ISOLATION AND CHARACTERIZATION OF NAPHTHAQUINONE PIGMENTS FROM *TORULA HERBARUM* (PERS.). HERBARIN AND DEHYDROHERBARIN

M. V. KADKOL, K. S. GOPALKRISHNAN and N. NARASIMHACHARI*

Antibiotics Research Center, Pimpri, Poona 18, India

(Received for publication January 16, 1971)

Torula herbarum (PERS.) LINK Ex. Fr. is a dermataceous fungus regularly associated with dry leaves and twigs of *Felia microphylla* LIEBM. In submerged cultures the organism produces weak antimicrobial substances when grown in CZAPEK's or in a malt extract medium. The temperature range for the growth of the organism was found to be 24~28°C and no growth occurred at 5° or 37°C. Nitrogen and carbon utilization studies indicated tyrosine and levulose to be the best sources, followed by ammonium tartrate and glucose respectively. Based on these studies a synthetic medium was developed for the optimum production of pigments.

Fermentation

Fermentation was carried out in shake flasks (500 ml) and in 5-liter fermentors (New Brunswick) through a seed inoculum at 24°C for 96~120 hours. The fermented broths were extracted at unacidified pH (4.5~5.0) into ethyl acetate and the extracts washed twice with water and concentrated under reduced pressure. When the concentrate was kept overnight at −5°C, a crystalline material separated which varied in color from batch to batch from light yellow to reddish yellow. Further concentration of the mother liquor yielded additional crystalline material.

Fig. 1. Preparative thin-layer chromatogram of herbarin mixture. Band 2 is herbarin.

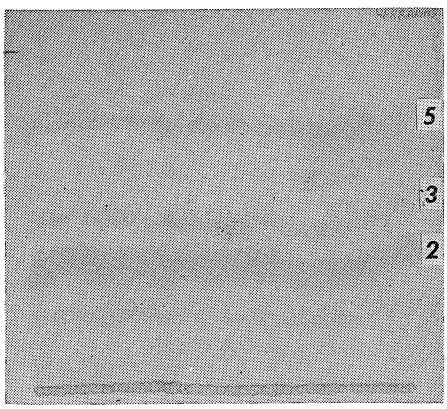


Table 1. Activity of hebarins against bacteria

Organism	Gram reaction	M. I. C. μg/ml
1) *Bacillus subtilis*	+	20
2) *B. mycoides*	+	30
3) *Sarcina lutea*	+	75
4) *Escherichia coli*	−	100
5) *Salmonella paratyphi*	−	75
6) *Klebsiella pneumoniae*	−	—
7) *Shigella sonnei*	−	100
8) *Xanthomonas argimone*	−	75
9) *X. citri*	−	60

* Present address : Galesburg State Research Hospital, Thudichum Psychiatric Research Laboratory, Galesburg, Illinois 61401, U.S.A.

Antimicrobial Activity

The pigment mixture obtained from the broth extract showed weak antibacterial and antifungal activity (Tables 1 and 2). It also showed antiamoebic activity against *Entamoeba histolytica* at a concentration of 20 μg/ml when tested by the method of THIRUMALACHAR *et al.*[1].

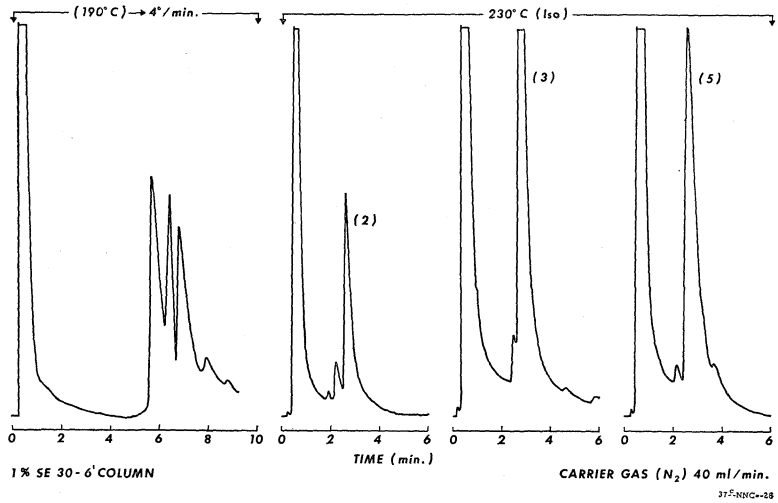
Table 2. Activity of hebarins against fungi

Organism	M. I. C. μg/ml
1) *Alternaria solani*	50
2) *Aspergillus niger*	75
3) *Cephalosporium purpuram*	75
4) *Curvularia* sp.	75
5) *Fusarium oxysporum* f. *vasinfectum*	75
6) *Helminthosporium* sp.	75
7) *Mucor* sp.	75
8) *Pythium debaryanum*	25
9) *Syncephalastrum* sp.	75
10) *Trichoderma viride*	75

Chromatography of the Pigments

Five distinct spots, three yellow and two red, were seen on thin-layer chromatograms (silica gel chromatogram 6061) with chloroform, and chloroform and acetic acid (50 : 1) as developing solvents. Preparative thin-layer chromatography (TLC) on silica gel thin-layer using multiple elution technique and also column chromatography (silica gel) were used for the separation of the pigments. A preparative chromatogram is shown in Fig. 1. The pigments were also separated by gas-liquid chromatography (GLC) on the SE-30 column (1 % or 3 %) either isothermally or with temperature programming (190°~195°/min.). The fractions obtained by TLC or column chromatography were monitored by the GLC technique. Some typical GLC chromatograms are shown in Fig. 2. The major pigments, one a bright yellow compound (herbarin), and the second, a red compound (dehydroherbarin), were obtained in adequate quantities for characterization. The physical and physio-chemical properties of herbarin and dehydroherbarin are given in Table 3.

Fig. 2. Gas liquid chromatogram of herbarin mixture, and of the thin-layer fractions 2, 3 and 5.



Herbarin

After separation by column chromatography or by preparative thin-layer chromato-

Table 3. Physico-chemical properties of herbarin and dehydroherbarin

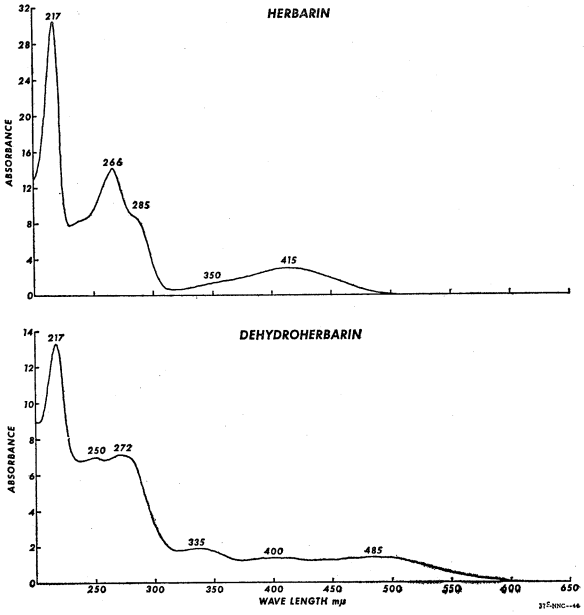
	Herbarin	Dehydroherbarin
M. P. (°C)	192～193	189～190
UV (EtOH)	λ_{max} 415, 266 and 216 nm (log E 3.48, 4.18 and 4.83) $\lambda_{infl.}$ 350, 285, 237 nm (log E 3.15, 3.94 and 3.91)	λ_{max} 485, 400, 335, 272, 250, 217 nm (log E 3.15, 3.15, 3.28, 3.85, 3.85 and 4.12)
IR (KBr disc)	3325 (OH), 1678, 1650 (C=O), 1607, 1572, 1567 cm^{-1} (C=C)	1675, 1667 (C=O), 1632, 1610, 1600, 1565 cm^{-1} (C=C)
NMR	(pyridine d_5) 2.61 (1H, d, J=2.5), 3.18 (1H, d, J=2.5), 4.9～5.0 (2H, m), 5.4 (1H, broad singlet), 6.17 (3H, s), 6.21 (3H, s), 6.86 (1H, m, J=18), 7.33 (1H, m, J=18 Hz), 8.26τ (3H, s)	($CDCl_3$) 2.75 (1H, d, J=2.5), 3.30 (1H, d, J=2.5), 4.18 (1H, d, J=1.0 Hz), 4.88 (2H, s), 6.05 (3H, s), 6.07 (3H, s), 8.02 τ (3H, s)
Mol. wt.	299 (osmometric)	
Mass spectrum (high resolution)	M^+=304.0905	M^+=286.0821
Elementary analysis	Found C 62.9, H 5.15 Calc. $C_{16}H_{16}O_6$: C 63.15, H 5.26 M^+=304.0947	C 67.30, H 5.0 $C_{16}H_{14}O_5$: C 67.51, H 4.9 M^+=286.0841

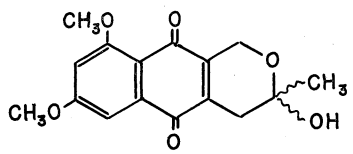
graphy, herbarin crystallized from methanol as long, bright yellow needles, m. p. 192～193°C. It was insoluble in aqueous sodium hydroxide and gave no color with ethanolic ferric chloride. In concentrated sulfuric acid a blood red color was developed which on standing turned brown. Molecular weight determination by osmometric method gave 299.

Analysis: Calculated for $C_{16}H_{16}O_6$, C 63.15, H 5.26.
Found, C 62.9, H 5.15.

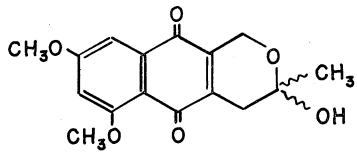
High resolution mass spectra: M^+=304.0905 ($C_{16}H_{16}O_6$ = 304.0947), M^+-H_2O=286.0846 ($C_{16}H_{14}O_5$ = 286.0841), M^+-CO=276.1028 ($C_{15}H_{16}O_5$=276.0998), (M^+-H_2O)-CH_3= 271.0643 ($C_{15}H_{11}O_5$=271.0607).

Fig. 3. UV spectra of herbarin and dehydroherbarin in EtOH.





I



II

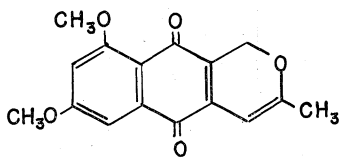
Optical activity: In ethanolic solution herbarin showed negligible rotation at D line, but ORD showed a negative cotton effect at 345 nm. Based on the UV, IR, NMR and mass spectral data and biogenetic considerations herbarin is assigned structure I or II.

Dehydroherbarin

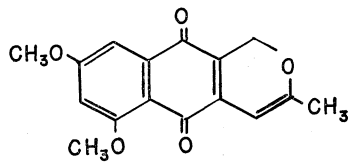
Separated by silica gel column chromatography, dehydroherbarin crystallized from methanol as bright red glistening needles, m.p. 186~188°C. It was soluble in ethyl acetate, chloroform, diethyl ether and insoluble in water. It was insoluble in aqueous sodium hydroxide, and did not give color with ethanolic ferric chloride.

Analysis: Calculated for $C_{16}H_{14}O_5$; C 67.51, H 4.90.
Found; C 67.30, H 5.0.

High resolution mass spectra M^+=286.0821, ($C_{16}H_{14}O_5$=286.0841), M^+-CH_3=271.0561 ($C_{15}H_{11}O_5$=271.0607), M^+-CO=258.0888 ($C_{15}H_{14}O_4$=258.0892). Based on its spectral characteristics and its relationship to herbarin, dehydroherbarin is represented by structures III or IV.



III



IV

When herbarin was heated in acetic anhydride solution at 100°C for 8 hours, dehydroherbarin was obtained. This sample was identical in all respects to the naturally occurring metabolite. Since the process employed in the extraction and purification of the fermentation broth is mild and since herbarin is stable under these conditions, dehydroherbarin isolated from the fermentation is a true metabolite and not an artifact.

The structures of herbarin and dehydroherbarin proposed here were derived from spectral data and biogenetic considerations are fully discussed elsewhere in this journal[2]. Several naphthaquinones have been reported as microbial metabolites[3,4,5,6]; however, this is the first report of their isolation from the genus *Torula* and from the family Dermatiaceae of the imperfect fungi.

Acknowledgements

One of us (M. V. K.) thanks Hindustan Antibiotics Ltd. for a Fellowship. We thank Dr. R. Nagarajan for the NMR and mass spectra.

References

1) Thirumalachar, M. J.; C. V. Radhakrishnan & A. P. B. Vores: Testing procedures of evaluation of antiamoebic drugs *in vitro* and in experimental animals. Hindustan Antibiot. Bull. 8: 201~204, 1966
2) Nagarajan, R.; N. Narasimhachari, M. V. Kadkol & K. S. Gopalkrishnan: Structure of herbarin. J. Antibiotics 24: 249~252, 1971
3) Arnstein, H. R. V. & A. H. Cook: Production of antibiotics by fungi. III. Javanicin, an antibacterial pigment from *Fusarium javanicum*. J. Chem. Soc. 1947: 1021~1028, 1947
4) Ruelius, H.W. & A. Gauhe: Fusarubin, a naphthaquinone pigment from Fusarien. Ann. Chem. 569: 38~59, 1950
5) Gerber, N. N. & B. Wiechawek: The structures of two naphthoquinone pigments from an actinomycete. J. Org. Chem. 31: 1496~1498, 1966
6) Howe, R. & R. H. Moore: 6-Ethyl-5-hydroxy-2, 7-dimethoxynaphthoquinone, a metabolite of *Hendersonula toruloidea nattrass*. Experientia 25: 474, 1969