THE FLAVONOIDS OF UMBELLULARIA CALIFORNICA (LAURACEAE)

by

HEATHER ANNE NEVILLE

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Department of **BOTANY**

The University of British Columbia
Vancouver, Canada

Date **July 21, 93**
ABSTRACT

An analysis of the flavonoids of *Umbellularia californica* was performed on 23 samples collected from throughout the range of the species in Oregon and California. The goals of the study were to identify the major leaf flavonoids and to establish the presence or absence of flavonoid profile differences between the two growth forms that exist in this monotypic genus. Using standard isolation techniques nine major flavonoids were identified representing a relatively simple profile of flavonol glycosides. Virtually no differences in profile were seen between the two forms. It was concluded that the flavonoid data do not provide any help in defining infraspecific taxa. The flavonoid profile identified in *U. californica* is consistent with profiles reported from other members of the Lauraceae.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHEMOSYSTEMATICS</td>
<td>5</td>
</tr>
<tr>
<td>FLAVONOIDS DEFINED</td>
<td>12</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>18</td>
</tr>
<tr>
<td>SOURCE OF PLANT MATERIAL</td>
<td>18</td>
</tr>
<tr>
<td>EXTRACTION PROCEDURES</td>
<td>19</td>
</tr>
<tr>
<td>TLC METHODS</td>
<td>21</td>
</tr>
<tr>
<td>ISOLATION OF VACUOLAR FLAVONOIDS</td>
<td>23</td>
</tr>
<tr>
<td>SUGAR ANALYSIS</td>
<td>24</td>
</tr>
<tr>
<td>SPECTROSCOPIC METHODS</td>
<td>25</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>28</td>
</tr>
<tr>
<td>FLOWER FLAVONOIDS</td>
<td>37</td>
</tr>
<tr>
<td>LEAF SURFACE COMPONDS</td>
<td>37</td>
</tr>
<tr>
<td>WOOD COMPONENTS</td>
<td>38</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>41</td>
</tr>
<tr>
<td>FLAVONOIDS OF UMBELLULARIA AND OTHER LAURACEAE COMPARED</td>
<td>44</td>
</tr>
<tr>
<td>GENERAL CONCLUSIONS</td>
<td>55</td>
</tr>
<tr>
<td>FUTURE STUDIES</td>
<td>56</td>
</tr>
<tr>
<td>V. LITERATURE</td>
<td>58</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Classes of Flavonoids and Possible Functions . . . 14
2. Collection Sites . . . . . . . . . . . . . . . . . . . . . . . . 18
3. Taxonomic Placement of Lauraceae Showing Genera from
   which Flavonoids have been Identified . . . . 46
4. Distribution of Flavonoids in the Lauraceae using
   the Taxonomic System of Kostermans . . . . . . 48
5. Flavonoid Occurrences in Lauraceous Genera . . . . . . 52
LIST OF FIGURES

1. A flavonoid study of three diploid *Asplenium* species and their allopolloid progeny ................. 11
2. Flavonoid Structure Guide .......................... 13
3. Collection Sites of *Umbellularia* .................. 17
4. U.V. fluorescence of one dimensional chromatogram of main flavonoids of *Umbellularia californica* versus glycoside standards ................................. 26
5. One dimensional chromatogram of cleaved sugars from isorhamnetin triglycoside ......................... 27
6. Two dimensional chromatogram of *Umbellularia californica* leaf extract ............................... 29
7. U.V. fluorescence of two one dimensional chromatograms of *Umbellularia californica* .................. 32
8. Major Flavonoids of *Umbellularia californica* ..... 36
9. U.V. fluorescence of one dimensional chromatogram of shrub wood fractions ............................ 40
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I. INTRODUCTION

The goal of this thesis was to identify the flavonoids of *Umbellularia californica* and to apply the data to taxonomic concerns within the species as well as to questions of relationships within the Lauraceae. There are two different growth forms of *U. californica* and if any differences in flavonoid profile exist between the two forms this would constitute further evidence for possible infraspecific taxonomic recognition. To attain this systematic goal both leaf and wood flavonoids were investigated. The thesis questions were:

(1) What are the flavonoids of *U. californica*?
(2) Do the flavonoid profiles indicate a difference between the two forms?
(3) How does the flavonoid profile of *Umbellularia* compare with flavonoids identified from other members of the Lauraceae and related families?

The thesis begins with information on *U. californica*. Background on the use of flavonoids in chemosystematics is presented to put the work in context. The body of the thesis comprises a description of the flavonoids of *U. californica* and their application to delineating groups within the species. Next, the present flavonoid findings are compared with those for other genera of the Lauraceae.
Finally the flavonoids of the Laurales are briefly discussed in relation to the known flavonoid chemistry of the Magnoliidae.

**Umbellularia** (Nees) Nuttall is a monotypic genus which consists of *U. californica* (Hook. & Arn.) Nutt. The species ranges from southern California to southwestern Oregon. In California the range is divided between (1) coastal and inner coast ranges and (2) foothills of the Sierra Nevada. *Umbellularia californica*, commonly known as California Bay, Bay tree, pepperwood, Bay laurel or Oregon myrtle, is often used for decorative furniture because of its hardness, attractive grain and capacity to take a high polish. The leaves are entire, oblong-lanceolate, alternate and shiny. Flowers are small, yellow-white and bisexual; the fruit is a drupe (Munz 1959). The leaves and wood are highly fragrant. The principal odour component is umbellulone, a monoterpen ketone known to irritate membranes and cause headache in some individuals (Drake & Stuhr 1935). Use of the leaves of *U. californica* as a condiment has also been reported (Lawrence et al. 1974).

A quantitative analysis of volatile constituents from *U. californica* was done by Lawrence and coworkers (1974). Umbellulone was shown to be the major component (39.0%) of the steam-volatile fraction from leaves. The next major component was 1,8-cineol (eucalyptol) at 23.4% followed by a
number of lesser components: terpinen-4-ol (5.6%), sabinene (4.8%), alpha-pinene (3.6%), thymol (3.3%), beta-pinene 2.3%), linalool (2.0%), gamma-terpinene (1.8%), myrcene (1.3%), and p-cymene (1.0%). Minor components (less than 1.0% each) included the following: trans-beta-farnesene, alpha-terpinyl acetate, alpha-terpinene, alpha-p-dimethylstyrene, benzaldehyde, limonene, terpinolene, methyl eugenol, selina-4,11-diene, alpha-phellandrene, guaia-6,9-diene, ethyl benzoate, delta-terpineol, beta-phellandrene, alpha-santalene, pinocarvone, alpha-selinene, beta-bisabolene, ethyl cinnamate, eugenol, verbenone and citronellol.

Pech and Bruneton (1983) reported three aporphine alkaloids from U. californica, domesticine, nor-domesticine and isoboldine, and bufotenine, which is 5-hydroxy-N,N-dimethyltryptamine.

The only report of flavonoids from U. californica appears in a study done in 1962 by Bate-Smith. This work, which only involved acid-hydrolyzed leaf extracts, showed the presence of kaempferol and quercetin. Bate-Smith’s study included representatives of the following genera as well: Apollonias, Cinnamomum, Cryptocarya, Laurus, Lindera and Persea.
**Umbellularia californica** consists of two growth forms, a full-size tree (up to 45 meters) with broad crown and single trunk that can be up a meter in diameter (tree form), and a shrubby form with several smaller trunks (up to 15 cm. diameter) that may attain a height of about six meters (shrub form). The tree form occurs mainly in lower coastal woodlands while the shrub form occurs in drier, inland canyons and chaparral. In several of these drier areas **U. californica** occurs on serpentine-derived soils which presents an edaphic challenge to many plants. **Umbellularia californica** is one of several taxa in the Californian flora that have evolved to inhabit serpentine soil (Kruckeberg 1985). The only infraspecific taxon described, however, is **U. californica** var. *fresnensis* Eastw. whose inflorescence and lower leaf surface were finely tomentulose. It was not specifically stated which growth form was involved (Munz 1959).

Rohwer (1986) pointed out that the name *Sciadiodaphne* Reichenbach had been proposed in 1841 for what we now recognize as **Umbellularia** (Nees) Nuttall (first appearance in 1842), but that it had never occurred with a specific epithet. Neither had it appeared in the *Index Nominum Genericorum* to that date. Rohwer proposed that **Umbellularia** be conserved since it refers to a well known decorative tree. The proposal to conserve was subsequently accepted (Nicholson 1991).
The genus can be regarded as relictual and palaeoendemic (Raven and Axelrod 1978). The origin of *Umbellularia* is unknown. Raven (1977) pointed out that there are many families in the Californian flora, Lauraceae included, some members of which appear to have originated in South America and others from Eurasia.

When the new genus arose is unknown, but it was shown to have existed between 15 and 20 million years ago based upon fossils found near Bridge Creek in north-central Oregon. The fossil study, done by Chaney (cited by Ornduff 1974), also showed that the frequency of occurrence of *U. californica* at the present time in the Muir Woods in Marin County (13%) is similar to what was found in the Oregon site (9%). If unchanged morphology implies evolutionary fitness for a particular niche, *U. californica* may be considered maximally suited to its environment.

**Chemosystematics**

A systematic analysis involves study of certain characteristics for the purpose of understanding relationships between organisms or groups of organisms. A thorough systematic analysis is behind every taxonomic delineation and is also useful for evolutionary considerations. Chemosystematics involves use of various
chemical types, usually stable secondary metabolites, in such an analysis. For example, if certain groups within a family contain a particular compound or compound type, which the others lack, some taxonomic delineation may be indicated. Acceptance of such delineations depends upon the extent to which such information agrees with data from morphological, anatomical, cytological and other studies.

Commonly used secondary metabolites include terpenoids, alkaloids and flavonoids. Of all the compounds used in chemosystematics, flavonoids are arguably the most useful for the following reasons:

(1) The flavonoid structure is inherently stable although certain types are comparatively less so. For example, anthocyanins are sensitive to changes in pH and chalcones can isomerize spontaneously to flavanones. However, herbarium specimens decades old have yielded excellent results, and in the extreme, flavonoids have been successfully extracted from certain fossilized plants (Giannasi and Niklas 1977, 1981). In the case of the fossil studies comparisons were possible between 20 million year old extinct and extant species.

(2) The extraction, purification and structural determination of flavonoids is relatively simple and does not require unusual or expensive equipment. A crude methanolic extract can be resolved into its components using column (CC) or thin layer chromatography (TLC). Useful
structural information can often be obtained from a compound’s chromatographic behaviour, its behaviour in UV light, and its reaction with chromogenic reagents. Purification can readily be done using TLC. Essential structural information can then be obtained using standard ultraviolet spectroscopic methods (Mabry et al. 1970; Markham 1982).

(3) Flavonoids occur in virtually all vascular plants including the angiosperms, gymnosperms and ferns. Flavonoids have also been found in some mosses and green algae. As very widespread secondary metabolites flavonoids are potentially useful at every taxonomic level.

(4) Flavonoids occur in all plant organs. The distinct tissue distribution of different types of flavonoids may coincide with their function. For example, the vividly coloured anthocyanins are usually concentrated in flowers where they serve to attract pollinators.

(5) Because there are several skeletal differences among flavonoids and because oxidation levels and substitution patterns can vary, the range of structures is extensive. This makes the flavonoid group ideal for systematic study; the molecular diversity rivals that of the plant kingdom, providing enough variation for meaningful comparisons.

(6) The use of flavonoids as systematic markers was recognized in the 1960’s (Bate-Smith 1962). Since then a substantial data base has been amassed by plant systematists around the world. The size of the data bases facilitates
application of these compounds to a variety of problems in the plant kingdom.

(7) The biosynthesis of flavonoids and genetic control of the various steps are well understood.

It should also be mentioned that flavonoids are useful in studies of hybridization because pigment profiles are usually inherited codominantly.

Examples of the use of Flavonoids in Systematic Studies

Family - On the basis of similarities in morphology the Ericaceae and Empetraceae are set apart from the related families Epacridaceae, Clethraceae and Diapensiaceae. Gossypetin (3,5,7,8,3',4'-hexahydroxyflavone), a comparatively uncommon flavonoid marker, occurs in members of Ericaceae and Empetraceae but not in the other families. As a character, gossypetin strengthens the apparent link between these two families (Moore et al. 1970).

Subfamily - The Gesneriaceae consists of two subfamilies, the Gesnerioideae and Cyrtandroideae. Two flavonoid distinctions between these subfamilies have been discussed by Harborne (1966). Members of the Gesnerioideae accumulate 3-deoxyanthocyanins as well as normal anthocyanins in both leaf and floral tissue whereas members of the Cyrtandroideae appear to make only normal anthocyanins. The other difference involves chalcones and aurones which have been
reported from members of the Cyrtandroideae but appear to be lacking in Gesnerioideae.

**Tribe** - Within the tribe Vicieae of the Fabaceae there are four genera that show apparent segregation patterns reflecting flavonoid variation. *Lathyrus* and *Pisum* are linked because they form the phytoalexin pisatin (a 6α-hydroxypterocarpan). *Vicia* and *Lens*, however, produce furanoacetylenes as phytoalexins (traces of pterocarpan have been found). The chemical distinction between *Lathyrus* and *Vicia* is striking, since the two genera are morphologically so similar (Robeson & Harborne 1977).

**Genus** - Apples and pears were once thought to belong to the same genus (*Pyrus* L.) because of many shared characteristics and the capacity to hybridize. Separate genera are now recognized, *Malus* for apple and *Pyrus* for pear, based on differences in flower and fruit morphology. Phenolic differences between the two groups support this view; *Malus* accumulates dihydrochalcones while the major phenolic compound in *Pyrus* is the non-flavonoid arbutin (hydroquinone glucoside) (Challice 1973).

**Species** - Of eleven species of *Chondropetalum* (Restionaceae) tested for flavonoids, seven exhibit one profile (myricetin, larycitrin and syringetin) while four exhibit a second (kaempferol, quercetin, gossypetin, gossypetin 7-methyl
ether and herbacetin 4′-methyl ether) (Harborne et al. 1985).

**Hybrids** - Flavonoid patterns were very useful in helping to unravel relationships between species of the fern *Asplenium* in the Appalachian Mountains (Smith and Levin 1963; Harborne et al. 1973). Plants of putative hybrid origin showed chromatographic patterns that were the sum of parental species; see Figure 1 below. Flavonoids were also found to be useful in a study of hybridization and subsequent backcrossing within the genus *Dubautia* on the Island of Hawaiʻi (Crins et al. 1988).

**Race** - The fern *Notholaena standleyi* consists of three forms characterized by plant size, geographic distribution and the nature of their leaf exudate chemistry. The 'gold' race plants exude mainly kaempferol 7-methyl ether and kaempferol 4′-methyl ether. 'Yellow' race plants produce kaempferol and its 3,7- and 7,4′-dimethyl ethers. The 'yellow-green' race accumulates the same kinds of kaempferol derivatives but the profiles are intermediate between the other two types (Seigler and Wollenweber 1983).

Flavonoid races were also observed in a study of *Lasthenia californica* (Bohm et al. 1989; Desrochers & Bohm 1993). Different flavonoid profiles were observed in several populations in California. The geographical
A flavonoid study of three diploid Asplenium species and their alloplloid progeny

Although (e)(A.x kentuckiense) has an altered chromosome number compared to its ostensible tetraploid (d)(A. rhizophyllum x A. montanum) and diploid (c)(A. platyneuron) sources, its ancestry is verified by a perfectly additive chromatographic pattern, including the "original" contributions from (a)(A.x rhizophyllum) and (b)(A. montanum).
patterning of these pigment types within the populations were invariant over a period of 10 years.

**Individuals** - Flavonoid differences among individuals within a species may be greater than differences among species or even among higher level taxa. Such a situation was seen in the Hawaiian members of the genus *Bidens* (Ganders et al. 1990). The rich and seemingly random infraspecific expression of flavonoids within this genus suggests genetic plasticity, possibly in response to the adaptive pressures of relatively recent colonization.

**Flavonoids Defined**

Flavonoids are phenolic compounds based on the 1,3-diphenylpropane skeleton (Figure 2). A number of structural classes exist based on whether the three-carbon bridge is cyclized (as in [2] where flavanones have $R = H$ and dihydroflavonols have $R = OH$) or not (as in [1], a chalcone). Further variation is based upon the level of oxidation of ring-C, the degree of oxygenation of rings-A and B, the level of glycosylation, the presence of O-methyl groups and certain other functionalities. Occurrences of flavonoid classes in plants range from very common, as in the case of flavones (in [2] $R = H^{2,3}$), flavonols (in [2] $R = OH^{2,3}$) and anthocyanidins [3], to comparatively rare, as in the case of flavonoid sulfates. Table 1 presents a
Flavonoid Structure Guide

[1]

[2]

[3]
general overview of where many of the flavonoid types occur within the plant and lists possible functions.

Table 1. Classes of Flavonoids and Possible Functions

<table>
<thead>
<tr>
<th>Class</th>
<th>Major Organ</th>
<th>Suggested Function</th>
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<tbody>
<tr>
<td>Flavones</td>
<td>Leaves</td>
<td>U.V. Screen</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Leaves</td>
<td>U.V. Screen, Hormone</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Flowers</td>
<td>Pollinator Attractant</td>
</tr>
<tr>
<td>Chalcones</td>
<td>Flowers</td>
<td>Pollinator Attractant</td>
</tr>
<tr>
<td>Aurones</td>
<td>Flowers</td>
<td>Pollinator Attractant</td>
</tr>
<tr>
<td>Isoflavonoids</td>
<td>Leaves, Roots</td>
<td>Phytoalexin</td>
</tr>
<tr>
<td>Dihydroflavonols</td>
<td>Leaves, Wood</td>
<td>Phytoalexin</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Leaves</td>
<td>Feeding Deterrent</td>
</tr>
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The first flavonoids, chalcones, are formed by the condensation of three molecules of malonyl CoA with one of p-coumaroyl CoA catalyzed by chalcone synthase (CS). The flavonoid A-ring arises by cyclization of the six-carbon polyketide portion of the precursor; the B-ring and three-carbon bridge arise from the p-coumaroyl unit. Chalcones are cyclized to flavanones through the action of chalcone isomerase (CI). An alternate cyclization of chalcones may occur to yield aurones which are characterized by having five-membered C-rings. The bridge double bond of chalcones may be reduced to give dihydrochalcones.
Flavanones serve as branch point intermediates. Loss of hydrogens from carbons-2 and 3 yields flavones (flavone synthase, FS). Flavanone 3-hydroxylase (F3H) catalyzes the hydroxylation of flavanones at C-3 to yield 3-hydroxyflavanones (dihydroflavonols). Loss of hydrogens from C-2 and C-3 of dihydroflavonols yields flavonols in a reaction analogous to the formation of flavones. Different enzymes are involved, however. A third fate of flavanones involves migration of the phenyl function from C-2 to C-3 to give isoflavones (isoflavone synthase).

Dihydroflavonols also serve as substrates for several different processes. Dehydrogenation to flavonols has already been mentioned. Dihydroflavonols may also undergo reduction of the carbonyl function to yield flavan-3,4-diols (dihydroflavonol 4-reductase) which can in turn be converted through a complex series of reactions to anthocyanidins. Flavan-3,4-diols are also involved in the biosynthesis of proanthocyanidins, so-called condensed tannins.

A-Ring hydroxyl groups at positions-5 and 7 are provided by the precursor acetyl CoA units while the 4' hydroxyl group comes from the p-coumaroyl CoA unit. Flavonoid 3'-hydroxylase catalyzes the placement of an OH group at position-3' of the B-ring to yield the 3',4'-dioxygenated, or quercetin type, substitution pattern. Further hydroxylation at position-5' by a related enzyme
yields the 3',4',5'-trioxygenated, or myricetin-type, substitution pattern. O-Methylation, to yield compounds such as isorhamnetin, is catalyzed by a series of flavonoid O-methyl transferases that appear to be highly selective with regard to the position methylated. S-Adenosylmethionine serves as the cofactor in this reaction.

O-Glycosylation is often the final step in flavonoid biosynthesis. Diglycosides, such as rutinosides (rhamnosylglucosides), are formed sequentially. The first reaction, in the formation of rutinosides, involves transfer of glucose unit from uridine diphosphoglucose (UDPG) to the 3-OH of quercetin catalyzed by flavonol 3-O-glucosyltransferase. The second sugar, rhamnose in this case, is placed on the glucose by a rhamnosyltransferase specific for the 6-hydroxyl group of glucose. Elongation of the diglycoside to a triglycoside would involve transfer of the next sugar in the same fashion.
Figure 3.
Collection Sites of Umbellularia

III  
II

IV

125°

125°

XIII, XIV

V-IX

40°

XXIII

X

35°

XII, XV-XVII

11

XVIII

XIX

XX, XXI

XXII

30°
II. MATERIALS AND METHODS

Source of Plant Material

_Umbellularia californica_ was collected from 21 sites in California, one in Oregon and one in West Vancouver, British Columbia. In the case of the Californian and Oregon samples plant material was collected from individual plants and placed in a plant press. The B.C. specimen was extracted without drying. Voucher specimens have been deposited in UBC. Two wood samples were collected, one from a shrub at the Mud Flat site (BAB-1960) and one from a large tree from Santa Clara County (Schofield s.n., Dec. 1992). Table 2 shows details of the collection sites. Figure 3 shows the collection sites.

Table 2. Collection Sites

<table>
<thead>
<tr>
<th>Pop'n.</th>
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<td>-</td>
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<td>S</td>
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<tr>
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<td>Pope Val Rd</td>
<td>Napa</td>
<td>1966</td>
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<tr>
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<td>Calistoga</td>
<td>Napa</td>
<td>WBS s.n.</td>
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<tr>
<td>XVIII</td>
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<td>San Mateo</td>
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<td>1975</td>
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<tr>
<td>XXIII</td>
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<td>Feather River</td>
<td>Butte</td>
<td>2006</td>
<td>?</td>
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1) Numbers are B.A. Bohm collections; WBS are W.B. Schofield collections.

2) T is tree form, S is shrub form.

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**Extraction Procedures**

When available, 20 grams of dry leaves were removed from the branches and immersed in dichloromethane for two minutes with intermittent stirring. For a few samples smaller amounts were available (see Table 2). This washing was repeated once. The combined dichloromethane extracts were filtered and evaporated to dryness under reduced
pressure in a rotary evaporator. Residues were taken up in ca. three mls of dichloromethane and stored in small vials.

After removal of the dichloromethane, the extracted leaves were air dried and crushed in liquid nitrogen to achieve maximum disruption of cells. The leaf powder of each sample was extracted at room temperature six times with ca. 400 ml of methanol for each extraction. This number of extractions were needed to remove all colour (chlorophyll) from the ground leaves. The pooled methanol extract from each sample was evaporated to dryness as above. The residue was shaken vigorously with two 250 ml volumes of boiling distilled water. The resulting aqueous extract was filtered through paper to remove waxy solids. Occasionally some chlorophyll or chlorophyll degradation products passed into the water extract. These caused no problems in subsequent stages of the isolation procedure. Flavonoids were removed from the aqueous extract by several extractions with water-saturated n-butanol. After evaporation of the combined butanol extracts of each sample, the residues were taken up in a few mls of methanol and stored in small vials.

Flowers (ca. five grams) of U. californica (pooled sample) were extracted with methanol and subjected to two-dimensional TLC as for the leaf extracts.
Ground debarked wood samples were extracted by soaking in methanol at room temperature for several days. Soaking with new methanol was repeated until the solution was no longer coloured. Combined methanolic extracts were evaporated to dryness under reduced pressure. The wood extract (shrub form) was chromatographed on LH-20 using methods to be described for vacuolar flavonoids. The wood extract from the tree was compared to the shrub using TLC.

**Thin-layer Chromatographic Methods**

The leaf surface extracts were chromatographed one dimensionally using a mixture of ethyl formate/ cyclohexane/ n-butyl acetate/ formic acid (50:25:23:2). The TLC plates used consisted of ca. 0.25 mm thick Polyamid 6.6 spread on glass. All plates were home-made.

Two solvent systems were used for the vacuolar flavonoids. The first, referred to as the aqueous system, consisted of water/n-butanol/acetone/dioxane (70:15:10:5). The second system, referred to as the organic system, consisted of dichloroethane/methanol/butanone/water (50:25:21:4). Two-dimensional chromatography was done by running plates in the first direction using the aqueous system and then in the second direction using the organic system. The plates were air dried between the first and second runs. To confirm the extent of glycosylation of the
isolated flavonoids commercial microcrystalline cellulose plates were used with 6% aqueous acetic acid. Sugar analyses were done using commercial silica gel K 60 plates developed in chloroform/methanol/water (16:9:2).

The butanol-soluble extract from each plant sample was spotted onto a thin layer plate and chromatographed in two directions. After thorough drying in the air the phenolic profile was viewed under ultraviolet light (366nm) under three conditions: (1) no spray, (2) fumed with ammonia, and (3) after spraying with 0.1% Naturstoffreagent (ethanolamine diphenylborinate) in 1:1 methanol/water (NR). The behaviour of each compound/spot was recorded and a copy of the chromatogram was made using tracing paper.

All of the plant samples were analysed in this way. Due to the similarity of all of the two-dimensional profiles the butanol extracts were combined (except for small quantities saved for reference purposes), evaporated to dryness and suspended in about 50 mls of 30% methanol in water and filtered through a pad of Celite filter-aid in preparation for chromatographic column loading.
Isolation of Vacuolar Flavonoids

A glass column (70 x 3.5 cm) was packed with a slurry of Sephadex LH-20 in 30% methanol in water. The extract was loaded onto the column with a Pasteur pipette. Thirty percent methanol was run through the column at an approximate rate of five mls per minute. Two fractions (ca. 200 mls) were taken before changing to 40% methanol. Three additional fractions, totalling ca. 200 mls, were taken; then fifty percent methanol was run for seven fractions (total volume ca 500 mls). Next sixty percent methanol was run for four fractions (ca. 300 mls total), seventy percent for five fractions (ca. 450 mls total), eighty percent for six fractions (ca. 450 mls total), and one hundred percent for two fractions (ca. 300 mls total). The final fraction consisted of 200 mls of acetone. All thirty fractions were evaporated to dryness under reduced pressure and the residues were taken up in a few mls of methanol and stored in capped vials.

These fractions were spotted on thin-layer polyamide plates and chromatographed in one direction in the aqueous solvent system. A duplicate plate was run in the organic system. As before, the behaviour of the spots was recorded under UV, etc.
Partition column chromatography was used to separate the compounds in LH-20 column fraction No. 9 (which contained the two major flavonoids). A 20 x 2.5cm glass column was packed with a slurry of microcrystalline cellulose-water (2:1) in a water-saturated mixture of ethyl acetate and petroleum ether (60:40). Final packing of the column was done under slight air pressure (air line). After loading the fraction-9 material the column was developed with mixtures of ethyl acetate in petroleum ether (60:40, 70:30, 80:20, 100:0). Approximately 100 ml fractions were taken. After evaporation to dryness and redissolving in a few mls of methanol, the 22 fractions were chromatographed one dimensionally in the organic system. Spots were visualized and recorded in the usual manner.

LH-20 Column fractions, other than No. 9, were resolved into component flavonoids using TLC. Final purifications of individual compounds was also done by preparative chromatography on polyamide plates using the solvents described above. In all cases bands of purified compounds were scraped off the thin-layer plates and eluted using methanol.

Sugar Analysis

The level of glycosylation of each flavonoid was determined, in part, by its TLC behaviour. Flavonoid
monoglycosides, diglycosides and triglycosides have characteristic mobility in the aqueous system with the monoglycosides being the slowest moving, triglycosides being the fastest moving and diglycosides being intermediate. See Figure 4.

Saccharide identity was determined by hydrolysis of flavonoid glycosides with trifluoroacetic acid, TLC of the cleaved sugars on silica gel using the chloroform/methanol/water system followed by spraying with a reagent that consists of thymol (0.9 g) in 19 mls 95% ethanol and 1.0 ml H$_2$SO$_4$. Heating the sprayed chromatograms for a few minutes at 105° develop the characteristic colours of the sugars. Appropriate standards are used for comparison. See Figure 5 for a graphic representation of a sugar analysis.

Spectroscopic Methods

Compounds were subjected to ultraviolet absorption analysis using standard techniques (Mabry et al. 1970). These techniques involve spectral shift reagents: sodium acetate, boric acid, aluminum chloride/HCl, and sodium methoxide. Mass spectral analysis involved standard electron impact mass spectrographic techniques (70ev) as described by Markham (1972).
Figure 4.
U.V. fluorescence of one dimensional chromatogram of main flavonoids of *Umbellularia californica* versus glycoside standards: 

- M = Quercetin-3-0-Glc,
- D = Isorhamnetin rutinoside, 
- T = Isorhamnetin-3-0-Gal-Rha-Rha.

(after reagent spray)

- ○ = green
- □ = blue
- ● = yellow

24g' = fraction 24, upper green
9y = fraction 9, yellow
Figure 5.
One dimensional chromatogram of cleaved sugars from isorhamnetin triglycoside (of fraction 9)
Two-dimensional TLC analysis of all 23 collections of *U. californica* revealed that they were virtually superimposable both qualitatively and quantitatively. Figure 6 is a graphical representation of the profile. Two kinds of spots were observed, blue fluorescent ones that are presumably lower molecular weight phenols, and UV absorbing ones (dark). The UV absorbing spots fluoresced green in the presence of ammonia fumes which indicated that they were either 5-OH flavones or 3-0-substituted flavonols with a 4’-OH (Markham 1982, p. 19). Upon spraying the chromatogram with NR some of the dark spots gave a green colour and some gave a yellow colour. Green indicates the presence of a 4’-hydroxyl group, while a yellow colour indicates 3’,4’-dihydroxylation.
Figure 6.
Two dimensional chromatogram of *Umbellularia californica* leaf extract.

- Dark before spray
- Green after spray
- Yellow after spray
Figure 6. (continued)

(Key to spot numbers)

1. Quercetin tetrarlycoside
2. Isorhamnetin tetrarlycoside
3. Quercetin triglycosides plus diglycosides
4. Isorhamnetin triglycosides plus diglycosides
5. Quercetin diglycosides plus monoglycosides
6. Quercetin monoglycoside
7. Isorhamnetin diglycoside
8. Quercetin monoglycoside
9. Isorhamnetin monoglycoside
10. Quercetin aglycone
11. Isorhamnetin or Kaempferol aglycone
12. Quercetin diglycoside or monoglycoside
13. Quercetin diglycoside
Column chromatography of the combined butanol-soluble phenolic fractions from 21 specimens (The West Vancouver, Claremont and Feather River collections were not used) gave the following general elution results: fractions 3 and 4, presumed tetra-glycosides; fractions 5-10, triglycosides; fractions 11-22, diglycosides; fractions 23-28 monoglycosides; and fractions 29 and 30, aglycones. Blue fluorescent compounds appeared in many fractions. See Figure 7 for a diagrammatic representation of these data.

Work on selected fractions resulted in isolation and identification of nine compounds. Kaempferol and quercetin derivatives were identified by means of their characteristic colour reactions with NR, their ultraviolet spectral characteristics and by direct comparison with known compounds. The structure of isorhamnetin, suggested by ultraviolet data, was confirmed by mass spectroscopy (molecular ion = 316, molecular ion minus methyl = 301, A-ring fragment = 152/153, B-ring fragment = 151).

The monoglycoside fractions yielded a flavonol and either glucose or rhamnose on hydrolysis. The compounds identified were kaempferol and quercetin 3-O-glucosides and kaempferol and quercetin 3-O-rhamnosides. The diglycoside fraction yielded two major compounds which gave either kaempferol or isorhamnetin on acid hydrolysis plus equal amounts of glucose and rhamnose. These compounds had Rf
Figure 7.
U.V. fluorescence of two one dimensional chromatograms of *Umbellularia californica* leaf vacuole column fractions 1–15 (after reagent spray) $S =$ Quercetin 3-0-Glc

1 5 10 15 $S$

Organic

Aqueous

○ = blue

○ = yellow

○ = green
Figure 7. (continued)
U.V. fluorescence of two, one dimensional chromatograms of *Umbellularia californica* leaf vacuole column fractions 16-30. (after reagent spray) S = Quercetin 3-O-Glc

= Blue
= Yellow
= Green
behaviour equal to that of rutinosides (rhamnosylglucosides) but confirmatory studies on the isolated diglycosides were not done. A very small amount of what has been assumed to be the equivalent quercetin 3-diglycoside was also observed. The triglycoside fraction gave two compounds that gave, on acid hydrolysis, either quercetin or isorhamnetin and glucose and rhamnose. Inspection of sugar chromatograms from these compounds suggested that glucose was present in higher amounts than rhamnose which would mean that these compounds were flavonol 3-O-(glucose, glucose, rhamnose). The order of arrangement of the sugars was not determined. Partial hydrolyses of these compounds, done using low concentrations of acid at room temperature for short periods of time, gave ambiguous results. Very small spots were seen in the 2D chromatograms that had higher Rf values in the aqueous solvent than the triglycosides. Attempts to obtain these presumed tetruglycosides failed, however. Figure 8 shows the structures of the flavonoids identified.

Attempts to relate the compounds identified with individual spots on the 2D chromatogram were only partially successful. Figure 6 shows an attempt to correlate the various flavonol glycosylation levels identified with spots on a representative chromatogram. The triglycoside spots are the most reliably assigned. It is also clear that tetruglycosides would be expected to run higher than the triglycosides in the aqueous solvent. The diglycosides
identified co-chromatographed with known flavonol diglycosides in the aqueous system so the assignment of the diglycoside region on the 2D TLC is reasonable. The monoglycoside region on the 2D chromatograms was more complex than the identified compounds would suggest. Insufficient plant material was available to solve this problem. Small amounts of the aglycones, presumably kaempferol, quercetin and isorhamnetin, were observed along the organic system axis on the 2D chromatograms and in LH-20 column fraction 30. Whether these compounds are naturally occurring or are artifacts of the isolation procedures was not investigated.
Kaempferol, \( R = H \)
Quercetin, \( R = OH \)
Isorhamnetin, \( R = OMe \)

Isorhamnetin 3-0-triglycoside (Glc, Glc, Rha)
Quercetin 3-0-triglycoside (Glc, Glc, Rha)
Isorhamnetin 3-0-diglycoside (Glc, Rha)
Kaempferol 3-0-diglycoside (Glc, Rha)
Quercetin 3-0-diglycoside (Glc, Rha)
Quercetin 3-0-monoglucoside
Kaempferol 3-0-monoglucoside
Quercetin 3-0-monorhamnoside
Kaempferol 3-0-monorhamnoside
**Flower Flavonoids**

Flowers from several collections were pooled and extracted with methanol. Chromatography of the concentrated extract gave a 2D profile that was similar to, but somewhat simpler than, the profile seen with the leaf samples. Also, there were fewer blue fluorescent spots in the flower profiles. As in the case of the leaf chromatograms, spraying with NR produced intense green and yellow spots (kaempferol and quercetin-type flavonols, respectively) in the triglycoside position. These were accompanied by small putative tetroglycosides. The monoglycoside position was occupied by intense green and yellow spots and it seems likely that the diglycosidic position was also comprised of the same flavonol pair. Traces of aglycones were also observed.

**Leaf Surface Compounds**

The dichloromethane extracts from the leaf surface of *Umbellularia* were chromatographed one dimensionally in the ethyl formate-cyclohexane solvent system. No flavonoid spots were seen in any of the samples. There was considerable streaking of blue fluorescent material in most fractions. No differences were noted between tree and shrub forms.
After several weeks a copious white crystalline precipitate had accumulated in each sample of the cuticular extract. This material was collected and subjected to infrared analysis. The IR spectrum exhibited bands that could be assigned to a carbonyl group, a cyclopropyl group and an ether linkage all of which are consistent with the two major compounds reported from the oil fraction of *Umbellularia*, namely umbellulone, which has both a cyclopropyl ring and a ketone group and eucalyptol which contains an ether linkage.

**Wood Components**

The shrub wood sample from Mud Flats, California (1960) yielded 39 fractions by column chromatography. The one dimensional chromatogram of these fractions, run using the dichloroethane-based organic system, showed several prominent compounds (see Figure 9). The compound in fraction-16 was dark before spraying and green with ammonia vapour, indicating a 3-O-substituted flavonol. The 3-position is not glycosylated, however, since migration in the aqueous system for all wood compounds was nil. The fraction-16 compound gave a yellow colour with NR suggesting a quercetin-type B-ring.
Fraction 22 contained two major compounds both of which were yellow-green fluorescent before spraying; neither showed any change with ammonia fumes. Spraying with NR gave yellow and green colours suggesting quercetin and kaempferol type aglycones, respectively.

The main compound in fraction 25 was blue before spraying and showed no change with ammonia, which could signify a 5-substituted isoflavone. This compound gave a reddish reaction with NR; this behaviour does not immediately suggest a structure.

Two-dimensional chromatograms of crude butanolic extracts of wood samples from tree and shrub forms were run using the dichlorethane solvent system in both directions. This served to show the profile similarity between the two samples. Tree and shrub samples appeared the same, therefore, column chromatography of the tree specimen was not done.
Figure 9.
U.V. fluorescence of one dimensional chromatogram of *Umbellularia californica* shrub wood fractions 1–39 (after reagent spray)  
S = Quercetin-3-0-Glc

![Graph](image-url)

- ⬤ = red
- ⬤ = yellow
- ⬤ = green
- ⬤ = blue
IV. DISCUSSION

The first two questions asked above concerned the nature of the flavonoids of *Umbelullaria californica* and whether any differences exist between the compounds present in the two growth forms of the species. Twenty-three collections of *U. californica* were made, 13 of the tree form and 9 of the shrub form (we did not see the Feather River sample). The 2D chromatograms of all of these were virtually superimposable. The only differences seen were minor quantitative ones. Samples of wood were obtained from two *U. californica* plants, one a tree and one a shrub. Again, no differences were seen in the phenolic profiles of the two growth forms.

The tree form of *U. californica* is a member of the wet coastal forest vegetation of southwestern Oregon south to central California, whereas the shrub form is found in drier habitats such as occur in the inner coast mountains of California. The species also occurs in the western foothills of the Sierra Nevada but only one sample was available from this area. Several of the drier sites occupied by *U. californica* are characterized by serpentine-derived soils. Kruckeberg (1984) includes the shrubby form of *U. californica* in his list of taxa that have local or regional indicator status for ultramafic substrates in California.
It is clear from the flavonoid analyses that neither edaphic features nor availability of water have any apparent effect on the flavonoid profile of the species. It is possible that other sources of information may indicate that sufficient differences exist between the two growth forms to argue for taxonomic recognition. Flavonoids do not. It is clear from the uniformity of pigment profiles in these growth forms that the flavonoid biosynthetic pathways in *U. californica* had been established before divergence of the two forms.

The evolutionary origin of *Umbellularia* is obscure (Raven 1977). It is possible that it may have had its origin from some element of the South American laureaceous flora. Lauraceae is well represented in the present flora of South America. It is also possible that *Umbellularia* may have had a Eurasian origin. *Laurus nobilus*, the true bay, is a native of the Mediterranean region.

Cronquist (1981) stated that fossil wood from late upper Cretaceous (Mastrichtian Epoch, 72 million yr. b.p.), from California, falls within the range of variation of the Lauraceae and that Eocene wood from Yellowstone may be lauraceous. Raven and Axelrod (1978) commented that the Eocene and early Oligocene floras (35-50 million yr b.p.) that existed from Washington southward comprised several subtropical families, Lauraceae included. *Persea*
(podalinia) and Umbellularia (californica) were components of the late Miocene age Remington Hill flora (Condit 1944) and the Pliocene age Oakdale and Turlock Lake floras (Axelrod 1944). Late Miocene (13 million yr. b.p.) fossils show that Persea, Nectandra, and Ocotea, as well as Umbellularia, were part of the oak-laurel forest in California (Axelrod 1977). Umbellularia has also been identified as a component of the late Pliocene age Mulholland and Oakdale floras dating to about 5 million yr b.p. (Axelrod 1944). Persea, Ocotea and Nectandra are large genera, with the latter two being well represented in the present South American flora (Mabberley 1987).

Flavonoids of some members of South American genera have been reported, although sampling is poor and in at least one case only aglycones were identified. From the fruits of Nectandra glabrescens Barbosa-Filho and coworkers (1989) obtained kaempferol and quercetin. Wofford (1974) reported kaempferol and quercetin 3-O-glucosides from several species of Persea, while Merici and coworkers (1982) identified quercetin 3-O-glucoside and 3-O-diglucoside, as well as the flavones apigenin and luteolin, from P. americana leaves. With the exception of the flavones reported from P. americana the flavonol derivatives identified from U. californica are similar to those described from these South American genera. It can be pointed out that quercetin 3-O-rhamnosylglucoside (rutin)
was also shown to exist in leaves of the European species *Laurus nobilus* (Makarov 1971).

An intriguing observation comes from the work of Koch and König (1981) on *Aniba rosaeodora* which is a South American species although it appears not to be known from the California fossil record. These workers identified kaempferol and quercetin 3-O-rhamnosylglucosides and a quercetin 3-O-tetraglycoside. The tetraglycoside gave equal amounts of glucose and rhamnose on acid hydrolysis. The flavonoid profile of *Umbellularia californica* includes rutinosides, flavonol 3-O-triglycosides with only glucose and rhamnose, and trace amounts of compounds that have the chromatographic properties of tetraglycosides. The amounts of these putative tetraglycosides were too small to allow purification, however. The comparative rarity of flavonol tetraglycosides makes this similarity between *Umbellularia* and *Aniba* noteworthy.

**Flavonoids of Umbellularia and Other Lauraceae Compared**

In the discussion below the flavonoids of the two growth forms of *U. californica* will be described. Following that will be a comparison of the flavonoids of *Umbellularia* with other genera in the family. Next, the flavonoid chemistry of the Lauraceae will be compared with the chemistry of other families of the Laurales. Finally, the
flavonoid chemistry of the Laurales will be discussed in terms of the known chemistry of the Magnoliidae.

Since the two growth forms of *U. californica* exhibit identical patterns of flavonol glycosides, the major ones of which are shown in Fig 8, no arguments for recognizing infraspecific taxa can be made.

In order to set the stage for comparisons at higher taxonomic levels, it is first necessary to present the appropriate taxonomic framework. Kostermans' (1957) taxonomic treatment of the family, which involves two subfamilies, five tribes and eight subtribes, will be used. The constitution of the Laurales and its position in the Magnoliidae will be given according to Cronquist (1981). These relationships are presented in Table 3.
Table 3. Taxonomic Placement of Lauraceae Showing Genera from which Flavonoids have been Identified

Class MAGNOLIOPSIDA
Subclass MAGNOLIIDAE
Order LAURALES

Amborellaceae  Idiospermaceae
Calycanthaceae  Lauraceae
Gomortegaceae  Monimiaceae
Hernandiaceae  Trimeniaceae

Lauraceae

Subfamily Cassythoideae  Only Cassytha (NFD\textsuperscript{1})
Subfamily Lauroideae
Tribe Perseeae
Subtribe Perseineae  Persea
Machilus (incl. in Persea by Kostermans)
Phoebe
Beilschmiediinae  Apollonias
Beilschmiedia
5 other genera (NFD)

Tribe Cinnamomeae
Subtribe Cinnamomineae  Actinodaphne
Cinnamomum
Nectandra (incl. in Ocotea by Kostermans)
Ocotea
Table 3 presents the taxonomic frameworks into which the Lauraceae and its constituent genera fit (after Kostermans and Cronquist). Table 4 presents a detailed account of flavonoid reports from members of the family taken from the literature and includes the current results from Umbellularia. Finally, Table 5 summarizes the flavonoid information in terms of generic profiles and presents the chemical information in terms of types of flavonoids or unique structural features present in each genus.
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<td>L. lucida (L)</td>
<td>-/5678-Flavone, -/5678/3′4′-MDO-Flavone, 57/68/3′4′-MDO-Flavone</td>
<td>Lee &amp; Tan (1965)</td>
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<tr>
<td>L. lucida (R)</td>
<td>Modified A-ring chalcones</td>
<td>Lee &amp; Tan (1965)</td>
</tr>
<tr>
<td>L. pipericarpa (R)</td>
<td>Modified A-ring chalcones</td>
<td>Kiang et al. (1962)</td>
</tr>
<tr>
<td>Species</td>
<td>Flavonoids</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------------------------------------------------------------</td>
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<tr>
<td><em>L. umbellata</em> (L)</td>
<td>2'4'6'/3'-Menthyl-chalcone, 2'6'/4'/3'-Menthyl-chalcone, 2'4'6'/-DiHchalcone, 2'6'/4'-DiHchalcone, 2'6'/4'/3'-Menthyl-chalcone, 57/-Flavanone, 5/7-Flavanone</td>
<td>Ichino (1989), Tanaka et al. (1984), Shimomura et al. (1988)</td>
</tr>
<tr>
<td><em>L. umbellata</em> (B)</td>
<td>No flavonoid data</td>
<td>'Tribe VI Hypodaphneae'</td>
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</table>

**Explanation of abbreviations:**
- Apig = apigenin; Lute = luteolin; K = kaempferol; Q = quercetin;
- DiHK = dihydrokaempferol; DiHQ = dihydroquercetin; Pel = pelargonidin;
- Me = methyl; MDO = methylenedioxy; Gly = glycoside; Glc = glucoside;
- Gln = glucuronide; Ara = arabinoside; Rhm = rhamnoside; Rut = rutinoside;
- L = leaves; B = bark; R = root; F = fruit; W = wood; T = all parts;
- Hydroxy positions/Methoxy positions/Other substituent(s)

*Tribe IV Cryptocaryeae*
- **Eusideroxylineae**
  - **Cryptocarya**
    - **C. bourdilloni** (R)
      - Modified A-ring chalcone
      - Govindachari et al. (1973)
Table 5. Flavonoid Occurrences in Lauraceous Genera

<table>
<thead>
<tr>
<th>Genus</th>
<th>Sp.²</th>
<th>CHL</th>
<th>FVN</th>
<th>DHF</th>
<th>ORD</th>
<th>CGL</th>
<th>FLV’OL</th>
<th>GLY</th>
<th>Cat</th>
<th>M</th>
<th>D</th>
<th>T</th>
<th>P</th>
<th>MET</th>
<th>MDO</th>
<th>BRD</th>
<th>XOX</th>
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</tbody>
</table>

1) CHL = chalcone; FVN = flavanone; DHF = dihydroflavonol; ORD = ordinary flavone (e.g. apigenin); CGL = C-glycosylflavone; FLV’OL = flavonol (K = kaempferol, Q = quercetin, I = isorhamnetin, A = azaleatin); GLY = glycoside (M = mono-, D = di-, T = tri-, P = tetra-); MET = O-methyl; MDO = methylenedioxy; BRD = B-ring deoxy flavonoid; XOX = extra A-ring oxygenation
2) Number of species for which flavonoid data are available/
   Number of species known for the genus
3) Quercetin 3-methyl ether
The first thing that is clear from Table 5 is that members of the family are primarily flavonol accumulators. The presence of flavonol derivatives as the major flavonoid type present in *Umbellularia* is consistent with this. Four differences from the base pattern of kaempferol and quercetin have been observed. First, *Umbellularia* and *Beilschmiedia mearsii* (Harborne and Mendez 1969) are the only taxa that have been shown to produce isorhamnetin (quercetin 3′-O-methyl ether). *Beilschmiedia* is further distinguished within the family by its capacity to methylate the 5-OH of quercetin to give azaleatin. The third difference is seen in *Phoebe cinnamomifolia* which has been shown to produce quercetin 3-methyl ether (Martinez O. et al. 1990). The fourth feature is the presence of O-methylated derivatives of the unusual B-ring deoxyflavonol galangin in *Aniba riparia* (Franca et al. 1976; Fernandes et al. 1978).

O-Methylated derivatives of other flavonoid types have also been reported from members of the family. O-Methylated flavanones were identified from *Aniba riparia* (Fernandes et al. 1978), *Lindera erythrocarpa* (Liu et al. 1973a, 1973b) and *L. umbellata* (Shimomura et al. 1988) while O-methylated flavones have been found in *Actinodaphne madraspatana* (Adinarayama and Gunsekara 1979) and *Lindera lucida* (Lee and Tan 1965). O-Methylated chalcones have also been reported from *Aniba riparia* (Fernandes et al. 1978) and from species

The most commonly observed flavonol glycosides are those that are linked through the 3-OH group. Most flavonol glycosides in the Lauraceae, including those from Umbellularia, are of this type. The only exceptional case is the report of kaempferol-3,7-diglycosides in leaves of Cinnamomum sieboldii (Nakano et al. 1983). The most common glycosides of flavones and flavanones, on the other hand, are linked through the 7-OH. This is the case with regard to luteolin 7-O-glucoside from leaves of Persea americana (Merici et al. 1992) and naringenin 7-O-glucoside from leaves of Litsea glutinosa (Mohan et al. 1977). Mohan and coworkers (1977) also identified the common anthocyanin pelargonidin 5-O-glucoside from L. glutinosa.

The present findings of kaempferol and quercetin derivatives in Umbellularia are in agreement with the general observations that simple flavonols are the major flavonoid group in the family which, in turn, is in agreement with observations that other members of the Laurales are characterized by such a simple profile. The profiles are not identical, however, as some other flavonoid types have been found in some of the other families. Sterner and Young (1980) found kaempferol and quercetin
glycosides in members of the Calycanthaceae, while kaempferol, quercetin and isorhamnetin have been reported from the Monimiaceae (Gornall et al. 1979; Bombardelli et al. 1976). Derivatives of kaempferol and quercetin (including isorhamnetin) along with C-glycoflavones have been reported from members of the Hernandiaceae (Gornall et al. 1979). Amborella trichopoda, the sole member of the Amborellaceae, accumulates glycosides of kaempferol along with procyanidin (Young 1982). Young and Sterner (1981) obtained quercetin glycosides as well as flavone derivatives and C-glycosyl-flavones from Idiospermum australiense, which is the sole member of the Idiospermacae.

Despite the variation seen in this group of families a fundamental feature is the absence of myricetin (3',4',5'-trihydroxy B-ring) derivatives. The lack of myricetin derivatives in members of the Magnoliiflorae (sensu Gornall et al. 1979) was discussed as a significant factor in distinguishing this group from Hamamelidiflorae and Dilleniiflorae (Gornall et al. 1979).

General Conclusions

What general evolutionary conclusions can be drawn from this work? One can speculate that several millions of years ago the adaptation of U. californica flavonoids to the environment reached its pinnacle and that conditions since
then have not changed with regard to the evolutionary pressures involved in shaping the flavonoid profile. The genetic basis for the growth forms of *U. californica* would appear to be more plastic compared to the supposedly fixed genes of flavonoids biosynthesis.

Since we don't have flavonoids from fossil *U. californica* plants, it is impossible to say with certainty just how far back the simple flavonol profile, defined by this work, existed. Studies of several genera of fossil plants, *Quercus*, *Celtis*, *Ulmus*, *Zelkova* (Giannasi and Crawford 1986) showed that these 15-20 million year old fossils exhibited essentially the same flavonoid profiles as extant species. Owing to the apparent stability of flavonoid patterns over time it is not unreasonable to assume that the present flavonoid profile of *Umbellularia* is representative of the plant over its history.

**Future studies.**

It would seem reasonable that a morphometric study of *U. californica* be undertaken in order to see if consistent differences between the two growth forms exist. Some differences in leaf shape observed in the field suggest such a study.
Variation in the size of some of the minor spots on chromatograms suggest that a quantitative study would be useful. This would be best done with plants from the range of the species maintained in a common garden environment. This would also offer an opportunity to study variation of volatile leaf components. Natural variation in volatile components has not been done.
V. LITERATURE


Giannasi, D. E. and Niklas, K. J. (1977) Flavonoid and other chemical constituents of fossil Miocene *Celtis* and *Ulmus* (Succor Creek Flora), Science 197: 765-767.


King, F. E. (1962) J. Chem. Soc. 1192. This citation was taken from R. Hegnauer, Vol. IV. Chemotaxonomie der Pflanzen, p. 367. A search in the journal showed that the citation was incorrect. Further searches for papers by King or for papers on Ocotea usambarensis were unsuccessful.


