Isolation of Allelopathic Substances in Lemon Balm Shoots

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To clarify the allelopathic system of lemon balm (Melissa officinalis L.), purification and isolation of allelopathic substances was undertaken in acetone extract of the shoots of the 30-d-old plants. Three growth-inhibiting substances were found in the neutral fraction of the extract after silica gel column chromatography, and most active substance was further purified and isolated by thin layer chromatography, C18 Sep Pack cartridge and reverse-phase high performance liquid chromatography. This substance inhibited the growth of cress (Lepidium sativum L.) at concentrations greater than 0.3 μg mL⁻¹ and the concentration required for 50% growth inhibition was 2.9 μg mL⁻¹. These results suggest that lemon balm shoots may contain at least three growth-inhibiting substances, which may be released into the environment, either as exudates from living tissues or leachates from residues of the plant, and may act as allelochemicals to neighboring plants.

Keywords: allelopathy, growth inhibitor, Lepidium sativum, Melissa officinalis, weed control

INTRODUCTION

Increasing use of commercial herbicide for weed control during the last 50 years has resulted in serious ecological and environmental problems. Public awareness and demand for environmentally safer herbicides with less persistence and less contaminating potential make searches for new weed control strategies (Olofsdotter et al., 1995; Einhellig, 1996; Seigler, 1996; Narwal, 1999).

Plants produce a hundred of secondary compounds. Many of these compounds are phytotoxic and have potential as herbicides or templates for new herbicide classes (Duke, 1986; Putnam, 1988; Gross and Parthier, 1994; Inderjit, 1996). Only a fraction of these compounds have been evaluated their herbicidal or bioregulator activity (Dodge, 1987; Einhellig and Leather, 1988).

Although much research has been conducted to evaluate the pharmacological effects of lemon balm (e.g. Van den Berg et al., 1997; Tagashira and Ohtake, 1998), information about the allelopathic system of the plants is limited. Recently, the possible allelopathic potential of an aqueous acetone extract obtained from shoots of lemon balm was reported (Kato-Noguchi, 2001). The objective of this research was isolation of allelopathic substances in the acetone extract of the lemon balm shoots.
MATERIALS AND METHODS

Plant material and extraction. Shoots of 30-d-old lemon balm (Melissa officinalis L.) were washed thoroughly with tap water and rinsed with distilled water. After blotting dry with filter paper (No 1; Toyo Ltd., Tokyo), the shoots (1 kg fresh weight) were homogenized with 5 L of 70% (v/v) cold aqueous acetone and the homogenate was filtered through filter paper (No. 1). The residue was homogenized again with 5 L of 50% (v/v) cold aqueous acetone and filtered. The two filtrates were combined and evaporated in vacuo at 35°C to give an aqueous residue. The residue was adjusted to pH 7.0 with 1 M phosphate buffer and partitioned three times with an equal volume of ethyl acetate. After drying over anhydrous Na2SO4, the ethyl acetate phase was evaporated to dryness in vacuo at 35°C (neutral fraction).

Purification of inhibitor. The neutral fraction was chromatographed on a column (2 × 60 cm) of silica gel (100 g, Silicagel 60, 70-230 mesh ; Merck, Darmstadt, Germany), and eluted stepwise with n-hexane that contained increasing amounts of ethyl acetate (10% per step, v/v ; 200 mL step−1). After the eluting solvent reached ethyl acetate, the elution was completed with 200 mL methanol. The biological activity of the fractions was determined using a cress bioassay as described below. After evaporation of the active fractions eluted from the silica gel column, the residue was applied as a strip to a thin layer chromatography (TLC) plate (Silicagel 60 GF254 ; Merck), and the plates was developed with a mixture of chloroform and acetic acid (95 : 5, v/v) for 15 cm. Then, ten equal segments of chromatogram were scraped off and eluted with a mixture of ethyl acetate and methanol (1 : 1, v/v). After evaporation of the active fractions, the residue was dissolved in 20% aqueous methanol (2 mL, v/v) and loaded onto reverse-phase C18 Sep-Pak cartridges (Waters Co., Milford, MA, USA). The cartridge was eluted with 20, 40, 60 and 80% aqueous methanol and methanol (20 mL each). After evaporation of the active fraction, the residue was finally purified by reverse-phase high performance liquid chromatography (HPLC) (0.8 cm i.d. × 30 cm; μBondasphere C18; Waters; eluted at a flow rate of 2 mL min−1 with 30% aqueous methanol, detected at 240 nm).

Bioassay. Each fraction from the chromatography described above was evaporated to dryness, dissolved in a small volume of a mixture of ethyl acetate and methanol, transferred onto a sheet of filter paper (No. 2 ; Toyo) in a 3 cm Petri dish and dried. The filter paper in the Petri dish was moistened with 1 mL of 3 mM potassium-citrate buffer (pH 7.0) containing 0.05% (v/v) Tween 20, and 10 seeds of cress (Lepidium sativum L.) were sown on the filter paper, and allowed to germinate and grown in the dark at 25°C as described by Kato-Noguchi (2000). Control seedlings were treated with plain solution without extracts. After 40 h, the root length of the seedlings was measured.

RESULTS AND DISCUSSION

The neutral fraction (3.8 g) of an acetone extract obtained from 30-d-old lemon balm shoots (1 kg fresh weight) was subjected to a chromatography on silica gel, and the biological activity of the eluted fractions was evaluated by the cress bioassay (Fig. 1). Inhibitory activity was detected in fractions 5 to 7 (elution with 40–60% ethyl acetate in n-hexane), fractions 9 to 10 (elution with 80–90% ethyl acetate in n-hexane), and fraction 12 (elution with methanol), respectively. However, the activity in fractions 5 to 7 was much greater than those in fractions 9 to 10, and fraction 10.

Fractions 5 to 7 were combined, evaporated and the residue was further purified by TLC. Inhibitory activity was detected in the fractions of Rf 0.3 to 0.5 in the cress bioassay (Fig. 2). The active residue was passed through C18 Sep-Pak cartridges and the activity was detected in
the fraction eluted with 40% aqueous methanol (data not shown). The active residue was finally purified by reverse-phase HPLC and the inhibitory activity was found in a peak fraction eluted between 40 and 43 min (Fig. 3), yielding an active compound (0.3 mg).

This compound inhibited the growth of cress roots at concentrations greater than 0.3 μg mL⁻¹ (Fig. 4). When the root length of cress was plotted against the logarithm of the concentrations, the response curve of the inhibitor was linear between 10 and 90% inhibition. The concentration required for 50% inhibition in the assay was 2.9 μg mL⁻¹ as interpolated from the response curve.

Chemicals with allelopathic activity are present in many plants and in many organs, including leaves, flowers, fruits, and buds (Rice, 1984; Putnam and Tang, 1986; Inderjit, 1996). Under certain condition, these compounds are released into the environment, either as exudates from living tissues or leachates from residues in sufficient quantities to affect the
In the present research, three growth-inhibiting substances were found by silica gel column chromatography from the neutral fraction of the acetone extract of 30-d-old lemon balm shoots (Fig. 1). Most active substance was purified and isolated by TLC, C_{18} Sep-Pack cartridge and reverse-phase HPLC (Figs. 2 and 3), although the active component was not characterized. These results suggest that lemon balm shoots may contain at least three growth-inhibiting substances. These substances may be released into the environment under certain condition and may act as allelochemicals to other plants (Rice, 1984; Einhellig, 1996; Seigler, 1996). For identification and characterization of the growth-inhibiting substances, large-scale purification of the inhibiting-substances in lemon balm shoots is now underway.
REFERENCES


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<和文抄録>

レモンバームのアレロパシー物質の分離精製

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レモンバーム（Melissa officinalis L.）のアレロパシー物質を明らかにするために、30日齢のレモンバームのシュートからアレロパシー物質の分離精製を試みた。レモンバームのシュートのアセトン抽出物から得た中性物質をシリカゲルのカラムクロマトグラフィーで12画分に分離し、それぞれをレタス（Lactuca sativa L.）の生物検定に供したところ、3種類の生長抑制物質が見出された。その内、1つの生長抑制物質は、薄層クロマトグラフィー、C18 Sep Pack カートリッジと逆相カラムHPLCにより分離された。この物質は、0.3 µg mL⁻¹以上でレタスの下胚軸の生長を抑制し、2.9 µg mL⁻¹でレタスの下胚軸の生長を50%抑制した。以上の結果は、レモンバームのシュートには少なくとも3種類の生長抑制物質が存在すること、これらの物質は何らかの方法で環境に放出され、他の植物の生長に影響を与える可能性があることを示唆している。