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PHARMACEUTICAL ANALYSIS OF PODOPHYLLIN CONSTITUENTS

by

VERN ANOZIE CHUKWUMAEZE OBIAJUNWA

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE.**

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate studies and Research for acceptance, a thesis entitled **PHARMACEUTICAL ANALYSIS OF PODOPHYLLIN CONSTITUENTS** submitted by **VERN ANOZIE CHUKWUMAEZE OBIAJUNWA** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE** in the FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES, UNIVERSITY OF ALBERTA

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Dr. F.M. Pasutto

Dr. G.B. Baker

Date Sept 26th 1995

TO MY MOTHER AGNES

ABSTRACT

Podophyllin constituents from *P. emodi* and *P. peltatum* were isolated using column chromatography on silicic acid and Celite® with chloroform-methanol or chloroform ethyl acetate mobile phase. The isolated constituents were characterized by TLC, MP,IR, NMR, UV, and HPLC. The constituents isolated and identified include α-peltatin, ß-peltatin, podophyllotoxin, 4'-demethylpodophyllotoxin, and podophyllotoxin glucoside.

HPLC quantitative analysis was used to determine the quantities of each of the constituents in podophyllin from *P. peltatum* and *P. emodi*. The results of the analyses showed differences between the actual quantities of the constituents and the estimated quantities previously reported from gravity elution chromatographic analysis. HPLC analysis gives reproducible, accurate, quantities of the constituents present in both samples of podophyllin. This present analysis is the first report of the quantification of 4'-demethylpodophyllotoxin from *P. peltatum*.

Podophyllin is a non-standardized drug for which there is a safety concern. The HPLC quantitative analysis will provide the essential data necessary for the standardization of podophyllin.

ACKNOWLEDGEMENTS

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A. INTRODUCTION

NATURAL PRODUCTS IN MEDICINE

The use of natural products has been a part of the healing arts and science since mankind first began to treat illness. The instinct by which ancient man acquired biologic knowledge in determining the plants and animals to use for food was applied equally in the discovery of medicinal plants (Tyler, 1988). The healing powers of certain herbs, roots, and juices were discovered through trial and error, and the information over time was passed from generation to generation. The use of natural products in medicine has undergone a number of evolutionary changes. Today, the developments in the methods of extraction, separation, isolation, and identification of chemical compounds have enabled researchers to identify the specific therapeutic agents of natural origin.

Some ancient remedies of natural product origin, with some modifications, are still being used in the modern health care system. Natural products provide about 40% of all new drugs in the market (Der Marderosian & Liberti, 1988) and they serve as prototypes for the development of new, better and more effective drugs.

Some of the common therapeutic agents derived from plants include digoxin from *Digitalis purpurea* or *D.*

lanata, colchicine from Autumn crocus, atropine from *Atropa belladonna*, and reserpine from *Rauwolfia canescens* (Ross & Brain, 1977).

Podophyllum peltatum L and its related species, *P. hexandrum* Royle of the Podophyllaceae family (formerly Berberidaceae) (Steiner, 1986) have a number of important constituents with a variety of pharmacological activities. The analyses of the resin (podophyllin) constituents are the objects of this research.

B. LITERATURE SURVEY

I. BACKGROUND INFORMATION ON PODOPHYLLUM PLANTS

P. peltatum and *P. hexandrum* (*P. emodi* Wall) are the two main species of podophyllum that are used in medicine (Leung, 1980). They are perennial herbs with erect stems that bear one or two large shield-like leaves. *P. peltatum*, also known as American Podophyllum, has common names such as mayapple, mandrake, American mandrake, devil's apple, wild lemon, and vegetable mercury. It is native to Eastern North America from Quebec to Florida and west to Minnesota and Texas. It occurs in moist and shady deciduous woods and marshy meadows. *P. hexandrum* is abundant in regions of Tibet, Afghanistan, Pakistan and Northern India and is also known as Indian podophyllum (Morton, 1977).

The podophyllum plant blooms to produce a solitary flower between March and May, and fruits that ripen between May and August. The fruit is an edible sweet-tasting apple with a banana flavour. Every other part of this plant, including the seed, is poisonous if ingested. *P. peltatum* grows to a height of 45 cm, and *P. hexandrum* may grow to height of 60 cm or more. The plants have creeping, jointed rhizomes that can be several metres in length and about 6 mm in thickness. The dried rhizomes and the roots are called podophyllum and contain podophyllum resin or podophyllin.

The plant has seasonal changes in the quantity of resin and it is most abundant if the roots and rhizomes are harvested in late July or August, after the leaves have fallen.

II. FOLKLORE AND OLDER MEDICINAL USES OF PODOPHYLLUM

The North American Indians used podophyllum for many medicinal purposes for several centuries before the arrival of the early settlers. An anecdotal account from an old Appalachian Indian Herbalist described the use of *P. peltatum* for the treatment of variety of ailments (Crellin & Philpott, Vol.II, 1990). Some of the uses include the treatment of liver and kidney disorders, and as a purgative especially for "the cleaning out" of the human system in spring. Podophyllum extracts were added to whisky or drunk as an infusion in tea, usually with the addition of ginger to make the extract less harsh. Infusions of podophyllum were given to patients together with senna leaves or wild ginger or dandelion roots to reduce the potency.

Cherokee Indians used drops of the juice of fresh rhizomes as ear drops to relieve deafness (Morton, 1977). Podophyllum was used for the treatment of typhoid fever, cholera, bladder and prostate problems, gonorrhea and syphilis. It was also used as an antidote for snake bites, as an emetic, and for the treatment of jaundice (Leung, 1980). Recent findings suggest that the extract has a direct

effect on herpes simplex type II, influenza A, and vaccinia viruses (Crellin & Philpott, Vol II, 1990).

III. ISOLATION OF PODOPHYLLIN

Podophyllum resin or podophyllin is obtained from the roots and rhizomes of podophyllum by alcohol extraction (Hartwell & Schrecker, 1958). The resin is obtained by slow percolation of the powdered podophyllum. The percolate is concentrated to a thin syrup by evaporation. The resin is precipitated with one percent HCl cooled to a temperature below 10°. The clear liquid is decanted and the precipitate washed several times with water and air dried. American podophyllum yields about 2 - 8% of podophyllin and Indian podophyllum yields between 6 - 12% of the resin (Morton, 1977; Merck index, 1989; Hartwell & Schrecker, 1958; Wallis, 1967). Podophyllin is an amorphous powder with a faint unpleasant odour and a bitter acrid taste. The colour varies from light brown to greenish yellow and it turns darker when exposed to light (Hartwell & Schrecker, 1958).

IV. PHARMACOLOGY OF PODOPHYLLIN

Podophyllin was used as a cathartic shortly after its discovery in 1834 (Vogelzang et al. 1982). It replaced most other medicinal uses of podophyllum. It was an active ingredient in several proprietary medicines including

Carter's Little Pills® (Carter Wallace NS Inc., Mississauga, Ontario) and anthelmintic agents (Miller, 1985; Vogelzang et al. 1982). However, by the 19th century podophyllin was replaced by less drastic and more effective medicinal agents (Jardine, 1980).

Interest in podophyllin was revived when Kaplan (1942) discovered that it was useful in the topical treatment of genital warts. This led to studies of other activities of podophyllin including its action on tumour tissues. King and Sullivan (1946 & 1947) showed that podophyllin had a colchicine-like effect as a spindle poison on dividing cells. The destructive effect of podophyllin on cancer cells was demonstrated on experimental animals by Hartwell and Shear (1947). The renewed interest in podophyllin led to the isolation and identification of several constituents.

V. CONSTITUENTS OF PODOPHYLLIN

Podophyllin is a mixture of biologically active lignans, lignan glucosides and some inactive flavonol pigments. Lignans can be defined as a family of plant products characterised by a carbon skeleton formed by dimerisation of two phenylpropanoid units at the ß-carbon atom of the propane side chain (Erdman, 1955; Pettit, 1977; Stahl, 1969). Podophyllotoxin (Figure 1) was the first podophyllin lignan to be isolated from *P. peltatum* by a

crude precipitation technique (Hartwell & Schrecker, 1958; Vogelzang et al. 1982). Podophyllotoxin was also later isolated from *P. emodi* and shown to be identical to podophyllotoxin from *P. peltatum* (Hartwell & Schrecker, 1958). The original structure proposed for podophyllotoxin

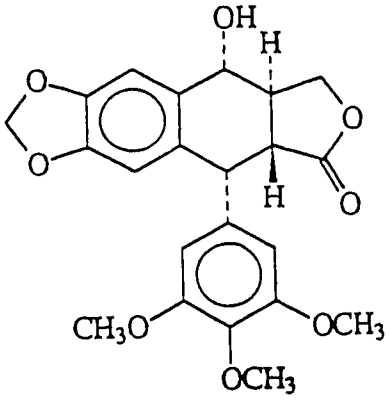


Figure 1. Podophyllotoxin

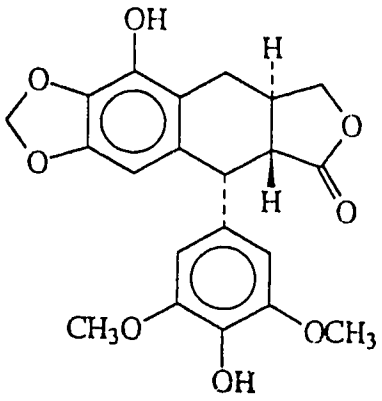


Figure 2. α-Peltatin

was later reversed after extensive structural studies by Hartwell and his collaborators (Hartwell & Schrecker, 1953a; 1953b).

α-Peltatin (Figure 2) and ß-peltatin (Figure 3), were isolated in 1947 and 1948 respectively from *P. peltatum*

(Hartwell, 1947; Hartwell & Detty, 1948). Alumina column chromatography with a 1:1 ratio of ethanol-benzene was used to separate the two peltatins (α- and ß-) and the podophyllotoxin. When the same chromatographic system was used for podophyllin from *P. emodi*, 4'-demethylpodophyllotoxin (Figure 4), picropodophyllin glucoside (Figure 5), and podophyllotoxin were isolated (Nadkarni et al. 1953). Purity of these compounds was significantly enhanced by chromatography with chloroform-methanol or chloroform-ether mobile phases (Hartwell & Schrecker, 1958) on a silica gel column. Desoxypodophyllotoxin (Figure 6) and dehydropodophy-

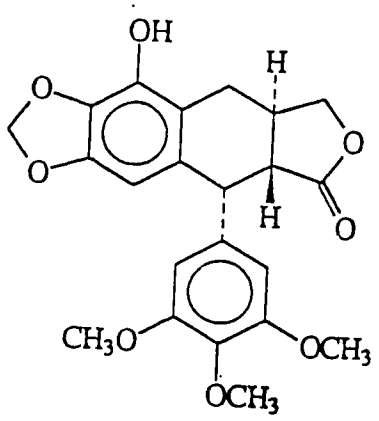


Figure 3. ß-Peltatin

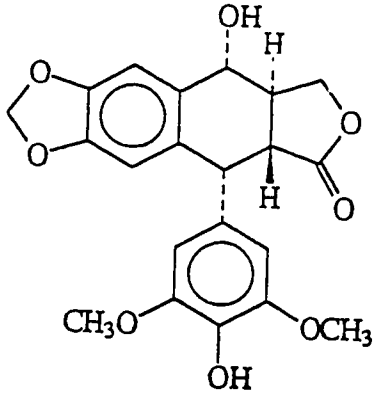


Figure 4. 4'-Demethylpodophyllotoxin

llotoxin (Figure 7) were isolated from *P. peltatum* but in very small quantities (Kofod & Jorgensen, 1954; 1955). Some of the reported podophyllin constituents from *P. peltatum* include podophyllotoxin (20%), α-peltatin (7%), ß-peltatin (13%), trace quantities of 4'-demethylpodophyllotoxin, and the glycosides in very small quantities (Hartwell & Schrecker, 1958). *P. emodi* contained podophyllotoxin (40%) and 2% of 4'-demethylpodophyllotoxin, with small quantities of the glycosides. These constituents were originally present in the plant as ß-D-glucoside (Merck Index, 1989) and were probably generated during the extraction process. The lignan glucosides have little biological activity (Jardine, 1980).

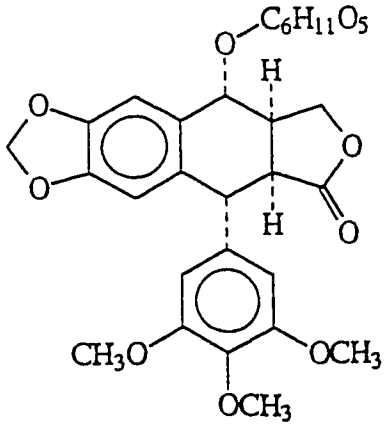


Figure 5.Picropodophyllotoxin Glucoside

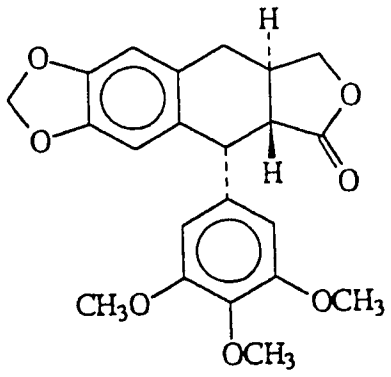


Figure 6.Desoxypodophyllotoxin

The lignan glucosides were isolated by chromatography with a more polar mobile phases. The isolated glucosides were not hydrolysed by maltase or reduced by copper reagents, which indicates that the sugars were pyranoses in the ß configuration (Hartwell & Schrecker, 1958). They were hydrolysed readily with ß-glucosidases, and the lignan and their sugars were identified after the hydrolysis (Nadkarni et al. 1953).

VI. PHARMACOLOGY OF PODOPHYLLIN CONSTITUENTS

1. INTRODUCTION

Pharmacological activities of podophyllin have now been identified in most of its isolated constituents. Podophyllotoxin was identified and confirmed to be responsible for the anti-genital wart activity (Marcus & Camisa, 1990). Desoxypodophyllotoxin have liver protective property against drug induced liver damage (Kiso, 1982), and anti-cancer effects were due to podophyllotoxin, α and ß-peltatin, and 4'-demethylpodophyllotoxin (Jardine, 1980). The purgative action of podophyllin were attributed mainly to the peltatins (Morton, 1977).

2. ANTI-WART ACTIVITY OF PODOPHYLLIN AND PODOPHYLLOTOXIN

Podophyllin is the drug of choice for the treatment of genital warts. It is most effective for treating moist condyloma which is relatively new and has not spread significantly (Von Krogh, 1981). Podophyllin is recomended for the treatment of external genital warts, perianal warts, and accessible meatal warts. Podophyllin has also been used for the treatment of benign papillomas such as plantar warts (Krogh, 1989). Podophyllin has been used to treat nasal and laryngeal papillomas (Benneth & Grist, 1985).

Podophyllotoxin, has been identified as the main constituent of podophyllin for anti-genital wart activity (Edwards, 1988; Marcus & Camisa, 1990). Podophyllotoxin is

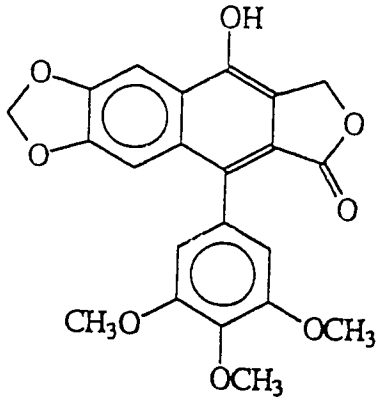


Figure 7. Dehydropodophyllotoxin

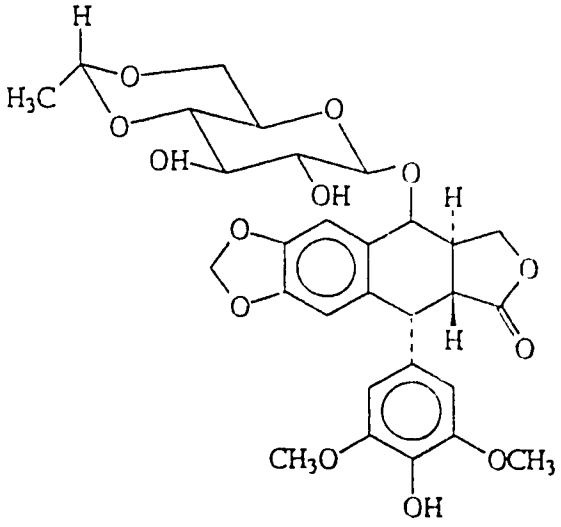


Figure 8. Etoposide (VP 16 213)

more effective and has fewer side effects than podophyllin in the treatment of genital warts (Lassus, 1987). Clinical studies reported a higher cure rate and a lower relapse rate with 0.5% podophyllotoxin than with 20% podophyllin. Podophyllotoxin as a pure, stable, and standardised drug may replace podophyllin for the treatment of genital warts (Edwards et al. 1988; Lassus, 1987).

3. ANTI-CANCER ACTIVITY OF PODOPHYLLOTOXIN AND ITS DERIVATIVES

Tumour-damaging properties of podophyllin were first documented by Bentley in 1862 (Vogelzang et al. 1982) and later by Hartwell and Shear (1947). The anti-cancer effects of podophyllin, and its constituent podophyllotoxin, were tested on experimental animal models with some success, but human trials were stopped because of excessive local and systemic toxicity of the drug (Vogelzang, 1982; Bettley, 1971).

Local side effects of podophyllin include blistering of normal skin and mucous membranes, severe necrosis, and scarring of the ano-genital area (Krogh, 1989; Miller, 1985). The systemic toxic effects of podophyllin are usually multisystemic and may occur when podophyllin is topically applied to a large area, in excessive amounts or is allowed to remain in contact for a long time (Miller, 1985).

Podophyllin is lipid soluble and is readily absorbed through the gastrointestinal system, skin, and mucous surfaces (Cassidy et al. 1982). Symptoms of toxicity include abdominal pain, fever, dizziness, vomiting, tachycardia, peripheral neuropathy, coma, loss of reflexes, and death (Rate et al. 1979; Stoehr et al. 1978). Bone marrow depression due to mitotic arrest at metaphase may occur as a result of an aggressive podophyllin treatment.

The disappointing results of the human trials of podophyllin and podophyllotoxin caused a decline in research interest, but efforts at chemical modifications did not stop. Earlier research studies established that although the lignan glucosides were 10^2 - 10^4 times less active than podophyllotoxin, they did not produce the noxious side

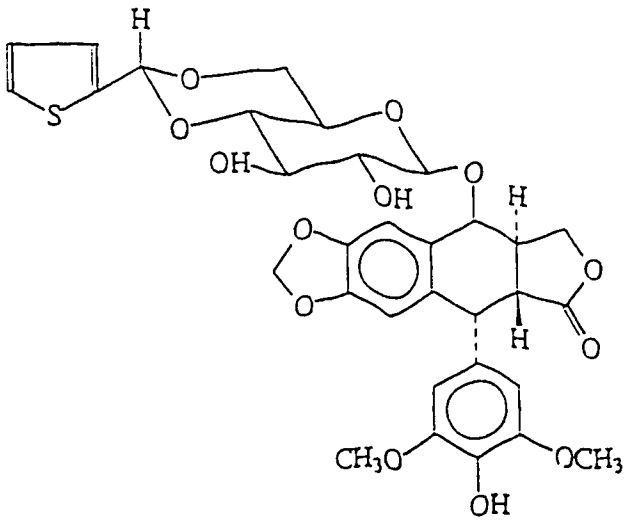


Figure 9. Teniposide (VM 26)

effects of their parent lignans (Jardine, 1980). Partial substitution of the glucoside residue by condensation with various aldehydes resulted in increased activities of the lignan glucosides, to the levels of podophyllotoxin, without increase in side effects. Podophyllotoxin benzylidine ß-D-glucopyranoside and 4'-demethylpodophyllotoxin benzylidine ß-D-glucopyranoside, generated from these chemical modifications, underwent clinical trials but were still found to be too toxic for clinical use.

Kuhn and Wartburg working for Sandoz Laboratories, developed a glucosidation procedure for the synthesis of epipodophyllotoxin from the processes of total synthesis of the podophyllotoxin (Jardine, 1980). Epipodophyllotoxin glycoside products were not very active, but the 4'-demethylepipodophyllotoxin products were. Some cyclic acetals and ketals of 4'-demethylepipodophyllotoxin ß-D-glucopyranoside showed superior anti-cancer activity with fewer side effects (Jardine, 1980; Vaitkevicius & Reed, 1966). From these groups of compounds, etoposide (VP 16-213, Figure 8) and teniposide (VM-26, Figure 9) were selected for clinical trials. They are 4'-demethyl-1-O-[4,6-O-(ethylidene-ß-D-glucopyranosyl]epipodophyllotoxin and 4'-demethyl-1-O-[4,6-O-(2-thienylidene)-ß-D-glucopyranosyl] epipodophyllotoxin respectively. Etoposide is used for treating of small cell lung cancer, testicular cancer, acute

non-lymphocytic leukaemia, ovarian cancer, and thyroid cancer. Teniposide, on the other hand, is used for paediatric cancers, Hodgkin's disease and non-Hodgkin's lymphomas such as reticulum cell sarcoma.

4. MECHANISMS OF ACTION OF PODOPHYLLOTOXIN AND ITS DERIVATIVES

The mechanisms of action of etoposide and teniposide are different from that of podophyllotoxin. Etoposide and teniposide inhibit the enzyme DNA topoisomerase II, which is involved in the unwinding of the DNA supercoil during replication. This inhibition results in cell death. Podophyllotoxin, on the other hand, is a spindle poison and its mechanism of action is cell division arrest at metaphase. It binds to tubulin subunits and prevents its polymerization into the microtubule that is required for cell division (Dewick, 1989).

5. LIVER PROTECTIVE PROPERTY OF DESOXYPODOPHYLLOTOXIN

Kiso et al. (1982) demonstrated that desoxypodophyllotoxin has a protective action against drug-induced liver damage in experimental animals. They used D-galactosamine to induce liver damage similar to viral hepatitis in rats. One group of rats was pre-treated and the other group was not pre-treated with desoxypodophyllotoxin before they were

given D-galactosamine. Histological examinations showed that rats not pre-treated with desoxypodophyllotoxin had focal necrosis and inflammatory infiltration in their hepatic tissues which were absent in the pre-treated group.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also used to monitor the degree of liver damage in the rats. The elevated concentrations of AST and ALT was an early indication of liver tissue damage (Schiff & Schiff, 1987). Rats not pre-treated with desoxypodophyllotoxin showed higher levels of AST and ALT than the pre-treated group. However, it is not certain if other constituents of podophyllin have this liver protective action as well. Further research is required to establish the mechanism of action.

VII. ANALYTICAL METHODS FOR PODOPHYLLIN

1. INTRODUCTION

Podophyllins obtained from both species of podophyllum have the same physical characteristics and appearance. However, the two differ in their lignan constituents and relative quantities. Podophyllins from the United States Pharmacopoeia and most other pharmacopoeias are obtained solely from *P. peltatum* but podophyllin of the British Pharmacopoeia can be derived from either *P. peltatum* or *P. emodi* (Drew et al. 1987). There are a number of tests used

to differentiate the podophyllin from the two sources.

2. CHEMICAL IDENTIFICATION OF PODOPHYLLIN

Podophyllins are soluble in aqueous potassium or sodium hydroxide to form yellow solutions. The solutions darken on standing and can precipitate resin using acid (Gathercoal & Wirth, 1936). Podophyllins from the two sources have different reactions in the presence of aqueous alcohol and potassium hydroxide. Podophyllin (0.4 g) from *P. peltatum* will gelatinize when mixed with 3 mL of 60% alcohol and 0.5 mL aqueous solution of potassium hydroxide, while podophyllin from *P. hexandrum* will not gelatinize (Gathercoal & Wirth, 1936 ; Wallis, 1967). The two podophyllins can also be differentiated through their reaction with aqueous copper acetate. Extracts from *P. peltatum* will give a bright green colour and extracts from *P. hexandrum* will give a brown precipitate (Gathercoal & Wirth, 1936; Wallis, 1967). For further differentiation of the two resins, 500 mg of podophyllin from *P. peltatum*, when shaken with 30 mL of a dilute solution of ammonia for 30 minutes will yield not more than 50 g of insoluble residue; podophyllin from *P. emodi* will yield between 180 - 250 g of the insoluble residue when shaken with ammonia solution.

VIII. ANALYTICAL METHODS FOR PODOPHYLLIN CONSTITUENTS

1. INTRODUCTION

Various solvent mixtures have been used in silica gel column chromatography to separate the lignans and their glucosides. The constituents of podophyllin are separated on the basis of their relative polarities in mobile phases. The glucoside separation is achieved with a more polar solvent gradient on silica gel while the aglycones require less polar solvents (Hartwell & Schrecker, 1958).

2. THIN LAYER CHROMATOGRAPHIC SEPARATIONS OF THE LIGNANS AND THE LIGNAN GLUCOSIDES OF PODOPHYLLIN CONSTITUENTS

Thin layer chromatography (TLC) methods have been used effectively to separate and visualize the lignans and their glucosides. The most effective TLC technique for the separation of the lignan and the glucoside seems to be stepwise developmental TLC (Stahl & Kaltenback, 1969). In this method, the TLC chromatogram is developed to two different heights with two different solvents. In the first step, a run of 6 cm with chloroform-methanol (90 : 10) will separate glucosides but move the lignans to the solvent front. The lignans are then separated using a less polar solvent mixture (chloroform : acetone - 65:35) and developing the chromatogram to a height of 12 cm. The

developments.

The separated compounds are first visualized under short-wave UV light and then sprayed with sulfuric acid-acetic anhydride (1:3). The sprayed chromatogram is heated for about 10 minutes at 100°. Most of the podophyllum lignans and their glucosides are seen under short wave UV light as fluorescence quenching spots on TLC plates containing fluorescent indicator. A spray reagent of silver nitrate (0.1N) can be used to differentiate the peltatins (α- and ß-), 4'-demethylpodophyllotoxin, podophyllotoxin, and the other lignans (Stahl, 1969). The 4'-demethyl-podophyllotoxin and α-peltatin give jet black spots at room temperature and ß-peltatin turns brownish black. Other lignans are shown as white oily spots which only after heavy spraying will turn brownish after several hours. The separation of the lignans and their glucosides on TLC plates can only be observed under UV light if the plates are prepared with fluorescent indicator.

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PODOPHYLLIN CONSTITUENTS

One of the earliest uses of HPLC to analyze the constituents of podophyllin lignans was by Cairnes et al. (1980). They developed a qualitative method for detecting desoxypodophyllotoxin in a crude plant extract of

Austrocedrus chilensis. In the experiment, the HPLC retention time of authentic desoxypodophyllotoxin was used for its identification in the crude extracts. The peak at the suspected retention time was increased in size when the crude extract was spiked with an authentic sample of desoxypodophyllotoxin. *Austrocedrus chilensis* is known to contain desoxypodophyllotoxin, but it was not physically isolated, identified, and analyzed in the investigation.

Lim and Ayres (1983) developed a series of mobile phases for the resolution of the seven known diastereoisomers of podophyllotoxin. A ternary mobile phase of acetonitrile-methanol-water (22.5 : 22.5 : 55), dimethyl sulfoxide-methanol-water (10 : 25 : 35) or a quaternary solvent mixture of acetonitrile-dimethyl sulfoxide-methanol-water (20 : 20 : 10 : 50) was required for the separation of diastereoisomers and functional group derivatives.

C. STATEMENT OF THE PROBLEM

Thin layer chromatography and gravity elution column chromatography have been useful methods of analysis for podophyllin constituents, but they are not precise quantitative methods. The estimations of podophyllin constituents were reported from either preparative TLC or gravity elution column chromatographic analysis (Hartwell & Detty, 1950; Nadkarni et al. 1953), and the amounts of the constituents vary from one report to another.

The inconsistency in the quantities of podophyllin constituents reported are caused by the imprecise methods of determination (Hartwell & Schrecker, 1958; Wallis, 1967; Gathercoal & Wirth, 1936; and Leung, 1980), resulting from loss of sample to the silica gel or alumina in preparative TLC or gravity elution column chromatography. The goal of the present investigation was to use HPLC to determine the quantities of the podophyllin constituents. The use of HPLC to measure directly the quantities of the constituents present in the resin may be a more definitive and precise method for quantitative determination.

D. EXPERIMENTAL

INSTRUMENTS:

1. Phillips PU 8740 Ultraviolet/Visible Spectrophotometer (Pye Unicam Ltd, Cambridge, Great Britain)
2. Nicolet FT Infrared Spectrophotometer (Nicolet Instrument Corporation, Madison, Wisconsin)
3. Bruker AM 300 FT Nuclear Magnetic Resonance Spectrophotometer (Spectrospin, Toronto, Canada)
4. Waters High Performance Liquid Chromatography Apparatus (Waters Associates, Milford, Massachusetts)

(a). Ultraviolet Detector (Waters model 468)

(b). Solvent Programmer (Waters model 660)

(c). Two HPLC Pumps (Water model 501)

5. Hewlett Packard 3390A Integrator (Hewlett Packard Co., Avondale, Pennsylvania)
6. Buchler Flash Evaporator (Buchler Instruments, Fort Lee, New Jersey)
7. Sartorius Electronic Analytical Balance (Sartorius Instruments Ltd, Surrey, Great Britain)
8. Micro Hammer-Cutter Mill (Glen mills, Clifton, New Jersey)
9. Corning pH Meter 220 (Corning, New York, New York)
10. Buchler Fractomette 220 Automatic Fraction Collector (Buchler Instruments, Fort Lee, New Jersey)

11. Mineralight UV lamp Model UVGL - 58 (Ultra-Violet Products Inc., San Gabriel, California)
12. Mettler FP1 Melting point Apparatus (Mettler Instrument Corporation, Princeton, New Jersey) (Melting points were uncorrected)
13. Eastman Chromatogram Chamber (Eastman Kodak Co., Rochester, New York)
14 Whatman C_{18} HPLC column (PXS 10/25) of partisil® 5 μm particle size (Whatman Inc., Clifton, New Jersey)

II. MATERIALS

1. Roots and Rhizomes of *Podophyllum peltatum* (Penick, New York, New York)
2. Podophyllin from *Podophyllum emodi* (Penick, New York, New York)
3. Silicic Acid 100 mesh (Mallinckrodt Chemical Works, Pointe Claire, Quebec)
4. Kieselgel silica gel for TLC (Merck, Switzerland)
5. Podophyllotoxin (Aldrich Chemical Co., Milwaukee, Wisconsin)
6. Washed sea sand (Fisher Scientific, Nepean, Ontario)
7. Pre-coated Analytical TLC Plates (silica gel 0.25mm) (Eastman Kodak Co., Rochester, New York)

III. SOLVENTS

1. Ethanol 95% and 85% (Commercial Alcohols Inc., Winnipeg, Manitoba)
2. Acetone (Anachemia, Toronto, Ontario)
3. Methanol (Mallinckrodt, Mississauga, Montreal)
4. Chloroform (Mallinckrodt, Mississauga, Montreal)
5. Dichloromethane (General Intermediates of Canada, Edmonton, Alberta)
6. Sulfuric acid (Allied Fisher Scientific, Toronto, Ontario)
7. Acetic anhydride (Caledon Laboratories Ltd, Georgetown, Ontario)
8. Ethyl acetate (Anachemia, Mississauga, Montreal)
9. Acetonitrile (OmniSolv, Toronto, Ontario)
10. Deuterochloroform (Adrich Chemical Co., Milwaukee, Wisconsin)

All solvents met A.C.S. specifications.

IV. PODOPHYLLIN EXTRACTIONS FROM *PODOPHYLLUM PELTATUM*

Dried roots and rhizomes of *P. peltatum* were ground with a micro hammer-cutter mill in the fumehood. Five hundred g of the obtained powder was soaked in 85% ethanol overnight. The material was extracted in a 1000 mL percolator using ethanol (85%), until it were exhausted of its resin. Two litres of the percolate obtained was evaporated to a thin syrup with a flash evaporator under a reduced pressure at 40°. The syrup was poured with constant stirring into a litre of cold water (below 10°) containing one percent hydrochloric acid. The resulting precipitate was allowed to settle and the clear liquid was decanted. The precipitate was washed with two portions of 500 mL of cold water and dried overnight at room temperature. See Figure 10 for the summary of the extraction method.

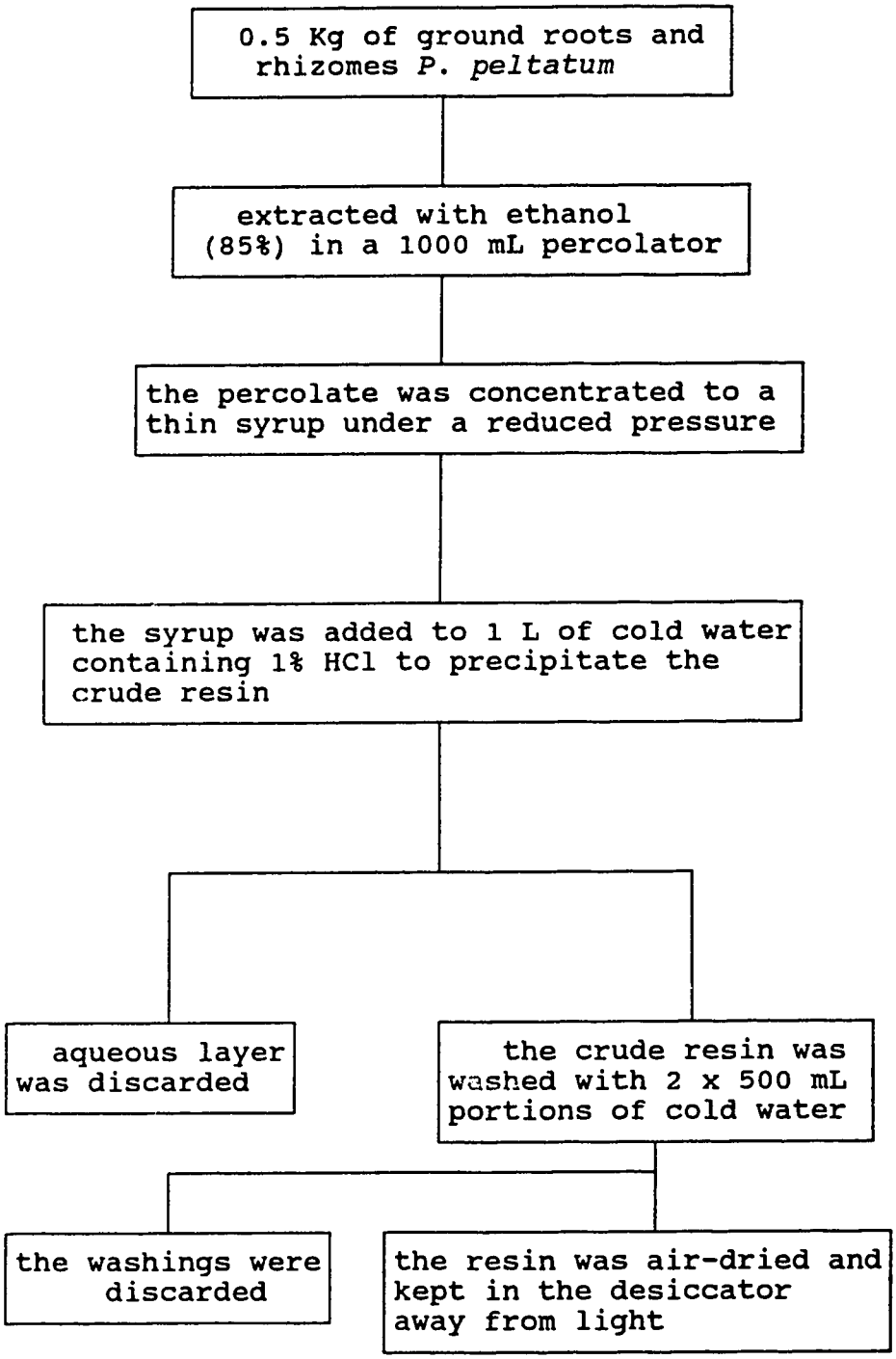


Figure 10. Flow-chart summary of podophyllin extraction from *P. peltatum*.

IV. SEPARATION OF PODOPHYLLIN FROM *P. EMODI*: METHOD ONE.

Eighty g of silicic acid and thirty g of Celite® were thoroughly mixed with two hundred mL of chloroform. The slurry was gravity packed into a chromatographic column (25 x 2 cm) with glass wool stuffed at the column outlet. One hundred mL of chloroform was added to the column prior to its packing. After packing, excess solvent was allowed to drain through the column outlet, leaving enough solvent to just cover the column surface. Two g of Indian podophyllin dissolved in a minimum volume of chloroform-methanol was applied to the column. Podophyllin is not completely soluble in methanol or chloroform but is soluble in the mixture of the two. The column surface was packed with washed sea sand to stabilize the surface against the in-coming mobile phase. Chloroform alone was used to elute the column until the eluting fractions were transparent under UV light. The column was eluted with chloroform-methanol in the composition given in Table 1.

The flow rate was manually adjusted to one mL per minute, and ten mL fractions were collected in a fraction collector. Collected fractions were evaporated to dryness under reduced pressure and re-dissolved with a few drops of methanol or chloroform for TLC analysis.

Table 1. Solvent compositions used for the chromatography of podophyllin from *P. emodi*: method one.

Chloroform (%)	Methanol (%)	Total Volume (mL)
98	2	300
96	4	200
94	6	200
92	8	200
90	10	200
86	14	200
82	18	200
80	20	200
75	25	200
70	30	200
50	50	200
10	90	200

V. THIN LAYER CHROMATOGRAPHY (TLC)

Two types of TLC, preparative and analytical were used for the investigation.

1. PREPARATIVE THIN LAYER CHROMATOGRAPHY

Preparative TLC plates were prepared with silica. A slurry of the silica gel in distilled water (1:2) was spread on 20 x 20 cm glass plates to a thickness of 0.75 mm. The plates were air-dried at room temperature overnight and heated in an oven for two hours at 100°. The activated plates were stored in a desiccator until used.

2. ANALYTICAL THIN LAYER CHROMATOGRAPHY

Pre-coated TLC plates (silica gel 0.2 mm) were used for the qualitative analysis. The fractions from the silicic acid column, in a minimum volume of methanol, were spotted 3 cm from the bottom of TLC plate and 1 cm apart. Mobile phase containing chloroform-methanol (90 : 10) was used to develop the chromatogram to the height of 6 cm and it was dried for thirty minutes at room temperature. The second mobile phase consisting of chloroform-acetone (65 : 35) was used to develop the chromatogram to the height of 12 cm. The separation on the chromatogram was first observed under the short-wave UV light and then sprayed with sulfuric acid-acetic anhydride (1 : 3). The sprayed chromatogram was heated for about 3 minutes at 100° instead of 15 minutes as reported by Stahl (1969). Fractions with the same Rf value and colour characteristics in the TLC analysis were combined for further analysis. The following section describes the

treatment of the major fractions from the above column.

FRACTION. A.1.1.

This fraction was the first eluted fraction from the column, with Rf value of 0.83. It had a single isolated spot with the same colour characteristics and Rf value as authentic podophyllotoxin. The fraction was evaporated to dryness under a reduced pressure. The resulting residue was dissolved in a minimum volume of ethanol (85%) in a ten-mL Erlenmeyer flask. The solution was concentrated under reduced pressure to a supersaturated state and allowed to crystallize at room temperature. The crystals were dried overnight in a desiccator and stored at 4°.

FRACTION. A.1.2.

The fraction had a major spot with the same Rf value and colour characteristics as the authentic podophyllotoxin but interfering constituents were visible on TLC under UV light. The fraction was concentrated and rechromatographed with forty g of silicic acid and fifteen g Celite®. The column was gravity packed as before, but initial elution was with 300 mL of chloroform. Chloroform-ethyl acetate was used in the ratios described in Table to elute the column. The fractions were collected and analyzed by TLC as described earlier. A single spot was observed when the isolated substance, podophyllotoxin, and A.1.1. were spotted together and chromatographed in TLC.

Table 2. Solvent compositions used for rechromatography of fraction A.1.2.

Chloroform (%)	Ethyl Acetate (%)	Total Volume (mL)
98	2	200
96	4	200
94	6	200
92	8	200
90	10	200
86	14	200
82	18	200
78	22	200
75	25	300

FRACTION. A.1.3.

The fraction had a single isolated spot under UV and TLC, with a Rf value of 0.64. The fraction was evaporated to dryness and redissolved in a minimum volume of ethanol for crystallization. This fraction failed to crystallize from ethanol, contrary to reported results (Hartwell & Schrecker, 1958), but readily crystallizes from chloroform.

FRACTION A.1.4.

This fraction contained yellow-coloured matter which gave a faint spot at the start of the chromatogram. No constituent was isolated from this fraction. The yellow coloured matter was a substantial constituent of podophyllin but it was not analyzed. See Figure 11 for the summary of the separation of podophyllin from *P. emodi*: method one.

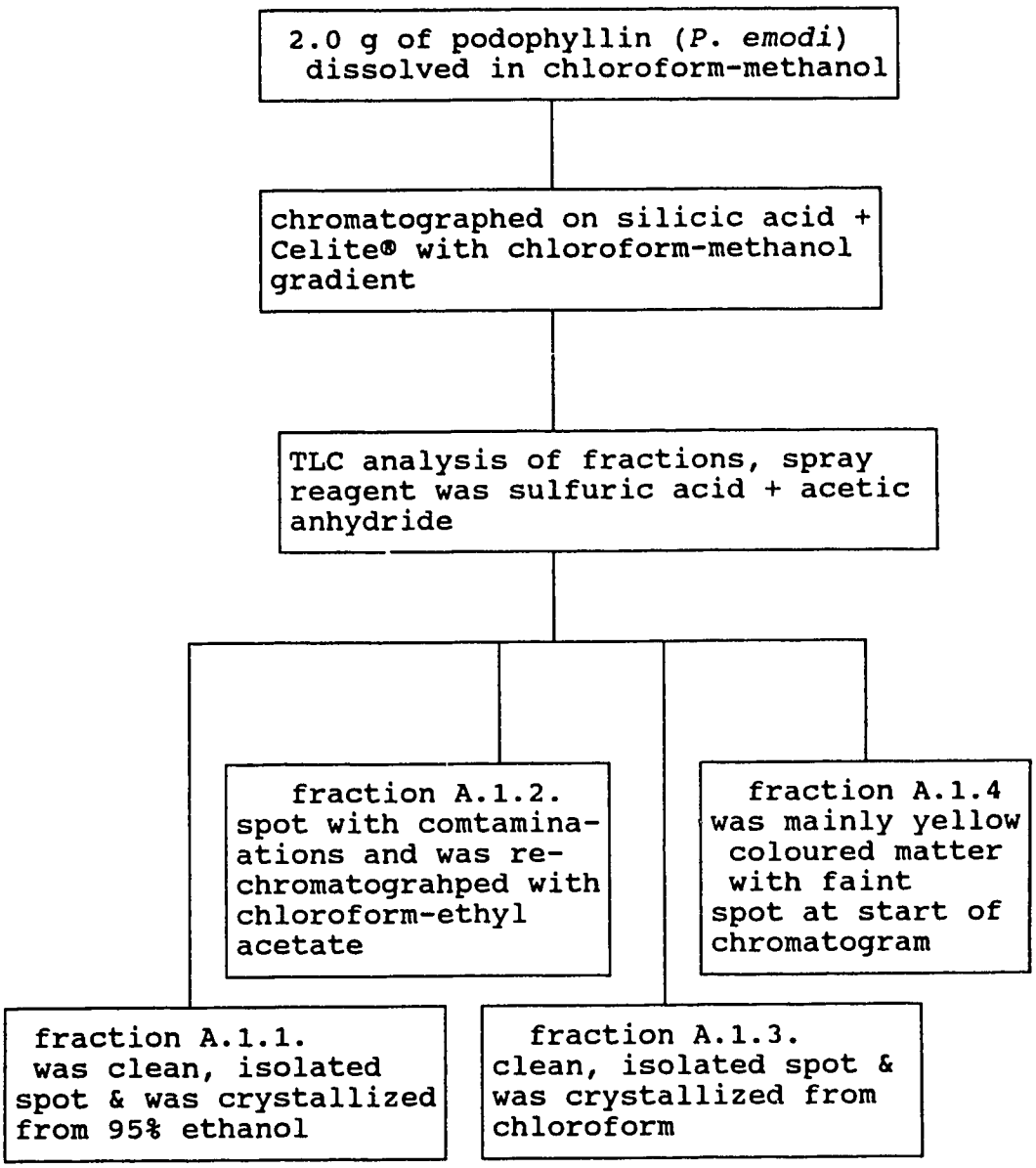


Figure 11. Flow-chart summary separation of podophyllin from *P. emodi*: method one.

VI. SEPARATION OF PODOPHYLLIN FROM *P. EMODI:* METHOD TWO.

One gram of Indian podophyllin was suspended in a separatory flask with forty mL of chloroform-water (1:1) was shaken well and allowed to settle. The chloroform and aqueous layer were removed from the separatory flask. A solid residue left was repeatedly partitioned with forty mL of equal volumes of chloroform and water until it disappeared. The aqueous extracts were combined and extracted three times with fifty mL of chloroform and then concentrated to a thin syrup of about ten mL. The chloroform extracts were combined and filtered with Whatman phase separating filter paper and evaporated to dryness. In TLC analysis, the chloroform extract had two major spots at the upper region of the chromatogram. The aqueous extract had a spot at the lower regions of the chromatogram with a minor trace spot in the upper region.

1. CHROMATOGRAPHIC ANALYSIS OF THE CHLOROFORM FRACTION

The chloroform extract was dissolved in a minimum volume of chloroform and was chromatographed with forty g of silicic acid and fifteen g Celite®. The sample was eluted with five hundred mL of chloroform and chloroform-ethyl acetate in the ratio outlined in Table 3. Fractions A.2.1. and A.2.2. were isolated.

Table 3. Solvent compositions used for the chromatography of podophyllin from *P. emodi*: method two.

Chloroform (%)	Ethyl Acetate (%)	Total Volume (mL)
97.5	2.5	200
95	5	200
92.5	7.5	200
90	10	200
87.5	12.5	200
85	15	200
82.5	17.5	200
80	20	200
75	25	300

FRACTION A.2.1.

The fraction had a single isolated spot with a Rf value 0.83 and it had the same characteristics as the podophyllotoxin. It was crystallized with aqueous ethanol (85%) and dried in a desiccator.

FRACTION A.2.2.

The fraction also had a single isolated spot with Rf value 0.64, but it was crystallized from chloroform.

2. CHROMATOGRAPHIC ANALYSIS OF THE AQUEOUS FRACTION

The aqueous fraction had some yellow-coloured matter that overlapped a greyish spot that was later identified to be glucoside. Fifty ml chloroform was used to precipitate the yellow coloured matter from the thin syrup of the aqueous fraction. The soluble portion was evaporated to dryness and redissolved in a minimum volume of methanol, for chromatography. The spot in this fraction was isolated by preparative TLC. The sample in a minimum volume of solvent was applied in a straight thin line 3 cm from the bottom of preparative TLC plates. The plates were developed to a height of 14 cm in a closed tank with a chloroform-methanol (90 : 10) mobile phase. The fraction A.2.3. below was isolated. The plates were air-dried at room temperature and observed under UV light. A small section of the chromatogram was sprayed with sulfuric acid : acetic anhydride (1 : 3) and heated for two minutes at 100°. An isolated band identified under UV light and from spraying was marked and scraped from the plates. The spot was eluted with methanol and concentrated for TLC analysis.

FRACTION A.2.3.

The fraction had a Rf value (0.20) and spot characteristic of podophyllin glycosides (Stahl, 1973). An enzymatic hydrolysis was performed as described in section XI.5.(a) for the analysis of the aglycone and the sugar of

the glucoside. See Figure 12 for the summary of the separation of podophyllin from *P. emodi*: method two .

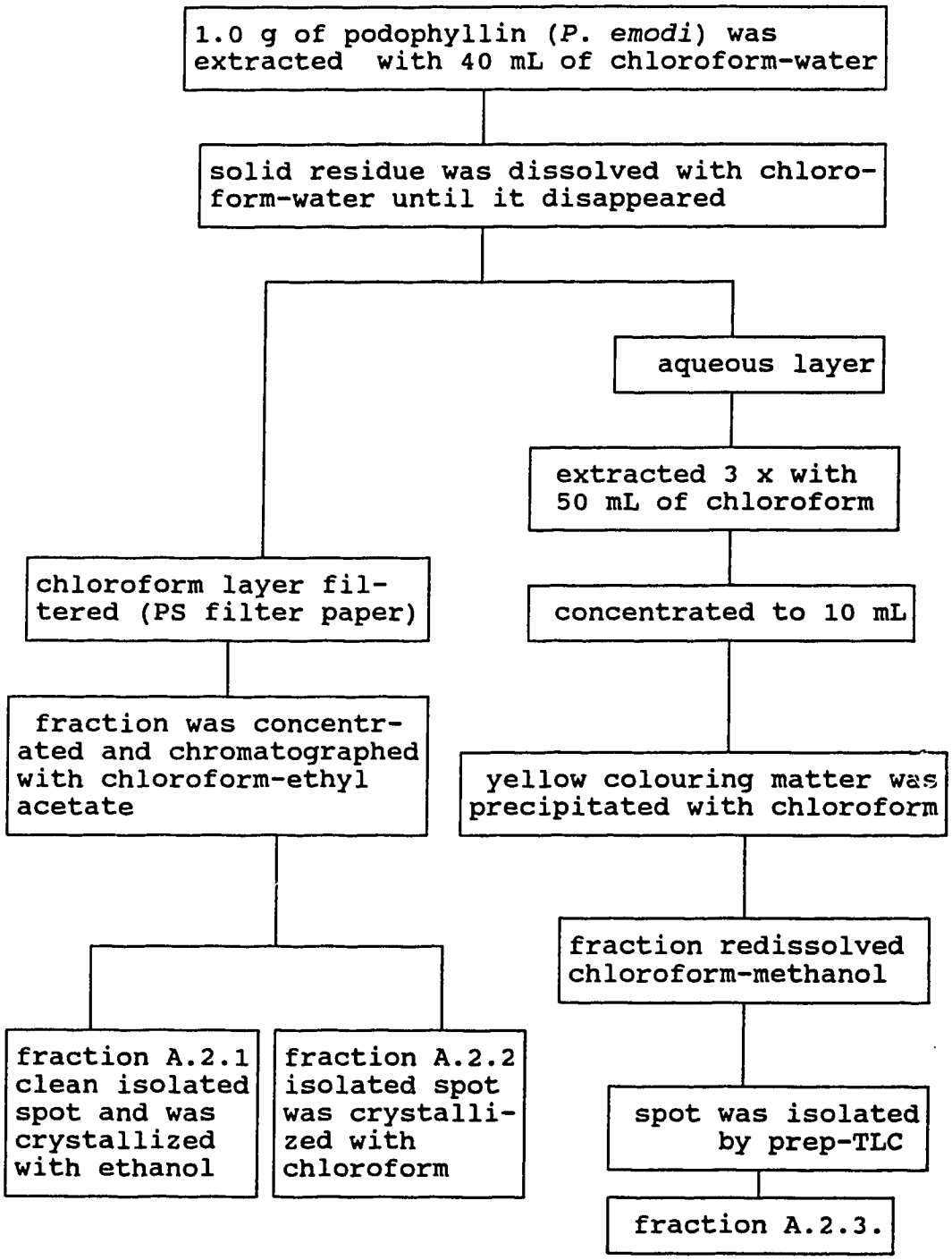


Figure 12. Flow-chart summary of separation of podophyllin from *P. emodi*: method two.

VIII. SEPARATION OF PODOPHYLLIN FROM *P. PELTATUM*: METHOD ONE.

One hundred g of silicic acid and thirty g of Celite® were made into a slurry with 200 mL of chloroform. The slurry was gravity packed into a 25 x 2 cm glass chromatographic columns. Two g of podophyllin from *P. peltatum* in a minimum volume of chloroform-methanol was applied to the column surface. Washed sea sand was used to stabilize the column surface. The column was eluted with one litre of chloroform followed by chloroform-methanol in the ratio contained in Table 4. The flow rate was manually maintained at one mL per minute and ten ml fractions were collected by the fraction collector. The elution was continued until the elute was consistently transparent under UV light and no spot could be identified in the TLC analyses.

Fractions were evaporated to dryness and re-dissolved with a few drops of methanol or chloroform for TLC and UV analysis as described earlier. Fractions with the same characteristics under UV and by TLC were combined for further analysis. The following fractions were isolated: B.1.1., B.1.2.,.B.1.3., B.1.4., B.1.5., B.1.6., and B.1.7..

Table 4. Solvent compositions used for the chromatography of podophyllin from *P. peltatum*: method one.

Chloroform (%)	Methanol (%)	Total Volume (mL)
98	2	300
96	4	200
94	6	200
92	8	200
90	10	200
86	14	200
82	18	200
80	20	200
75	25	200
70	30	200
50	50	200
90	10	200

FRACTION. B.1.1.

This fraction had a major spot with a Rf value 0.84 and with some contamination from the spots below it. It was

evaporated to dryness and rechromatographed with forty g of silicic acid and fifteen g Celite®. The sample was eluted with 300 mL of chloroform followed by chloroform-ethyl acetate in the ratio outlined in Table 5. A single spot (reddish in colour), with Rf value slightly higher than podophyllotoxin was isolated.

Table 5. Solvent compositions used for rechromatography of fraction B.1.1..

Chloroform (%)	Methanol (%)	Total volume (mL)
98	2	200
96	4	200
94	6	200
92	8	200
90	10	200
85	15	200
80	20	300

FRACTION. B.1.2.

The fract[redacted] consisted of spots from B.1.1. and B.1.3.. It was concentrated and separated by preparative TLC.

Methanol was used to elute the components from silica gel. Fractions corresponding to B.1.1. and B.1.3. were identified and added to their respective mother fraction.

FRACTION. B.1.3.

The fraction had a single isolated spot that had the same Rf value 0.83 and colour characteristics as authentic podophyllotoxin in TLC analysis. A single spot was observed when this fraction and authentic podophyllotoxin were spotted together and chromatographed. The crystallization of the fraction from ethanol was as described earlier.

FRACTION. B.1.4.

The fraction consisted of spots from B.1.3. and B.1.5. and was again separated by preparative TLC like fraction B.1.2.. Fractions corresponding to B.1.3. and B.1.5. were added to their mother fractions.

FRACTION. B.1.5.

The fraction had a single isolated spot with Rf value 0.76. The fraction had a reddish spot similar to B.1.1. but it was just the podophyllotoxin spot and it was crystallized from 85% ethanol.

FRACTION. B.1.6.

The major spot had a Rf value of 0.64 with yellow, tailing contaminations. The fraction was concentrated and chloroform was used to precipitate most of the yellow colouring matter. The residue was evaporated to dryness and dissolved in a minimum amount of chloroform-methanol and

rechromatographed on twenty g silicic acid and five g Celite®. Sample elution started with four hundred mL of chloroform-methanol in a 98 : 2 ratio. The sample was eluted with the solvent compositions shown in Table 6.

Table 6. Solvent compositions used for rechromatography of fraction B.1.6.

Chloroform (%)	Methanol (%)	Total Volume (mL)
94	6	200
90	10	200
86	14	200
82	18	200
78	22	200
75	25	200

FRACTION B.1.7.

The fraction contained mainly the yellow colouring matter with a minor spot at the bottom of the chromatogram. It was evaporated to dryness and chloroform was used to precipitate the yellow colouring matter. However, crystals were not isolated from this fraction. See Figure 13 for the summary of the separation of podophyllin from *P. peltatum*: method one.

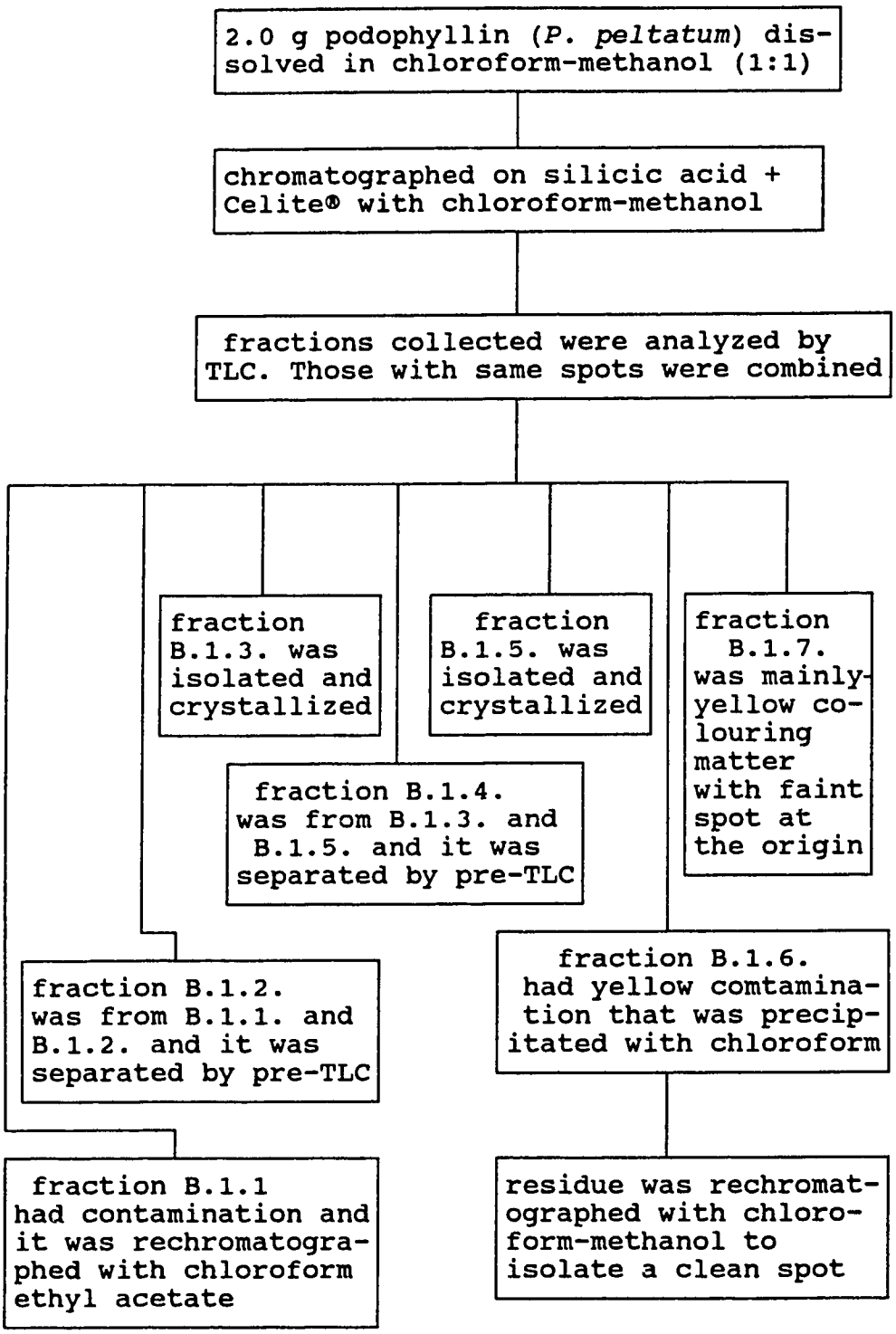


Figure 13. Flow-chart summary of separation of podophyllin from *P. peltatum*: method one

IX. SEPARATION OF PODOPHYLLIN FROM *P. PELTATUM*: METHOD TWO.

One gram of American podophyllin was suspended in a separatory flask containing forty mL of chloroform-water (1:1). The mixture was shaken well and the chloroform and the aqueous layer were removed from the flask. The solid residue was repeatedly partitioned with 40 mL of equal volumes of chloroform and water until it disappeared. The aqueous layer was extracted three times with fifty mL of chloroform. The combined fractions of chloroform were filtered with Whatman phase separating filter paper and concentrated under reduced pressure. TLC of the chloroform fraction showed spots at the upper region of the chromatogram. The aqueous layer was also evaporated to a thin syrup and its TLC showed a spot at the lower region of the chromatogram.

1. CHROMATOGRAPHIC ANALYSIS OF THE CHLOROFORM FRACTION

The chloroform fraction was chromatographed with forty g of silicic acid and fifteen g Celite®. The sample was eluted with one litre of chloroform followed by chloroform-ethyl acetate mixed in the ratios outlined in Table 7. The flow rate was maintained at one mL per minute and the following fractions were isolated: B.2.1, B.2.2., B.2.3., B.2.4., and B.2.5..

Table 7. Solvent compositions used for the chromatography of podophyllin from *P. peltatum*: method two.

Chloroform (%)	Ethyl Acetate (%)	Total Volume (mL)
98	2	200
96	4	200
94	6	200
92	8	200
90	10	200
86	14	200
82	18	200
78	22	200
75	25	300

FRACTION B.2.1.

This fraction had a single isolated spot with a Rf value just above the authentic podophyllotoxin. It was crystallized from ethanol (85%) .

FRACTION B.2.2.

This fraction had a Rf value and spot characteristics of the authentic podophyllotoxin. It was again crystallized from ethanol (85%).

FRACTION B.2.3.

The fraction had an isolated spot that was below that of podophyllotoxin and it was crystallized from ethanol (85%).

FRACTION B.2.4.

The fraction contained an isolated brownish spot lower than the B.2.3. spot and it had a minor yellow contamination that was removed by preparative TLC. It was crystallized from chloroform instead of ethanol.

2. CHROMATOGRAPHIC ANALYSIS OF THE AQUEOUS FRACTION

The aqueous extract was concentrated and chloroform was used to precipitate the yellow colouring matter. The residue was evaporated to dryness, re-dissolved in methanol and separated by preparative TLC as described earlier. A single spot was isolated.

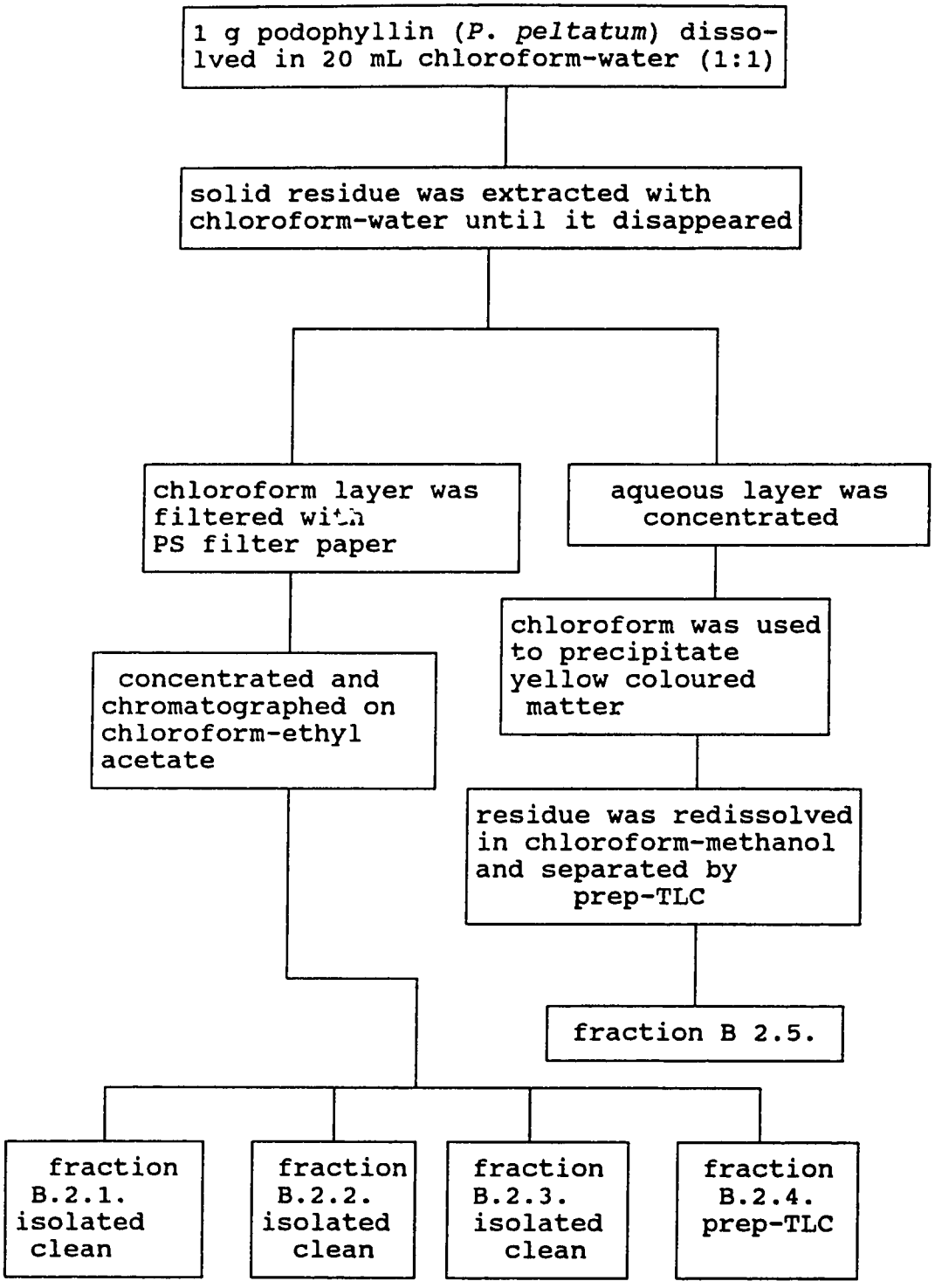


Figure 14. Flow-chart summary of separation of podophyllin from *P. peltatum*: method two.

FRACTION B.2.5.

The fraction had a spot with a Rf value of 0.20. This fraction had a TLC spotting characteristic of a glycoside, and ß-glycosidase enzyme hydrolysis was carried out as described in section VI-(3).

RESULTS

1. ANALYTICAL DATA FOR ß-PELTATIN

Fractions B.1.1. and B.2.1 from *P. peltatum* were identified as ß-peltatin from the following analyses.

IR Spectrum Data:

λ max(KBr) cm^{-1} 3500 - 3400 (OH); 3000 - 2900 (aliphatic stretch); 1770 (C=O of lactam); 1620 & 1600 (C=C of aromatic); 1510 (C-O bend); 1490 (CH_2 bend); 1420 (CH_3 stretch).

NMR Spectrum Data:

δ ($CDCl_3$) 6.38 (singlet, integration 2) for proton H-2'& H-6';6.25 (singlet, integration 1) for proton H-8; 5.98 (doublet, integration 2) for proton O-CH_2-O; 4.95 (singlet) for proton OH-5; 4.65 (doublet, integration 1) for proton H-1; 4.55 (quadruplet, integration 1) for proton H-3aα; 3.95 (quadruplet, integration 1) for proton H-3aß; 3.85 (singlet,

integration 3) for proton O-CH_3-4'; 3.80 (singlet, integration 6) for proton O-CH_3-3' & 5'; 3.22 (quadruplet, integration 1) for proton H-4α; 2.72 (multiplet, integration 2) for proton H-2 & H-3; 2.52 (quadruplet, integration 1) for proton H-4ß.

UV Spectrum data: [illegible] EtOH) (log ε 3.10) 288 nm.

TLC Rf Value: [illegible]

Melting Point: 240 - 243° obtained [242° reported (Hartwell & Schrecker, 1958)].

2. ANALYTICAL DATA FOR α-PELTATIN

Fractions B.1.5. and B.2.3. from *P. peltatum* were shown as α-peltatin according to the following analyses.

IR Spectrum Data:

λ max(KBr) cm^{-1} 3400 - 3300 (OH); 3050 - 2950 (aliphatic stretch); 1750 (C=O of lactam); 1610 (C=C of aromatic); 1520 (C-O bend); 1450 (C-H bend); 1320 (C-O bend).

NMR Spectrum Data:

δ ($CDCl_3$) 6.40 (singlet, integration 2) for proton H-2' & H-6'; 6.25 (singlet, integration 1) for proton H-8; 5.95 (doublet, integration 2) for proton O-CH_2-O; 5.44 (singlet) for OH-4' proton; 4.95 (broad peak) for proton OH-5; 4.65

(doublet, integration 1) for proton H-1; 4.58 (quadruplet, integration 1) for proton H-3aα; 4.00 (quadruplet, integration 1) for proton H-3aß; 3.79 (singlet, integration 6) for proton O-CH_3-3' & 5'; 3.20 (quadruplet, integration 1) for proton H-4α; 2.70 (multiplet, integration 2) for proton H-2 & H-3; 2.55 (quadruplet, integration 1) for proton H-4ß.

UV Spectrum: λ max(EtOH) (log ε 3.02) 287 nm.
TLC Rf Value: ca 0.70.
Melting Point: 240 - 242° obtained [242° reported (Hartwell & Schrecker, 1958)].

3. ANALYTICAL DATA FOR PODOPHYLLOTOXIN

Fractions A.1.1., A.1.2., and A.2.1., from *P. emodi* and fractions B.1.3. and B.2.2. from *P. peltatum* were identified as podophyllotoxin according to the following analyses.

IR Spectrum Data:

λ max(KBr) cm^{-1} 3500 - 3450 (OH); 3100 - 2900 (aliphatic stretch); 1770 (C=O of lactam); 1600 (C=C of aromatic); 1510 (CH_2 bend); 1490 (CH_3 bend); 1410 (C-O bend).

NMR Spectrum Data:

δ ($CDCl_3$) 7.10 (singlet, integration 1) for proton H-5; 6.52 (singlet, integration 1) for proton H-8; 6.35 (singlet, integration 2) for proton H-2' & 6'; 6.03 (doublet, integration 2) for proton O-CH_2-O; 4.85 (doublet, integration 2) for H-4ß proton; 4.65 (multiplet, integration 1) for protons H-1 & H3aα; 4.11 (triplet, integration 1) for H-3aß; 3.85 (singlet, integration 3) for proton O-CH_3-4'; 3.78 (singlet, integration 6) for proton O-CH_3-3' & 5'; 2.8 (multiplet, integration 2) for proton H-2 & H-3; 1.63 (singlet) for proton OH-4.

UV Spectrum: λ max(EtOH) (log ε 3.54) 293 nm.
TLC Rf Value: ca 0.83 the same as authentic podophyllotoxin.
Melting Point: 118° obtained [114 - 118° reported for solvated (Hartwell & Schrecker, 1958)].

4. ANALYTICAL DATA FOR 4'-DEMETHYLPODOPHYLLOTOXIN

Fractions A.1.3. and A.2.2. from *P. emodi* and fractions B.1.6. and B.2.4. from *P. peltatum* were found to be 4'-demethylpodophyllotoxin according to the following analyses.

IR Spectrum Data:

λ max(KBr) cm^{-1} 3550 - 3400 (OH); 3100 - 2800 (aliphatic stretch); 1750 (C=O of lactam); 1610 (C=C of

aromatic); 1480 (C-H bend); 1220 (C-O bend of ester); 1120 - 1000 (primary alcohol).

NMR Spectrum Data:

δ ($CDCl_3$) 7.14 (singlet, integration 1) for proton H-5; 6.54 (singlet, integration 1) for proton H-8; 6.40 (singlet, integration 2) for proton H-2' & 6'; 6.01 (doublet, integration 2) for proton O-CH_2-O; 5.43 (singlet) for proton OH-4'; 4.75 (doublet, integration 1) for H-4ß proton; 4.60 (multiplet, integration 1) for protons H-1 & H3aα; 4.10 (triplet, integration 1) for H-3aß; 3.80 (singlet, integration 6) for proton O-CH_3-3' & 5'; 2.85 (multiplet, integration 2) for proton H-2 & H-3; 1.30 (singlet) for proton OH-4.

UV Spectrum: λ max(EtOH) (log ε 3.40) 293 nm.
TLC Rf Value: ca 0.63.
Melting Point: 247 - 250° obtained [250 - 252° reported (Hartwell & Schrecker, 1958)].

5. IDENTIFICATION OF THE PODOPHYLLIN GLUCOSIDE

Fraction A.2.3. from *P. emodi* and fraction A.2.5. *P. peltatum* were identified as podophyllotoxin glucoside according to the analysis of the chloroform fraction (aglycone) and sugar from the enzyme hydrolysis as described below.

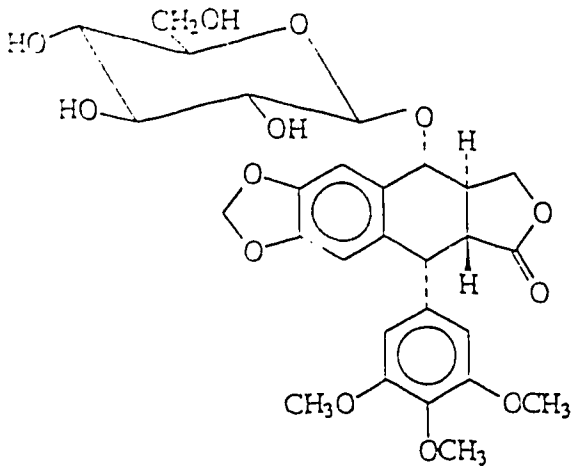


Figure 15. Podophyllotoxin Glycoside

(a). ENZYME HYDROLYSIS OF PODOPHYLLIN GLYCOSIDES (Nadkarni et al, 1953)

ß-Glycosidase enzyme in acetate buffer at pH 5.0 was mixed with the glycoside in the ratio of 1 : 2 of enzyme to glycoside. The mixture was incubated at 37° for twenty hours. At the end of the incubation, chloroform was used to extract the aglycone while the sugar was left in the mother liquor. The chloroform fraction was evaporated to dryness and the aglycone was identified by TLC and HPLC analysis.

The identification of the sugar was by TLC on cellulose. The sugar fraction was evaporated to dryness and redissolved in a minimum volume of water with few drops of methanol. The sample was chromatographed on a TLC plate beside a D-glucose standard. The mobile phase was formic

acid : acetone : tert-butanol : water (15 : 25 : 35 : 25) (Damonte et al. 1971). The spray reagent was prepared by dissolving 4 g of diphenylamine, 4 mL aniline, and 20 mL of 85% phosphoric acid in 200 mL of acetone (Stahl, 1969). The sprayed chromatogram was heated at 85° for about two and half minutes instead of ten minutes reported by Stahl (1969) and Damonte et al. (1971). Various sugars give different colour characteristics.

(b). ANALYSIS OF AGLYCONE FROM THE HYDROLYSIS

The aglycone in the chloroform fraction had the same Rf value and light brown colour characteristic of podophyllotoxin. When authentic podophyllotoxin and the chloroform extract of the two hydrolyses were spotted together and chromatographed on TLC, only a single spot was observed under UV light and on TLC analysis. When the same chloroform extracts were spotted and chromatographed with other aglycones, two spots were observed. In HPLC analysis, the chloroform fraction contained a product with the same retention time as the authentic podophyllotoxin; when they were injected together, only one chromatographic peak was observed.

(c) ANALYSIS OF THE SUGAR FROM THE HYDROLYSIS

Identification of the sugar was by TLC on cellulose as described earlier. The sugar obtained from the hydrolysis

and the authentic D-glucose had the same Rf value of 0.54 and both of them gave a dark grey colour indicative of glucose on the TLC plate. Analysis of the fractions from hydrolysis showed that the hydrolysed fraction was podophyllotoxin glucoside.

6. HPLC QUANTITATIVE ANALYSIS OF PODOPHYLLIN CONSTITUENTS

HPLC analyses of these compounds was performed on an apparatus from Waters Associates previously described in the Instrumentation section. The column used was Whatman PXS ODS -3 column described in the instrumental section. Acetonitrile-water gradient was the mobile phase used and the gradient was over a 20 minute period from 20 to 60% acetonitrile. The flow rate was 1 mL per minute and detection was by UV absorption at 254 nm on a Waters 486 tunable absorbance detector. The injection volume was 10 μl of the various concentrations used in the analyses.

The injection samples for the quantitative analysis were prepared by dissolving 5 mg of the sample in 50 mL acetonitrile - water (1:1). From this concentration of 0.1 mg/mL, serial dilutions of 0.05 mg/mL, 0.025 mg/mL, 0.0125 mg/mL, and 0.00625 mg/mL were made. A calibration curve of these concentrations versus the peak area was generated by regression analysis. The peak area from 0.05 mg/mL of crude podophyllin was used to determine the percentage of the aglycone constituents present in podophyllin. For the

glucoside, the concentration of podophyllin used was 0.1 mg/mL. See Table 8 for the results.

Table 8. Results from the HPLC quantitative analysis of the podophyllin constituents. The result was from an average of three chromatographic analysis.

Compounds	R Squared (r^2)	% in *P. emodi*	% in *P. peltatum*
Podophyllotoxin	0.999719	39.04	19.22
ß-Peltatin	0.999619	-	9.45
α-Peltatin	0.999746	-	9.97
4'-Demethylpod-ophyllotoxin	0.988201	2.32	.58
Podophyllotoxin Glucoside	0.986752	6.28	0.27

Figure 16. Calibration curve for podophyllotoxin

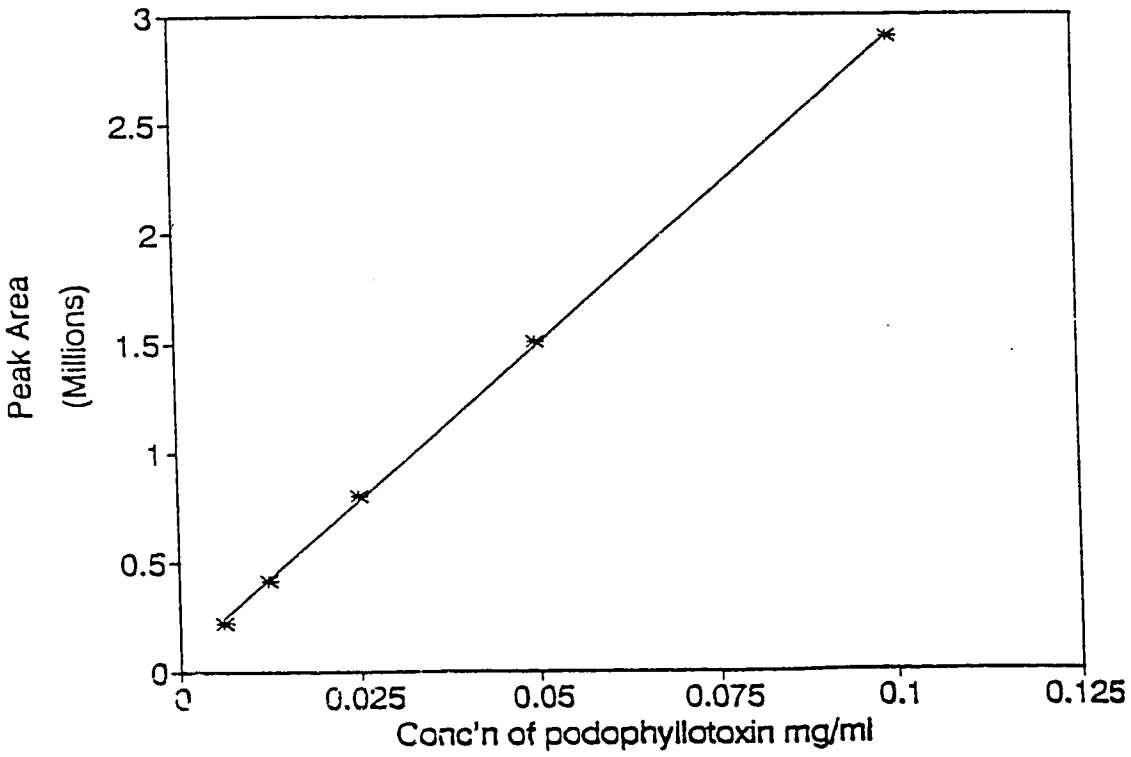


Figure 17. calibration curve for ß-Peltatin.

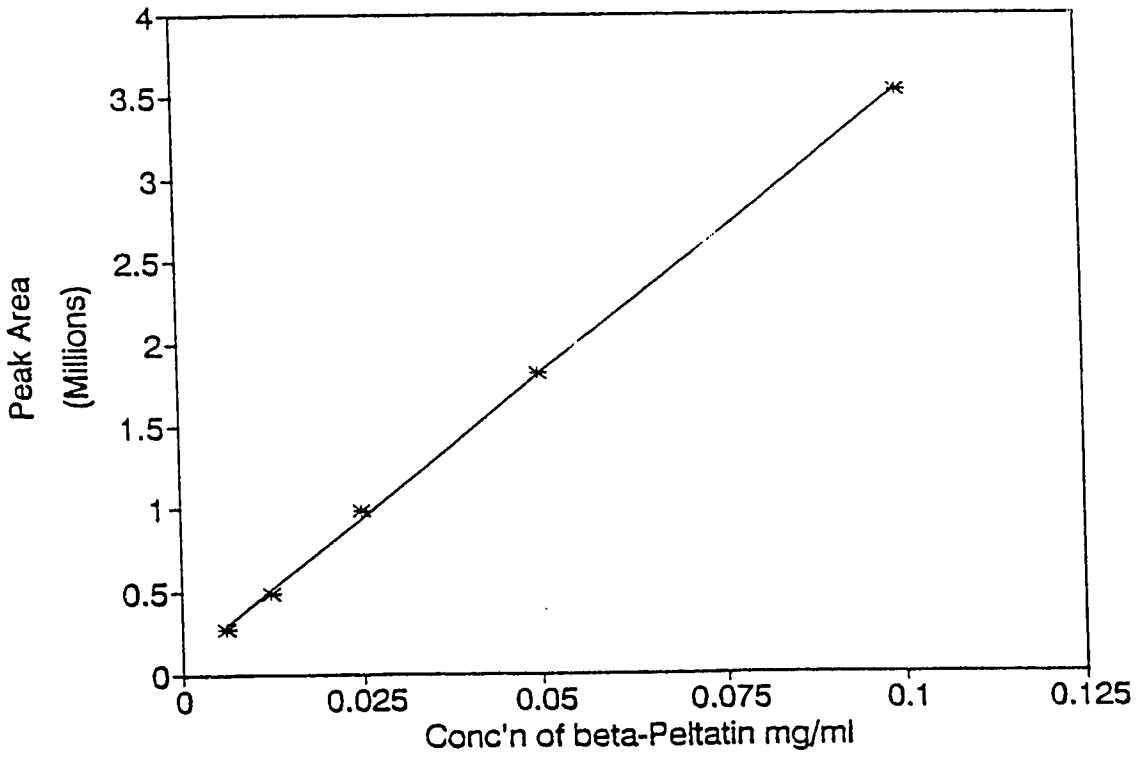


Figure 18. calibration curve for α-Peltatin.

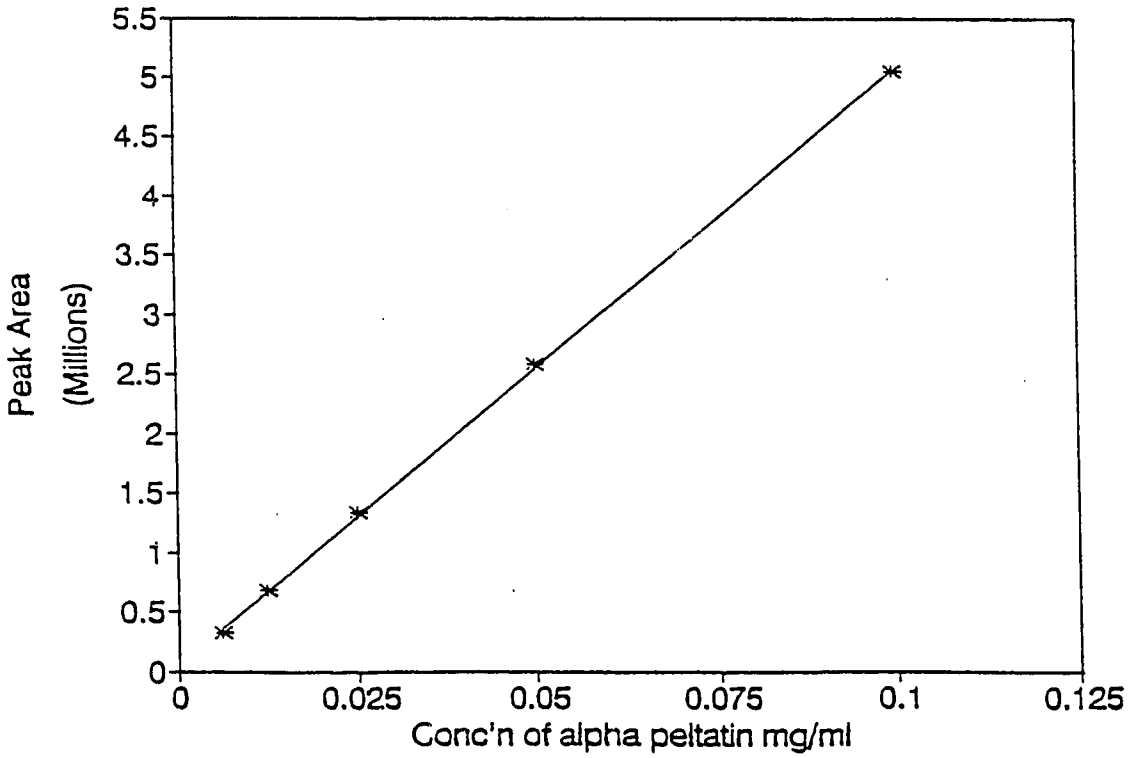


Figure 19. Calibration curve for 4'-Demethylpodophyllotoxin

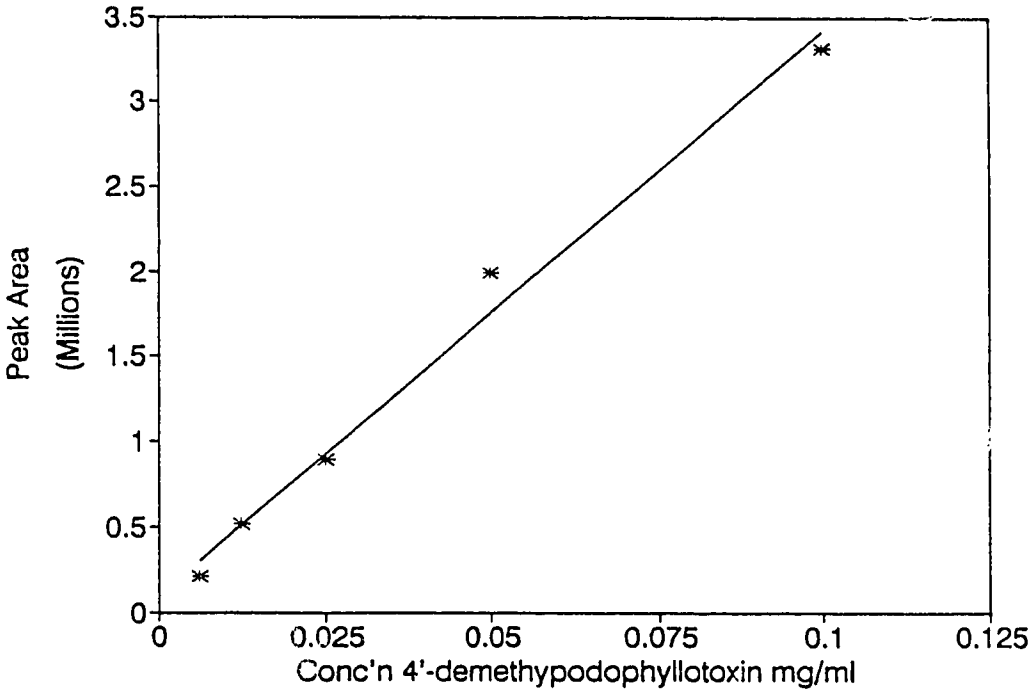
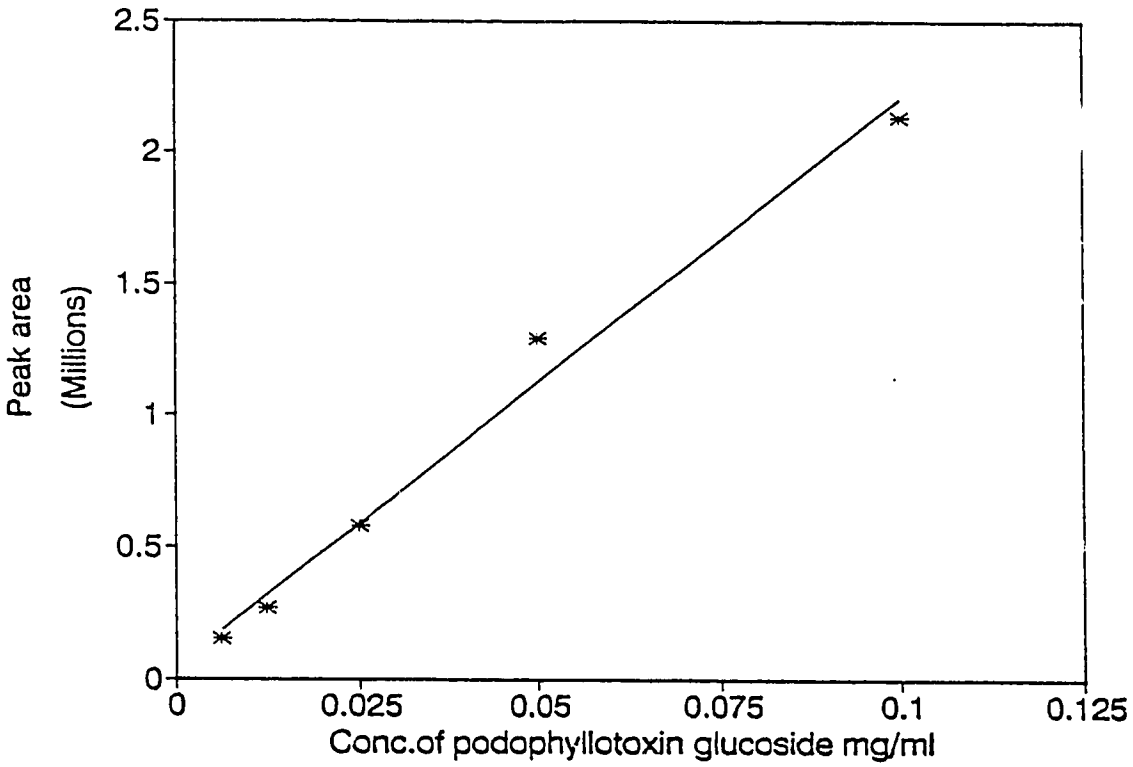


Figure 20. Calibration curve for Podophyllotoxin glucoside



E. DISCUSSION

Podophyllin from *P. emodi* (Indian podophyllin) was obtained commercially and podophyllin from *P. peltatum* (American podophyllin) was extracted from roots and rhizomes. The yield of podophyllin was 3.12%, which was within the expected 3 - 6% yield from *P. peltatum* (Leung, 1980). Podophyllin from the two main sources of podophyllin was used for the present quantitative analysis of the constituents.

Podophyllin from *P. peltatum* and *P. emodi* can be easily differentiated by TLC analysis (Stahl, 1973). Two reddish spots at Rf 0.84 and 0.7 in podophyllin from *P. peltatum* were not present in Podophyllin from *P. emodi*. These two spots, α- and ß-peltatin, are known to be present in American podophyllin and not in Indian Podophyllin (Hartwell & Detty, 1950; Leung 1980).

Podophyllin is a mixture of compounds that vary in polarity as well as other chemical properties. Gravity elution chromatography and TLC was used for many years to separate these constituents. A range of solvents and solvent mixtures has been used to separate the constituents (Stahl, 1973; Jackson & Dewick, 1984; Hartwell & Detty, 1950). Investigators interested in aglycones usually use less polar solvents while those interested in the glycosides use more

polar solvents like methanol or ethanol. Chloroform-methanol was primarily used for the analysis, however, chloroform-ethyl acetate mixture were used in cases where methanol might be too polar. The choice of the mobile phase was to achieve the separation of the aglycone and the glycoside with a two solvent mixture system.

Alumina or silica gel have been used for the separation of the podophyllin constituents in gravity elution chromatography by most investigators (Lee et al. 1986; Hartwell & Detty 1950; Treppendahl & Jakobsen, 1980). Alumina is very polar and will retain the more polar constituents (Treppendahl & Jakobsen, 1980). Silicic acid (100 mesh) and Celite® were used in the present analysis because they showed better separation of the constituents (Hartwell & Schrecker, 1958). Celite® was used to moderate the mobile phase flow rate.

1. THIN LAYER AND GRAVITY ELUTION CHROMATOGRAPHIC SEPARATION OF PODOPHYLLIN CONSTITUENTS

The purpose of the gravity elution chromatographic separation of podophyllin was to obtain pure samples of the constituents for the quantitative analysis. Two methods were used for the separation. The first method (method one) was a more conventional method of chromatographing podophyllin with a mobile phases of varying polarity. Most of the

constituents were eluted by this method but the eluted fractions required repeated rechromatographing followed by preparative TLC in some cases. The isolation of the constituents took a long time and was tedious. Lignan glucosides were not crystallized from this method.

In method two, podophyllin was partitioned between equal volumes of chloroform and water. After a repeated extractions with water and chloroform, the polar glucoside was extracted into the aqueous layer while less polar aglycones were in the chloroform layer, as was revealed by TLC analysis. Weiss et al. (1975) used a similar method for the isolation of cytotoxic lignans from *Linum album*; however, this method was developed without prior knowledge of their work. The isolation of constituents was much faster and less tedious with this method than in method one, and the fractions did not require rechromatographing. Preparative TLC was used in a few cases to remove contaminants from fractions or to remove the yellow colouring matter. Lignan glycoside was isolated by preparative TLC of the aqueous fraction. It should be noted that about 5% of the sample material was lost during the partitioning and the extraction processes.

Separation of the constituents was monitored by analytical TLC which rapidly gave easily identifiable coloured spots for the constituents. Constituents show

various coloured spots on silica gel TLC plates when sprayed with sulfuric acid-acetic anhydride spray reagent (See figure 21). α- and ß-Peltatin show reddish spots that sharply contrast with the dark brown spots of podophyllotoxin and 4'-demethylpodophyllotoxin. The yellow colouring matter normally extended from the spot corresponding to 4'-demethylpodophyllotoxin to the starting point of the chromatogram. The glycoside has a greyish spot that is visible from the yellow colouring smear. The precise Rf values of the constituents were not used for identification purposes because they varied slightly from one chromatographic run to another but their positions relative to the authentic podophyllotoxin were constant. The separation of the constituents by TLC was also monitored under short wave UV light.

Crystallization of each constituent was carried out once it was certain that the isolated constituent was in pure state. TLC analyses, using various mobile phases, and visualization under UV light were used for reconfirmation of purity. All of the isolated aglycone constituents were crystallized from aqueous ethanol except 4'-demethyl-podophyllotoxin. 4'-Demethylpodophyllotoxin was crystallized from chloroform after repeated attempts to crystallize it from ethanol have failed. Its crystallization from chloroform was a chance discovery and contrary to the

reports of the previous investigators (Hartwell & Schrecker, 1958; Nadkarni et al. 1953).

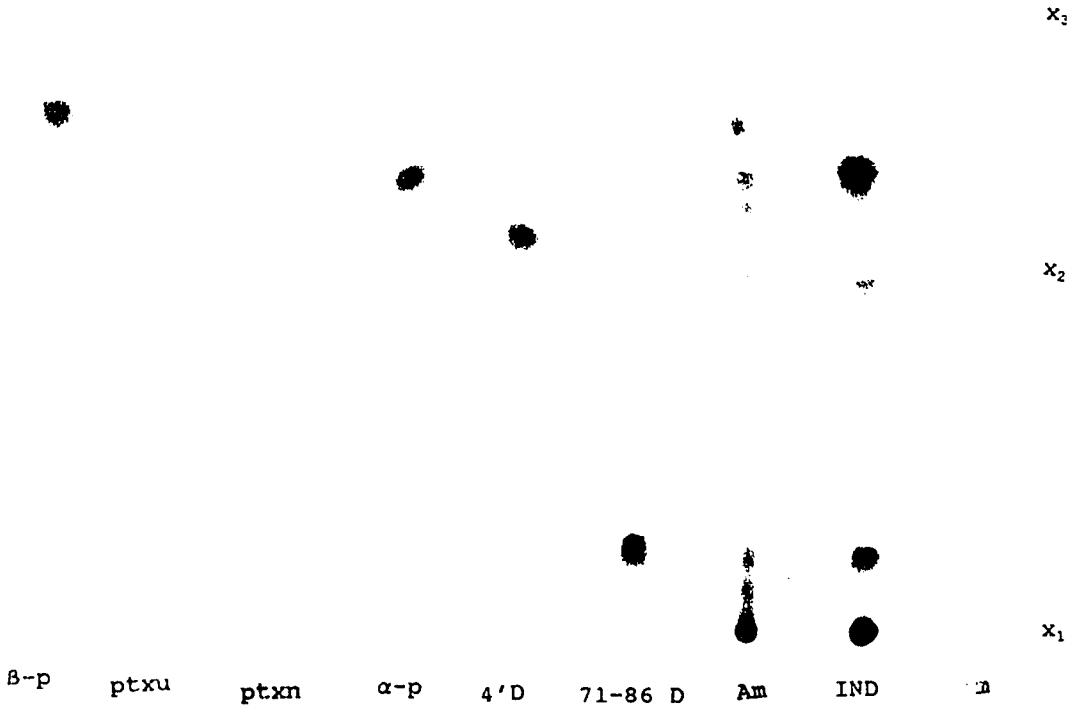


Figure 21. ß-p is for ß-peltatin, ptxn is for podophyllotoxin, ptxu is for authentic podophyllotoxin, α-p is for α-peltatin, 4'D is for 4'-demethylpodophyllotoxin, 71-86 D is for the glucoside, Am is podophyllin from *P. peltatum*, IND is podophyllin from *P. emodi*, Ycm is for the yellow colouring matter. x_1 is the origin, x_2 is the end of first run, x_3 is the end of second run.

SPECTROSCOPIC ANALYSIS

The samples for NMR analysis were dissolved in deuterochloroform. The isolated podophyllotoxin had the same NMR spectrum as the authentic podophyllotoxin. The chemical shift assignments for the isolated podophyllin constituents were in agreement with data and spectra reported by Brewer et al. (1979); Jackson & Dewick, (1984); and Loike et al. (1978). The chemical shift assignments for the methylenedioxy and methoxy protons were made based on both the chemical shift positions and the integrated intensity. The methoxy protons at 3' and 5' have the same chemical environment and therefore had a single peak with an integrated intensity of 6. Podophyllotoxin and ß- peltatin had a second methoxy at 4' position with an integrated intensity of 3, that was absent in α-peltatin and 4'-demethylpodophyllotoxin. α-Peltatin and 4'-demethylpodophyllotoxin had peaks indicating two phenolic groups for α-peltatin at δ 5.95 and δ 4.95, and one phenol and aliphatic alcohol for 4'-demethylpodophyllotoxin at δ 4.75 and δ 1.30.

The isolated podophyllotoxin had the same IR spectrum as authentic podophyllotoxin. The IR spectral data obtained for the isolated constituents were in agreement with those reported by Jackson & Dewick (1984). The carbonyl group of the lactam ring was prominent in all of the spectra.

HPLC QUANTITATIVE ANALYSIS OF THE PODOPHYLLIN CONSTITUENTS

The results obtained from HPLC quantitative analysis of podophyllin constituents differ from most of the values reported over the years for these constituents (Leung, 1980; Morton, 1977; Hartwell & Detty 1950; Hartwell & Detty 1950; Hartwell 1947; Hartwell & Schrecker 1958; Nadkarni et al. 1953; and Stoll et al. 1954). The result from the current analysis showed podophyllin from *P. peltatum* contained 19.2% of podophyllotoxin while podophyllin from *P. emodi* contained 39.0% of podophyllotoxin. These values differ from the quantitative estimates obtained from gravity elution chromatography for podophyllotoxin that ranged from 9.1 to 20% from *P. peltatum* and 40 - 50% from *P. emodi* (Leung, 1980; Morton, 1977; Treppendahl & Jakobsen, 1980; Hartwell & Schrecker, 1958). These estimates are not precise and depended on the experience and method of the investigator. Treppendahl and Jakobsen (1980) reported a determination of 15% of podophyllotoxin from *P. peltatum* resin with normal phase HPLC. However, they did not provide information on their experimental methods and their results has not been confirmed or reported elsewhere by other investigators.

The quantity of ß-peltatin obtained from the analysis was 9.4%, which differs from 4% and 4 - 7.8% obtained by Hartwell and Detty (1948; 1950) from gravity elution chromatography. Other reports of the isolated quantities of

ß-peltatin varied from 5 to 13% (Treppendahl and Jakobsen, 1980; Morton, 1977). However, no trace of ß-peltatin was detected in podophyllin from *P. emodi* by either HPLC or TLC analysis.

Podophyllin from *P. peltatum* contained 9.97% of α-peltatin according to the current analysis. The obtained quantity again differs from the values reported by other investigators, but not by as much as was the case of ß-peltatin. Hartwell (1947) obtained 9% from gravity elution chromatography while Treppendahl & Jakobsen, (1980) reported a yield of 10.7% from normal phase HPLC analysis. No trace of α-peltatin was obtained in podophyllin from *P. emodi* in this analysis.

The quantities of 4'-demethylpodophyllotoxin contained in *P. peltatum* and *P. emodi* were 1.58% and 2.32%, respectively. The estimated quantity of 4'-demethyl-podophyllotoxin previously obtained by gravity elution chromatography from *P. emodi* was 1.7% (Nadkarni et al. 1953; Hartwell & Schrecker, 1958). Trace quantities of 4'-demethylpodophyllotoxin in *P. peltatum* were suggested in a number of references (Hartwell & Schrecker, 1958; Morton, 1977). Its existence in *P. peltatum* was positively confirmed by Jackson and Dewick (1984). The present analysis is the first report of the actual quantity of 4'-demethylpodo-phyllotoxin in podophyllin from *P. peltatum*.

The reports of isolated podophyllotoxin glycoside from the two kinds of podophyllin ranged from 0.5 to 1.0% by the gravity elution chromatography (Stoll et al. 1954; Hartwell & Schrecker, 1958). The current HPLC analyses shows that there is actually 6.28% and 0.27% of podophyllotoxin glucoside present in podophyllin from *P. emodi* and podophyllin from *P. peltatum*, respectively. The difference between the estimates from gravity elution chromatography and the value obtained from this analysis was very profound. The difference was greater than that observed for the aglycones. Under-estimation of the quantity of podophyllotoxin glucoside present in podophyllin might have been caused by excessive retention of the polar glucosides by polar silica gel or alumina normally used in the separation of the constituents.

The results obtained by this HPLC quantitative analysis a reflect a more accurate measure of the quantities of these podophyllin constituents. Gravity elution chromatography used to obtain these reported quantities was not an accurate and reproducible method of quantification (Nadkarni et al. 1953; Hartwell & Detty, 1950). Sample materials lost during chromatography were usually not accounted for, and the effectiveness of the method largely depends on the experience of the investigator. The use of HPLC offers a more reliable, accurate, and reproducible method for

quantitative determination of podophyllin constituents. In the present analysis, the quantity of each of the constituents was determined directly from the crude podophyllin. A calibration curve of the concentration versus the peak area of each of the constituents was made (see figure 16 to 20). The retention time of the constituents were used to aid in the identification of crude podophyllin (see figure 22 and 23). The concentration of podophyllin used for the determination of the aglcones was 0.05 mg/mL while 0.1 mg/mL was used to determine the concentration of the glucoside. A higher concentrations of podophyllin were used for the glucoside because it was assumed to be in much lower concentration than the aglycones.

Podophyllin and its constituents are used in medicine for the treatment of various skin disorders (Edwards et al. 1988). Podophyllotoxin, a constituent of podophyllin, is the lead drug for the synthesis of the anticancer agents etopside and teniposide. Other podophyllin constituents may soon become lead drugs for the synthesis of other pharmacological agents. It is therefore important to establish accurate estimate of the quantities of these constituents present in the two types of podophyllin. A knowledge of the quantities of the constituents in podophyllin will be of great benefit to those who require podophyllin and its constituents in large quantities.

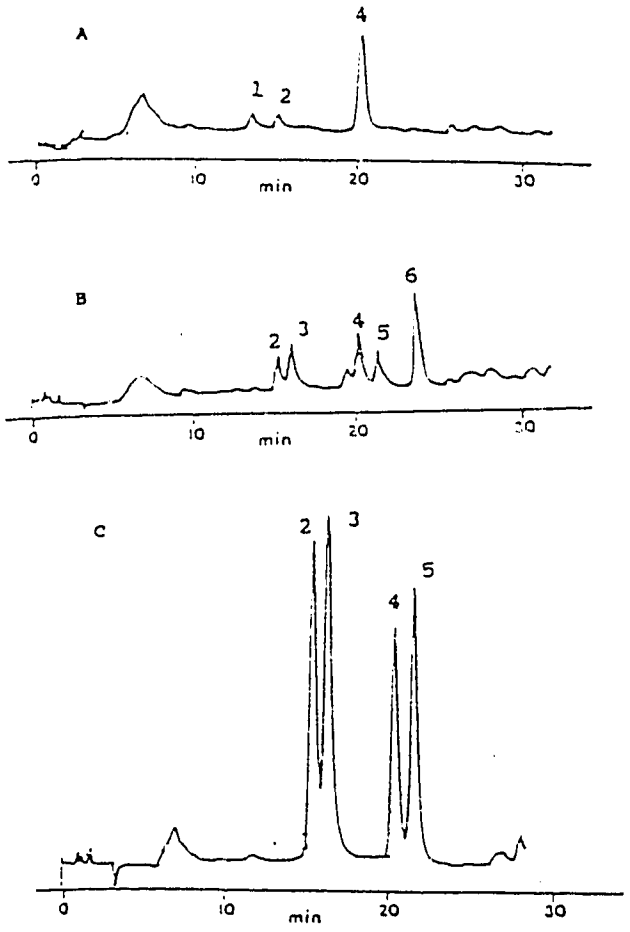


Figure 22. HPLC spectra of podophyllin and the isolated aglycone constituents. A is the spectrum of crude podophyllin from *P. emodi*, B is the spectrum crude podophyllin from *P. peltatum*, and C is the spectrum of the isolated constituents. Peak 1 is an undentified constituent, peak 2 is for 4'-demethylpodophyllotoxin, peak 3 is for α-peltatin, peak 4 is for podophyllotoxin, peak 5 is for ß-peltatin, and peak 6 is an undentified constituent.

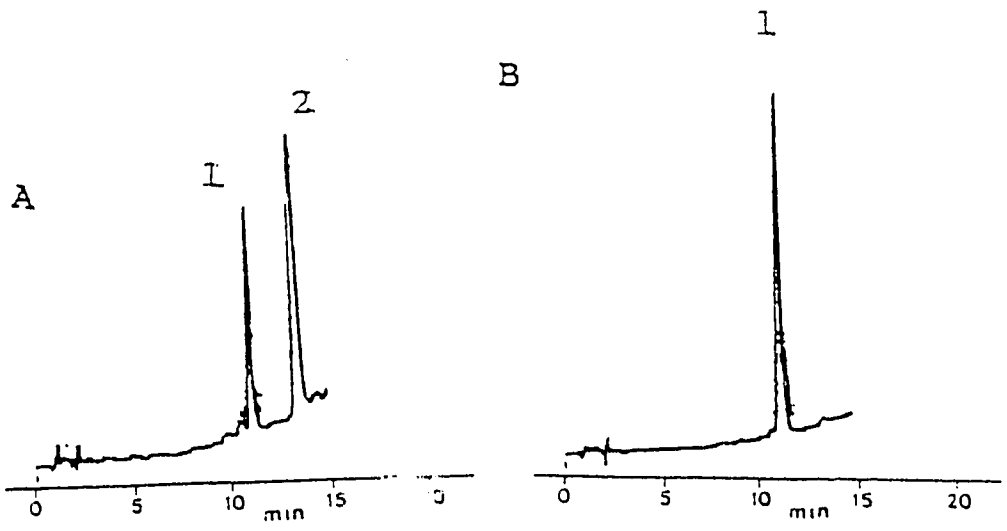


Figure 23. HPLC spectral of podophyllin and the isolated glucoside. A is the crude podophyllin from *P. peltatum* (podophyllin from P. emodi had the same spectra). Peak 1 is for podophyllotoxin glucoside and 2 is an unidentified constituent.

The method developed in this analysis can be applied to the analysis of podophyllin-type lignans present in other plant species and podophyllin constituents in pharmaceutical formulations. This method of analysis and the data obtained from it can be used to standardize non-standardized podophyllin.

CONCLUSION

Chloroform-methanol (or ethyl acetate) elution of podophyllin loaded on silicic acid column was used to isolate the constituents of podophyllin from *P. peltatum* and *P. emodi*. Two separation methods were used. Method one was the more conventional method of separation of podophyllin constituents while method two had some modifications that made separation of constituents much easier and faster.

The isolated constituents were identified by NMR, IR, UV, TLC, HPLC, and their melting points, and they were β-peltatin, podophyllotoxin, α-peltatin, 4'-demethylpodophyllotoxin, and podophyllotoxin glucoside. The glucoside was identified by the analysis of the aglycone and the sugar from the enzymatic hydrolysis. The aglycone had the same characteristics as the authentic podophyllotoxin in its TLC, HPLC, and melting point. The sugar was identified as D-glucose from its colour reaction on cellulose TLC with aniline-diphenylamine-phosphoric acid spray.

The quantities of the podophyllin constituents obtained from this analysis differed with those previously obtained from gravity elution chromatography or preparative TLC. Values obtained in this HPLC analysis are a more accurate measure of the quantities of podophyllin constituents in the two samples of podophyllin. HPLC quantitative analysis should therefore replace the use of gravity elution chromatography or TLC for quantitative estimates because gravity elution chromatography or TLC are not precise for estimating quantities.

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