Novel *Mentha spicata* Clones with Enhanced Rosmarinic Acid and Antioxidant Activity

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**Abstract**

Phenolic compounds are a diverse class of secondary metabolites that exhibit antioxidant activity. Mints contain moderate levels of these compounds, and a number of *Mentha spicata* plant clones, biochemically selected in vitro for elevated phenolics, were characterized for phenolic acid composition, rosmarinic acid content and antioxidant activity. Total phenolics of mints grown under field conditions ranged from 19 mg/g DW to 67 mg/g DW, which is twice the concentration found in the controls. Levels of rosmarinic acid, a potent and effective HIV-1 integrase and reverse transcriptase inhibitor, were as high as 122 mg/g DW, greater than twice the amount present in control plants. A significant correlation was found between elevated phenolics and antioxidant activity, and a direct linear correlation between rosmarinic acid and free radical quenching. Commercially, these high-output antioxidant mint clones potentially can provide an effective, low cost source of natural antioxidants for medicinal purposes. Since these fast growing, perennial spearmint genotypes are easy to cultivate, can sustain several harvests annually, and generate high levels of rosmarinic acid, they could deliver a cost-effective, sustainable source of a powerful HIV drug for emerging nations.

**INTRODUCTION**

Oxidation in biological organisms is a normal consequence of metabolism, resulting in formation of detrimental free radical by-products and reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and singlet oxygen. These chemical species induce oxidative stress, which occurs when the body is unable to contain the excess ROS by its cellular antioxidant enzyme systems and extracellular antioxidant compounds. Left uncompensated, the ROS possess an unpaired electron that attacks DNA, lipids in cell membranes or structural and functional proteins. Therefore, oxidative damage plays a key role in human cardiovascular disease, cancer initiation, aging, inflammatory diseases and neurological disorders (Lampe, 1999).

Plants have established various self-defense mechanisms to protect metabolic functions against harmful free radicals generated by oxidation. Several groups of secondary metabolites in higher plants have the capacity to react as antioxidants, and a primary group of antioxidants is the phenolics (Robards et al., 1999). Rosmarinic acid is a potent phenolic antioxidant occurring in the Mint family, Lamiaceae (Fig. 1). This compound has unique properties, having antiviral (HIV-1), antibacterial, and anti-inflammatory activities (Mazumder et al., 1997; Szabo et al., 1999; Hooker et al., 2001).

*Mentha spicata* (spearmint) is a fast growing perennial crop in the Mint family that biosynthesizes significant amounts of rosmarinic and phenolic acids. Clonal lines of spearmint specifically selected for superior rosmarinic and phenolic levels could provide an inexpensive source of these potent antioxidants. Biochemical characterization of these clonal lines would identify environmentally stable overproducers of these antioxidants for potential commercial and medical uses.
MATERIALS AND METHODS

Plant Material
Spearmint clones (n = 44) were obtained from Burpee Seed, Warminster, PA (coded as MSH) and Lake Valley Seed, Arapahoe Boulder, CO (coded as HMS). Plant clones selected for high phenolics were performed via tissue culture using either proline or Pseudomonas as the selection agent (Kwok and Shetty, 1998). Tissue culture explants were transferred to soil and propagated plants were transferred to small plots (1 x 1.5 m) at the University of Guelph Research Station, Arkell, Ontario. Plants were harvested five times: July, August, and October 2000; July and August 2001. Dried leaves were separated from stems and homogenized in a Proctor-Silex mini grinder for 30 seconds and stored in glass vials.

Extraction Methods
Phenolics were extracted by placing dried sample (10 mg) in 2.5 mL 95% ethanol and placed in the dark at 4°C for 48-72 h. The extracts were centrifuged at 12,000 xg and the supernatant stored at 4°C for phenolic analysis as required. For rosmarinic acid, plant tissue was extracted with 50% methanol/water in a water bath at 55°C for 2.5 h. Samples were centrifuged at 12,000 xg and stored at 4°C until analysis.

Total Phenolics Assay
Total phenolics were determined as described by Chandler and Dodds (1983). Briefly, extracts (1 mL) were diluted with water/ethanol (1:5) and mixed with Folins reagent (0.5 mL) before the addition of 5% potassium carbonate (1 mL). Samples were placed in the dark for 1 h and centrifuged (2 min, 4,500 xg) to remove particulates. Absorbance was recorded on a Beckmann double beam spectrophotometer at 725 nm. A standard gallic acid curve was used to calculate the concentrations of the extracts. All samples and standards were performed in triplicate.

Total Rosmarinic Acid Assay
Following the procedure of Yang and Shetty (1998), the ethanolic extract (1 mL) was placed in a clean test tube and diluted 10 fold with 50% aqueous methanol. The sample was transferred directly to a cuvette and the absorbance read at 333 nm. The concentration of rosmarinic acid was based on the following equation: 
\[ C = \frac{A}{\varepsilon \times d} \]
where, \( C \) = concentration, \( A \) = absorbance, \( \varepsilon \) = extinction coefficient of Rosmarinic acid (19 000 M\(^{-1}\) cm\(^{-1}\)), and \( d \) = dilution factor. All samples were run in triplicate.

Antioxidant Activity: DPPH Radical Scavenging Assay
The radical scavenging activity was measured by monitoring the reduction of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) in the presence of the spearmint extracts. Briefly, a DPPH solution (1.0 x 10\(^{-4}\) M in ethanol) was prepared 24 h prior to each experiment. The DPPH free radical solution (240 µL) was placed into a 96 well microtiter plate and the sample extracts (10 µL) were added to the free radical solution. The plate was shaken gently to ensure mixing and then placed in a Wallace Victor 1,420 multilabel counter (microplate reader) set at 37°C and 490 nm. The disappearance of the DPPH free radical was monitored over a 15 min period and samples were measured in triplicate. Gallic and rosmarinic acids were used as standards. The radical scavenging activity was expressed as a percent quench of the DPPH radical, calculated by 
\[ \left(\frac{T_0 - T_{15}}{T_0}\right) \times 100 \]

RESULTS AND DISCUSSION

Total Phenolic Content of Selected Spearmints
The highest levels of phenolics in the 44 spearmint clones examined ranged from 51 to 73 mg/g DW depending on the harvest date. In general, the August harvests showed
the highest mean production of phenolics across all clones, and showed a significant
difference between spearmint lines at the different harvest dates. This variation can
largely be attributed to genotypes and environmental influences (Skoula et al., 2000;
McAuley, 2002). Overall, ten spearmint clones were identified with consistently high
phenolic production over the 5 sampling periods (Table 1).

The phenolics concentrations are higher than those reported for culinary herbs
such as *Thymus vulgar* and *Rosmarinus officinalis* (1-7 mg/g DW), and berries such as
crowberry (50.8 mg/g DW), strawberries and blueberries (0.9 to 4.7 mg/g FW)
(Kahkonen et al., 2001; Kalt et al., 1999). High levels of phenolics in spearmint clones
are important indicators, since phenolic levels have been directly correlated with high
antioxidant potential, making these clones prime candidates for high antioxidant capacity.

**Rosmarinic Acid Content of Mentha spicata Clones**

All the 44 *M. spicata* clones were assessed for rosmarinic acid content, an
important antioxidant, antibacterial, and antiviral agent. Clones MSH-05 and MSH-2
showed high overall rosmarinic acid levels accompanied by significant variability at
harvest dates, whereas clones MSH-06 and MSH-27 showed high levels, but relatively
low variability (Table 2). The high content and low seasonal variability of MSH-06 and
MSH-27 makes these clones ideal for commercial production of rosmarinic acid.

Romasinic acid content varied with the time of harvest. Analyses showed that
rosmarinic acid content was higher in all spearmint clones harvested in either July or
August with a decrease in the tissues collected in October (Table 2). A direct linear
correlation ($r = 0.97$) was found between decreasing levels of rosmarinic acid and
decreasing temperature. The decrease in rosmarinic acid was not evident in the total
phenolics analyses where levels of total phenolics remained fairly constant (Table 1). This
effect could be related to the response of plant metabolism to cold temperatures. Exposure
to cold temperatures is known to enhance phenolic metabolism at the earliest synthesized
phenolics so as to make available these compounds for protective lignin and suberin
biosynthesis (Solecka and Kacperska, 1995). Cold temperatures would divert caffeic acid
from rosmarinic acid biosynthesis, lowering the levels in tissues, which may explain the
decrease in rosmarinic acid observed in the October harvest.

Romasinic acid is a known HIV-1 reverse transcriptase inhibitor (IC$_{50} = 90 \mu$M,
NERT assays, Hooker et al., 2001) and is one of the few compounds that can effectively
inhibit HIV-1 integrase (IC$_{50} = 5 \mu$M, Mazumder et al., 1997). Therefore, extracts of
these spearmint clones could potentially serve as new, low cost, multi-target AIDS
therapy.

**Antioxidant Capacity of Spearmint Clones**

The antioxidant capacity of the spearmint extracts was calculated on the ability of
the extracts to quench the stable free radical DPPH. High concentrations of antioxidants
in all 44 clones had an excellent capacity to quench free radicals, resulting in a total
quench of the DPPH radical in some extracts in vitro.

Analyses of lower concentrations of the extracts in the DPPH assay (0.2-5 \(\mu\)g
gallic acid equivalents) showed variability in the effectiveness to quench the free radical.
A positive correlation existed between total phenolic content and antioxidant capacity ($r =
0.92$). Similarly, a direct correlation was found between rosmarinic acid content and the
antioxidant capacity ($r = 0.80$). This indicates that the total components of the spearmint
extract may act synergistically as an antioxidant.

To prove this synergistic potential, quench curves were generated for three of the
top spearmint clones that had both high phenolics and rosmarinic acid content. A direct
comparison of the quench curves of pure rosmarinic acid with the clonal lines MSH-11,
MSH-13 and MSH-14 clearly showed a superior quench curve for the spearmint extracts
(Fig. 2). If the observed antioxidant capacity was derived only from the rosmarinic acid
content, the curves would be expected to be similar, however, the extracts are clearly
superior, indicating strong synergism among the components in the extracts.
The phenolics that contribute to this synergistic antioxidant capacity in the spearmint extracts remains unknown, however, current HPLC profiling of the extracts in our laboratory may lead to the elucidation of key components responsible for superior antioxidant activity in these novel spearmint clones.

**CONCLUSIONS**

Genetically fixed spearmint clones have been identified to deliver high levels of rosmarinic acid and total phenolics. The bio-production of rosmarinic acid is dependent on temperature whereas total phenolic content was not affected indicating that cold temperatures affect phenylpropanoid metabolism. The antioxidant capacity of the spearmint clonal extracts tested is superior to rosmarinic or gallic acid, a synergistic interaction of the phenolics occurs to increase antioxidant capacity. The high levels of rosmarinic acid make these clones an excellent source of a known naturally occurring multi-target HIV-1 inhibitor, and could provide an economical source of a potent alternative antiviral drug for emerging nations.

**Literature Cited**


Tables

Table 1. Total phenolic content of ten best spearmint (*Mentha spicata*) clones over 5 field harvest dates 2000-2001. Total phenolics are expressed as mean ± standard deviation in mg/g dry weight.

<table>
<thead>
<tr>
<th><em>Mentha spicata</em> Clone</th>
<th>Phenolics (mg/g DW) at five harvest dates</th>
<th>Mean Phenolic level (mg/g dry weight)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Jul-00</td>
<td>Aug-00</td>
</tr>
<tr>
<td>MSH-14</td>
<td>53.22</td>
<td>66.44</td>
</tr>
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<td>MSH-13</td>
<td>61.75</td>
<td>53.95</td>
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<td>MSH-05</td>
<td>47.54</td>
<td>50.71</td>
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<td>MSH-02</td>
<td>43.78</td>
<td>49.12</td>
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<td>MSH-24</td>
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<tr>
<td>MSH-03</td>
<td>41.73</td>
<td>45.88</td>
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</table>

Table 2. Variation of rosmarinic acid content (mg/g dry weight) among the top 10 spearmint (*Mentha spicata*) clones at 5 field harvest dates 2000-2001.

<table>
<thead>
<tr>
<th>Spearmint clone</th>
<th>Rosmarinic acid (mg/g DW) at five harvests</th>
<th>Mean rosmarinic acid (mg/g dry weight)</th>
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<tr>
<td></td>
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<td>MSH-05</td>
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<tr>
<td>MSH-03</td>
<td>51.58</td>
<td>78.00</td>
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</tbody>
</table>
Figures

Fig. 1. Structure of rosmarinic acid.

Fig. 2. Quench curves for pure rosmarinic acid and three spearmint (Mentha spicata) clones, MSH-11, MSH-13 and MSH-14. The curves indicate that the antioxidant capacity of the spearmint extracts is far superior to that of pure rosmarinic acid. For a direct comparison quench curves for the spearmints are reported in rosmarinic acid equivalents.