Reprinted from: Trends in new crops and new uses. 2002. J. Janick
and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

The American Mayapple and its Potential for Podophyllotoxin Production*

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INTRODUCTION

Podophyllotoxin is the starting material for the semi-synthesis of the anti-cancer drugs etoposide, teniposide and etopophos. These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemias. It is also the precursor to a new derivative CPH 82 that is being tested for rheumatoid arthritis in Europe, and it is the precursor to other derivatives used for the treatment of psoriasis and malaria. Several podophyllotoxin preparations are on the market for dermatological use to treat genital warts. Since the total synthesis of podophyllotoxin is an expensive process, availability of the compound from natural renewable resources is an important issue for pharmaceutical companies that manufacture these drugs.

Currently, the commercial source of podophyllotoxin is the rhizomes and roots of *Podophyllum emodi* Wall. (syn. *P. hexandrum* Royle), Berberidaceae, an endangered species from the Himalayas. In recent studies, we concluded that the leaf blades of the North American mayapple (*P. peltatum* L.) may serve as an alternative source of podophyllotoxin production. Since leaves are renewable organs that store lignans as glucopyranosides, podophyllotoxin can be obtained by conversion of podophyllotoxin 4-*O*-*β*-*D*-glucopyranoside into the aglycone using our buffer extraction procedure. This extraction procedure of *P. peltatum* leaves yields podophyllotoxin in amounts similar to the ethanol extraction of *P. emodi* rhizomes and roots.

IMPORTANCE OF PODOPHYLLOTOXIN AND ITS DERIVATIVES

Lignans are biosynthetically derived from the phenylpropanoid pathway, which are ubiquitously distributed among plant species and play important roles in plant defense (Fukuda et al. 1985; Figgitt et al. 1989). Of more restricted taxonomic distribution, the aryltetralin lignans are found in high amounts in plants of the genus *Podophyllum*. Among them, podophyllotoxin is the most important due its biological activity blocking mitosis (Loike and Horwitz 1976a; Loike et al. 1978) and its use as the starting compound of the semi-synthetic chemotherapeutic drugs etoposide, teniposide, and etopophos (Stähelin and von Wartburg 1991) (Fig. 1). These antineoplastic pharmaceuticals block DNA toposisomerase II (Loike and Horwitz 1976b; Horwitz and Loike 1977; Minocha and Long 1984) and have been used for the treatment of small and large cell lung, refractory testicular, stomach, pancreatic cancers, and myeloid leukemias (Ekstrom et al. 1998; Holm et al. 1998; Ajani et al. 1999).

The successful derivatization of podophyllotoxin into these potent antineoplastic drugs, etoposide and teniposide, has generated interest in structure optimization to produce new derivatives with superior pharmacological profiles and broader therapeutic uses. Numerous derivatives varying the etoposide basic structure have been proposed, synthesized, and clinically tested. Etopophos is a new etoposide phosphate designed to overcome the limitations associated with the poor solubility of etoposide. Etopophos can be administered intravenously at higher doses and rapidly converted by phosphatase in the plasma to etoposide, thus constituting an improvement in the treatment (Schacter 1996). NK 611, TOP 53, and GL 311 are among the most promising derivatives that attempt to increase the biological activities becoming more potent drugs than etoposide. These derivatives are in the first phase of clinical trials or pre-clinical developments (Huang et al. 1996; Pagani et al. 1996; Utsugi et al. 1996; Imbert 1998; Raßmann et al. 1999).

The administration of podophyllotoxin-derived drugs causes complex physiological reactions beyond inhibition of DNA topoisomerase and tubulin polymerization. A mixture of benzylidinated podophyllotoxin glycosides is a new drug (CPH 82) for the treatment of rheumatoid arthritis and psoriasis. Arthritis patients treated with CPH 82 have shown a reduction of the inflammatory process within three months of therapy in

*This work was funded by USDA Seed grant 97-35501-4886, Strengthening award grant 99-01739, and the Specific cooperative agreement No. 58-6408-7-012.

first and second phases of clinical trials in Europe (Lerndal and Svensson 2000). These results suggest that CPH 82 is a safe and efficacious drug for rheumatoid arthritis with gastrointestinal inconveniences as side effects (Bjorneboe et al. 1998).

The therapeutic value of podophyllotoxins as mitosis inhibitors has other medicinal applications including uses as anti-malarial and anti-fungal agents with immune modulator activities (Leander and Rosen 1988; Pugh et al. 2001). Therefore, many synthetic chemists have devoted their efforts in developing new routes to the total synthesis of podophyllotoxin. This is, however, a low yield process due to the large number of steps involved (Bush and Jones 1995). Currently, the preferred source of podophyllotoxin is the Indian *Podophyllum* species, and it has acquired the status of a man-made endangered species due to intense collection and lack of cultivation. To secure podophyllotoxin supply, we have examined the North American mayapple (*P. peltatum* L.) and its potential for podophyllotoxin production.

PODOPHYLLUM AS THE SOURCE OF PODOPHYLLOTOXIN

The genus *Podophyllum* (Berberidaceae) has two species that are the most commercially exploited sources of podophyllotoxin; *P. emodi* Wall. (syn. *P. hexandrum* Royle) in India and Nepal and *P. peltatum* L. in the United States. Extracts of dried rhizome of Bankakri and mayapple were used by Himalayans and the North American native populations as cathartics and cholagogues respectively. In 1947, Hartwell and Shear demonstrated that a single dose of resin was effective in reducing tumors, but severe abdominal pains were associated with the treatment. Extracts containing natural lignan glycosides from *Podophyllum* were tested to eliminate side effects and provide better pharmacological results. The glycosides not only showed lower toxicity but also lower anticancer activity. These results led to the derivatization of podophyllotoxin, which lead to the development of etoposide and teniposide (Fig. 1).

After the major discovery of the anticancer properties of podophyllotoxin derivatives, Meijer (1974) reported that the US annual demand for *P. peltatum* rhizomes was more than 130 tons in 1970. The commercial interest turned to *P. emodi* when these rhizomes were found to contain more podophyllotoxin than the *P. peltatum* (Jackson and Dewick 1984).

Podophyllum emodi is a perennial rhizomatous herb found in the Himalayas growing in the understory of subalpine forests (Bhadula et al. 1996). Due to high demand in the international market for the past three decades, there has been a sharp decline in *P. emodi* populations and it has acquired endangered species status (Foster 1993). As of today, many Indian research institutions are making a great effort to rescue the species.

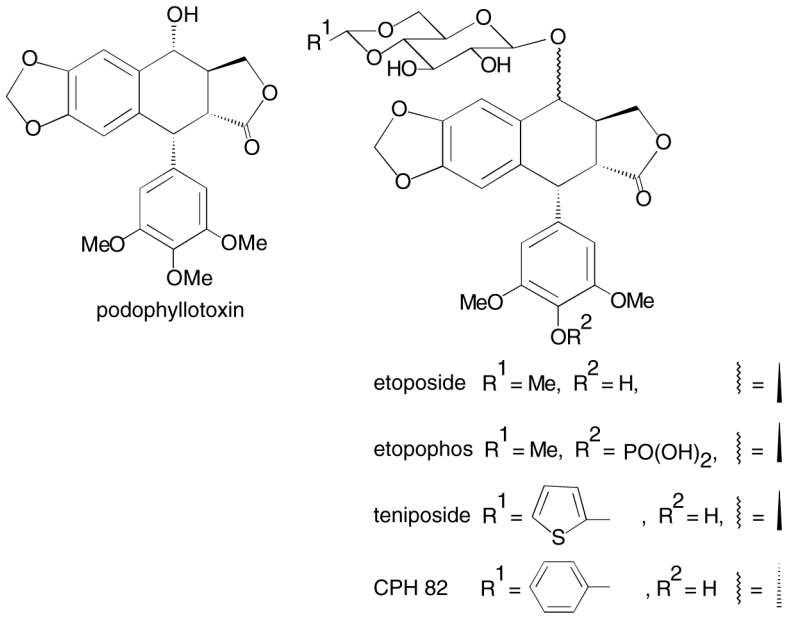

Fig 1. Structures of (-)- podohyllotoxin and its derivatives.

The Department of Botany at the University of Delhi is collecting specimens for population biology and genetic diversity study. The populations are being characterized at the cytogenetic level to assess the existing genetic diversity. Representative specimens are being transplanted to experimental plots in the Himalayan foothills (Bhadula et al. 1996).

Researchers at the G.B. Pant Institute of Himalayan Environment and Development in Garhwal have approached the replenishing of *P. emodi* by propagating the natural stocks using rhizome cuttings, viable seeds, and plants regenerated from embryogenic calli (Nadeem et al. 2000). Researchers at the National Center for Natural Products Research (NCNPR) are also attempting to secure podophyllotoxin supply by developing the American mayapple into an alternative source.

Our findings suggest that buffer extraction of leaf blades of *P. peltatum* yields podophyllotoxin in amounts similar to the ethanol extraction of *P. emodi* rhizomes and roots (40.0 mg g^{-1} on a dry weight basis) (Jackson and Dewick 1985; Canel et al. 2001). Leaf blades of *P. peltatum* store podophyllotoxin *4*-O-β-D-glucopyranoside and in the process of extraction by buffer, the glucopyranoside is converted into the aglycone (Canel et al. 2000b). Thus, leaves may serve as a rich source of podophyllotoxin, and since leaves are renewable organs the American mayapple has potential to become a sustainable crop.

THE BIOLOGY OF THE AMERICAN MAYAPPLE

Podophyllum peltatum, the American mayapple also known as mandrake, grows in large colonies in eastern North America from Quebec and Minnesota to Florida and Texas (Meijer 1974). Its wide-range of distribution shows that *P. peltatum* can survive under varying growing conditions, adapting well from the extreme low winter temperatures of northern climates to the high summer temperatures of the southern US. It is a rhizomatous perennial, 46 to 61 cm (1.5 to 2') in height, with a petiole bearing one or two leaves with round blades up to 30 cm (12") in diameter. Mayapple is described as self-incompatible but some researchers believe that colonies in the wild may come from a single seedling, thus one genotype grows in clonal patches (Laverty and Plowright 1988). In contrast, Policansky (1983) reported that mayapple colonies are comprised of more than one genotype and intra-population crosses resulted in lower seed set than inter-population crosses. This evidence suggests that mayapple is at least partially self-incompatible.

Plants remain juvenile for 4 to 5 years and during this time there is upward growth of the underground bud. The rhizome grows horizontally from that single bud and ends with a terminal bud after reaching maturity. Each terminal bud produces a shoot next season. The rhizome continues to develop annually, producing elongated internodes between nodes. Each node is a complex structure composed of a compressed stem, a main bud that develops into the next season's growth, and minor buds that can develop and continue rhizome growth if the terminal bud is lost. Roots develop at the base of the node and can also arise from the internodal tissue near the terminal bud of the rhizome.

A single shoot arises from each node of the rhizome and are either asexual, producing a single leaf, or sexual, producing a forked petiole with two leaves and a solitary flower (Geber et al. 1997). Both asexual and sexual growths emerge in early spring before trees produce leaves and then senesce by midsummer. Landa et al. (1992) showed that photoassimilates produced during the growing season and stored to roots, rhizomes, and nodes at the onset of leaf senescence are translocated to newly developing growing points in the following spring. Plants senescing later produce longer and heavier rhizomes (de Kroon et al. 1991). According to Watson and Lu (1999), *P. peltatum* shoot senescence is affected by several factors such as vigor of the rhizome system, the genotype, and the environment to which the plant was exposed.

Vegetative propagation of mayapple is by rhizome cuttings or by micropropagation using the terminal bud as the source of explant-inducing adventitious buds with 70% to 90% success rate in soil acclimatization (Moraes-Cerdeira et al. 1998). As for growing mayapple, one may expect that shade will provide the best condition for growth since numerous reports on colonies describe the plant as an understory species. Mayapple, however, is one of the first species to sprout in the spring before the leaves of taller trees are fully developed and gathering enough energy to support the yearly growth. Our results revealed that plants yielded more podophyllotoxin under the sun than plantings under the shade-house (Moraes et al. 2001). In addition colonies can be seen growing under sunny conditions along roadsides.

THE AMERICAN MAYPPLE PODOPHYLLOTOXIN RICH-CHEMOTYPES

Seventeen sites were surveyed in April 1998 in six different states, to identify high-yielding genotypes of *P. peltatum*. Samples of 18 accessions were collected and separated by organ parts consisting of leaves, rhizomes, and roots. Ground tissues were extracted by the procedure described in the method of Canel et al. (2000a), and results showed that mayapple accessions can be classified in two chemotype groups: podophyllotoxin-rich types with blades yielding between 85% to 94% podophyllotoxin of the total lignan content; and the peltatin-rich types with 65% to 80% of the total lignan being α-peltatin (Moraes et al. 2000).

Podophyllotoxin-rich accessions are of particular interest because biomass with high purity represents a significant economy in the process of purification. These accessions were proven to be stable podophyllotoxin chemotypes and confirmed as chemotypes by cultivating them in different growing conditions for three consecutive years and harvesting their blades for lignan extraction (Moraes et al. 2001). Comparison of the different chemotypes showed an inverse relationship between podophyllotoxin content and α-peltatin content. This was also noticed in our previous report on our US partial survey of the natural range of *P. peltatum* and later confirmed by the cultivation studies.

Podophyllum peltatum accessions with podophyllotoxin-rich leaf biomass were identified and transplanted to different growing conditions by vegetative cuttings. Results indicate that the lignan profile in leaves does not change over time or due to environmental conditions. Podophyllotoxin and α-peltatin contents in the blades seem to be stable with an inverse relationship between these compounds. Podophyllotoxin-rich leaf accessions showed low biosynthetic capability to synthesize α- and β-peltatin and the opposite was also true, indicating that selection and cultivation of high-yielding podophyllotoxin leaf biomass may reduce production costs.

CONSIDERATIONS AND PERSPECTIVES FOR PODOPHYLLOTOXIN PRODUCTION FROM THE AMERICAN MAYAPPLE

As the population ages in America, cancer is expected to be one of the leading causes of death. It is estimated that the world market in cancer-fighting pharmaceuticals will grow at a rate of approximately 8.0% annually with expected sales to reach US$14.7 billion in 2002. The National Cancer Institute (NCI) publishes a list of 167 clinical trials using etoposide in chemotherapeutic cocktails either as the positive control or as an investigative new treatment even though the US sales of podophyllotoxin derivatives has dropped since 1999. Future demand of podophyllotoxin derivatives may be affected by additional approvals within the anticancer drug market and also by the new therapeutic targets currently being investigated.

Bioprospecting for podophyllotoxin in ten genera *Podophyllum, Juniperus, Teucrium, Hyptis, Dysosma, Linum, Nepeta, Jeffersonia, Thymus*, and *Thuja* has been reported in the literature, and these were evaluated using our extraction procedure and an HPLC separation method (Bedir et al. 2001). *Podophyllum peltatum* and *P. emodi* are the richest sources and continue to have the greatest potential for cultivation as a specialty crop. Another species, *Juniperus virginiana,* yielded lower podophyllotoxin content (4.2 mg g^{-1} on a dry weight basis) than in *Podophyllum,* however, leaves are a highly abundant by-product of the lumber industry.

Comparing the three sources *P. emodi, P. peltatum,* and *J. virginiana,* plants grown from rhizome cuttings of *P. emodi* were estimated to take at least 5 years to produce rhizomes in fair sizes and plants raised from seedlings would take even longer (Choudhary et al. 1998). The long growing cycle required to produce rhizome biomass for extraction may affect farmers' decision on planting the Indian species. Although, leaves of *J. virginiana* are in great abundance, the cost of processing and extracting a low-yielding biomass may be as high as the cost of cultivating a high-yielding source such as *P. peltatum*.

For cultivation, *P. peltatum* may be a better candidate for sustainability as a specialty crop since leaf blades rich in podophyllotoxin are renewable organs. With proper management, it appears that production and subsequent harvest of leaf material from field plantings will take far less time than for rhizome material. Future developments concerning the American mayapple will depend on many things, such as demand for drugs derived from podophyllotoxin, costs associated with propagation and cultivation of, and costs associated with alternative sources such as *Juniperus virginiana*.

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