Journal of Natural Products
Vol. 47, No. 5, pp. 872-874, Sep-Oct 1984

ANTITUMOR ALKALOIDS IN CALLUS CULTURES OF *OCHROSIA ELLIPTICA*

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The genus *Ochrosia* is composed of some forty different species that grow on islands in the Indian and Pacific Oceans, mainly on New Caledonia and Madagascar. The leaves of *Ochrosia elliptica* Labill. contain four alkaloids: ellipticine, 9-methoxyellipticine, isoreserpiline, and elliptinine (1). The first two alkaloids have been shown to display tumor-inhibiting activity (2), and it has been demonstrated that this antitumor activity is due to the intercalation of alkaloid in DNA (3).

The present study was undertaken to compare the alkaloid composition of callus tissue with that of the plants in vivo. As far as we are aware, no studies have been made of the biosynthetic capabilities of *O. elliptica* tissues grown in culture.

EXPERIMENTAL

PLANT MATERIAL.—*O. elliptica* (Apocynaceae) leaves were removed from the plant growing in the Museum National d'Histoire Naturelle of Paris, France; *O. elliptica* seeds were kindly supplied by the Jardin de Aclimatacion de la Oratova, Puerto de la Cruz, Canary Islands. The seeds were surface sterilized in 2% sodium hypochlorite (10 min) and 70% EtOH (2 min) and then germinated on Knop agar medium in sterile Petri dishes. Seedlings were used when they were 2 months old.

TISSUE CULTURE.—Cultures were raised from stem root and leaf explants obtained from young seedlings. Calli have been initiated on Gamborg B5 medium (4) supplemented with kinetin (0.1 ppm) and α-naphthalene acetic acid (NAA, 1 ppm) or 2,4-dichlorophenoxyacetic acid (2,4-D, 1 ppm). The pH was adjusted to 5.5 with KOH (0.1 N) before autoclaving it at 110° for 20 min. Explants were also cultured on Linsmaier and Skoog medium (5), supplemented with 3% glucose and the hormones of B5 as mentioned above.

Tissues were subcultured on different media supplemented with various factors: carbon sources (3%), growth hormones in different concentrations, coconut water (5%), casein hydrolysate (0.1%), and gel. Various compounds—polyvinylpyrrolidone, 0.01% (6), phloroglucinol, 0.1% (7), charcoal, 0.3% (8), and bovine serum albumin 1% (6)—were also incorporated in the media to reduce browning. The best medium used for subcultures of *O. elliptica* callus was B5 agar medium supplemented with 2% sucrose, 0.1% casein hydrolysate, 1 ppm kinetin, and 10 ppm 2,4-D, and this medium was used further in all studies.

All the cultures have been maintained at $24 \pm 1°$ in continuous fluorescent light (2000 lux) and subcultured every 3 weeks on fresh medium.

ANALYSIS OF STANDARDS.—Ellipticine (25 mg), 9-methoxyellipticine (23 mg), isoreserpiline (20 mg), and elliptinine (2 mg) were obtained by extraction from *O. elliptica* leaves (770 g dry weight) and purification, following the procedure used for alkaloids of *Ochrosia balansae* by Bruneton and Cavé (9) with slight modification. Ellipticine and 9-methoxyellipticine were also obtained by synthesis according to the technique of Dalton and co-workers (2).

All the alkaloids obtained as standard compounds from leaves and by synthesis were comparable in all the spectral and physicochemical properties: uv, ir, pmr, ms, and mp (1,2,10).

ISOLATION OF ALKALOIDS.—Callus tissues (57 g, 6 weeks old) were freeze-dried (3 g), powdered, rendered alkaline (28% NH_4OH), and extracted in a Soxhlet apparatus with CH_2Cl_2 (400 ml) for 6 h. After evaporating the extract to dryness, it was dissolved in 2% H_2SO_4 (50 ml) and extracted with CH_2Cl_2 (6×100 ml). The aqueous layer was adjusted to pH 10 with 28% NH_4OH and extracted with CH_2Cl_2 (6×100 ml). The organic phase, after drying with Na_2SO_4, was evaporated under reduced pressure. The residue was dissolved in EtOH and applied to Silicagel-60 plates (E. Merck, Darmstadt, W. Germany). EtOH (95%) or CH_2Cl_2-MeOH (9:1, v/v) was used as developing solvent. The unknown samples were compared with standard compounds.

The preparative tlc resulted in the separation of two alkaloids identified as ellipticine: Rf, 0.52; uv λ max (EtOH) 227, 237, 245, 275, 286, 295, 332, 346, 384; fluorimetry λ exc. 287, 328, 398 λ em. 444, 514, eims[1] (70 eV) *m/z* 247 (19%) (M+1), 246 (100) (M^+), 245 (32) (M-1) 231 (17.5) (M-CH_3) and cim (NH_3), *m/z* 248 (22%)

[1]Mass spectra were determined with a V.G. micromass 305 F spectrometer operating at 70 eV.

TABLE 1. *Ochrosia elliptica* alkaloid content in leaves, in vitro tissues, and nutrient medium. At each passage, three-week-old tissue from 20 culture tubes were mixed and three samples were analyzed for their alkaloid content.

	Leaves	In vitro tissues at passages					Culture medium at passage
		10th	11h	12th	13h	15th	10th
Ellipticine	50[a]	60	47	45	65	54	7
9-methoxyellipticine	120	150	135	105	135	150	3

[a]$\mu g/g^{-1}$ dry weight.

(M+2), 247 (100) (MH^+), 246 (11.5) (M^+); 9-methoxyellipticine: Rf, 0.45; uv λ max (EtOH) 244, 274, 288, 304, 335, 352; fluorimetry, λ exc. 412, λ em. 470; eims (70 eV), *m/z* 277 (3%) (M+1), 276 (100) (M^+) 275 (9) (M-1), 261 (50) (M-CH_3) and cim (NH_3) *m/z*, 278 (21.5) (M+2), 277 (100) (NH^+) 276 (25) (M^+). The two other alkaloids have been tentatively identified as elliptinine and isoreserpiline by a comparison with the standards obtained from *O. elliptica* leaves.

The powder remaining after Soxhlet extraction with CH_2Cl_2 was hydrolyzed by 5% H_2SO_4 (reflux, 1 h), and this extract was analyzed as above. An alkaloid with the same Rf as isoreserpiline was noted in very small amounts. The media remaining after growth of calli were also studied. They were freeze-dried and extracted with MeOH.

QUANTITATIVE ANALYSIS OF ELLIPTICINE AND 9-METHOXYELLIPTICINE.—The alkaloid content of callus tissues was determined by fluorimetry.[2] After separation on Silicagel-60 plates with 95% EtOH, ellipticine and 9-methoxyellipticine were quantified either after elution by MeOH (ellipticine: λ exc. 398, λ em. 444; 9-methoxyellipticine: λ exc. 412, λ em. 470) or directly on the plates.[2] Calibration graphs were linear over a tested range from 0.01 to 0.40 μg for the two alkaloids,

RESULTS AND CONCLUSION

O. elliptica calli were easily developed on B5 or Linsmaeir and Skoog's agar media, but in the latter callus growth was slower. Initial tissues were chlorophyllous and compact. However, subsequent subcultures every 3 weeks on the B5 initiation medium were rapidly accompanied by browning and local necrosis. Replacement of agar by Biogel-P6 (11) or culture on a filter paper (12) or addition of polyvinylpyrrolidone, or phloroglucinol, or charcoal or bovine serum albumin, did not reduce the browning. Glucose in place of sucrose or NAA in place of 2,4-D reduced the growth. Benzyladenine slightly improved the color and texture, but tissue growth was poor.

Tissues grew satisfactorily on B5 medium supplemented with kinetin (0.1 ppm), 2,4-D (10 ppm), and casein hydrolysate (0.1%). On this medium, tissues were cream colored and granular. Browning was reduced in successive passages. Tissues of leaf origin grew well as compared to tissues isolated from root and stem. Therefore, only leaf calli have been used as experimental material.

The presence of ellipticine and 9-methoxyellipticine has been confirmed by comparative Rf, uv, fluorimetric spectra, and mass spectra studies. Isoreserpiline and elliptinine were tentatively identified by comparison with standards.

Alkaloid complexation has been reported in in vitro cultures of some members of the Apocynaceae (13). A second CH_2Cl_2 extract of *O. elliptica*, after hydrolysis, produced only traces of isoreserpiline. The result shows that no alkaloid complexation occurred in these tissues.

The fluorometric method developed in this laboratory can be used successfully for the detection of these compounds in minor quantities. The quantitative results of alkaloid production obtained with *O. elliptica* cultures by this method in different passages are presented in Table 1. It is evident from the

[2]Spectrofluorimeter JOBIN YVON JY 3D and Shimadzu tlc scanner.

results that callus tissues produced almost the same amount of ellipticine and 9-methoxyellipticine as recorded in in vivo tissues. Alkaloids were also detected in the culture medium in traces. Studies are in progress to produce these antitumor alkaloids in a bioreactor.

ACKNOWLEDGMENTS

Leaves of *O. elliptica* and a gift of isoreserpiline were kindly provided by Dr. J. Garnier. 9-Methoxyellipticine was provided by Dr. N. Dat Xuong.

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Received 28 November 1983