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Research Article

Antioxidant activity of mustard green and Thai rat-tailed radish grown from cold plasma treated seeds and their anticancer efficacy against A549 lung cancer cells

Khanit MATRA[1], Worachot SAENGHA[2], Thipphiya KARIRAT[2], Kannika NAKHOWONG[2], Pimsupa PATTANU[2], Piyatida KITKAYUN[2], Theerayut BUBPAMALA[2], Benjaporn BURANRAT[3], Teeraporn KATISART[4], Vijitra LUANG-IN[2*]

[1]*Department of Electrical Engineering, Faculty of Engineering, Srinakharinwirot University, Nakhon Nayok 26120, Thailand; khanit@g.swu.ac.th*
[2]*Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Mahasarakham 44150, Thailand; vijitra.l@msu.ac.th (*corresponding author); worachot207@gmail; thipphiya.k@gmail.com; 60010813114@alumni.msu.ac.th; 60010813057@alumni.msu.ac.th; 60010813120@alumni.msu.ac.th; 60010813118@alumni.msu.ac.th*
[3]*Faculty of Medicine, Mahasarakham University, Mahasarakham 44000, Thailand; buranrat@gmail.com*
[4]*Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham 44150, Thailand; tkatisart@gmail.com*

Abstract

Lung cancer is Thailand's second-highest cause of mortality. Mustard green (MG) and rat-tailed radish (RTR) 7-day-old microgreens were previously shown to exhibit cytotoxicity against MCF-7 and HepG7 cells. However, both plants have yet to be tested on A549 lung cancer cells. This study evaluated the antioxidant activity of MG and RTR plants grown from cold plasma treated seeds at different early growth periods and their anticancer activities against A549 lung cancer cells. Plant seeds primed with cold plasma at 21 kV (for MG) and 19 kV (for RTR) for 5 min were grown on vermiculite for 14, 21 and 28 days. Results showed that RTR-P and MG-P (plasma-treated seeds) showed significantly higher ferric reducing antioxidant power (FRAP) and total antioxidant phosphomolybdate activity than RTR-C and MG-C (control seeds). Highest cytotoxicity (*E*max) of 95.41% against A549 cells was found in MG-P of 14 days at 72 h exposure with IC_{50} value of 67.11 μg/mL. Lower IC_{50} of 30.93 μg/mL was found in RTR-P of 14 days at 72 h exposure and *E*max of 93.38%. MG-P and RTR-P had significantly more pronounced effects on apoptosis and migration-related gene expressions (*Bax, Bcl-2, caspase-3, p21, MMP-9* and *cyclin D1*) and also protein expressions (caspase-3, cytochrome c and p21). The RTR-P extract was more cytotoxic and antiproliferative than MG-P in human lung cancer cells. Cold plasma played a key role in enhancing cytotoxicity in these two plants with improved chemopreventive benefits for consumers.

Keywords: A549 cells; apoptosis; *Brassica juncea*; flavonoid; non-thermal plasma; phenolic acid; *Raphanus sativus*

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Introduction

Lung cancer accounts for 14.1% of all cancers diagnosed in Thailand, with 70% of cases discovered after disease progression (Reungwetwattana *et al.*, 2020). In 2018, mortality rate for lung cancer in Thailand was 18.7%, second highest after liver cancer at 20.3%. Non-small cell lung cancer and small cell lung cancer (NSCLC and SCLC, respectively) are the two most common types of lung cancer based on the pathology. Eighty-five percent of all cases of lung cancer are caused by NSCLC as the most frequent type (Ettinger *et al.*, 2012).

Surgical removal and radiation therapy are critical components of Thailand's multimodality lung cancer treatment (Reungwetwattana *et al.*, 2020). Key issues and obstacles in cancer treatment in Thailand are medication accessibility and a shortage of specialized healthcare workers such as medical oncologists, radiation oncologists and thoracic surgeons.

Recently, increased research has been conducted on natural compounds that can be used as innovative anticancer medicines against lung cancer. Cold plasma treatment on plant seeds grown into 7-day-old microgreens of *Brassica juncea* (L.) Czern and Coss (mustard green; MG) and *Raphanus sativus* var. *caudatus* (Thai rat-tailed radish; RTR) has proved a novel tool to increase bioactive compounds, bioactivities and cytotoxicities against breast cancer and liver cancer cells (Luang-In *et al.*, 2021; Saengha *et al.*, 2021). Increased risk of lung cancer has been linked to free radical-induced oxidative damage (Sharifi-Rad *et al.*, 2020), with effective and natural alternatives to minimize oxidative damage and promote human health now focusing on the manufacture of antioxidant supplements or foods.

No studies have documented the use of MG or RTR as bioactive compounds in the context of lung cancer cells. Here, we examined the antioxidant activity and cytotoxic effects of MG and RTR grown from untreated seeds and cold plasma-treated seeds on A549 human lung cancer cells.

Materials and Methods

Plant cultivation and cold plasma treatment

Punthawee mall shop (https://www.pwcmallonline.com) provided the MG and RTR seeds. As previously reported, MG and RTR seedlings (100 seeds/replicate/treatment) were treated in triplicate with cold plasma voltage of 21 kV for MG and 19 kV for RTR for 5 min at the Faculty of Engineering Srinakharinwirot University in Nakhon Nayok Province (Luang-In *et al.*, 2021; Saengha *et al.*, 2021). The seeds were sprayed with 20 mL of deionized water every day and germinated in trays at 25 °C (12 h light/12 h dark cycle) with light intensity regulated at 42 μmol/s/m^2 until harvest at 14, 21 and 28 days. The plants were carefully cut 1 cm above the vermiculite surface for further analysis. RTR-P and MG-P were grown from plasma-treated seeds while RTR-C and MG-C were grown from control seeds.

Plant extract preparation

The freeze-dried plants (10 g) were combined with 500 mL of 80% methanol, homogenized and incubated for 24 h at 37 °C with constant shaking at 250 rpm. The samples were then centrifuged at 10,000g for 15 min and filtrated. The supernatants were freeze-dried and then reconstituted in ethanol to obtain plant extract solution at a stock concentration of 20 mg/mL for use in the antioxidant assays.

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) reagent was prepared by combining 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-Tris(2-pyridyl)s-triazine solution in 40 mM HCl, and 20 mM iron(III) chloride solution

in 40 mM HCl in a 1:1:10 (v/v) ratio, respectively (Luang-In *et al.*, 2021). After adding the FRAP reagent (180 µL), the extract solution (20 µL) was properly mixed and incubated in the dark for 30 min. The absorbance of the samples was measured at 593 nm and compared to the reference iron (II) sulfate (Sigma, St. Louis, MO, USA).

Phosphomolybdate assay (total antioxidant capacity)

Phosphomolybdenum was employed to measure the total antioxidant capacity (Umamaheswari and Chatterjee, 2007). A 0.1 mL aliquot of plant extract solution was mixed with 1.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and stirred. A water bath was used to incubate the test tubes for 90 min at 95 °C. The absorbance was measured at 765 nm using Trolox as a reference.

Cancer cell culture

The A549 (ATCC ® CCL-185™) human lung adenocarcinoma cells were obtained from the American Type Culture Collections (Manassas, VA, USA). The Dulbecco© Modified Eagle Medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) to grow A549 cells at 37 °C with 5% CO_2. The DMEM was replenished every two or three days. The cell lines were washed in pH 7.2 phosphate-buffered saline (PBS) and trypsinized with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA).

Plant extraction for cell cultures

Plant extraction was carried out following Luang-In *et al.* (2021). Fresh plants (50 g) were pulverized and mixed at 250 rpm for 2 h at 37 °C in 0.1 M citrate phosphate buffer (50 mL), pH 7.0. Dichloromethane was then added in a 1:1 ratio to the mixtures for 30 min. The mixtures were centrifuged for 15 min at 10,000g before being filtered, evaporated and freeze-dried. To obtain plant extract solutions, the dried materials were resuspended with 1% dimethyl sulfoxide (DMSO)(Fisher Scientific, Loughborough, UK) at 1,000 µg/mL. The extract solution was then filtered through a 0.22 µm millipore syringe filter to ensure sterility. Different concentrations of plant extracts were prepared in serial dilution in DMEM including 3.91, 7.81, 15.6, 31.3, 62.5, 125 and 250 µg/mL for MTT assay. For other assays, serial dilutions of interest were prepared in DMEM from a stock solution in the same manner.

Cytotoxicity assay

The A549 cells (5×10^3 cells/mL) were grown in 96-well plates for 24 h at 37 °C in 5% CO_2. The plant extract (0-250 µg/mL) was exposed to cancer cells for 24, 48 and 72 h. Next 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(0.5 mg/mL) was added to the wells and incubated for 4 h before being withdrawn and replaced with DMSO (200 µL) to determine mitochondrial function, a key indicator of cellular health. Tetrazolium MTT salt (yellow) is converted to MTT formazan (purple) in living cells by the mitochondrial dehydrogenase enzyme. The absorbance (A) was measured at 590 nm. *E*max (maximum cytotoxicity (%)) and half maximal inhibitory concentration (IC_{50}) values were also measured.

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

An IC_{50} result of less than 50 µg/mL indicates a high level of cytotoxicity. Furthermore, IC_{50} values of 50-100, 100-200 and 200-300 µg/mL indicate moderate, weak and extremely weak cytotoxicity, respectively.

Hematoxylin and eosin (H&E) staining for cell morphology

Morphological effects of the plant extracts on cancer cell nuclear and cytoplasmic machinery were studied qualitatively using H&E staining. Different mitotic phases of mitosis can be identified using H&E staining of the nucleus (blue-purple) and cytoplasm (pinkish-red) (Boyd *et al.*, 2018). Control and treated cells (5×10^3 cells/mL) were grown for 24 h in 24-well microplates. Afterward, the cell medium was withdrawn and the cells were washed with 1×PBS twice. The cells were then fixed with cold 70% ethanol for 20 min before being rinsed twice with 1×PBS. After 15 min of hematoxylin (H) staining, three washes with 1×PBS were applied. After that, another 15 min of eosin (E) staining was conducted, followed by three further washings in 1×PBS. The stained cells were dried and mounted with neutral gum. An inverted light microscope (NIB-9000, Xenon, China) was used to examine cell morphology at magnifications of 100 and 400.

The HPLC analysis of phenolics and flavonoids

The 14-day-old MG and RTR plants showing the highest cytotoxicity toward A549 cells were chosen for HPLC analysis of bioactive contents**.** The samples (1 g) were extracted for 12 h at 37 °C in HCl/methanol (1:100) at 150 rpm in the dark (Chumroenphat *et al.*, 2021). The filtrates were then re-extracted using a vacuum and 40 °C evaporation. Analysis of phenolic acids and flavonoids was conducted using a Shimadzu LC-20AC pump, SPD-M20A diode array detector and Inertsil ODS-3, C18 column. Acetic acid pH 2.74 (solvent A) and acetonitrile (solvent B) were used as the mobile phase at a flow rate of 0.8 mL/min. The gradient elution conditions followed Kubola and Siriamornpun (2011). Phenolic acids and flavonoids were detected using ultraviolet (UV) diode arrays at wavelengths of 280 and 370 nm, respectively on the column at 38 °C. External standard curves were used to determine the content. Retention periods and UV spectra of the samples were compared to the standard compounds.

Clonogenic assay

A colony formation test was conducted to examine the influence of plant extracts on cancer cell development. Viable cancer cells (800 cells/well) were planted in 6-well plates before exposure to plant extracts (0, 6.25, 12.5, 25, 50 and 100 µg/mL) for 24 h. The cells were then rinsed with PBS and placed in fresh DMEM for 14 days of culture. The DMEM medium was then discarded, and the cells were rinsed, fixed and stained for 1 h with 0.5% crystal violet. After washing, the colonies were photographed using a digital camera (Nikon D50).

Migration wound healing assay

A549 cells with 100% confluency were given scratch wound markings followed by rinsing with PBS. After exposure to plant extract (50 µg/mL), the cells were allowed to migrate for 24 and 48 h. An inverted microscope was used to view the wound coverage, with the percentage of wounds covered determined as:

$$\text{Relative wound coverage (\%)} = \frac{(\text{wound area at 0 h} - \text{wound area at T h})}{(\text{wound area at 0 h})} \times 100$$

Real-time polymerase chain reaction (PCR) analysis

Real-time PCR was used to examine apoptotic and migration-related mRNA alteration components after 24 h of treatment with MG and RTR. The A549 cells ($2x10^5$ cells/well) were grown at 37 °C for 24 h in 6-well plates before exposure to plant extracts (50 µg/mL) for 24 h at 37°C. Total ribonucleic acid (RNA) was isolated using TRIzol™ reagent (Thermo Fisher Scientific, IL, USA) and later converted to complementary deoxynucleic acid (cDNA) by reverse transcriptase (iScript™ Reverse Transcription Supermix, Bio-Rad, Hercules, CA, USA). The Fast Start Essential DNA Green Master (Roche Applied Science) was used in a QuantStudio real-time PCR for quantitative RT-PCR (Applied Biosystems, Foster City, CA, USA). Primers

for *Bax, Bcl2, caspase-3, p21, MMP-2, MMP-9* and *cyclin D1* are presented in Table 1 with real-time PCR conditions set as follows: denaturation at 94 °C for 10 min, annealing at 60 °C for 10 s, extension at 72 °C for 10 Ås for 45 cycles and then normalized to GAPDH expression levels.

Table 1. Primers for real-time PCR analysis

Gene	Primer	Sequence (5'->3')	Size (bp)
GAPDH	Forward	CACTGCCAACGTGTCAGTGGTG	121
	Reverse	GTAGCCCAGGATGCCCTTGAG	
Bax	Forward	TGCTTCAGGGTTTCATCCAG	170
	Reverse	GGCGGCAATCATCCTCTG	
Bcl2	Forward	AGGAAGTGAACATTTCGGTGAC	149
	Reverse	GCTCAGTTCCAGGACCAGGC	
caspase-3	Forward	GGCGCTCTGGTTTTCGTTAAT	121
	Reverse	TCCAGAGTCCATTGATTCGCT	
p21	Forward	AGTCAGTTCCTTGTGGAGCC	109
	Reverse	GCATGGGTTCTGACGGACAT	
MMP-2	Forward	TTGACGGTAAGGACGGACTC	153
	Reverse	ACTTGCAGTACTCCCCATCG	
MMP-9	Forward	TTTGACAGCGACAAGAAGTG	208
	Reverse	CAGGGCGAGGACCATAGAGG	
cyclin D1	Forward	ATCTCTGTACTTTGCTTGCT	564

Protein extraction and Western blot analysis

Western blot was used to examine apoptotic and migration-related protein alteration components after 24 h of treatment with MG and RTR. The A549 cancer cells were sown in 6-well plates and exposed to plant extracts (50 μg/mL) for 24 h. The cells were then lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitor cocktail (Roche, Penzberg, Germany) for 30 min on ice, and centrifuged at 14,000g for 15 min at 4 °C to remove any debris. A BCA protein kit test was used to measure protein content in the supernatant (Thermo Fisher Scientific, IL, USA). The gel was electrophoretically transferred to a nitrocellulose membrane and a protein sample of 25 μg was resolved by SDS-PAGE utilizing 12% polyacrylamide gel electrophoresis (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Tris buffered saline with 0.1% Tween-20 (TBST) was used to block the membranes for 1 h at room temperature before primary antibody (Boster Biological Technology, Inc., Wuhan, China) incubation. The membranes were washed three times with TBST and incubated for 1 h at room temperature with goat anti-rabbit and goat anti-mouse secondary Ab conjugated with horseradish peroxidase (1:5000). For chemiluminescent detection, the membranes were washed in TBST and subjected to Amersham ECL TM Prime. MageQuant TL 400 was used to visualize the unique protein band densities. All experiments were performed in triplicate using β-actin expression as a housekeeping protein loading control.

Statistical analysis

Mean ± standard deviation (SD) data were collected in three separate experiments. The statistical program SPSS (demo version) was used to perform one-way analysis of variance (ANOVA) and Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$.

Results

Antioxidant activity

The FRAP value of 26.79 mg Fe(II)/g extract from MG-P at 21 days was significantly higher than MG-C and MG-P values for other days (Figure 1A). Likewise, the highest total antioxidant phospholybdate activity (TAPA) of 48.45 mg TE/g extract) was found in MG-P at 21 days (Figure 1B). However, FRAP values of RTR-P at 14, 21 and 28 days were similar with the highest value of 32.17 mg Fe(II)/g extract at 14 days (Figure 1C). Similarly, TAPA values at 14, 21 and 28 days were similar, with the highest 56.26 mg TE/g extract at 21 days (Figure 1D). The growth period that gave the highest antioxidant activity was 21 days for both plants. All MG-P and RTR-P had significantly higher antioxidant activity than their MG-C and RTR-C counterparts, indicating that cold plasma treatment on seeds enhanced the antioxidant activity of the plants.

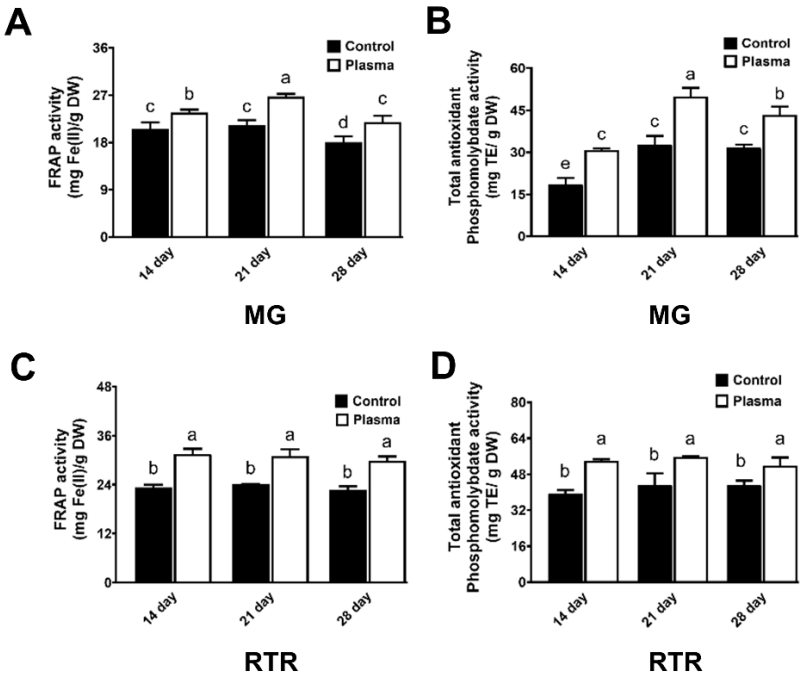

Figure 1. Antioxidant activity assessment; (A) FRAP value of MG; (B) Total antioxidant activity of MG; (C) FRAP value of RTR; (D) Total antioxidant activity of RTR
Statistical significance ($p < 0.05$) is shown by different letters

Cytotoxicity

Results in Table 2 showed that MG-P at 14 days had the highest *E*max of 95.41%, with IC_{50} value 67.11 μg/mL.

The lowest IC_{50} value of 59.68 μg/mL was found in MG-C at 21 days. Likewise, RTR-P at 14 days and 21 days displayed similar and highest *E*max values of 93.38% and 93.65%, respectively with the lowest IC_{50} value of 30.93 μg/mL from RTR-P at 14 days. The IC_{50} value of RTR-P at 14 days was 30.93 μg/mL, demonstrating high cytotoxicity against A549 cells. The 14-day RTR-P showed considerable promise as a source of natural anticancer medicines. Thus, 14-day-old plants of both MG and RTR (from control and plasma-treated seeds) were selected for further experiments.

Decrease in A549 cell density and increase in nuclear condensation and apoptotic bodies were detected in H&E staining of treated A549 cells for both control and plasma-treated seeds when plant extract concentration increased (Figure 2). These findings showed that MG and RTR extracts induced apoptosis in A549 cells in a dose-dependent manner.

The H&E staining revealed marked morphological alterations in MG and RTR-treated cells in comparison with untreated cells, and compromised cell density in a dose-dependent manner (Figure 2). Untreated cells were confluent, with dense population and normal division of the majority of cells in the interphase, while treated cells increased in the metaphase showing visible apoptotic characteristics such as shrunken cells, membrane blebbing and apoptotic bodies indicative of cell death via apoptosis (Figure 2).

Table 2. *E*max (%) and IC_{50} of MG and RTR extract on A549 cells

Treatment	Incubation time (h)	MG extract on A549 cells		RTR extract on A549 cells	
		*E*max (%)	IC_{50} (µg/mL)	*E*max (%)	IC_{50} (µg/mL)
Control 14 days	24 h	93.00 ± 0.22^{b}	129.50 ± 2.13^{i}	91.18 ± 0.51^{b}	98.40 ± 4.02^{g}
	48 h	94.33 ± 0.12^{a}	91.92 ± 3.54^{e}	93.29 ± 0.26^{a}	86.17 ± 8.86^{f}
	72 h	94.22 ± 0.11^{a}	90.73 ± 7.21^{e}	93.20 ± 0.08^{a}	70.94 ± 3.58^{e}
Control 21 days	24 h	91.37 ± 0.56^{b}	113.27 ± 1.86^{g}	90.79 ± 0.58^{b}	126.33 ± 2.06^{i}
	48 h	93.69 ± 0.24^{a}	84.65 ± 2.93^{d}	93.12 ± 0.07^{a}	97.71 ± 7.05^{g}
	72 h	93.83 ± 0.19^{a}	59.68 ± 1.94^{a}	92.89 ± 0.08^{a}	77.32 ± 3.93^{e}
Control 28 days	24 h	92.02 ± 0.84^{b}	125.80 ± 6.56^{h}	91.92 ± 0.27^{b}	139.70 ± 5.05^{j}
	48 h	91.52 ± 0.73^{b}	97.16 ± 2.94^{c}	92.57 ± 0.38^{a}	111.90 ± 6.56^{h}
	72 h	93.04 ± 1.37^{b}	103.10 ± 3.04^{f}	92.21 ± 0.19^{a}	111.23 ± 7.32^{h}
Plasma 14 days	24 h	90.97 ± 0.50^{c}	91.01 ± 0.73^{e}	90.83 ± 1.18^{b}	43.03 ± 3.37^{c}
	48 h	93.75 ± 0.20^{a}	69.04 ± 4.26^{b}	92.39 ± 0.15^{a}	34.37 ± 2.88^{b}
	72 h	95.41 ± 0.07^{a}	67.11 ± 6.34^{b}	93.38 ± 0.01^{a}	30.93 ± 0.36^{a}
Plasma 21 days	24 h	90.74 ± 0.59^{c}	104.43 ± 4.41^{f}	92.50 ± 0.19^{a}	74.77 ± 1.77^{e}
	48 h	88.46 ± 2.67^{d}	103.27 ± 4.38^{f}	92.60 ± 0.08^{a}	57.43 ± 5.69^{d}
	72 h	90.89 ± 0.67^{c}	91.72 ± 7.66^{e}	93.65 ± 0.18^{a}	34.92 ± 4.23^{b}
Plasma 28 days	24 h	89.32 ± 1.19^{d}	104.29 ± 6.52^{f}	91.91 ± 0.08^{b}	144.53 ± 4.82^{k}
	48 h	$91.62\pm0.1b^{a}$	110.89 ± 7.28^{g}	91.21 ± 0.40^{b}	131.57 ± 5.01^{j}
	72 h	90.45 ± 0.95^{c}	71.81 ± 2.40^{c}	90.45 ± 0.95^{b}	71.81 ± 2.40^{e}

$^{a, b, c \ldots}$ represent statistical significance in the columns at $p < 0.05$.

The HPLC analysis of bioactive contents

Results showed that cold plasma impacted bioactive contents in MG and RTR (Table 3). The only flavonoid detected in all samples as myricetin was richer in RTR. MG contained five phenolic acids. Ferulic acid was present in RTR only. Cold plasma significantly increased the contents of chlorogenic acid, caffeic acid and ellagic acid in MG-P. Likewise, cold plasma seed treatment enhanced ferulic acid and ellagic acid contents in RTR-P.

Table 3. Flavonoid and phenolic acid content

Sample	Compound (mg/g DW)			
	MG-C 14 days	MG-P 14 days	RTR-C 14 days	RTR-P 14 days
Flavonoid				
Myricetin	$0.0046\pm0.0001^{A,c}$	$0.0047\pm0.0011^{B,c}$	$0.0162\pm0.0014^{A,a}$	$0.0110\pm0.0012^{A,b}$
Phenolic acid				
Catechin	$0.0034\pm0.0009^{B,a}$	$0.0018\pm0.0002^{C,a}$	ND	ND
Chlorogenic acid	$0.0016\pm0.0005^{C,b}$	$0.0019\pm0.0003^{C,a}$	ND	ND
Caffeic acid	$0.0008\pm0.0001^{D,b}$	$0.0012\pm0.0002^{D,a}$	ND	ND
Syringic acid	$0.0003\pm0.0000^{E,a}$	$0.0002\pm0.0000^{E,b}$	ND	ND
Ferulic acid	ND	ND	$0.0006\pm0.0001^{C,b}$	$0.0026\pm0.0009^{B,a}$
Ellagic acid	$0.0034\pm0.0009^{B,d}$	$0.0066\pm0.0010^{A,c}$	$0.0092\pm0.0012^{B,b}$	$0.0107\pm0.0011^{A,a}$

$^{a, b \text{ and } c}$ represent statistical significance in rows at $p < 0.05$. $^{A, B, C \ldots}$ represent statistical significance in columns at $p < 0.05$.
Results are expressed as mean ± S.D. across three independent experiments. ND = not detected.

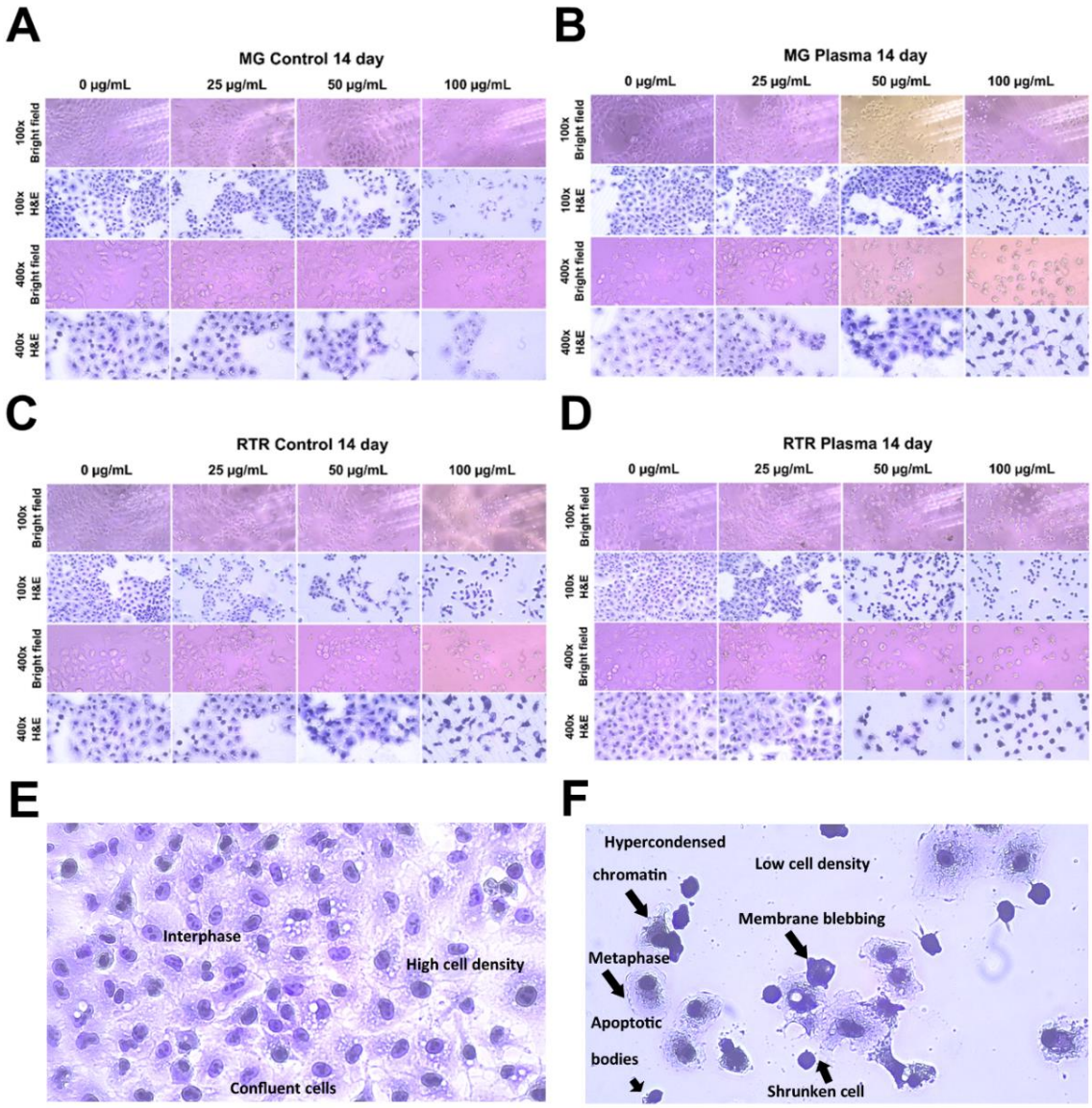

Figure 2. Effect of MG and RTR extracts of 14-day-old plants on cell morphology of A549 cells; (A) MG of 14 days from control seeds; (B) MG of 14 days from plasma-treated seeds; (C) RTR of 14 days from control seeds; (D) RTR of 14 days from plasma-treated seeds; (E) Enlarged image of untreated A549 cells; (F) Enlarged image of RTR Plasma treated A549 cells
Arrows show characteristics of apoptosis

Antiproliferative and antimigratory activity

Results showed that 14-day-old MG control had an IC_{50} value of 107.57 µg/mL, and significantly higher than 67.26 µg/mL of 14-day-old MG plasma (Figure 3A and 3B). Likewise, 14-day-old RTR control displayed a higher IC_{50} value of 78.73 µg/mL than RTR plasma of 19.59 µg/mL (Figure 3C and 3D). This indicated that RTR exhibited a significantly stronger antiproliferative effect against A549 cells than MG.

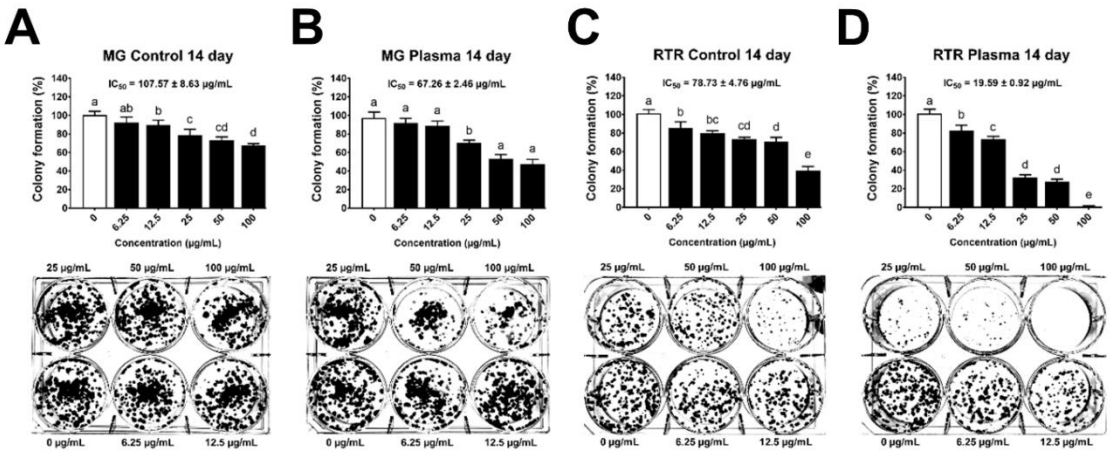

Figure 3. Effect of MG and RTR extract of 14-day old plants on colony formation of A549 cells; (A) MG of 14 days from control seeds; (B) MG of 14 days from plasma-treated seeds; (C) RTR of 14 days from control seeds; (D) RTR of 14 days from plasma-treated seeds

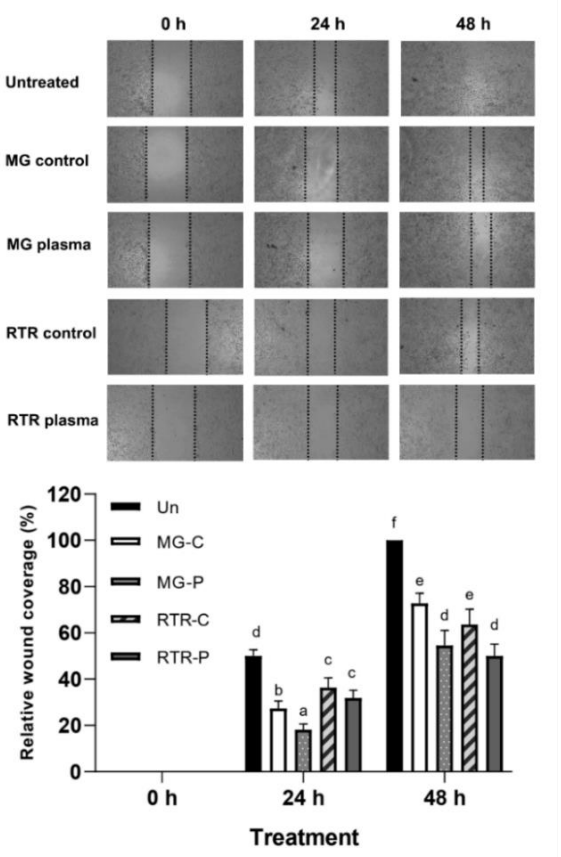

Figure 4. Antimigratory activity of MG and RTR extracts of 14-day old plants using a wound healing assay; C = control; P = Plasma. Different letters indicate statistical significance in the bars at $p < 0.05$

Both MG and RTR extracts substantially inhibited A549 cell migration and decreased wound coverage. After only 24 h, the control covered 50% of the migratory area, whereas the treated samples only covered 40% (Figure 4). After 48 h, cells treated with MG-C and RTR-C extracts covered 72.7% and 63.6 % of the migratory region, respectively compared to 100% for the control. In MG-P and RTR-P, wound coverage decreased to

54.5% and 50.0%, respectively indicating higher antimigratory impacts (Figure 4). When compared to untreated cells, percentages of migratory regions of MG and RTR-induced A549 cells were considerably lower.

Apoptosis and migration-associated gene and protein expressions

Real-time PCR findings revealed that MG-P and RTR-P substantially affected all examined genes *Bax, Bcl-2, caspase-3, p21, MMP-9* and *cyclin D1* compared to untreated cells, except for *MMP-2* in MG (Figure 5A and 5B).

Similarly, MG-P and RTR-P significantly influenced cytochrome c, caspase-3 and p21 protein expression compared with untreated cells (Figure 5C and 5D). However, RTR-P resulted in more pronounced changes (more fold increase/decrease) in gene and protein expressions than MG-P in accordance with lower IC_{50} values of RTR-P in MTT and clonogenic assays.

Bax, caspase-3 and *p21* mRNA levels significantly increased, indicative of apoptosis induction and activation of the caspase-cascade response, while *Bcl-2*, *MMP-9, MMP-2* and *cyclin D1* levels decreased, indicative of apoptosis induction, antimigratory activity due to downregulation of MMP-2 and MMP-9 and cell cycle arrest at G1/S transition. MG-P and RTR-P exhibited potential as antitumor drugs.

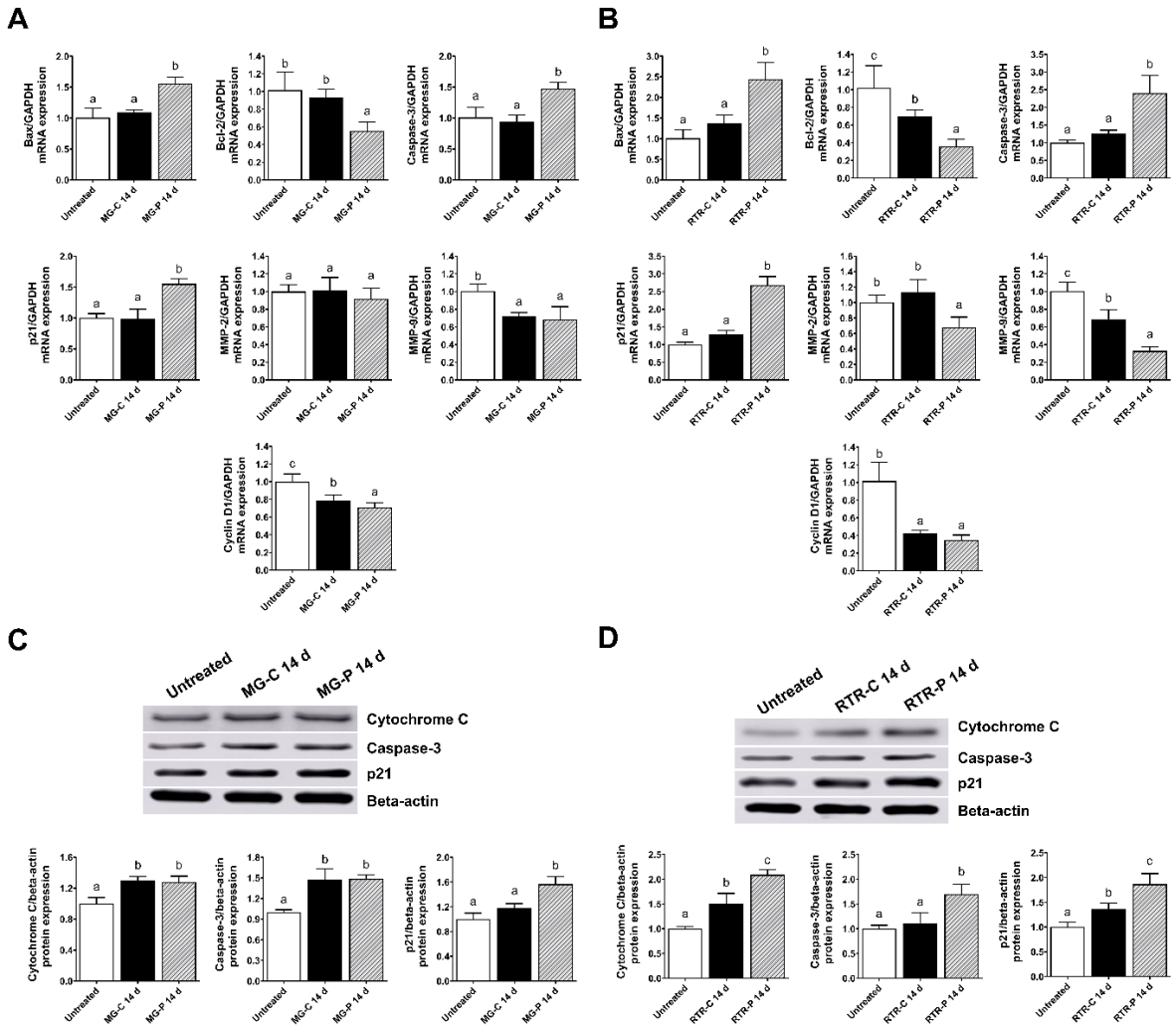

Figure 5. Effect of 14-day old MG and RTR extracts on gene and protein expressions; (A) Gene expressions from MG; (B) Gene expressions from RTR; (C) Protein expressions from MG; (D) Protein expressions from RTR. C = control; P = plasma

Discussion

This is the first report detailing the antioxidant activity of MG and RTR plants grown from cold plasma treated seeds at the early stages of growth (14, 21 and 28 days) and their anticancer activities against A549 lung cancer cells. Key findings highlighted that RTR-P and MG-P (plasma-treated seeds) had significantly higher antioxidant and anticancer activity against A549 cells. The MG-P of 14 days at 72 h with IC_{50} value of 67.11 μg/mL displayed the highest cytotoxicity at 95.41%, while RTR-P of 14 days at 72 h and 93.38% cytotoxicity had the lowest IC_{50} of 30.93 μg/mL. Cold plasma treatment on seed priming produced plants with higher impacts on apoptosis and migration-related gene expressions (*Bax, Bcl-2, caspase-3, p21, MMP-9* and *cyclin D1*) and also protein expressions (caspase-3, cytochrome c and p21). MG and RTR plant extracts displayed apoptotic-inducing properties and antiproliferative and antimigratory activities that were considerably enhanced by cold plasma treatment on seeds. RTR was more effective against A549 cells than MG.

Two experiments were performed to assess antioxidant activity. The FRAP test measures the ability of antioxidants to reduce ferric iron. The ferric-TPTZ complex is transformed into a blue ferrous-TPTZ complex by the transfer of a single electron from antioxidant molecules. Antioxidant capacity of the plant extracts was assessed using the phosphomolybdate method (Wan *et al.*, 2011). A green phosphomolybdenum V complex with maximum absorbance of 695 nm is formed when antioxidants convert Mo (VI) to Mo (V). The FRAP value of 26.79 mg Fe(II)/g extract from MG-P at 21 days was significantly higher than MG-C and MG-P values for the other days (Figure 1A) and when compared to MG-P of 7 days (13.57 mg Fe(II)/g extract) in our previous study (Saengha *et al.*, 2021). The FRAP values of RTR-P at 14 days of 32.17 mg Fe(II)/g extract (Figure 1C) were significantly higher than RTR-P at 7 days (21.72 mg Fe(II)/g extract) reported in Luang-In *et al.* (2021).

Cold plasma treatment on seeds enhanced the antioxidant activity of several plants. Basil (*Ocimum basilicum* L. cv. Genovese Gigante) showed improved antioxidant activity following seed priming with 10 and 15 kV cold plasma for 10 min (Abarghuei *et al.*, 2021). In theory, plasma-reactive oxygen species might increase the antioxidant activity of phenolic compounds (Ramazzina *et al.*, 2015). Cold plasma on seed priming may alter polyphenol production in plants during the sprouting phase, which also produces metabolites including flavonoids, phenolic acids, proanthocyanidins and ellagitannins (Schendel, 2018). A deposit of bioactive constituents such as polyphenols occurs as a consequence of radical oxidant activity and physiological processes inside seeds (Ji *et al.*, 2015). However, the reasons why only certain types of compounds increase while others decrease or remain unchanged are unclear.

RTR-P was more effective in cytotoxicity (Table 2), antiproliferation (Figure 3) and antimigration (Figure 4) of A549 cells than MG-P. Ferulic acid was only found in RTR and myricetin was richer in RTR than in MG (Table 3). Cold plasma enhanced the contents of ferulic acid and ellagic acid in RTR-P. Ferulic acid derivatives were previously used to restrict A549 cell growth and metastasis, and also induced cell cycle arrest in the G0/G1 stage. Based on Western blotting data, ferulic acid derivatives increased the expression of Bax but inhibited MMP-2 and MMP-9 (Yue *et al.*, 2019). Myricetin also displayed anticancer activity by delaying cell cycle progression and ROS-dependent mitochondria-mediated death in A549 cells (Rajendran *et al.*, 2021).

Recently, HPLC chromatograms of pod extract in dichloromethane from Thai rat-tailed radish showed the presence of protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid and ferulic acid (Yongpradoem and Weerapreeyakul, 2020). Compared to our results, 14-day-old RTR plant extracts in ethanol from RTR contained myricetin, ferulic acid and ellagic acid (Table 3). Differences in the contents may be due to the solvent, different parts of plants and plant growth periods used for extraction. Polyphenols such as gallic acid, sinapic acid, caffeic acid, vanillic acid, *p*-coumaric acid, *p*-hydroxybenzaldehyde, ferulic acid, rutin, catechin, protocatechuic acid, *p*-hydroxybenzoic acid and chlorogenic acid have been identified in different mustard varieties (Tian and Deng, 2020). The polyphenol content of mustard differed significantly in various parts of

the plant. The MG contained the five phenolic acids catechin, chlorogenic acid, caffeic acid, syringic acid and ellagic acid and a flavonoid, myricetin (Table 3), as found in previous studies.

The ethanolic extract of *Brassica nigra* seeds showed significant growth-inhibitory impact, reducing the viability (IC_{50} of 32.02 µg/mL) and clonogenic survival of A549 cells and inducing apoptosis, as demonstrated by elevated caspase-3 activity, while migratory and invasive capabilities of A549 cells were also inhibited by the downregulation of MMP-2 and MMP-9 (Ahmed *et al.*, 2020).

Compared with a previous report (Ahmed *et al.*, 2020), our IC_{50} value of 59.68 µg/mL from MG-C of 21 days (Table 2) was much higher than from *B. nigra* seed extract, indicating that the seed extract was more effective as an antitumor. Isothiocyanates, phenolic acids and flavonoids of both plants may contribute to the cytotoxic property. The active ingredients of MG were allyl isothiocyanate and 3-butenyl isothiocyanate (Saengha *et al.*, 2021). *B. nigra* seeds contained allyl isothiocyanate, an essential constituent with anticancer activities in human lung cancer. Isothiocyanates are formed by the hydrolysis of their precursor molecules, glucosinolates. Thus, MG phytochemicals may have synergistic effects on antiproliferative, proapoptotic and antimigratory actions.

Ethanolic extracts of roots and leaves from Saudi Arabian-grown white radish *R. sativus* suppressed the growth of A549 and MCF-7 cells by inducing apoptosis (Noman *et al.*, 2021). An IC_{50} value of 217 µg/mL against A549 cells was observed in roots, whereas a value of 250.6 µg/mL was obtained in leaves, suggesting that the root extract had greater cytotoxic ability. Raphasatin and other flavonoid chemicals in RTR may be responsible for this antiproliferative action (Sangthong *et al.*, 2017; Luang-In *et al.*, 2021).

A colony formation assay, commonly known as a clonogenic test, is an *in vitro* test for cell survival that measures the ability of a single cell to endure and multiply by establishing colonies over a lengthy period of time (Franken *et al.*, 2006). As a result of its phenotypic implications, this is an essential trait that may need many cell divisions to develop. A short-term cytotoxicity test like the MTT assay cannot reveal drug resistance, which is why a clonogenic test is important (Sumantran, 2011). From our results, A549 cells had no resistance over the course of 14-day treatment (Figure 3) against both plant extracts, which could be used as potential anticancer reagents. Cold plasma treatment on seeds also enhanced the antiproliferative activity of plant extracts.

Cell migration is commonly studied using the wound healing test since this is low-cost with a practical design (Bobadilla *et al.*, 2019). Once the cell monolayer is confluent, the area in the monolayer is scratched. The cells are allowed to migrate and the scratched region is recolonized. Pharmacological agents with molecular mechanisms that modulate cell migration and hence drive treatment regimens are intensively studied using this experimental technique (Decaestecker *et al.*, 2007; Walter *et al.*, 2010). E-cadherin depletion and increased metalloproteinases (MMP-2 and MMP-9) are hallmarks of cancer invasion and metastasis (Gonzalez-Avila *et al.*, 2019). The transcriptional downregulation of *MMP-2* and *MMP-9* by MG-P and RTR-P extracts (Figure 5A and 5B) provided further evidence of the possible prevention of A549 cell migration (Figure 4).

One of the most well-studied aspects of cell homeostasis is apoptosis, or programmed cell death, which is present in a wide range of organisms. Several molecules, including members of the Bcl-2 family of proteins, control the caspase cascade, a critical step in cell death. Caspase family proteases may be triggered by the death receptor-mediated route as well as by the mitochondrial-dependent mechanism.

Here, caspase-3 was activated by MG-P and RTR-P in real-time PCR and Western blot analyses (Figure 5), indicating that the cells had gone through apoptosis. An analogous set of outcomes was achieved using mustard oil-derived allyl isothiocyanate (Fadhil *et al.*, 2020). As we hypothesized, MG-P and RTR-P induced cell death by activation of the caspase cascade.

The abnormal growth of cancer cells is attributed to deregulation of the cell cycle, which results in misregulation of cell cycle-related proteins. Cell cycle progression, especially the G1/S transition phase, is tightly controlled by cyclin D1. Cyclin D1 is overexpressed in breast carcinoma, correlating with early cancer onset and tumor progression (Montalto *et al.*, 2020). When the p21 protein interacts with several stimuli,

including p53, the DNA repair process may cause the cell cycle to be arrested. Apoptosis-inhibiting p21 interacts with a variety of molecules and transition factors to prevent tumor growth. Results showed that MG-P and RTR-P inhibited the development of cancer by increasing p21 level (Figure 5).

Several additional variables influence caspase activation. Among these, Bcl-2 family members have a key function in either suppressing (Bcl-2, Bcl-xL) or encouraging cell death (Bax, Bak)(Kale *et al.*, 2018). In our study, the mRNA level of *Bax* increased, while the *Bcl-2* level decreased (Figure 5A and 5B). The heme protein cytochrome c, a proapoptotic protein, was also upregulated based on Western blot. Cytochrome c is released into the cytoplasm during apoptosis and binds to and activates Apaf-1, enabling the ring-like apoptosome to be formed and ATP binding. Caspase-3, 6 and 7 are all important participants in the execution phase of cell death, while caspase-9 is responsible for their activation (Li *et al.*, 1997). MG-P and RTR-P induced apoptosis in human lung cancer cells *in vitro* through a molecular mechanism partially explained by our findings.

Based on our data we conclude that MG-P and RTR-P may decrease the growth of A549 human lung cancer cells and cause apoptosis. Caspase cascades may be activated and *Bax, caspase-3* and *p21* expressions may be increased, whereas *Bcl-2, Bcl-2, MMP-9, MMP-2* and *cyclin D1* expressions may be decreased as a result of stimulating apoptosis. *MMP-2* and *MMP-9* expressions may also be downregulated as part of the molecular mechanism. Our results support the use of MG-P and RTR-P as chemopreventative functional foods.

Conclusions

Cold plasma technology for seed priming is a novel tool and still in its infancy, with many potential applications in horticulture. MG and RTR cold plasma treated seeds showed a substantial reduction in the rate of cell division of human lung adenocarcinoma A549 cells, while MG-P and RTR-P stimulated apoptosis and caspase activation and limited migratory activity, highlighting their promise as anticancer functional foods.

Authors' Contributions

The authors are responsible for any claims arising from the content of this article and will be held liable for any damages. Testing was carried out by K.M., W.S., T.K., K.N., P.P., P.K., and T.B. The tests were devised by B.B. T.K. collected and sorted the cancer cells. V.L. conducted the experiments, evaluated the data and wrote the text. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Abarghuei FM, Etemadi M, Ramezanian A, Esehaghbeygi A, Alizargar J (2021). An application of cold atmospheric plasma to enhance physiological and biochemical traits of Basil. Plants 10(10):2088. *https://doi.org/10.3390/plants10102088*

Ahmed AG, Hussein UK, Ahmed AE, Kim KM, Mahmoud HM, Hammouda O, ... Bishayee A (2020). Mustard Seed (*Brassica nigra*) extract exhibits antiproliferative effect against human lung cancer cells through differential regulation of apoptosis, cell cycle, migration, and invasion. Molecules (Basel, Switzerland) 25(9):2069. *https://doi.org/10.3390/molecules25092069*

Bobadilla A, Arévalo J, Sarró E, Byrne HM, Maini PK, Carraro T, ... Alarcón T (2019). *In vitro* cell migration quantification method for scratch assays. Journal of the Royal Society, Interface 16(151):20180709. *https://doi.org/10.1098/rsif.2018.0709*

Boyd LS, Gozuacik D, Joubert AM (2018). The *in vitro* effects of a novel estradiol analog on cell proliferation and morphology in human epithelial cervical carcinoma. Cellular and Molecular Biology Letters 23:10. *https://doi.org/10.1186/s11658-018-0079-z*

Chumroenphat T, Somboonwatthanakul I, Saensouk S, Siriamornpun S (2021). Changes in curcuminoids and chemical components of turmeric (*Curcuma longa* L.) under freeze-drying and low-temperature drying methods. Food Chemistry 339:128121. *https://doi.org/10.1016/j.foodchem.2020.128121*

Decaestecker C, Debeir O, Van Ham P, Kiss R (2007). Can anti-migratory drugs be screened *in vitro*? A review of 2D and 3D assays for the quantitative analysis of cell migration. Medicinal Research Reviews 27:149-176. *https://doi.org/10.1002/(ISSN)1098-1128*

Ettinger DS, Akerley W, Borghaei H, Chang AC, Cheney RT, Chirieac LR, ... Gregory KM (2012). Non-small cell lung cancer. Journal of the National Comprehensive Cancer Network 10(10):1236-1271. *http://dx.doi.org/10.6004/jnccn.2012.0130. PMid:23054877*

Fadhil AB, Saleh LA, Altamer DH (2020). Production of biodiesel from non-edible oil, wild mustard (*Brassica juncea* L.) seed oil through cleaner routes. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 42(15):1831-1843. *https://doi.org/10.1080/15567036.2019.1604893*

Franken NAP, Rodermond HM, Stap J, Haveman J, Bree CV (2006). Clonogenic assay of cells *in vitro*. Nature Protocols 1:2315-2319.

Gonzalez-Avila G, Sommer B, Mendoza-Posada DA, Ramos C, Garcia-Hernandez AA, Falfan-Valencia R (2019). Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. Critical Reviews in Oncology/Hematology 137:57-83. *https://doi.org/10.1016/j.critrevonc.2019.02.010*

Ji SH, Kim T, Panngom K, Hong YJ, Pengkit A, Park DH, ... Park G (2015). Assessment of the effects of nitrogen plasma and plasma-generated nitric oxide on early development of *Coriandum sativum*. Plasma Process and Polymers 12:1164-1173. *https://doi.org/10.1002/ppap.201500021*.

Kale J, Osterlund E, Andrews D (2018). BCL-2 family proteins: changing partners in the dance towards death. Cell Death and Differentiation 25:65-80. *https://doi.org/10.1038/cdd.2017.186*

Kubola J, Siriamornpun S (2011). Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai gac (*Momordica cochinchinensis* Spreng). Food Chemistry 127(3):1138-1145. *https://doi.org/10.1016/j.foodchem.2011.01.115*

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91(4):479-89. *https://doi.org/10.1016/S0092-8674(00)80434-1*

Luang-In V, Saengha W, Karirat T, Buranrat B, Matra K, Deeseenthum S, Katisart T (2021). Effect of cold plasma and elicitors on bioactive contents, antioxidant activity and cytotoxicity of Thai rat-tailed radish microgreens. Journal of the Science of Food and Agriculture 101(4):1685-1698. *https://doi.org/10.1002/jsfa.10985*

Montalto FI, De Amicis F (2020). Cyclin D1 in cancer: A molecular connection for cell cycle control, adhesion and invasion in tumor and stroma. Cells 9(12):2648. *https://doi.org/10.3390/cells9122648*

Noman Omar M, Nasr FA, Alqahtani AS, Mohammed A, Cordero Mary Anne W, Alotaibi Amal A, Asmatanzeem B, ... Daoud A (2021). Comparative study of antioxidant and anticancer activities and HPTLC quantification of rutin in white radish (*Raphanus sativus* L.) leaves and root extracts grown in Saudi Arabia. Open Chemistry 19(1):408-416. *https://doi.org/10.1515/chem-2021-0042*

Ramazzina I, Berardinelli A, Rizzi F, Tappi S, Ragni L, Sacchetti G, Rocculi P (2015). Effect of cold plasma treatment on physico-chemical parameters and antioxidant activity of minimally processed kiwifruit. Postharvest Biology and Technology 107:55-65.

Rajendran P, Maheshwari U, Muthukrishnan A, Muthuswamy R, Anand K, Ravindran B, ... Chung WJ (2021). Myricetin: versatile plant-based flavonoid for cancer treatment by inducing cell cycle arrest and ROS-reliant mitochondria-facilitated apoptosis in A549 lung cancer cells and in silico prediction. Molecular and Cellular Biochemistry 476(1):57-68. *https://doi.org/10.1007/s11010-020-03885-6*

Reungwetwattana T, Oranratnachai S, Puataweepong P, Tangsujaritvijit V, Cherntanomwong P (2020). Lung Cancer in Thailand. Journal of Thoracic Oncology 15(11):1714-1721. *https://doi.org/10.1016/j.jtho.2020.04.024*

Saengha W, Karirat T, Buranrat B, Matra K, Deeseenthum S, Katisart T, Luang-In V (2021). Cold plasma treatment on mustard green seeds and its effect on growth, isothiocyanates, antioxidant activity and anticancer activity of microgreens. International Journal of Agriculture and Biology 25(3):667-676. *https://doi.org/10.17957/IJAB/15.1715*

Sangthong S, Weerapreeyakul N, Lehtonen M, Leppanen J, Rautio J (2017). High-accuracy mass spectrometry for identification of sulphur-containing bioactive constituents and flavonoids in extracts of *Raphanus sativus* var. *caudatus* Alef (Thai rat-tailed radish). Journal of Functional Foods 31:237-247.

Schendel RR (2018). Phenol content in sprouted grains. In: Feng H, Nemzer B, Devries J (Eds). Sprouted grains: Nutritional value, production, and applications. Elsevier, London pp. 247-315.

Sharifi-Rad M, Anil Kumar NV, Zucca P, Varoni EM, Dini L, Panzarini E, ... Sharifi-Rad J (2020). Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. Frontiers in Physiology 11:694. *https://doi.org/10.3389/fphys.2020.00694*

Sumantran VN (2011). Cellular chemosensitivity assays: an overview. Methods in Molecular Biology (Clifton, N.J.) 731:219-236. *https://doi.org/10.1007/978-1-61779-080-5_19*

Tian Y, Deng F (2020). Phytochemistry and biological activity of mustard (*Brassica juncea*): a review. CyTA - Journal of Food 18(1):704-718. *https://doi.org/10.1080/19476337.2020.1833988*

Umamaheswari M, Chatterjee TK (2007). *In vitro* antioxidant activities of the fractions of *Coccinnia grandis* L. leaf extract. African Journal of Traditional, Complementary and Alternative Medicines 5(1):61-73.

Walter MNM, Wright KT, Fuller HR, MacNeil SM, Johnson WEB (2010). Mesenchymal stem cell-conditioned medium accelerates skin wound healing: an *in vitro* study of fibroblast and keratinocyte scratch assays. Experimental Cell Research 316:1271-1281. *https://doi.org/10.1016/j.yexcr.2010.02.026*

Wan C, Yu Y, Zhou S, Liu W, Tian S, Cao S (2011). Antioxidant activity and free radical-scavenging capacity of *Gynura divaricata* leaf extracts at different temperatures. Pharmacognosy Magazine 7(25):40-45. *https://doi.org/10.4103/0973-1296.75900*

Yongpradoem P, Weerapreeyakul N (2020). Evaluation of antioxidant activity and inhibition of tyrosinase activity of *Raphanus sativus* var. *caudatus* Alef extract. Walailak Journal of Science and Technology 17(8): 838-850.

Yue SJ, Zhang PX, Zhu Y, Li NG, Chen YY, Li JJ, ... Duan JA (2019). A ferulic acid derivative FXS-3 inhibits proliferation and metastasis of human lung cancer A549 cells via positive JNK signaling pathway and negative ERK/p38, AKT/mTOR and MEK/ERK signaling pathways. Molecules 24(11):2165. *https://doi.org/10.3390/molecules24112165*