ($P < 0.05$)

Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

The results showed that the anti-colony formation of MG microgreen extracts was dose-dependent in both MCF-7 (Fig. 4A) and HepG2 (Fig. 4B). MG microgreen extracts from cold plasma-treated seeds at 21 kV exerted the most potent antiproliferative effect on MCF-7 (IC_{50} = 23.23 ± 0.23 μg/mL) (Fig. 4A) and HepG2 cells (IC_{50} = 20.44 ± 0.56 μg/mL) (Fig. 4B).

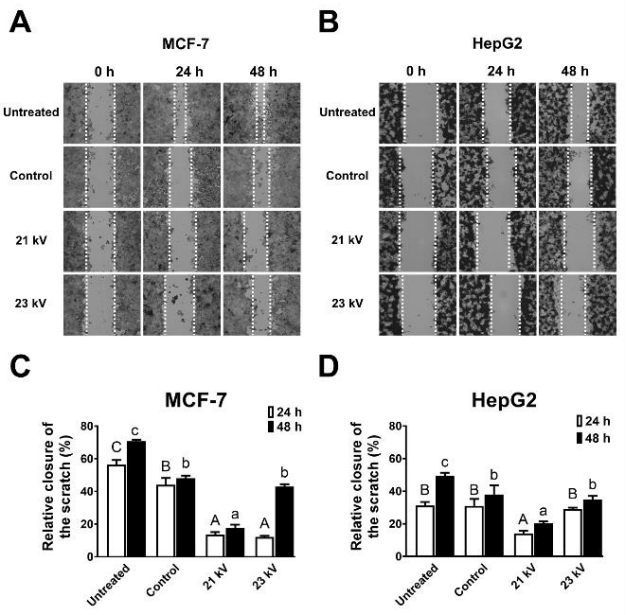

Fig. 5: Wound healing assay: **A**) MCF-7, **B**) HepG2, C Relative closure of the scratch (%) of MCF-7 and D Relative closure of the scratch (%) of HepG2. Different letters above columns indicate significant differences ($P < 0.05$). Uppercase letters for 24 h and lowercase letters for 48 h. Untreated = Cancer cells without any treatment; Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

Antimigratory effect of MG microgreens

Antimigratory effect of MG microgreen extracts was tested using wound healing assay. The results clearly demonstrated that MG microgreen extracts (25 μg/mL) from cold plasma-treated seeds at 21 kV inhibited both MCF-7 (Fig. 5A) and HepG2 (Fig. 5B) cell migration after 24 and 48 h. The antimigratory effect from cold plasma-treated seeds at 21 kV was more pronounced than those from cold plasma-treated seeds at 23 kV, control seeds and untreated cells.

Next, gelatinase zymography was carried out to assess the protein expression of invasion-linked matrix metalloproteinase 2 (MMP 2) and matrix metalloproteinase 9 (MMP 9) relating to cancer migration. The results showed that MG microgreen extracts (25 μg/mL) from cold plasma-treated seeds at 21 kV significantly inhibited both MMP 2 and MPP 9 protein expressions in both MCF-7 (Fig. 6A) and HepG2 (Fig. 6B) cells after 48 h exposure to 25 μg/mL of microgreen extract significantly more than those from cold plasma-treated seeds at 23 kV, control seeds and untreated cells.

Gene expressions in cancer cell death

Gene expressions related to apoptotic pathway in cancer cells were investigated using RT-PCR technique. All MG microgreen extracts increased *Bax* gene expressions in both

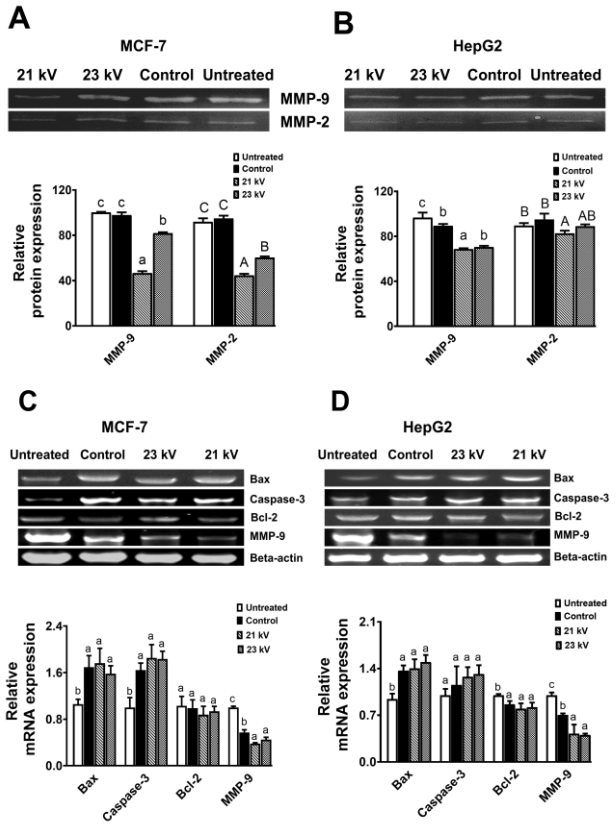

Fig. 6: Protein expression of MMP-2 and MMP-9 and gene expressions: **A**) Protein expression of MCF-7, **B**) Protein expression of HepG2, **C**) Gene expressions of MCF-7 and **D**) Gene expressions of HepG2. Data represented as mean ± SE of three independent experiments. Different letters above columns indicate significant differences ($P < 0.05$)

Untreated = Cancer cells without any treatment; Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

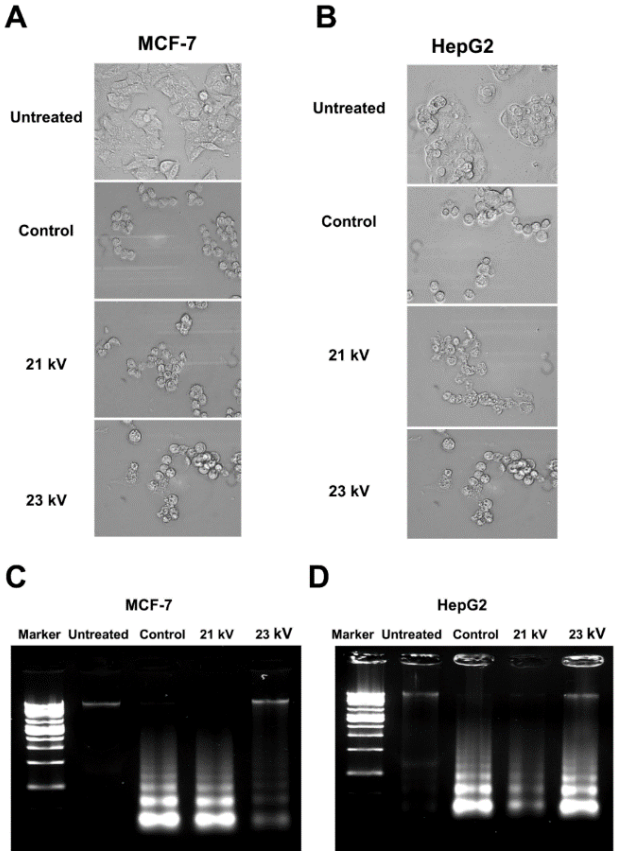

Fig. 7: Cancer cell morphology and DNA fragmentation after 24 h exposure to MG microgreen extracts: **A**) MCF-7 morphology, **B**) HepG2 morphology, **C**) DNA fragmentation of MCF-7 and **D**) DNA fragmentation of HepG2

Untreated = Cancer cells without any treatment; Control = MG microgreens from control seeds (no plasma-treated); 21 kV = MG microgreens from plasma-treated seeds at 21 kV; 23 kV = MG microgreens from plasma-treated seeds at 23 kV

MCF-7 (Fig. 6C) and HepG2 (Fig. 6D) after exposure to 50 μg/mL microgreen extract for 24 h which were significantly higher than those of the untreated group. MG microgreen extracts from plasma-treated seeds caused more inducing effect to *MMP-9* gene expressions than those from control seeds in both cancers, but no difference was found in other genes (Fig. 6C–D). The most potent effect was from MG microgreen extracts from plasma-treated seeds at 23 kV.

Cancer cell morphology and DNA fragmentation

Under phase-contrast microscopy, cell death and morphological changes in cancer cells were detected. The sizes of MG microgreen extract-treated MCF-7 and HepG2 cells appeared smaller than those untreated cells after 24 h (Fig. 7A–B). Both cancer cells shrank, and the membrane blebbed. The organelle condensation appeared as black spots inside the cancer cells and cells were broken into smaller cells.

The results of DNA fragmentation showed that 100 μg/mL of all MG microgreen extracts led to typical ladder pattern of internucleosomal DNA fragmentation from both MCF-7 (Fig. 7C) and HepG2 (Fig. 7D) on agarose gel when compared to the unbroken DNA from the untreated cells. In cancer cell death, MG microgreen extracts were able to cause apoptosis as observed by DNA fragmentation.

Discussion

The results from this work led to the acceptance of our hypothesis that cold plasma treatment on MG seeds was able to increase seed germination, ITC content, bioactive compounds and anticancer activity of MG microgreens when compared to those grown from the control seeds without plasma treatment.

Cold plasma treatment at 23 kV resulted in the highest germination percentage possibly because 23 kV was optimal to enrich the seed surface with oxygen-containing functional groups as in the previous report (Amnuaysin *et al.* 2018)

and thus increased water absorption ability and loosening of the seeds (Müller *et al.* 2009) which may increase seed germination and growth (Jiayun *et al.* 2014). In addition, cold plasma treatment at 21 and 23 kV on MG seeds was able to increase ITC content when compared to that of control MG. This increase may be the result of cold plasma ability to induce glucosinolate synthetic genes and thus ITC production. Previously, 10 m*M* $CaCl_2$ priming as abiotic stressor or elicitor was able to induce *BrST5b* (*sulfotransferase 5b*) and *BrAOP2* (*2-oxoglutarate-dependent dioxygenase 2*) gene expressions for increased glucosinolate biosynthesis in broccoli sprouts (Yang *et al*. 2016) and thus enhanced corresponding ITC products.

Hydroxyl (OH) radical, singlet oxygen, Ar- and N^2-excited-species generated during cold plasma treatment may induce reactive nitrogen species (RNS) and reactive oxygen species (ROS) generation along with UV radiation, shock wave, and photons (Matra 2018). These species may stimulate stress in MG seeds/microgreens leading to increased glucosinolate biosynthesis (subsequent hydrolysis to ITCs) or antioxidant enzymes or non-enzymatic antioxidants (Bußler *et al*. 2015; Zhang *et al*. 2017). ROS was found to stimulate stress-causing synthesis of 4-hydroxyglucobrassicin and neoglucobrassicin glucosinolates (Torres-Contreras *et al.* 2018) and another previous report showed that light stimulated plant myrosinase that hydrolyzed glucosinolates to ITCs (Yamada *et al*. 2003). Similar to the previous reports of mature MG plants (Olivier *et al*. 1999; Arora *et al*. 2016), the two dominant ITCs found in MG microgreens in this study were AITC and 3-butenyl ITC.

Plasma-treated seeds at 23 kV gave the highest values of TPC and TFC. Cold plasma treatment at 23 kV on seeds may act as an abiotic stressor by generating RNS and ROS and thus induce TPC and TFC synthesis in MG microgreens. It is known that the phenylpropanoid pathway produces phenolic compounds in plants and can be stimulated by environmental stressors and elicitors (Kim *et al*. 2006; Giorgi *et al.* 2009; Yuan *et al*. 2010).

The cytotoxic effect results showed that the lowest IC_{50} value towards MCF-7 at 72 h was observed in 23 kV (32.44 ± 1.64 μg/mL). However, for HepG2, the lowest IC_{50} value at 72 h was found in control (28.58 ± 1.04 μg/mL). The highest cytotoxic effect from 23 kV may be due to the highest ITC, TPC and TFC contributing to the most potent anticancer effect towards MCF-7 at 72 h. For antiproliferative effect, MG extracts from 21 kV treatment gave the lowest IC_{50} values towards MCF-7 (23.23 ± 0.23 μg/mL) and HepG2 (20.44 ± 0.56 μg/mL) followed by those from 23 kV treatment (31.28 ± 1.05 μg/mL and 29.53 ± 1.22 μg/mL, respectively). This suggested that the highest DPPH scavenging activity from MG microgreen extracts from 21 kV treatment may have an important role in antiproliferation of both cancer cells. In addition, lower IC_{50} values of MG microgreen extracts from 21 and 23 kV treatment were able to inhibit colony formation when compared to those used to induce cytotoxicity suggesting that MG microgreen extracts from 21 and 23 kV treatment at low concentrations were more suitable for longer-term use in antiproliferation against MCF-7 and HepG2.

The most potent bioactive compound responsible for anticancer effect of MG microgreen extracts was assumed to be ITC. According to the previous reports, 3-butenyl ITC extract from *Brassica juncea* showed IC_{50} of 0.049 μL/mL against HepG2 and 0.666 μL/mL against MCF-7 (Arora *et al*. 2016). When compared to our results of crude MG microgreen extract, the higher IC50 values of 19.11 ± 0.35 μg/mL for HepG2 and 28.35 ± 0.23 μg/mL for MCF-7 after 72 h exposure were found suggesting lower efficacy than those of the previous work. ITC and bioactive compounds may stimulate caspase-3 activity and thus induce apoptosis (Steelman *et al*. 2004). AITC was able to prohibit MCF-7 cell proliferation and reduce *Bcl-2* gene expression (Sayeed *et al*. 2018). AITC was found to activate caspase-8, -9 and -3, deactivate anti-apoptotic protein Bcl-2 and activate pro-apoptotic protein leading to apoptosis in MCF-7 (Bo *et al*. 2016) and induce DNA fragmentation (Murata *et al.* 2000). These results of the previous studies were in accordance with our results in that *caspase-3* gene expression increased in MCF-7 and *Bcl-2* gene expression decreased in HepG2 upon MG microgreen treatments from control, 21 kV and 23 kV when compared with the untreated cells. In addition, 3-butenyl ITC from *B. juncea* L. Czern var. Pusa Jaikisan exhibited cytotoxicity on cervical cancer, liver cancer, and breast cancer with reduction in MMPs protein expression that contributed to antimigration of cancer cells (Gottlieb *et al*. 2003; Kim *et al*. 2011; Arora *et al*. 2016). This was also comparable to our finding in that MMP 2 and MMP 9 protein expressions significantly decreased in MCF-7 and HepG2 upon MG microgreen treatments from 21 kV (with more pronounced effect) and 23 kV.

Conclusion

Overall MG microgreen extracts from cold plasma-treated seeds at 21 kV was most effective regarding cytotoxicity against MCF-7 at 48 h and HepG2 at 24 h and 48 h, antiproliferation and antimigration against both cancer cells. This is the first report to highlight the significant influence of cold-plasma treatment on MG seeds and food functionality of the corresponding MG microgreens including increased bioactive contents, DPPH antioxidant activity and anticancer properties. Cold plasma can be considered as an innovative and clean technology for food and agricultural applications especially microgreen industry starting from the seeds to developing functional foods with enhanced antioxidant and chemopreventive benefits.

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Author Contributions

VL designed, conducted the experiments, analyzed data and wrote the manuscript. WS and TK conducted the experiments. TK and KM contributed samples, materials, or data. BB and SD designed the experiments. All authors listed have read and approved the manuscript for publication.

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