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Variation in leaf toughness and phenolic content among five species of Australian rain forest trees

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Abstract

Several leaf characteristics, including toughness and total phenols and condensed tannins, were measured in Australian rain forest leaves of different ages and related to observed herbivory rates. In most cases, toughness and chemical toxicity increased as leaves aged, and corresponding insect grazing decreased. Herbivory losses ranged from 4.8% to 32.5% leaf area losses, and were more positively correlated with toughness than with phenolics. It is suggested that a suite of factors, including physical and chemical characteristics of leaves as well as spatial and temporal factors, interact to create variation in grazing intensities.

Introduction

Several physical and chemical characteristics of leaves have been related to herbivory in recent literature (Fox & Macauley 1977; Feeny 1968, 1970; Onuf 1978; Coley, in press). The physical attribute of toughness (whether due to cuticle, pubescence or sclerophylly) may mechanically limit an insect's chewing capacity, with preference exhibited for soft leaf tissue (e.g. Feeny 1970; Tanton 1962). Secondly, leaves may accumulate chemical substances that lower the palatability of leaf tissue (reviewed in Feeny 1976; Rhoades & Cates 1976). In particular, the properties of tannins and total phenols in leaf tissue have been widely reported as an index of plant chemical defence (e.g. eucalypt leaves; Macauley & Fox 1980), and the methods for their extraction are well established (Ribereau-Gayon 1972). Other leaf characteristics have been implicated in plant herbivore relationships (e.g. nitrogen, White 1968; Onuf 1978; phenology, Feeny 1970), but are not evaluated here.

Observations of herbivory in Australian rain forest canopies revealed high variability on several spatial and temporal scales (Lowman 1982a). Rates ranged from 4.8% to 32.5% leaf area losses between species; young leaves were more heavily grazed than mature leaves, and lower canopy, shade leaves were preferred over upper canopy, sun leaves. The details of herbivory studies are reported elsewhere (Lowman 1982a). In this study, several leaf characteristics are examined that may relate to grazing patterns observed in the canopies. The analyses were designed to quantify leaf toughness and phenolic content, and relate them to observed herbivory rates at four levels of variability: (1) among rain forest tree species; (2) among individuals of one species; (3) among leaves of different ages; and (4) between sun and shade leaves.

Methods

Leaf samples

Leaves were collected from five species of rain forest trees in three rain forest formations in central New South Wales, Australia: *Ceratopetalum apetalum* (coachwood) an evergreen canopy tree of the warm temperate rain forests; *Doryphora sassafras* (sassafras), an evergreen canopy species of both subtropical and warm temperate formations, and an understory tree in cool temperate rain forests: *Dendrocnide excelsa* (giant stinging tree), a subtropical canopy dominant but with unusually short-lived leaves (approximately six months as compared to two years for most rain forest species); *Nothofagus moorei* (Antarctic beech), the single species dominant of cool temperate rain forest canopy in New

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South Wales; and *Toona australis* (red cedar), a deciduous canopy tree of both subtropical and warm temperate formations. Leaf samples were collected from mature canopy trees in three national parks: Dorrigo, 30° 30′ S (subtropical or complex notophyll vine forest), New England, 30° 10′ S (cool temperate or mossy microphyll fern forest) and Royal, 34° 10′ S (warm temperate or simple notophyll vine forest; Webb 1959).

Leaf age classes were defined based on preliminary field observations (Fig. 1):

(1) Young leaf usually from 0–2 weeks old; after budburst but before attaining full expansion.

(2) Youthful leaf — from 2–4 weeks old; leaf full-sized but lacking in the attributes of a mature leaf such as heavy cuticle or deep green pigmentation.

(3) Mature leaf — from approximately one month after budburst until the end of its first year; fully grown and structurally developed leaf.

(4) Old leaf — mature leaves during their second year; distinguished from previous class by position on branch, by darker (shade leaf) or yellower (sun leaf) pigment, or by initial signs of epiphylly or senescence.

(5) Senescent leaf — leaf in the process of dying and just prior to abscission; distinguished by basal position on shoot, loss of chlorophyll, or onset of decomposition.

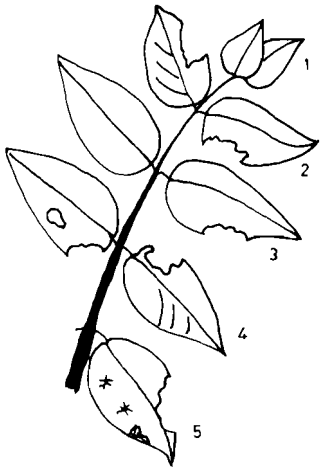

FIG. 1. Leaf age classes as defined for use in rainforest leaf analyses: 1 = young leaf (emerging); 2 = youthful (just emerged); 3 = mature (first year); 4 = old leaf (second year); 5 = senescent leaf (dying).

Leaves were collected and measured during September-October, when all age classes were present in the canopy. Since most Australian rain forest trees underwent a major leaf flush in spring and leaf fall during summer, both old and new leaves were present during spring (Lowman 1982a). The only exception was *T. australis*, whose canopy is deciduous and thus homogeneous in leaf age structure; leaves were sampled as the canopy aged (September for classes 1 and 2, January for class 3, June for class 5, and class 4 nonexistent since the leaves did not exist for a 2nd year).

Herbivory rates

Herbivory rates were measured by marking leaves of replicate branches in the field and monitoring grazing activities from 1979–1982. These long term observation methods, designed to measure both partial and total defoliation of individual leaves are described in detail elsewhere (Lowman 1982a) and only summarized here. At least three branches at intervals along vertical transects on each of three trees were monitored monthly for each species at each site, and grazing losses were compared at different spatial and temporal scales. Mean herbivory losses were calculated for each species (representing an average of hundreds of leaves from different shoots, trees, sites, canopy heights and light regimes), and used here to examine the effectiveness of leaf toughness and phenolics as plant defences. Missing leaf area was measured with a Lambda portable area meter, and hole area expressed as percentage of total (potential) leaf size.

Leaf toughness

Three leaves of each of the five defined age classes (Fig. 1) were clipped randomly from the canopies of the five species. In addition, both sun and shade leaf samples were collected from *C. apetalum* and *D. sassafras* since these species had morphologically different leaves with respect to light regime; and additional leaves from three individuals and three sites were collected to examine spatial variation of *D. sassafras* as it was a widely distributed rain forest species. Age class 4 (second year) leaves did not exist for *T. australis* and *D. excelsa* since the leaves of these species senesced in less than a year.

A penetrometer was constructed according to the design of Feeny (1970). The device consisted of a brass 5 mm diameter plunger that pierced a leaf

surface when adequate pressure was applied. Unlike Feeny's model, where sand provided the weight to activate the plunger, water was instead trickled into a beaker of known weight until the plunger penetrated the sample. Water was easy to control and obtain in the field. Leaves were measured in the penetrometer immediately upon collection. Five sections of tissue in each leaf were sampled between main veins in the adaxial surface. The total weight (g) of the beaker plus water required to penetrate the leaf provided an index of leaf toughness. All weights were transformed into $\log_{10}$ form for homogeneity of variance, and multiple factor analyses of variance were performed on the data, with factors of species and leaf class regarded as fixed (Snedecor & Cochrane 1967).

Leaf phenolic substances

Leaves of the five age classes (Fig. 1) were picked from two individuals of the five species during September-October, since all age classes were present simultaneously in the canopy at that time. (The only exception was *T. australis*, whose old leaves were picked in June, since its canopy is deciduous and thus homogeneous in age structure.) In addition, sun and shade leaf populations were sampled from *C. apetalum* and *D. sassafras*, because both showed strong differences in leaf morphology with light regime.

The leaves were dried overnight at 80°C and stored at −12°C. Prior to extraction, they were re-dried at 85°C, ground in a Wiley mill, and placed in a desiccator over silica gel. Portions (100 mg) were extracted in gently boiling (80°C) 50% v/v aqueous methanol for 15 min and cooled to room temperature for a further 45 min. Each extract was filtered through an ignited (500°C, overnight) Whatman GF/C filter paper and made up to 100 ml with 50% v/v methanol in a volumetric flask.

Aliquots (1000 µl see Appendix 1) of the methanolic extract were diluted to 10.0 ml with distilled water and analysed for total phenols using the Folin-Ciocalteau phenol reagent with tannic acid as the standard. Tannin concentrations which gave final absorbances up to $A_{750} \simeq 1.0$ with the Folin Ciocalteau reagent were free from enhanced absorbances at 750 nm due to the action of the alkaline supporting medium (Na_2CO_3) on the dissolved organic substances, a phenomenon found by Box (unpublished) for natural water samples. Condensed tannins were determined using vanillin and HCl. The method of Broadhurst & Jones (1978) was modified by doubling all the volumes to allow the use of 4 cm cuvettes, and standardized with (+)−catechin hydrate.

Results

Leaf toughness

Leaf toughness was significantly different among species ($F_{4,140} = 2747.1$, $P < 0.001$, data log-transformed: Fig. 2). In general, *T. australis* had the softest leaves, followed by *D. excelsa*, *N. moorei*, *D. sassafras* and *C. apetalum* in order of increasing toughness. Leaf toughness increased with age up to class 3 (except in *D. excelsa*) and then decreased slightly between maturity (class 3 and 4) and senescence (class 5). Leaf toughness did not increase during age class 3; rather, the toughness values between leaves of 4 and 11 months were similar if not slightly decreased toward old age (class 4). Most species showed a dramatic increase in toughness after age class 1 (young leaf), with *N. moorei* leaves becoming 10 times tougher between age classes 1 and 3. *D. excelsa* was an exception to this pattern: its young leaves (class 1) were tougher than its senescent leaves (class 5), probably owing to the high density of surface hairs on the unfolding leaf and the paper-like texture of old leaves.

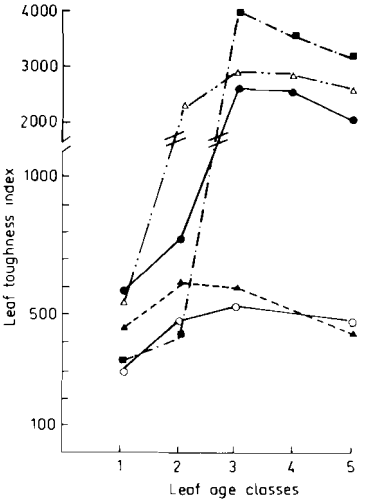

FIG. 2. Leaf toughness value for different ages of five rainforest trees, as measured with a penetrometer. Points represent means of five replicates of three leaves. Lead toughness index is the weight in grams required to puncture leaf; (see Fig. 1 for definition of leaf age classes (■) *N. moorei*; (△) *C. apetalum*; (●) *D. sassafras*; (○) *T. australis*; and (▲) *D. excelsa*.

When leaves grown under different light regimes were compared, sun leaves were consistently tougher than shade leaves in both *D. sassafras* and *C. apetalum* (Fig. 3). Both types of leaves, however, showed a similar pattern of increasing toughness with age.

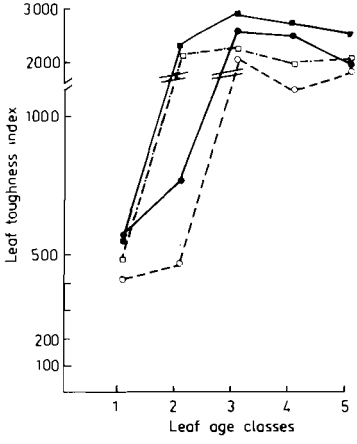

FIG. 3. Leaf toughness values for different ages of sun (■, ●) and shade (□, ○) leaves of two rainforest trees, *C. apetalum* (squares) and *D. sassafras* (circles), as measured with a penetrometer. Points indicate mean values of five replicates of three leaves (see Fig. 1 for definition of leaf age classes).

In addition to the factors of species and age, *D. sassafras* (sun leaves) were examined for variability among individuals and sites. Leaf samples from three individuals at each of three rain forest formations differed significantly in toughness levels with site ($F_{2,36} = 82.94$, $P < 0.001$) but not with individuals ($F_{2,36} = 0.6006$, n.s.). The site differences correspond to elevational changes of the rain forests sampled where toughness (T) increased with altitude; warm temperate (22 m, $T = 1556$) < subtropical (800 m, $T = 1789$) < cool temperate (1200 m, $T = 2330$).

Leaf phenolic substances

Total phenols (TP) and condensed tannins (CT) expressed as percentage of dry weight of leaf material varied significantly among species ($F_{4,125} = 351.01$, $P < 0.001$; Fig. 4). *Toona australis* and *D. sassafras* had the highest phenol concentrations, whereas *D. excelsa* leaves had very low levels. TP differed significantly between two individuals of *T. australis* ($F_{1,16} = 21.28$, $P < 0.001$) whereas two individuals of *N. moorei* had statistically similar amounts of TP ($F_{1,20} = 2.85$, n.s.). For both species, however, the interaction between age and individuals was significant, implying that much of the individual variation related to the dependence of these factors.

Phenol contents were significantly different among the five age classes of leaves ($F_{4,125} = 7.54$, $P < 0.001$). Four species showed an accumulation of both TP and CT with age albeit with some fluctuations; only *D. excelsa* decreased with age. Sun leaves had up to twice the levels of TP and CT than shade leaves.

Herbivory rates

The mean amounts of leaf material grazed annually from the canopies of rain forest trees (expressed as % leaf area lost) ranged from 4.8% (*T. australis*) to 32.5% (*D. excelsa*) with intermediate levels of 26.1% (*C. apetalum*), 30.7% (*N. moorei*) and 14.8% (*D. sassafras*) (Table 1). Grazing intensity varied considerably among age classes and leaves and among species. Other factors that affected grazing losses included light regime, sites and canopy heights of leaves, but these factors are considered in greater detail elsewhere (Lowman 1982a). At least 90% of the cumulative leaf area loss occurs during age classes 1 and 2 for the long-lived, evergreen rain forest trees (*N. moorei, D. sassafras* and *C. apetalum*). In contrast, *T. australis* and *D. excelsa*, which have shorter-lived, soft leaves, were grazed throughout the lifespan of their leaves, with less preference for young leaf tissue. Leaf toughness values were strongly correlated to grazing losses among different ages of leaves in several species (*N. moorei* and *D. sassafras* with r^2 values of 0.92 and 0.90, respectively). Phenolic contents were less closely correlated to grazing levels, with *D. sassafras* showing the highest r^2 value of only 0.55.

Discussion

The ratio of CT to TP gives an indication of the relative proportions of hydrolysable and condensed tannins. The ratio will be affected by factors such as the reaction of the Folin-Ciocalteau reagent with substances other than tannins (e.g. compounds with reducing properties, particularly those with phenolic hydroxyl groups); the varying reactivity of CT to the two reagents depending on the degree of poly-

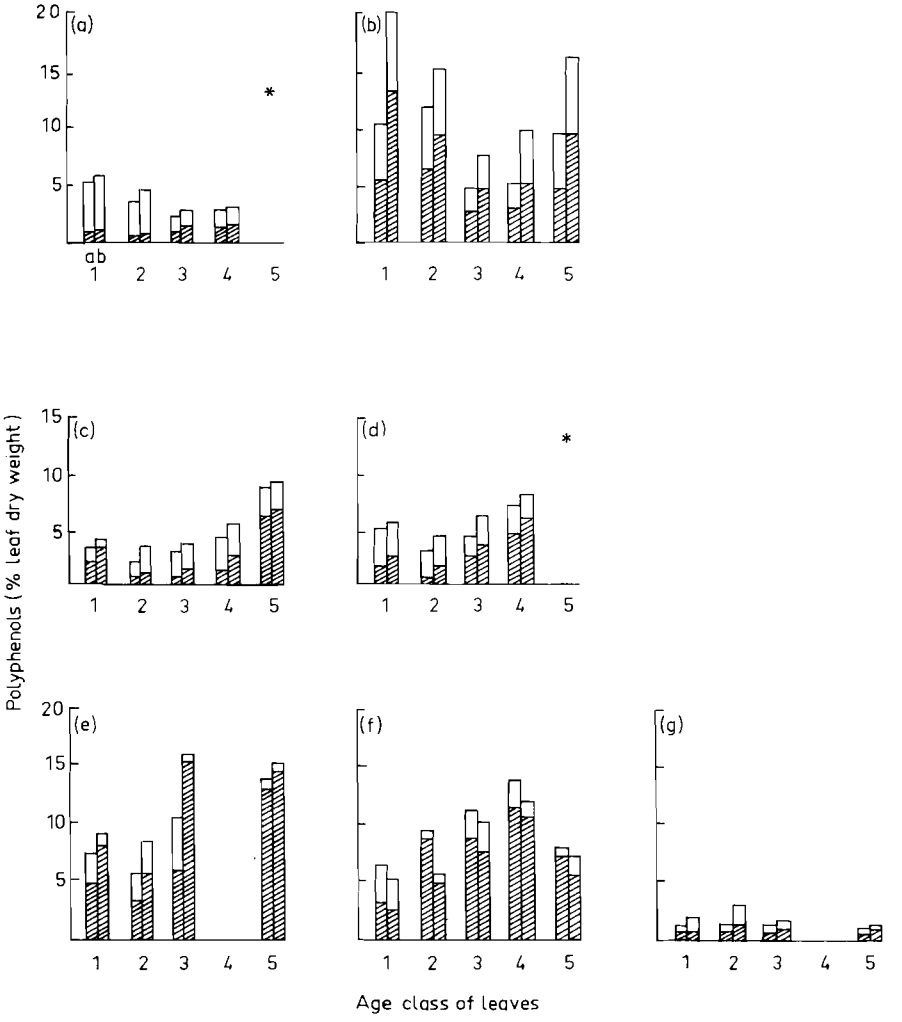

FIG. 4. Variation in total phenols (represented by an entire bar) and condensed tannins (shaded section) among five age classes in two Individual trees (labelled a & b), expressed as percentage of dry weight of leaves of five rainforest canopy tree species (see Fig. 1 for age class definitions): (a) *D. sassafras* (shade), (b) *D. sassafras* (sun), (c) *C. apetalum* (shade), (d) *C. apetalum* (sun), (e) *T. australis*, (f) *N. moorei*, (g) *D. excelsa*. * Indicates no sample.

merization (Goldstein & Swain 1963; Lewak 1968); differences in the reactivity of condensed and hydrolysable tannins to the Folin-Ciocalteau reagent (e.g. 2000 μg l^{-1} of tannic acid and catechin had $A^{4\ cm}_{750}$ values of 0.41 and 0.51, respectively); and a lack of standardization using tannins extracted from each of the five species used in this study. Thus, the ratio should not be taken as being absolute but rather used as an indication of the variation between species or age classes of leaves.

There were no clearcut relationships of grazing levels with either toughness or phenolic content of leaves (Table 1). In four of five species, however, changes in leaf toughness with age showed a stronger correlation to proportions grazed than changes in phenolics. Low leaf toughness was usually associated with high herbivory (e.g. young *D. sassafras* and *N. moorei* leaves) but not always (e.g. all ages of *T. australis* leaves). In *T. australis*, the extremely high phenolics appear to protect the leaf tissue

TABLE 1. Differences in herbivory among five different ages and species of Australian rain forest leaves (expressed as the mean proportion of leaf area grazed by insect herbivores; $n = 100$ leaves)

	Proportion of leaf area grazed (%) Age Class						Regressions (r^2)	
Species	1	2	3	4	5	Herbivory (%)	Phenols	Toughness
N. moorei	13.0 (38)[1]	17.0 (54)	1.5 (7)	0.1 (0.5)	0.1 (0.1)	30.7	0.30	0.90
T. australis	0.03 (1)	1.0 (23)	2.9 (64)	—[2]	0.9 (13)	4.8	0.20	0.68
D. excelsa	6.8 (20)	10.1 (34)	5.7 (19)	—	8.0 (27)	32.5	0.19	0.68
C. apetalum	10.5 (42)	12.5 (48.6)	3 (7)	0.6 (1)	0.5 (.4)	26.1	0.40	0.40
D. sassafrasa	6.2	7.5 (43)	0.3 (52)	0.3 (2)	0.2 (2)	14.5 (1)	.55	0.90

[1]Numbers in brackets are the percentage of total grazing for the species which occurred in that age class. ([2]—) Indicates that the leaf age class did not exist in the canopy.

effectively from grazing despite their softness. In *D. excelsa*, neither characteristic was effective as defence, and subsequent grazing levels at all ages were high. The polyphenolic levels were extremely variable, however, between individual trees of one species (e.g. sun leaves of *D. sassafras*), suggesting the need for more extensive analyses of intraspecies leaf variability.

In general, observed herbivory rates were explained best by a combination of both toxins and toughness in conjunction with the seasonal patterns of tree canopies (Lowman 1982a) and insect herbivore populations (Lowman 1982b). For example, young beech leaves (age class 1) had low toughness values and moderate polyphenol levels, and suffered higher levels of grazing than mature beech leaves. In addition to this physical and chemical vulnerability, the young beech leaves flushed synchronously during September, thereby creating an apparent food supply to a herbivore (Feeny 1976). With beech leaf flush, a host specific herbivore (*Novocastria nothofagi* larvae, Chrysomelidae) also emerged and severely defoliated young beech leaves (Selman & Lowman in press). Presumably the beetle larvae are physiologically adapted to tolerate beech toxins at the levels contained in young leaves yet find the mature leaves less palatable; and synchronization of the insect phenology with leaf flush ensures an abundant food supply.

Young shade leaves of *D. sassafras* and *C. apetalum* had low levels of phenolics and toughness and high grazing pressure; toughness and toxicity increased in mature leaves, and grazing intensity subsequently decreased. Insect abundance was also greatest during peak times of leaf flushing (Lowman 1982b). Nutritive contents of young leaf tissue may be proportionally high, rendering it preferable to grazers (Moran & Hamilton 1980). Further studies on the relationship of rain forest leaf nitrogen and grazing intensity are under way, since it has been suggested that insect grazing may relate to leaf nitrogen levels (e.g. White 1978; Fox & Macauley 1977).

Rain forest leaves had lower amounts of polyphenols than leaves of the adjacent eucalypt woodlands (see Fig. 1, Macauley & Fox 1980). The proportions of CT tended to be higher in all rain forest leaves, however, and the absolute amounts of TP lower. No rain forest leaves were as heavily grazed as eucalypts where up to 75% leaf area losses have been reported (Journet 1981), implying that other factors such as leaf nutritive qualities, abundance and seasonality of herbivores, or herbivore predators and parasites in rain forests versus sclerophyll woodlands may be involved.

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Appendix I. The Folin-Denis reagent (Folin & Denis 1912) has been widely used for estimating total phenols in tannin extracts (Swain & Hillis 1959; Ribereau-Gayon 1972; Fox & Macauley 1977). Criticisms of this reagent led to the development of the Folin-Ciocalteau reagent (Folin & Ciocalteau 1927) in which the same chemicals are refluxed for longer in a more acid medium to which lithium is added to increase the solubility of the molybdic and tungstic complexes (Kloster 1974). The methodology and performance characteristics associated with the use of the Folin-Ciocalteau reagent lead to the inclusion of both hydrolysable and condensed tannins; the latter were independently estimated using vanillin and HCl.

The conversion of flavan-3, 4-diols (leucoanthocyanidins) into coloured anthocyanidins by heating in dilute acid has been used as means of estimating condensed tannins (Swain & Hillis 1959; Goldstein & Swain 1963). The method does not determine the flavan-3-ol (catechin) group of condensed tannins (Ribereau-Gayon 1972) and suffers from a poor yield of anthocyanidins (White 1958; Ribereau-Gayon 1972). Vanillin reacts with under-activated phloroglucinol or resorcinol nuclei of flavans in acid medium to give a coloured product (Goldstein & Swain 1963) and the reaction can be used to determine flavan-3-ols and flavan-3, 4-diols, the two major classes of condensed tannins.

Several aspects of our methods are explained below in an effort to clarify the method for analyses of leaf polyphenolic contents.

Effect of methanol concentration on total phenols determination

Distilled water and 50% v/v methanol were mixed in varying proportions to give a 10 ml treatment and the procedure for the determination of total phenols carried out. It was found that 1 to 2 ml of 50% v/v methanol in a sample of 10 ml (equivalent to 10 ml of 10% v/v methanol) did not interfere with the total phenols methodology.

Final methanol concentrations greater than 10% v/v methanol resulted in a white precipitate. For this study of leaf tannins a maximum aliquot of 1000 µl methanolic extract was used for the total phenols determinations.

Comparison of extraction methods

A comparison was made of the effectiveness of different methods of tannin extraction from leaves of the five species — *C. apetalum* (sun leaves, age class 4), *D. excelsa* (age class 3), *D. sassafras* (shade leaves, age class 5), *N. moorei* (age class 4) and *T. australis* (age class 3). Samples (100 mg) of the dried ground leaves were extracted for varying times in three solutions:

(1) 100 ml distilled water at 97°C in a boiling water-bath,

(2) 100 ml boiling (*c.* 80°C) 50% v/v methanol, and

(3) 100 ml v/v methanol at room temperature for 25 h in a dark cupboard.

The extracts obtained by methods (1) and (2) were allowed to attain room temperature before filtration.

TABLE AI. Effect of different procedures on the extraction of total phenols from leaves

Extraction procedure	Total phenols extracted (% dry weight) De	Ds	Ca	Nm	Ta
Water (97°C)					
15 min	2.31	4.34	5.79	6.64	15.02
30 min	2.60	4.38	6.33	6.37	14.55
60 min	3.11	4.36	6.25	6.56	15.83
300 min	4.80	4.96	6.95	6.48	15.05
50% methanol (boiling)					
1 min	2.01	5.58	6.02	7.76	17.10
5 min	2.25	5.79	6.21	7.84	16.91
15 min	2.50	5.67	6.27	7.84	17.06
30 min	2.68	5.71	6.31	7.91	17.18
50% methanol (room temperature)					
25 h	1.49	5.17	6.08	8.11	12.16

Species as follows: De — *D. excelsa*, Ds — *D. sassafras*, Ca — *C. apetalum*. Nm — *N. moorei*. Ta — *T. australis*.

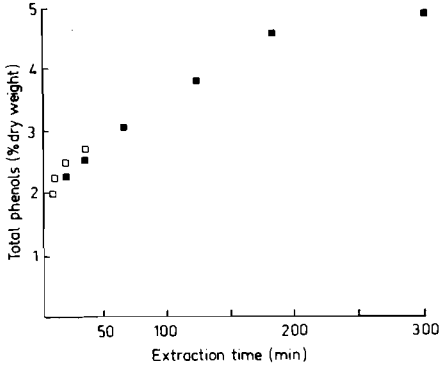

FIG AI. Tannins (measured as total phenols) extracted by 50% boiling methanol (□) and water (97°C (■) from *D. excelsa* leaves for varying extraction periods.

Total phenols were determined with the Folin-Ciocalteau reagent.

The results (Table AI) show that 50% methanol at room temperature extracted variable amounts of total phenols in comparison with boiling 50% methanol. Boiling 50% methanol extracted more total phenols from the leaves of *C. apetalum, D. sassafras, N. moorei* and *T. australis* than hot water and the duration of exposure had little effect on the concentrations of total phenols extracted with either hot water or boiling methanol. The levels of total phenols extracted from leaves of *D. excelsa* using either hot water or boiling methanol increased with time (Fig. AI) and approached an asymptote after 5 h in hot water.

An extraction of 15 min in boiling 50% methanol was chosen as a compromise for this study since relative, rather than absolute, tannin concentrations were needed.

Potential interference due to proteins

The Folin-Ciocalteau reagent is reduced by a number of substances besides phenols (Box unpublished), including some amino-acids (tyrosine, tryptophane, alanine, histidine, cysteine) which are incorporated into proteins. Potential interference from this source was examined by determining total phenols in 5 ml methanolic extracts of leaves from the five species (see section on comparison of extraction methods) after treatment with 5 ml 10% trichloracetic acid at 4°C overnight (5 ml distilled water was added to a parallel series of controls). After centrifuging, all the methanolic extracts ex-

TABLE AII. Investigation of protein interference in the estimation of total phenols in leaf extracts by precipitation with trichloracetic acid

Species	Total phenols (% dry weight) Without TCA[1]	With TCA[2]
D. excelsa	2.75	2.87
D. sassafras	6.37	6.44
C. apetalum	6.85	6.91
N. moorei	7.68	7.80
T. australis	17.83	18.10

[1]Equal volumes of methanolic extract and distilled water. [2]Equal volumes of methanolic extract and 10% trichloracetic acid.

posed to trichloracetic acid showed a very small white precipitate at the bottom of the centrifuge tube. The levels of total phenols in the extracts after centrifuging (Tale AII) were similar whether the extracts had been treated with trichloracetic acid (proteins precipitated) or distilled water (proteins present). This indicated that protein interference was not significant for the leaves of the five species examined.