Podophyllotoxin Concentration in Junipers in the Big Horn Mountains in Wyoming

Valtcho D. Zheljaskov¹

University of Wyoming, Sheridan Research and Extension Center, 663 Wyarno Road, Sheridan, WY 82801

Charles L. Cantrell

USDA, Agricultural Research Service, NPURU, University, MS 38677

Mateus Augusto Donega

Escola Superior de Agricultura “Luiz de Queiroz”—Universidade de São Paulo, Av. Pádua Dias, 11-13418-900 Piracicaba, Brazil

Tess Astatkie

Dalhousie University, Faculty of Agriculture, Truro, Nova Scotia, B2N 5E3, Canada

Bonnie Heidel

University of Wyoming, WY Natural Diversity Database, 1000 E. University Avenue, Laramie, WY 82071

Additional index words. *Juniperus horizontalis*, *Juniperus scopulorum*, anticancer

Abstract. Podophyllotoxin is used for the production of the anticancer drugs etoposide, etopophos, and teniposide. Currently, podophyllotoxin is extracted from the Himalayan mayapple (*Podophyllum hexandrum* Royle). Some junipers and other species also contain the same natural product and have been explored as a domestic source for this compound. The objective of this study was to screen junipers in the Big Horn Mountains in Wyoming for podophyllotoxin. Twenty junipers (18 accessions of *Juniperus horizontalis* Moench. and two accessions of *J. scopulorum* Sarg.) were sampled in Mar. 2012 and analyzed for podophyllotoxin. Podophyllotoxin concentration in the samples varied from 0.058% to 0.673% with five accessions having podophyllotoxin concentration above 0.5%. This study demonstrated wide variation of podophyllotoxin in *J. horizontalis* and *J. scopulorum* in the Big Horn Mountains. Some of the accessions had greater than 0.5% podophyllotoxin making them a feasible source for podophyllotoxin extraction.

Podophyllotoxin is used as a chemical precursor for the production of the anticancer drugs etoposide, etopophos, and teniposide, which are used for the treatment of lung cancer, testicular cancer, neuroblastoma, and hepatoma (Imbert, 1998; Koulman et al., 2004; Stahelin and Wartburg, 1991). In addition, some other derivatives of podophyllotoxin were found to be promising in the treatment

of psoriasis, malaria (Leander and Rosen, 1988; Lerndal and Svensson, 2000), and also as antiviral agents (Hammonds et al., 1996). Currently, the only commercial source for podophyllotoxin is the Himalayan mayapple (*Podophyllum hexandrum* Royle, syn. *P. emodi* Wall ex. Honigberger), an endangered species in India.

Researchers identified a number of other species that contain podophyllotoxin. Much research has been focused on the American mayapple (*P. peltatum* L.), a native species in North America. Despite numerous studies on American mayapple in Poland, Russia (Bogdanova and Sokolov, 1973; Saraeva, 1952), and in the United States (Cushman et al., 2005; Meijer, 1974; Moraes et al., 2002; Zheljaskov et al., 2009), this species was not domesticated as a result of its limitations.

Eastern red cedar (*Juniperus virginiana*) has also been investigated as a source for podophyllotoxin (Canel et al., 2001; Cushman et al., 2003; Gawde et al., 2009; Hartwell et al., 1953). Rocky mountain juniper (*J. scopulorum*) also contains podophyllotoxin (Cantrell et al., 2013; Renouard et al., 2011) although in lower concentrations than Eastern red

cedar. Our preliminary studies in the Big Horn Mountains found that some accessions of creeping juniper (*J. horizontalis* Moench.) had much greater concentrations of podophyllotoxin than reported for any other juniper species. Therefore, the objective of this study was to screen different accessions of creeping juniper in the Big Horn Mountains and establish the range of podophyllotoxin concentrations.

Materials and Methods

Plant collection. In Mar. 2012, 20 different junipers (18 accessions of *Juniperus horizontalis* Moench. and two accessions of *J. scopulorum* Sarg.) were sampled in the Big Horn Mountains of Wyoming. The sampling area was chosen based on our preliminary studies that found accessions with high podophyllotoxin, and this area is characterized by a very high density of creeping junipers. Samples were taken from naturally occurring junipers situated at least 30 m away from any roads. From each plant, ≈1 kg of fresh material was collected. Only mature leaves of the juniper plants were sampled. The GPS coordinates were recorded for every plant accession that was sampled. The collected samples were dried in a shady aerated area at temperatures between 22 and 28 °C until constant weight. Subsamples from each collection were identified by Bonnie Heidel, a botanist at the Wyoming Natural Diversity Database, University of Wyoming, and representative vouchers were deposited in the University of Wyoming Rocky Mountain Herbarium.

Podophyllotoxin. The extraction and purification of podophyllotoxin from the collected juniper samples was as previously described (Canel et al., 2001). Approximately 40 mg of each dry tissue sample was incubated at 20 °C with 0.6 mL of 25 mM potassium phosphate buffer (pH of 7.0) on an Eppendorf Thermomixer R for 30 min at 750 rpm. Subsequently, 0.6 mL of ethyl acetate was added, and the incubation continued for an additional 5 min in the same manner. The aqueous and organic partitions were separated by centrifuge for 15 min (Savant speed vac, svc 200). The organic layer was removed using a Pasteur pipette and evaporated under a stream of N₂, leaving the organic soluble material to be dissolved in 0.8 mL of methanol and analyzed by high-performance liquid chromatography (HPLC).

Podophyllotoxin in the purified extract was analyzed using an HPLC system (Agilent 1200 series consisting of a vacuum degasser, quaternary pump, ALS autosampler, a photodiode array detector, and an Agilent Eclipse XDB-C18, 4.6 × 150 mm, 5-μm column). The injection volume for all samples and for the podophyllotoxin standard was 10 μL and standards and samples were analyzed at room temperature. The analytical method was isocratic (28% acetonitrile:72% deionized water containing 0.1% trifluoroacetic acid) for 20 min followed by a 5-min column wash with methanol and re-equilibration.

Received for publication 4 Sept. 2012. Accepted for publication 1 Oct. 2012.

This research was funded in part by the University of Wyoming startup funding awarded to Dr. Zheljaskov.

We thank the Forest Service of the U.S. Department of Agriculture for issuing us a permit to sample junipers in the Bighorn National Forest. We thank Mr. Travis Fack, Natural Resource Specialist, and Mr. Clarke McClung, Tongue District Ranger, for helping with the permit. We thank Mr. Lyn Ciampa and Mrs. Ekaterina Jeliazkova for their help with the collections of junipers in the Big Horn Mountains.

¹To whom reprint requests should be addressed; e-mail vjeliazk@uwyo.edu; valtcho.pubs@gmail.com.

Table 1. Mean Podophyllotoxin concentration (%) from the 20 *Juniperus* accessions,^z elevation, and GPS coordinates of each collection site.

Accession no., species	Podophyllotoxin (%)	Elevation (m)	GPS coordinates
130, <i>J. scopulorum</i> Sarg.	0.156 h	2072	Lat. N 44°37.077', long. W 107°05.166'
131, <i>J. horizontalis</i> Moench	0.457 d	2070	Lat. N 44°37.108', long. W 107°05.072'
132, <i>J. horizontalis</i> Moench	0.478 cd	2049	Lat. N 44°37.097', long. W 107°04.925'
133, <i>J. horizontalis</i> Moench	0.312 e	1990	Lat. N 44°37.056', long. W 107°04.063'
134, <i>J. horizontalis</i> Moench	0.531 bcd	1978	Lat. N 44°37.041', long. W 107°04.685'
135, <i>J. horizontalis</i> Moench	0.327 e	1948	Lat. N 44°37.004', long. W 107°04.666'
136, <i>J. horizontalis</i> Moench	0.159 h	1945	Lat. N 44°37.006', long. W 107°04.676'
137, <i>J. horizontalis</i> Moench	0.151 h	1942	Lat. N 44°36.998', long. W 107°04.679'
138, <i>J. scopulorum</i> Sarg.	0.558 b	1909	Lat. N 44°37.040', long. W 107°05.323'
139, <i>J. horizontalis</i> Moench	0.067 ij	1899	Lat. N 44°37.497', long. W 107°05.287'
140, <i>J. horizontalis</i> Moench	0.287 ef	1893	Lat. N 44°37.495', long. W 107°05.274'
141, <i>J. scopulorum</i> Sarg.	0.165 h	1888	Lat. N 44°37.484', long. W 107°05.280'
142, <i>J. horizontalis</i> Moench	0.253 efg	1839	Lat. N 44°37.440', long. W 107°05.246'
2-117N, <i>J. horizontalis</i> Moench	0.058 j	1793	Lat. N 44°37.493', long. W 107°05.134'
2-117O, <i>J. horizontalis</i> Moench	0.565 b	1814	Lat. N 44°37.517', long. W 107°05.152'
2-118N, <i>J. horizontalis</i> Moench	0.555 bc	1961	Lat. N 44°37.033', long. W 107°04.660'
2-118O, <i>J. horizontalis</i> Moench	0.673 a	1961	Lat. N 44°37.033', long. W 107°04.660'
2-118P, <i>J. horizontalis</i> Moench	0.138 hi	1961	Lat. N 44°37.033', long. W 107°04.660'
2-118Q, <i>J. horizontalis</i> Moench	0.215 fgh	1963	Lat. N 44°37.032', long. W 107°04.670'
2-118R, <i>J. horizontalis</i> Moench	0.202 gh	1970	Lat. N 44°37.037', long. W 107°04.675'

^zMeans sharing the same letter are not significantly different at the 5% level.

Analytes were detected at 220 nm with a reference of 450 nm by a photodiode array detector.

Quantitation was performed by means of an external standard calibration curve obtained from standard solutions of pure podophyllotoxin, purchased from Sigma-Aldrich (St. Louis, MO). Linearity was imposed by using response factors and regression coefficients independently. Response factors (RFs) were calculated using the equation $RF = DR/C$, where DR was the detector response in peak area (PA) and C was the analyte concentration. The target podophyllotoxin peak was confirmed by retention time and ultraviolet spectroscopy from 200 to 500 nm. The RF of the target chemical constituent was used to determine the percent for each sample using the equation: $(PA/RF/C) * 100 = \%$.

Statistical analysis. The effect of 20 juniper tree accessions on podophyllotoxin (%) was determined by conducting a one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute Inc., 2008). The validity of the ANOVA model assumptions, namely normal distribution and constant variance assumptions on the error terms, was verified by examining the residuals as described in Montgomery (2009). Because the effect of accession was highly significant ($P < 0.01$), further multiple means comparison was done using Tukey's honestly significant difference method at the 5% level. The more conservative Tukey's method was used because of the low experimental error and to protect the Type I experimentwise error rate from overinflation resulting from the relatively large number of accessions.

Results and Discussion

As expected, podophyllotoxin concentration in the collected samples varied in wide ranges from 0.058% to 0.673% (Table 1). Five accessions had podophyllotoxin concentration above 0.5%, two accessions had a

podophyllotoxin concentration between 0.45% and 0.5%, two accessions had a podophyllotoxin concentration between 0.3% and 0.4%, four accessions had a podophyllotoxin concentration between 0.2% and 0.3%, five accessions had a podophyllotoxin concentration between 0.1% and 0.2%, and two accessions had a podophyllotoxin concentration between 0.05% and 0.1%.

In this study, we found seven accessions of creeping juniper with podophyllotoxin concentrations above 0.5%. Previously, Cantrell et al. (2013) reported 0.254% and 0.138% podophyllotoxin in two accessions of creeping juniper (*J. horizontalis*). In a recent study in a wider range of the Big Horn Mountains, Zheljzkov et al. (2013) found podophyllotoxin concentration range of 0.27–0.073% in *J. horizontalis*, 0–0.40% in *J. scopulorum*, and none in *J. communis*.

Previous research on Eastern red cedar found podophyllotoxin concentration to be between 0.1% and 0.34% (Gawde et al., 2009) and 0.1% to 0.18% (Cushman et al., 2003). However, Cantrell et al. (2013) in a recent study reported 0.48%, 0.044%, 0.28%, and 0.66% in four different accessions of *J. virginiana*.

Apparently, the range of podophyllotoxin concentrations in creeping juniper in the Big Horn Mountains in this study was either higher or similar to the ones reported for Eastern red cedar. Hence, creeping juniper accessions found in the Big Horn Mountains seem promising as a source for podophyllotoxin.

Literature Cited

- Bogdanova, V.P. and V.S. Sokolov. 1973. Developmental features of *Podophyllum peltatum* L. and *Podophyllum emodii* Wall. in cultivation conditions in the Lvov province. *Herba Polonica*. 19:379–384.
- Canel, C., F.E. Dayan, M. Ganzera, I.A. Khan, A. Rimando, C.L. Burandt, Jr., and R.M. Moraes. 2001. High yield of podophyllotoxin from leaves of *Podophyllum peltatum* by in situ conversion of podophyllotoxin 4-O- β -D-glucopyranoside. *Planta Med.* 67:97–99.

- Cantrell, C.L., V.D. Zheljzkov, W.A. Osbrink, A. Castro-Ruiz, V. Maddox, L.E. Craker, and T. Astatkie. 2013. Podophyllotoxin and essential oil profile of juniperus and related species. *Ind. Crops Prod.* 43:668–676.
- Cushman, K.E., M. Maqbool, and P.D. Gerard. 2005. Mulch type, mulch depth, and rhizome planting depth for field-grown American mayapple. *HortScience* 40:635–639.
- Cushman, K.E., M. Maqbool, P.D. Gerard, E. Bedir, H. Lata, and R.M. Moraes. 2003. Variation of podophyllotoxin in leaves of Eastern red cedar (*Juniperus virginiana*). *Planta Med.* 69:477–478.
- Gawde, A.J., V.D. Zheljzkov, V. Maddox, and C.L. Cantrell. 2009. Bioprospection of Eastern red cedar from nine physiographic regions in Mississippi. *Ind. Crops Prod.* 30:59–64.
- Hammonds, T.R., S.P. Denyer, D.E. Jackson, and W.L. Irving. 1996. Studies to show that with podophyllotoxin the early replicative stages of herpes virus type 1 depend upon functional cytoplasmic microtubules. *J. Med. Microbiol.* 45:167–172.
- Hartwell, J.L., J.M. Johnson, D.B. Fitzgerald, and M. Belkin. 1953. Podophyllotoxin from *Juniperus* species. *Savinin. J. Amer. Chem. Soc.* 75:235–236.
- Imbert, F. 1998. Discovery of podophyllotoxins. *Biochimie* 80:207–222.
- Koulman, A., W.J. Quax, and N. Pras. 2004. Biotechnology of medicinal plants: Vitalizer and therapeutic: Podophyllotoxin and related lignans produced by plants. In: Ramawat, K.G. (ed.). Science Publishers, Enfield, NH.
- Leander, K. and B. Rosen. 1988. Medicinal used for podophyllotoxin. US patent 4,788,216.
- Lerndal, T. and B. Svensson. 2000. A clinical study of CPH 82 vs. methotrexate in early rheumatoid arthritis. *Rheumatology (Oxford)* 39: 316–320.
- Meijer, W. 1974. *Podophyllum peltatum*—Mayapple: A potential new cash crop plant of eastern North America. *Econ. Bot.* 28:68–72.
- Montgomery, D.C. 2009. Design and analysis of experiments. 7th Ed. Wiley, New York, NY.
- Moraes, R.M., H. Lata, E. Bedir, M. Maqbool, and K. Cushman. 2002. The American mayapple and its potential for podophyllotoxin production, p. 527–532. In: Trends in new crops and new uses. Proc. of the Fifth National Symposium, Atlanta, Georgia, USA, 10–13 Nov. 2001. ASHS Press, Alexandria, VA.
- Renouard, S., T. Lopez, O. Hendrawati, P. Dupre, J. Doussot, A. Falguieres, C. Ferroud, D. Hagege, F. Lamblin, E. Laine, and C. Hano. 2011. Podophyllotoxin and deoxypodophyllotoxin in *Juniperus bermudiana* and 12 other *Juniperus* species: Optimization of extraction, method validation, and quantification. *J. Agr. Food Chem.* 59:8101–8107.
- Saraeva, P.I. 1952. Mayapple, p. 273–274. In: Saraeva, P.I. (ed.). Medicinal plants and their cultivation. Medgiz, Moscow, Russia.
- SAS Institute Inc. 2008. SAS/STAT® 9.2 user's guide. SAS Institute Inc., Cary, NC.
- Stahelin, H.F. and A.V. Wartburg. 1991. The chemical and biological route from podophyllotoxin to etoposide. *Cancer Res.* 51:5–15.
- Zheljzkov, V.D., B. Avula, A.M. Jones, V. Maddox, and D.E. Rowe. 2009. Lignan and nutrient concentrations in American Mayapple (*Podophyllum peltatum* L.) in the eastern United States. *HortScience* 44:349–353.
- Zheljzkov, V.D., C.L. Cantrell, M.A. Donega, and T. Astatkie. 2013. Bioprospecting for podophyllotoxin in the Big Horn Mountains, Wyoming. *Ind. Crop Prod.* 43:787–790.