LC-UV-Electrospray-MS-MS Mass Spectrometry Analysis of Plant Constituents Inhibiting Xanthine Oxidase

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Purpose. A previous screening showed that *Amyena scandens* Danser (Loranthaceae) efficiently inhibited XOD. The aim of this study was to identify the compounds with anti-XOD properties. For this purpose, Electrospray Tandem Mass Spectrometry (ESI-MS-MS) coupled with UV and Diode Array LC techniques were employed.

Methods. Leaves were delipidized with petroleum ether and extracted with acetone:water 70:30, v:v. The extract was fractionated into the ethyl acetate and water soluble phases. Chemical investigation was performed following the bioactivity guided fractionation. Two fractions with anti-XOD activity were isolated by silica gel column chromatography of the ethyl acetate phase and analyzed by LC-UV-ESI-MS-MS.

Results. The compounds identified with authentic standards were: catechin, epicatechin, epicatechin-3-gallate, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, and isorhamnetin-3-O-glucoside. Other constituents, only partially characterized, were a procyanidin dimer, a procyanidin trimer, three dimers epi/catechine-epi/catechine gallate and isorhamnetin-O-deoxyhexose. The anti-XOD activity was mainly due to galloyl-containing oligomeric proanthocyanidins.

Conclusions. The coupling of UV Diode Array-HPLC with ESI-MS-MS represents a versatile tool for the rapid characterization of compounds in complex mixtures, avoiding time-consuming previous isolation.

KEY WORDS: xanthine oxidase inhibitors; flavonoids; proanthocyanidins; electrospray mass spectrometry; *Amyena scandens*.

INTRODUCTION

Xanthine oxidase (XOD; xanthine:oxygen oxidoreductase) is a cytoplasmic enzyme converting xanthine to uric acid, which can precipitate gout attacks. Prevention of gout attacks can be accomplished with antihyperuricaemic therapy with either uricosuric drugs or xanthine oxidase inhibitors (1). XOD is also the enzymic tissue generator of superoxide ions which react with hydrogen peroxide to generate hydroxyl radicals. Its role has been advocated in endothelial-derived cell injury at sites of inflammation (2). The involvement of this enzyme has been proposed in ischemia/reperfusion damage in tissues (3,4). Oxygen-derived free radicals are also implicated in kidney damage (5), lung disease (6), allergies, atherosclerosis, diabetes, and aging (7). Following metabolic oxidation of ethanol, the high levels of NADH inhibit xanthine dehydrogenase resulting in a shift of purine oxidation to XOD, thereby promoting the generation of oxygen free radical species (8).

Therefore an approach to the treatment of pathologies where oxygen-free radicals play an adverse role may be the development of drugs with anti-XOD activity. Several compounds present in plants (flavonoids, xanthones, coumarins, proanthocyanidins) are reported to be inhibitors of XOD (9–13) and to posses anti-oxidant and anti-radical properties. A series of 38 plants from New Caledonia were subjected to screening for anti-XOD activity with the purpose of selecting plant species highly active for further phytochemical investigation (14). The preliminary screening indicated that *Amyena scandens* Dansen (Loranthaceae) was one of the most active and worthwhile to be investigated further.

In the present study, we applied LC-UV-Electrospray Tandem Mass Spectrometry (LC-ESI-MS-MS) for the analysis of the plant constituents exhibiting anti-XOD properties. This work demonstrates that the coupling of UV-Diode Array-HPLC with ESI-MS-MS represents an improved and versatile technique, which allows the rapid identification of compounds in complex mixtures thus avoiding tedious isolation procedures of previously known substances.

MATERIALS AND METHODS

Plant Material

Plant leaves were collected in September by Dudley Nicholls, ORSTOM Center, Noumea and dried in an oven at 40°C. Plant identity was verified at the herbarium of the ORSTOM Center of Noumea and a voucher specimen was deposited at the Laboratory of Natural Substances of Biological Interest, Noumea, New Caledonia.

Reagents

XOD (EC 1.1.3.22) was obtained from buttermilk (Grade III, 1.3 Units/mg. prot.), xanthine and quercetin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (+)-Catechin, (−)-epicatechin, (−)-epicatechin-3-gallate, and the flavonoid-3-O-glycosides were from Extrasynthèse (Lyon, France). All other reagents and silica gel-60 (230-400 mesh) were from Merck (Darmstadt, Germany); HPLC grade solvents were purchased from Romil Ltd. (Cambridge, UK) and Labscan Ltd. (Dublin, Ireland).

Assay of XOD Activity

The enzyme activity was measured spectrophotometrically following the conversion of xanthine to uric acid at 295 nm for 3 min, as reported by Robak *et al.* (13). The assay mixture contained 0.1 M K-phosphate buffer pH 7.8, 10 μM EDTA, 0.1 mM xanthine, and XOD 0.04 Units/ml, final volume 1 ml. The plant materials were tested at concentrations of 25, 50, and 100 μg/ml, dissolved in DMSO (less than 1% solvent in the assay volume). At this concentration, the solvent did not affect the enzymatic assay. Nevertheless, all control samples were added with the same amounts of DMSO used to test the plant materials. Quercetin-3-

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O-glucoside, quercetin-3-O-rhamnoside, and isorhamnetin-3-O-glucoside were tested for enzyme inhibition at 1–100 μM. The assay mixture was preincubated for 5 min at 25°C with the test material before adding the substrate. XOD inhibitory activity was expressed as the percentage of inhibition, calculated as (1-B/A)×100 where A is the enzyme activity without the test material and B the enzyme activity with the test material. Quercetin 10 μM was used as a reference inhibitor.

Fractionation of the Plant Material

The leaves (100 g) were powdered, delipidized with 400 ml of petroleum ether 40–60°, and extracted twice with 300 ml of acetone:water 70:30, v:v. The extract was filtered, dryed in a vacuum, and weighed for the determination of the w/w yield (24%). Chlorophyll was removed from the extract by elution on a cellulose column with methanol:water 70:30, v:v (5 g of extract, 45 g of cellulose, and 400 ml of solvent mixture). The recovery was 80% of the loaded material. The crude extract devoid of chlorophyll was fractionated in the ethyl acetate soluble phase and the water soluble phase (19% and 66% of the crude extract, respectively). The material obtained at each purification step was tested for XOD assay. Extracts were kept at −20°C until use and dissolved in dimethylsulfoxide to a concentration of 1 mg/ml for the biological tests.

Column Chromatography

500 mg of the ethyl acetate soluble phase were loaded on silica gel-60 (75 g, column Ø 3 cm). The elution was as follows: 300 ml of diethyl ether:ethyl acetate 1:1, 300 ml of diethyl ether:ethyl acetate 3:7, 300 ml of ethyl acetate, 300 ml of ethyl acetate:methanol 7:3, 300 ml of ethyl acetate:methanol 1:1, 300 ml of methanol, 300 ml of methanol:water 1:1, and 300 ml of water. The recovery was 86%. Fractions sharing the same tlc pattern were combined and tested for XOD inhibition assay (fractions I-VII).

UV-HPLC Analysis

HPLC analyses were performed on a Jasco liquid chromatograph model PU-980, equipped with a UV detector 875, using a C18 Nucleosil column (250 × 4.6 mm i.d., 5 μ), Alltech (Alltech Italia s.r. Milan, Italy). The Diode Array detector was a Beckman 168. Samples were analyzed under the following conditions: λ 280 nm, gradient acetonitrile–water (containing 0.03% formic acid) from 10% to 90% CH_3CN in 30 min, followed by 10 min of isocratic elution with 90% CH_3CN; flow rate 0.7 ml/min.

Electrospray Mass Spectrometry

An LCQ mass spectrometer (Finnigan Mat) equipped with an ES ion trap was used. Microsoft Windows-NT based software LCQ Navigator was used to control the instrument and for data acquisition and processing. The operating parameters were as follows: source voltage 5kV; ES capillary voltage −20V; capillary temperature 200°C; collision energy 10–15% of 5V depending on the compounds. The MS instrument was interfaced with a liquid chromatograph Hewlett Packard 1050. Conditions for HPLC runs were as reported in the previous paragraph and conditions for infusion of pure samples are the same as described, except that the HPLC system was replaced by a syringe pump delivering a 3–4 $\mu l \cdot min^{-1}$ sample flow.

The active fractions recovered from the silica gel column were analyzed by injecting into the LC-MS for recording of the total ion current trace and for the acquisition of the molecular ion of the peaks. The MS–MS spectra were obtained by direct inlet into the ion source. The structure determination of the compounds was obtained by comparing the spectra with those of authentic compounds and by co-chromatography in HPLC.

RESULTS AND DISCUSSION

Quercetin 10 μM used as positive control of xanthine oxidase assay gave an enzymatic inhibition of 75 ± 2.2%, mean ± s.e. of 12 experiments. The inhibition of XOD by the crude extract and the fractionated material is reported in Table I. No activity was lost while removing chlorophyll from the extract and the inhibition was associated with the ethyl acetate phase. From the silica gel column chromatography of the ethyl acetate phase, seven fractions were obtained. Anti- XOD activity was concentrated in fractions **II** and **V**. The compounds in fraction **II** were eluted with diethyl ether:ethyl acetate 30:70 and the compounds in fraction **V** eluted with ethyl acetate:methanol 70:30. HPLC coupled with a DAD detector gave information about the chemical structure of the compounds present in the two fractions (Figs. 1 and 2), providing UV spectra of the peaks in a selected wavelength range. This approach allowed the classification of the polyphenolic compounds. The information from the ESI-MS-MS provided additional data on the structures. The assignement of the structures for the flavonoid and flavan-3-ol derivatives was unambiguous, since these exhibited characteristic UV spectra (Figs. 1 and 2). The LC-ESI-MS procedure, using the same chromatographic conditions, allowed comparison of the UV-HPLC trace with the chromatogram of the total ion current trace (Fig. 3). With the ESI technique, in general, only the molecular ion is formed; however, the induction of fragmentation by in-source collision dissociation (MS-MS) with the generation of specific daughter ions, yields a significant amount of structural information of the compounds to be identified. Furthermore, the selectivity of the MS detection allowed the resolution of multicomponent peaks.

Table I. Inhibition of XOD by Leaf Extract and Fractions at Various Concentrations

Plant material	XOD % inhibition μg/ml		
	25	50	100
Crude extract	30.5	58.9	83.7
Crude extract (no chlorophyll)	35.7	66.5	85.6
Ethyl acetate phase	51.3	72.3	85.4
Silica gel fractions			
I		33.3	
II		**70.7**	
III		44.1	
IV		25.9	
V		**68.3**	
VI		20.9	
VII		18.9	

Note: Quercetin 10 μM gave an inhibition of 75 ± 2.2. Mean ± s.e. of twelve experiments.

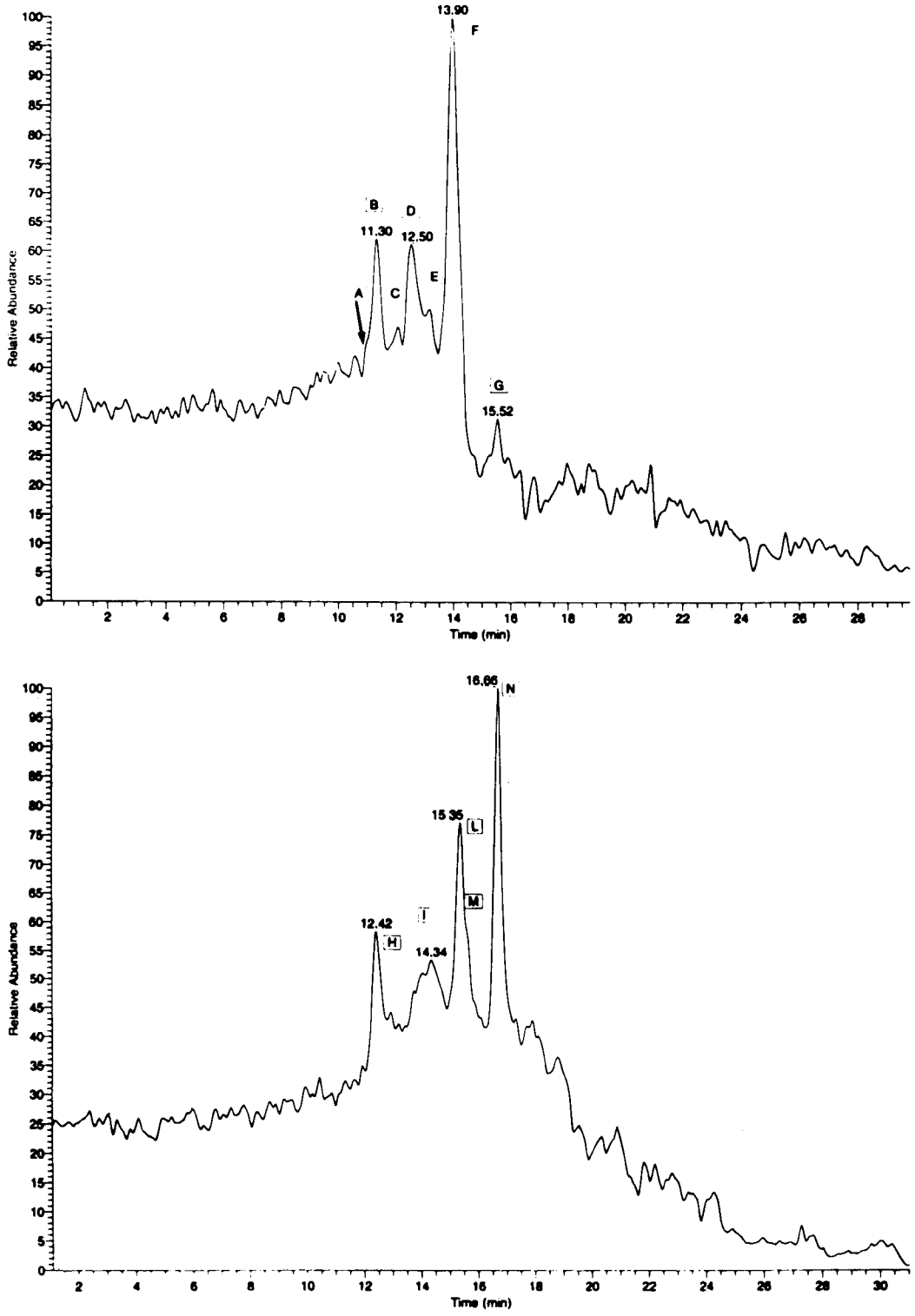

Fig. 1. Negative LC/ESI-MS ion current trace of Fraction II (upper pannel) and Fraction V (lower pannel) obtained using the chromatographic conditions described for HPLC analysis.

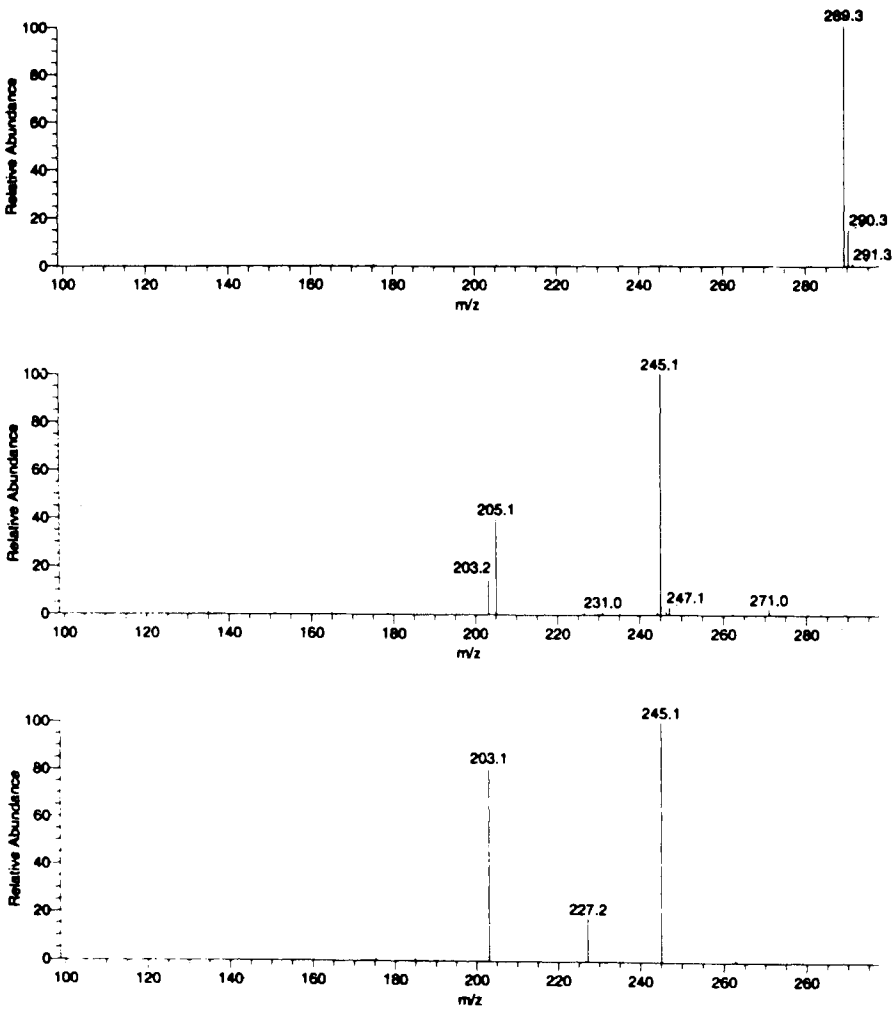

Fig. 2. Mass spectrum of (+)-catechin. Upper panel: full ms $[M-H]^-$; central panel: ms-1 fragments obtained by collision of the pseudomolecular ion; lower panel: ms-2 fragments generated by collision of the ion at m/z 245.

Fraction II

The UV spectrum of fraction II, with an Abs at 280 nm indicated that it was constituted by flavan-3-ol derivatives (15). This information was further validated by the single point spectrum of the HPLC peaks recorded with the Diode-array detector. Only a peak at r.t. 19.97 showed a UV spectrum suggesting the presence of a compound with a flavonoidic nature (16). The LC-ESI-MS allowed separation of seven compounds (**A**-**G**; Fig. 1, upper panel). The main fragments of the ESI spectra of the compounds are listed in Table II. The mass spectra of compounds **A** and **C** exhibited $[M-H]^-$ fragments at m/z 289, which correspond to the monomer (+)-catechin and (−)-epicatechin (mol. wt. 290). Daughter ions formed from m/z 289 were 245, 205, and 179; the ion at m/z 203 was obtained by collision of m/z 245 (Fig. 2). The fragmentation pattern of the two epimers was indistinguishable; therefore, the structure was assigned by r.t. comparison with the pure compounds. For compound **B**, the signal at m/z 577 corresponded to the deprotonated $[M-H]^-$ molecular ion of a procyanidin dimer of the B-type. The daughter ion at m/z 425 originated from the loss of 152 amu, which was already described in the FAB mass spectra of these compounds (17). The negative ESI mass spectrum of compound **F** exhibited the $[M-H]^-$ fragment at m/z 441, consistent with a flavanol monogallate (mol. wt. 442). Losses from the $[M-H]^-$ ion occur at m/z 289 (−152 amu), and m/z 271 (−170 amu) and m/z 169, indicative of the cleavage of the galloyl group. This type of ionization is similar to the one obtained by FAB (17). The structure of epicatechin -3-gallate was assigned by co-chromatography with the available authentic compound. Compounds **D** and **E** showed the $[M-H]^-$ at m/z 729 suggesting a dimeric structure containing one flavan-3-ol unit (catechin/epicatechin) and one flavanol gallate unit (catechin gallate/epicatechin gallate). This was confirmed when the pseudomolecular ion m/z 729 was subjected to CID. The fragments at m/z 577 and 559 corresponded to the loss of −152 amu, −170 amu as seen above for compound **F**. The fragment at m/z 441 corresponding to flavanol-gallate moiety is generated by the cleavage of the flavan-3-ol $[M-H-289]^-$. For compound **G**, on the basis of the $[M-H]^-$ pseudomolecular ion at m/z 723, the hypothesis of a dimer constituted by dihydroflavonol-O-

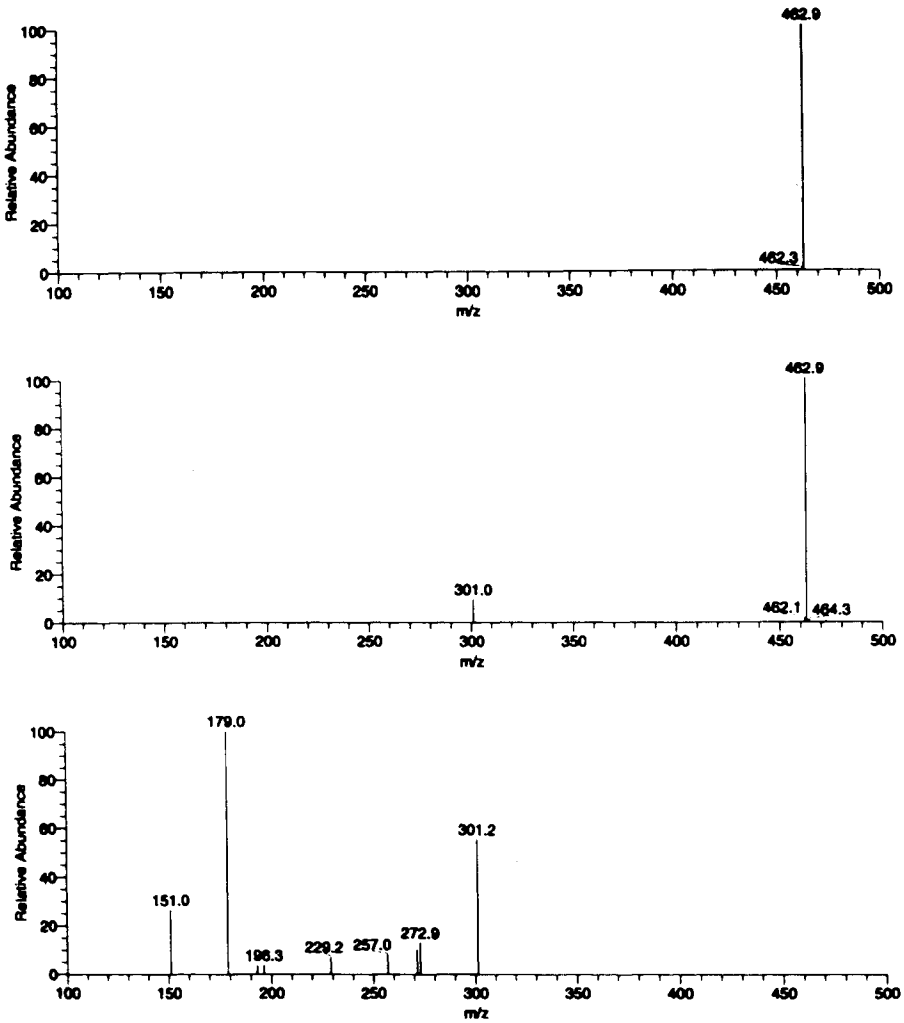

Fig. 3. Mass spectrum of quercetin-3-O-glucoside. Upper panel: full ms $[M-H]^-$; central panel: ms-1 $[A-H]^-$ fragment obtained by collision of the pseudomolecular ion; lower panel: ms-2 fragments generated by collision of the $[A-H]^-$ ion at m/z 301.

pentose linked to a flavan-3-ol unit is suggested. This compound was very resistent to CID and no other fragments could be obtained from the ion at m/z 571 (loss of 152 amu).

Fraction V

The UV spectrum of the fraction V showed three Abs at 267, 280, and 343 nm. The single point spectrum of the HPLC peaks, recorded with the DAD provided more information about the chemical structure: two peaks at r.t. 15.16 and 16.8 min gave typical absorbance of the flavan-3-ol derivatives (15); two peaks at r.t. 17.00 and 19.14 could be classified as flavonoids (16). The LC-ESI-MS allowed identification of five compounds (**H-N**; Fig. 1, lower pannel). The main mass fragments of the compounds are listed in Table III. Compound **H** gave a similar

Table II. Retention Times (by LC-MS) and Main Ions in the ESI-MS-MS Mass Spectra of Compounds in Fraction II

Peak	r.t. total ion current trace	ESI-MS-MS DATA			Structure
		Full ms $[M-H]^-$	ms-1	ms-2	
A	11.03	289	245, 205 179	203	catechin
B	11.30	577	425		procyanidin dimer
C	12.00	289	245, 205 179	203	epicatechin
D-E	12.50–13.00	729	603, 577 559, 441	559	flavan-3-ol-flavanol gallate
F	13.90	441	289, 271 169	245	epicatechin-3-gallate
G	15.52	723	571		?

Table III. Retention Times (LC-MS) and Main Ions in the ESI-MS-MS Mass Spectra of Compounds in Fraction V

Peak	r.t. total ion current trace	ESI-MS-MS DATA			Structure
		Full ms $[M-H]^-$	ms-1	ms-2	
H	12.42	729	603, 577 441	559	flavan-3-ol-flavanol gallate
I	13.75	1017	891,865, 847, 729, 603		procyanidin trimer*
	14.30	463	301	179, 151	quercetin-3-O-glucoside
L	15.35	447	301	179, 151	quercetin-3-O-rhamnoside
M	15.60	477	357, 315 314	285, 286, 271	isorhamnetin-3-O-glucoside
N	16.66	461	357,315 314	285, 286 271	isorhamnetin-O-deoxyhexose

* The trimer is composed by two units of flavan-3-ol (catechine or epicatechine) and one unit of flavanol gallate.

fragmentation pattern as compounds **D** and **E**, therefore, the same dimeric structure is suggested. Compound **I** was a mixture of two unresolved components. The signal at m/z 1017 most likely corresponded to the pseudomolecular ion of a trimer constituted by two flavan-3-ol units (catechin/epicatechin; mol. wt. 2 × 290-2) and one flavanol gallate (catechin gallate/epicatechin gallate; mol. wt. 442). The fragment at m/z 729 is generated by the loss of 289 amu from the $[M-H]^-$ and corresponded to the cleavage of the carbon–carbon bond of the flavan-3-ol moiety (mol. wt. 290) (13).

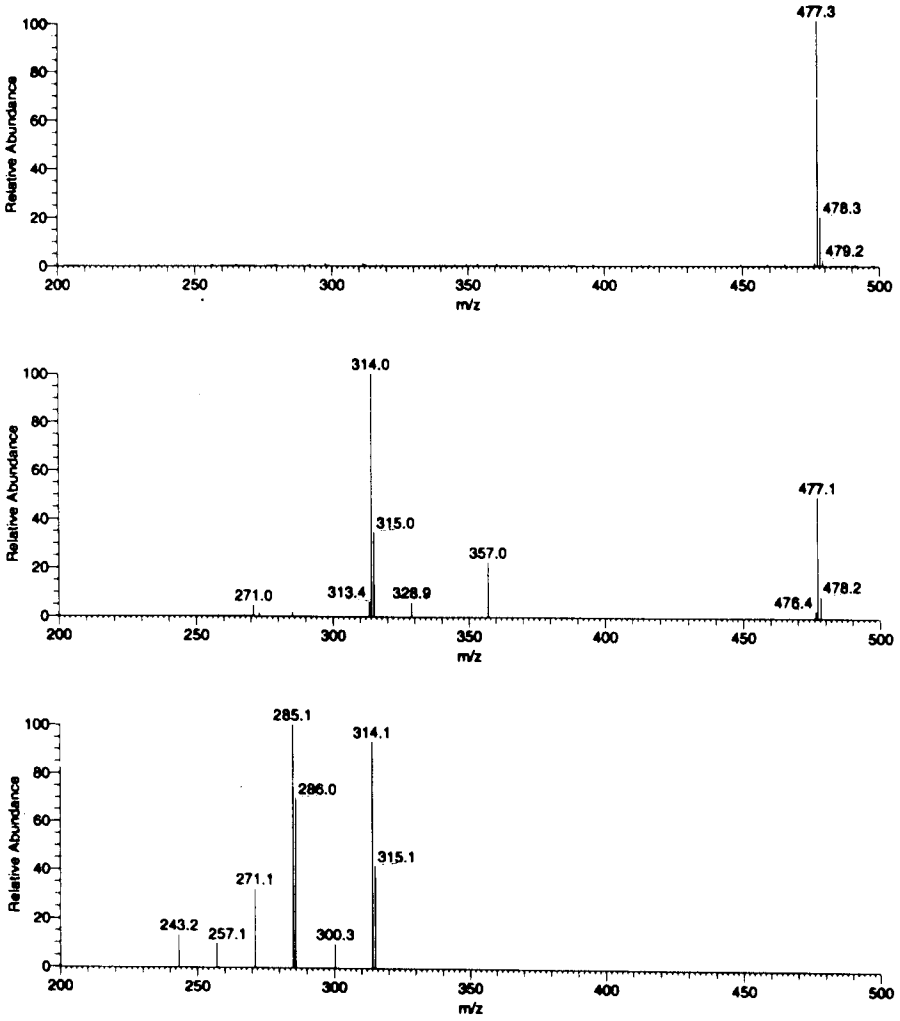

Fig. 4. Mass spectrum of isorhamnetin-3-O-glucoside. Upper panel: full ms $[M-H]^-$; central panel: ms-1 fragments obtained by collision of the pseudomolecular ion; lower panel: ms-2 fragments generated by collision of the $[A-H]^-$ ion at m/z 315.

The trimer co-eluted with a compound which exhibited a deprotonated $[M-H]^-$ negative ion at m/z 463, which suggested the structure of quercetin-O-hexose. The in-source collision generated the aglycon ion $[A-H]^-$ m/z 301, and the fragments m/z 179 and 151, typical of quercetin-3-O-glucoside, used as a reference (Fig. 3). Compounds **L** gave the $[M-H]^-$ negative ion at m/z 447, the aglycon ion $[A-H]^-$ m/z 301 and daughter ions as described above, thus indicating the structure of quercetin-O-deoxyhexose. The final assignment of quercetin-3-O-rhamnoside was obtained by co-chromatography with the authentic compound.

The compounds **M** and **N**, with $[M-H]^-$ negative ions at m/z 477 and 461 were glycosides of the same aglycone. The loss of 162 and 146 amu, respectively, corresponded to the cleavage of glucose and of a deoxyhexose, most likely rhamnose. The collision of the $[M-H]^-$ ions (m/z 477 and 461) gave rise to the $[M-H-\text{sugar}]^-$ and $[M-2H-\text{sugar}]^-$ fragments 315 and 314 amu. The fragments at m/z 285 and 286 are formed from the loss of the methoxy group $[A-H-30]^-$ and $[A-H-29]^-$. A similar fragmentation pattern was obtained with isorhamnetin-3-O-glucoside (Fig. 4). The loss of 120 amu (fragment $[M-H-120]^-$ at m/z 357), in general, is known to be highly specific for *C*-glycosylflavones (18,19). However as shown in the mass spectrum of isorhamnetin 3-O-glucoside, it may also occur for *O*-glycosyl derivatives, even if this fragmentation of the sugar moiety is not as abundant as for the *C*-glycosides (19). A similar fragmentation of the sugar moiety, shifted by 16 amu $[M-H-104]^-$, was observed in the mass spectrum of compound **N**. However, the possibility of impurities of *C*-glycosyl derivatives could not be ruled out. Co-chromatography with the pure compound allowed the definition of the structure of **M** as isorhamnetin-3-O-glucoside. Due to the lack of availability of isorhamnetin-3-O-rhamnoside, the final structure assignment to compound **N** was not possible with the available information.

Effect of Flavonol-3-O-glycosides on Xanthine Oxidase

To be able to relate anti-XOD activity to the found compounds, the available pure substances (Fig. 5) were tested. XOD was not affected by quercetin-3-O-glucoside and quercetin-3-O-rhamnoside at concentrations up to 100 μM. Isorhamnetin-3-O-glucoside 100 μM gave 22.7% inhibition; at lower concentrations, it was inactive.

CONCLUSIONS

The fractions of the leaf extract of *Amyena scandens* which displayed anti-XOD properties are a mixture of *O*-glycosylflavonols, low molecular weight proanthocyanidins, and the corresponding monomers. According to the literature, (+)-catechine, (−)-epicatechine, (−)-epicatechin-3-gallate (Fig. 5), and procyanidin B-2 and C-2 are devoid or have low (IC_{50}>40μM) XOD inhibitory activity (10,11,20). From our experimental data, it is shown that the contribution of *O*-glycosylflavonols to the inhibitory activity is also negligible, since only isorhamnetin-3-O-glucoside exhibited some activity, but at high concentrations (100 μM = 48 μg/ml). Then, according to the literature data and to our results, it is possible to relate the anti-XOD activity of fractions **II** and **V** to the galloyl containing oligomeric proanthocyanidins. Quantitative analysis of the active constituents was not performed because of the lack of suitable reference compounds. However, from the TIC traces (Fig. 1) it appears that in fraction **II**, epicatechin-3-gallate is predominant; fraction **V** conversely consists mainly of *O*-glycosylflavonols. Isorhamnetin-3-O-glucoside in fraction **V** can contribute only to a small extent, since the amount of this compound in fraction **V** does not reach the concentration required to exhibit the effect (100 μM to produce 22% inhibition).

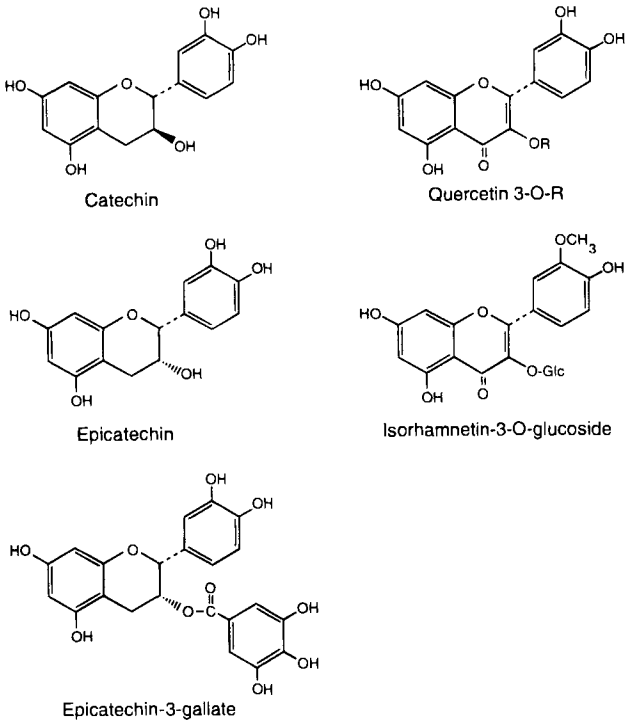

Fig. 5. Structure of the compounds identified by co-chromatography. R = glucose or rhamnose

Coupling UV-DAD-HPLC with ESI-MS-MS allowed a partial on-line identification of several compounds in a complex mixture, avoiding previous isolation. For flavonoid glycosides, significant structural information can be obtained, even if the MS method alone does not allow determination of the nature of the sugar moiety and positional isomers. The accuracy of assignment for three out of four flavonol-glycosides was obtained by using reference compounds analyzed by HPLC co-chromatography. In the case of the flavan-3-ol derivatives, direct data about the molecular weight of the oligomers are obtainable, thus providing information about the unit composition of the polymeric chain. Due to the lack of commercially available reference compounds, the correct definition of the oligomeric structure could not be achieved.

Even if LC-UV-ESI-MS-MS has the limitations mentioned above, the development and application of these techniques is important for rapid characterization of previously identified bioactive substances in plants, a process known as dereplication (21), therefore increasing the chances for finding novel bioactive plant metabolites. As recently shown by Nawwar *et al.* (22), who detected a new ellagitannin in a mixture of 28 compounds without preliminary sample manipulation, HPLC-ESI MS-MS analysis is a remarkable and efficient approach for searching new compounds. The same technique can also be helpful in

following isolation steps until the pure compound is obtained. The electrospray interface has the advantage of increased stability and efficiency of ionization with respect to thermospray and is the method of choice for biopolymers but also for polar natural products unstable under thermal stress (21,23).

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