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Taxol Concentration In *Taxus* Cultivars[1]

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Abstract

Taxol is a naturally occurring diterpene alkaloid which has significant anticancer activity. In order to assess the full chemotherapeutic potential of the drug, the National Cancer Institute (NCI) requires sufficient material for the treatment of 12,000 patients with a variety of cancers. The current principal source of the compound is the bark of the Pacific yew tree, *Taxus brevifolia*. While NCI's short range goal may be achieved through the processing of *T. brevifolia* bark, it is imperative to identify alternative or supplemental sources of taxol for environmental, economic, and humanitarian reasons. One potential source of the drug is biomass from commercial *Taxus* plantings. In this study needles from 14 cultivars of *Taxus* were analyzed for taxol content. Samples were obtained from 7- to 12-year-old plants at four commercial nurseries, as well as 15-year-old plantings from the Connecticut Agricultural Experiment Station's test plots. Taxol concentrations in the needles varied from a low of 118 ppm in *T. cuspidata* 'Sieboldii' to a high of 882 ppm in *T. x media* 'Nigra' on a dry weight basis.

Index words: taxol, *Taxus*

Species used in this study: *T. baccata* 'Repandens' Parsons ex Rehd.; *T. cuspidata* Sieb. & Zucc.; *T. cuspidata* 'Capitata' Kammerer; *T. cuspidata* 'Brevifolia' (strain of *T. cuspidata* 'Nana' Sieb. & Zucc.); *T. cuspidata* 'Sieboldii' Sieb. & Zucc.; *T. cuspidata* 'Greenwave' Sieb. & Zucc.; *T. x media* 'Brownii' Rehd.; *T. x media* 'Densiformis' Rehd.; *T. x media* 'Halloriana' Rehd.; *T. x media* 'Hatfieldii' Rehd.; *T. x media* 'Hicksii' Rehd.; *T. x media* 'Nigra' Sieb. & Zucc. (also listed as *T. cuspidata*); *T. x media* 'Tauntonii' Rehd.; *T. x media* 'Wardii' Rehd.

Significance to the Nursery Industry

An excellent summary of the history of taxol and future directions of taxol research has been published (1). Briefly, it should be noted that taxol sources other than *T. brevifolia* bark must be identified in order to supply this anticancer drug through an environmentally nondestructive route and in the quantities required for expanded clinical trials. *Taxus* biomass supplied by the nursery industry may achieve both of these goals. We have examined the variation in taxol content across fourteen *Taxus* cultivars. The needles from *T. x media* 'Nigra' were found to contain amounts of taxol higher than the typical levels reported in *T. brevifolia* bark. *T. x media* 'Nigra' remains the most favorable cultivar when the taxol levels are expressed on the basis of taxol quantity

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per twig weight. This basis results in taxol levels for *T. x media* 'Tauntonii' and *T. cuspidata* 'Greenwave' which may make these cultivars additional candidates for "taxol specific" *Taxus* cultivation.

Introduction

From 1958 to 1980 the National Cancer Institute sponsored a program for the identification of anticancer activity in extracts derived from plant material. As a result of this screening, anticancer activity was detected in the extracts from the Pacific yew, *Taxus brevifolia* Nutt. (Taxaceae). In 1971 the compound responsible for this activity, taxol, was identified and characterized (2). It has been determined that taxol is a mitotic poison with a unique mechanism which enhances the association of the protein tubulin into microtubules (3). Data from limited clinical trials on patients with ovarian cancer have spurred interest in expanding the trials to additional patients and other types of cancers. The expansion is restricted by the limited availability of the drug (1). The U.S. Food and Drug Administration (FDA) has approved for use in human studies taxol derived solely from *T. brevifolia* bark, which may contain as much as 800 ppm of the drug (4). Since *T. brevifolia* Nutt. is a slow-growing tree that must be sacrificed to extract the compound and, additionally, is part of the habitat of the Northern spotted owl, it is desirable to identify other *Taxus* species containing taxol at levels high enough to warrant their use as alternative and/or supplemental sources of the drug.

Although Wani *et al.* alluded to the presence of taxol in two cultivated yews, *T. cuspidata* and *T. baccata* (2), and some data have been published on *T. cuspidata* (5, 6), *T. baccata* (5, 6), *T. media* (5, 6), and *T. chinensis* (7), no systematic study of the taxol content of common field grown *Taxus* cultivars has been reported. The present study is intended to compare the quantity of taxol in the needles from 14 cultivars grown at three commercial nurseries in Connecticut and one in Rhode Island and 15-year-old *Taxus* plantings at The Connecticut Agricultural Experiment Station. The results are reported both on a dry needle weight basis and normalized to twig weight. The extraction and clean-up method is described in detail. Quantitation is based on high performance liquid chromatography (HPLC) with photodiode array detection.

Materials and Methods

Samples were collected in December 1991 and March 1992. The December 1991 material was used for the development of the extraction and clean-up method only. Samples were collected of twigs with needles from the preceding season's growth ("feathers") on 7- to 12-year-old plants selected at random from throughout the cultivar block. At each of the cooperating nurseries approximately 6 to 20 plants of each cultivar were sampled. The cut field material was stored in tightly sealed plastic bags at 4°C (40°F) and analyzed within three weeks of collection.

A flow chart of the extraction and clean-up method used in our laboratory, which is a modification of previously reported methods (5, 8), is presented in Fig. 1. The fresh needles were manually stripped from the twigs and composited for each cultivar from a given nursery. A weighed amount of the fresh needles was put into the blender jar of a Waring laboratory blender and processed at high speed for 1 minute. A portion of the ground needles was removed

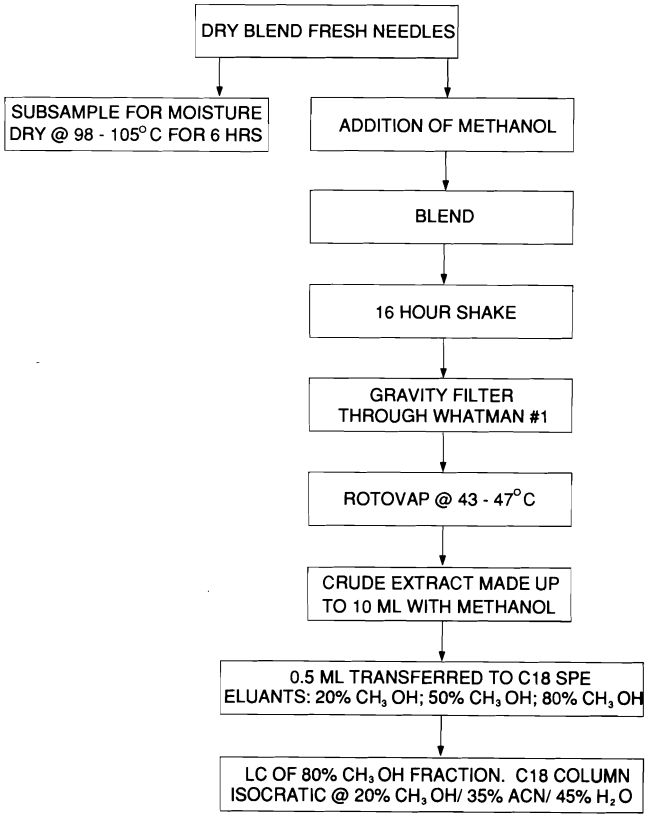

Fig. 1. Flow chart for the *Taxus* needle extraction and clean-up procedure used.

and weighed for determination of moisture content. HPLC grade methanol (J. T. Baker, Phillipsburg, NJ) was added to the needles remaining in the blender jar in a ratio of 100 ml methanol to 10 g needles and the whole was blended at high speed for 2 min. This mixture was transferred to a 500 ml Erlenmeyer flask which was sealed with a ground glass stopper and shaken for 16 hr at ambient temperature, 18–24°C (65–75°F). At the end of this time the mixture was gravity filtered through Whatman #1 filter paper into a 250 ml round bottom flask. The solution was reduced to approximately 5 ml by roto-evaporation in a water bath at 43–47°C (109–117°F). The dark green solution was transferred to a 10 ml concentrator tube and the round bottom flask was washed with several small portions of methanol which were added to the concentrator tube to bring the final solution volume to 10 ml. High capacity C18 solid phase extraction cartridges (catalogue number 7020-07, J. T. Baker) were conditioned with ethyl acetate, methanol, and distilled-deionized water and were not permitted to run dry after the final water wash. One-half milliliter of the extraction solution was transferred to the conditioned SPE cartridge and the solution allowed to soak into the adsorbent almost completely. The cartridge was then washed with 2 ml of distilled-deionized water, followed by 8 ml of 20% methanol-80% water, 10 ml of 50% methanol-50% water, and 10 ml of 80% methanol-20% water. Taxol was eluted in the final 80% methanol fraction.

Liquid chromatography of the final eluate was carried out using a Perkin-Elmer (Norwalk, CT) binary LC250 system equipped with an ISS-100 autoinjector, a PE LC235 diode array detector with λ A set at 230 nm, and a PE 1020

personal integrator. The analytical column was preceded by a 0.5 μm stainless steel in-line filter (Upchurch Scientific, Oak Harbor, WA) and a C18 guard column (Pickering Laboratories, Mountain View, CA). The eluants were A: 100% methanol and B: 44% acetonitrile/56% water. Isocratic elution with a mobile phase of 20% A at a flow rate of 0.5 ml/min on a Waters (Milford, MA) μBondapak C18 (10 μm) 300 × 2 mm analytical column provided resolution of taxol from cephalomannine in under twenty minutes. Authentic taxol, cephalomannine, and baccatin III were obtained from NCI. Stock solutions of the standards were prepared in HPLC grade methanol and diluted appropriately. Calibration curves were acquired daily by the external standard method using peak height from two calibration levels and the origin and data were processed on PE 1020 software. Injection volume for both the standards and samples was the same, 10 μl, and standards were in the 2.0 ng/μl to 35.0 ng/μl range. Retention time was checked at the end of a run of five to twelve samples by reinjection of a standard. Carry-over contamination from one injection to the next is eliminated on this system, but, as a precaution, injection of the higher taxol standard was usually followed by an injection of methanol. Correlation coefficients for the calibration curves were typically 0.999 and the daily response factors agreed to within 10%.

Moisture content on the subsample of ground needles was determined from the weight comparison before and after drying to constant weight at 98–105°C (208–221°F) in a drying oven. Needles were typically found to contain ≈60% moisture.

Results and Discussion

Table 1 is a summary of the three *Taxus* species and their representative cultivars included in this study, the nurseries at which each was sampled, along with the identification of the cooperating nurseries. Five cultivars were sampled at a single site and the remaining nine cultivars were sampled at two or more sites (Table 1 & Figure 3).

The extraction and clean-up method used in our laboratory and summarized in Fig. 1 has advantages over earlier reported techniques (5, 8). The methanolic extract of the needles contains a large amount of polar, coextracted plant material, which is removed typically by evaporating the methanol extract to dryness, reconstituting the residue in dichloromethane, and washing the dichloromethane solution with water. This step is often accompanied by formation of an emulsion. Breaking the emulsion by means of centrifuging can be time consuming and diminish the percent recovery of the target taxanes. These disadvantages were eliminated in our laboratory by evaporating the methanol extract to a small volume, transferring a portion of this extract to a high capacity C18 SPE cartridge, and removing polar coextractives with a generous water wash of the cartridge.

Table 1. *Taxus* cultivars and nurseries included in this study.

baccata	*cuspidata*	*media*
'Repandens'[d]	'Capitata'[a,b,c,d]	'Brownii'[a,b,c,d]
	'Brevifolia'[d]	'Densiformis'[a,b,c,d]
	'Sieboldii'[c]	'Halloriana'[d]
	'Greenwave'[a,c]	'Hatfieldii'[d]
		'Hicksii'[a,b,c,d]
		'Nigra'[c,d]
		'Tauntonii'[b,c,d]
		'Wardii'[c]

Sources:
[a]The Robert Baker Companies, West Suffield, CT
[b]Gardner's Nurseries, Rocky Hill, CT
[c]Imperial Nurseries, Granby, CT
[d]The Rhode Island Nurseries, Newport, RI
**T. cuspidata* 'Brevifolia' included in this study is identified by Rhode Island Nurseries as a strain of *T. cuspidata* 'Nana'.

Table 2 is a summary of the precision and accuracy of the method. These were assessed through replicate extraction of needles, replicate SPEs of the crude extract, replicate injections, as well as recovery of taxol spiked into a needle sample prior to extraction, taxol spiked directly onto the SPE cartridge, and taxol spiked into the crude extract prior to SPE.

An overlay of the chromatograms of a taxol standard (dotted line) and an 80% methanolic SPE eluate (solid line) acquired with λ A at 230 nm is presented in Fig. 2. The diode array detector permits the comparison of the UV spectrum from 195 nm to 365 nm of the authentic taxol with the UV spectrum of the peak at retention time (RT) 15 minutes in the eluate chromatogram. The two spectra match over the entire UV wavelength range. Further confirmation that the peak at RT 15 min is taxol comes from the spike recovery data (see Table 2) and the peak purity index, a mathematical comparison of the spectra at peak start (20% of full height) and peak end (20% of full height) calculated by the PE235 detector. This value was at least 1.7 or better for the peak identified as taxol in all chromatograms. Finally, under the LC conditions enumerated above, authentic cephalomannine, which can interfere with taxol quantitation, elutes at RT 13.5 min.

Taxol concentrations in the fourteen cultivars examined in this study are plotted in Fig. 3. The values plotted are averages across all replicate injections, replicate SPEs, and replicate needle extractions. All taxol values have been expressed on the basis of dry weight of needles, that is, cor-

Table 2. Typical accuracy and precision values for the method.

Replicate injections	Replicate SPE	Replicate extractions	Taxol needle spike	Taxol SPE spike
796 ppm ± 72 (n = 2)	882 ppm ± 86 (n = 3)	284 ppm ± 46 (n = 2)	100% recovery	111% recovery[z]
430 ppm ± 7 (n = 2)	524 ppm ± 11 (n = 2)	489 ppm ± 3 (n = 2)		98% recovery[y]
511 ppm ± 27 (n = 2)	472 ppm ± 28 (n = 2)	650 ppm ± 25 (n = 2)		
247 ppm ± 13 (n = 2)	346 ppm ± 25 (n = 2)			
562 ppm ± 36 (n = 3)				

[z]Spike consisted of methanolic taxol standard transferred directly onto conditioned SPE cartridge.
[y]Sample was prepared by spiking methanolic taxol standard into crude extract prior to transferring the extract to conditioned SPE cartridge.

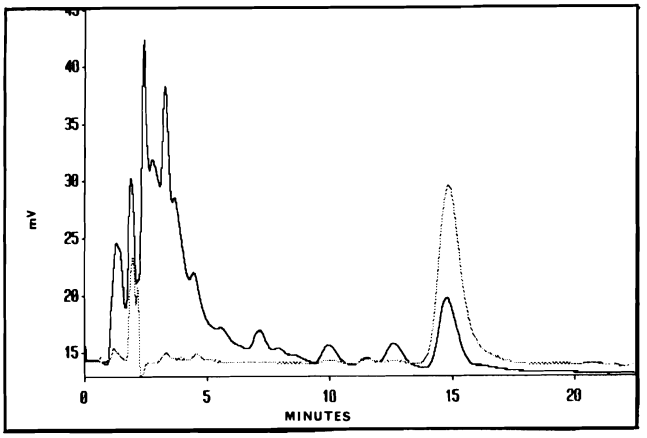

Fig. 2. Chromatogram recorded at 230 nm of 560 ng of taxol injected on-column (dotted trace) and the 80% methanol SPE fraction from the extraction of *T. x media* 'Tauntonii' needles.

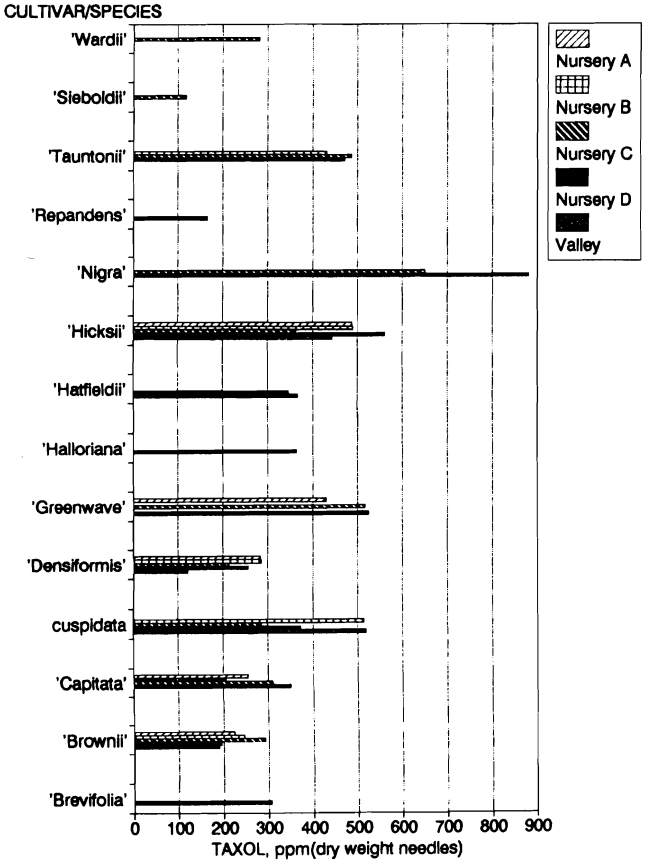

Fig. 3. Taxol content of *Taxus* needles expressed on a dry needle weight basis.

rected for the needle moisture content, to permit comparison with other forms of biomass. Several conclusions can be drawn from the data in Fig. 3. First, examination of Fig. 3 in relation to Table 1 shows that there is no apparent correlation between taxol concentration and species. For example, *T. cuspidata* includes a cultivar with high taxol content, 'Greenwave', and a cultivar with low taxol content, 'Sieboldii'. Second, the taxol content in the needles from *T. x media* 'Nigra' from two different sites exceeds the needle taxol content of any other cultivar in this study. These levels are in excess of the previously reported needle taxol content of *T. brevifolia* (<200 ppm) (4), *T. baccata* (30 ppm) (5), and *T. media* (20 ppm) (5), and are comparable to the highest reported level of taxol in *T. brevifolia* bark, which is just below 800 ppm for shaded trees (4). Third, genetic composition appears to be the determinative factor for taxol content in *Taxus*. Thus, taxol content for a given cultivar is relatively stable across the commercial nurseries participating in this study. For example, the taxol content of *T. cuspidata* 'Greenwave' varies by less than 20% over three sites. Nevertheless, subtle influences of different cultivation practices, soils, and climatic conditions on taxol content remain to be investigated. Finally, there does not appear to be a correlation between plant age and taxol content. Taxol concentrations in needles from the fifteen-year-old plants located at the Connecticut Agricultural Experiment Station's test plots (designated "Valley" in Fig. 3) are in the same range as concentrations in needles from the younger nursery plants.

During the course of this investigation it became apparent that the quantity of needles on a plant was in part a function of cultivar. Therefore, low taxol concentration in the needles may be compensated for by high quantity of needles for a particular cultivar. In an attempt to assess the taxol content of the biomass obtainable from various cultivars, we determined the fresh needle to fresh twig weight ratio for several of the samples included in this survey. These ratios are given in Table 3. If the taxol content of the dry needles is then expressed on the basis of fresh twig weight, the graph in Figure 4 is obtained. Although it should be noted that we sampled only *new* growth in our study, the four cultivars included in Figure 4 provide the most favorable values of taxol per twig weight. This factor together with the rate of plant growth will be important considerations in the selection of cultivars for "taxol specific" cultivation. The ultimate goal of this selection process is the maximization of taxol yield per acre.

These four cultivars, 'Densiformis', 'Greenwave', 'Nigra', and 'Tauntonii' will be included in our continued field and greenhouse studies of the taxol content of *Taxus* cultivars. We are examining several factors which may affect taxol concentration in the cultivars, including plant sex, soil conditions, and time of year in the field studies and environmental stress in the greenhouse studies. We are collecting samples from individual plants rather than composite samples, to determine the taxol variation within a cultivar population. For *T. brevifolia* Nutt. such variation is reported to be significant (6). We also intend to investigate the use of electrospray ionization with tandem quadruple mass spectrometry for the qualitative and quantitative analysis of taxanes in extracts of *Taxus*. This technique should offer improved accuracy over the recently reported HPLC with low resolution thermospray mass spectrometry for the quantitation of taxol in *Taxus* extracts (9).

Table 3. Needle to twig weight ratios for selected cultivars.

Cultivar	Nursery A	Nursery B	Nursery C	Nursery D
'Densiformis'	2.5	2.5	2.5	2.5
'Greenwave'	1.5	—	1.8	—
'Nigra'	—	—	1.6	1.7
'Tauntonii'	—	3.3	3.1	1.7

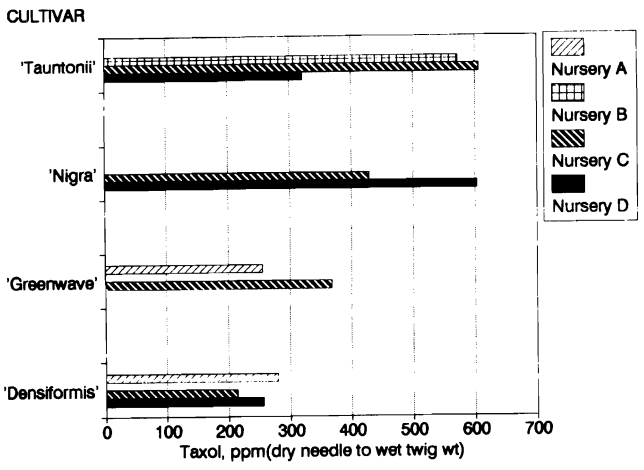

Fig. 4. Taxol content of dry needles per fresh twig weight for selected *Taxus* cultivars.

The data reported here support the conclusion that needles from *Taxus* species cultivated for landscape usage are an alternative taxol source to the bark from *T. brevifolia* Nutt. The plants are rapid-growing, their needles are a renewable resource, and, for selected cultivars, the taxol content is higher than that of the bark and needles from the Pacific yew.

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