Taxanes in *Taxus baccata* Pollen: Cardiotoxicity and/or Allergenicity?

Maurice Vanhaelen[1]
Jean Duchateau[2]
Renée Vanhaelen-Fastré[1]
Mondher Jaziri[1]

Original Paper

Abstract

Diterpenic alkaloids belonging to taxine (yield: 0.18% dry weight) and taxoids (paclitaxel, baccatine III and 10-deacetylbaccatine III, cumulated yield: 0.004% dry weight) were isolated from *Taxus baccata* L. pollen. Moreover, taxoids conjugated to macromolecules were also detected by ELISA. According to these data and to semi-quantitative measurements of pollen production, a hundred-year-old yew during its flowering time releases several grams of taxanes contained in 16 to 20 kg of pollen. Toxicity assays on mice indicated that any risk of acute toxicity resulting from *Taxus* pollen inhalation and subsequent taxine absorption is unlikely. On the other hand, anti-paclitaxel IgG were above all detected in sera of subjects displaying hypersensitivity reactions during the pollination periods of *Taxus* sp. and *Betula* sp. This natural anti-paclitaxel IgG acquisition by individuals living in the distribution areas of these trees could be at the origin of atopic manifestations. We also postulate that such a natural sensitization could have a marked influence on the tolerance to anticancer taxoids.

Key words
Taxus baccata · Taxaceae · taxoid · taxine · immunoglobulin · allergy · toxicity

Introduction

Several alkaloids, collectively referred to as taxine, have been the first isolated diterpenic constituents isolated from *Taxus* sp. (Taxaceae) [1]. Their activity on sodium and calcium channels explains the fearsome cardiotoxicity of yew [2]. The structures of several of them have been established [3]. In addition to taxine, paclitaxel (Taxol®) was further isolated from the bark of the Pacific yew, *Taxus brevifolia* Nutt. (Taxaceae) [4] and has reached with docetaxel (Taxotere®), a hemisynthetic taxoid, a key position among drugs used in cancer chemotherapy [5]. Paclitaxel has also been found with other taxanes in several species of the genus *Taxus* and in different parts of the trees including seed, needle, wood and root, with the exception of the red aril which is, therefore, devoid of toxicity [6].

A recent report related to a *Taxus* intoxication case emphasized the long and legendary history of yew as a poison [7]. According to Dioscoride, yew emits poisonous fumes, and sleeping or eating under this tree could bring about death [8]. These assertions might be related to the sudden liberation of great amounts of the airborne pollen emitted by yew during its pollination period [9] and to the occurrence of strong toxic and/or allergenic constituents which have been isolated from most parts of yew [10].

Starting from this hypothesis, a phytochemical study was carried out on *T. baccata* L. pollen to further clarify the toxicity and/or allergenic risks related to its release into the atmosphere.

Affiliation
[1] Laboratory of Pharmacognosy and Bromatology, Pharmaceutical Institute, Université Libre de Bruxelles, Bruxelles, Belgium
[2] Department of Immunology, CHU Brugmann, Université Libre de Bruxelles, Bruxelles, Belgium

Correspondence
Professor Maurice Vanhaelen · Laboratory of Pharmacognosy and Bromatology · Pharmaceutical Institute · Université Libre de Bruxelles · CP 205–4, Bd du Triomphe · 1050 Bruxelles · Belgium ·
E-Mail: vanhaele@ulb.ac.be · Fax: +32 2 6505282 · Phone: Tel.: +32 2 6505279

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Material and Methods

General experimental procedures

Silica gel 60 (Merck, 40 – 63 mesh) was used for column chromatography. Analytical TLCs were carried out on silica gel $60F_{254}$ precoated plates of 0.25 mm thick. Preparative TLC separations were achieved on 20 × 40 cm plates coated with Si gel $60F_{254}$ (2 mm layer thickness). HPLC was performed on a Hewlett-Packard 1100 instrument. ^{1}H-NMR spectra were recorded on a Varian-Unit 600 instrument. The MS were obtained on a Kratos MS9 spectrometer.

Plant material and semi-quantitative determination of pollen production

Leaves and pollen were collected in March 1998 from a male *Taxus baccata* L. tree (Taxaceae) growing in the "Jardin Expérimental Jean Massart", Université Libre de Bruxelles, Belgium. A voucher specimen (N° 2001/1) has been deposited at the herbarium of the "Université Libre de Bruxelles" (BRLU). About 5% of the branches of a hundred-year-old yew were collected at random in March during its early flowering time. The branches were then allowed to dry for two days on glazed paper. After drying, the plant material was sifted and the amount of pollen produced by the flowers was calculated by gravimetry.

Extraction of taxine from pollen and leaves of *T. baccata*

Pollen and air-dried grounded leaves (60 g each) were briefly extracted by maceration under stirring with 3 × 150 ml of MeOH at 4 °C. After concentration to 200 ml, 200 ml 0.05 N H_2SO_4 were added to the MeOH solution. The solution was freed of organic solvent by evaporation under reduced pressure below 35 °C and extracted by 3 × 75 ml Et_2O. After alkalinisation with NH_4OH, the aqueous suspension was extracted with 3 × 75 ml Et_2O. The evaporation of the combined organic solvents provided the crude taxine extract. The TLC of this extract was achieved with $CHCl_3$/MeOH, 9.6 : 0.4, and the alkaloids were visualized by using either Dragendorff and iodoplatinate reagents, or a 3% sulphuric acid ethanolic solution and heating at 120 °C for 5 min, R_f taxine B: 0.20. The HPLC of the taxine constituents was achieved on a Superspher Si 60 Lichrocart column (125 × 4 mm) (Merck) with a gradient of 1,2-dichloroethane (A) and of 1,2-dichloroethane/tetrahydrofuran, 70 : 30 (B) (t_R taxine B: 17 min). The run started with 97.5% A - 2.5% B and was raised to 70.0% A – 30.0% B within 15 min followed by isocratic elution with B within a further 15 min. The flow rate was 1 ml/min and the detection achieved at 280 nm with online UV spectra determination by a diode array detector operating between 220 – 380 nm for UV spectroscopic comparisons.

Extraction, isolation and quantitative determination of taxoïds from *T. baccata* pollen

T. baccata pollen (ca. 165 g) was macerated in 1 l MeOH for 48 h. The MeOH extract was evaporated after filtration; the residue was redissolved in a small volume of MeOH and an equal volume of H_2O was added. The sequential extraction of the aqueous MeOH solution was achieved by *n*-hexane, CH_2Cl_2 and EtOAc. Each of these phases was evaporated to dryness and analyzed by ELISA using antipaclitaxel IgG [11] and anti-10-deacetylbaccatin III IgG [12]. The CH_2Cl_2 extract, which represented more than 90% of the total immunoresponse in both ELISA assays, was separated into 40 fractions (15 ml each) by chromatography on a silica gel column (15 g) successively eluted with *n*-hexane, $CHCl_3$, EtOAc and MeOH (100 ml each). Fractions 15 – 16 eluted by $CHCl_3$ and fractions 23 – 24 eluted by EtOAc showed the highest immunosignal by ELISA. Fractions 15 and 16 were pooled (42 mg) and submitted to preparative TLC using EtOAc/*n*-hexane 4 : 1 yielding 5 subfractions. On the basis of the t_R values and the UV spectra, the isolation of paclitaxel and baccatin III from subfraction 2 was achieved by reversed phase HPLC on a Hibar Lichrosorb® RP-18 column (7 μm, Merck) using CH_3CN/H2O/MeOH 45 : 40 : 20 as mobile phase, 1 ml/min. The same experimental conditions were applied to fractions 23 and 24 (38 mg) which afforded by preparative TLC 7 subfractions. 10-Deacetylbaccatin III was isolated by HPLC from subfraction 5. The quantitative determination of paclitaxel, baccatin III and 10-deacetylbaccatin III was achieved by HPLC using the above experimental conditions mentioned for their isolation.

Extraction of the taxoids conjugates from *T. baccata* pollen

T. baccata pollen (ca. 1 g) was macerated in 20 ml H_2O for 24 h at 37 °C. The suspension was centrifuged at 3000 g and further extracted by 4 × 20 ml CH_2Cl_2. The crude aqueous extract was further purified by gel filtration on a Sephadex G25 column (Pharmacia Biotech, Sweden) eluted with phosphate buffer saline (PBS : $Na_2H\ PO_4$ 0.01 M, NaCl 0.15 M, pH 7.2).

Human sera

Samples of peripheral veinous blood were taken according to the standard procedure. The sera were conserved at –20 °C and included in a private collection of the Immunology Department, CHU Brugman, Université Libre de Bruxelles, Place van Gehuchten 4, B-1020 Bruxelles, Belgium.

ELISA (enzyme-linked immunosorbent assays)

ELISA for paclitaxel and for 10 deacetylbaccatin III were performed as previously described [11], [12].

Determination of the acute toxicity of taxine isolated from *T. baccata* leaf and pollen on mice

Animal ethics committee approval was obtained for the study. Male albinos mice were obtained from Iffa Credo, France. These animals were housed in a room apart from other colonies and were maintained with food and water available at liberty.

30 mg of the crude taxine extract were dissolved in 1 ml MeOH which was further diluted with a volume of 0.01 N HCl exactly calculated in order to salify the bases. Then, the aqueous MeOH solution was evaporated under reduced pressure (1 mm) at room temperature. The hydrochlorides were then dissolved in a 0.9% NaCl aqueous solution and injected (0.3 ml) at five different concentrations (0.1, 1.0, 5.0, 15.0 and 30 mg/kg) by a single *i. p.* injection to groups of 4 mice (weight 25 – 30 g). The controls were treated by the same volumes of a 0.9% NaCl aqueous solution.

Results and Discussion

The first part of our study was devoted to the comparative analysis of taxine extracted from *T. baccata* leaf and pollen. Preliminary semi-quantitative measurements of pollen production by yew showed that a hundred-year-old *T. baccata* specimen releases into the atmosphere 16 to 20 kg of pollen during the flowering

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time. Quantitative determinations, achieved by gravimetry after careful extraction of the crude unstable alkaloid content, indicated that taxine concentration in leaf (yield: 1.31% dry weight) was seven fold higher than in pollen (yield: 0.18% dry weight). TLC and HPLC methods developed for the qualitative analysis of these extracts showed that the complexity of taxine mixture from pollen (Fig. **1A**) was much higher in comparison with that from leaf (Fig. **1B**) which contains mainly taxine B, one of the most toxic alkaloids found in yew [13].

Toxicity assays on mice provided evidence that any risk of acute toxicity of taxine resulting from *Taxus* pollen inhalation and subsequent taxine absorption is unlikely. Indeed, the penetration of several grams of pollen into the airways should be required to induce toxic symptoms even if the toxicity of the other taxanes is taken into account. The LD (lethal dose) 100 of the leaf alkaloid extract administrated intraperitonialy to mice was 15 mg (equivalent to 1.1 g of leaf)/kg and the administration, in the same conditions, of the pollen extract at 30 mg (equivalent to 17 g of pollen)/kg, induced no effect. This difference was assigned to the higher concentration of taxine B among the other alkaloids of the leaf. The mice death could result in heart failure as suggested by current investigations on taxine cardiotoxicity [14]. Further characterization of the pollen taxine was postponed as the yield of the numerous alkaloids was low.

The isolation and the characterization of taxoids in *T. baccata* pollen constituted the second part of this study. Several chromatographic steps resulted in the isolation of several taxoids among which paclitaxel ($1.0 \cdot 10-3$% dry weight), baccatin III ($1.0 \cdot 10-3$% dry weight) and 10-deacetylbaccatin III ($2.0 \cdot 10-3$% dry weight). Their detections were hindered by great amounts of flavonoids and ELISA's [11], [12] were extensively used to guide pollen extract fractionation (Fig. **2**) and to achieve their isolations. ^{1}H-NMR spectrometry and CIMS methods combined to chromatographic comparisons with authentic samples using TLC and HPLC were used to ascertain their structure. From quantitative data obtained by HPLC, we have estimated that, within one flowering season, a hundred-year-old yew could release up to 160 mg of free paclitaxel into the atmosphere. As far as we know, this is the first report on the occurrence of paclitaxel and other taxanes in *Taxus baccata* pollen.

The potential sensitization by environmental taxoids through the penetration of pollen and subsequently of its constituents into human airways constituted the third part of this study. According to the distribution area of *Taxus* sp. which are exclusively found in the northern hemisphere temperate zones [15], the comparative search for anti-paclitaxel IgG in sera samples, as a marker of sensitization, was performed among subjects living in Belgium, in Congo and in Peru. The ELISA screening of these sera was achieved by using a synthesized paclitaxel-ovalbumin molecule as a coating conjugate.

Anti-paclitaxel IgG were exclusively detected in sera from Belgian subjects as opposed to sera from subjects living in the southern hemisphere (Table **1**). Positive control sera consisted in rabbit hyperimmune sera obtained after immunization with

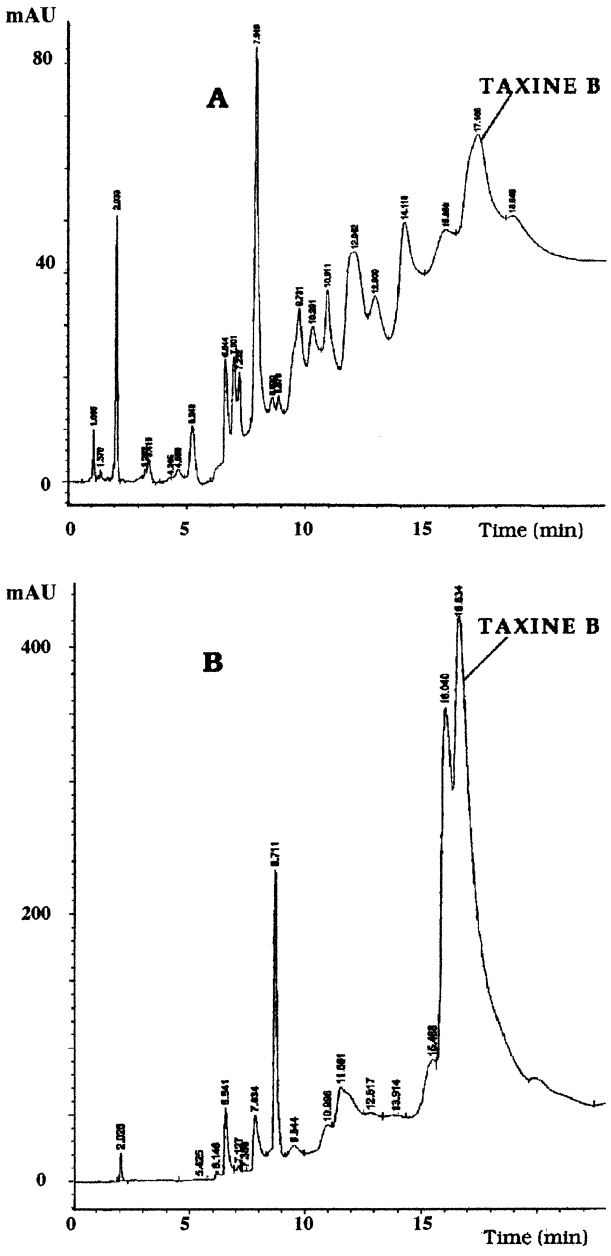

Fig. **1** Comparison of the HPLC profiles of taxine isolated from pollen (**A**) and from leaf (**B**) of *Taxus baccata* .

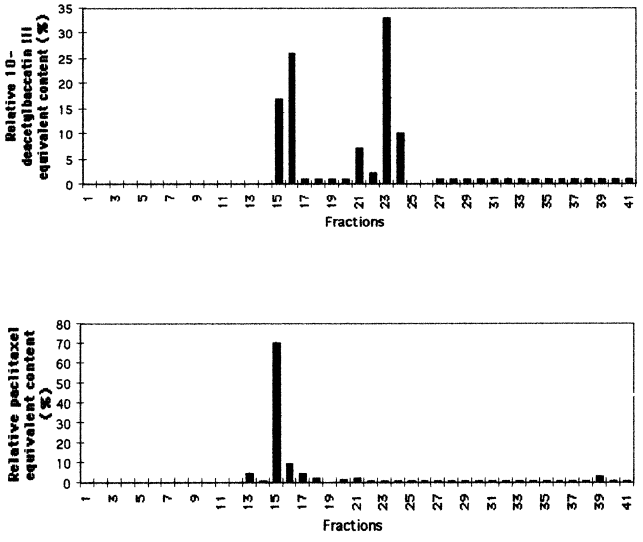

Fig. **2** Distribution of the immunoreactive constituents in the CH_2Cl_2 extract of *Taxus baccata* pollen.

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Table **1** Relative frequency of anti-paclitaxel IgG in sera samples from subjects living in the northern hemisphere (Belgium) and in the southern hemisphere (Congo and Peru) using an immobilized antigen in the solid phase of an ELISA

Tested subjects	*Relative frequency of anti-paclitaxel IgG*
Northern hemisphere (Belgian subjects)	
Unselected population of blood donnors (n = 63)	8.8%
Atopic patient showing allergy to Betula sp. (n = 47)	21.4%
Southern hemisphere	
Subjects from Congo (n = 40)	0%
Subjects from Peru (n = 10)	0%

2′-succinyl-paclitaxel-bovine serum albumin [12] and negative control consisted in the use of ovalbumin as a coating conjugate. Moreover, to discard any potential interference related to the carrier protein recognition, the same procedure was applied with another hapten-ovalbumin conjugate as immobilized antigen. This conjugate was previously used for the detection of artemisinin, an antimalarial sesquiterpene from *Artemisia annua* L. and was synthesized according to the same conjugation method used for paclitaxel [16]. The results of the assay indicated that this conjugate was not recognized by the human sera under investigation.

To further document the potential sensitization by *Taxus* sp. pollen, we tested two subgroups of Belgian subjects (Table **1**): an unselected population of blood donors and a cohort of atopic patients displaying a positive allergy to *Betula* sp. pollen as confirmed by the detection of specific IgE (data not shown). This last group of patients might well include either misdiagnosed cases of *Taxus* allergy, or polysensitized subjects reacting to more than one tree pollen. Indeed, *Betula* sp. pollination period (from April till half May, in Belgium) partially overlaps with that of *Taxus* sp. (from half March till the end of April, in Belgium). Anti-paclitaxel IgG, were detected in sera from both groups but with a significantly higher incidence in the second one (Table **1**). Binding of human IgG expressed a marked preference for protein-conjugated taxoids as confirmed by the use of an indirect competitive inhibition enzyme immunoassay. Human antipaclitaxel IgG did not show any cross reactivity with free forms of taxoids. This could reflect a lower functional affinity of these IgG to free taxoids as compared to the hyperimmune rabbit IgG.

Two hypotheses could be postulated to explain the origin of the antipaclitaxel IgG detected in the human sera. Free paclitaxel and/or taxoids could be released from *Taxus* pollen in contact with the nasal mucous; their further binding to human proteins could induce the production of specific immunoglobulins. Alternatively, a preliminary conjugation of a part of the taxoids to carriers such as pollen proteins or polysaccharides could occur. Experiments were thus carried out with an aqueous *T. baccata* pollen fraction first extracted with dichloromethane to discard the free taxoids. Aliquots from both extracts were assayed by ELISA using three different sera, rabbit anti-paclitaxel antiserum, rabbit anti-10-deacetylbaccatin III antiserum and human sera of the Belgian patients displaying positive responses to the synthesized paclitaxel-protein conjugate.

The presence in the CH_2Cl_2 fraction of both paclitaxel and 10-deacetylbaccatin III was confirmed by the indirect competitive immunoassay using rabbit anti-taxoids antisera. As previously observed, these free taxoids did not inhibit the human IgG binding.

The high molecular weight constituents of the crude aqueous fraction were further purified by chromatography on a Sephadex column and showed, by using ELISA, a significant binding with both the rabbit anti-taxoid antisera and the sera of atopic patients. These data definitively confirmed the presence in yew pollen of taxoids bound to high molecular weight which could be implicated in sensitization reactions.

The reported findings could contribute to a better understanding of hypersensitivity reactions observed during the flowering period of yew or other plants containing taxoids. Indeed, taxoids discovery in hazel tree was recently reported [17] and the flowering period of this shrub whose pollen allergenicity is well known [18] coincides with that of yew and birch. As for the other parts of this plant, the pollen could also contain these diterpenoids. Moreover, several involvements in chemotherapy with taxoids could be postulated such as drug biodisponibility modifications or increasing risk for allergic reactions during the cures as a result of a previous contact with taxoids transported by *Taxus* sp pollen. Anaphylactoid reactions were indeed previously observed in patients undergoing treatment with paclitaxel [19], [20].

From a wide point of view, the use of bioactive secondary metabolites from plants or derivatives in therapy has to be carefully considered. Unexpected effects could occur when these constituents are also accumulated in airborne pollen that allows their penetration in the human airways and the further sensitization of the patients.

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