Isolation and Identification of Antimicrobial Furocoumarins from Parsley

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ABSTRACT

Photoactive furocoumarins extracted from four varieties of fresh and freeze-dried parsley leaves inhibited a DNA repair-deficient Escherichia coli in a photobiological assay. Using media-modified assays, the human pathogens E. coli O157:H7 and Listeria monocytogenes, the spoilage microorganism Erwinia carotovora, and Listeria innocua were also inhibited. Pseudomonas fragi was not inhibited. Minimum concentrations of Forest Green parsley powder in agar which showed inhibition ranged from 0.12% to 8.0% depending on the microorganism. Ultraviolet light (UV) at 365 nm for 60 min used to photoactivate the furocoumarins in the bioassay had little effect on L. monocytogenes and L. innocua. A slight UV inhibitory effect was detected with E. carotovora. Furocoumarins, psoralen, 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), oxyypeucedanin and isopimpinellin were identified using gas chromatography-mass spectrometry. Psoralen, 8-MOP, and 5-MOP were quantified. A difference in relative furocoumarin concentration (weight of furocoumarin per weight of dry parsley leaves) for all varieties of parsley was revealed. The concentration of 5-MOP was significantly greater than 8-MOP (P < 0.05), but not significantly greater than psoralen. Psoralen and 8-MOP were not significantly different in concentration.

Key words: Parsley, Petroselinum crispum, furocoumarins, antimicrobial, inhibition

A number of food plants belonging to the Umbelliferae (parsley, parsnip, carrots, dill, and celery) and Rutaceae (grapefruit, limes, and oranges) families produce the phytalexins, furocoumarins. These compounds are synthesized continuously at low levels and at much higher concentrations when plants are stressed by environmental factors, including bacterial and fungal infections, changes in temperature, and exposure to metal ions (2). Furocoumarins are formed via the shikimate and chorismate metabolic pathways. These pathways are commonly used in the formation of some essential amino acids and secondary plant metabolites (10). It has long been known that furocoumarins are capable of inducing phototoxic lesions on human skin exposed to the compounds and ultraviolet light. However, little data is available regarding their toxicity when they are consumed. Humans may have mechanisms to degrade and detoxify furocoumarins, but more data must be obtained to fully understand their human toxicological implications (17).

Although furocoumarins have been tested as antimicrobials against a variety of bacteria and fungi, recent research focused on identifying organisms that were sufficiently sensitive to detect furocoumarins (1, 4). Furocoumarins inhibit microbial growth by reacting with DNA and disrupting DNA replication. This reaction occurs when both the furocoumarin and DNA are exposed to ultraviolet light at 365 nm. DNA monoadducts and interstrand cross-links are formed during the reaction (11, 12, 13, 14). Little work has focused on determining if furocoumarins could possibly be used in food products to protect against foodborne illnesses or to prevent microbial spoilage of food. In this study, varieties of parsley were screened for furocoumarins using a sensitive biological assay for photosensitive molecules (1). The ability of furocoumarins to inhibit the foodborne pathogens and spoilage organisms Listeria monocytogenes, Escherichia coli O157:H7, Erwinia carotovora, Pseudomonas fragi, and Listeria innocua were also tested. The furocoumarin compounds were then identified and quantified using gas chromatography-mass spectrometry procedures.

MATERIALS AND METHODS

Production and harvest

Parsley (Petroselinum crispum) varieties were obtained from Alf Christianson, Mount Vernon, WA, and were selected on the basis of genetic differences and various physical leaf characteristics. These included Dark Green Italian (flat leaf), Evergreen (double curl), Forest Green (triple curl), and Garland (double and triple curl). Seeds were planted on a carrot and parsley farm in Cedar, MN, during the summer of 1992 with soil type and growing conditions typical of Minnesota parsley grown for the fresh market.

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The varieties were planted in late May and were harvested over a 3-week period from the end of August through the 2nd week of September. Dates of harvest were based on plant height (6 to 8 in), as recommended by the grower. Twenty plants including the root were harvested randomly from the rows. Samples were refrigerated (4°C) for a maximum of 24 h after harvest.

Sample preparation

Stems of the parsley were removed approximately 2.5 cm below the leaf of the parsley plant. The leaves were washed with tap water to remove soil and rinsed with deionized water. Excess surface water was removed from the leaves by spinning in a vegetable juicer (ACME Juicer Co., Sierra Madre, CA) without a blade (3,700 rpm for 10 s). Leaves from the 20 parsley plants were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The frozen parsley powder was stored for future use in plastic “zip-lock” freezer bags at -80°C.

Extraction

A 10-g sample of the frozen parsley powder was extracted (swirled for 30 s, let stand for 5 min at 20°C) three times with 50 ml of ethyl acetate (total volume 150 ml) (4). The extract was filtered through Whatman filter paper #4 into an Erlenmeyer flask. The flask was then placed in a 35°C water bath, and the ethyl acetate was removed by evaporation under nitrogen.

Thin-layer chromatography (TLC)

The dried extract was dissolved in 11 ml of methanol. Extract (10 μl) was spotted in the lower right corner of a 20 by 20 cm plastic-backed silica gel 60 plate (#5748-7, E. Merck, Darmstadt, Germany). The plates were developed with chloroform for 15 min at 20°C and then rotated 90°. The plates were developed in a mixture of hexane, pentane, and ethyl acetate (30:30:35, vol/vol) for 15 min at 20°C and allowed to dry for 24 h at 20°C in a hood to ensure that all the solvents had evaporated from the plates and would not interfere with the microbial inhibition analysis. The plates were illuminated with a hand-held 365-nm ultraviolet light (UVP, San Gabriel, CA). Fluorescing spots were circled with a pencil to mark their location.

Bioassay

The bioassay procedure was a modification of a method by Ashwood-Smith et al. (1). Areas of the TLC plate containing the furocoumarins (compared to standards: see below) were cut out and placed silica-side down in square petri plates containing brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI). The BHI plates were equilibrated to room temperature before the furocoumarin plates were added to eliminate variation in diffusion rates due to temperature differences. The position of the circles marking the presence of furocoumarins were marked with a permanent marker on the underside of the petri plate so that any zones of inhibition could be correlated with the presence of furocoumarins. The furocoumarins from the silica gel were allowed to diffuse into the agar for 10 min prior to removal of the TLC plate. The BHI plates were then overlaid with soft agar (15.0 g of Bacto Tryptone, 9.0 g of Bacto Agar, and 5.0 g of potassium chloride per liter), seeded with 10⁶ CFU/ml of E. coli Bs-1, a DNA repair-deficient organism (O. Ceska, Department of Biology, University of Victoria, Victoria, B.C., Canada).

After the soft agar solidified, the plates were exposed to a UV lamp (Spectronics, Westbury, NY) at 365 nm for 60 min at 20°C. The UV source was set 12 in. from the surface of the petri plates. Nonirradiated plates (controls) were covered with aluminum foil and remained at 20°C for 60 min. After exposure, the experimental samples were also wrapped in foil and both experimental and control samples were incubated at 37°C for 24 h. After incubation, the plates were examined for zones of inhibition. The bioassay was repeated in duplicate for all varieties of parsley.

Inhibition of other organisms

The bioassay method was repeated with slight modifications to test the effectiveness of furocoumarins in inhibiting other organisms: Listeria innocua, L. monocytogenes, Scott A, Pseudomonas fragi, Erwinia carotovora subsp. carotovora ATCC 495, and E. coli O157:H7. Cultures were obtained from the American Type Culture Collection or the University of Minnesota culture collection. The base agar was changed from BHI agar to nutrient agar (Difco) for E. carotovora and to tryptic soy agar (TSA) (Difco) for P. fragi, L. innocua, and L. monocytogenes. Changes in media were based on recommendations published by ATCC. In addition, potassium chloride was omitted from the soft agar overlay for L. innocua, L. monocytogenes, P. fragi, E. carotovora, and E. coli O157:H7. Twenty microliters of extract was spotted on the thin-layer chromatography plate instead of 10 μl.

Parsley in agar

Frozen Forest Green parsley powder was incorporated into BHI agar at a concentration of 8.0% (wt/wt) to determine if microbial inhibition could be obtained without purifying the compounds. The agar-parsley powder mixture was steamed for 15 min and autoclaved for 20 min at 121°C. The agar was tempered to 45°C and poured into petri plates in a sterile hood. Care was taken to swirl the flask to ensure that the parsley was evenly distributed into the plates. Approximately 10⁶ CFU of each culture, incubated in the appropriate medium for 24 h at 37°C, was spread plated on the agar-parsley plate. The inoculum was allowed to absorb into the agar before the plates were exposed to UV light at 365 nm for 60 min. After irradiation, the plates were incubated at 37°C for 24 h. Two sets of control plates (parsley with no UV treatment, and UV treatment with no parsley) were prepared in addition to the experimental plates (parsley with UV light). Inhibition was scored subjectively by describing changes in colony growth density or numbers. Experiments were repeated decreasing the concentration to 4.0, 2.0, 1.0, 0.6, 0.4, 0.3, 0.12 and 0.06%. Concentrations immediately above and immediately below the minimum inhibitory concentration were replicated.

 Extraction of furocoumarins from agar

In order to prove that furocoumarins were at least partially responsible for microbial inhibition, it was necessary to extract furocoumarins from the agar. A 500-ml quantity of the soft agar overlay (used in the bioassay) containing 15 g of frozen Forest Green parsley powder was steamed for 20 min and then heated at 121°C for 15 min. The agar was tempered to 45°C, diluted 1:2 with 200°F deionized water and filtered through Whatman #1 filter paper to remove pieces of parsley. The filtrate was extracted with 300 ml of ethyl acetate. The resulting ethyl acetate-water emulsion was centrifuged for 10 min at 4,000 × g. The ethyl acetate layer was separated from the aqueous layer and evaporated to 1 ml. The extract was spread along the bottom of thin-layer chromatography plates (20 by 20 cm, E. Merck silica gel 60 plastic-backed plate) and developed in chloroform for 20 min at room temperature. Fluorescent bands, visible under 365-nm UV light, were scraped off the plates and reextracted in 20 ml of methanol. The silica gel was removed from the extract by centrifugation at 4,000 × g for 6 min. The methanol extract was decanted and evaporated under nitrogen gas to 40 μl. This extract was spotted on another silica gel plate along with the furocoumarin standards 5-methoxyisoporsalen (5-MOP), 8-methoxyisoporsalen (8-
MOP), and psoralen (Sigma Chemical Co., St. Louis, MO). The plate was developed in chloroform for 20 min and allowed to dry. Spots from the diluted agar extract were compared with the furocoumarin standards.

Separation of UV and furocoumarin effects
It was necessary to determine if UV light at 365 nm had an effect on the survival of the microorganisms. Controls without parsley and exposed to UV light were evaluated along with samples containing parsley and exposed to UV light.

Inocula of L. monocytogenes, L. innocua, and E. carotovora were serially diluted from 10^6 to 10^2 in 0.1% peptone and spread plated (0.1 ml) on two sets of plates containing BHI agar and one set of plates containing BHI agar plus 4% Forest Green parsley. The inocula were allowed to be absorbed into the agar at room temperature. The set of plates containing 4.0% Forest Green parsley were irradiated with 365-nm UV light for 60 min at 20°C. One set of control plates were treated with UV under the same conditions. Another set of control plates were not exposed to UV light and remained at room temperature covered with foil. After irradiation, all plates were covered with aluminum foil and incubated for 48 h at 37°C. The tests were performed in duplicate.

Gas chromatography (GC)-mass spectrometry: preliminary extractions
Utilizing information from the separation on the silica gel 60 plates, an attempt was made to identify the spots by modifying the TLC purification procedure described by Christakopoulos et al. (6). Frozen parsley extract was evaporated from 11 ml to approximately 2 ml and streaked along the bottom of several silica-gel plates. One set of plates was developed in chloroform for 15 min at room temperature, yielding two fluorescent bands. The other set of plates, developed in hexane/pentane/ethyl acetate (30:30:35) for 15 min at room temperature, also yielded two fluorescent bands. The bands were scraped off the chloroform and hexane/pentane/ethyl acetate plates with a stainless-steel spatula and placed in separate 50-ml centrifuge tubes along with 20 ml of acetone. The acetone-silica gel mixtures were placed in an ME 4.6 ultrasonicator (Metter Electronic, Pasadena, CA) for 5 min and then centrifuged at 4,000 × g for 6 min to separate the solvent from the silica gel. The acetone extract was decanted and evaporated to 10 μl. The concentrated extract (1 μl) was injected into a Kratos MS25 gas chromatograph-mass spectrometer (Kratos Analytical, Ramsey, NJ). The GC-MS interface was kept at 265°C. The compounds were separated on a DB1 column (J&W Scientific, Folsom, CA) (length 30 m, inside diameter 0.32 mm, 0.25-μm coating) using helium gas as the carrier. The GC-MS was temperature programmed from 60 to 280°C at a rate of 10°C/min and remained at 280°C for an additional 10 min to remove any residual compounds. The samples in duplicate were injected in the splitless mode and the injector was purged 30 s into each run. Samples were injected in duplicate.

GC-mass spectrometry large extractions
A larger quantity of freeze-dried (lyophilized) parsley extract was also analyzed using gas chromatography-mass spectrometry. In preparation for freeze drying, the leaves of the parsley varieties were rinsed with distilled deionized water, blotted dry, and stored at −20°C. The day before freeze drying, the parsley was placed at −62°C. Each variety was lyophilized at −40°C in a Virtis Freeze Mobile 25 freeze dryer (Virtis Co., Gardiner, NY). A vacuum of no less than 200 millitorr (ca. 26.6 Pa) was used. During freeze drying, the parsley was stored in 600-ml and 1,200-ml freeze-drying bottles. Initial weights were recorded and the bottles were weighed periodically until the weights no longer changed, indicating completion of dehydration. Total freeze-drying time was approximately 24 h.

Garland (100 g), Dark Green Italian (138.06 g), Evergreen (76.4 g), and Forest Green (121.87 g) freeze-dried parsley were extracted separately with two 2-liter batches of ethyl acetate over 2 days. The ethyl acetate extract was evaporated to dryness and dissolved in 1,250 ml of methanol. Water (2,083 ml) was added to create a 60% (vol/vol) water/methanol solution. Each water/methanol solution was extracted with a total of 4 liters of hexane to remove oils and waxes (5). The 4 liters of hexane were added 1 liter at a time to the flasks, shaken vigorously, and allowed to sit for 15 min. The hexane was removed and the water/methanol solution was evaporated under gaseous N₂ to remove the methanol from the solution. The remaining aqueous solution was reextracted twice with 1 liter of ethyl acetate (total volume, 2 liters of ethyl acetate). The ethyl acetate extracts were evaporated in a 35°C water bath under gaseous N₂ to a final volume of 4.0 ml for Garland, 6.5 ml for Dark Green Italian, 4.0 ml for Evergreen, and 4.2 ml for Forest Green parsley. A 1-μl portion of each of the concentrated parsley extracts was injected into the gas chromatograph-mass spectrometer under the same conditions as samples prepared in the preliminary extractions. Psoralen (0.0020 g), 8-MOP (0.0044 g), and 5-MOP (0.0046 g) standards were prepared separately in 10 ml of each of ethyl acetate and injected in the gas chromatograph-mass spectrometer prior to the injection of samples. Standards were injected each day that the samples were run to account for day-to-day fluctuations in gas chromatograph-mass spectrometer sensitivity. Mass spectra and chromatography data were collected. Psoralen, 8-MOP, and 5-MOP were quantified by comparing chromatograph peak heights with the peak heights of the standards.

Statistical analysis
An analysis of variance, using the MacAnova statistical program (15), was conducted. Utilizing the quantification data from the GC-mass spectrometry large extractions, duplicate measurements of all furocoumarin for the four parsley varieties were analyzed.

RESULTS AND DISCUSSION

Bioassay
All varieties of parsley exhibited antimicrobial activity against the screening organism, E. coli Bs-1. During thin-layer chromatography, three fluorescent spots were detected for all parsley varieties. Rf values are presented in Table 1. Inhibition of E. coli Bs-1 occurred at all three spots. Inhibition was defined as a zone of clearing greater than 0.5 cm in diameter. Spot 2 generally resulted in a larger zone of inhibition (1.0 to 1.5 cm in diameter) in the bioassay. Spots 1

<table>
<thead>
<tr>
<th>Spot</th>
<th>Chloroform</th>
<th>Hexane/pentane/ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09 ± 0.07</td>
<td>0.42 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.20 ± 0.09</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.24 ± 0.11</td>
<td>0.74 ± 0.08</td>
</tr>
</tbody>
</table>
and 3 produced zones with diameters between 0.5 and 1.0 cm.

**Inhibition of other organisms**

When the bioassay was modified to test furocoumarin antimicrobial activity against other organisms, inhibition zone(s) greater than 0.5 cm in diameter were observed for *E. carotovora*, *L. innocua*, *L. monocytogenes*, and *E. coli* O157:H7. Only one inhibition zone, at spot 2, was obtained when testing gram-negative *E. coli* O157:H7. Spots 1 and 3 did not inhibit the cells as effectively (data not shown). No inhibition was demonstrated for the gram-negative *P. fragi*.

Generally, gram-negative organisms are more resistant to hydrophobic antimicrobial substances than are gram-positive organisms such as *Listeria* spp. This result may be explained by the difference in structure between gram-positive and gram-negative microorganisms. The outer cell membrane of gram-negative microorganisms has several lipid-containing compounds, e.g., lipopolysaccharides. These compounds have the potential for screening out or possibly delaying the adverse effect of hydrophobic antimicrobial agents on the cell membrane (7, 8). Because gram-positive microorganisms have no outer cell membrane, they have a tendency to be more susceptible to hydrophobic antimicrobial agents. The hydrophobic furocoumarins were probably retained in the outer cell membrane of *P. fragi* to a greater extent than in that of the gram-positive organisms and did not diffuse well into the cell to react with the DNA.

**Parsley in agar**

Minimum inhibitory concentrations (MICs) of Forest Green parsley were 1.0, 1.0, 8.0, 0.12 and 0.12% for *L. innocua*, *L. monocytogenes*, *E. coli* O157:H7, *E. carotovora*, and *E. coli* Bs-1, respectively. At the MIC, experimental plates had large areas of inhibition with no colony formation. Isolated colonies were present between the areas. Microbial inhibition on experimental plates was differentiated from controls by irregular areas of clearing indicating a visible furocoumarin effect. The colonies were too numerous to count and had nonuniform growth. Control samples had dense lawns of growth. Inhibition zones were reduced or disappeared when the parsley concentration was reduced to 4.0% for *E. coli* O157:H7, 0.6% for *L. monocytogenes* and *L. innocua*, and 0.04% for *E. coli* Bs-1 and *E. carotovora*. While these results could not be compared statistically because they could not be quantified, they do indicate a possible link between the parsley in agar and microbial inhibition.

**Extraction of furocoumarins from agar**

Spots from the diluted agar extract and from the furocoumarin standards (8-MOP, 5-MOP, and psoralen) moved equal distances on the TLC plate. It can be concluded that the fluorescent compounds identified in the bioassay are the fluorescent compounds in the agar. This does not prove that there is not another compound in the parsley contributing to the antimicrobial properties, but the results indicate that the furocoumarins are likely an inhibitor.

**Separation of UV and furocoumarin effects**

UV light at 365 nm should have little or no antimicrobial effect (18). This was demonstrated for *L. monocytogenes* and *L. innocua* since the number of colonies on plates exposed and not exposed to UV light were similar, $1.8 \times 10^5$ to $2.6 \times 10^5$ and $2.5 \times 10^4$ to $3.1 \times 10^4$ CFU per plate, respectively. However, a lawn of growth on the controls does not rule out the possibility that UV light alone may have some inhibitory effect. UV light at 365 nm did seem to have an inhibitory effect on *Erwinia carotovora* with a reduction of $8.9 \times 10^4$ to $3.5 \times 10^3$ with exposure.

**GC-mass spectrometry preliminary extractions**

Spot 1 was identified as oxypeucedanin (m/z: 286, 202, 174, 145, 85). Spot 2 was identified as a combination of compounds: 8-MOP (m/z: 216, 201, 188, 173, 145), 5-MOP (m/z: 216, 201, 188, 173, 145), and psoralen (m/z: 186, 158, 130, 102). The presence of more than one compound in spot 2 may explain why it generally yielded a larger zone of clearing in the bioassay. These compounds have been isolated previously by Ceska et al. (4). Unlike the results of Ceska et al. (4), oxypeucedanin was isolated from the variety Dark Green Italian, a flat leaf parsley. This compound was not previously identified in flat leaf parsley. Standards were run to identify 8-MOP, 5-MOP, and psoralen. Mass spectra of 5-MOP and 8-MOP appear to be identical and they elute from the column within seconds of each other. Spot 3 could not be identified using this method. It was suspected that the compound(s) which made up this third spot were present at a concentration too low for identification.

**GC-mass spectrometry large extractions**

Larger samples were extracted to roughly quantify the furocoumarin compounds and compare values with those found in other studies. Again, oxypeucedanin, 8-MOP, 5-MOP, and psoralen were identified in all the varieties of parsley along with another compound, isopimpinellin (m/z: 246, 231, 203, 188, 175, 160, 147). This suggests that isopimpinellin is spot 3. Isopimpinellin has also been previously identified in varieties of parsley (4). Furocoumarin concentrations (Table 2) were higher than $1.62 \pm 1.61$, $2.29 \pm 0.63$ and $6.59 \pm 1.81 \mu g/g$ of dry commercial flakes for psoralen, 8-MOP, and 5-MOP, respectively, reported by Chaudhry et al. (5). Their preparative purification and quantitative methods utilized high-performance liquid chromatography (HPLC). Variety, drying method, and storage conditions were not indicated. Greater concentrations in our research may have been retained due to few purification steps, direct harvesting from the field, and immediate freezing in liquid nitrogen. Bier et al. (3) recently reported the concentration of psoralen, 8-MOP, and 5-MOP in four commercially dried parsley flakes ranging from 30.5 to 108.9, 2.4 to 58.3, and 50.2 to 166.1 µg/g, respectively, as analyzed by HPLC. Psoralen has been described as a transient precursor of the other two compounds and its levels vary with the stage of biosynthesis interrupted at harvest (5).

Innocenti (9), who used one-dimensional TLC and spectrophotometric analysis of the eluted bands, observed...
TABLE 2. Gas chromatography-mass spectrometry quantification of furocoumarins of four parsley varieties from large extraction using ethyl acetate

<table>
<thead>
<tr>
<th>Furocoumarin (µg/g of dry parsley flakes)</th>
<th>Garlic</th>
<th>8-Methoxypsoralen</th>
<th>5-Methoxypsoralen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsley variety</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Garland</td>
<td>52.72</td>
<td>9.60</td>
<td>479.20</td>
</tr>
<tr>
<td>Dark Green Italian</td>
<td>7.01</td>
<td>3.85</td>
<td>71.75</td>
</tr>
<tr>
<td>Evergreen</td>
<td>16.54</td>
<td>3.19</td>
<td>33.33</td>
</tr>
<tr>
<td>Forest Green</td>
<td>5.75</td>
<td>3.24</td>
<td>124.75</td>
</tr>
<tr>
<td></td>
<td>4.32</td>
<td>2.52</td>
<td>43.82</td>
</tr>
</tbody>
</table>

*Values from duplicate injections are presented. Oxypeucedanin and isopimpinellin were identified but concentrations could not be calculated as pure standards were not available.

seasonal variation in furocoumarin concentration. Levels of 5-MOP were the greatest between July and September, 169 to 200 µg/g (dry weight) and declining in October to 81.5 µg/g. Rodighiero and Allegri (16) used similar methods and reported levels of 5-MOP in dried parsley which ranged from 90 µg/g in the winter months to 990 µg/g during the summer months.

The concentrations of furocoumarins determined in our research are within the reported ranges. They were greater than those of Chaudhary et al. (5) and less than those of Bier et al. (3) and other research cited. Other factors such as different growing conditions, infestation, varieties, times of harvest, and analytical methods may also have contributed to differences in furocoumarin concentrations among values reported.

In our study variances in furocoumarin concentrations increased as the concentration increased. Therefore, a transformation of the raw data was required. Taking the log of the data provided a residual plot that was structureless. Peak trailing may have contributed to the unequal variance. Peak trailing is the phenomenon in which the compound does not elute all at once, failing to yield a sharp, definitive peak on the gas chromatogram. The compound continues to elute after the majority of it has passed the detector. trailing peaks can cause variability because the trailing portion does not contribute to the peak height (what was measured in calculating concentrations). This may have been the major factor causing the differences between duplicate determinations. Background noise from the equipment is another source of variability. Often noise increases proportionally with the concentration measured.

There was a statistically significant difference in the relative concentration of furocoumarins for all varieties (*P* < 0.01). 5-MOP was significantly more abundant than 8-MOP. However, psoralen and 8-MOP were not significantly different in concentration. Also, 5-MOP was not statistically more abundant than psoralen. These concentration trends are generally consistent for the data reported by Chaudhary et al. (5) and Bier et al. (3), especially for the relationship between the concentration of 5-MOP (greater) and 8-MOP (less).

Minimum inhibitory concentrations of furocoumarins from parsley in agar

Approximate concentrations (ng/g) of furocoumarins in BHI agar were calculated from gas chromatography-mass spectrometry data from the large extraction experiment (Table 3). Averages of the duplicates of each Forest Green furocoumarin were multiplied by the weight of parsley powder (converted to dry basis) in 15 g of BHI agar per plate. This number was divided by 15 to calculate the weight per gram of agar. The results of these calculations may only be considered as approximate and require more precise measurements for confirmation. However, in this BHI agar medium, the inhibitory concentration of the total of the three furocoumarins measured ranged from 13.9 to 923 ng/g depending on the bacteria. Extremely low concentrations of furanocoumarins were effective in this specific system.

Conclusions

All varieties of parsley contained photoactive furcoumarins which inhibited the growth of *E. coli* BS-1, *E. coli* O157:H7, *E. carotovora*, *L. monocytogenes*, and *L. innocua* using the bioassay method.

Statistically significant differences in concentrations of the furocoumarins from variety to variety could not be drawn from this study (*P* = 0.632). Further furcoumarin analyses replicated over several growing seasons would need to be performed before measured differences in furcoumarin production between varieties would be meaningful. Although the plants were grown in the same field, and attempts were made to limit variation in exposure to environmental stresses, it is possible that one or several varieties could have been exposed to more sun, moisture, fertilizer, or stress-inducing herbivorous animals than others which in turn affected furocoumarin synthesis. Concentrations of the furocoumarins also varied from report to report in the literature, likely due to seasonal, environmental and analytical differences. The consistency of identifying these compounds in parsley and the possibility that concentrations of furcoumarins could be optimized by controlling environmental conditions or genetic material are more important for future work than the uniformity of the concentrations among research reports. Reduction of variability among duplicate

Minimum inhibitory concentrations of furocoumarins from parsley in BHI agar on five strains of bacteria

<table>
<thead>
<tr>
<th>Furocoumarin</th>
<th><em>L. innocua</em></th>
<th><em>L. monocytogenes</em></th>
<th><em>E. coli</em> O157:H7</th>
<th><em>E. carotovora</em></th>
<th><em>E. coli</em> BS-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Methoxypsoralen</td>
<td>101</td>
<td>101</td>
<td>843</td>
<td>12.7</td>
<td>12.7</td>
</tr>
<tr>
<td>8-Methoxypsoralen</td>
<td>3.5</td>
<td>3.5</td>
<td>29</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>Psoralen</td>
<td>6.1</td>
<td>6.1</td>
<td>51</td>
<td>0.80</td>
<td>0.80</td>
</tr>
</tbody>
</table>

*Calculated from the lowest concentration to produce a clear area of inhibition on plates of BHI agar incubated at 37°C for 24 h. Average values of duplicate injections in the gas chromatography/mass spectrometry analysis were used.*

TABLE 3. Approximate minimum inhibitory concentrations of furocoumarins from Forest Green parsley in BHI agar on five strains of bacteria
GC-mass spectrometry analyses requires modification of the method used.

Results from this study seem to suggest photoactivated furocoumarins from parsley may inhibit some pathogenic and spoilage bacteria in food systems. Current research in this laboratory is confirming the microbial inhibition and effective concentrations of these compounds from additional Umbelliferae and Rutaceae in a model food system.

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