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ORIGINAL RESEARCH

LC-ESI-MS analysis of taxoids from the bark of *Taxus wallichiana*†

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ABSTRACT: LC-ESI-MS analysis was carried out for taxoid profiling of partially purified methanol extracts of the stem bark of *Taxus wallichiana* growing in different regions of the Himalayas (Kashmir, Himachal Pradesh, UP hills, Sikkim and Arunachal Pradesh). Cone voltage fragmentation of the protonated, ammonium or sodium cationized molecular species resulted in diagnostic fragment ions. Thus, information about the number and nature of substituents and the taxane skeleton (whether it is normal or rearranged) was readily available from the LC-ESI-MS spectra. The rearranged 11(15→1)-*abeo*-taxanes showed a characteristic elimination of the hydroxyisopropyl along with an acetoxy group. The identification of the taxoids was achieved by comparison of the ESI mass spectra with those of the authentic taxoids available to us or by interpreting the ESI mass spectra. The results were also corroborated by MS/MS analysis of the partially purified extract injected directly into the ESI source. Paclitaxel, its analogues and their xylosides are present in samples from all the regions. An interesting observation is the detection of a large number of basic taxoids having nitrogen-containing side chains. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Taxanes are a group of drugs that include paclitaxel (Taxol®) and docetaxel (Taxotere®), which are used in the treatment of cancer. It was the discovery of paclitaxel (Wani *et al.*, 1971) in the stem bark of *Taxus brevifolia* Nutt. that promoted the chemical and pharmacological studies of taxane diterpenoids (taxoids). Paclitaxel, a complex diterpene amide, is now considered a prototype for a new class of chemotherapeutic agents known as microtubule-stabilizing anticancer agents (Miller and Ojima, 2001). Over 300 taxoids have so far been isolated from *Taxus* genus including the Himalayan yew, *T. wallichiana*. Worldwide, indiscriminate felling of trees has resulted in the depletion of a resource of great pharmacological potential. It is therefore essential to make use of the limited resources wisely and as completely as possible and also to find bio-renewable resources. This cannot be achieved without profiling the taxoid content of the yew trees. Compared to other species, *T. wallichiana* has not yet been fully investigated.

The Himalayan yew is a slow growing conifer, an evergreen tree, usually 6 m or so in height and 1.5–1.8 m in girth, found in the temperate Himalayas at altitudes between 1800 and 3300 m (CSIR, 1976). In contrast to the European yew (*Taxus baccata* L.), the Himalayan yew has a remarkable history of medicinal use. It is also used as a colouring material and as an incense. There are also important differences between the secondary metabolites of *T. baccata* and *T. wallichiana*. For example, C-13, C-14-oxygenated taxoids and apocarotenoids have been isolated only from the latter species (Appendino, 1995). Differences in the acylation pattern of taxoids have also been observed since the compounds of this type isolated from *T. wallichiana* generally bear an acetyl and not a benzoyl group at C-2. Furthermore, taxine, a mixture of basic alkaloids responsible for the poisonous properties of the European yew, has not yet been detected in the Himalayan yew. The total alkaloid content in *T. wallichiana* is also reported to be one of the lowest in *Taxus* species (Poupat *et al.*, 2000). So far, only one nitrogen-containing taxoid has been detected in *T. wallichiana* (Chattopadhyay *et al.*, 1996b). Our earlier study on the profiling of taxoids in the needles of *T. wallichiana* using MS/MS techniques had revealed the presence of several taxane alkaloids (Madhusudanan *et al.*, 2001a). Moreover, we have so far isolated and characterized about 30 taxoids of different structural types from the needles, leaves, stem bark, heartwood and roots of *T. wallichiana* (Chattopadhyay and Sharma, 1995; Chattopadhyay *et al.*, 1994, 1995, 1996a–e, 1997, 1998, 1999a,b). Continuing the work we have tried to

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Abbreviations used: MCA, Multichannel analyses.

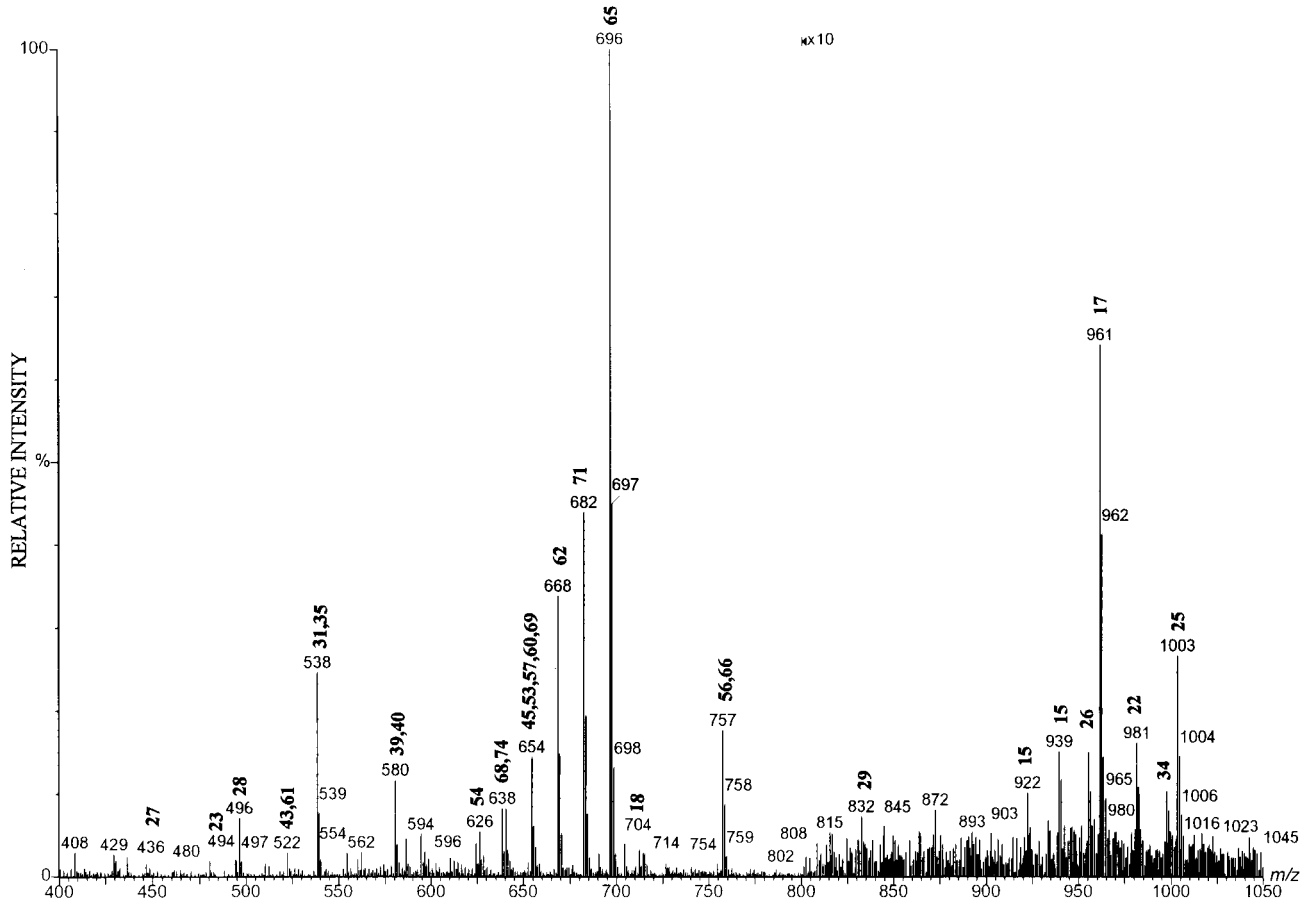

Figure 1. Electrospray ionization mass spectrum of partially purified methanolic extract of stem bark of *Taxus wallichiana* from UP Hills (U) at a cone voltage of 20 V.

profile the taxoid content in the extracts of stem bark of the Himalayan yew. It was also of interest to look for basic alkaloids in the bark extract. The taxoid profile of the extracts from different locations in the Himalayan region would help to identify the proper source of specific target molecules required for the semi-synthesis of paclitaxel or its second-generation analogues with improved efficacy, less toxicity, improved water solubility and pharmacokinetic properties.

Isolation and characterization of minor taxoids present in plant extracts or cell culture extracts by conventional means is extremely difficult because of the occurrence of several components in trace quantities. With the advent of techniques such as atmospheric pressure chemical ionization (APCI; Bruins, 1994) or electrospray ionization (ESI; Fenn *et al.*, 1990), mass spectrometry can now rapidly provide detailed analytical information for the analysis of such mixtures. Separation and identification can be achieved in the same analysis using hyphenated techniques such as LC-MS and MS/MS. LC-MS combines all the steps of the bench-scale protocol for the identification of components in complex mixtures. Several reports have appeared in the literature, demonstrating the applicability of LC (Mroczek *et al.*, 2000), LC-MS and MS/MS techniques in the analysis of taxanes (Griffini *et al.*, 1993; Hoffmann *et al.*, 1998; Hoke *et al.*, 1994; Kerns *et al.*, 1994, 1995; Madhusudanan *et al.*, 1997; Chang *et al.*, 2000).

Like FAB ionization, electrospray ionization also allows the application of a number of ionizing agents to effect metal cationization or ammonium cationization. In the presence of ammonium acetate, ESI gives the ammonium adduct ions of the various components in a mixture. A preliminary experiment with *T. wallichiana* extracts has indicated that at a cone voltage <20 V no fragment ions but only $[M + NH_4]^+$ ions are observed except in the case of amino taxoids which give protonated molecular ions. The molecular species can then be characterized by their MS/MS spectra. However, isomeric components in the mixture can not be resolved by this technique. It was therefore decided to carry out the profiling of taxoids in the bark extracts of *T. wallichiana* growing in different Himalayan regions by LC-ESI-MS.

EXPERIMENTAL

The stem bark of *T. wallichiana* were collected from different

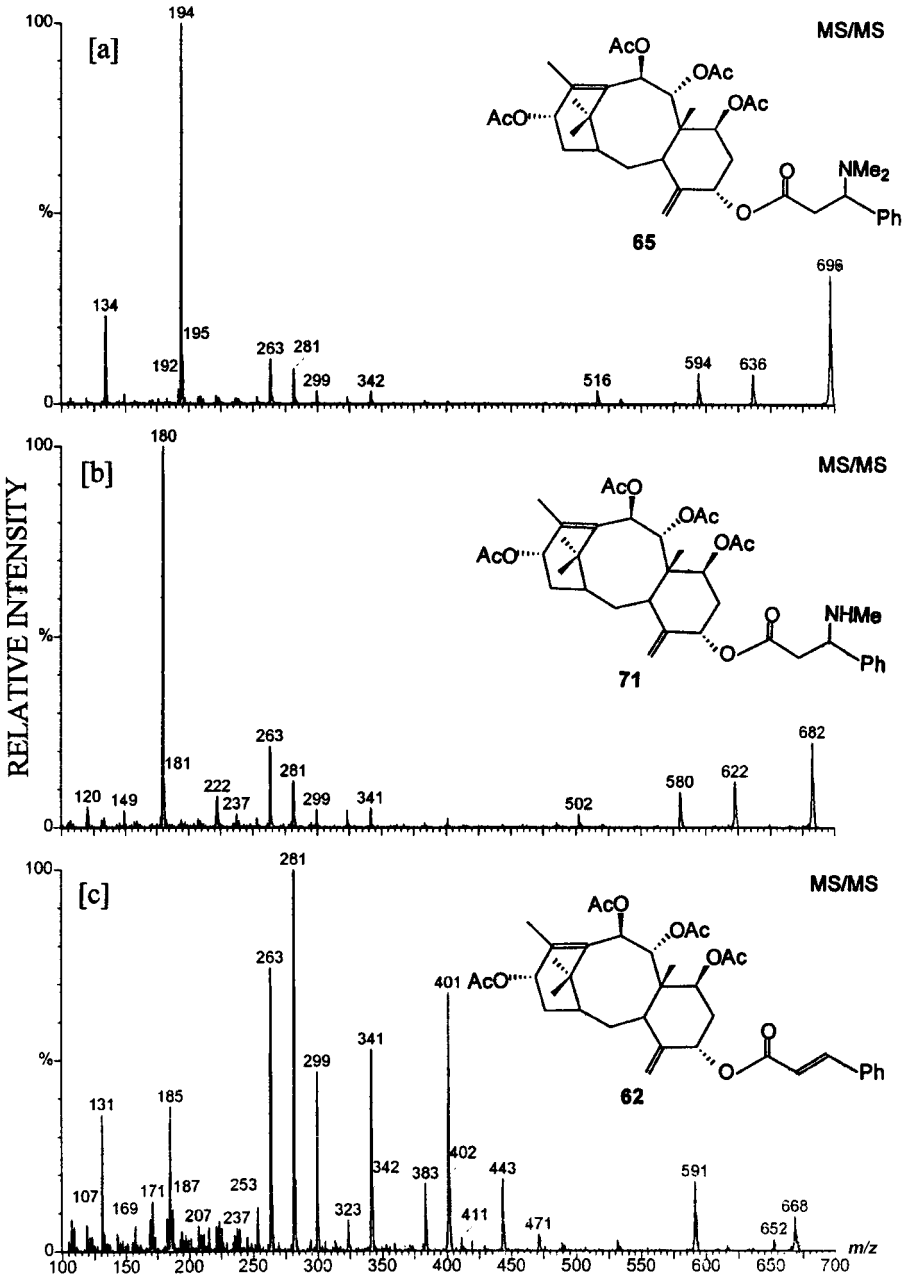

Figure 2. MS/MS (cone voltage 20 V) and LC-MS (cone voltage 80 V) spectra of $[M + NH_4]^+$ or $[M + H]^+$ ions of authentic taxoids: (a) **65** ($[M + H]^+$ at *m/z* 696, collision energy 20 eV), (b) **71** ($[M + H]^+$ at *m/z* 682, collision energy 20 eV), and (c) **62** ($[M + NH_4]^+$ at *m/z* 668, collision energy 15 eV). The LC-MS spectra of these components are given in (d), (e) and (f), respectively. (*continued opposite*).

regions stretching from Kashmir in the western Himalayas to Arunachal Pradesh in the eastern Himalayas. Accordingly, five samples from Kashmir (K), Himachal Pradesh (H), UP hills (U), Sikkim (S), Arunachal Pradesh (A) were collected during October 1997. The plant materials were identified by the Botany Division of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India and voucher specimen numbers are CIMAP KB-1, GGB-10, UPB-1, SB-1 and AB-105.

Extraction of taxoids. The stem bark of *T. wallichiana* after collection were dried and ground to a particle size of 3–5 mm. An aliquot of 20 g of ground bark was extracted with 50 mL (×3) of methanol during 48 h at room temperature (25°C). The methanol extracts were pooled and concentrated under reduced pressure to dryness (2 g). The residue was stirred with 50 ml water (pH 6.5) and extracted with hexane (3 × 50 ml). The hexane extract was discarded. The aqueous layer was then extracted with chloroform (3 × 50 ml). The chloroform extracts were combined and dried over anhydrous sodium sulphate, concentrated and redissolved in chloroform:methanol (1:1, 25 ml). The resulting solution was

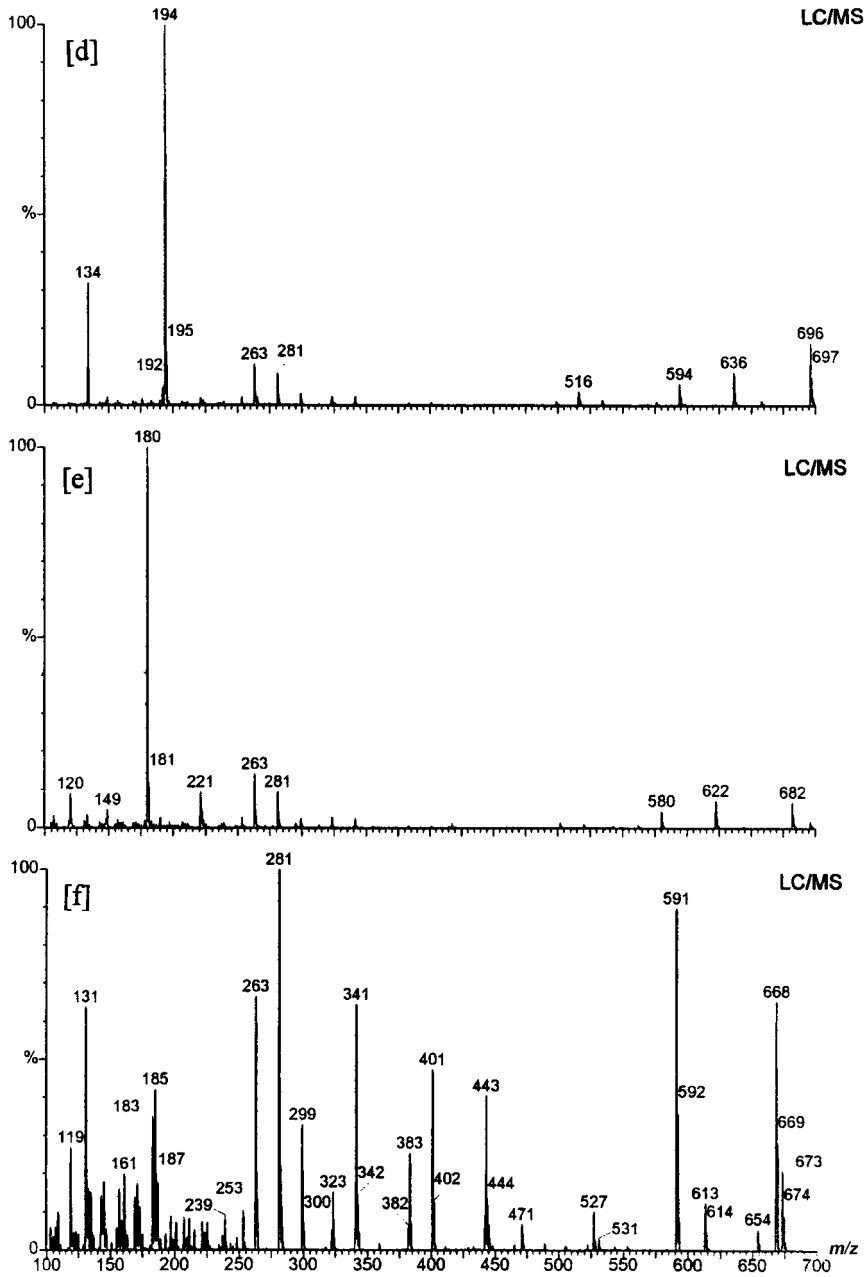

Figure 2. *Continued.*

filtered through Whatman filter paper and concentrated. The residue was used for LC-ESI-MS and MS/MS analyses. The authentic standards used in this study were isolated earlier from *T. wallichiana* and identified and fully characterized.

LC-ESI-MS. The HPLC separation was performed on a JASCO 980 LC using a Waters Spherisorb, 5 μm ODS 2 analytical column (4.6 × 250 mm) and a linear gradient elution with acetonitrile:1 mM ammonium acetate in water:methanol (2:2:1) as solvent A and acetonitrile:methanol (2:1) as solvent B (100% A up to 15 min, from 100% A to 100% B at 30 min and 100% B until 45 min) at a flow rate of 1.5 mL min^{-1}. The LC effluent was split in the ratio 10:1 to pass it through a UV detector at 280 nm and the mass spectrometer.

The electrospray mass spectra and MS/MS spectra were recorded on a Micromass Quattro II triple-quadrupole mass spectrometer (Micromass, UK) equipped with an electrospray ionization source. Heated dry nitrogen at 80°C was used as the nebulizing and drying gas at flow rates of 10 and 250 L h^{-1} respectively. The ESI source potentials were: capillary 3.5 kV; lens at 0.5 kV; cone at 80 V; and skimmer lens offset at 5 V. The mass spectrometer was scanned at the rate of 500 mass units per second. Mass resolution was set at approximately 1000. Data acquisition and processing were carried out using MassLynx 3.3 software supplied with the instrument. Calibration was carried out using NaI/CsI solution. For direct introduction of the sample, solutions were prepared in methanol containing 1 mM ammonium acetate at a concentration of approximately 10^{-4} M. The samples were

Table 1. Taxoids from bark extracts by LC/MS

Sample no.	Component tentatively identified	Retention Time	Molecular weight	MS data	Distribution K	H	U	S	A
1	Taxayuntin G	3.1	570	593(46), 588(9), 535(46), 475(100), 457(25), 415(68), 355(65), 295(74), 277(20), 253(20)	—	—	—	×	×
2	Taxuspine O	3.2	508	531(25), 526(22), 491(32), 473(57), 431 (12), 371(19), 353(17), 31(100), 311(36) 293(62), 275(32), 253(23), 211(41)	×	×	—	—	—
3	Taxayuntin J	3.5	612	635(49), 630(2), 613(5), 577(17), 517(49), 475(20), 457(62), 397(21), 357(100), 337 (21), 295(26), 277(20)	—	—	—	×	×
4	10,13-Deacetyl-*abeo*-baccatin IV	3.7	568	591(25), 586(14), 551(4), 533(11), 491(9), 473(21), 431(19), 423(11), 373(77), 313 (100), 253(26), 227(37)	×	×	×	—	—
5	19-Debenzoyl-19-acetyl taxinine M	3.7	624	647(28), 642(100), 607(13), 547(15), 50(12), 487(27), 445(20), 385(16), 325(14), 283(11), 265(8)	—	—	—	×	×
6	1-Hydroxy-5-deacetyl baccatin I	4.2	610	633(89), 628(49), 551(44), 491(20), 473(32), 431(47), 371(100), 353(62), 11(60), 293(64)	—	—	—	×	×
7	Taxchin A	4.3	638	661(43), 656(22), 639(30), 579(56), 537(9), 477(100), 459(25), 17(46), 357(32), 297(74), 279(37)	×	×	×	×	×
8	5-Decinnamoyl taxagi-fine	4.5	566	589(14), 584(100), 549(9), 489(16), 429(10), 387(12), 369(19), 327(22), 309(19), 197(5)	—	—	—	×	×
9	Baccatin III	4.6	586	609(53), 604(40), 587(21), 527(19), 509(21), 467(9), 449(16), 405(33), 387(17), 345(72), 327(83), 299(22), 287(21), 133(27)	×	×	—	×	×
10	2-Benzoyloxy-7,9,10, 13-tetraacetoxy-4(20), 11 -taxadiene	4.9	640	663(96), 658(80), 623(28), 603(49), 543(15), 401(16), 341(15), 281(42), 263(36), 105(100)	×	×	×	×	×
11	7-Xylosyl-10-deacetyl taxol D	5.2	909	927(6), 910(19), 659(9), 509(14), 252(100)	—	×	—	×	×
12	2,5,9-Trihydroxy-10, 13-diacetoxy-4(20), 11-taxadiene	5.4	436	459(15), 454(100), 377(80), 317(14), 299 (20), 281(5)	×	—	—	—	—
13	Taxayuntin F	5.6	630	653(25), 648(15), 613(22), 595(16), 535 (21), 493(15), 475(14), 433(15), 415(10), 373(98), 353(8), 313(100), 293(23), 253 (29), 227(26), 211(12), 105(28)	—	×	—	×	×
14	Baccatin IV	5.7	652	675(58), 670(100), 615(22), 593(19), 555 (30), 533(12), 495(7), 311(38), 293(37)	×	×	×	×	×
15	7-Xylosyl-10-deacetyl cephalomannine	6.1	921	922(1), 772(1), 641(1), 529(2), 509(3), 264 (100)	×	×	×	×	×
16	Taxayuntin E	6.4	630	653(50), 648(14), 629(7), 613(7), 595(25), 553(9), 535(22), 493(17), 435(94), 375(100), 353(22), 315(20), 297(37), 253(26), 227(28), 105(70)	—	—	—	×	×
17	7-Xylosyl-10-deacetyl taxol	6.8	943	961(2), 944(20), 926(3), 812(1), 794(3), 659(6), 641(4), 509(10), 286(100)	×	×	×	×	×
18	Taxinine M	7.0	686	709(100), 704(79), 669(11), 609(12), 549 (14), 507(5), 445(19), 385(10), 325(22)	×	×	×	×	×

Table 1. continued.

Sample no.	Component tentatively identified	Retention Time	Molecular weight	MS data	Distribution K	H	U	S	A
19	10-Deacetyl cephalomannine	7.3	789	812(21), 790(54), 772(6), 540(11), 499 (20), 477(30), 459(10), 417(7), 399(11), 315(10), 297(58), 279(21), 264(100)	×	×	—	×	×
20	5-Decinnamoyl taxinine J	7.4	578	601(42), 596(15), 541(22), 519(21), 501 (14), 417(16), 399(23), 357(25), 339(38), 321(15), 297(54), 279(100), 261(27)	×	×	—	×	—
21	13-Deacetyl baccatin VI	7.5	672	695(31), 690(26), 533(21), 491(12), 473 (25), 431(17), 373(100), 313(93), 293(23), 253(21), 227(20), 211(9), 105(5)	×	×	×	—	—
22	7-Xylosyl cephalomannine	7.8	963	986(86), 981(78), 964(100), 874(23), 776 (33), 730(25), 569(35), 264(74), 246(86)	×	×	×	×	×
23	Taxinine A	7.9	476	499(14), 494(13), 477(9), 417(40), 357(43), 339(25), 315(22), 297(100), 279(55)	×	×	—	—	—
24	10-Deacetyl taxol	7.9	811	834(2), 829(1), 812(10), 794(4), 527(3), 509(5), 286(100), 105(4)	×	×		×	×
25	7-Xylosyl taxol	8.6	985	1008(79), 1005(74), 986(43), 854(28), 794(19), 569(10), 551(15), 509(25), 286(96), 268(100), 240(43), 105(16)	×	×	×	×	×
26	7-Xylosyl-10-deacetyl taxol C	8.9	937	960(3), 938(14), 920(3), 855(4), 806(2), 788(3), 659(10), 641(6), 557(3), 517(5), 509(12), 280(100)	×	×	×	×	×
27	5-Hydroxy-9,10-dia-cetoxy-13-oxo-4(20), 11-taxadiene	9.2	418	441(70), 419(55), 401(10), 359(45), 341(15), 299(65), 281(100), 263(15)	×	×	×	—	—
28	2-Deacetyl-5-decinnamoyl taxinine E	10.1	478	501(22), 496(43), 461(33), 419(44), 401(19), 359(14), 341(43), 299(62), 281(100), 263(19), 185(75)	×	×	×	×	—
29	Cephalomannine	10.2	831	832(24), 796(9), 754(7), 569(19), 509(13), 264(100), 246(14)	×	×	×	×	×
30	10-Deacetyl *epi*-cephalomannine	10.4	789	81293), 807(25), 790(13), 264(100)	—	—	—	×	×
31	2-Deacetoxy-5-decinnamoyl taxinine J	11.0	520	543(19), 538(19), 483(30), 461(42), 401(23), 359(11), 341(46), 299(35), 281(100), 263(19), 185(27)	×	×	×	×	×
32	Paclitaxel	11.4	853	876(20), 871(62), 54(72), 569(19), 509(27), 286(100), 268(21), 240(18), 105(9)	×	×	×	×	×
33	10-Deacetyl *epi*-taxol	11.6	811	834(6), 829(37), 812(10), 794(8), 509(4), 286(100), 268(5), 240(7), 105(23)	—	—	—	×	×
34	7-Xylosyl taxol C	11.8	979	1002(10), 997(10), 980(28), 920(19), 848(37), 788(27), 569(25), 551(23), 509(22), 262(100), 234(20)	—	—	×	×	—
35	10-Deacetyl yunnanaxane	16.1	520	543(86), 538(19), 521(10), 461(32), 343(47), 283(93), 265(13), 161(100), 135(39)	—	—	—	×	×
36	*Epi*-taxol	16.7	853	876(19), 854(12), 794(19), 776(9), 569(39), 551(21), 509(100), 286(24), 168(40)	—	—	—	×	—
37	2-Hydroxy-2′-deacetoxyaustrospicatine	17.0	711	734(6), 712(100), 752(15), 279(5), 261(6), 194(25), 134(12)	×	×	×	—	—
38	2-Hydroxy-5,10,14-triacetoxy 4(20), 11-taxadiene	17.9	462	485(100), 480(10), 463(10), 403(27), 343(15), 283(51), 265(7), 161(80), 135(50)	×	×	×	×	×

Table 1. continued.

Sample no.	Component tentatively identified	Retention Time	Molecular weight	MS data	Distribution K	H	U	S	A
39	5,7,9,10,13-Pentaacetoxy 4(20), 11-taxadiene	18.3	562	585(83), 580(11), 525(28), 503(19), 485(36), 443(9), 401(40), 383(10), 341(54), 299(37), 281(100), 263(33), 185(41)	—	—	—	×	×
40	Yunnanxane	19.7	562	585(72), 580(43), 503(8), 485(31), 403(6), 385(42), 343(35), 325(19), 283(64), 265(100), 161(60), 135(28)	×	×	×	×	×
41	2-Acetoxy-2′,7-dideacetoxy-1-hydroxy austrospicatine	20.6	711	734(5), 712(41), 652(10), 592(5), 279(2), 261(3), 194(100)	×	—	—	—	—
42	7,9,10,13-Tetraacetoxy-5-(3′-acetylamino-3′-phenyl)propionyloxy-4(20), 11-taxadiene	20.8	709	732(80), 727(77), 710(20), 672(93), 650(27), 304(63), 281(26), 263(100), 251(47), 208(72)	—	×	×	—	—
43	Taxusin	21.3	504	527(100), 522(5), 467(6), 345(19), 325(10), 283(8), 265(95), 223(9), 133(34)	×	×	×	—	×
44	9-O-Benzoyl-9,10-dideacetyl (15-1)-*abeo* baccatin VI	21.9	734	757(60), 752(21), 717(51), 699(46), 657(100), 597(59), 535(17), 477(62), 417(63), 353(21), 293(21), 53(13), 105(46)	—	—	—	×	×
45	Hydroxy-triacetoxy-5-(3′-dimethylamino-3′-phenyl)propionyloxy-4(20), 11-taxadiene	22.1	653	654(30), 594(3), 297(12), 263(9), 194(100)	×	—	—	—	—
46	N-Methyl taxol C	22.3	861	884(9), 862(63), 734(12), 639(33), 569(47), 509(43), 294(100), 276(21)	—	—	—	×	—
47	1-Dehydroxy baccatin VI	22.3	698	721(100), 661(12), 639(37), 579(19), 537(47), 499(15), 415(9), 373(14), 355(14), 313(23), 295(36)	—	—	—	×	×
48	7-Deacetoxy-2′,9,10,13-tetraacetoxy-2-hydroxy-5-(3′-methyl amino-3′-phenyl)propionyloxy-4(20),11-taxadiene	22.5	697	720(100), 715(8), 680(27), 660(15), 638(16), 578(10), 560(20), 401(26), 383(10), 341(33), 23(19), 99(14), 81(54), 263(47), 238(74), 220(26)	×	×	×	—	—
49	2-Deacetyl-2-benzoyl-13-acetyl taxayuntin	22.7	776	799(100), 794(51), 655(62), 637(58), 595(59), 535(62), 415(59), 355(49), 297(32)	—	—	—	×	×
50	Taxchin B	23.2	768	791(100), 769(10), 709(74), 667(26), 649(22), 607(7), 589(8), 399(5), 339(4), 279(14), 261(8), 131(6)	×	—	—	—	—
51	2,7-Dibenzoyloxy-5,20-epoxy-1-hydroxy-4,9,10,11-tetraacetoxy tax-11-ene	23.2	776	799(100), 794(84), 777(5), 759(19), 717(64), 699(49), 657(53), 597(51), 535(14), 475(12), 415(85), 355(60), 105(20)	—	—	—	×	×
52	9-Benzoyl-9-deacetyl-11(15-1)*abeo* baccatin VI	23.9	776	797(69), 794(81), 717(52), 699(60), 657(100), 597(49), 535(15), 477(72), 417(72), 353(19), 293(16), 253(11), 105(38)	—	—	—	×	×
53	Hydroxy-triacetoxy-5-(3′-dimethylamino-3′-phenyl)propionyloxy-4(20), 11-taxadiene	24.4	653	654(42), 594(12), 401(6), 341(6), 281(20), 194(100), 134(22)	×	—	—	—	—
54	Taxezopidine G	24.5	608	631(47), 626(19), 609(6), 591(75), 531(5), 471(11), 411(5), 383(10), 323(25), 299(20), 281(100), 263(78), 185(37), 131(47)	×	×	×	—	—

Table 1. continued.

Sample no.	Component tentatively identified	Retention Time	Molecular weight	MS data	Distribution K	H	U	S	A
55	Taxinine J	24.5	708	731(31), 726(30), 687(18), 649(43), 591(18), 517(100), 501(37), 415(36), 399(46), 357(77), 355(56), 339(51), 321(35), 295(86), 279(98), 261(68), 131(81)	—	—	—	×	×
56	2′,7,9,10,13-Pentaace-to-xy-5-(3′-methylami-no-3′-phenyl)propionyl-oxy-4(20), 11-taxadiene	24.7	739	762(100), 757(10), 702(26), 680(21), 638(19), 620(17), 578(15), 560(16), 443(19), 401(56), 383(25), 341(49), 323(25), 299(21), 281(73), 263(90), 238(62), 220(46), 185(22), 131(23)	×	×	×	—	—
57	Hydroxy-triacetoxy-5-(3′-dimethylamino-3′-phenyl)propionyloxy-4(20), 11-taxadiene	24.7	653	654(27), 594(5), 534(2), 474(2), 237(2), 194(100), 134(14)	×	×	—	—	—
58	2-Acetoxy-2′-deacetoxy austrospicatine	24.8	753	754(17), 694(4), 517(4), 385(9), 279(7), 261(8), 194(100), 134(19)	—	—	—	×	×
59	2-Deacetoxy taxinine B	25.6	606	629(30), 624(6), 607(6), 547(35), 459(48), 417(17), 399(16), 357(27), 339(15), 297(100), 279(42), 131(14)	×	×	×	×	×
60	Hydroxy-triacetoxy-5-(3′-dimethylamino-3′-phenyl)propionyloxy-4(20), 11-taxadiene	25.8	653	654(27), 594(16), 194(100), 134(19)	×	—	×	—	—
61	Taxayunnanine C	26.3	504	527(72), 522(15), 505(14), 445(38), 343(30), 283(72), 265(9), 161(100), 135(47)	×	×	×	×	×
62	2-Deacetoxy taxinine J	26.6	650	673(18), 668(62), 591(83), 531(3), 471(9), 443(38), 401(46), 383(24), 341(64), 279(35), 281(100), 263(72), 185(40), 131(59)	×	×	×	×	×
63	2′-Deacetyl austrotax-ine	27.0	725	726(19), 684(4), 666(2), 569(6), 552(7), 401(4), 341(6), 292(9), 281(14), 263(17), 210(100), 194(59)	×	—	—	—	—
64	2,5,10-Triacetoxy-14-(2-methyl butyryloxy)-4(20), 11-taxadiene	27.2	546	569(48), 564(14), 445(3), 385(26), 343(5), 325(15), 283(23), 265(100), 223(10), 133(27)	×	—	—	×	—
65	2′-Deacetoxy austrospi-catine	27.3	695	718(3), 696(16), 594(10), 534(5), 516(5), 341(4), 299(4), 281(10), 263(12), 194(100), 134(32)	×	×	×	×	×
66	2,7,9,10,13-Pentaace-toxy-5-(3′-methylamino -3′-phenyl)propiony-loxy-4(20),11-taxadiene	27.8	739	762(13), 757(100), 680(28), 638(9), 620(9), 560(5), 500(4), 222(17), 180(100)	—	—	×	×	—
67	1-Deoxy-9,10-diacetyl taxine B	28.2	651	674(8), 652(51), 357(6), 297(35), 194(100)	×	×	×	×	×
68	9,10,13-Triacetoxy-5-(3′-dimethyl amino -3′-phenyl)pro-pionyloxy-4(20),11-taxadiene	28.5	637	638(11), 578(7), 518(1), 283(3), 265(7), 194(100), 134(43)	×	×	×	×	—
69	Hydroxy-triacetoxy-5-(3′-dimethylamino -3′-phenyl)propionyloxy-4(20), 11-taxadiene	29.6	653	654(25), 299(8), 281(3), 194(100), 134(5)	×	×	×	×	×
70	9,10,13-Triacetoxy-7-hydroxy-5-(3′-methyl amino -3′-phenyl)pro pionyloxy-4(20), 11-taxadiene	30.2	639	640(27), 580(16), 180(100)	×	—	×	—	—

Table 1. continued.

Sample no.	Component tentatively identified	Retention Time	Molecular weight	MS data	Distribution K	H	U	S	A
71	7,9,10,13-Tetraace toxy-5-(3′-methyl amino -3′-phenyl)pro pionyloxy-4(20), 11-taxadiene	30.9	681	682(10), 622(12), 580(7), 520(1), 502(3), 323 (5), 281(11), 263(16), 221(12), 180(100)	×	×	×	×	×
72	9,10,13-Triacetoxy-5-(3′-methyl amino -3′-phenyl)pro-pionyloxy-4(20), 11-taxadiene	32.0	623	624(15), 564(3), 283(4), 265(8), 221(12), 180 (100)	×	×	×	×	×
73	9,13-Dioxo-10-hydroxy-5-(3′-methyl amino -3′-phenyl)propionyloxy-4(20), 11-taxadiene	33.9	493	494(100), 180(98)	—	—	—	×	×
74	7,9,10-Triacetoxy-13-oxo-5-(3′-methyl amino -3′-phenyl)pro pionyloxy-4(20), 11- taxadiene	34.1	637	638(30), 357(5), 315(3), 297(22), 279(11), 180(100), 120(7)	×	×	×	×	×
75	7,9,10-Triacetoxy-13-hydroxy-5-(3′-methyl amino -3′-phenyl)propionyloxy-4(20), 11-taxadiene	37.1	639	640(20), 622(3), 580(2), 341(5), 299(10), 281 (11), 263(9), 221(12), 180(100)	×	×	×	—	—

infused into the ESI source from a Harvard Apparatus Model 11 syringe pump at a flow rate of 5 μL min^{-1}. MS/MS spectra of various ions were performed in an r.f.-only hexapole lens. Argon was admitted at a pressure so as to achieve 30% transmission of the precursor ion. The collision energy was 25 eV. The ESI-MS and MS/MS spectra were collected at a scan rate of 300 mass units per second in the multichannel analyser (MCA) mode. The spectra were accumulated over 25 scans.

RESULTS AND DISCUSSION

Ammonium cationization at low cone voltage followed by MS/MS was utilized earlier by us to profile the taxoids in the partially purified extracts of the needles of *T. wallichiana* from different Himalayan regions (Madhusudanan *et al.*, 2001b). As an illustrative example, a similar spectrum obtained from the bark extract of the sample from UP hills is given in Fig. 1, which clearly shows the molecular species present in the mixture. However, since isomeric compounds having same molecular weights cannot be resolved using this technique it was decided to analyse the bark extract samples by LC-ESI-MS. In order to get structural information directly from LC-MS runs the cone voltage was raised to 80 V in the present study. This resulted in the simultaneous observation of $[M + NH_4]^+$ and $[M + Na]^+$ ions for most of the components. Some components predominantly produced $[M + Na]^+$ or $[M + H]^+$ ions depending upon their structural features. All the spectra contained fragment ions indicative of the type of taxoid. The similarity of the MS/MS and LC-MS spectra can be confirmed from the examples of MS/MS and the corresponding LC-MS spectra given in Fig. 2. The TIC traces obtained for the diffferent samples from Kashmir, Himachal Pradesh, UP hills, Sikkim and Arunachal Pradesh are given in Fig. 3. The distribution of taxoids and the components identified tentatively from the mass spectral fragmentation pattern are given in Table 1. Except for a few, all are listed in a recent review (Baloglu and Kingston, 1999). Structurally diagnostic ions which help to identify the taxoids and their abundances in the LC-MS spectra are also included in Table 1.

Several structural types of taxoids are known, the most common being the class with a C4(20) double bond. Others include taxoids with C4(20) epoxide, oxetane ring, opened oxetane or oxirane ring and taxoids with C12(16) oxido bridge and C4(20) double bond. A growing number of taxoids having 11(15→1) *abeo*-taxane skeleton are also being isolated. Along with NMR spectroscopy mass spectrometry plays an important role in structure elucidation of taxoids. As explained in our previous publications (Madhusudanan *et al.*, 1997, 2001a,b), normal taxoids having C4(20) double bond give characteristic fragments at *m/z* 265/283, 263/281 or 261/279 depending upon whether the taxane skeleton has four, five or six substituents, respectively. The (15→1) *abeo*-taxoids exhibit similar peaks at *m/z* 221/239 or 219/

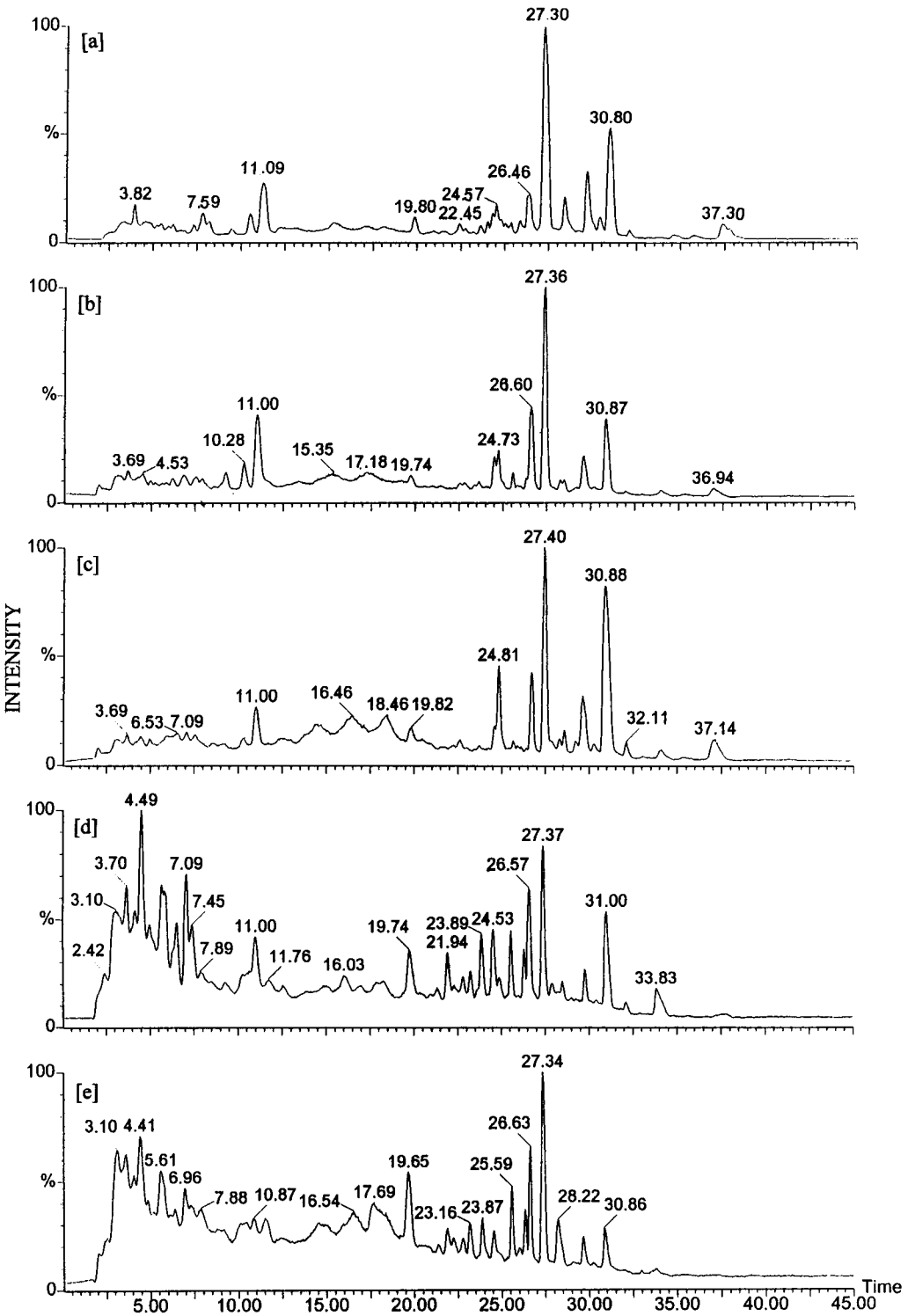

Figure 3. TIC (*m/z* 170–1050) ESI-LC-MS of the partially purified methanolic extracts of stem bark of *T. wallichiana* from (a) Kashmir, (b) Himachal Pradesh, (c) UP hills, (d) Sikkim and (e) Arunachal Pradesh.

237. Moreover, (15→1) *abeo*-taxoids also give rise to abundant peaks due to elimination of 118 Da depending upon the presence of suitable acetoxy functions. Oxetane-containing taxoids do not give any similar characteristic ions. Peaks at *m/z* 105 and 131 corresponding to benzoyl and cinnamoyl ions, respectively, and peaks due to elimination of benzoic and cinnamic acids indicate the occurrence of benzoyl and cinnamoyl derivatives.

The presence of nitrogen-containing substituents is indicated by abundant even mass fragment ions and also often by abundant $[M + H]^+$ ions. Accordingly, the presence of peaks at *m/z* 180, 194, 210, 238, 252, 264, 280 and 286 are indicative of side chains containing nitrogen. Almost half of the 75 taxoids for which tentative structures are given belong to basic or amide type taxoids. Twenty-two basic taxoids and 14 taxol derivatives (amides) were identified on the basis of their mass spectral characteristics. The intense signals at 27.3 and 30.9 min. in the TIC (Fig. 1) correspond to the basic taxoids having molecular weights (mw) 695 (**65**) and 681 (**71**), respectively. Both contain four acetoxy groups, C4(20) exocyclic methylene group and a β-aminoacid ester side chain at C5. The β-aminoacid has *N*-dimethyl group in the former and *N*-methyl group in the latter as evidenced by the abundant fragment ions at *m/z* 194 and 180, respectively. Basic taxoid containing one more acetoxy function at C2′ and having mw 753 (**58**) is detected at 24.8 min.

Analysis of LC-MS data has revealed the presence of a regular amino taxoid, **42** (mw 709) with four acetoxy groups and a β-aminoacid ester side chain having *N*-acetyl group (*m/z* 208). Another basic taxoid detected had an mw of 697 (**48**) with three acetoxy groups, one hydroxy group and a β-aminoacid ester side chain having *N*-methyl amino and 2′-acetoxy groups (*m/z* 238). 2′-Deacetyl austrotaxine, **63** (mw 725) having four acetoxy groups, a keto function and C5 β-aminoacid ester side chain with *N*-dimethyl and 2′-hydroxy groups (*m/z* 210), was detected only in the sample from Kashmir.

The basic taxane eluting at 28.2 min having mw 651 (**67**) could be 1-deoxy diacetyl taxine B (Appendino *et al.*, 1993). Another basic taxane eluting at 33.9 min gave a simple mass spectrum showing only two peaks at *m/z* 494 ($[M + H]^+$) and *m/z* 180 (β-aminoacid ester side chain). The molecule seems to contain two carbonyl functions and a hydroxyl group and could be identified as 9,13-dioxo-10-hydroxy-5(3′-methylamino-3′-phenyl)-propionyloxy-4(20),11-taxadiene (**73**).

The presence of many isomeric basic taxoids could be confirmed by LC-MS analysis. For example, there are five isomeric basic taxoids (mw 653) with a C4(20)-exocyclic methylene group and a hydroxy group at C5 esterified with a β-amino acid. One of them could be taxuspine Z, reported earlier from the Japanese yew *Taxus cuspidata* (Shigemori *et al.*, 1997). The other four isomers also showed the presence of a β-amino acid side chain with an *N*-dimethyl group (*m/z* 194), three acetoxy groups and a hydroxy group. Thus, on the basis of LC-MS data, it can be inferred that the four taxoids are isomers of taxuspine Z. These are still not reported from *Taxus* species. This study has also confirmed the presence of two isomeric basic taxoids with mw 639 and another one with an mw of 623 (**72**). All three contain an amino acid ester side chain (*m/z* 180) and belong to regular taxoid. LC-MS data also confirm that the taxoids with mw 639 contain three acetoxy and one hydroxy groups whereas, the one with mw 623 is devoid of the hydroxy group. The basic taxoids eluting at 24.7 (**56**) and 27.8 (**66**) min have the same mw 739. Both have five acetoxy groups. However, the former has an acetoxy group at 2′ position of the C5 β-amino acid ester side chain containing *N*-methyl group (*m/z* 238), whereas the latter has the acetoxy group at C2 and a C5 β-amino acid ester side chain with N-methyl group (*m/z* 180). Isomeric austrospicatine derivatives having mw 711 (**37** and **41**) could be detected at 17.0 and 20.6 min, respectively. Two basic taxoids with mw 637 (**68** and **74**) were detected at 28.5 and 34.1 min. The ESI mass spectrum of the former indicated the presence of only four substituents (*m/z* 265/283) including a β-aminoacid ester side chain at C5 having *N*-dimethyl group (*m/z* 194). The other eluting at 34.1 min showed the presence of a C5 β-amino acid ester side chain having an *N*-methyl group (*m/z* 180). The fargment peaks at *m/z* 279/297 in their ESI mass spectra indicated the presence of a keto function. Hence the compound is identified as 7,9,10-triacetoxy-13-oxo-5-(3′-methylamino-3′-phenyl) propionyloxy-4(20),11-taxadiene (**74**). The detection and tentative identification of such a large number of nitrogen-containing taxanes from a single genus has not been reported so far.

The study also revealed the presence of several xyloside derivatives. The xylosides identified along with the side chain fragment in parentheses are: 7-xylosyl-10-decaetyl paclitaxel, **17** (*m/z* 286), 7-xylosyl-10-decaetyl cephalomannine, **15** (*m/z* 264), 7-xylosyl paclitaxel, **25** (*m/z* 286), 7-xylosyl-10-decaetyl taxol C, **26** (*m/z* 280), 7-xylosyl-10-deacetyl taxol D, **11** (*m/z* 252) and 7-xylosyl taxol C, **34** (*m/z* 280). 10-Deacetyl taxol xylosides are now considered important analogues of taxol as they can be converted into 10-decaetyl taxol and, in turn, to paclitaxel through chemical processes (Chattopadhyay *et al.*, 1999b)

The other amide type taxoids detected and identified include 10-deacetyl cephalomannine (**19**), 10-decaetyl *epi*-cephalomannine (**30**), 10-deacetyl taxol (**24**), 10-decaetyl *epi*-taxol (**33**), cephalomannine (**29**), Paclitaxel (**32**), *epi*-paclitaxel (**36**) and *N*-methyl taxol C (**46**).

Apart from these, several regular, rearranged or oxetane/epoxide-containing taxanes were detected and identified tentatively based on ESI MS fragmentation pattern. The taxanes eluting at 5.6 and 6.4 min have the same mw 630 and may correspond to taxayuntin F (**13**) and E (**16**), respectively, as observed in the analysis of the needle extracts (Madhusudanan *et al.*, 2001a,b). There are three isomeric compounds having mw 776 eluting at 22.7, 23.2 and 23.9 min. Based on the MS fragmentation pattern these could be identified as 2-deacetyl-2-benzoyl-13-acetyl taxayuntin (**49**), 2,7-dibenzoyloxy-5,20-epoxy-

1-hydroxy-4,9,10,13-tetraacetoxy tax-11-ene (**51**) and 9-benzoyl-9-decaetyl-11(15→ 1) *abeo* baccatin VI (**52**), respectively.

The isomeric taxoids taxusin (**43**) and taxayunnanine C (**61**) having mw 504 and eluting at 21.3 and 26.3 min, respectively, show characteristic differences in their mass spectra. The C14 oxygenated taxayunnanin C shows an abundant fragment ion at *m/z* 161, whereas it is absent in the mass spectrum of taxusin. Similar differences are also noticed in the spectra of isomeric taxanes 5,7,9,10,13-pentaacetoxy-4(20),11-taxadiene (**39**) and the C14-oxygenated yunnanxane (**40**). Moreover, the spectrum of the former shows peaks at *m/z* 263/281 characteristic of five substituted taxanes, whereas that of the latter shows peaks at *m/z* 265/283 characteristic of four substituted taxanes.

The present study also reveals that unlike the needles, the stem bark contains more of regular taxoids. Rearranged taxoids were predominant in the needle extracts, whereas only 10 rearranged taxoids could be detected in the bark samples. Forty-four of the taxanes had C20-exocyclic methylene group.

CONCLUSION

The results confirm that LC-ESI-MS is a convenient and reliable method for the direct profiling taxoids in plant extracts. The different types of taxoids and the number and nature of substituents can be easily identified from the mass spectral fragmentation pattern. The technique is of immense value in the search for other pharmaceutically important paclitaxel precursors as well as second generation paclitaxel derivatives. This study has identified as many as 75 taxoids including the ones already reported earlier from this plant. The detection and identification of over 36 aminotaxoids may give a new direction to the analysis of *T. wallichiana* species.

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REFERENCES

Appendino G. The phytochemistry of the yew tree. *Natural Product Report* 1995; **12**: 349–360.

Appendino G, Tagliapietra S, Ozen HC, Garibaldi P, Gabetta B and Bomabrdelli E. Taxanes from the seeds of *Taxus baccata*. *Journal of Natural Products* 1993; **56**: 514–520.

Baloglu E and Kingston DGI. The taxane diterpenoids. *Journal of Natural Products* 1999; **62**: 1448–1472.

Bruins AP. Atmospheric pressure ionization mass spectrometry. *Trends in Analytical Chemistry* 1994; **13**: 37–43.

Chang Y, Abliz Z, Li L-J, Fang Q-C and Takayama M. Study on fragmentation behaviour of 5/7/6-type taxoids by tandem mass spectrometry. *Journal of Mass Spectrometry* 2000; **35**: 1207–1214.

Chattopadhyay SK, Tripathi VK, Thakur RS, Sharma RP and Jain SP. Isolation of taxol, 10-deacetyl baccatin III and (−)betuligenol from *Taxus baccata*. *Indian Journal of Chemistry* 1994; **33B**: 409–411.

Chattopadhyay SK and Sharma RP. A taxane from the Himalayan yew *Taxus wallichiana*. *Phytochemistry* 1995; **39**: 935–936.

Chattopadhyay SK, Sharma RP, Appendino G and Gariboldi P. A rearranged taxane from the Himalayan yew. *Phytochemistry* 1995; **39**: 869–870.

Chattopadhyay SK, Saha GC, Kulshrestha M, Sharma RP and Kumar S. Studies on the Himalayan yew *Taxus wallichiana.* Part II. *Indian Journal of Chemistry* 1996a; **35B**: 175–177.

Chattopadhyay SK, Kulshrestha M, Saha GC, Sharma RP and Kumar S. Studies on the Himalayan yew *Taxus wallichiana.* Part III. First report on isolation of a basic taxane. *Indian Journal of Chemistry* 1996b; **35B**: 508–509.

Chattopadhyay SK, Saha GC, Kulshrestha M, Sharma RP and Kumar S. Studies on the Himalayan yew *Taxus wallichiana.* Part IV. Isolation of dihydrotaxol and dibenzolylated taxoids. *Indian Journal of Chemistry* 1996c; **35B**: 754–756.

Chattopadhyay SK, Saha GC, Sharma RP, Kumar S and Roy R. A rearranged taxane from the Himalayan yew. *Phytochemistry* 1996d; **42**: 787–788.

Chattopadhyay SK, Kulshrestha M, Saha GC, Sharma RP, Jain SP and Kumar S. The taxoid constituents of the heartwood of *Taxus wallichiana*. *Planta Medica* 1996e; **62**: 482.

Chattopadhyay SK, Saha GC, Kulshrestha M, Sharma RP and Roy R. Studies on the Himalayan yew *Taxus wallichiana.* Part V. Isolation of a new rearranged taxoid. *Indian Journal of Chemistry* 1997; **36B**: 831–832.

Chattopadhyay SK, Saha GC, Kulshrestha M, Tripathi V and Sharma RP. The taxoid constituents of the roots of *Taxus wallichiana*. *Planta Medica* 1998; **64**: 287–288.

Chattopadhyay SK, Tripathi V, Shawl AS, Roy R and Joshi BS. Studies on the Himalayan yew *Taxus wallichiana*. Part VIII. Isolation of additional taxoids and panasterone from the leaves of *T. wallichiana*. *Indian Journal of Chemistry* 1999a; **38B**: 874–876.

Chattopadhyay SK, Sharma RP and Kumar S. *Process for production of taxol*. 1999b. US Patent no. 5,856,532, 5 January, 1999.

CSIR 1976. *The Wealth of India. A Dictionary of Indian Raw Materials and Industrial Products*, Vol. 10. CSIR: New Delhi; 132–134.

Fenn JB, Mann M, Meng CK, Wu SF and Whitehouse CM. Electrospray ionization. Principles and practice. *Mass Spectrometry Review* 1990; **9**: 37–70.

Griffini A, Peterlongo F, De Bellis P and Pace R. Desorption chemical ionization and thermospray mass spectra of taxol and related compounds. *Fitoterapia* 1993; **64**: 53–81.

Hoffmann A, Khan W, Worapong J, Strobel G, Griffin D, Abrogast B, Barofsky D, Boone RB, Ning L, Zheng P and Daley L. Bioprospecting for taxol in angiosperm plant extracts. *Spectroscopy* 1998; **13**: 22–32.

Hoke SH, Cooks RG, Chang C-J, Kelly RC, McGuire MT and Snader KM. Determination of taxanes in *Taxus brevifolia* extracts by tandem mass spectrometry and high-performance liquid chromatography. *Journal of Natural Products* 1994; **57**: 277–286.

Kerns EH, Volk KJ, Hill SE and Lee MS. Profiling taxanes in *Taxus* exctracts using LC/MS and LC/MS/MS techniques. *Journal of Natural Products* 1994; **57**: 1391–1403.

Kerns EH, Volk KJ, Hill SE and Lee MS. Profiling new taxanes using LC/MS and LC/MS/MS substructural analysis technique. *Rapid Communications on Mass Spectrometry* 1995; **9**: 1539–1545.

Madhusudanan KP, Chattopadhyay SK, Saha GC, Kulshrestha M, Sharma RP and Kumar S. Fast atom bombardment mass spectrometry for the characterization of taxanes. *Journal of Mass Spectrometry* 1997; **32**: 216–224.

Madhusudanan KP, Chattopadhyay SK, Tripathi V, Sashidhara KV

and Kumar S. MS/MS Profiling of Taxoids from the Needles of *Taxus wallichiana*. *Phytochemical Analysis* 2001a; **13**: 18–30.

Madhusudanan KP, Chattopadhyay SK and Srivastava S. Elimination of 118 Da: a characteristic fragmentation in the MS/MS spectra of 11(15 → 1)-*abeo*-taxanes. *Journal of Mass Spectrometry* 2001b; **37**: 91–98.

Miller ML and Ojima I. Chemistry and chemical biology of taxane anticancer agents. *The Chemical Record* 2001; **1**: 195–211.

Mroczek T, Glowniak K and Hajnos M. Screening of pharmaceutically important taxoids in *Taxus baccata* var. *Aurea* Corr. with CC/SPE/HPLC-PDA procedure. *Biomedical Chomatography* 2000; **14**: 516–529.

Poupat C, Hook I, Gueritte F, Ahound A, Guenard D, Adeline M-T, Wang X-P, Dempsey D, Breuillet S and Potier P. Neutral and basic taxoid contents in the needles of *Taxus* species. *Planta Medica* 2000; **66**: 580–584.

Shigemori H, Wang XX, Yoshida N and Kobayashi J. Taxuspines X-Z, new taxoids from Japanese yew *Taxus cuspidata*. *Chemical Pharmeutical Bulletin* 1997; **45**: 1205–1208.

Wani MC, Taylor HL, Wall ME, Coggon P and McPhail AT. Plant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of the American Chemistry Society* 1971; **93**: 2325–2327.

