Taxanes from Shells and Leaves of *Corylus avellana*

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Paclitaxel is an effective antineoplastic agent originally extracted in low yield from the bark of *Taxus brevifolia*. Although it was generally considered a particular metabolite of *Taxus* sp., paclitaxel was recently found in hazel cell cultures. The aim of the present work was to verify whether hazel differentiated tissues could be used as a commercial source of paclitaxel and other taxanes. Thus, shells and leaves of hazel plants were analyzed by ELISA and HPLC-MS. Both shell and leaf extracts contained taxanes. Among these, paclitaxel, 10-deacetylbaccatin III, baccatin III, paclitaxel C, and 7-epipaclitaxel were identified and quantified. Hazel extracts also showed biological activity, inhibiting metaphase to anaphase transition in a human tumor cell line. The level of total taxanes in leaves was higher than in shells collected in the same period from the same plants. However, the finding of these compounds in shells, which are considered discarded material and are mass produced by many food industries, is of interest for the future availability of paclitaxel and other antineoplastic compounds.

Paclitaxel is the generic name of Taxol, a chemotherapeutic agent with a broad spectrum of activity.[1–3] Presently, most of the drug for clinical use is produced by semisynthesis,[4] starting from a natural precursor, 10-deacetylbaccatin III, that is more readily available from the needles of yew species as a renewable source.[5,6] The same precursor is also used to produce docetaxel (Taxotere), a synthetic analogue with biological activity similar to paclitaxel. Numerous efforts are also addressed to obtain paclitaxel and its precursors from plant cell cultures of Taxus.[7] However, the cost of this drug remains very high, despite several attempts to increase its production.[7–9]

Taxus species and endophytic fungi have been considered the only sources to be exploited for the commercial supplies of paclitaxel and its precursors generally named taxanes.[10,11] The finding of paclitaxel in hazel species was initially thought to be derived from endophytic fungi living inside these plants.[12] However, our recent report on the recovery of taxanes in *in vitro* cell cultures suggested that hazel species possess the metabolic pathway(s) for taxane biosynthesis.[13]

The aim of the present study was to explore the possibility of employing plant material from a more abundant and available species than yew as a new source of taxanes. With this purpose, secondary metabolites extracted from leaves and shells of *Corylus avellana* L. (Betulaceae family) were analyzed for the presence of taxanes by ELISA and HPLC-MS. Extracts were also tested for their ability to inhibit metaphase to anaphase transition in *in vitro* tumor cells.

Results and Discussion

Methanolic extracts from leaves and shells of hazel were first screened for the presence of taxanes by ELISA analysis. Both shell and leaf extracts have been found to contain taxanes. Although ELISA did not permit either to identify or to quantify the single taxane, it is generally used to screen for the presence of these compounds.[14] Samples were considered to be positive for the content of taxanes when the value of the absorbance, calculated for hazel extracts, corresponded to a paclitaxel concentration more than 0.5 ng/mL on the standard curve.

In order to identify the main taxanes contained in hazel tissues, several extracts, already shown to be positive for the presence of taxanes by ELISA, were analyzed by HPLC-MS. Particularly, hazel shell extracts were found to contain paclitaxel, 10-deacetylbaccatin III, baccatin III, paclitaxel C, and 7-epipaclitaxel. Other taxanes were also identified, but not individually quantified, due to the limited separation under the chromatographic condition used. Several taxanes were then assembled according to their retention time (t_R): 10-deacetyl-7-xylosylcephalomannine and 10-deacetyl-7-xylosylpaclitaxel (t_R = 21.90–22.43 min); taxinine M, 10-deacetyl-7-xylosylpaclitaxel C, 10-deacetylpaclitaxel, and 7-xylosylpaclitaxel (t_R =23.53–25.19 min); cephalomannine and 10-deacetyl-7-epipaclitaxel (t_R =27.19–28.80 min).

The extracted ion chromatogram and MS/MS spectra of paclitaxel contained in hazel shell extracts are reported in Figure 1 as an example.

Shells were recovered from different plants of *C. avellana* (H1, H2, and H3) cultivated in three different sites. Our results suggested that these plants were different in taxane production. Particularly, total taxanes found in H1 were significantly higher ($p < 0.05$, t test) than total taxanes contained in H2 and H3 (Table 1). This difference was mainly attributed to the higher level of paclitaxel, baccatin III, 7-epipaclitaxel, and assembled taxanes (10-deacetyl-7-xylosylcephalomannine and 10-deacetyl-7-xylosylpaclitaxel) contained in H1 compared to H2 and H3. On the other hand, H3 contained a level of paclitaxel C significantly higher than H1. No significant difference in the level of secondary metabolites was found between H2 and H3. Hazel plants used in the present study were grown in three sites: climate, height, and land composition could be responsible for the different production of secondary metabolites in these plants. However, additional analyses will be performed in order to elucidate whether H1 also differs from H2 and H3 in genotype.

The biological activities of hazel extracts were investigated by evaluating their capability to inhibit metaphase to anaphase transition in cell culture. Table 2 shows the effect of shell methanolic extracts on human tumor SK-Mes-1 cells. A 10 nM Taxol treatment was also included as a positive control. Previous experiments treating SK-Mes-1 cells with different amounts of Taxol, ranging from 0.2 nM to 10 μM, indicated that 10 nM was the most suitable

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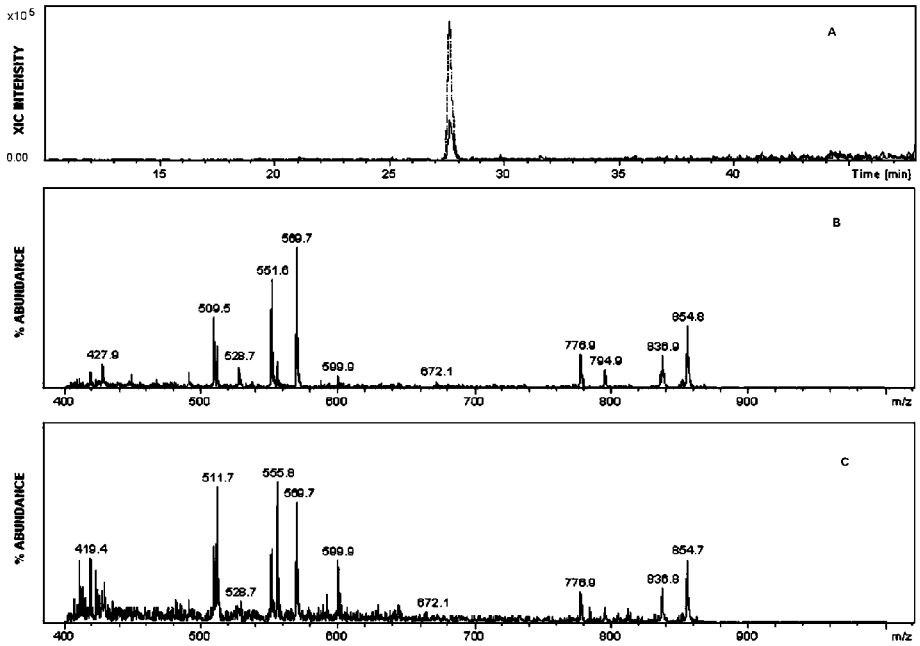

Figure 1. HPLC-MS analysis of the methanolic extract recovered from hazel shells. Extracted ion currents of paclitaxel in standard (---) and in sample (—) (A); MS/MS spectra of paclitaxel in standard (B) and in sample (C).

Table 1. Taxanes Contained in Hazel Shell Extracts

	mean ± standard deviation (μg/g dry weight)		
taxanes[a]	H1 (4)[b]	H2 (4)[b]	H3 (6)[b]
1	10.83 ± 2.43	1.75 ± 1.01	4.66 ± 2.73
2	13.21 ± 6.61	19.03 ± 13.93	29.08 ± 42.89
3	108.43 ± 9.80	43.46 ± 40.91	10.40 ± 8.77
4 + **5**	206.26 ± 131.64	52.64 ± 73.61	36.48 ± 57.50
6 + **7** + **8** + **13**	29.01 ± 5.96	36.36 ± 25.70	36.63 ± 37.98
9 + **10**	23.70 ± 3.31	15.77 ± 17.96	52.56 ± 42.04
11	0.87 ± 0.85	4.27 ± 2.17	6.49 ± 4.49
12	1.08 ± 0.60	0.11 ± 0.01	0.32 ± 0.24
total	393.40 ± 142.75	173.39 ± 91.33	176.62 ± 150.86

[a] **1**: paclitaxel; **2**: 10-deacetylbaccatin III; **3**: baccatin III; **4**: 10-deacetyl-7-xylosylcephalomannine; **5**: 10-deacetyl-7-xylosylpaclitaxel; **6**: 10-deacetyl-7-xylosylpaclitaxel C; **7**: 10-deacetylpaclitaxel; **8**: 7-xylosylpaclitaxel; **9**: cephalomannine; **10**: 10-deacetyl-7-epipaclitaxel; **11**: paclitaxel C; **12**: 7-epipaclitaxel; **13**: taxinine M. [b] Hazel type (no. of samples).

Table 2. Effect of Hazel Shell Extracts on Metaphase/Anaphase Transition

treatment	anaphases/metaphases mean ± standard deviation	p^a
control	0.159 ± 0.080	
Taxol (10 nM)	0.026 ± 0.033	<0.001
shell extracts	0.054 ± 0.004	<0.02

[a] p value estimates of significance by Fisher's exact test data are the mean of at least 3 experiments.

dose to identify a change in the anaphase/metaphase ratio in this cell type. Higher doses caused the complete block in metaphase with a lack of anaphases, preventing a precise evaluation of the ratio. A decrease in the anaphase/metaphase ratio was observed in Taxol and shell extract treated cells.

As known, the yield of secondary metabolites from plants is largely influenced by extraction and purification methods. In fact, paclitaxel, 10-deacetylbaccatin III, and basic taxanes are generally recovered using different procedures.[5,15]

In the present study hazel shells were first extracted with MeOH. An aliquot was subsequently purified through a nylon filter, while another aliquot was purified with *n*-hexane. HPLC analysis of methanolic extracts, either purified through a nylon filter or by hexane, showed that there is no difference in the recovery of taxanes

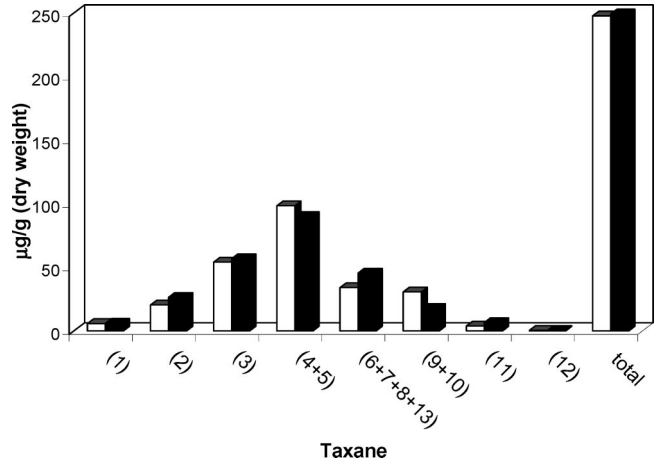

Figure 2. Taxanes recovered from hazel shell extracts purified through a nylon filter (□) or by hexane (■). **1**: paclitaxel; **2**: 10-deacetylbaccatin III; **3**: baccatin III; **4**: 10-deacetyl-7-xylosylcephalomannine; **5**: 10-deacetyl-7-xylosylpaclitaxel; **6**: 10-deacetyl-7-xylosylpaclitaxel C; **7**: 10-deacetylpaclitaxel; **8**: 7-xylosylpaclitaxel; **9**: cephalomannine; **10**: 10-deacetyl-7-epipaclitaxel; **11**: paclitaxel C; **12**: 7-epipaclitaxel; **13**: taxinine M.

with these two different methods (Figure 2). Thus, methanolic leaf extracts were processed by filtration through a nylon filter.

Figure 3 shows taxanes contained in leaves and shells collected from H2 and H3 plants during August. Although a certain variability existed, these results suggested that leaves contained a higher level of taxanes than shells ($p < 001$, t test). The difference is particularly evident for baccatin III, the pool including taxinine M, 10-deacetyl-7-xylosylpaclitaxel C, 10-deacetylpaclitaxel, and 7-xylosylpaclitaxel, and 7-epipaclitaxel. No difference was observed for the recovery of paclitaxel.

Plant secondary metabolites, as well as taxanes, are generally influenced qualitatively and quantitatively by many factors, including season. Future studies will be performed in order to determine taxane production in leaves collected during different periods of the year. Particularly, the analysis of leaves from H1 plants will deserve attention, as they have been shown to contain higher levels of taxanes in their shells.

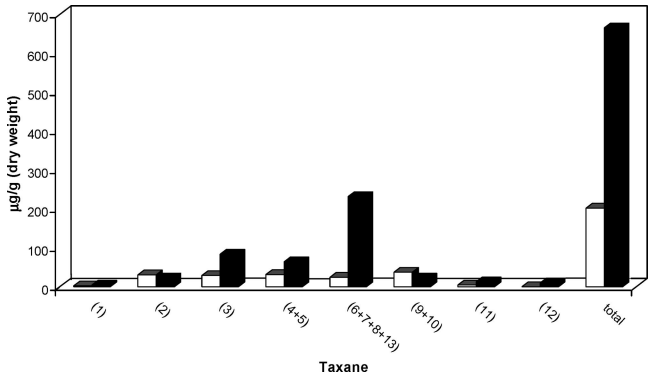

Figure 3. Taxanes recovered from hazel shells (□) and leaves (■). **1**: paclitaxel; **2**: 10-deacetylbaccatin III; **3**: baccatin III; **4**: 10-deacetyl-7-xylosylcephalomannine; **5**: 10-deacetyl-7-xylosylpaclitaxel; **6**: 10-deacetyl-7-xylosylpaclitaxel C; **7**: 10-deacetylpaclitaxel; **8**: 7-xylosylpaclitaxel; **9**: cephalomannine; **10**: 10-deacetyl-7-epipaclitaxel; **11**: paclitaxel C; **12**: 7-epipaclitaxel; **13**: taxinine M.

Even if the amount of paclitaxel recovered from differentiated tissue of hazel plants was lower than that recovered from taxus tissues, it is worth noting that hazel is a widely available and fast growing species. Moreover, the finding of paclitaxel and other taxanes in shells is of interest in consideration of the fact that they are mass produced by many Italian food industries as discarded material and that shell disposal requires labor intensive procedures, besides posing environmental concerns.

Thus, hazel shells could become a new commercial source of paclitaxel and related compounds, to be used both as new therapeutic agents and as new precursors for paclitaxel and docetaxel semisynthesis.

Experimental Section

General Experimental Procedures. For taxane identification, samples were analyzed by an Agilent 1100 HPLC system coupled to a 1100 MSD ion trap mass spectrometer, equipped with an electrospray ion source. The column was an Agilent Zorbax C18 (0.5 × 100 mm), and the gradient was a standard H_2O/CH_3CN from 30% to 100% in 40 min. Formic acid (0.1%) was added to both the H_2O and CH_3CN. Mass spectra were acquired in positive ion mode. Identification of taxanes was accomplished by comparison of t_R and mass fragmentation against the following standards (Hauser): paclitaxel, 10-deacetylbaccatin III, baccatin III, 10-deacetylpaclitaxel, 7-xylosylpaclitaxel, 10-deacetyl-7-xylosylcephalomannine, 10-deacetyl-7-xylosylpaclitaxel, 10-deacetyl-7-xylosylpaclitaxel C, cephalomannine, 10-deacetyl-7-epipaclitaxel, 7-epipaclitaxel, paclitaxel C, and taxinine M, which were prepared in MeOH. Taxanes were quantified by a Thermo Finnigan Surveyor LC Pump Plus HPLC system equipped with a PDA Plus detector using a Phenomenex Curosil PFP 5 μm particle size (250 × 4.6 mm) column. The gradient was CH_3CN/H_2O from 25% to 75% in 40 min at 1.0 mL/min. Peaks were recorded over the UV range of the absorption, and the chromatograms were plotted at 227 and 228 nm. Quantifications were carried out by reference to a calibration curve prepared with a dilution of 50 μg/mL reference sample of the above standards. The detection limit was 0.25 μg/mL.

Plant Material. Shells derived from adult *C. avellana* trees cultivated in three Northern Italian sites (Ventimiglia, Genova, Alessandria), differing in climate and height, were used for taxane extraction. Voucher specimens (H1-H2-H3/2005) were deposited at the Department of Pharmaceutical Sciences of the University of Genova. Plants from Ventimiglia (H1) possessed red leaves in spring and green leaves in summer; the leaves of plants from Genova (H2) and Alessandria (H3) are always green. Only leaves from H2 and H3 were analyzed for the presence of taxanes. Hazel branches, with leaves and nuts, were collected in July and August 2005.

Leaf and Shell Extraction. Leaves were separated from stems and dried at room temperature. Nuts were crushed and the shells were separated from seeds. About 100 g of dried leaves and shells was separately extracted with MeOH containing 0.01% HOAc for 2–3 days in the dark on a 100 rpm shaker. Extracts were filtered through a paper filter and evaporated to dryness *in vacuo*. After evaporation residues were re-extracted with MeOH to reach a concentration of 3 g/mL of shell or leaf dry weight. Shell methanolic extracts were partitioned and processed by two different procedures. An aliquot of methanolic extract was mixed with *n*-hexane (MeOH/*n*-hexane, 1:1). The *n*-hexane phase was removed and the methanolic extract was processed for taxane analysis. An aliquot of methanolic extract was purified by filtering through a 0.2 μm Gelman nylon membrane filter before being processed for taxane analysis.

Leaf extracts were only purified by filtering through a 0.2 μm nylon membrane filter and processed for taxane analysis. Each analysis was repeated at least four times.

Taxane Evaluation. A competitive solid-phase enzyme-linked immunosorbent assay (ELISA) (TAO1, Hawaii Biotech Inc.) was employed for the detection of taxanes. Free taxanes present in MeOH extracts were detected by inhibition of the reaction between solid-phase paclitaxel−protein conjugate and antitaxane antibody. The assay was performed following the procedures recommended by suppliers, using 96-well flat bottom plates (Corning Incorporated, Costar) coated with paclitaxel−protein antigen. The plates were blocked with 1% (w/v) bovine serum albumin (Sigma) in PBS, and excess antigen was washed away. Solid-phase-bound paclitaxel was incubated with 50 μL of each extract, paclitaxel standard, and a specific antitaxane rabbit antibody. Antibody bound to the solid-phase-bound paclitaxel was detected by using an alkaline-phosphatase-conjugated secondary antibody, using alkaline phosphate substrate, *p*-nitrophenyl phosphate (Sigma), as chromophore-generating substance. Absorbance of each well was read on a dual-wavelength Mithras LB 940 ELISA reader (Berthold Technologies). Taxanes were determined in each sample using a curve plot generated with paclitaxel standard. Samples with values higher than 0.5 ng/mL were considered positive for the presence of taxanes.

Biological Activity. Experimental details for evaluating the effects of hazel extracts on metaphase to anaphase transition have been described in a previous paper.[13]

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