Journal of Natural Products
Vol. 51, No. 1, pp. 152-154, Jan-Feb 1988

ISOLATION OF DEOXYSHIKONIN, AN ANTIDERMATOPHYTIC PRINCIPLE FROM *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

G. HONDA,* F. SAKAKIBARA, K. YAZAKI, and M. TABATA

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan

Various plant tissue cultures have been reported to show antimicrobial activities (1–6) as well as activity against plant viruses (7,8). In the present study, we have carried out a screening test of culture lines derived from five species of boraginaceous plants in an attempt to isolate antidermatophytic as well as antibacterial substances. This paper chiefly deals with the isolation of deoxyshikonin from cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. as a compound showing the strongest antidermatophytic activity.

Of the extracts of 36 cell lines tested, 22 (61%) showed growth inhibitory activities against one or more of the test microorganisms (Table 1). None of these extracts, however, was active against *Escherichia coli* NIHJ IFO 12734, and only the extract of *Echium lycopsis* was active against *Candida albicans* IFO 0197. The red cell lines of *L. erythrorhizon* producing shikonin derivatives were active against *Staphylococcus aureus* 209P IFO 12732 and *Trichophyton rubrum* IFO 5808, whereas all 10 of the non-pigmented cell lines of this species were inactive.

It has been reported that the pigmented cultured cells of *L. erythrorhizon* contain the same red naphthoquinone pigments (shikonin derivatives) as those found in the root bark of the original plant (10). It is also known that several naphthoquinones and benzoquinone derivatives from *L. erythrorhizon* as well as *E. lycopsis* cultures have antibacterial activities (13), but these compounds have not been examined with respect to the antidermatophytic activity. The present study has demonstrated for the first time that deoxyshikonin is the main MeOH-soluble antidermatophytic principle of *Lithospermum* cells.

As shown in Table 2, deoxyshikonin proved to inhibit the growth of four dermatophytes (MIC 6.25–25 μg/ml), *Saccharomyces sake* IFO 0305 (MIC 6.25 μg/ml), and for four Gram-positive bacteria deoxyshikonin showed weak activities (MIC 50–200 μg/ml). Except against *S. sake*, the activity of shikonin was somewhat weaker or equal to deoxyshikonin. It is possible, therefore, that the antifungal activity is decreased by the presence of a hydroxy group at the 1′ position of the side chain. Neither deoxyshikonin nor shikonin inhibited the following Gram-negative bacteria and yeasts even

TABLE 1. Antimicrobial Activities of Extracts from Cultured Cell Lines of Boraginaceous Plants.

Plant species	No. of cell lines	Red pigment formation	Microorganism			
			Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Trichophyton rubrum*
Borago officinalis L.	1	−	0[a]	0	0	0
Echium lusitanicum L.	1	−	0	0	0	0
Echium lycopsis L.	1	+	1	0	1	1
Lithospermum erythrorhizon Sieb. et Zucc.	21	+	21	0	0	21
	10	−	0	0	0	0
Plagiobothrys arizonicus Greene	1	−	0	0	0	0

[a]Number of active culture lines.

TABLE 2. Antimicrobial Activities of Deoxyshikonin, Shikonin, and Two Reference Drugs.

Microorganism	Minimum inhibitory concentration (μg/ml)			
	Deoxy-shikonin	Shikonin	Chloram-phenicol	Griseo-fulvin
Gram-positive bacteria				
Staphylococcus aureus 209P IFO 12732	200	>200	12.5	—[a]
Micrococcus roseus IFO 3764	100	200	25	—
Micrococcus luteus IFO 12708	50	100	6.2	—
Bacillus subtilis PCI 219 IFO 3513	200	>200	6.2	—
Fungi				
Saccharomyces sake IFO 0305	6.2	3.1	>200	—
Trichophyton rubrum IFO 5808	25	25	—	3.1
Trichophyton mentagrophytes IFO 5809	12.5	25	—	3.1
Trichophyton tonsulans var. *sulfureum* IFO 5945	12.5	25	—	3.1
Microsporum gypseum IFO 8307	6.2	12.5	—	1.5
Epidermophyton floccosum IFO 9045	6.2	6.2	—	1.5

[a]Not tested.

at the highest concentration tested (200 μg/ml): *E. coli* NIHJ IFO 12734, *Pseudomonas aeruginosa* IFO 12689, *Serratia marcescens* IFO 12648, *C. albicans* IFO 0197, *Candida guilliermondii* IFO 0454, *Candida mycoderma* IFO 0162, *Candida tropicalis* IFO 0006, and *Candida utilis* IFO 0454.

In the experiment employing the agar streak method followed by the liquid dilution method, the survival of *Trichophyton mentagrophytes* IFO 5809 was confirmed even at the deoxyshikonin concentration of 100 μg/ml. Therefore, it is considered that the activity of deoxyshikonin against dermatophytes is not fungicidal but fungistatic.

EXPERIMENTAL

PLANT MATERIAL AND CULTURE METHOD.—Callus cultures used in the present experiments were originally derived from germinating seeds of five boraginaceous species (Table 1) cultured on Linsmaier-Skoog's basal agar medium (9) containing 10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-5} M kinetin. These calli have been subcultured on the same medium containing 10^{-6} M indole-3-acetic acid and 10^{-5} M kinetin every 4 weeks for more than 4 years. All the cultures were maintained at 25° in the dark.

MICROORGANISMS.—All strains of the microorganisms used in this study were purchased from the Institute for Fermentation (IFO), Osaka, Japan.

ANTIMICROBIAL SCREENING.—The paper disc method was employed for antimicrobial screening. A powdered sample (1 g) of cultured cells was soaked in 5 ml MeOH for 3 days at room temperature. A portion (20 μl) of the supernatant was applied to a paper disc (4.5 mm in diameter) with a microsyringe and air-dried to remove the solvent. The disc was then placed on the surface of an agar nutrient medium on which test microorganisms had been plated. Heart infusion agar medium (Difco) was used for *S. aureus* 209P IFO 12732 and *E. coli* NIHJ IFO 12734 and Sabouraud's agar medium (Nissui) for *C. albicans* IFO 0197 and *T. rubrum* IFO 5808. The antimicrobial activity was detected after an incubation period of 24 h at 37° for bacteria and yeasts and of 7 days at 27° for dermatophytes. When a test extract failed to inhibit the growth of a microorganism, it was regarded as inactive.

ISOLATION OF ANTIDERMATOPHYTIC PRINCIPLE.—Dried cells (1.5 kg) of cell line M18 of *L. erythrorhizon* cultured in M9 liquid medium (9) for 14 days were extracted with MeOH (20 liters) three times. The MeOH was evaporated under reduced pressure to give an extract (512 g) which was found to be inhibitory to the growth of *T. mentagrophytes* IFO 5809 in the in vitro assay. When the extract was divided into ethereal and aqueous layers, all the activity moved into the former. The Et_2O extract (98 g) was separated into 18 fractions (300 ml each) by Si gel cc (Wako gel C-200, 1 kg) using $CHCl_3$-MeOH (9:1) as developing solvent, and each fraction was tested against *T. mentagrophytes* by the paper disc method. The active fractions (1–3) were rechromatographed on the same gel (500 g) with $CHCl_3$-EtOAc (9:1) as developing solvent. Of 29 fractions (100 ml each) so obtained, fractions 3–5 showing a strong antidermatophytic activity were

separated by preparative tlc (Kiesel gel G 90, 2 mm thick; developing solvent $CHCl_3$). One of the red pigments located at R_f 0.68 was found to be the most active substance by the paper disc method and was recrystallized from EtOH to yield needles (8.5 mg, mp 92–92.5°). This compound was identified (uv, ir, ^1H nmr, and mmp) as deoxyshikonin by comparison with an authentic sample.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC).—MIC values were determined by the agar dilution streak method (11,12) using the heart infusion medium for bacteria or Sabouraud's medium for yeasts and dermatophytes (9.5 ml), to which the MeOH solution (0.5 ml) of a test sample or a reference drug (chloramphenicol and griseofulvin) was added. The conditions of incubation were the same as those described earlier.

EXAMINATION OF FUNGICIDAL CONCENTRATION.—A MeOH solution (0.25 ml) of deoxyshikonin (25–2000 μg/ml) was added to Sabouraud's liquid medium (4.75 ml), and a piece of filamentous mycelia of *T. mentagrophytes* IFO 5809 was put on the test medium. After incubation for 7 days at 27°, the solution was streaked on a newly prepared Sabouraud's agar medium and incubated for 7 days at 27° in order to detect the fungicidal concentration.

LITERATURE CITED

1. M.C. Mathes, *Science*, **40**, 1101 (1963).
2. M.C. Mathes, *Lloydia*, **30**, 177 (1967).
3. G. Campbell, E.S.C. Chan, and W.G. Barker, *Can. J. Microbiol.*, **11**, 785 (1965).
4. P. Khanna and E.J. Staba, *Lloydia*, **31**, 180 (1968).
5. P. Khanna, M. Suchendra, and T.N. Nag, *Lloydia*, **34**, 168 (1971).
6. I.A. Veliky and R.K. Latta, *Lloydia*, **37**, 611 (1974).
7. M. Misawa, M. Hayashi, and H. Tanaka, *Biotechnol. Bioeng.*, **17**, 1335 (1975).
8. S. Takayama, M. Misawa, K. Ko, and T. Misato, *Physiol. Plant.*, **41**, 313 (1977).
9. E.M. Linsmaier and F. Skoog, *Physiol. Plant.*, **18**, 100 (1965).
10. Y. Fujita, Y. Hara, T. Ogino, and T. Suga, *Plant Cell Rep.*, **1**, 59 (1981).
11. M. Ookoshi, *Chemotherapy*, **22**, 1126 (1974).
12. G. Honda, M. Tabata, and M. Tsuda, *Planta Med.*, **37**, 172 (1979).
13. M. Tabata, M. Tsukada, and H. Fukui, *Planta Med.*, **44**, 234 (1982).

Received 15 May 1987