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THE PHENOLICS AND A HYDROLYSABLE TANNIN POLYPHENOL OXIDASE OF *MEDINILLA MAGNIFICA*

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Key Word Index—*Medinilla magnifica*; Melastomataceae; hydrolysable tannin polyphenol oxidase; phenolic acids; quantitative determination.

Abstract—The production of viable meristem cultures of *Medinilla magnifica* has proved to be very difficult. This may be due, in part, to a pronounced 'browning' response of the tissues on cutting. For this reason the phenolic compounds and the hydrolysable-tannin polyphenol oxidase from *Medinilla* were studied. The distribution of the compounds was: simple phenols 19%, flavonoids 5%, hydrolysable tannins 69%, condensed tannins 7%. Amongst the simple phenols and phenolic acids, the following were identified: phloroglucinol, *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid, gallic acid (both in free and bound form the most abundant simple phenol), syringic acid, *trans*-*p*-coumaric acid, *trans*-ferulic acid and *trans*-caffeic acid. No kaempferol or quercetin or their derivatives were detected but condensed tannins are present. Methods for the extraction, fractionation and quantitative determination of phloroglucinol and the phenolic acids, as well as correction factors for losses during the extraction, alkali treatment and derivatization, are presented in a supplementary publication.* With regard to the hydrolysable tannin polyphenol oxidase activity of *Medinilla* stems, the enzyme(s) is rather specific since at neither of its two pH optima (6 and 7) could a classical polyphenol oxidase activity be detected. The enzyme was strongly inhibited by 2-mercaptoethanol. Preliminary experiments have further shown that in addition to the hydrolysable tannins of the tissue, the ferrous ions of the medium, and oxygen together with the hydrolysable tannin polyphenol oxidase could play a role in the browning response. Ways to overcome this difficulty have been suggested.

INTRODUCTION

The common method for the propagation of *Medinilla magnifica*, a cultivated ornamental, is by the rooting of cuttings. However, this method is not always successful and the production of this ornamental plant, which is in great demand, is thus declining. The use of tissue culture as a supplementary technique for the mass propagation of *Medinilla magnifica* has therefore been considered.

Isolated epidermal and subepidermal tissues [1], root tips and apices from buds have been used for the production of suitable tissue cultures [2] but, so far, no viable meristem cultures of *Medinilla* have been obtained. This is apparently due in part to the formation of a black-brown pigment which rapidly develops on the cut-ends when the Murashige–Skoog medium is used [3]. This suggests that *Medinilla* contains high concentrations of phenols which are oxidized on cutting [4]. However, the rapid black-brown pigmentation of the tissues could also be due to an interaction of the plant phenols with ferrous ions in the culture medium or to quinone–protein interactions [5, 6].

In order to obtain more information on the phenolics of *Medinilla*, the simple phenols, phenolic acids, hydrolysable and condensed tannins of the whole plant have been quantitatively analysed. Flavonoids (probably mainly proanthocyanidins) proved to be present only in relatively small amounts and neither kaempferol, quercetin nor their derivatives could be detected. In addition, the polyphenol oxidase and hydrolysable tannin polyphenol oxidase activity of leaves and stems have been studied. Following the discovery of relatively high amounts of phenols, especially hydrolysable tannins, in the whole plant and of a rather specific hydrolysable tannin polyphenol oxidase activity in the stem tissues, suggestions have made for the modification of the culture technique of *Medinilla* tissues to reduce the formation of pigment.

RESULTS

The leaves and stems of *Medinilla magnifica* were extracted with boiling MeOH–EtOH (1:1) under nitrogen and after concentration the extract partitioned between *n*-butanol and 6% Na_2CO_3. After removal of Et_2O-soluble substances, the aqueous layer was acidified to pH 3.5 and re-extracted with Et_2O giving fraction A (free phenolics). The acidic aqueous layer was basified with concentrated NaOH until 2 M and refluxed for 2 hr,

* In a supplementary publication, flow sheets are given for the detailed extractions of the phenolic acids; SUP 90049 (21 pp.) deposited at the National Lending Library, Boston Spa, Yorkshire, LS 237 BQ, U.K., from whom copies can be obtained.

acidified and extracted with Et_2O to give fraction B (carbonate-soluble alkali-labile bound phenolics). The *n*-butanol layer from the above was refluxed in 2 M NaOH for 2 hr, the cooled hydrolysate extracted with *n*-BuOH followed by Et_2O and the aqueous layer then acidified as above and re-extracted with Et_2O to give fraction C (carbonate-insoluble, alkali-labile bound phenolics). Fraction D was obtained from the residue insoluble in MeOH–EtOH after basic hydrolysis, acidification, ether extraction, partitioning the ether solubles between Na_2CO_3 (pH 9) and Et_2O; acidifying the carbonate layer and re-extracting with Et_2O (alcohol-insoluble, alkali-labile bound phenolics). Full details are given in the supplementary publication.

The following phenol and phenolic acids were identified in the different fractions of the MeOH–EtOH (1:1) extract and the insoluble residue of *Medinilla magnifica*: in the free state (extract A): protocatechuic, vanillic, gallic, syringic, *p*-coumaric, caffeic and ferulic acids. In extract B: phloroglucinol, *p*-hydroxybenzoic, vanillic, gallic and ferulic acids. In extract C: *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids. In fraction D: phloroglucinol, protocatechuic, vanillic, gallic, *p*-coumaric and ferulic acids.

Each compound was identified by GLC, TLC (in different solvent systems), fluorescence or absorption in UV light, UV spectrum and colour reaction with diazotized *p*-nitroaniline. The results from quantitative analyses were as far as possible corrected for losses occurring during the extraction procedures and during the hydrolysis of bound compounds with 2 M NaOH. Losses that incurred during GLC and TLC were also corrected for. In this connection, the more or less pronounced stability of the phenols in alkaline medium proved to be very important. The stability of the identified compounds in sodium carbonate solution or boiling 2 M NaOH, as well as the corresponding overall correction factors, are therefore given in the supplementary publication (see also [7, 8]). The 'overall correction factors' embrace corrections for losses during extraction, sodium carbonate treatment (and if required also for alkaline hydrolysis with 2 M NaOH), preparation of the TMSi-derivatives of the phenols, GLC separation, hydrolysis of the collected TMSi-derivatives, TLC separation, and elution and final quantitative determination of each compound. Due to the extreme instability of the cinnamic acid derivatives (e.g. ferulic acid) in acid medium [8, 9], no acidic hydrolysis of the fractions has been performed.

Table 1 shows the quantitative determination of the identified phenols. The results obtained (expressed in μg/g dry wt) were corrected as described. Table 2 represents the total amount of extractable phenols, flavonoids, and hydrolysable and condensed tannins, found in the MeOH–EtOH (1:1) extracts and in the aqueous extracts from *Medinilla*. These latter results, which were obtained by means of the procedure described by Marigo [10], are expressed in mg gallic acid equivalent per g dry wt. The presence of condensed tannins was also proved by the red colour formation after heating the extracts (2 hr at 90–95°) with 5% *n*-BuOH–HCl [11].

It is apparent that polyphenol oxidases are present in *Medinilla*. Due to the presence of high concentrations of phenolics, especially tannins, it was decided to prepare the enzymes by the acetone powder procedure. The powder was extracted with acetate buffer (pH 5.7) and freeze-concentrated and purified by chromatography on Sephadex G25 and DEAE-cellulose.

Table 3 reports the activity of the purified 'hydrolysable tannin polyphenol oxidase' from *Medinilla* stems in μmol O_2 consumed/min/mg protein. An analogous enzyme preparation from leaves proved to be inactive. The stem enzyme showed two pH maxima: the first and most pronounced being at 6 (K_m with tannic acid as substrate = 0.21%) and the second at 7 (K_m = 2.0%). (For the expression of the K_m values in % concentration see Discussion.) The blank values were higher with increasing pH from pH 6.3 and so determinations of enzyme activity were made at pH 6.

The inhibition of the hydrolysable tannin polyphenol oxidase by some typical polyphenol oxidase inhibitors [12] is shown in Table 4.

DISCUSSION

Only the two structurally related compounds vanillic and *trans*-ferulic acid occur in all fractions. Although the *cis* and *trans* forms of the substituted cinnamic acids can be readily separated by GLC [13], only the *trans* forms were found to be present, presumably due to the fact that all extractions and chromatographic separations (except for GLC) were performed under orange light which prevents *trans–cis* interconversion [14]. Next to the identified phenolics, several other unidentified substances, giving rise to a colour reaction with diazotized *p*-nitroaniline, were detected (see supplementary publication). However, from the intensity of the colour reactions it was concluded that most of these compounds occur only in minor quantities.

Amongst the free and (or) bound phenols, gallic acid is by far the predominant compound (87.5% of the total free and 97.9% of the total bound phenols; (Table 1), although the free fraction represents only 0.34% of the sum of both the free and total alkali-labile bound amounts of the phenolic acids.

With the exception of syringic and caffeic acid, all identified compounds were shown to occur in much higher concentration in the bound than in the free form (Table 1). This is especially the case for *p*-coumaric and ferulic acids. With other methods of extraction and analysis it is believed that the small amounts of the free acids would not be detected. It is also striking that fraction B, which contains almost three times as much gallic acid than D, contains less ferulic acid (ferulic acid about 10 times more present in D than in B), *p*-coumaric acid and phloroglucinol. It is quite possible that the higher gallic acid content of B with regard to fraction D reflects the extraction of hydrolysable tannins with EtOH–MeOH (1:1).

Extraction and distribution experiments with commercial tannic acid have shown that the hydrolysable tannins remain mainly in fraction B. The presence of the higher amounts of hydroxycinnamic acid in the alcohol-insoluble residue is further in agreement with the findings of El-Basyouni *et al.* [15], who suggested that alcohol-insoluble derivatives of the phenolic cinnamic acids in wheat act as intermediates in lignification. Moreover, the same authors proposed that an insoluble enzyme ester of the above acids could well be one of the intermediates although it was not suggested that the total amount of hydroxycinnamic acids obtained from the alcohol-insoluble fraction would be bound to enzymes. More

Table 1. Quantitative analysis of the phenolic compounds identified in *Medinilla magnifica**

Phenolic compound	(A) Free phenols	(B) Alcohol and carbonate soluble, diethyl ether-insoluble, alkali-labile bound phenols	(C) Alcohol-soluble and carbonate-insoluble, alkali-labile bound phenols	Alcohol-insoluble, alkali-labile bound phenols	Total alkali-labile bound phenols
		μg/g dry wt			
Phloroglucinol	—	55.1 $S\bar{x} = 9.2$	—	108.7 $S\bar{x} = 15.8$	163.8
p-Hydroxybenzoic acid	—	25.1 $S\bar{x} = 2.9$	3.0 $S\bar{x} = 0.1$	—	28.1
Vanillic acid	1.3 $S\bar{x} = 0.1$	30.0 $S\bar{x} = 1.7$	2.8 $S\bar{x} = 0.2$	10.2 $S\bar{x} = 1.4$	43.0
Protocatechuic acid	9.4 $S\bar{x} = 0.5$	—	—	26.2 $S\bar{x} = 3.8$	26.2
Syringic acid	9.0 $S\bar{x} = 0.5$	—	—	—	—
Gallic acid	198.0 $S\bar{x} = 18.7$	43 493.9 $S\bar{x} = 1390.7$	—	15 204.7 $S\bar{x} = 1403.4$	58 698.6
p-Coumaric acid	1.0 $S\bar{x} = 0.1$	—	25.0 $S\bar{x} = 0.4$	388.4 $S\bar{x} = 24.5$	413.4
Ferulic acid	2.9 $S\bar{x} = 0.2$	54.2 $S\bar{x} = 2.9$	18.6 $S\bar{x} = 0.6$	526.7 $S\bar{x} = 19.4$	599.5
Caffeic acid	4.7 $S\bar{x} = 0.3$	—	—	—	—

* Analysis of the whole plant; the results (means of at least six different determinations) are expressed in μg/g dry wt. The final figures were obtained after application of the overall correction factors.

Table 2. Quantitative determination of the extractable total phenols, flavonoids, hydrolysable and condensed tannins present in *Medinilla magnifica*

Phenolic compounds	Extracts				
	MeOH–EtOH extract	Aqueous layer No. 1	Aqueous layer No. 2	Total	Distribution of the total phenol content (%)
Phenols	16.3	0.5	2.0	18.8 (79%)	19
Flavonoids	0	1.0	4.1	5.1 (21%)	5
Total non-tannins	16.3	1.5	6.1	23.9 (100%)	24
Hydrolysable tannins	60.2	9.2	1.0	70.4 (91%)	69
Condensed tannins	2.0	0.5	4.6	7.1 (9%)	7
Total tannins	62.2	9.7	5.6	77.5 (100%)	76
Total phenol content Σ (tannins + non-tannins)	78.5	11.2	11.7	101.4	100

(After separation, the final analyses were performed with Folin–Ciocalteu reagents; the results being expressed in mg gallic acid equivalent per g dry weight).

Table 3. Hydrolysable-tannin polyphenol oxidase activity (pH 6.0) from *Medinilla magnifica* stems using different phenols as substrate

Substrate (at 3.3 mM)	Enzyme activity after DEAE-cellulose chromatography* (10^{-2} μmol O_2 consumed/min/mg protein)
Tannic acid†	381
Catechol	5
Phloroglucinol	10
Protocatechuic acid	3
Gallic acid	15
Chlorogenic acid	3
Tyrosine	10
DL-Dopa	16
Max. error (oxygen monitor system)	31

* At pH 7.0 (the second pH optimum of the enzyme preparation) analogous results were obtained.
† MW tannic acid (BDH) 1701.23.

Table 4. Inhibition of the hydrolysable tannin polyphenol oxidase by some typical phenolase inhibitors

Inhibitor	Concentration tested (mM)	% Inhibition
Sodium diethyldithiocarbamate	3.3	14
Salicylhydroxamic acid	3.3	7
Disodium-EDTA	3.3	12
L(+)-Cysteine	3.3	28
N-Caffeoylglycine	3.3	11
Hydroxylamine HCl	3.3	41
	10.0	62
Glutathione	0.5	4
	1.0	24
	3.3	50
	5.0	53
	10.0	83
2,3-Dimercapto-1-propanol (BAL)	0.5	17
	1.0	63
	3.3	88
	5.0	90
	10.0	89
Sodium metabisulphite	0.5	39
	5.0	93
2-Mercaptoethanol	0.05	5
	0.1	29
	0.3	87
	0.5	92

information on the occurrence of alcohol-insoluble phenolic acid–protein combinations may be found in a review by Van Sumere *et al.* [6]. The total amounts of extractable phenols, flavonoids, condensed and hydrolysable tannins present in *Medinilla magnifica* (Table 2) were obtained by the method of Marigo [10]. This method, which was checked with varying test mixtures, proved to give acceptable results. The results in Table 2 show that 76.0% of the total extractable phenols of *Medinilla* occur as tannins. The hydrolysable tannins (69% of the total phenols) represent by far the most abundant fraction. This latter figure seems to be in agreement with the high amounts of gallic acid (74.1% of the total amount alkali-labile bound gallic acid and 73.8% of the total amount of gallic acid) found in *Medinilla* (Table 1). In both the MeOH–EtOH and the aqueous extracts, condensed tannins were present as was shown by the formation of anthocyanidin after heating the extracts with 5% *n*-butanol–HCl [11]. However, in the absence of precise information with regard to the type

and degree of polymerization of the procyanidins [11], no assumption on the value $E^{1\%}_{1cm}$ has been made. Additional work on the fractionation and characterization of the tannins is now in progress.

In reference to the polyphenol oxidase* activity (Table 3) of *Medinilla* leaves and stems it is rather interesting that only the latter show a hydrolysable tannin polyphenol oxidase activity. This enzyme(s), which to our knowledge has never been described before, seems to be rather specific, because at either of its pH optima it proved to be active with commercial tannic acid as substrate, although it did not show any activity with a series of classical polyphenol oxidase substrates. For this reason the name hydrolysable tannin polyphenol oxidase is proposed. It was further shown with the Marigo procedure [10] that the commercial substrate contained ±95% hydrolysable tannins while, according to the manufacturer's label, the MW should be 1701.23. Nevertheless, the K_m values (at each pH optimum) of the enzyme have been intentionally expressed in percentage concentration because further analysis of the commercial substrate, by column chromatography, HPLC [14], proved that it contained a series of ±8 more or less analogous components (some in minor amounts) with different molecular sizes.

Although the isolation of each tannic acid component has already been performed, the commercial tannic acid was still used as a substrate because several of the isolated components proved to be very unstable or less suitable for the determination of enzyme activity. Even under nitrogen some of the fractions showed a rather rapid brown-black pigmentation which is most likely due to a reaction with traces of oxygen. With other purified fractions, the activity of the enzyme varied between 30 and 270%. (The activity obtained with the complete tannic acid mixture was taken as 100%). Therefore, until the different components of the tannic acid mixture have been further characterized and some of them even proved to be pure and acceptable substrates for the hydrolysable tannin polyphenol oxidase, the K_m values of the foregoing enzyme are expressed in % concentration of the more stable and readily soluble mixture of commercial tannic acid.

With regard to potential inhibitors of the hydrolysable tannin polyphenol oxidase (Table 4), it may further be concluded that mercapto compounds especially (e.g. 2-mercaptoethanol, 2,3-mercapto-1-propanol, glutathione) are inhibitory. Indeed, at 5×10^{-4} M 2-mercaptoethanol depressed the enzyme activity by 92%, while 3.3 mM disodium-EDTA decreased enzyme activity by only 12%.

With regard to the difficulties in obtaining viable meristem cultures from growing buds of *Medinilla*, the brown-black colouring at the cut ends of the tissues is most likely due to two different reactions: (a) an oxidation of hydrolysable tannins mediated by the ferrous ions of the Murashige–Skoog medium; (b) an oxidation of the same tannins by means of the hydrolysable tannin polyphenol oxidase.† The onset of both reactions can be delayed at least *in vitro*. After addition of 0.17% hydrolysable tannins to the Murashige–Skoog medium at room temperature a brown colouration was obtained within 30–60 min and after 2 days the colour of the medium was black. Quadrupling the EDTA concentration of the Murashige–Skoog medium (the final sodium EDTA · $2H_2O$ concentration being 4×10^{-4} M) delayed the brown-black colouration for more than 2 weeks. Substitution of the ferrous ions by ferric ions retarded colour formation even further. The same was true when citric acid (2×10^{-4} M) and a quadruple amount of EDTA were used. The beneficial effect of EDTA is certainly due to chelation of the ferrous ions. In addition, ferric ions show a specifically much higher stability with *O*-containing ligands, while citrate has further been suggested to be the ligand that binds ferric ions during xylem transport [17]. Preliminary experiments with *Medinilla* leaf fragments (1 cm^2) and growing buds on solidified (0.6% agar) Murashige–Skoog medium, which contained the quadruple amount of EDTA, showed that development of a brown colouration in the medium underneath the plant material could be delayed by increasing the concentration of chelating agent. It has further been shown that the replacement of $FeSO_4 \cdot 7$ aq. by Fe-EDTA or $FeCl_3$ in the Murashige–Skoog medium (eventually other chelating agents for ferrous ions could also be tried [18–21]) is beneficial.

According to Brown [22] ferric ion complexes with oxygen donors and the binding by phenolic groups is very specific for ferric iron. Thus it seems that the search for optimal chelating agents which do not affect plant growth and (or) the replacement of ferrous ions by ferric ions, together with acceptable inhibitors of the hydrolysable tannin polyphenol oxidase, and growth of the cultures at lower temperature, etc., may to a great extent overcome the 'brown-blackening' response in tissue cultures of growing buds of *Medinilla*. Inhibition of the foregoing response is further improving the prospects for producing tissue cultures for the mass-propagation of the ornamental plant.

EXPERIMENTAL

Plant material. Potted *Medinilla magnifica* plants obtained from cuttings originating from 30-year-old stock were grown in a green house (temp. 25°; humidity, 75%) under normal photoperiodic conditions. Plants of the same size (30–35 cm) were selected and used for analysis.

Extraction of the plant material for the quantitative determination of the phenols and phenolic acids. The plants were cut just above soil level. They were homogenized under orange light (Perspex ICI Nc. 300) in hot MeOH–EtOH (1:1) and the extract was worked up according to the scheme outlined in the text, which is given in detail in the supplementary publication, for free phenolic acids (fraction A)‡; MeOH–EtOH (1:1) and sodium carbonate-soluble but diethyl ether-insoluble alkali-labile bound phenols and phenolic acids (B); and EtOH–MeOH (1:1)-soluble and carbonate-insoluble, alkali-labile bound phenolic acids (C). The residue gave rise to EtOH–MeOH (1:1)-insoluble, alkali-labile bound phenols and phenolic acids (D). During the

* The names polyphenol oxidase and hydrolysable tannin polyphenol oxidase have been selected with reference to the recent review paper by Mayer and Harel [16].

† During the action of the enzyme the colour of the tannic acid solution changed from light yellow to brown.

‡ Lyophilization of the plant material as employed by Balsa *et al.* [23] should be omitted because low MW compounds such as phenolic acids, peptides, etc., may be removed from the concentrate by the high vacuum [24]. Some of the compounds could further be recovered from the ice trap of the all glass freeze-drying apparatus which was used during the experiments.

extractions, the phenolic compounds remained once or twice for 20 min in a sodium carbonate soln. In this alkaline medium some of the compounds are unstable and their recoveries from the sodium carbonate solution are time-dependent. Therefore each extraction was performed according to an identical time schedule.

Analysis of free and bound phenols and phenolic acids. The free and bound phenols and phenolic acids of *Medinilla* were analysed as described in the supplementary publication (see also [7]). For the qualitative and quantitative determination of the bound phenols and (or) phenolic acids, the extracts were first hydrolysed by reflux under N_2 using 2 M NaOH [7]. In each case a final Et_2O extract was obtained which was further analysed by a combination of GLC and TLC [7, 13] or METC [8, 25, 26] (see also supplementary publication).

GLC. The phenolic compounds were first converted to volatile trimethylsilyl derivatives (TMSi-derivatives with *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)) [7, 13]. The volatile esters and (or) ethers of the phenols were then separated using previously described procedures [7, 13].

Collection and hydrolysis of the separated TMSi-derivatives. The derivatives were, as far as possible, collected as a single peak and then hydrolysed by adding a drop of water [13]. When the concn of a phenolic acid in an extract proved to be low, up to 3 collections of the same peak were made in the same tube.

TLC and qualitative identification of the phenols. The hydrolysed TMSi-fractions were further analysed by TLC on cellulose MN or mixed layers of Si gel G and cellulose MN (1:1) [8, 26, 27]. For this purpose the following solvent systems were used: toluene–HCO_2Et–HCOOH (TEF; 5:4:1); $CHCl_3$–HOAc–H_2O (CAW; 4:1:1); toluene–HOAc (TA; 9:1); C_6H_6–HOAc (BeA; 95:5) and 2% HOAc. For qualitative identification, the plates were viewed under UV both before and after treatment with 2 M NaOH. Thereafter, the chromatograms were treated with diazotized *p*-nitroaniline [27].

Presence of free phenolic acids. The occurrence of free phenolic acids in *Medinilla* was proved as follows: the MeOH–EtOH extract was first treated with petrol (bp 100–140°) and the remaining alcoholic layer was concd in vacuum to dryness. After further drying over P_2O_5 and NaOH, 501.3 mg material (corresponding with 10.4 g fr. wt) was treated with 10 ml BSTFA and the TMSi-derivatives were prepared in a sealed vial [7, 13]. The volatile derivatives were then transferred to a specially constructed distillation–sublimation apparatus (see supplementary publication) and the excess reagent was removed by normal distillation (2 hr; temp. range 20–140°). After cooling until room temp., vacuum distillation (0.1 mm Hg; temp. range 30–170°) was then performed during 3–4 hr. The distilled and (or) sublimed TMSi-compounds that were partly retained at the cold trap [24] were removed from it by refluxing with acetonitrile. Subsequently the TMSi-derivatives and solvent were transferred to a tared bottle and the soln was blown to dryness in N_2 (dry wt 82 mg). After renewed treatment with BSTFA (100 μl per mg material), the GLC separation, collection, hydrolysis and TLC analysis of the separated TMSi-derivatives were performed as described [7, 13]. When the concn of a phenolic acid proved to be low, several collections of the same peak were made in the same tube.

Quantitative TLC and determination of the phenols. When known amounts of standards were applied alongside the hydrolysed TMSi-fractions, quantitative results could be obtained by combining TLC with fluorimetry (vanillic, syringic, *p*-coumaric, caffeic and ferulic acid), spectrophotometry (*p*-hydroxybenzoic acid) or colorimetry (phloroglucinol, protocatechuic and gallic acid). Vanillic, *p*-coumaric and ferulic acid were determined as described [7, 8]. Syringic acid (excitation max 324 nm; fluorescence max 370 nm) and caffeic acid were also analysed with the same technique although 0.6% w/v) $AlCl_3 \cdot 6H_2O$ was employed as an eluant for the latter compound; the excitation and fluorescence maxima of the metal complex being respectively 384 and 488 nm. Phloroglucinol, protocatechuic and gallic acid were eluted with 5% Na_2CO_3 and subsequently determined by colorimetry at 760 nm using Folin–Ciocalteu reagent [28].

Overall correction factors. Radioactive compounds, and if unavailable, pure unlabelled substances, were used to check the extraction schemes, alkaline instability of some of the compounds and chromatographic recoveries. Further details about the overall correction factors may be found in the supplementary publication.

Extraction of the plant material for the quantitative determination of the total extractable phenol, flavonoid, hydrolysable- and condensed-tannin content. 75 g (fr. wt) of *Medinilla* leaves and stems were mixed in hot MeOH–EtOH (1:1) and refluxed in N_2 for 2 hr. After centrifugation, the residue was again extracted 4 × as before. The extracts were combined and the residue was treated as described below. To the combined MeOH–EtOH extracts, petrol (bp 100–140°) was added (removal of chlorophyll). This last treatment was repeated twice. The MeOH–EtOH layer was then concd, diluted with H_2O and again concd until an aq. soln remained. Subsequently, the phenol and tannin content of the latter was determined by the procedure of Marigo [10]. The above-mentioned residue was further extracted with H_2O for 2 hr at room temp. After centrifugation, this treatment was repeated twice. The aq. fractions were pooled (aq. fraction No. 1) and further analysed for phenols and tannins as described by Marigo [10]. Finally, the remaining residue was extracted with boiling H_2O (refluxed in N_2 for 2 hr). After centrifugation, this treatment was also repeated twice and the extracts (aq. fraction No. 2) were further analysed for phenols and tannins as indicated above. (The MeOH–EtOH and aq. extracts have also been qualitatively analysed for condensed tannins by the method of Bate-Smith [11].)

Preparations of a hydrolysable tannin polyphenol oxidase from stems of Medinilla. After the removal of the leaves the stems were macerated in a blender for 10 min with (2.5 ml/g) cold Me_2CO (−15°) [29]. The suspension was centrifuged (1900 *g*, 4°) and the residue was homogenized, with cold Me_2CO (2.5 ml/g). After centrifugation, the residue was dried on Whatman No. 1 paper at 4°. The dried Me_2CO powder was stirred for 20 min with 0.02 M acetate buffer, pH 5.7 (5 ml/g of original tissue wt) and the suspension kept overnight. The dark brown supernatant was freeze-dried to a vol. of *ca* 0.15–0.2 ml/g original (crude extract). This extract was partly purified by gel filtration through Sephadex G25 (medium) (column 55 × 2.5 cm) 0.01 M K phosphate buffer, pH 7.0, being used as an eluant. The crude extract separated into 3 peaks, only the first peak showing hydrolysable tannin polyphenol oxidase activity. Thereafter the enzyme (total 20.8 mg) was further purified by DEAE-cellulose chromatography (Whatman DE 32; column 15 × 2.5 cm). Fraction vols were approximately 5 ml. Elution of the column was begun with K phosphate buffer (0.02 M, pH 8) until fraction 115, thereafter a gradient of K phosphate buffer (0.02 M, pH 8 until 0.5 M, pH 5.7) was applied. Four different protein peaks were obtained; the whole enzyme activity being located in the first peak (between fraction 10 and 25). The protein content of the enzyme peak (colourless protein) was 0.052 mg/ml (total 4.2 mg). The specific activity of the purified hydrolysable tannin polyphenol oxidase was 5.63 μmol O_2 consumed/min/mg protein; the total activity being 23.4 μmol O_2 consumed/min (recovery of the total enzyme activity 92%).

Protein content. The protein content was determined by the method of Hartree [30].

Enzyme assay. The enzyme assay was based on the measurement of rate of O_2 uptake with tannic acid (BDH) as a substrate. Oxygen consumption was measured at 37° by means of a Clark-type oxygen electrode. The reaction mixture contained 1 ml 10 mM tannic acid (BDH, MW 1701.23) and 1 ml 0.15 M K phosphate buffer, pH 6.0. After 3 min stirring, 1 ml of the enzyme preparation (boiled enzyme for blank) was added. The results (corrected for suitable blanks) are expressed in μmol O_2 consumed/min/mg protein.

Inhibitors of the hydrolysable tannin polyphenol oxidase. Varying final concns of the inhibitors were separately prepared in buffer. The inhibition of the enzyme is expressed as a % of the maximum activity of the untreated enzyme.

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