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POLYPHENOLS AND CHEMICAL DEFENCE OF THE LEAVES OF *QUERCUS ROBUR**

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Key Word Index—*Quercus robur*; Fagaceae; leaves; phenolic components; seasonal variations; insect predation.

Abstract—Changes in the phenolic content and profile of leaves of pedunculate oak (*Quercus robur*) were determined through two growing seasons. The results are used to formulate an alternative explanation for the apparent relationship between changes in phenolic content and insect predation.

INTRODUCTION

The role of polyphenols (syn. vegetable tannins) in the overall economy of higher plants remains a fascinating field of study and indeed speculation [1–3]. The biosynthesis of polyphenols, in so far as its details are known, involves a substantial input of energy and it might therefore be expected on the basis of the precepts of modern chemical ecology [4] that there was a compensating gain to the plants which metabolize them. It has been suggested [5] that vegetable tannins, which are synthesized in bulk, are part of the chemical defence strategy of 'apparent' plants. This property derives it is argued from their characteristically repellent taste and their ability to form insoluble complexes with proteins in the digestive tracts of herbivores rendering the protein thereby nutritionally unavailable [6–8]. This view has developed as a result of several observations but most notably those of Feeny and Bostock [4, 9] who studied the changes in the tannin content of oak (*Quercus robur* L.) leaves throughout a growing season. This species is heavily infested by insects in the spring, but is rarely subject to serious predation after mid-June. Feeny and Bostock [9] showed that the level of hydrolysable tannins remained approximately constant throughout the season but that condensed tannins (proanthocyanidins) did not appear until late May. They concluded that the period of highest insect attack on oak leaves (early spring) corresponds to the time when the total tannin content is at a minimum and condensed tannin is absent (or almost so). Feeny later suggested [4] that leaf toughness is probably the chief proximate factor in mature oak leaves which deters attack whilst, of several possible ultimate factors, the seasonal decline in the availability of nitrogen (due both to the decrease in leaf protein and the concomitant increase in leaf tannins) is probably one of the most significant. As part of a continuing study of polylphenol metabolism in higher plants the changes in the polyphenol metabolic 'fingerprint' of several plants throughout the growing season has been examined. Results obtained with the leaves of pedunculate oak (*Quercus robur* L.) are here described. They corroborate several of the important findings of Feeny and Bostock [9] and considerably amplify others. Attention nevertheless is finally drawn to an alternative explanation to that suggested by Feeny [5] concerning the causal relationship between polyphenols (tannins) and the predation of oak by insects in spring.

RESULTS AND DISCUSSION

Oak leaves (*Quercus robur*) were collected from the same tree at 2–3 weekly intervals from late April (nascent buds), May (olive bronze leaflets, 1–2 cm, in length), June (mature leaves) through to October when senescence commenced. The leaves were extracted as described in Fig. 1 to give four principal fractions—(i) residual plant debris (I and II); (ii) ethyl acetate soluble phenols and (iii)

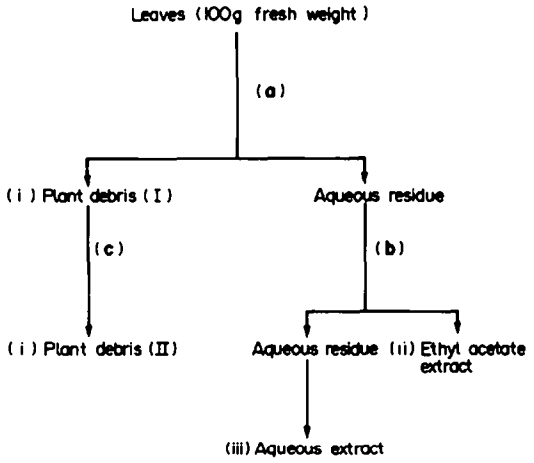


Fig. 1. Extraction of Oak leaves (*Quercus robur* L.). (a) Leaves extracted (× 3) in high speed blender (3 min) with methanol (500 ml). Plant debris (i) filtered off. Methanol residue reduced at 30° to 25–50 ml bulk and filtered to remove lipids and chlorophyll. (b) Aqueous residue extracted (× 10, 75 ml) with ethyl acetate. Solvent removed at 30° to give ethyl acetate extract (ii). Water removed at 30° from residue to give aqueous extract (iii). (c) Plant debris (I) stirred at 20° for 7 days with acetone–water (1:1, v/v, 250 ml). Filtration gives plant debris (II) (i).

*Part 2 in the series plant polyphenols and chemical defence. For Part 1, see ref. [1].

the final water extract containing the residual water soluble phenols. Fractions (ii) and (iii) which comprise all the freely soluble phenolic metabolities of oak leaf were analysed by HPLC and by two dimensional paper chromatography (Figs 2 and 3). *Comparative* quantitative changes in phenolic metabolities throughout the growing season were followed by various colorimetric methods—(a) esters of hexahydroxydiphenic acid (ellagitannins [10]) which are of greatest significance in oak by the method of Bate-Smith [11–11b] using nitrous acid under nitrogen (blue colour, λ_{max} 600 nm); (b) esters of gallic acid by treatment with potassium iodate solution at 0° [12] and (c) proanthocyanidins by conversion to anthocyanidins in hot mineral acid in *n*-butanol [13, 14]. The composition of each of the fractions (i–iii) and the seasonal changes in these fractions are discussed below.

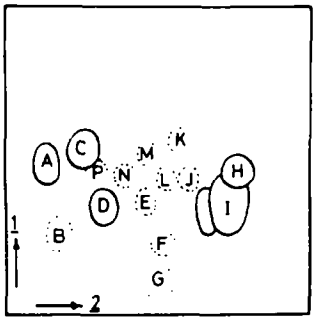


Fig. 2. Ethyl acetate-soluble phenols of oak leaves (*Quercus robur* L.). Principal components: A, unidentified ellagitannin (nascent bud); C, pedunculagin: D, casuarictin: H, (+)-catechin: I, Flavonol glycosides. Minor components: B, F-unidentified galloyl/hexahydroxydiphenoyl esters; J, (+)-gallocatechin; N, procyanidin B-3; M, P, K, proanthocyanidins, E, tellimagrandin-2; G, ellagic acid. This fraction consisted of ~ 1.2% (nascent bud) to ~ 1.8% (mature leaf) of the fresh weight of oak leaf.

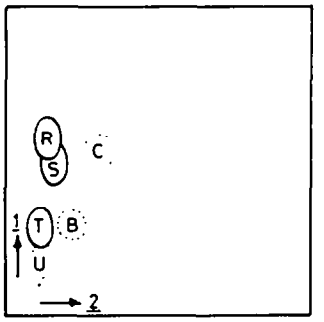
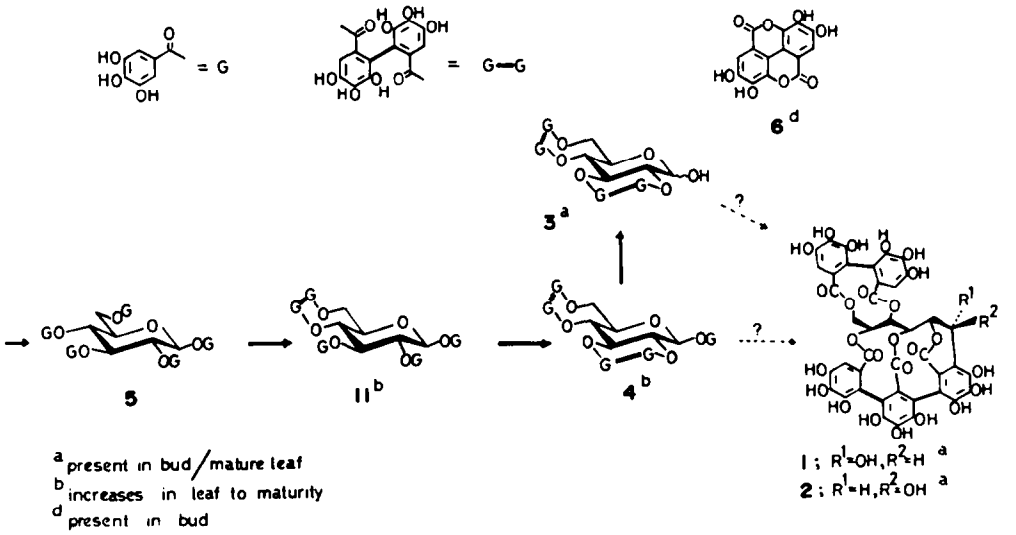


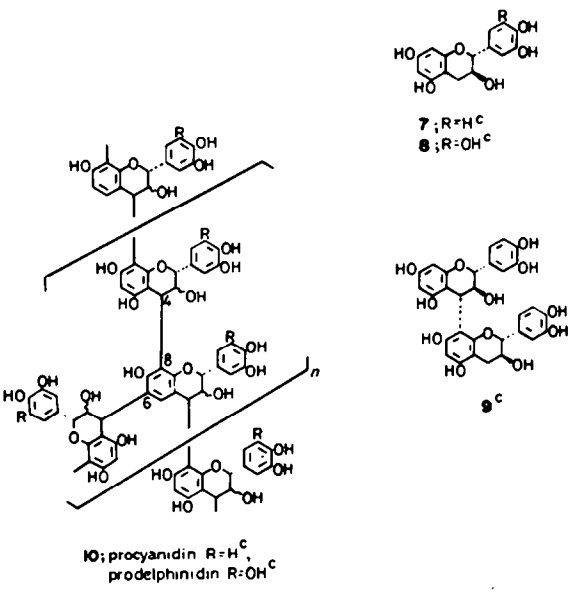
Fig. 3. Water soluble phenols of oak leaves (*Quercus robur* L.). Principal components: R, vescalagin; S, castalagin; T, unidentified hexahydroxydiphenoyl esters. Minor components: C, pedunculagin; B, U, unidentified hexahydroxydiphenoyl esters. This fraction consisted of ~ 3.0% (nascent bud) to 7.5% (mature leaf) of the fresh weight of oak leaf.

The water soluble phenols (iii)

This fraction in oak is somewhat unusual and distinctive, since for a great many plant species the water fraction retains very few residual phenolic metabolites. In the case of oak however the water extract contains almost exclusively hydrolysable tannins. All the major components (Fig. 3) responded to colour reactions (HNO_2, KIO_3 tests) which identified them typically as esters of hexahydroxydiphenic acid and/or gallic acid. Several components notably vescalagin (**1**), its diastereoisomer castalagin (**2**) and pedunculagin (**3**) were isolated and identified by ^1H and ^{13}C NMR spectroscopy and by cochromatography with authentic samples, (in ref. [9] these are *probably* identical with D_1, D_2 and H respectively in Fig. 1). It is worthy of comment that both **1** and **2**, which formally differ by just six hydrogen atoms from their presumed biosynthetic precursor β-penta-*O*-galloyl-D-glucose (**5**), are so freely soluble in water whilst **5** is relatively sparingly



Scheme 1.



[c] Increases in leaf (late May onwards)

Scheme 2.

soluble. Although the water-soluble fraction increases relatively in quantity throughout the growing season the increase within this of phenolic materials (maximally 10–15%) occurs largely in the initial phase of growth (May–June), (subsequent increases in weight of this fraction per unit weight of leaf are probably accounted for by water soluble materials of a carbohydrate structure). This observation differs slightly from that of Feeny [4] who states that 'the level of hydrolysable tannin remained approximately constant from April throughout the season'. The water extract however represents the major freely soluble polyphenolic (tannin) metabolities of oak leaf, it consists almost exclusively of the esters (**1**, **2**) and their, as yet, unidentified congeners, (Fig. 3).

The ethyl acetate soluble phenols(ii)

This fraction (Fig. 2) contained a number of components which were readily identified by isolation, ^{1}H and ^{13}C NMR spectroscopy, and cochromatography, namely pedunculagin (**3**), casuarictin (**4**), ellagic and (**6**), (+)-catechin (**7**) and (+)-gallocatechin (**8**), procyanidin B-3 (**9**, cat-4-α-8-cat, also identified by toluene-α-thiol degradation [15]), procyanidin B-1 (*epi*-4-β-8-cat) and flavonol glycosides based on quercetin. The presence of free ellagic acid in small amounts in all these extracts might well be due to its adventitious formation as an artifact by hydrolysis during isolation. However the relatively high concentrations found in the nascent bud are not wholely explicable on this basis. Other compounds noted in this fraction were unidentified hexahydroxy-diphenoyl esters (Fig. 2 A, B, F) and various minor soluble oligomeric procyanidins and/or prodelphinidins (Fig. 2 K, M, N, P). Quantitatively the polyphenols of greatest significance in this fraction are the ellagitannins pedunculagin (**3**), casuarictin (**4**) and their congeners and, when the leaf matures (late June–July), (+)-catechin (**7**). Some interesting relative changes occur amongst the phenolic components of this fraction during the growing season—suggestive of possible biogenetic inter-relationships. In the nascent bud (late April) (+)-catechin (**7**), pedunculagin (**3**) and the unidentified ester(s) of hexahydroxydiphenic acid (Fig. 2 A) predominate. As the leaves break (May) then the latter ester(s) rapidly diminished in relative concentration and casuarictin (**4**) rapidly increases to an amount roughly equivalent to that of pedunculagin (**3**). As the growing season extends to Autumn the relative concentration of casuarictin (**4**) declines slowly once again. Amongst the various flavonoids (+)-catechin (**7**), (+)-callocatechin (**8**) and the associated proanthocyanidins show a rapid fall in relative and absolute concentration as the leaves emerge (May) and the flavonol glycosides (Fig. 2 I) appear and remain in approximately the same concentration throughout the growth of the leaf. However as the leaves mature (late May–June) the flavon-3-ols (**7**, **8**) increase once again in relative concentration as do the various proanthocyanidins (Fig. 2 K–N). These quite marked changes in the flavonoids are suggestive of a change in the redox potential of the leaf tissue as it emerges (May) and then matures (late May–June).

The plant debris I and II(i)

This fraction is frequently ignored in plant studies but its significance in relation to the proanthocyanidins of leaf and other tissue has recently been pointed out [16]. Very small amounts of soluble polymeric proanthocyanidins were released from the plant debris (I) by acetone–water treatment and as in many other plants the polymeric forms of the proanthocyanidins appear to remain indissoluble and largely bound directly to the insoluble plant tissue. Treatment of the debris with mineral acid in butanol [13, 14] and chromatography in Forestal solvent [17] showed that the anthocyanidins released were cyanidin and delphinidin in the approximate ratio of 2:1—indicative of a mixed proanthocyanidin structure (**10**). Relative quantitative changes were followed using the same reaction and measurement of the absorption at 540 nm. Polymeric proanthocyanidins were not found in the plant debris in the emerging leaf (early May) but appeared in late May–June and *increased steadily* as the leaf matured to a maximum in July–August.

These results show many similarities to those reported by Feeny and Bostock [9], although the principal metabolities have now been identified. However notwithstanding the similarities an alternative interpretation of these more detailed observations *in toto* and their relationship to insect predation is feasible and should not only be entertained but seriously examined in the light of the continuing debate [1] concerning the role of plant polyphenols in chemical defence. The predominant polyphenols of the vegetable tannin class in the leaf of *Q. robur*. are at all stages of growth undoubtedly those of the hydrolysable (ellagitannin [10]) group. If quantitative changes in the vegetable tannins of oak leaf tissue have a causal relationship with changes in insect predation then it would seem entirely reasonable to anticipate that these would be quantitatively reflected in the principal group–those of the hydrolysable class. Such major changes were however not observed during two (1984, 1985) growing seasons in the principal ellagitannins (Figs 2, 3 C, D, R, S) of oak leaf. Consonant, with the results of Feeny and Bostock [4, 9] the most significant changes amongst the polyphenols (tannins) takes place amongst the com-

paratively minor group (in oak)—the various proanthocyanidins by far the greater proportion of which are bound, apparently indissolubly, to plant tissue. We have recently [16] drawn attention once again to the early observations of Sir Robert and Lady Robinson [18] on the 'insoluble' proanthocyanidins of plant tissues and confirmed by subsequent workers in this field. Thus for example Hillis and Swain [19] have shown that the 'leucoanthocyanins' of plum leaves can be divided into three classes, the first two being successively extractable with absolute, followed by aqueous methanol and the third, remaining in the residue, being non-extractable by these or other neutral solvents. Likewise Bate-Smith [20] in an investigation of the proanthocyanidins of herbaceous Leguminosae noted that in most of the species examined the amount extracted, even under the most favourable conditions, is only a small proportion of the total present. These observations are, we believe, of considerable relevance to the questions surrounding the purported relationships between polyphenols and chemical defence. In a recent paper [16] it was suggested that these insoluble forms of plant proanthocyanidins are bound by covalent linkages to polysaccharide (or other) structures in the developing cellular matrix. Attention was also drawn to the possible analogies with the structure of lignin and the process of lignification in plant tissues.

In this context it now seems entirely appropriate and pertinent to consider again some of the early observations of Bate-Smith [21] on the botanical significance of plant proanthocyanidins. Bate-Smith noted that these polyphenolic substances were frequently associated with the character and quality of 'woodiness' in plants. He also strongly hinted at a possible relationship between proanthocyanidin metabolism and lignification and hence to a putative structural role in the plant for these oligomeric polyphenolic metabolites. In his studies Feeny [4] concluded that leaf toughness is the chief proximate factor in deterrence to insect predators of oak leaf. This opinion is also entirely consistent with an alternative interpretation of the apparent correlation of declining insect predation with proanthocyanidin formation—namely that leaf toughness develops simultaneously with the initiation of the development of a cell structure which has enhanced 'woody' characteristics [19] and in which concomitantly proanthocyanidin oligomers and polymers are deposited as an integral part of these structures. The suggested interpretation of decreased insect predation in oak in early summer is that this is therefore related to changes in cell structure, texture and toughness of the leaf and in the development of which the formation of proanthocyanidin polymers play an intimate role. This alternative perspective on this problem incidentally supports the conclusions of Coley [22] who noted that leaf toughness was most highly correlated with levels of herbivory, followed by fibre content and finally nutritive value.

As a final note, it may be mentioned that the ethyl acetate soluble phenols (fraction ii) from the leaves of several other species of oak obtained from Kew Gardens have been examined during the course of this work. Many bear a close relationship to the phenolic profile found in *Quercus robur*–namely *Q. cerris*, *Q. macracantha*, *Q. infectoria*, *Q. alba*, *Q. ilex*, *Q. lusitanica*, *Q. aegilops*, *Q. castaneifolia*, *Q. petraea* and *Q. hispanica*. All however, in mature leaves (July), contained additionally rather more significant quantities of tellimagrandin 2 (**11**). The phenolic metabolites of *Q. borealis*, although largely of the ellagitannin type, are significantly different from those of the other species and are the subject of further study in this laboratory.

EXPERIMENTAL

Isolation procedure. Leaves (100 g) were gathered from a single tree (~ 10 years) of pedunculate oak (*Quercus robur*) growing at 300 m on a north facing slope. Extracts were prepared as outlined in Fig. 1 within 12 hr of leaf collection. Leaf (100 g) was macerated with cold MeOH (3 × 500 ml) in a high-speed food mixer and the plant debris (i) finally removed and air dried. The methanolic solution was evapd at 30° to *ca* 50 ml bulk, pptd chlorophyll, fats and waxes were removed by gravity filtration and the precipitate washed (3 × 10 ml H_2O). The combined aqueous solution was extracted (10 × EtOAc, 75 ml) to give the EtOAc-soluble phenols (ii) and, after removal of H_2O by ethanol azeotrope, the water soluble phenols (iii).

Analysis. Paper chromatography (27.5 cm^2) was carried out using solvent systems (1) 6% HOAc and (2) ^tBuOH–HOAc–H_2O (14:1:5). Chromatograms were developed as described previously and compounds identified by cochromatography [15, 16, 23]. Quantitative analysis of classes of polyphenol were carried out by known procedures 11–14.

Large scale extractions (2000 g leaf) to give fractions (ii) and (iii) were carried out followed by chromatography on Sephadex LH-20 to give the components C, D, H–J, N, R, S which were identified by ^1H and ^{13}C NMR spectroscopy and chemical degradation (N) [15]. HPLC analysis was carried out using a Bondapak ODS C_{18} column as previously described [16].

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