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THE PRESENCE OF TAXOL AND RELATED COMPOUNDS IN *TAXUS BACCATA* NATIVE TO THE UKRAINE (CRIMEA), GEORGIA, AND SOUTHERN RUSSIA

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ABSTRACT

Twenty-eight leaf and twig samples and one bark sample of *Taxus baccata* from the Ukraine (Crimea), Russia, and Georgia were analyzed for taxane compounds. Taxol and the related diterpenoid 10-deacetylbaccatin III (baccatin III) were present in all samples and 20.7% of those samples exhibited a taxol content nearly equal to or greater than that obtained from the bark of the Pacific Yew, *T. brevifolia*. *Taxus baccata* is a potential significant source of taxol, the most promising new drug identified in the last twenty years for the treatment of selected forms of cancer.

Key words: *Taxus, Taxus baccata, Taxus brevifolia,* taxol, yew.

INTRODUCTION

The U.S. National Cancer Institute (NCI) has been engaged for nearly 30 years on a worldwide, broad-scale screening program of plant and animal species for new active compounds capable of treating one or more forms of cancer. In the late 1960s, a crude extract obtained from the bark of the Pacific Yew tree, *Taxus brevifolia* Nutt., was demonstrated as having cytotoxic activities against several murine tumors. This finding initiated activities which led to the identification of taxol, a complex diterpene amide, as a new antileukemic and antitumor agent (Wani et al. 1971). Once identified and isolated, taxol became the subject of numerous studies including those showing that it was a novel antimicrotubule agent. It prevents mitosis from occurring by stimulating microtubule formation and stabilizing them against being dismantled, thus making cell division impossible (Schiff, Fant, and Horwitz 1979; Schiff and Horwitz 1980). In 1977, NCI decided to take the steps necessary to develop taxol for preclinical and Phase I clinical trials. Extensive toxicological and molecular pharmacological studies of taxol were made before clinical trials on humans were initiated in 1983 (Rowinsky, Cazenave, and Donehower 1990). Encouraging results from Phase I trials led to more extensive Phase II trials. This paper is not intended to be a review of the development and use of the drug taxol, as this has been thoroughly dealt with by Rowinsky et al. (1990).

A major obstacle in obtaining adequate supplies of taxol is the inadequacy of the current source, the Pacific Yew. While taxol is present in most parts of *Taxus brevifolia* (wood, bark, twigs, leaves, and seeds), its greatest concentration is in the bark. Unfortunately, taxol occurs in exceedingly low concentrations. The

current isolation procedures produce 1 kg of taxol from approximately 25,000 lb of dried bark (Cragg and Snader 1991). Approximately 10 lb of dry bark can be obtained from a single mature tree. Stripping the bark of these trees results in their death. Pacific Yews are slow-growing, long-lived trees often found in the understory of dense conifer forests (Bolsinger and Jaramillo 1990). Thus, excessive harvesting of this yew could result in the extinction of some populations and loss of a portion of its inherent genetic diversity. Issues relating to the conservation of the Pacific Yew were treated by Scher (1991). Cragg and Snader (1991) pointed out that 2500 trees would have to be cut and stripped of their bark just to yield 1 kg of taxol. The immediate anticipated demand for taxol is between 250 to 300 kg per year which translates into an annual harvest of approximately 700,000 trees. Thus, the need for an alternate long-term source of taxol is patently clear.

The logical alternative would appear to be the laboratory synthesis of taxol. In fact, the synthesis of taxol is a high priority, but this effort is hampered because of taxol's complex molecular structure. Complete laboratory synthesis may not be attainable (Kingston, Samaranayaka, and Ivey 1990); however, partial synthesis of taxol can be made from more readily available precursors. For example, taxol and the related taxotere have been synthesized from baccatin III (Lavelle, Fizames, and Gueritte-Voegelein 1989). Baccatin III can be obtained from the leaves, twigs, and bark of the European Yew, *Taxus baccata* L.

As a result, the survey for taxol and related compounds from which taxol can be synthesized has expanded to European and Asian species of *Taxus*. At the initiation of this work, little was known about the presence or abundance of taxol and related compounds in the populations of *Taxus baccata* in Russia, Georgia, and the Crimea. Most of the samples of the European Yew (*Taxus baccata*), the Japanese Yew (*Taxus cuspidata* Sieb. & Zucc.), the hybrid *Taxus* × *media* Rehd., and several ornamental forms analyzed in the United States came from commercially grown stock (Vidensek, Lim, Campbell, and Carlson 1990; Witherup, Look, Stasko, Ghiorzi, and Muschik 1990). Witherup et al. reported dry weight percent of taxol of 0.001% for stems and 0.003% for the needles of a *Taxus baccata* cultivar. Vidensek et al. reported a comparable 0.003% taxol content in the leaves of *T. baccata* obtained from specimens growing in the U.S.

The European Yew, *Taxus baccata,* is widespread throughout Europe. It reaches its easternmost distribution in the European of the former U.S.S.R. where it is found along the Baltic seacoast and the Carpathian Mountains. This yew occurs at lower elevations of the Greater Caucasus Mountains but is more frequent in the northwestern portion of this chain (Sokolov, Svyazeva, and Kubly 1977). It is disjunctly distributed in the Crimean highlands. It then extends into Turkey along the Black Sea and is scattered in western and south-central parts of that country. This yew is also known from a few scattered locations in northern Iran near the Caspian Sea (Browicz 1982).

Because of its slow growth and hundreds of years of harvesting in the Caucasus Mountains and Crimean highlands, old growth *Taxus baccata* forests are rare and the overall numbers of trees greatly reduced. As a result, this species is listed in the official Red Data Book of the U.S.S.R. (Borodin 1984) and in the Red Book of the RSFSR (Golovanov et al. 1988). These two volumes detail the recognized and protected rare and endangered species. It was excluded for unknown reasons from the Red Data Book of the Ukraine (Sitnik et al. 1980).

In this paper, populations from near the southeast limits of the natural range

of *T. baccata* were examined for taxol and related compounds. Results of the first phase of sampling are presented here.

MATERIALS AND METHODS

Twenty-nine samples from 12 native populations of *Taxus baccata* were collected in late April and early May and in September, 1991, in southern Crimea, Georgia, and southern Russia for analysis for taxane compounds. These samples, consisting of leaves and young twigs and bark in one case, were collected fresh and allowed to dry on paper over three to five days exposed to ambient air temperatures and air circulation. Once dried, the samples were packed in paper bags. Dry weight samples of approximately 20–50 g were obtained from each collection. Each sample represented a single tree. Voucher specimens were collected for the samples and deposited at RSA-POM. Because one species of *Taxus* occurs in this area of the former U.S.S.R. and it does not closely resemble any other native conifer, the precise identity of the samples is certain.

The samples were shipped immediately after return to the United States to the Natural Products Branch of the National Cancer Institute in Frederick, Maryland. The samples were analyzed for taxane compounds (taxol, cephalomannine, and baccatin III) in the Chemical Synthesis and Analysis Laboratory of Program Resources, Inc., under contract with NCI. Taxol and related compounds were isolated and quantified using high-performance liquid chromatography techniques (Witherup, Look, Stasko, McCloud, Issaq, and Muschik 1989). This procedure involves the use of cyano- and phenyl-bonded silica gel phases in the reverse phase mode to separate taxol from closely related natural products present in crude methanol extract and methylene chloride solubles derived from the samples of *T. baccata.* Approximately 10 g samples were analyzed from each collection to insure comparable results.

Collection sites for the samples are: Georgia: Borzhoni State Zaprovednik, 150 km W of Tbilisi, *Elias 12311, 12312, 12313*; Russia: North Ossetia State Zapovednik, 50 km SW of Vladikarkaz, *Elias 12314, 12315, 12316, 12317, 12318, 12319, 12320*; Ukraine (Crimea): Dzurla Valley, east of Alushta, upper part of Alaca River, *Elias & Sazonov 12321, 12322, 12323*; Edge of Ai-Petri, along Ai-Petri Road, *Elias & Sazonov 12324, 12325, 12326*; Headwaters of Kok-Koz River, ca. 35 km NW of Yalta, *Elias & Sazonov 12327*; along trail to Bolshoi Canyon, ca. 35 km NW of Yalta, *Elias & Sazonov 12328, 12329*; North slope of main range of Crimean Mountain Babugan, State Crimean Reservation and Hunting areas, Valley of the River Alma, *Elias & Sazonov 12330, 12331*; Chatyr Dag Mountain, 19 km NW of Alushta, *Elias & Sazonov 12334, 12335*; Chatyr Dag, mountain plateau above Alushta, *Elias & Korzhenevsky 12338, 12348*; near Khapkhal Waterfall, beech forest above village Generalskoe, *Elias & Sazonov 12383A, 12383B*; Karabi Mountain, 52 km NE by road from Yalta, West of Lelenogorskoe, limestone plateau, *Elias & Korzhenevsky 12405, 12406, 12408.*

RESULTS

The results of the analysis are presented in Table 1. While every attempt was made to analyze 10-g dry-weight samples, sample weight variations occurred and two samples were less than 10 g; therefore, a comparison of percentages of taxol,

Table 1. Taxane Compounds in *Taxus baccata.*

Sample[1] (collection no.)	Taxol (mg)	Taxol (%)	Cephalo-mannine (mg)	Cephalo-mannine (%)	Baccatin III (mg)	Baccatin III (%)	Dry weight (g)
Georgia							
12311	0.573156	0.0057	0.315720	0.0032	0.444453	0.0044	10.00
12312	0.881406	**0.0122**[2]	0.480088	0.0067	0.632651	0.0088	7.20
12313	0.871707	0.0085	0.560096	0.0055	0.662389	0.0065	10.20
Russia							
12314	0.334744	0.0033	0.178986	0.0018	0.249869	0.0025	10.00
12315	1.254378	**0.0125**	0.633133	0.0063	0.845322	0.0085	10.00
12316	0.371536	0.0037	0.183747	0.0018	0.219998	0.0022	10.00
12317	0.629319	0.0063	0.463934	0.0046	0.556666	0.0056	10.00
12318	0.902121	0.0089	0.292585	0.0029	0.664961	0.0065	10.17
12319	0.593009	0.0059	0.296134	0.0030	0.405680	0.0041	10.00
12320	1.185623	**0.0119**	0.791693	0.0079	0.966335	0.0097	10.00
Ukraine (Crimea)							
12321	0.552863	0.0055	0.258680	0.0026	0.466888	0.0047	10.00
12322	0.942311	**0.0093**	0.821564	0.0081	0.872567	0.0086	10.18
12323	0.301117	0.0030	0.256795	0.0025	0.395228	0.0039	10.00
12324	0.609510	0.0061	0.503888	0.0050	0.532522	0.0053	10.00
12325	0.494746	0.0049	0.445797	0.0045	0.467697	0.0047	10.00
12326	0.421205	0.0042	0.256719	0.0026	0.348915	0.0035	10.00
12327	0.719868	0.0072	0.399232	0.0040	0.496004	0.0050	10.00
12328	0.358953	0.0035	0.255774	0.0025	0.313608	0.0031	10.12
12329	1.568079	**0.0151**	1.081966	0.0104	1.398649	0.0134	10.38
12330	0.263058	0.0026	0.342974	0.0034	0.428524	0.0042	10.00
12331	0.311937	0.0030	0.198303	0.0019	0.330534	0.0031	10.34
12334	0.184533	0.0018	0.103422	0.0010	0.232644	0.0023	10.00
12335	0.236137	0.0022	0.078238	0.0007	0.349435	0.0032	10.60
12338	0.381952	0.0038	0.201179	0.0020	0.218342	0.0022	10.00
12383A	1.484108[3]	**0.0148**	0.650632	0.0065	0.479396	0.0048	10.00
12383B	0.658977	0.0080	0.233746	0.0028	0.272236	0.0033	8.26
12405	0.878369	0.0088	0.633565	0.0063	0.652249	0.0065	10.00
12406	0.207821	0.0021	0.276154	0.0028	0.255802	0.0026	10.00
12408	0.618034	0.0062	0.576759	0.0058	0.600912	0.0060	10.00

[1] Collection numbers are those of Thomas S. Elias.

[2] Numbers in bold face indicated a taxol content equal to or greater than the taxol extracted from the bark of *Taxus brevifolia.*

[3] Represents a bark sample.

cephalomannine, and baccatin III are more meaningful than the actual amounts obtained. The data reveal several important facts.

First, they confirm the presence of taxol and related compounds in *T. baccata* native to the Crimea, Georgia, and Russia. All 29 samples contained taxanes, although the quantities varied considerably. However, a comparison must be made with the taxol obtained from bark samples of *T. brevifolia* before any significance can be placed upon the results obtained from the leaves and young twigs of *T. baccata.* Dr. Gordon Cragg of the National Cancer Institute informed us that the taxol content of *T. brevifolia* is usually 0.01% (personal communication). Vidensek et al. (1990) reported average taxol content in dry-weight samples of the bark to be 0.015%. They also found average taxol content of 0.0015% in

the leaves and 0.0012% in the twigs. In this species, taxol occurs at the greatest levels in the bark; therefore, the presence of taxol in nearly equal or greater amounts in the leaves and twigs of other *Taxus* species must be considered significant. Current studies as yet unpublished on the taxol content of some cultivated forms of *Taxus* are yielding promising results (Douglas Daly, personal communications).

Six samples (20.7% of the total collections) exhibited taxol contents in the leaves and twigs of *Taxus baccata* equal to or greater than that obtained from the bark of *T. brevifolia.* An additional three samples (*12313, 12318,* and *12405*) contained quantities of taxol close to the levels obtained from bark samples of the Pacific Yew. Of the six samples, one is from Georgia, two from Russia, and three from the Crimea.

A second important fact is the considerable variation in taxol content among individual trees within and between populations of the European Yew. For example, the sample (*12329*) with the largest amount of taxol (0.0151%) was 8.2 times greater than the sample (*12334*) with the least amount (0.0018%). There does not appear to be a pattern to the concentrations of taxol within a population. For example, the three samples (*12311–12313*) from Georgia were collected along a small stream in the Borzhomi State Zapovednik. They were all growing in the same habitat, approximately one kilometer separating the first from the third collection. Despite their proximity, the percentage of taxol in the second collection (*12312*) was double that of the first collection (*12311*).

Similar variation was observed in the collections obtained in the Northern Ossetia State Nature Reserve on the north flank of the Caucasus Mountains in southern European Russia. These seven samples were taken from a scattered population of several hundred trees. Despite the common habitat, sample *12315* contained up to four times the level of taxol as did nearby trees. Likewise, sample *12320* contained twice as much taxol as did collection *12319,* a neighboring tree. All of the trees in the Ossetia Reserve were growing in very similar habitats, a mature beech (*Fagus orientalis* Lipsky) forest with *Prunus, Alnus, Tilia,* and *Fraxinus,* at approximately 1000 m elevation.

Similar patterns of variation in taxol content were obtained from samples from the Crimean highlands. Trees with high taxol content (*12322, 12329,* and *12383A*) occurred near trees with two to five times less taxol even though all of the trees lived within the same large population.

Leaves, young twigs, and bark samples were made from one collection (*12383*). Taxol obtained from the bark samples was almost twice the level of taxol extracted from the leaves and twigs. These results were expected since taxol has been shown to be in higher concentrations in the bark of other *Taxus* species, particularly *T. brevifolia.*

There was no noticeable difference in the content obtained from the samples collected in the spring (*12311–12335*) compared to those collected in the fall (*12338–12408*). However, a larger sampling program is required through the year to confirm this and to detect any seasonal differences in taxol content.

It is interesting to note a possible correlation in taxol content with different growth forms due to ecological factors. The Karabi Mountain, a large limestone plateau located about 60 km NE of Yalta, is characterized by numerous funnel-shaped depressions and deep holes. The softer limestone has eroded away in many places, leaving small to enormous sink holes and even caves and caverns. A low-

growing shrubby form of *Taxus baccata* occurs near the rim of some of the larger sink holes and occasionally on the plateau. These individuals are contrasted with the definite tree forms of *T. baccata* which grow in the sink holes. Both forms are sexually mature, producing abundant seeds, and are assumed to be of approximately the same age. The two samples (*12405* and *12408*) from the xerophytic shrub form contained three to four times as much taxol as the tree form (sample *12406*) growing under more mesophytic conditions. The present sample size is too small to verify if this growth-form condition correlates statistically with taxol content. A thorough sampling of both forms is underway.

Also noteworthy is the average taxol content of the 28 leaf and twig samples of *T. baccata* obtained from its native habitat in the Crimea, Russia, and Georgia was 0.0063% or twice the levels reported by Witherup et al. (1990) and Vidensek et al. (1990) for *T. baccata* samples obtained from cultivated material grown in the United States. At this time, it is impossible to know if this difference is due to the drying and processing procedures or the effect of being brought into cultivation.

Higher levels of baccatin III occurred in those samples with high levels of taxol. Baccatin III levels from five Crimean samples exceeded taxol content; however, overall, baccatin III content was lower than taxol content. Despite this, the ability of hydrolyzing all taxanes to baccatins before semisynthesis to taxol effectively increases potential taxol production by approximately 80%.

DISCUSSION

Currently, the only source of taxol approved by the U.S. Food and Drug Administration (FDA) for use in preclinical and clinical trials for treating cancer comes from the bark of *Taxus brevifolia.* The trees must be destroyed in order to obtain the bark. It should be noted that the *T. brevifolia* does have the ability to regenerate from stump sprouts. The demand for sufficient taxol for the preliminary studies as well as Phase I and Phase II clinical trials required the harvesting of approximately 100,000 trees to date. The trees are not abundant enough nor are they fast growing enough to sustain a large-scale harvesting program for bark. Therefore, the only large-scale approach now feasible is to harvest leaves and twigs in a periodic pruning of selected forms with high taxol content. Unfortunately, the taxol content in the leaves of *T. brevifolia* is one tenth to one fifth the amount found in the bark (Gordon Cragg, personal communication). Also, cuttings of this species are known to be difficult to root and for the length of time needed to develop a satisfactory root system. Furthermore, the FDA would have to approve the use of taxol from the leaves and twigs, a process which can take several years.

It is important to note that even though taxol content is higher in bark than in young twigs or leaves, twigs and leaves are much more abundant than bark. Furthermore, twigs and leaf regeneration can be viewed as a seasonably renewable resource unlike the bark.

Clearly, additional sources of taxol are needed, particularly sources which do not contribute to a serious reduction of the number of viable populations of a species. The most logical candidates are species of *Taxus* that are closely related to *T. brevifolia* and can be shown to have high taxol content. The data obtained in this paper demonstrate significantly greater amounts of taxol and baccatin III

in the leaves and young twigs of *T. baccata* from the Crimea, Georgia, and southern Russia than is present in comparable foliage of *T. brevifolia,* and even some trees of *T. baccata* containing levels of taxol equal to or greater than that found in the bark of *T. brevifolia.* Thus, the European Yew, *Taxus baccata,* can be a significant source of taxol and baccatin III. This yew can be grown on large-scale plantations to produce taxol in large quantities. It can be easily grown in other countries for taxol production on a sufficiently inexpensive scale to make this drug available to a wide range of people throughout the world.

The technology is available for the extraction of taxol and baccatin III from leaves and twigs. Since *T. baccata* is easier to propagate, via cuttings, and grow on a larger scale than is *T. brevifolia,* it is feasible to identify native wild tree selections of *T. baccata* with high taxol content, propagate cuttings from those trees, and develop large orchards managed solely for the production of taxol and baccatin III. This is especially true for selected regions of the Crimea, Georgia, and Russia.

To accomplish this, a rigorous sampling program is needed to: (1) determine the identity and location of as many individual trees with high taxol content as is feasible, (2) determine seasonal variation in taxol concentrations, and (3) attempt to correlate growth forms and habitat with taxol content. In this process, we should be able to determine if taxol content is genetically controlled or affected by climatic and edaphic factors.

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