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Extraction and quantification of “condensed tannins” as a measure of plant anti-herbivore defence? Revisiting an old problem

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Abstract Contents of phenolic compounds in leaf extracts often serve as a measure of plant anti-herbivore defence. This method suffers from the multifunctionality of phenolics and from problems with their colorimetric quantification. Here we present further evidence for the pertinence of these problems. Contents of condensed tannins (CCT) were spectrophotometrically quantified in leaf extracts of 11 closely related mimosoid species, and *Spodoptera littoralis* caterpillars were reared on artificial diet containing these extracts. The relationship of CCT with caterpillar growth differed considerably among plant species, since both positive and negative correlations were detected. There was, however, a negative correlation of CCT with fungal spore germination, indicating a role of these compounds in resistance to fungi. Detailed knowledge on the structure and biological function of defensive compounds and on the overall composition of leaves is required to estimate a plant's defensive efficacy against a particular group of enemies.

Introduction

The defensive effects of phenolic compounds are often emphasised in theories on plant–herbivore interactions

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(Feeny 1976; Bryant et al. 1983; Coley et al. 1985; Jones and Hartley 1999) and have repeatedly been demonstrated to play important roles in plant resistance (Mole and Waterman 1987a, b; Cheeke 1989; Bennett and Walls-grove 1994; Shirley 1996; Mueller-Harvey 2001; Schofield et al. 2001). Since the different classes of phenolics can be extracted and quantified easily, they are often used as a general measure of a plant's investment in, or efficacy of, anti-herbivore defence. Since 1995, more than 200 studies have quantified tannins or total phenolics in the context of plant anti-herbivore defence, or have studied phenolic compounds in order to estimate changes in plant defence under elevated CO₂.

Condensed tannins (proanthocyanidins) form a heterogeneous group of flavonols that are widespread in the plant kingdom and are generally characterised by their capacity to precipitate proteins (Appel 1993; Ayres et al. 1997). This trait can lead to the inhibition of enzymes, e.g. in insects' digestive tracts, or to the formation of insoluble complexes with dietary proteins, thereby reducing the nutritive value of plant material (Appel 1993). Some herbivorous insects can exhibit specific counter-adaptations against this function (Konno et al. 1997). Several studies reported that total tannin content and/or total phenolics were negatively correlated with growth rates of feeding herbivores (overview in Appel 1993). Tannins are metabolically stable and have low turnover rates, and their effect has been assumed to be positively correlated with their concentration. Therefore, tannins are regarded as an example of 'quantitative defences', which lower herbivore growth rates and thereby increase the risk of predation or parasitisation of herbivores (Feeny 1976; Price et al. 1980).

However, the defensive role of tannins has been a matter of discussion for over 20 years (Bernays 1978, 1981; Martin and Martin 1982; Mole and Waterman 1987a, b). Several studies failed to reveal clear relations between content of phenolic compounds in leaves and growth rates of insects feeding on them (Martin and Martin 1982; Dudt and Shure 1994; Ayres et al. 1997; Ossipov et al. 2001). The arguments most often formu-

lated to explain these results have been (1) the comparison of unrelated species which differed too much from each other in their phenolic profile as to allow useful comparisons, (2) the use of entire leaves, which are defended by many other compounds in addition to tannins, (3) the evolution of counter-adaptations by herbivores (Bernays 1978, 1981; Konno et al. 1997), and (4) the use of colorimetric methods that are affected by cross-reactions with compounds other than tannins (Schofield et al. 2001).

Although these technical problems are well known, colorimetric methods are still widely used to quantify tannins, most probably due to a lack of good alternatives. The present study was conducted to re-visit this problem. To focus more directly on the interaction between herbivores and phenolics we tried to avoid most of the technical problems by which earlier studies were plagued. In order to check for alternative defensive properties of the phenolics extracted by our method, biotests on fungal spore germination were performed.

1. Different myrmecophytic and non-myrmecophytic species of the genus *Acacia* (Fabaceae, Mimosoideae) and three additional species of related genera were selected. Belonging to the same subfamily, and in most cases to the same section of the same genus, these species all are closely related to each other. Myrmecophytic *Acacia* species are defended by mutualistic ants (Janzen 1966, 1967), invest heavily in this 'biotic' defence, and are therefore expected to have a lowered direct chemical defence (Rehr et al. 1973; Seigler and Ebinger 1987; Heil et al. 2000). Because many *Acacia* species are known to have high concentrations of tannins (see, for example, Readell et al. 2001), the species selected for this study could be expected to cover a broad spectrum of phenolic contents within a group of closely related species.
2. Leaf extracts were prepared and were added to standardised caterpillar food, in order to exclude effects of other quantitative defences, such as fibre content, on the growth rates of the tested herbivore.
3. Egyptian cotton worm (*Spodoptera littoralis*) caterpillars were used as herbivores. *Spodoptera* is a generalist herbivore capable of feeding on many different plants. It originates from Africa. This allowed us to exclude any problems potentially arising from co-evolutionary adaptations of the tested herbivore to some specific compounds of the plant species investigated.
4. In contrast to other colorimetric methods used to quantify phenolic compounds (see Martin and Martin 1982; Mole and Waterman 1987a, b; Schofield et al. 2001 for overviews), the method used here has the advantage that the reaction product of DMCA (4-dimethylaminocinnamaldehyde) with catechin shows an absorbance maximum between 632 and 640 nm, while other aldehydes lead to absorption at shorter wavelengths. The DMCA reagent can therefore be used for specific detection of catechin and proanthocyanidins (Treutter 1989).

Material and methods

Plant material

Leaves of five ant-*Acacia*, three non-myrmecophytic *Acacia* and three other mimosoid shrub species were collected in Mexico in March and April 2000 and in April 2001. In 2000, material from the *Acacia* myrmecophytes, *A. hindsii* Benth., *A. cornigera* (L.) Willd., *A. globulifera* Safford and *A. chiapensis* Safford, was collected from sites on the Isthmus of Tehuantepec (state of Oaxaca). Samples of the non-myrmecophytic mimosoid shrubs *Prosopis juliflora* (Swartz) DC., *Leucaena leucocephala* (Lamarck) de Wit, and *Mimosa tenuiflora* (Willd.) Poir. originated from the same site. Samples of the myrmecophyte *A. collinsii* Safford and of the non-myrmecophyte *A. macracantha* Humb. et Bonpl. were collected near Coba, on the Yucatan peninsula (state of Quintana Roo). Samples of the non-myrmecophyte *Acacia farnesiana* (L.) Willd. originated from the valley of Oaxaca (state of Oaxaca). Additional samples of *A. farnesiana* (hereinafter '*A. farnesiana* Isth.') were collected in 2001 at the Isthmus of Tehuantepec (the two populations of *A. farnesiana* are hereinafter treated as two 'species' for statistical and graphical purposes). Samples of another non-myrmecophyte, *A. cochliacantha* Humb. et Bonpl. ex Willd. were collected in 2001 near Puerto Escondido on the Pacific coast (state of Oaxaca). All sites were extensively used pastures or similarly structured, open secondary shrublands. All plants used were shrubs of heights between 1.5 and 2.5 m and grew in the full sun. Species were determined following Standley (1920), Rudd (1966), Janzen (1974), Seigler and Ebinger (1995), and Hughes (1998) and by comparison with specimens held at the Herbario MEXU at UNAM (Mexico City). Voucher specimens are deposited at the Herbario MEXU and in the personal collection of M. Heil. Sample identification numbers at Herbario MEXU are: *A. hindsii* 1026602, *A. cornigera* 1026606, *A. globulifera* 1026599, *A. chiapensis* 1026610, *A. collinsii* 996387, *A. macracantha* 996388, *A. farnesiana* 996385, *A. cochliacantha* 1026604, *P. juliflora* 1026609, *L. leucocephala* 996380, and *M. tenuiflora* 996382.

Sample treatment and quantification of tannins

For each sample, about 100 g of fresh leaf material was collected from different twigs of an individual shrub (sample sizes varied from three to 12 shrubs per species; see Table 1 for sample sizes). Leaves were selected to give a representative mixture of age classes as present on the respective shrub. Those showing visible damage due to herbivory or pathogens or bearing extensive mechanical damage were avoided. The leaves were dried in the dark at air temperatures between 25 and 35°C within 48 h to constant weight, first openly and then in airtight boxes over silica gel. The material then was transported to Germany, and was ground with an IKA MF

Table 1 Correlations (Spearman rank correlation) between caterpillar growth rates and total tannin content (CCT) within each species studied. *n* sample size, *r* coefficient of correlation

	Species	<i>n</i>	<i>r</i>	<i>P</i>
Positive correlations	<i>Prosopis j.</i>	3	1.00	<0.001
	<i>A. farnesiana</i>	7	0.65	0.055
	<i>Leucaena l.</i>	7	0.61	0.074
	<i>A. farnesiana</i> Isth.	8	0.65	0.040
Negative correlations	<i>A. hindsii</i>	12	-0.45	0.060
	<i>Mimosa t.</i>	5	-0.97	0.002
	<i>A. cornigera</i>	10	0.04	>0.10
	<i>A. macracantha</i>	7	0.09	>0.10
	<i>A. chiapensis</i>	10	-0.31	>0.10
	<i>A. collinsii</i>	8	-0.31	>0.10
	<i>A. globulifera</i>	11	-0.18	>0.10
<i>A. cochliacantha</i>	6	-0.06	>0.10	

10 mill (IKA, Germany) to fine powder and sieved (mesh size 1 mm).

A small amount (3 g) of dry powder was extracted in 40 ml methanol (48 h at approximately 25°C). After filtration, the filtrate was re-extracted twice, each time using 20 ml methanol. These three extracts were combined and evaporated at 40°C and under light vacuum (continuously decreasing from ~400 mbar to 150 mbar). The residue was re-dissolved in 40 ml methanol and then further diluted 1:10 in ethanol (1 ml extract + 9 ml ethanol). 100 µl of the diluted extracts were thoroughly mixed with 1 ml DMCA solution (0.1% DMCA in methanol-HCl 9:1 v/v) (McMurrough and McDowell 1978; Treutter 1989). DMCA was purchased from Fluka (Neu-Ulm, Germany). Absorption at 640 nm was measured after 5 min of colour development at room temperature in a Unicam spectrophotometer (UV/Vis Spectrophotometer, Unicam, Cambridge, UK), and concentration of condensed tannins (ConcCT) in the extract was calculated based on a calibration curve established with catechin as a standard [calibration curve: catechin ($\mu\text{g ml}^{-1}$) = $12.88 \cdot x^2 + 36.6 \cdot x$, $r^2 = 0.997$, x = absorption relative to 1 ml DMCA solution mixed with 100 µl methanol : ethanol 1:1 (v/v)]. Contents of condensed tannins (CCT) in leaves were calculated by relating the concentrations as found in the extracts to the dry weight of the extracted leaf material.

Biotests with caterpillars

Neonates (newly hatched first-instar caterpillars) of Egyptian cotton worm, *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) were used. The animals had been reared for at least four generations on artificial diet consisting of finely homogenised white beans (*Vicia faba* var. 'Hang down') saturated with an equal amount (v/v) of water. To this diet, 18 mg g⁻¹ ascorbic acid, 18 mg g⁻¹ Nipagin, 8 mg g⁻¹ formaldehyde and 1 mg g⁻¹ Gentamycin were added to inhibit bacterial and fungal infection.

The same diet was used for the biotests. A 3 ml sample of extract was allowed to dry overnight at room temperature, and the residue was mixed thoroughly with 3 g of diet in order to create a homogeneous distribution of tannins in the artificial diet. A group of 20 neonates were placed on this food for a period of 2 weeks. Each day some water (~0.5 ml) was added to keep the food moist. The same amount of food was used to rear caterpillars, and the food was never used up earlier than 3 weeks. Food limitation thus did not occur during the biotests. The number of surviving caterpillars and their total weight was measured after 2 weeks. Controls (20 caterpillars on 3 g diet without extract) were kept in the same rooms and for the same time span. Relative growth rates were calculated by subtracting the total weight of control caterpillars from the total weight of caterpillars fed with extract-containing diet. The initial weight of neonates was much less than 1% of the caterpillar's final weight; the latter value was therefore regarded as representing total weight gain. Three replicates per extract (i.e. per individual plant) and three controls were performed and their results were averaged to obtain one value for relative growth rate (comparing the weight increase of 60 caterpillars reared on extract-containing food to 60 control caterpillars) for each plant extract and, therefore, each individual plant.

Biotests with fungi

For the tests on biological activity against fungi we used cultures of *Gliocladium roseum* (Link) Bainier (Ascomycota: Pezizomycotinae, see Schroers et al. 1999 for a recent taxonomic reclassification and ecological description). *Gliocladium roseum* is a widespread, soil-borne fungus occurring in decomposing plant material and on living plants of a broad spectrum of species. It is widely used as a biocontrol agent (see, for example, Perello et al. 1997; Yu and Sutton 1997). Due to its ecology, no specific adaptations to chemical defences of *Acacia* were to be expected.

Sterile water (10 ml) was added to the culture tube and shaken gently. The resulting suspension was adjusted to a density of 10⁵ to

10⁶ spores ml⁻¹. In total, 40 extracts (representing 40 different plant individuals) were selected to give a representation of the total range of CCT as found in our plants. Extracts were diluted 1:10 with water, and aliquots of 20 µl were placed in microtitre plates and mixed with 180 µl spore suspension (three replicates per sample, i.e. per extract). Controls consisted of 180 µl spore suspension mixed with 20 µl of methanol:water (1:10 v/v). After incubation in the dark at 30°C for 24 h, we counted numbers of germinated and not-germinated spores using Malassez counting chambers (10 cells each containing about 25–30 spores were counted per sample). From these data we calculated relative germination rates and averaged them for each sample according to the method explained for caterpillars.

Results

Contents of condensed tannins (CCT) in leaves as estimated from methanolic extracts differed strongly among the samples of each single species and among different species. The 'species' was a source of significant variation in the data set (univariate ANOVA: $n=94$, $df=11$, $F=24.89$, $P<0.001$). Lowest CCT were found in leaves of *A. farnesiana* (mean of seven samples: 0.02 mg g⁻¹ dw), highest contents in *A. macracantha* (21.18 mg g⁻¹, $n=11$). In single samples, the range in CCT was from 0.01 mg g⁻¹ in a sample of *A. farnesiana* up to 37.6 mg g⁻¹ in *A. macracantha*. In 74 of 94 samples, CCT in leaves were lower than 5 mg g⁻¹.

Growth rates were significantly different between caterpillars reared on diets containing extracts of different species (univariate ANOVA: $n=94$, $df=11$, $F=4.76$, $P<0.001$). Most extracts (67 of 94) had a positive effect on caterpillar growth, i.e. caterpillars reared on food containing these extracts gained more weight than those reared on controls (Fig. 1). For example, mean final weight of 20 caterpillars reared on *A. cochliacantha* was 0.37 g and on *L. leucocephala* 0.32 g higher than on controls. Average caterpillar growth was negatively affected by extracts of only three species (*A. macracantha*, *A. farnesiana* Isth., and *M. tenuiflora*). Overall, there was no significant correlation of caterpillar growth rate with the concentration of condensed tannins in the extracts, ConcCT ($r=-0.07$, $P>0.10$, $n=94$, Spearman rank correlation). Clearer patterns occurred when species were regarded separately (Fig. 1, Table 1). On four species (*A. farnesiana*, *A. farnesiana* Isth., *L. leucocephala* and *P. juliflora*), caterpillar growth was significantly and positively correlated with ConcCT (see Table 1, Fig. 1A). In contrast, caterpillars in biotests on two species (*A. hindsii* and *M. tenuiflora*) showed a growth response that was negatively correlated with ConcCT (Fig. 1B). In the remaining six species, no trends towards a correlation of ConcCT with caterpillar growth could be detected (Fig. 1C, Table 1).

Rates of spore germination differed strongly among the various extracts, ranging from 102% (slightly better germination rate than in control) to 8%. ConcCT of less than 1 mg ml⁻¹ in the original extract had no detectable effect on spore germination, while higher ConcCT increasingly inhibited spore germination (Fig. 2). The

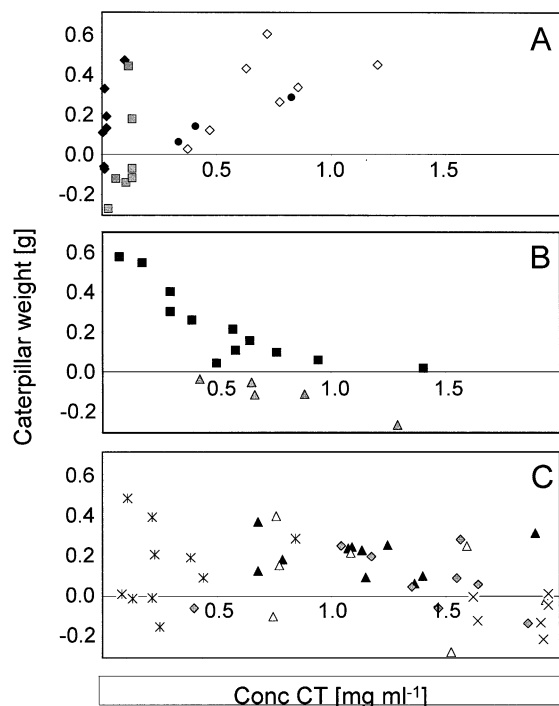


Fig. 1A–C Effects on caterpillar growth of extracts of different Mimosoideae differing in their concentration of condensed tannins (*ConcCT*). *ConcCT* was determined spectrophotometrically and is given in milligrams per millilitre of plant extract. Growth of *Spodoptera* caterpillars containing these extracts was determined after 14 days and is expressed here as the difference to weight of control caterpillars. See Table 1 for sample sizes and significance of correlation. **A** Species showing positive correlations, i.e. *A. farnesiana* (diamond black), *A. farnesiana* (Isth.) (square grey), *Prosopis juliflora* (circle black), and *Leucaena leucocephala* (diamond open). **B** Species showing negative correlations, i.e., *A. hindsii* (square black) and *Mimosa tenuiflora* (triangle grey). **C** Species showing no clear relation, i.e., *A. globulifera* (triangle black), *A. chiapensis* (diamond grey), *A. collinsii* (triangle open), *A. cornigera* (asteriks), and *A. macracantha* (cross)

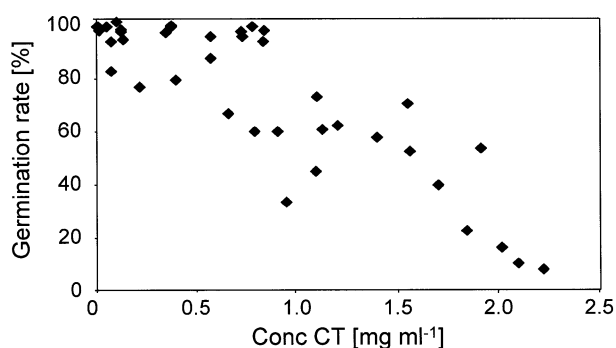


Fig. 2 Effects on spore germination of the fungus *Gliocladium roseum* of extracts of different Mimosoideae differing in their concentration of condensed tannins (*ConcCT*). Results are expressed as percentage germination rate of spores after 24 h in extract-containing spore suspension (100%: germination rate in controls, i.e. extract-free suspension). Sample number = 40 extracts. Each value appearing in this figure represents the mean of three replicate experiments

ConcCT of the extracts and spore germination rate were significantly and negatively correlated (Spearman rank correlation: $r = -0.809$, $P < 0.001$, $n = 40$).

Discussion

Our results demonstrate that the anti-herbivore effects of leaves on a generalist herbivore cannot be directly predicted from their content of condensed tannins (*CCT*) as quantified in methanolic extracts, even though effects of other quantitative defences, such as fibre content, were excluded. For nine species, caterpillars reared on diets containing extracts grew better than controls, while extracts of three species inhibited caterpillar growth. Correlation tests showed that caterpillar growth was significantly and negatively correlated with the concentration of condensed tannins, *ConcCT*, in extracts of two species, but positively correlated in four species.

Interestingly, the average effect of extracts of a given species on mean caterpillar weight was independent of the effects of *ConcCT* on caterpillar growth. For example, extracts of *A. hindsii* had a positive effect on caterpillar growth (i.e. caterpillars reared on food containing extracts of this species gained in all cases more weight than control caterpillars), while within this species *ConcCT* was negatively correlated with caterpillar growth (Fig. 1B). In contrast, all extracts of *M. tenuiflora* had a negative effect on caterpillar growth, and among samples of this species, growth rate was also negatively correlated with *ConcCT* (Fig. 1B). Furthermore, even among two populations of the same species, *ConcCT* had different average effects on caterpillar growth (positive in the case of *A. farnesiana* and negative in *A. farnesiana* Isth., see Fig. 1A), while within samples of both populations *CCT* was positively correlated with caterpillar growth. All these data show that predictions of the efficacy of anti-herbivore defence that are based on measurements of tannin or total phenolic content are oversimplified.

Similar results have already been presented in earlier studies, but are still largely ignored in the ecological practice and literature. A general problem seems to be that the protein-precipitating activity of tannins (which in general is assumed to be the main reason for their biological activity) is not closely correlated with the quantity of these compounds as determined by chemical methods (Martin and Martin 1982; Mole and Waterman 1987a, b). This led to the statement that techniques for 'chemically determining tannins' are difficult to use 'when studying a range of taxonomically distinct sources' (Mole and Waterman 1987b), and to the general recommendation to use methods based on protein precipitation to study tannins in ecological contexts (Martin and Martin 1982, 1983; Mole and Waterman 1987a).

However, the species used in the present study are all closely related to each other, yet the defensive efficacy of a leaf extract, in terms of its effect on growth of a generalist herbivorous caterpillar, could not be predicted

from CCT. Martin and Martin (1982) had compared six closely related oak species and were not able to relate contents of phenolics or proanthocyanidins to protein precipitation activity. It therefore remains open whether protein precipitating activity of plant extracts is directly and only a consequence of tannins at all. Surprisingly few studies have tried to demonstrate biological activity of specific tannins directly (but see Ayres et al. 1997).

Two factors may explain the large variation in the effect of a given CCT on caterpillar growth (Fig. 1). First, growth rates of herbivores are affected both by 'beneficial' dietary compounds such as carbohydrates, lipids and proteins, and by 'digestion inhibitors' or toxins. Several *Acacia* species, as well as *L. leucocephala* and *M. tenuiflora*, serve as suitable forage plants due to their comparably high contents of proteins (Garcia et al. 1996). However, *L. leucocephala* contains a non-proteinogenic amino acid, mimosine, which can serve as an effective anti-herbivore defence (Quirk et al. 1988). Several ant- and non-ant acacia species contain cyanogenic glycosides (Rehr et al. 1973). Predicting the nutritive value of leaves based on quantification of one single substance class therefore cannot be expected to result in tight correlations. Secondly, even the effect of isolated tannins can differ greatly depending on concentration, structure, and the particular mixture of tannin molecules (Ayres et al. 1997). Their in vitro effect is both tannin-specific and protein-specific (Asquith and Butler 1986; Scalbert 1991), and their biological activity therefore depends on the type of herbivore, too (Bernays 1978; Ayres et al. 1997).

In contrast with the tests on caterpillar growth, the effect of condensed tannins on spore germination was obvious (Fig. 2): ConcCT was significantly and negatively correlated with spore germination rates. This finding, although still very preliminary, supports a function in the plants' resistance to pathogens rather than to herbivores (Scalbert 1991; Hain et al. 1993; Taiz and Zeiger 1998; Hammerschmidt 1999a, b).

Many studies that only quantified condensed tannins or total phenolics have failed to reveal a close correlation (or sometimes any correlation at all) between content of phenolics and effects on the investigated herbivores (this study; Ayres et al. 1997; Goverde et al. 1999; Cornelissen and Fernandes 2001; Ossipov et al. 2001). Detailed knowledge on molecule structures and mixtures is required in order to determine the specific effect of phenolics in plant defence (Scalbert 1991; Ayres et al. 1997), and to estimate their suitability in studies focused on plant investment in defence against herbivores.

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edged. The experiments conducted in this study comply with the laws of the countries where studies were carried out.

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