Investigations on *Hoya species*, II. Latex Lipids and Leaf Phenolics of *Hoya bella* Hook*

Wim J. Baas and Gerard J. Niemann

Botanical Laboratory, University of Utrecht, The Netherlands

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In *Hoya bella* latex a number of triterpenols were found, both in the free form and as esters. The main triterpenols were β-amyrin, cycloartenol, lupeol and the rarely occurring isobauerenol; esters of these alcohols which included cinnamic acid, propionic acid and isovaleric acid were identified. A homologous series of alkanes with carbon chain length ranging from C-20 to C-31 was found. In leaf extracts acylated flavonol glycosides as well as free ferulic acid occurred; the concentration of the latter decreased with increasing leaf age.

Introduction

Owing to their pharmacological significance a number of Asclepiadaceae have already been investigated for their chemical constituents. The family is particularly noted for the occurrence of cardenolides, saponin-like bitter principles and triterpenoids [1]. The Asclepiadaceae have scarcely been investigated for other compounds such as the polyphenolics. As far as the closely related Apocynaceae are concerned a similar situation exists. In both families triterpene alcohols and esters have been found in the latices of many species; in the Apocynaceae triterpenes also occur in the cuticular wax [1], but the Asclepiadaceae have hardly been investigated in this connection. As far as the phenols are concerned caffeic acid, the flavonols kaempferol and quercetin, and leucocyanidin generally seem to be present in both families [1, 2]. Kozjek *et al.* [2] emphasize the fact that Asclepiadaceae and Apocynaceae contain no C-glycoflavones or xanthones; in this respect these families differ from other families of the Contortales.

In our laboratory investigations on Asclepiadaceae are restricted to the genus *Hoya* and are particularly concerned with the metabolism and physiological significance of triterpenoids and phenolics. In *H. australis* triterpenols were found both in latex and in leaf cuticular wax [3, 4]; however, there was a great difference in their composition and in their relation to the age of the plant part investigated. Whereas the triterpene composition of leaf wax was found to be very variable with age, that of latex appeared independent of age, the place of collection and the growth conditions of the specimen investigated [5]. In a screening experiment we found that the latex composition of an individual specimen was constant whereas the composition of latices of various *Hoya* species differed considerably. This discovery led us to conduct the present series of investigations. Among the different *Hoya* species there also seems to be great variability in leaf phenolics, which led us to consider possible taxonomic significance of the terpenoids and the phenolics.

Knowledge about *Hoya* triterpenoids is based only on *H. australis* [3–5] and on the description of the isolation of β-sitosterol from *H. carnosa* leaves [6]. Our information about phenolics stems from an indication of the possible presence of leucocyanidin and questionable occurrence of kaempferol and quercetin in *H. carnosa* leaves [2] and from the description of chlorogenic acid in *H. bandanensis* [7].

Materials and Methods

Plant material: Latex was obtained from leaf stalks of *Hoya bella* Hook., cultivated in greenhouses. From the same plants (clonal material) young and old leaves were collected in March 1978 for the extraction of phenolic compounds. A voucher specimen was deposited at the Institute for Systematic Botany, University of Utrecht.

Extraction and separation: The latex total lipid [8] was analysed unsaponified by GC-MS. Phenolic extracts were obtained by extraction with acetone,

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Requests for reprints should be sent to Dr. G. J. Niemann, Botanical Laboratory, University of Utrecht, Lange Nieuwstraat 106, Utrecht, The Netherlands.

after the leaves had been dipped in chloroform to remove wax lipids. The acetone extracts were freed from lipids with petrol ether, concentrated and extracted with butanol. The butanol extracts were then separated by repeated band-chromatography on Whatman No. 1 chromatography paper.

Instrumentation: GC-MS analyses were carried out with a Varian Mat CH 7 chromatograph, on a 1% OV-1 column, temperature programmed from 160 ° to 300 °C/4 °/min. 5-α-Cholestane was used as internal standard. For high-performance liquid chromatography (HPLC) of the phenolic extracts a Dupont 830 chromatograph was used with a 4.2 × 240 mm Zorbax ODS column eluted with a gradient (concave 2) of 45% methanol in water to 100% methanol, both with 0.1% phosphoric acid, at 50 °C. The compounds were detected by UV at 254 and 360 nm on a fixed wavelength detector and a Dupont 837 spectrometer. Vitexin was used as internal standard.

Identification: The latex compounds were identified by GC-retention and by mass spectrum. Isovaleric acid was identified by Warnaar [11]. The phenolics by R_f, HPLC-retention, UV spectral data inclusive shifts and by alkaline and/or acid hydrolysis and degradation products [9].

Results and Discussion

Fig. 1 shows a typical gas chromatogram of the unsaponified total latex lipid fraction, in which 34 separated or partly separated peaks can be distinguished. In this chromatogram the first part is formed by a homologous series of alkanes, starting with the C-20 alkane (Peak 1) and regularly increasing by a methylene unit via C-21 (Peak 2) to a C-31 compound (Peak 12). In the second part of the chromatogram, the first series of somewhat higher peaks after the internal standard 5α-cholestane, the free triterpenols and sterols are found, partly mixed with acetates. We identified 24-methylene lophenol (peak 13), lupeol (14), cycloartenol (15), isobauerenol (16, a double peak) and pseudo-taraxasterol (17) and the acetates of β-amyrin (16) and possibly germanicol (same peak), lupeol (17, together with pseudo-taraxasterol) and isobauerenol (18). The main esters were the isovalerates, which form the next part of the chromatogram and include β-amyrin isovalerate (21) and very high concentrations of the lupeol- (22) and isobauerenol (23) isovalerates. We found lower concentrations of propionates (isobauerenol, peak 20), benzoates (lupeol, peak 25, isobauerenol, peak 26)

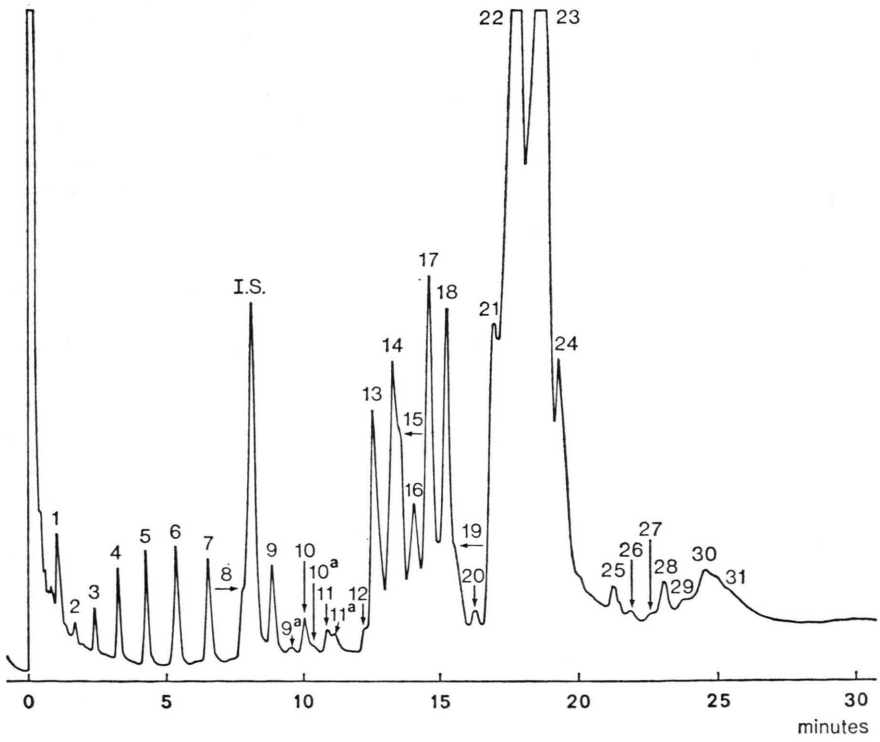

Fig. 1. Gas-chromatogram of the unsaponified latex of *Hoya bella* on a 1% OV-1 column, temperature programmed at 4 °/min from 160–300 °C.

and *cis*-(β-amyrin, peak 27, lupeol, peak 28) and *trans*-cinnamates (β-amyrin, peak 29, lupeol peak 30, isobauerenol peak 31). As mentioned previously [5], the *cis* cinnamates have to be regarded as artifacts resulting from *cis-trans* isomerization.

When compared with the latex composition of other Asclepiadaceae [1] including *Hoya australis* [5], the occurrence of the rare triterpenol isobauerenol in relatively high concentration in *Hoya bella* is very striking. The absence of α-amyrin is also rather surprising. Minor quantities may however be present, but will not be detected by the simultaneous elution of lupeol derivatives.

Fig. 2 represents the HPLC chromatograms of phenolic extracts of young (2A) and old (2B) leaves respectively. From this figure a rather high variation in the relative concentration of the five peaks is apparent. This phenomenon, however, has to be interpreted carefully. One of the first compounds we isolated and identified from young *H. bella* leaves was ferulic acid; the relative concentration of this compound decreases tremendously with leaf-ageing. Nevertheless, in the HPLC chromatograms the relative contribution of peak 3, with which ferulic acid co-chromatographs, increases due to the simultaneous increase in another as yet unidentified phenol with the same retention.

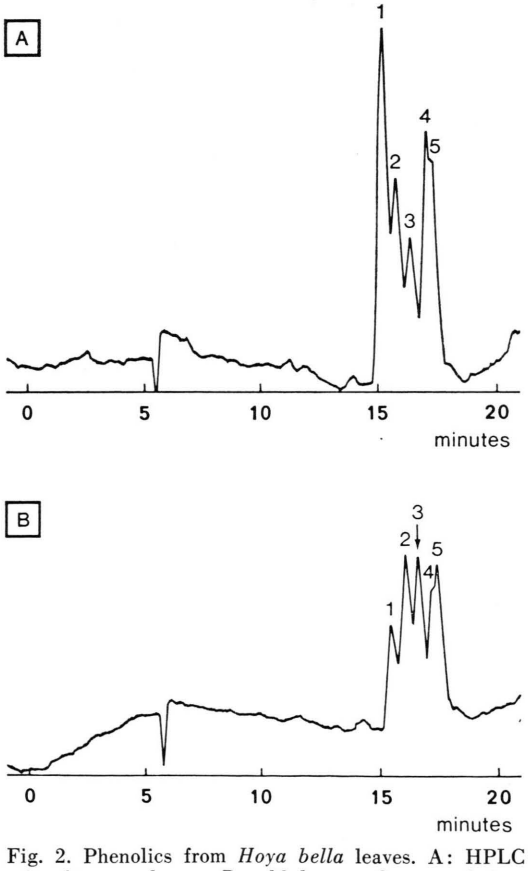

Fig. 2. Phenolics from *Hoya bella* leaves. A: HPLC analysis of young leaves, B: old leaves. Compound 1 co-chromatographs with vitexin, 2 with C(=isorhamnetin-3-ferulyldiglycoside) and 3 with ferulic acid.

A rather high increase with ageing in the relative concentration was also found for the main flavonoid spot on the paper chromatograms. On further analysis these flavonoids appeared to be a mixture of mainly acylated flavonol glycosides of which only trace amounts were left after purification. Some R_f-values and spectral data have been summarised in Table I.

The compounds A, B, C and D all appeared to be acylated flavonol glycosides, which released ferulic acid on alkaline hydrolysis. In addition, compounds A and B yielded kaempferol and C and D isorhamnetin and all four gave low concentrations of a corresponding glycoside, for which some data have been summarised in Table I. Only for compound B was a sufficient amount available for further analysis of the diglycoside obtained on alkaline hydrolysis; this compound appeared to be identical with kaempferol-3-diglucoside. Thus, B is kaempferol-3-(ferulyl-diglucoside). The 255 peak in the UV spectrum was caused by an impurity of one of the isorhamnetin analogues. Compounds A, C and D are a kaempferol-3-(ferulyldiglycoside), an isorhamnetin-3-(ferulyldiglycoside) and an isorhamnetin-3-(ferulylmono-?-glycoside) respectively. It is very interesting that the typical ferulic 323 UV peak is found only in compounds A and D but not in B and C, which may indicate that the attachement of ferulic acid in A and D is at the sugar connected with the flavonol and in B and C it is at the second sugar of a disaccharide. Compound E appeared to be a kaempferol-3-diglycoside, with glucose and arabinose released on acid hydrolysis, possibly indicating a kaempferol-3-vicianoside.

The combination of a decrease in concentration of ferulic acid and an increase of the ferulyl-flavonol glycosides suggests that the younger leaves use ferulic acid for synthesising the acylated compounds that occur especially in older leaves.

During HPLC analysis co-chromatography was found with the C-glycoside vitexin (compound 1, Fig. 2). However, so far no further evidence for the

Table I. Chromatographic and UV-spectral data relating to *Hoya bella* flavonoids.

Compound	TBA	BuAW	15HAc	H_2O	Phenol	UV
A	38	51	64	55	61	*263 323* 337^s 362^s
B	57	60	64	55	81	255 *263 343*
C	53	59	67	33	77	*251* 262^s *339* 349^s
D	55	60	65	28	79	*245*^{s} *261* 297^s *323* 350^s
E	53	55	45	22	67	*265 349*
alk. hydr. A			53			*262*^{s} 308^s 348^s
alk. hydr. B		58	57			*267* *348*
alk. hydr. C		55	65			*255* 266^s *355*
alk. hydr. D		45	46			*255* 268^s 318^s 367^s
kaempferol-3-glucoside	69	72	42	14	71	*265 249*
kaempferol-3-diglucoside *	40	58	59		49	

* R_f-values from ref. [10].

occurrence of this compound has been obtained. Nevertheless, a possible classification based on the absence of C-glyco-flavones (2) has to be amended since from other *Hoya* species vicenins and other C-glycosides have been identified (unpublished results).

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