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ANTIFUNGAL AGENTS: IN VITRO AND IN VIVO ANTIFUNGAL EXTRACT FROM THE COMMON DAISY, *BELLIS PERENNIS*

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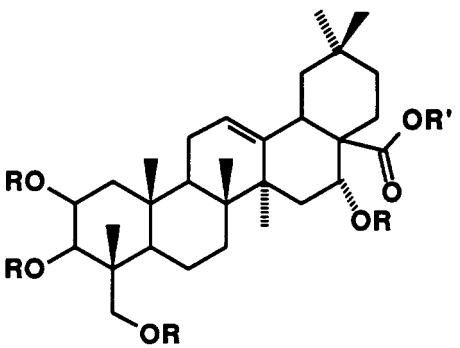
In preliminary general screening experiments, we have studied the antifungal activity of 49 crude extracts of higher plant species growing in Brittany (1–3). This work has led to selection of the EtOH extract of the common daisy *Bellis perennis* L. (Compositae) (4), which showed activity both in vitro and in vivo against *Ceratocystis ulmi* (=*Graphium ulmi*), the fungus responsible for Dutch elm disease. This activity was traced to a saponin fraction (5).

The greatest antifungal activity was in an *n*-BuOH extract obtained from the crude EtOH extract. The residue that was obtained after evaporation of the *n*-BuOH showed several spots on tlc. Cc followed by preparative tlc allowed isolation of the most active material that had $R_f = 0.42$ in EtOAc-HCOOH-H_2O (10:2:3) and that showed a yellow color after chlorosulfonic acid spray. Microhydrolysis experiments were performed on tlc plates suspended in acidic vapors before development; three sugars were thus liberated, first rhamnose, then xylose and glucose. Three genins were observed in tlc, among which the least polar one was identified. The presence of two minor tlc spots after hydrolysis means either that the material was not homogeneous before hydrolysis or that rearrangements had occurred. ^{1}H nmr of this genin, **1**, showed signals for six angular methyls at high field and 5 or 6 broad resonances between 3 and 6 ppm. It gave the tetra-acetyl derivative **2** whose ^{1}H-nmr spectrum displayed signals for an isolated CH_2OAc and for four other protons, two of which were superimposed at 5.4 ppm. In C_6D_6, however, these signals were resolved into a broad singlet at δ 6.11 ppm, a quartet at δ 5.6 ppm ($J = 3.5$ Hz), a triplet at δ 5.46 ppm ($J = 3.5$ Hz), and a sharp doublet at 5.15 ppm ($J = 3.5$ Hz); the doublet collapsed into a singlet upon irradiation of the quartet. Compound **2** yielded a monomethyl ester **3** after ethereal CH_2N_2 treatment. Compound **3** gave a weak molecular ion at *m/z* 686 ($C_{39}H_{58}O_{10}$), which successively lost two $C_2H_4O_2$ units (*m/z* 626 and 566); further fragmentation was observed at *m/z* 507 (loss of $C_2H_3O_2$) and 499 (loss of CH_2OAc). As usual in triterpenes, the mass spectrum was dominated by the two ubiquitous ions *m/z* 260 and 201, which confirmed the presence of an angular methyl ester and of a latent unsaturation in the D and E rings of the molecule.



1 R=R'=H
2 R=Ac, R'=H
3 R=Ac, R'=Me

These data led to the conclusion that **1** was a tetrahydroxy acid of the oleanane group. This was confirmed by ^{13}C nmr,

which showed all but two of the requisite carbon resonances for such a structure. Due to uncontrolled presence of inorganic impurities and to unfavorable relaxation times, the acid carbonyl was not observed. In addition, C-12 was hidden under one of the solvent triplets (C_5D_5N). Among known triterpenoids that could fit the above mentioned data, polygalacic acid (2β,16α,24-trihydroxyoleanolic acid) was the closest match, and excellent agreement was found between the ^{13}C-nmr spectrum of **1** and of the genin of polygalasin D (6). Finally, determination of **1** was secured by direct comparison of **1** with polygalacic acid and of **2** with the peracetate of polygalacic acid (7). It must be pointed out that polygalacic acid is rare in nature, and this is the first time it has been found as a genin of an antifungal agent, the isolated saponin being the most active component of the in vivo active crude EtOH extract.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were recorded at 500 MHz on a Bruker WM 500 and at 300 MHz on a Bruker AC 300. ^{13}C-nmr spectra were obtained on a Bruker WH 270 at 68.9 MHz. Mass spectra were measured on a JEOL D 300 and on a Varian Mat-311.

EXTRACTION AND ISOLATION.—The whole wild fresh plant of *B. perennis* L. (3 kg), gathered near Rennes, was washed with H_2O and throughly extracted with boiling EtOH. A voucher specimen is deposited in our laboratory. These first extractions and further separations were monitored for antifungal activity. After filtration, the EtOH was removed in vacuo, and the residue was extracted in a Soxhlet apparatus first with $CHCl_3$ and then with EtOH. The EtOH extract was evaporated to dryness and partitioned between H_2O and *n*-BuOH. After drying and evaporation of the *n*-BuOH, 9 g of crude saponin mixture was obtained. The crude mixture (150 mg) was chromatographed on a column of Si gel eluted with $CHCl_3$ and with a gradient of MeOH in $CHCl_3$. All the fractions were assayed against *C. ulmi* and pooled according to activity. The most active fraction (5 mg) was eluted with $CHCl_3$-MeOH (2:1).

DETERMINATION OF IN VITRO ANTIFUNGAL ACTIVITY.—In vitro antifungal activities were measured on Petri dishes inoculated in the center with young mycelium; nutrient medium was Sabouraud's (malted gelose). The substances to be tested were deposited as solutions into holes bored at the periphery of the dish (1,3,5).

DETERMINATION OF IN VIVO ANTIFUNGAL ACTIVITY.—In vivo testing was monitored with EtOH crude extracts. These extracts, filtrated on Millex 0.45 μm, were deposited in sterile flasks, each containing 15 ml. Twenty-one diseased elms were treated. Extracts were injected once a month over a 4 month period, through a mastic plug into four cylindrical holes on the bottom of the trees. In comparison with control diseased elms, evolution of fungus infection has been stopped (4).

ACID HYDROLYSIS OF THE MAJOR SAPONIN.—The major bioactive saponin (150 mg) was refluxed for 1 h in 30 ml 0.5 N HCl. Extraction with EtOAc, drying, and evaporation yielded a mixture that was purified by preparative tlc. A pure solid **1** (20 mg) was thus obtained.

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