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# Mycoassay of Fluorescent Fractions from Seven Essential Oils

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The presence of a wide array of fluorescent compounds in a number of essential oils analyzed by thin layer chromatography (TLC) has been reported by several investigators (Touchstone and Dobbins 1977; Morozumi 1978; Wagner et al 1983). Several of the fluorescent compounds such as eugenol, cinnamic aldehyde, coumarin, and thymol detected in essential oils have been reported to inhibit mycelium growth in species of mycotoxigenic fungi (Bullerman et al 1977) and pseudomycelium production by common food spoilage and industrially important yeasts (Conner and Beuchat 1984).

In the present study the fluorescent fractions of seven essential oils were separated, isolated, and tested for antifungal activity against the mycelium growth of Aspergillus, Mucor, and Rhizopus species.

## MATERIALS AND METHODS

Food-grade quality essential oils, which included bay, cinnamon bark, cinnamon leaf, clove, pimenta berry, pimenta leaf, and thyme, were used in this study (Fritzsche, Dodge and Olcott; New York, New York).

Strains of species of Aspergillus, Mucor and Rhizopus were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and the Department of National Health and Welfare (Ottawa, Canada). Stock cultures were maintained on slants of potato dextrose agar (PDA). Routine subcultures were made every four weeks.

Standard 20 x 20 cm chromatography plates coated with a layer (0.25 or 0.50 mm) of silica gel G (Supelco; Bellefonte, PA) were activated at 100 C for one hour and then held in a desiccator until needed.

Ten microliters of essential oil were spotted on silica gel G plates and developed in benzene:chloroform

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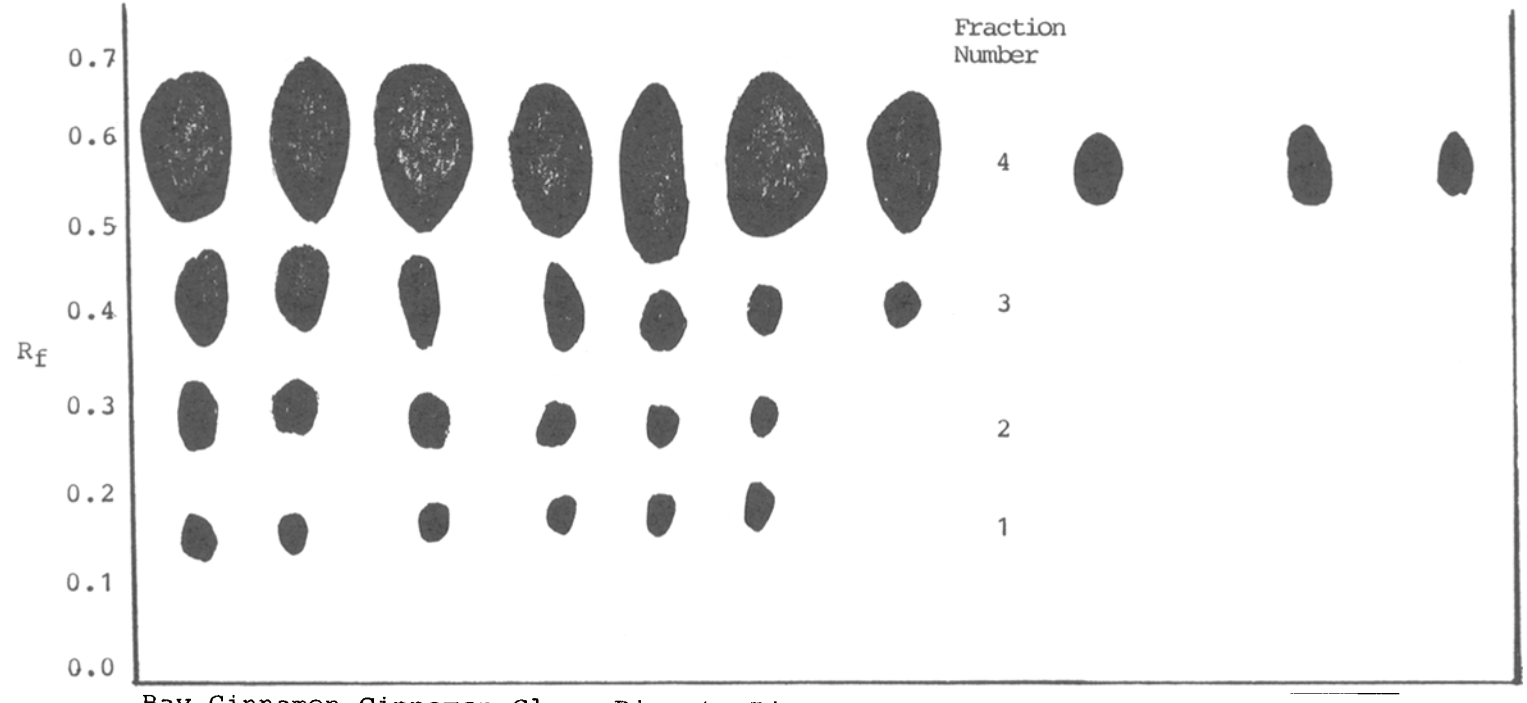
(50:50, v/v). After development, the plates were air-dried and examined in visible light and under ultraviolet (UV) light at 254 and 366 nm before and after exposure to vanillin-sulfuric acid reagent (Wagner et al 1983) or iodine vapor (Touchstone and Dobbins 1978). The zones containing the fluorescent fraction were scraped from the plates and eluted with 1 ml of absolute methanol. Ten microliters of the eluted fluorescent fractions were re-chromatographed on TLC plates for further purification ans subsequent identification. Technical grade (99.5% pure) authentic samples which included thymol, cinnamic acid, cinnamic aldehyde, coumarin, and eugenol were purchased from Pfaltz and Bauer (Stamford, CN). The authentic reference samples were co-chromatographed along side of the fluorescent compounds isolated from the second chromatographic separation.

Fungitoxicity of the four fluorescent fractions from the seven essential oils were evaluated against ten species of Rhizopus, three of Mucor, and eight of Aspergillus. One hundred microliters of methanolic fraction from the first chromatographic separation were added to each filter paper disk (Whatman No. 2, 8 mm dia). The disk was placed in the center of a petri plate containing 10 ml of potato dextrose agar. For the control, filter paper disks supplemented with 100 ul of methanol were used. Test fungus plugs were transferred to the centers of the filter paper disks located on the PDA plates and allowed to grow at 27 C for seven days. A 5-mm diameter fungus-agar-disk from the leading edge of a culture was used for the inoculum. Two plates per treatment per fungus were replicated three times. Experimental data were analyzed statistically using the Duncan's new multiple rank test (P=0.05).

## RESULTS AND DISCUSSION

Chromatographic separation of fluorescent substances and data for the seven essential oils are given in Figure 1 and Table 1. Four similar fluorescent fractions were detected in all of the oils except thyme, where two fractions were detected. A fluorescent fraction with an $R_f$ value of 0.15 appeared bluish, while fractions 2 and 3 with $R_f$ values of 0.30 and 0.45 respectively, were blue in the majority of the oils. The largest of the fractions, fraction 4, with an $R_f$ value of 0.63 appeared blue in all the essential oils with the exception of cinnamon bark, where an additional bright blue fluorescent center was also present. Re-chromatographic separation of the fractions revealed that several fluorescent components were present in the majority of the fractions except




Solvent System: Benzene:Chloroform (50:50, v/v)

Figure 1. Chromatographic separation of fluorescent fractions in seven essential oils.

Table 1. Occurrence of fluorescent fractions in seven essential oils.

| Number of Fluorescent Fraction | $R_f$ values in Benzene: Chloroform | Fraction color under UV light (366 nm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Bay | Cinnamon Bark | Cinnamon Leaf | Clove | Pimenta Berry | Pimenta Leaf | Thyme |
| 1 | .15 | Bluish | Light Bluish | Light Bluish | Light Bluish | Light Bluish | Light Bluish | - |
| 2 | .30 | Blue | Blue | Blue | Blue | Dark Blue | Blue | - |
| 3 | .45 | Blue | Blue | Blue | Blue | Dark Blue | Light Bluish | Dark Blue |
| 4 | .63 | Blue | Dark Blue and Bright Blue Center | Blue | Blue | Bluish | Bluish | Blue |
| Cinnamon Aldehyde | .60 | | | | | | | |
| Eugenol | .63 | | | | | | | |
| Thymol | .62 | | | | | | | |

| Number of Fluorescent Fraction | $R_f$ values in Benzene: Cloroform | Fraction color under UV light (366 nm) after chemical treatment | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Bay | Cinnamon Bark | Cinnamon Leaf | Clove | Pimenta Berry | Pimenta Leaf | Thyme |
| 1 | .15 | Dark Blue | Dark Blue | Dark Blue | Dark Blue | Dark Blue | Dark Blue | - |
| 2 | .30 | Blue | Blue | Blue | Dark Blue | Blue | Blue | - |
| 3 | .45 | Blue | Blue | Blue | Blue | Dark Blue | Blue | Dark Blue |
| 4 | .63 | Blue | Bright Blue Center | Blue | Blue | Blue | Blue | Blue |

Solvent System: Benzene:Chloroform (50:50, v/v)
Symbol - : no fluorescent fraction detected.

fraction 1. While the identity of a number of fluorescent compounds in the fractions could not be resolved, several fluorescent compounds known to possess antifungal activity, such as eugenol, cinnamic aldehyde, and thymol, were identified in fraction 4. Eugenol ($R_f$ 0.63) was detected in all of the essential oils except thyme, cinnamic aldehyde ($R_f$ 0.60) in cinnamon bark and leaf, and thymol ($R_f$ 0.62) in thyme only. To confirm the identification of eugenol, thymol, and cinnamic aldehyde in fraction 4, authentic reference samples were co-chromatographed along side of the isolated fluorescent components detected in the chromatographic separation of fraction 4. When tested against three genera of fungi, only fluorescent fraction 4 exhibited fungistatic-fungicidal activity. In general, the growth inhibitory effect of fluorescent fraction 4 varied depending upon the genus. The duration of inhibiton (day) of fungal growth was determined macroscopically and recorded. Fluorescent fraction 4 was most effective, however, in inhibiting mycelial growth in Rhizopus and least effective in the genus Mucor (Tables 2, 3, and 4).

Table 2. Antifungal effect of fluorescent fraction four from seven essential oils on Mucor Species

| Fungi | Duration of growth inhibition (day) Fluorescent Fraction Four | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | B | CB | CL | Cl | PB | PL | T |
| Mucor hiemalis Wehmer ATCC 8690 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| M. mucedo (Linn.) Fresenius ATCC 9836 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| M. racemosus f. racemosus Schipper ATCC 7935 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |

C-Control, B-Bay, CB-Cinnamon Bark, CL-Cinnamon Leaf, Cl-Clove, PB-Pimenta Berry, PL-Pimenta Leaf, and T-Thyme

Table 3. Antifungal effect of fluorescent fraction four from seven essential oils on *Aspergillus* Species

| Fungi | Duration of growth inhibition (day) Fluorescent Fraction Four | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | B | CB | CL | Cl | PB | PL | T |
| *Aspergillus flavus* Link ATCC 15546 | 0 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| *A. flavus* Link ATCC 15547 | 0 | 1 | 2 | 2 | 1 | 1 | 1 | 1 |
| *A. flavus* Link ATCC 15548 | 0 | 1 | 2 | 1 | 2 | 1 | 1 | 1 |
| *A. flavus* Link ATCC 26945 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| *A. flavus* Link ATCC 28539 | 0 | 1 | 2 | 2 | 1 | 1 | 1 | 1 |
| *A. flavus* Link ATCC 36182 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *A. parasiticus* Speare ATCC 26862 | 0 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| *A. parasiticus* Speare ATCC 28285 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 |

C-Control, B-Bay, CB-Cinnamon Bark, CL-Cinnamon Leaf, Cl-Clove, PB-Pimenta Berry, PL-Pimenta Leaf, and T-Thyme

Table 4. Antifungal effect of fluorescent fraction four from seven essential oils on *Rhizopus* Species

| Fungi | Duration of growth inhibition (day) Fluorescent Fraction Four | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | B | CB | CL | Cl | PB | PL | T |
| *Rhizopus arrhizus* Fischer ATCC 6204 | 0 | 2 | 3 | 3 | 4 | 3 | 3 | 2 |
| *R. chinenis* Saito ATCC 12276 | 0 | 1 | 3 | 3 | 3 | 2 | 2 | 1 |
| *R. circinans* van Teighem ATCC 1225 | 0 | 1 | 3 | 3 | 4 | 2 | 2 | 1 |
| *R. japonicus* Vuillemin ATCC 24863 | 0 | 1 | 3 | 3 | 4 | 2 | 1 | 1 |
| *R. kazanensis* Hanzawa ATCC 8998 | 0 | 1 | 4 | 3 | 3 | 1 | 1 | 1 |
| *R. oryzae* Went:Prinsen-Geerlings ATCC 22957 | 0 | 2 | 3 | 3 | 3 | 2 | 2 | 2 |
| *R. pygameus* Naoumoff ATCC 1159 | 0 | 1 | 4 | 4 | 3 | 2 | 2 | 1 |
| *R. stolonifer* (Ehren:Fr.) Lind ATCC 12939 | 0 | 3 | 2 | 3 | 3 | 3 | 1 | 1 |
| *R. tritici* Saito ATCC 1230 | 0 | 1 | 3 | 2 | 3 | 2 | 2 | 1 |
| R. 66-81-2 | 0 | 1 | 3 | 3 | 3 | 3 | 3 | 2 |

C-Control, B-Bay, CB-Cinnamon Bark, CL-Cinnamon Leaf, Cl-Clove, PB-Pimenta Berry, PL-Pimenta Leaf, and T-Thyme

The common pattern of fluorescent compounds in the majority of the seven essential oils is of interest since these same seven oils were earlier reported as effective antifungal agents against toxigenic and nontoxigenic fungi (Thompson and Cannon 1986; Thompson 1986). The fungistatic-fungicidal activity of the fluorescent compounds merits continued research in view of the renewed interest of finding naturally occurring fungitoxicants that are biodegradable.

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