AN INVESTIGATION OF THE ANTIVIRAL ACTIVITY OF *PODOPHYLLUM PELTATUM*

E. BEDOWS

School of Public Health and School of Medicine, The University of Michigan, Ann Arbor, MI 48109

and

G. M. HATFIELD

College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109

ABSTRACT.—The antiviral activity of an aqueous extract of *Podophyllum peltatum* was investigated. The extract was fractionated by reversed-phase chromatography, and podophyllotoxin was found to be the most active component in inhibiting the replication of measles and herpes simplex type I viruses. β-Peltatin and desoxypodophyllotoxin produced marginal antiviral effects, while α-peltatin and picropodophyllotoxin were inactive at the levels tested.

Several years ago an aqueous extract of *Podophyllum peltatum* Linne (Berberidaceae) was reported to have antiviral activity against herpes simplex (type II), influenza A, and vaccinia viruses (1). Of the 178 plants screened for antiviral effects in this study, *P. peltatum* was one of the most active. Although the authors speculated that water soluble lignan glycosides (2–4) may have been responsible for the effects noted, the nature of the active constituent(s) has not been established. The following investigation is concerned with the fractionation and identification of the principal antiviral constituents of this plant.

Measles virus was selected for the primary antiviral bioassay because of its novel cytopathic effect (cpe). As with other paramyxoviruses, measles virus produces syncytia which are distinctly different from cell rounding due to general toxicity. Thus, when measles virus was used, it was possible to distinguish between the cytoxicity produced by extract constituents and virus-induced cpe. This was considered to be important in this study because of the well-documented cytotoxic effects of *P. peltatum* lignans (2). However, antiviral activity was not judged solely on the basis of suppressed cpe in treated cultures. The ability of cultured green monkey kidney (Vero) cells to produce infectious progeny virus was also determined. The infectivity of extract-treated cells (plaque forming units/ml or pfu/ml) was compared with untreated controls, and significant antiviral activity was considered to be present if an order of magnitude drop in pfu/ml was detected. As shown in table 1, an aqueous extract of *P. peltatum* rhizomes exhibited a one hundred-fold drop in measles virus pfu/ml when tested at 10 μg/ml (maximum tolerated dose).

The same concentration of crude extract residue also produced a significant, albeit less dramatic, inhibition of herpes simplex (type I) virus (table 1), thus confirming, in part, the observations made by May and Willuhn (1). Since herpes simplex virus-induced cpe involves cell rounding, it was virtually impossible to differentiate between virus- and extract-induced changes in Vero cell morphology. Thus, no attempt was made to assess anti-herpes simplex activity against this criterion.

Fractionation of the aqueous extract was accomplished by high pressure liquid chromatography (hplc) with a reversed-phase (C–18) column (5). A typical chromatogram of the aqueous extract is shown in figure 1. This system allowed the separation of nonglycoside lignan constituents (podophyllotoxin and related compounds) from other components of the aqueous extract (2–4). The initial separation yielded four fractions; of these, the lignan-containing material (fraction C) was the only one to exhibit antiviral effects at the concentration employed (table 1).

TABLE 1. Antiviral bioassay results.

Materials Assayed	Concentration	Non-Infected Cells		Measles Virus-Infected Cells			HSV-Infected Cells
		Percent Cell Rounding	Percent Viable[a]	Percent Cell Rounding	Percent CPE[b]	Infectivity[c] Percent Control (pfu/ml)	Infectivity[d] Percent Control (pfu/ml)
None (control)	—	<5	97.3	<5	100	100 (2.5×10^6)	100 (4.5×10^7)
Aqueous extract	10 μg/ml	90	88.9	90	50	2.4 (6.1×10^4)	6.1 (2.8×10^6)
Fraction A	4 μg/ml[e]	<5	94.5	<5	100	100 (2.6×10^6)	not tested
Fraction B	4 μg/ml	<5	93.8	<5	100	100 (2.6×10^6)	not tested
Fraction C	4 μg/ml	50	77.0	90	40	15.4 (4.0×10^5)	not tested
Fraction D	4 μg/ml	<5	96.5	<5	100	100 (2.6×10^6)	not tested
Podophyllotoxin	1 μM	80	91.7	>95	25	0.9 (2.3×10^4)	11.7 (5.3×10^6)
Picropodophyllotoxin	1 μM	15	89.9	5	100	41.5 (1.1×10^6)	84.4 (3.8×10^7)
α-Peltatin	1 μM	5	91.9	>95	25	47.5 (1.2×10^6)	31.1 (1.4×10^7)
β-Peltatin	1 μM	5	85.4	50	100	15.1 (3.9×10^5)	21.7 (9.8×10^6)
Desoxypodophyllotoxin	1 μM	30	90.7	50	70	19.8 (5.2×10^5)	16.2 (7.3×10^6)

[a]Determined by trypan blue exclusion; average of triplicate assays.
[b]Percent of non-rounded cells incorporated into measles virus-specific syncytia.
[c]Virus released from 2×10^6 infected cells; average of three plaque assays.
[d]Virus released from 2×10^6 infected cells; average of two plaque assays.
[e]Dissolved in DMSO (1% final concentration in culture).

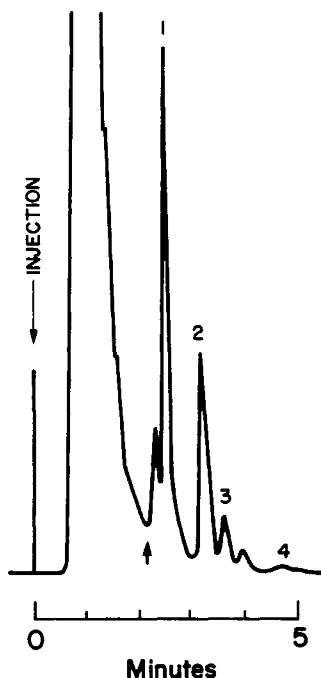

FIG 1. Fractionation of an aqueous extract sample of *Podophyllum peltatum*. See the test for a description of sample preparation and hplc conditions. Fraction B was collected from time zero to the point indicated by the arrow. Fraction C was then collected to 5 min. Peaks: 1=α-peltatin; 2=podophyllotoxin; 3=β-peltatin; 4=desoxypodophyllotoxin.

Analytical hplc of the antiviral fraction showed that the principal lignan present was α-peltatin (0.4% of the aqueous extract residue). Smaller quantities of β-peltatin, podophyllotoxin and desoxypodophyllotoxin were also detected. These compounds were tested for antiviral activity (table 1). Podophyllotoxin, which produced a one hundred-fold reduction in measles virus pfu/ml, was found to be the most active. β-Peltatin and desoxypodophyllotoxin produced marginal antiviral effects ($\leq$ten-fold drop in pfu/ml), while α-peltatin was essentially inactive. The antiviral effects on herpes simplex virus were not as pronounced, but followed the same trend with podophyllotoxin being the most active of the lignans tested (table 1). Although not all of the minor constituents of the lignan fraction were identified and tested, podophyllotoxin appeared to be primarily responsible for the antiviral effects noted for the aqueous extract in this study.

The observation that α-peltatin does not significantly reduce the production of infectious virus, despite diminishing measles virus-specific cpe, demonstrates that these two effects do not always correlate. Others have also reported that paramyxovirus-induced cpe may form without virus production (6,7) and *vice versa* (8). Thus, a bioassay based on the reduction of infectious virus titers, as employed in this investigation, is a more reliable method for assessing antiviral activity against paramyxoviruses than one based solely on the suppression of cpe.

Epimerization of podophyllotoxin to picropodophyllotoxin has been shown to readily occur under mild conditions (2) and was noted following prolonged refrigeration of solutions during this investigation. Because of this, the antiviral effects of picropodophyllotoxin were also determined, and it was found to be inactive against both test viruses (table 1). However, the extent of epimerization

occurring during the antiviral assays was found to be less than 10%, as determined by hplc.

The antiviral effect observed with podophyllotoxin is likely due to the disruption of cellular microtubules. Consistent with this proposal is the observation that cells change shape in response to podophyllotoxin treatment (9). Furthermore, other mitotic poisons, such as colchicine and the vinca alkaloids, have also been shown to either reduce virus production (10) or retard virus release from infected cells (11–12). All of these spindle fiber poisons are known to specifically bind to the tubulin subunit of microtubules (9). The therapeutic potential of these compounds as antiviral agents appears to be limited due to their highly cytotoxic nature. However, further study of their mechanism of action may prove to be valuable in understanding the role that cellular organelles play in virus replication.

Following the submission of this manuscript, it was learned that another group of investigators recently reported antiherpetic (type 1) activity for podophyllotoxin and related lignans (13–14). Although the antiviral bioassay employed in these studies was based entirely upon cpe analysis, their conclusions essentially support those presented here.

EXPERIMENTAL

PLANT MATERIAL.—Rhizomes of *Podophyllum peltatum* were collected in late July, 1981, near Ann Arbor, Michigan. The plant material was frozen at −20° until used. A herbarium sample was deposited in The University of Michigan Herbarium.

EXTRACTION AND FRACTIONATION.—The rhizomes were freeze-dried prior to extraction. Ten grams of the dried material was ground (20 mesh) and mixed with 100 ml water. This mixture was stirred and heated to boiling for 20 min (1). When cooled to 40° and centrifuged (8000 × *g*) for 30 min, a clear light yellow extract was obtained. The extract was freeze-dried and yielded 50 mg of residue per ml. This material was stored at −20° until used.

The extract residue was fractionated by an hplc system which consisted of a Waters Associates 6000A solvent pump, 660 flow programmer, U6K sample injector, 440 UV detector (254 nm) and Fischer Recordal Series 5000 recorder. The system employed a 4.6 mm (ID) × 25 cm Partisil 10 ODS 3 ®(C18, Whatman) column preceded by a 3.2 mm (ID) × 5 cm precolumn containing Co:Pel ODS® (C18 pellicular, Whatman). The solvent system contained acetonitrile and water (40:60) and was flow programmed as described below.

Samples of the extract residue were prepared by shaking 50 mg portions of the residue with 5 ml of the hplc solvent for 5 min at room temperature. The mixture was centrifuged (1500 × *g*, 10 min), and the supernatant was removed for hplc fractionation. The insoluble portion of the extract residue (30% by weight) was freeze-dried and was designated fraction A.

Hplc fractionation of the supernatant consisted of dividing the column eluate into two fractions as shown in figure 1. Fraction B (48%) was eluted at 3 ml/min. The flow rate was then increased to 5 ml/min and fraction C (9%), which contained the principal lignan constituents of the extract, was collected. To insure that all components of the extract were removed from the column, it was successively eluted with 50 ml of acetonitrile, methanol and water following the fractionation of 10 mg of aqueous extract residue. The residue from this column flush was designated fraction D (13%).

ANALYTICAL HPLC.—The system described above was utilized with flow programming to increase resolution. The solvent flow rate was increased from 2 ml/min to 7 ml/min over a 5 minute period on a Waters Associates 660 flow programmer (program 8). A sample of the aqueous extract residue (prepared as described above) was analyzed for the following lignan constituents: α-peltatin (k=3.1), podophyllotoxin (5.0), picropodophyllotoxin (5.1), β-peltatin (5.8) and desoxypodophyllotoxin (10.3). Peaks were identified by cochromatography with standards and the amount of α-peltatin present was established by relating peak height to the amount present in the injected sample on a standard curve.

BIOASSAY FOR ANTIVIRAL ACTIVITY.—Vero cells were grown in 8 oz (ca. 240 ml) prescription bottles and were either not infected or were infected with measles virus at a multiplicity of infection of 0.5 (15). Virus was allowed to adsorb for 1 hr at 37°. Following adsorption, nonadsorbed virus was removed by washing with calcium-free diluent; 12 ml of Eagle's minimal essential medium (mem) containing 10% fetal bovine serum (15), and the material being tested for antiviral effects, was added. The cultures were allowed to incubate at 37° until 100% of the cells in virus-infected samples containing no test materials exhibited syncytia. At this time, other samples were also examined for cpe and abnormal cellular morphology (rounding). Noninfected cells were also trypsinized and their viability determined by trypan blue exclusion. Infected samples were then quick-frozen at −70° and stored for further use.

Thawed samples were clarified by centrifugation (1500 × *g*, 5 min) and virus titers were assessed by the following plaque assay technique. Vero cells grown in 25 cm^2 culture flasks were inoculated with 0.1 ml of varying dilutions of the virus-infected cell suspension to be

assayed. After virus adsorption (1 hr, 37°), the cell monolayers were overlaid with mem containing 2% fetal bovine serum and 2% Bactoagar (Difco). Infected cultures were incubated 5 days at 37°, at which time 3 ml of a 0.02% solution of neutral red was added and plaques were counted 4–6 hours later. The results of these bioassays are shown in table 1. Assays involving herpes simplex virus (type I) were performed in an identical manner except that infected plaque assay samples were incubated 4 days prior to the addition of neutral red.

The concentration of test materials added to cell cultures was set at a level which left approximately 90% of the cells viable during the test period (maximum tolerated dose). Similar samples (i.e., fractions A–D and individual lignans) were tested at levels (4 μg/ml and 1 μM, respectively) which allowed potency comparisons within a group.

ACKNOWLEDGMENTS

The authors wish to thank Drs. M. Suffness and J. D. Douros, as well as the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, for samples of α-peltatin, β-peltatin, podophyllotoxin, desoxypodophyllotoxin and picropodophyllotoxin.

Received 27 February 1982

LITERATURE CITED

1. G. May and G. Willuhn, *Drug Res.*, **28**, 1 (1978).
2. J. L. Hartwell and A. W. Schrecker, *Fortschr. Chem. Org. Naturst.*, **15**, 83 (1958).
3. M. Kuhn and A. VonWartburg, *Helv. Chim. Acta*, **46**, 2127 (1963).
4. A. VonWartburg, M. Kuhn and H. Lichi, *Helv. Chim. Acta*, **47**, 1203 (1964).
5. D. A. Cairnes, D. G. I. Kingston and M. Madhusudana Rao, *J. Nat. Prod.*, **44**, 34 (1981).
6. J. V. Baublis and F. E. Payne, *Proc. Soc. Exp. Biol. Med.*, **129**, 593 (1968).
7. A. M. Breschkin, B. Walmer and F. Rapp, *Virology*, **80**, 441 (1977).
8. C. Mandansky and M. Bratt, *J. Virol.*, **26**, 724 (1978).
9. S. N. Timasheff and L. M. Grisham, *Annu. Rev. Biochem.*, **49**, 565 (1980).
10. N. R. Farnsworth, G. H. Svoboda and R. N. Blomster, *J. Pharm. Sci.*, **57**, 2174 (1968).
11. R. S. Spendlove, E. N. Lennette, J. N. Chin and C. O. Knight, *Cancer Res.*, **24**, 1826 (1964).
12. E. Bedows, unpublished.
13. T. Markkanen, M. L. Makinen, J. Nikoskelainen, J. Ruohonen, K. Nieminen, P. Jokinen, R. Raunio and T. Hirvonen, *Drugs Exptl. Clin. Res.*, **7**, 691 (1981).
14. T. Markkanen, M. L. Makinen, E. Maunuksela and P. Himanen, *Drugs Exptl. Clin. Res.*, **7**, 711 (1981).
15. E. Bedows and F. E. Payne, *J. Virol.*, **37**, 103 (1981).