Petroselinum crispum has antioxidant properties, protects against DNA damage and inhibits proliferation and migration of cancer cells

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Abstract

BACKGROUND: Petroselinum crispum (English parsley) is a common herb of the Apiaceae family that is cultivated throughout the world and is widely used as a seasoning condiment. Studies have shown its potential as a medicinal herb. In this study, P. crispum leaf and stem extracts were evaluated for their antioxidant properties, protection against DNA damage in normal 3T3-L1 cells, and the inhibition of proliferation and migration of the MCF-7 cells.

RESULTS: The dichloromethane extract of P. crispum exhibited the highest phenolic content (42.31 ± 0.50 mg GAE g⁻¹) and ferric reducing ability (0.360 ± 0.009 mmol g⁻¹) of the various extractions performed. The extract showed DPPH radical scavenging activity with an IC₅₀ value of 3310.0 ± 80.5 μM. Mouse fibroblasts (3T3-L1) pre-treated with 400 μM of the extract showed 50.9% protection against H₂O₂-induced DNA damage, suggesting its potential in cancer prevention. The extract (300 μg mL⁻¹) inhibited H₂O₂-induced MCF-7 cell migration by 41% ± 4%. As cell migration is necessary for metastasis of cancer cells, inhibition of migration is an indication of protection against metastasis.

CONCLUSION: Petroselinum crispum has health-promoting properties with the potential to prevent oxidative stress-related diseases and can be developed into functional food.

INTRODUCTION

Petroselinum crispum (Mill) Nyman ex AW Hill, commonly known as English parsley, is a culinary and medicinal herb of the Apiaceae family that grows up to 30–100 cm high. The herb has been used to flavor the cuisines of South East Asia, China, India, South America and Mexico. Although native to Europe and western Asia, the herb is now cultivated and consumed throughout the world. The leaves and stems, either fresh or dried, as well as the seeds, have been employed in the food, pharmaceutical and cosmetic industries. In folk medicine, the aerial part of P. crispum is used to treat hemorrhoids, the stem for urethral inflammation, and the root is used to pass kidney stones and improve brain function and memory. Additionally, P. crispum is used as a carminative, stomachic, emmenagogic, abortifacient and nutritive agent. Studies have shown that P. crispum has hypoglycemic, diuretic, hypolipidemic, antimicrobial, anticoagulant and hepatoprotective activities.

The chemical composition and pharmacological properties of P. crispum have been previously reported in various studies. The herb contains flavonol glycosides of quercetin, apio, myristicin and luteolin. Terpenes, phthalides, furanocoumarins, apiin, carotenoids, ascorbic acid and tocopherol are also present in P. crispum. Supplementation of diets with fresh P. crispum leaves increases the antioxidant capacity of plasma in rats and decreases oxidative stress in humans. Zheng et al. reported the inhibition of benzo[a]pyrene-induced tumorigenesis in the lungs of mice by myristicin, a major volatile aromatic constituent of parsley leaf oil. The vast health-promoting properties associated with P. crispum warrant further study. Previous investigations

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on *P. crispum* mostly focused on its antioxidant properties.\(^\text{14}\) As phenolic compounds and antioxidant activities depend on variety, location and growth conditions of the plant, data on the antioxidant activity of *P. crispum* are still relevant and useful. The effect of *P. crispum* leaves and stems on the two most common cancers in humans – breast cancer and colon cancer – are unclear and lacking thus far. The main aim of this work was to investigate the antioxidant activities and protection against DNA damage by extracts of *P. crispum* leaves and stems and their inhibition of the proliferation and \(H_2O_2\)-induced migration of the breast cancer cell line MCF-7. To the best of our knowledge, this is the first study reporting on the effects of *P. crispum* on DNA protection and inhibition of MCF-7 cell migration.

### EXPERIMENTAL

#### Materials and methods

Analytical-grade solvents were purchased from Fisher Scientific (Loughborough, UK). Dimethyl sulfoxide (DMSO) and \(H_2O_2\) were purchased from Univar (Ingleburn, NSW, Australia). Chemicals, polyphenol standards (gallic acid, quercetin, rutin), proteinase K and RPMI-1640 were obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Lonza (Basel, Switzerland) and fetal bovine serum (FBS) was obtained from iDNA Biotechnology, Singapore.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin–Ciołkowska phenol reagent, tris(hydroxymethyl)aminomethane and ethidium bromide were purchased from Bio-Rad (Hercules, CA, USA). TRizol \(\text{®}\) reagent was purchased from Life Technologies (Carlsbad, CA, USA). RNase A and DNA ladders were obtained from Thermo Scientific (Carlsbad, CA, USA). Ultrapure water from a Milli-Q-plus filter system (Millipore, Molsheim, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Sigma-Aldrich (St Louis, MO, USA). Ultrapure water from a Milli-Q-plus filter system (Millipore, Billerica, MA, USA) was used throughout the experiments.

#### Cell lines and cell culture conditions

Two human breast adenocarcinoma cell lines – MCF-7 and MDA-MB-231 – and the human colorectal adenocarcinoma cell line HT-29 were used in the antiproliferative study. The MCF-7 cell line expresses the estrogen receptor whereas MDA-MB-231 and the human colorectal adenocarcinoma cell line HT-29 were used in the antiproliferative study. The MCF-7 cell line was grown in RPMI-1640. MDA-MB-231 and 3T3-L1 cells were routinely cultured in RPMI-1640. MCF-7 and HT-29 cancer cells were cultured in DMEM. The cells were supplemented with 10% fetal bovine serum (FBS), 100 U mL\(^{-1}\) penicillin and 100 μg mL\(^{-1}\) streptomycin. Cells were grown at 37 \(^\circ\)C in a humidified incubator with 5% \(CO_2\).

#### Plant material

Fresh *P. crispum* leaves and stems were purchased from the local market in Kuala Lumpur, Malaysia. The plant was identified by Dr M Sugumaran, Institute of Biological Sciences, University of Malaya, and the voucher specimen (KLU47745) was deposited in the University of Malaya herbarium. The leaves and stems were washed under running tap water and finally rinsed with distilled water. The plant parts were then freeze dried, weighed, ground into fine powder and stored at \(-20\) \(^\circ\)C until extraction.

#### Preparation of *P. crispum* extracts

Powdered leaves and stems of *P. crispum* were extracted through sequential extraction using hexane, dichloromethane, ethyl acetate, methanol and water. Briefly, 120 g powdered leaves and stems were extracted in 600 mL hexane (1:5 w/v) for 6 h at 40 \(^\circ\)C on a hotplate stirrer. Extracts were then filtered through Whatman no. 1 filter paper and the resulting residue was re-extracted twice with fresh hexane. The remaining residue was subsequently extracted three times each with dichloromethane, followed by ethyl acetate, methanol and water. Each filtrate (except for the aqueous extract, which was concentrated to dryness in a freeze-dryer) was concentrated to dryness under reduced pressure at 40 \(^\circ\)C using a rotary evaporator. The dried extracts were stored at \(-20\) \(^\circ\)C. For bioassays, the dried extracts were dissolved in DMSO and diluted in ultrapure water to make appropriate extract concentrations. The final concentration of DMSO in reaction mixtures was less than 1%. All dissolved extracts were kept at 4 \(^\circ\)C throughout the experiments.

#### Determination of total phenolic content

The total phenolic content of *P. crispum* extracts was determined using the Folin-Ciołkowska method,\(^{15}\) with some modifications. Briefly, 500 μL of 1:10 Folin–Ciołkowska phenol reagent was added to 10 μL of sample (dissolved in 10% DMSO), standard or positive control. The mixture was mixed and allowed to stand for 5 min before the addition of 350 μL of 10% sodium carbonate. The resulting reaction mixture was incubated in the dark at room temperature for a further 2 h. Absorbance was then measured at 765 nm using a spectrophotometer. Gallic acid (50–500 μg L\(^{-1}\) in 10% DMSO) was used as the standard. Rutin and quercetin were used as positive controls. Results were expressed in milligrams of gallic acid equivalents (GAE) per gram of dried extract. All experiments were carried out in triplicate.

#### Ferric reducing antioxidant power (FRAP) assay

Ferric reducing activity of *P. crispum* extracts was estimated based on the assay by Benzie and Strain\(^\text{16}\) with slight modifications. A working reagent was prepared fresh by mixing 10 mL of 300 mmol L\(^{-1}\) acetate buffer with 1 mL of 10 mmol L\(^{-1}\) 2,4,6-tripryidyl-s-triazine (TPTZ) in 40 mmol L\(^{-1}\) hydrochloric acid and 1 mL of 20 mmol L\(^{-1}\) ferric chloride hexahydrate (FeCl\(_3\).6H\(_2\)O). The freshly prepared FRAP reagent was pre-warmed at 37 \(^\circ\)C for 5 min, after which a blank reading was taken at 595 nm using a plate reader. Subsequently, 3 μL sample (dissolved in 10% DMSO), standard or positive control and 9 μL water were added to 90 μL of the FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent and again at 4 min after the start of the reaction. The change in absorbance in the 4 min reaction was calculated by comparison with a FeSO\(_4\).7H\(_2\)O standard curve (100–1000 μmol L\(^{-1}\)) tested in parallel. Rutin and quercetin were used as positive controls. Results were expressed as micromoles of ferric reducing activity of the extracts per gram of dried extract. All experiments were carried out in triplicate.

#### 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The radical scavenging activity of *P. crispum* extracts was determined by the DPPH radical scavenging assay,\(^{17}\) with some modifications. Petroselinum crispum extract (20 μL) was added to 120 μL of 0.04 mg mL\(^{-1}\) DPPH solution in methanol. The extracts tested...
ranged from 0 to 5000 μg mL⁻¹ (dissolved in 10% DMSO). The solutions were mixed well and incubated in the dark for 30 min. The reduction of DPPH absorption was measured at 515 nm using a plate reader. Rutin and quercetin were used as positive controls. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated according to the following equation:

\[
\text{Percentage inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100
\]

The results were expressed as half-maximal inhibitory concentration (IC₅₀), i.e. the concentration of the plant extract required to scavenge 50% of the total DPPH radicals available.

**Inhibition of proliferation (MTT assay)**

The antiproliferative activity of *P. crispum* extracts on MCF-7, MDA-MB-231 and HT-29 cancer cell lines was estimated using the MTT assay as described by Mosmann. Briefly, cells supplemented with 5% FBS were seeded (5 × 10⁵ cells per well) in 96-well plates and were allowed to grow at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h incubation, the cells were treated with different concentrations of extract (0–500 μg mL⁻¹) for a further 48 h. Vehicle-control wells with cells only and diluent-control wells with similar DMSO concentrations as in the treatment were included. After incubation, 10 μL of 5 mg mL⁻¹ MTT bromide in phosphate-buffered saline (PBS) were added to each well. The plates were reincubated for 4 h, after which media and MTT were removed by aspiration. DMSO (100 μL) was added to each well to dissolve the formazan crystals. Absorbance was read using a microtiter plate reader at 595 nm. All measurements were performed in triplicate. The percentage inhibition of cell proliferation was calculated using the following formula:

\[
\text{Percentage inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{treated}})}{\text{Absorbance}_{\text{control}}} \times 100
\]

**Comet assay**

The DNA protective effect of *P. crispum* was estimated using the comet assay. Mouse fibroblasts (3T3-L1) were cultured in 12-well culture plates (1 × 10⁵ cells per well) for 24 h. The cells were then treated with dichloromethane extracts of *P. crispum*, at concentrations of 100–400 μg mL⁻¹ for a further 24 h. The control contained DMSO instead of extract. After pre-treatment, cells were treated with 100 μmol L⁻¹ of H₂O₂ (final concentration in the well) for 60 min on ice to induce DNA damage. Cells were then harvested using a cell scraper, centrifuged and resuspended in 1 mL PBS. The cell suspension (25 μL) was mixed with 75 μL of 0.6% low-melting agarose and the suspension was spread on a frosted microscope slide pre-coated with 250 μL of 0.8% normal melting agarose, covered with a cover slip, and then allowed to solidify on ice for 10 min. The cover slips were removed and the slides were immersed in cold lysis solution containing 1% SDS, 2.5 mol L⁻¹ NaCl, 100 mmol L⁻¹ Na₂ ethylenediaminetetraacetic acid (Na₂EDTA), 1% Triton X-100 and 10% DMSO (with the DMSO added just before use) for 1 h at 4 °C in the dark. The slides were arranged in an electrophoresis tank filled with pre-chilled electrophoretic buffer (1 mmol L⁻¹ Na₂EDTA and 300 mmol L⁻¹ NaOH) and incubated for 20 min. Electrophoresis was conducted in the same buffer at 25 V (300 mAh) for 20 min. The slides were washed with 0.4 mol L⁻¹ Tris–HCl (pH 7.5) and stained with 20 μg mL⁻¹ ethidium bromide for viewing under a fluorescence microscope. The comet tail length was measured using an ocular micrometer. A total of 50 individual cells were screened per slide. The assay was carried out in triplicate. Results were expressed in percent DNA protection, calculated using the following formula:

\[
\text{DNA protection} (%) = \frac{(\text{taillength}_{\text{control}} - \text{taillength}_{\text{treatment}})}{\text{taillength}_{\text{control}}} \times 100
\]

**Scratch motility assay**

The inhibitory effect of *P. crispum* on MCF-7 cell migration was tested using the scratch motility assay. MCF-7 cells (3.5 × 10⁵ cells per well) were seeded in a 24-well plate and grown for 24 h. The confluent cell monolayer was then scratched vertically with a pipette tip, washed twice with PBS and incubated with media containing *P. crispum* dichloromethane extract (0, 200, 300, 400, 500 and 600 μg mL⁻¹) with 5% FBS. H₂O₂ was added into each well at a final concentration of 1 μmol L⁻¹ in the cell suspension to stimulate the proliferation and migration of MCF-7 cells. The number of cells in the denuded area were photographed and counted at 0 and 24 h incubation. The experiment was performed in triplicate. The percentage inhibition was calculated as described by Sato and Rifkin. Percentage inhibition = 100 – ([cell no. in denuded area of sample / cell no. in denuded area of control] × 100).

**Trypan blue dye exclusion assay**

A hemocytometer-based trypan blue dye exclusion cell quantitation and viability assay was used to confirm the antiproliferative activity of *P. crispum*. MCF-7 cells were seeded in a six-well plate (1.5 × 10⁶ cells per well) supplemented with 5% FBS and allowed to grow at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h incubation, the cells were treated with different concentrations of extract (0–800 μg mL⁻¹) for a further 48 h. Diluent-control wells with similar DMSO concentrations as in the treatment were included. Following treatment, the cells were collected using 0.25% trypsin–EDTA, pelleted and resuspended in medium. The cells were then stained with an equal volume of 0.2% (w/v) trypan blue dye solution and the number of viable cells was counted using a hemocytometer under an inverted microscope. All measurements were performed in triplicate.

**DNA fragmentation analysis**

Agarose gel electrophoresis was used to investigate DNA fragmentation in cells treated with *P. crispum*. MCF-7 cells (2 × 10⁶ mL⁻¹) were grown in a 75 cm² culture flask for 24 h. The cells were then treated with *P. crispum* dichloromethane extract (500 or 800 μg mL⁻¹) for 24 or 48 h. After treatment, cells were lysed and the DNA was extracted using TRIzol® reagent according to the manufacturer’s protocol. Extracted DNA was treated with RNase A (3 mg mL⁻¹) at 37 °C for 1 h and protease K (200 μg mL⁻¹) at 50 °C for 2 h. The purified DNA was stored at −20 °C until DNA electrophoresis. The experiment was performed in triplicate. Isolated DNA samples were subjected to electrophoresis in 1.8% (w/v) agarose gel (in TAE buffer) impregnated with 0.5 μg mL⁻¹ ethidium bromide and run at 90 V for 50 min. The gel was observed under UV illumination and visualized using a gel documentation system (UVP, USA).
conditions can influence the extraction of phenolic compounds, such as solvent polarity, extraction procedures and the stage of plant development. Leaves and stems proved to be the highest yield of phenolic compounds. In a study by Luthria et al., they investigated the influence of particles size on phenolic compound extraction of Petroselinum crispum leaves and stems. They reported that phenolic values ranging from 9.63±2.60 to 42.31±0.50 mg GAE g\(^{-1}\) (Table 1). The dichloromethane extract displayed the highest phenolic content (P < 0.05) among the extracts. The nature of the extracting solvent is one of the most important factors in the extraction of antioxidants, thus explaining the various phenolic values from different extracts of P. crispum leaves and stems.

A study by Luthria et al. investigated the influence of particle size on phenolic compound extraction of P. crispum with ethanol:water, 50:50 (v/v), using a pressurized liquid extractor. They reported phenolic values ranging from 18.3 to 22.9 mg GAE g\(^{-1}\). In our study, we obtained a similar result with a particle size of 75 µm, which yielded a phenolic content of 3310.0±80.5 µg mL\(^{-1}\). The dichloromethane extract exhibited the highest FRAP value (0.360±0.009 mmol g\(^{-1}\)). Among the extracts tested, the dichloromethane extract of P. crispum exhibited the highest FRAP value (0.360±0.009 mmol g\(^{-1}\), P < 0.05). Pearson correlation analysis was performed to assess the relationship between phenolic content and ferric reducing activities of the leaf and stem extracts. A statistically significant positive correlation was identified between FRAP and phenolic content of P. crispum (r = 0.875, P < 0.01; Table 2). This indicates that phenolic compounds present in P. crispum contributed to their ferric reducing activities. The reductive ability of the extracts suggests their ability to donate electrons to reduce ferric tripyridyltriazine (Fe\(^{3+}\)-TPTZ) to the ferrous complex (Fe\(^{2+}\)-TPTZ). This implies that P. crispum extracts may provide antioxidative protection from free radicals in actual biological systems by donating electrons to radicals and breaking radical chain reactions from causing diseases related to chronic oxidative stress.

### Ferric reducing antioxidant power

The FRAP values of P. crispum extracts are presented in Table 1. Among the extracts tested, the dichloromethane extract of P. crispum showed antioxidant activities in the β-carotene bleaching assay (EC\(_{50}\) = 5.12 mg mL\(^{-1}\)) and DPPH scavenging assay (EC\(_{50}\) = 80.21 mg mL\(^{-1}\)). The dichloromethane hydrodistilled extract showed an IC\(_{50}\) value of 12.0±0.10 mg mL\(^{-1}\) in the DPPH scavenging assay. In our study, the P. crispum extracts showed better DPPH radical scavenging activity (Table 1) compared to the two studies mentioned above.

### RESULTS AND DISCUSSION

#### Total phenolic content

Phenolics are secondary metabolites that are ubiquitously present in plants. Positive correlations between antioxidant activities present in medicinal plants with their total phenolic content have been reported. In our study, the phenolic values of P. crispum extracts ranged from 87.0±0.009 mg GAE g\(^{-1}\) to 42.7±2.3 (leaf and stem) 4485.0±78.0 (positive control) (Table 1). The dichloromethane extract displayed the highest phenolic content (P < 0.05) among the extracts. The nature of the extracting solvent is one of the most important factors in the extraction of antioxidants, thus explaining the various phenolic values from different extracts of P. crispum leaves and stems.

A study by Luthria et al. investigated the influence of particle size on phenolic compound extraction of P. crispum with ethanol:water, 50:50 (v/v), using a pressurized liquid extractor. They reported phenolic values ranging from 18.3 to 22.9 mg GAE g\(^{-1}\). In our study, we obtained a similar result with a particle size of 75 µm, which yielded a phenolic content of 3310.0±80.5 µg mL\(^{-1}\). The dichloromethane extract exhibited the highest FRAP value (0.360±0.009 mmol g\(^{-1}\)). Among the extracts tested, the dichloromethane extract of P. crispum exhibited the highest FRAP value (0.360±0.009 mmol g\(^{-1}\), P < 0.05).

Pearson correlation analysis was performed to assess the relationship between phenolic content and ferric reducing activities of the leaf and stem extracts. A statistically significant positive correlation was identified between FRAP and phenolic content of P. crispum (r = 0.875, P < 0.01; Table 2). This indicates that phenolic compounds present in P. crispum contributed to their ferric reducing activities. The reductive ability of the extracts suggests their ability to donate electrons to reduce ferric tripyridyltriazine (Fe\(^{3+}\)-TPTZ) to the ferrous complex (Fe\(^{2+}\)-TPTZ). This implies that P. crispum extracts may provide antioxidative protection from free radicals in actual biological systems by donating electrons to radicals and breaking radical chain reactions from causing diseases related to chronic oxidative stress.
Antioxidant, DNA-protective and anticancer properties of Petroselinum crispum

A strong and significant positive correlation was seen between DPPH scavenging activity and phenolic content of P. crispum ($r = 0.910, P < 0.01$; Table 2). This shows that phenolic compounds of P. crispum could be responsible for the observed DPPH radical scavenging activity, since these compounds can readily donate hydrogen atoms to the radical. From the antioxidative study, it is observed that the dichloromethane extract displayed highest phenolic content and FRAP value while exhibiting best DPPH radical scavenging activity among the extracts of P. crispum leaves and stems.

Antiproliferative activity
Extracts of P. crispum (0–500 μg mL$^{-1}$) were tested for their effect on the proliferation of MCF-7, MDA-MB-231 and HT-29 cells, using the MTT assay. Generally, extracts of P. crispum leaves and stems exhibited weak cytotoxic activity with percent inhibitions below 50% (Fig. 1). Among the five extracts analyzed, the dichloromethane extract exhibited the best antiproliferative activity. At the highest concentration tested (500 μg mL$^{-1}$), the dichloromethane extract showed a percentage inhibition of 48.4% ± 1.8%, 25.5% ± 3.0% and 49.9% ± 1.0% on MCF-7, MDA-MB-231 and HT-29 cells, respectively (Fig. 1). The ethyl acetate, methanol and aqueous extracts showed less than 20% inhibition, even at 500 μg mL$^{-1}$ of extract. The different cytotoxic effects of the various extracts in this study suggest the importance of using solvents of differing polarity in order to extract compounds with various polarities that contribute to different biological activities of the plant extract. Each extract of P. crispum behaved differently against the cell lines. The distinct effects of these extracts may be due to the phytodiversity or different mechanisms associated with the compounds present in the extracts and the various susceptibility levels of cell lines to the plant extracts.$^{27,28}$

In a study by Yoshikawa et al.$^{29}$ the methanolic extract from the aerial parts of P. crispum (1 and 10 μg mL$^{-1}$) was shown to have potent estrogenic activity and increased MCF-7 cell proliferation. In our study, the methanol extract did not increase MCF-7 cell proliferation but exhibited a very weak antiproliferative effect on MCF-7 cells. Our study on P. crispum leaves and stems showed that the dichloromethane extract displayed best antioxidant and antiproliferative activities. Hence we selected the dichloromethane extract for further analysis.

DNA protective activity
Within living cells, reactive oxygen species are constantly being generated as normal by-products of mitochondrial respiration. Uncontrolled levels of reactive oxygen species can cause severe damage to macromolecules, especially DNA, leading to degenerative diseases such as cancer.$^{30}$ H$_2$O$_2$ is an oxidizing agent which produces reactive hydroxyl radicals that can induce strand breaks associated with DNA damage.$^{20}$ The comet assay is a quick, simple and sensitive method for the evaluation of DNA damage, mainly single-strand and double-strand breaks in individual cells. The comet tail length is associated with DNA damage. Greater tail length signifies greater DNA damage.$^{19}$

Figure 1. Anti-proliferative activities of Petroselinum crispum extracts on cancer cell lines, MCF-7, MDA-MB-231 and HT-29. Results are presented as means ± SD (n = 3). Values within the same cell line with different letters (a–c) are significantly different at $P < 0.05$ from the different extracts. Extract concentration tested: 0–500 μg mL$^{-1}$.
Table 3. Protection from H2O2-induced DNA damage in 3T3-L1 fibroblasts pre-treated with Petroselinum crispum extract

| P. crispum dichloromethane extract (μg mL⁻¹) | DNA protection (%) |
|---------------------------------------------|--------------------|
| 100                                         | 19.0 ± 6.1*        |
| 200                                         | 23.1 ± 6.9*        |
| 300                                         | 37.9 ± 7.8*        |
| 400                                         | 50.9 ± 6.6*        |

Results are presented as means ± SD (n = 3).

*P < 0.05 compared to control (without extract treatment), as tested by Student’s t-test.

Cells pre-treated with the dichloromethane extract of *P. crispum* at concentrations of 100–400 μg mL⁻¹ showed a significant dose-dependent increase in DNA protection (*P < 0.05*) compared to the control of H2O2 treatment alone (Table 3). At 400 μg mL⁻¹ of extract pretreatment, DNA damage was reduced by 50.9% ± 6.6% compared to the control, indicating 50.9% DNA protection. The high phenolic content in the dichloromethane extract of *P. crispum* as shown in Table 1 may be responsible for the observed DNA protective effect. A study on spices (ginger, caraway, cumin, star anise and fennel) has shown a strong positive correlation between DNA protection and phenols.20 Phenolics in *P. crispum* can lower H2O2 levels or hydroxyl radicals by increasing the levels of H2O2-detoxifying enzymes in cells, thus preventing DNA damage.21 Studies have shown that supplementation of diets with fresh *P. crispum* leaves can increase antioxidant capacity of rat plasma,11 protect against mitochondrial oxidative damage in the mouse brain10 and decrease oxidative stress in humans.12 Our study shows that the *P. crispum* extract protected 3T3-L1 fibroblasts against H2O2-induced DNA damage, suggesting that appropriate addition of the herb in the daily diet might reduce the effects of free radical-induced carcinogenesis, hence affording some protection against cancer.

Inhibition of H2O2-induced MCF-7 cell migration using the scratch motility assay

Metastasis is the most characteristic aspect of malignant neoplasm and is the leading cause of the ineffectiveness of chemotherapeutic drugs and cancer deaths. The scratch motility assay tests the ability of *P. crispum* extracts to inhibit migration of

![Figure 2](image-url)
cancer cells in the denuded area, thus indicating defense against metastasis.\textsuperscript{32} \( \text{H}_2\text{O}_2 \) was included in this experiment to induce the proliferation and migration of MCF-7 cells. The concentration of \( \text{H}_2\text{O}_2 \) (1 \( \mu \text{mol} \text{L}^{-1} \)) used in this assay has been previously tested in our laboratory and showed increased cell migration and proliferation.\textsuperscript{20}

In this study, the scratch motility assay displayed the ability of \textit{P. crispum} to suppress \( \text{H}_2\text{O}_2 \)-induced migration of MCF-7 cells in a denuded area (Fig. 2). Treatment with \( P. \text{crispum} \) extract at \( 300 \mu \text{g mL}^{-1} \) resulted in the highest inhibition of MCF-7 migration (41\% \pm 4\%). At higher concentrations of \( P. \text{crispum} \) extract, the inhibitory effect on cell migration decreased; at the highest concentration tested, \( 600 \mu \text{g mL}^{-1} \), the inhibition of migration was the lowest (18\% \pm 7\%). The dichloromethane extract of \( P. \text{crispum} \) inhibited the migration of MCF-7 cells, but not in a directly proportional manner to the concentration of extract. It is interesting to note that the inhibition of proliferation induced by the extract was highest at the highest concentration of \( 500 \mu \text{g mL}^{-1} \). As cell migration is necessary for metastasis of cancer cells, inhibition of migration is an indication of protection against metastasis. \textit{Petroselinum crispum} prevented migration of MCF-7 cells, thus showing potential in preventing metastasis. The flavonoids present in \( P. \text{crispum} \), apigenin and luteolin,\textsuperscript{9} have been reported as chemopreventive agents of metastasis due to their ability to prevent tumor cell motility and invasion.\textsuperscript{33} Phenolics present in \( P. \text{crispum} \) might lower \( \text{H}_2\text{O}_2 \) levels or hydroxyl radicals by increasing the levels of \( \text{H}_2\text{O}_2 \)-detoxifying enzymes in cells such as glutathione peroxidase,\textsuperscript{11} thus preventing cancer cell proliferation and migration induced by \( \text{H}_2\text{O}_2 \). Antioxidants present in \( P. \text{crispum} \) can maintain \( \text{H}_2\text{O}_2 \) levels in cells within physiological levels and may be associated with the prevention of cancer cell proliferation and migration.

**Trypan blue dye exclusion**

Trypan blue is taken up by dead cells that have lost their membrane permeability barrier or dye exclusion capacity, while the intact plasma membrane of live cells excludes the dye.\textsuperscript{34} To assess the antiproliferative effect of \( P. \text{crispum} \) on MCF-7 cells, trypan blue exclusion counts were conducted on cells treated with 0–800 \( \mu \text{g mL}^{-1} \) of dichloromethane extract for 48 h. A significant dose-dependent decrease in live cell number (\( P < 0.05 \)) was observed in cells treated with the dichloromethane extract of \( P. \text{crispum} \) compared to the control (Fig. 3). This is shown by the lower number of live cells counted as the concentration of dichloromethane extract treatment increased. Using the trypan blue exclusion assay, the percent of viable cells relative to untreated control was 55.04\% \pm 0.75\% at 500 \( \mu \text{g mL}^{-1} \) of dichloromethane extract treatment, whereas at the highest concentration of extract treatment (800 \( \mu \text{g mL}^{-1} \)) cell viability decreased to 30.10\% \pm 1.48\%, indicating the antiproliferative activity of \( P. \text{crispum} \) dichloromethane extract on MCF-7 cells. This antiproliferation profile by trypan blue exclusion assay further confirmed the inhibitory effect of \( P. \text{crispum} \) on MCF-7 cell proliferation analyzed using the MTT assay (Fig. 1).

**DNA fragmentation analysis**

DNA fragmentation is a hallmark of apoptosis. In agarose gel electrophoresis, apoptotic cells demonstrate a characteristic DNA ‘ladder’ pattern at \( \sim200 \) bp intervals, while necrotic cells are observed as a ‘smear’ of randomly degraded DNA.\textsuperscript{35} However, internucleosomal DNA fragmentation is not universal as it may not always occur during apoptosis.\textsuperscript{36}

In this study, apoptotic DNA fragmentation was analyzed using agarose gel electrophoresis. DNA isolated from untreated control cells exhibited one clear band that pointed to the presence of living cells with intact DNA strand (Fig. 4). A typical DNA ladder pattern was not evident in MCF-7 cells treated with dichloromethane extract of \( P. \text{crispum} \) (500 or 800 \( \mu \text{g mL}^{-1} \)) for 24 or 48 h. Instead, a smear pattern of DNA fragmentation was seen in cells treated for 48 h, compared to 24 h. The results could indicate that \( P. \text{crispum} \) dichloromethane extract kills MCF-7 cells by necrosis in a time-dependent manner, where random DNA fragmentation occurs through the release of lysosomal DNases to form a ‘smear’ on agarose gels.\textsuperscript{37} Conversely, studies have reported that MCF-7 cells can undergo apoptosis without showing DNA fragmentation due to lack of caspase-3, which is responsible for this feature.\textsuperscript{38} Thus, using the results from DNA fragmentation analysis...
alone would not be definitive to accurately ascertain the mode of cell death (whether by apoptosis or necrosis, or both), in which *P. crispum* kills MCF-7 cells. Further work will be needed for more in-depth investigation into the mechanism of cell death induced by *P. crispum*.

**CONCLUSIONS**

The dichloromethane extract of *P. crispum* leaves and stems showed antioxidant activities and also inhibition of proliferation and cell migration in MCF-7 cells. The extract also protected against DNA damage induced by H$_2$O$_2$.

Regular addition of *P. crispum* in the daily diet as food or supplements can help strengthen the antioxidant systems of the body and reduce the effects of free radical-induced carcinogenesis, cancer and subsequent metastasis caused by prolonged and excessive oxidative stress.

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