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Search for new antifungal compounds from higher plants

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Abstract: The increasing occurrence of opportunistic systemic mycoses associated with the use of immunosuppressive drugs and AIDS has led to new efforts in the search for novel antifungal compounds. At the same time, there is continuing interest in the discovery of antifungal agents which are effective against plant pathogenic fungi. Since the plant kingdom provides a useful source of lead compounds of novel structure, a wide-scale investigation of species from the tropics has been undertaken. TLC bioautography allows the screening of plant material and subsequent bioassay-guided fractionation and isolation.

Treatments with immunosuppressive drugs and the spread of AIDS have meant that diseases caused by weaknesses in the immunitary systems of humans are becoming more and more prevalent. Associated with these problems is an increasing predisposition to fungal attack. The infections commonly observed in the immuno-compromised host include candidiasis (*Candida albicans* and other species) of the oesophagus and mouth, cryptococcosis (*Candida neoformans*) and aspergillosis (*Aspergillus flavus*, *A. fumigatus*, *A. niger*). As there are few really effective antifungal preparations currently available for the treatment of systemic mycoses and as the efficacy of existing drugs is rather limited, it is important to find new sources of antifungal agents.

In the past, efforts have been mainly directed towards compounds active against plant pathogenic fungi (and research along these lines in agrochemistry is still of high interest) but it has been demonstrated that plant-derived constituents may offer potential leads for novel agents against systemic mycoses (1).

Although other techniques exist, bioautography is the method of choice when searching for antifungal compounds from plants. This is because it allows the combination of a bioassay *in situ* and, at the same time, localization of active constituents on the TLC plate employed for the assay. Spore-producing fungi, such as *Aspergillus*, *Penicillium* and *Cladosporium* spp. can all be employed as target organisms in direct bioautographic procedures (2, 3). After migration of an extract or sample on a TLC plate, the plate is sprayed with the microorganism and incubated in a humid atmosphere. Zones of inhibition appear where spore growth is prevented by the active constituents of the plant extract.

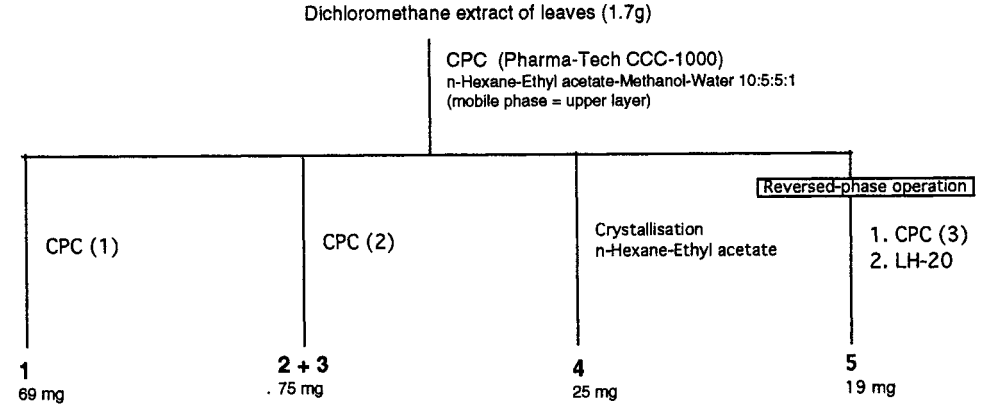
Bioautography with the plant pathogenic fungus *Cladosporium cucumerinum* has been employed to guide the fractionation and isolation of numerous new natural products with fungicidal activity from African and other tropical plants (4). These compounds have many different chemical structures, varying from naphthoquinones to saponins.

Since direct bioautography is not possible with yeasts such as *Candida albicans*, a simple and rapid agar overlay assay has been developed (5). This technique, which is a hybrid of direct and contact bioautography, relies on the transfer of active compounds by a diffusion process from the stationary phase into an agar layer containing the microorganism. When the plate is sprayed with methylthiazoyltetrazolium chloride (MTT), an MTT formazan is produced and inhibition zones are observed against a purple background.

In addition to the screening of African plants, a series of 153 crude extracts of plants from Panama (representing 28 species from 21 families) has been investigated for antifungal activity against *Cladosporium cucumerinum* and *Candida albicans*. It was found that 15% of the extracts showed activity against one of the microorganisms and 9% of the extracts were active against both in the bioautographic assays. The most promising extracts originated from plants used in traditional medicine and these are presently being studied - *Bursera simaruba* (Burseraceae), *Gliricidia sepium* (Leguminosae), *Piper auritum* (Piperaceae) (6).
With respect to new antifungal agents from plant sources, certain active compounds have recently been isolated using the bioassay-guided fractionation approach.

DIPLOLOPHIUM BUCHANANI (UMBELLIFERAE)

A dichloromethane extract of the leaves of this plant, which grows in Malawi, central Africa, was found to inhibit growth of *C. cucumerinum*. Three phenylpropanoids, myristicin (**1**), elemicin (**2**), isoelemicin (**3**) and a furanocoumarin, oxypeucedanin (**4**) were isolated by means of the recently introduced liquid-liquid chromatographic technique of centrifugal partition chromatography (CPC) (7) alone, while the furanocoumarin oxypeucedanin hydrate (**5**) was obtained by a combination of CPC and gel filtration (Fig.). Antifungal activities of the pure compounds against *C. cucumerinum* are as follows (expressed as minimum amounts required to inhibit growth of spores in the TLC bioassay): **1**, 20 µg; **2** + **3**, 8 µg; **4**, 1 µg; **5**, 10 µg. The value for **4** compares quite favorably with those for the reference compounds miconazole (1 µg) and the triazole antifungal agrochemical propiconazole (0.1 µg).



CPC (1): Pharma-Tech CCC-1000. Solvent n-hexane-t-butylmethylether-acetonitrile 5:1:5 (mobile phase=upper phase). Flow-rate 3 ml/min. Sample 130 mg. Detection 254 nm.
CPC (2): Pharma-Tech CCC-1000. Solvent n-heptane-acetonitrile-methanol 6:3:1 (mobile phase=upper phase). Flow-rate 3 ml/min. Sample 125 mg. Detection 254 nm.
CPC (3): Pharma-Tech CCC-1000. Solvent chloroform-methanol-ethyl acetate-water 5:6:3:4 (mobile phase =upper phase). Flow-rate 3 ml/min. Sample 343 mg. Detection 254 nm.

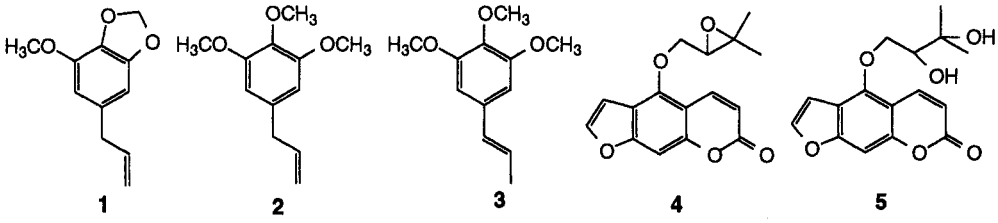
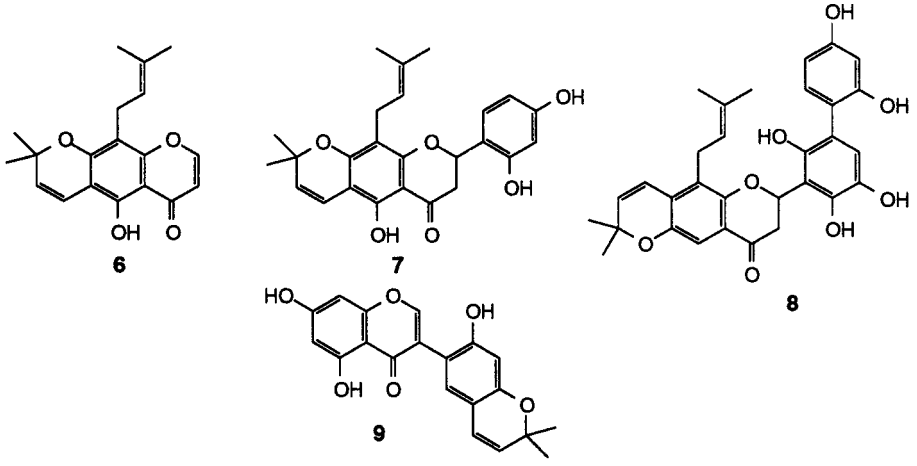


Fig. Isolation of constituents of *Diplolophium buchanani* leaves.

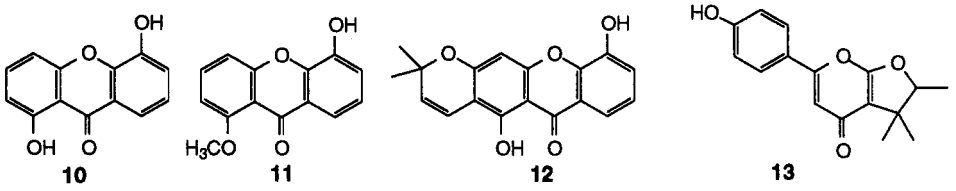
ERIOSEMA TUBEROSUM (LEGUMINOSAE)

This is a Chinese medicinal herb used in Yunnan province. Activity against *C. cucumerinum* and *C. albicans* was found in the dichloromethane extract of the roots. A chromone (**6**), two flavanones (**7**,**8**) and a new isoflavone (**9**) were isolated from this extract by a combination of open-column chromatography, medium pressure LC on an RP-18 support and gel filtration on Sephadex LH-20. The minimum quantity of all four polyphenols required to inhibit growth of *C. cucumerinum* in the assay was 5 µg.



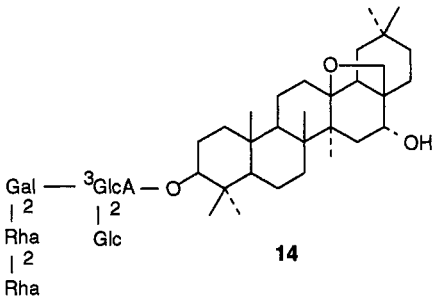
HYPERICUM BRASILIENSE (GUTTIFERAE)

The genus *Hypericum* is known to furnish antifungal compounds - *H. revolutum*, for example, is a source of chromenyl ketones which are effective against *C. cucumerinum* (8). *Hypericum brasiliense*, from Brazil, as its name suggests, is a source of the antifungal xanthones **10** - **12** and the novel γ-pyrone hyperbrasilone **13** (9). The minimum quantity of compounds **11** - **13** which was necessary to inhibit growth of the fungus on the TLC plates was 3 µg, while for **10**, the value was 0.25 µg. Simple xanthones are not known for their antifungal properties and the very low inhibitory quantity recorded for **10** justifies a more extensive search in this class of compounds for potential leads.



RAPANEA MELANOPHLOEOS (MYRSINACEAE)

A methanolic extract of leaves of *R. melanophloeos*, a tree found in southern Africa, was both fungicidal against *C. cucumerinum* and molluscicidal against the schistosomiasis-transmitting snail *Biomphalaria glabrata*. Bioactivity-guided fractionation of this extract was performed, first by open-column silica gel chromatography and then by using a combination of gel filtration, low-pressure and high-pressure liquid chromatography on RP-18 stationary phase. A triterpene saponin, sakurasosaponin (**14**), was isolated. This was active in the *C. cucumerinum* TLC bioassay at 1 µg and also against *B. glabrata* snails. It would appear that the 13ß,28-epoxy moiety is important for the bioactivity, since analogues with the heterocycle open were devoid of activity (10).



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SEARCHING FOR ANTIFUNGAL COMPOUNDS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

This multidimensional approach to chromatography is an important new addition to the analytical techniques used by the phytochemist. By combining the separation power of HPLC and the ability of MS to give useful structural information, it is possible to obtain preliminary data about the composition of a plant extract before any costly and time-consuming isolation work is performed. LC-MS provides a rapid method for avoiding known fungicidal substances since these can be screened out at the beginning of any investigation. In the case of xanthones, for example, a combination of on-line UV analysis of the HPLC effluent and determination of molecular weights of the individual components of a mixture can help enormously in the tentative attribution of structure. When post-column derivatization is added, a good idea of the substitution pattern can be obtained. For certain xanthones from *Chironia* species (Gentianaceae), the combination of LC-UV and LC-MS (thermospray interface) allowed a full on-line structure determination. This identification procedure was possible without isolation of the pure products (11).

CONCLUSIONS

The use of simple bioautographic techniques enables a rapid screening of plant extracts for fungicidal activity. Once an activity has been located, the extract can be analysed by LC-MS to establish whether new or known compounds and/or substance classes are involved. Activity-guided fractionation can then be performed to isolate the antifungal agents. This approach has been successfully employed for the separation of novel bioactive compounds from African, South American and Asian plants.

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