Simple Micromethod for Detecting Antifungal Activity

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A bioassay that can be carried out on thin-layer chromatograms is described for rapid detection of antifungal or general cytotoxic activity with microquantities of test compound.

An antifungal bioassay has been developed in this laboratory for evaluation of scarce compounds of natural origin. The assay requires only a very small quantity of test compound, appears to be inherently more utilisable than older procedures (1, 2, 4) for sparingly water-soluble materials, and provides an index of effects on mycelial growth as well as on spore germination.

The test suspension of spores and mycelium was obtained from a 10-day-old culture of Helminthosporium sativum grown on commercial potato dextrose (Biological Laboratories) at 26 °C in continuous darkness. The suspension was prepared by pipetting 5 ml of fresh potato-dextrose broth (300 g of potato, 20 g of dextrose, and 1,000 ml of water) fortified with 1% sodium glycolcholate onto the culture surface. Spores and mycelial fragments were then suspended by scraping the culture surface with a glass rod. The suspension was decanted, and an equal volume of a 2% agar solution at 50 °C was added. The resulting mycelial-spore suspension (50 to 75 spores per 100× microscopic field) was immediately pipetted onto microscopic slides so that a 2-mm agar film was deposited on the surface.

Solutions of the compound to be tested were dried on clean slides as spots 5 to 8 mm in diameter. Discs of 1 cm in diameter were cut from the inoculated agar slides and placed over the spots containing the test compound. The agar-disc slides were incubated in a moist chamber at 26 °C for 2 to 4 hr, after which germinated and ungerminated spores were counted (50 spores) and recorded. Inhibition of germ tube and mycelial growths were also evaluated by comparing, on a scale from 0 to 4, the growth of treated and untreated spores and mycelium. Evaluations were made every 24 hr until mycelial mats formed in the untreated controls (2 to 3 days).

In experiments designed to compare the new method with the standard antifungal slide-germination test (1), phenyl mercuric acetate (PMA) was used in the amounts of 5, 1, 0.5, 0.25, and 0.1 μg. An identical LD₅₀ value of 0.29 μg was obtained by both methods. Replicate experiments with PMA also plotted LD₅₀ values of 0.29 μg with the new method, indicating its quantitative reproducibility. Spore counts were facilitated in the new method since the agar discs could be removed from the test compound spot, eliminating interference from fungicide particles. The method also permits easy recovery of test compound that did not diffuse into the agar discs. In addition, the relationship between exposure time of a fungicide to the toxic effects it produces could be studied by this method but would appear to be an impractical approach with the other reported methods. The advantages clearly establish the new method as a valuable tool in evaluating antifungal activity, especially where small amounts of water-insoluble compounds are concerned.

In another experiment, the scope and sensitivity of the new antifungal test were compared with the agar-plate paper-disc bacterial bioassay (3). This assay involves impregnating a paper disc with test compound and placing it onto the surface of nutrient agar which has been inoculated with a test bacterium. If compounds are toxic, zones of growth inhibition appear around the paper discs. Bacterial toxicity has been often used as a preliminary screen for general cytotoxicity when dealing with limited amounts of natural products. Therefore, comparing the fungal and bacterial bioassays for their ability to detect a more generalized cytotoxicity is of particular value to those investigators interested in pesticidal natural products. For this study, appropriate amounts of the following compounds were tested: PMA, 1.0 to 0.001 μg; 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), 400 to 50 μg; 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT), 400 to 50 μg; Chlordane (1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methan-
TABLE 1. **Comparison of antifungal toxic thresholds for selected bioactive compounds**

| Test compound            | Toxic threshold of antifungal bioassay (µg) | Toxic threshold of antibacterial bioassay (µg) |
|--------------------------|--------------------------------------------|----------------------------------------------|
|                          | Spore inhibition | Mycelial mat inhibition | Staphylococcus aureus inhibition | Sarcina lutea inhibition |
| PMA (fungicide)          | 0.25            | 0.25                      | 0.001                           | 0.001                     |
| 2,4,5-T (herbicide)      | 140             | 200                       | 200                             | 100                        |
| DDT (insecticide)        | 250             | 400                       | NE (400)                        | NE (400)                   |
| α1-Chlordane (insecticide) | 200         | NE* (400)                 | NE (400)                        | NE (400)                   |
| Mustard gas (mutagenic agent) | 10           | 25                        | NE (100)                        | NE (100)                   |
| Alternariol (mycotoxin)  | 250             | 500                       | 500                             | 500                        |
| Alternariol + AME (mycotoxins) | 62.5 + 62.5   | 250 + 250                 | 2.5 + 2.5                      | 2.5 + 2.5                  |
| Kojic acid (mycotoxin)   | 200             | 100                       | 100                             | 100                        |
| HIF factor (phytotoxin)  | 300             | 400                       | NE (400)                        | NE (400)                   |

* Toxic threshold is the minimum amount of test compound that gives a prescribed toxic effect.
* No effect.

Silica gel was removed from the edge of the spot. A spore-germination bioassay disc prepared as already described was placed onto the outlined PMA spot. Areas on the thin-layer chromatogram which had been developed in the solvent system but had no compound present were used as controls. The thin-layer plates were incubated in a moist chamber for 5 hr, and the per cent inhibition of spore germination was recorded. Again the LD₅₀ = 0.29 for PMA, whereas the controls germinated 100%. This evidence indicates that the adaptation of the new method can be carried out on thin-layer chromatograms with no loss of sensitivity. Therefore, it appears that the new antifungal assay in combination with thin-layer chromatography can be used to isolate and purify antifungal substances present in crude mixtures.

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