Flavonoid and Other Chemical Constituents of Fossil Miocene *Celtis* and *Ulmus* (Succor Creek Flora)

Abstract. *Organic solvent extractions of green-colored fossil* Celtis *sp. and* Ulmus *sp. leaves (36 to 25 × 10^6 years old) indicate the preservation of quercetin-3-O-glycosides, apigenin and luteolin carbon glycosides, methyl pheophorbide a, and a consortium of other organic constituents. Comparisons between referable fossil and extant taxa indicate a high level of phytochemical fidelity. The preservation of* O-*glycosides indicates a mild postdepositional environment during fossilization.*

Organic solvent extractions of green-colored angiosperm leaves from the Succor Creek Flora (36 to 25 × 10^6 years old) have yielded kaempferol, dihydrokaempferol, methyl pheophorbide a, and a consortium of organic acids (*1*). The preservation of flavonoids and chlorophyll derivatives in these and related sediments indicates an unusual postdepositional environment (*2*). In this report we discuss the paleobiochemistry of additional fossil taxa (*Celtis* and *Ulmus*) found in these strata and attempt to draw phytochemical correlations between respective fossils and extant genera.

The rocks containing Succor Creek fossils have the same stratigraphic position as the intermontane beds which Kirkham (*3*) assigns to the Payette formation; while Chaney (*4*) confirms their occurrence as interbeds in the Columbia River basalt, subsequent work suggests that this flora is Oligocene to Miocene in age (*5*). In contradistinction to the coarse sediments common in the Payette Flora (upper Miocene), which are micaceous sands and gravels, the Succor Creek beds are mostly opaline shales with occasional tephra lenses. The former strata, weathering to a reddish hue, show alteration due to contact with basaltic lava flows. The biochemical analyses given in this and an earlier report (*1*) were derived from material preserved in the uppermost pyroclastic ashfall (tephra) lenses, which showed no evidence of basalt contact or igneous intrusion. On the basis of their state of chemical and morphological preservation, as well as available geologic data, the Succor Creek "green leaves" are thought to have been preserved in a unique microgeologic environment which was the result of rapid cool volcanic ash deposition. The lack of apparent cuticular and chemical decomposition suggests that burial and subsequent fossilization occurred rapidly. Data derived from the thermal decomposition rates and products of selected flavonoids indicate that these fossils did not experience temperatures greater than 80°C or extreme *p*H shifts (*2*).

Small quantities of fossil *Celtis* sp. and *Ulmus* sp. leaves preserved as cuticular compressions were obtained from freshly cleaved rock surfaces, ground into a fine powder, and extracted in absolute methanol. Two-dimensional descending paper chromatography of these extracts, ultraviolet spectral analysis of compounds, and hydrolysis experiments were carried out by standard techniques (*1*, *6*, *7*).

Paper chromatograms of fossil *Celtis* sp. (Fig. 1, B and C) showed the presence of eight dark-absorbing compounds when viewed under ultraviolet light. Spectral analysis (*6*) and hydrolysis experiments (*7*) showed that nearly all of these compounds are apigenin or luteolin carbon glycosides—that is, *C*-glycosylflavones (Table 1). As a means of verifying these identifications, leaves of a single specimen of the closely related *Celtis occidentalis* were also chromatographed (Fig. 1, A and C). The extant *C. occidentalis* contains only *C*-glycosylflavones (Table 1). While several of these glycoflavones (compounds 1, 3, 4, 7, and 8) are shared in common with the fossil taxon, others appear unique to the extant material (compounds 9, 10, 10a, 11, 12, and 13).

Acid hydrolysis experiments with HCl (*6*, *7*) showed that the chemical similarity between the extinct and extant *Celtis* taxa is closer than is apparent from the chromatograms. Unlike flavonoid *O*-glycosides, which are hydrolyzed to the referable sugars and aglycon during acid hydrolysis (*6*), flavonoid *C*-glycosides are not hydrolyzed. Instead, they undergo a Wessely-Moser rearrangement (isomerization) in which the central pyrone ring opens, followed by ring closure on either of the two phenolic hydroxyl groups *ortho* to the carbonyl group. This results in a mixture of two isomers; for example, a 6-*C*-glycosylflavone will give rise to its 8-*C*-glycosyl isomer and vice versa (*8*).

Celtis occidentalis apparently does not produce compound 2 (8-*C*-glycosylapigenin), which does appear in fossil material (Table 1). Strong acid hydrolysis

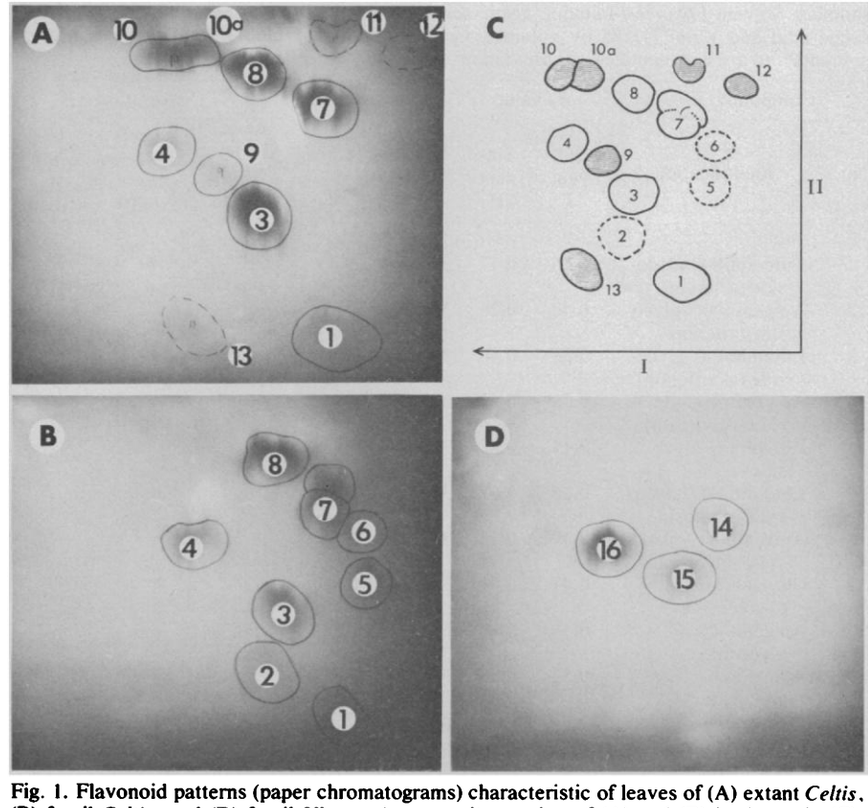

Fig. 1. Flavonoid patterns (paper chromatograms) characteristic of leaves of (A) extant *Celtis*, (B) fossil *Celtis*, and (D) fossil *Ulmus*. A composite version of (A) and (B) is shown in (C), where clear and shaded spots refer to fossil and extant patterns, respectively (spots encircled in broken lines are for compounds found in fossil *Celtis* only). Solvents I and II are described in the legend of Table 1. For further details see text.

of compound 4 (6-*C*-glycosylapigenin), however, produces a mixture of both compound 4 and the acid hydrolysis product compound 2 (the 6-*C* and 8-*C* isomers, respectively). While both isomers appear only in the fossil material, the potential for producing both in the extant taxon exists in vitro at least, if not in vivo.

Similarly, mild acid hydrolysis of compound 8 from both living and fossil *Celtis* leaves yields compound 2, indicating that compound 8 possesses an *O*-glycoside attached to the *C*-glycosyl moiety. Subsequent rigorous acid hydrolysis of the resulting compound 2 gives rise to its alternative *C*-glycosyl isomer (compound 4). While the extant *Celtis* lacks compound 2, it appears that the taxon may simply "package" the compound as an 8-*O*-glycosyl-*C*-glycoside rather than the simpler 8-*C*-glycoside. A similar relationship exists between compound 7 and compounds 1 and 3. The degree of glycosylation apparently belies a greater similarity between the fossil and extant taxa.

It should be noted that specific production of either 6- or 8-*C*-glycosylflavones is known in other angiosperms (such as *Lemna*) (*9*) and thus illustrates a real genetic specificity. The development of this type of biosynthetic specificity as a form of evolution in *Celtis* is certainly suggested from comparisons of fossil and extant genera. The alternative possibility, that the formation of additional *C*-glycosyl isomers observed in the fossil material is due to diagenesis during fossilization does not seem to be borne out by further experiments in our laboratory. Authentic flavonoids were subjected to simulated geothermolysis (*2*) and were found to require more vigorous conditions for diagenesis than are thought to have occurred at the Succor Creek locality (*4*).

The chromatographic profile on fossil *Ulmus* sp. (Fig. 1D), in contrast to that of *Celtis*, shows three compounds which were identified as quercetin-3-*O*-glycosides (Table 1). Glycoflavones typical of the *Celtis* specimens are absent from fossil *Ulmus*. This is not unexpected, since previous work on 20 extant *Ulmus* species (*10*) showed only the presence of quercetin-3-*O*-glycosides. Bate-Smith (*11*) has found in other elm taxa the related flavonol, kaempferol, in glycosidic form. The preservation of quercetin-3-*O*-glycosides in fossil *Ulmus* is remarkable, since *O*-glycosides are easily hydrolyzed under acidic conditions (*3*). This, along with the lack of free flavonol aglycons in fossil *Ulmus*, shows that fossilization conditions attending sediment maturation were probably mild and indicates the validity of intertaxonomic phytochemical correlations.

Combined gas chromatography–mass spectroscopy (GC-MS) of organic solvent extract (*1*) of fossil *Celtis* and *Ulmus* was used to identify *n*-alkanes, fatty acids, steroid derivatives, and other organic constituents. Fatty acid/*n*-alkane distributions in both taxa were unimodal and showed carbon preference indices indicative of mild, if any, diagenesis. *Celtis* and *Ulmus* specimens had fatty acid ranges of C_{23} to C_{30} (maxima at C_{26} and C_{28}) and C_{24} to C_{32} (maxima at C_{26} and C_{30}), respectively, while *n*-alkanes ranged from C_{25} to C_{35} (maxima at C_{29} and C_{31}) in *Celtis* and C_{23} to C_{36} (maxima at C_{29} and C_{32}) in *Ulmus*. These data are remarkably consistent with those on referable extant leaf material (*12, 13*). Elution with a mixture of benzene and methanol (3:1) followed by column and preparative $AgNO_3$-SiO_2 thin-layer chromatography indicated a sterane mixture. Isolated constituents, identified by GC-MS, included 5β-cholesterane (*Ulmus*), stigmasterane (*Ulmus* and *Celtis*), and a tentatively identified sitosterol derivative (*Celtis*). These compounds are thought to be derivatives of referable steroids and as such are consistent with the phytochemistries of respective extant taxa (*12–14*). 7-Hydroxycalamenene (*14*), a ubiquitous elm heartwood compound, was not detected in any of the material examined.

The differential sterane and fatty acid/*n*-alkane profiles seen in fossil *Celtis* and *Ulmus* support the observed phytochemical differences based upon flavonoid chemistry. While fatty acid/*n*-alkane profiles of fossil taxa are not significantly different from one another, neither are they different in extant material. In contradistinction, differential sterane profiles in *Celtis* and *Ulmus* are pronounced. Gas chromatograms of *Ulmus* fossils indicated a wide range of tri- and tetracyclic triterpanes ($C_{27}H_{48}$ to $C_{34}H_{68}$) with major peaks at $C_{29}H_{52}$ (11.6 percent), $C_{30}H_{52}$ (11.8 percent), and $C_{40}H_{78}$ (16.0 percent). *Celtis* gas chromatograms were significantly less complex and showed major peaks referable to $C_{29}H_{52}$ (12.0 percent) and $C_{40}H_{78}$ (12.0 percent).

Chloroform extracts of fossil material (*1, 15*) yielded methyl pheophorbide a as determined by visible-light absorption

Table 1. Identification, spectral data, and distribution of flavonoid glycosides in fossil and extant species of *Celtis* and *Ulmus*. Complete spectral data for all compounds are available on request. The compounds are identified tentatively and remain to be compared with authentic samples. Solvent I was *tert*-butanol, acetic acid, and water (3:1:1 by volume), solvent II was acetic acid and water (15:85 by volume). Compounds 6, 11, and 13 were present in trace amounts and are not identified. Abbreviation: s, shoulder or inflection.

Compound		R_F value		Spectral maxima in methanol (nm)	Distribution		
No.	Identification	Solvent I	Solvent II		*Celtis* sp. (fossil)	*C. occidentalis* (extant)	*Ulmus* sp. (fossil)
				Celtis *sp.*			
1	Luteolin-8-*C*-glycoside (orientin)	0.26	0.17	347, 268, 256	+	+	
2	Apigenin-8-*C*-glycoside (vitexin)	0.40	0.26	332, 270	+		
3	Luteolin-6-*C*-glycoside (isoorientin)	0.38	0.40	347, 268, 256	+	+	
4	Apigenin-6-*C*-glycoside (isovitexin)	0.56	0.53	333, 271	+	+	
5	Luteolin-*C*-glycoside	0.14	0.42	343, 268, 256	+		
7	Luteolin-8-*O*-glycosyl-*C*-glycoside	0.26	0.67	348, 268, 256	+	+	
8	Apigenin-8-*O*-glycosyl-*C*-glycoside	0.39	0.75	331, 271	+	+	
9	Chrysoeriol-*C*-glycoside	0.47	0.51	342, 268^s, 254^s		+	
10	Apigenin-*C*-glycoside	0.56	0.80	331, 270		+	
10a	Apigenin-*C*-glycoside	0.50	0.80	331, 270		+	
				Ulmus *sp.*			
14	Quercetin-3-*O*-glycoside	0.41	0.54	352, 270^s, 256			+
15	Quercetin-3-*O*-glycoside	0.48	0.43	356, 268^s, 255			+
16	Quercetin-3-*O*-glycoside	0.61	0.49	356, 266^s, 257			+

spectra and R_F values. The vivid green color of these fossil leaves is the result of localized accumulation of this chlorophyll derivative.

The morphological and chemical states of preservation of the Succor Creek specimens provide a basis for making phytochemical correlations between respective fossil and living taxa. The preservational states reported here are comparable to, but significantly different from, those seen in other angiosperm green-leaf localities—for instance, in the Geisel Valley leaf impressions. The presence of chlorophyll derivatives in Geisel Valley (middle Eocene) fossils has been interpreted (*15*) as the result of methyl pheophorbide a accumulation in sites previously occupied by leaves of various taxa. In consequence, such fossils, while providing excellent chemical data, reflect a nonselective concentration of organic constituents (similar to that of an ion-exchange column) derived from many genera rather than one. Such states of preservation preclude phytochemical correlations on the generic level. The fossils collected from the Succor Creek ashfall deposits are cuticular compressions showing a high level of cellular fidelity. The existence of significantly different biochemical profiles between taxa (*Celtis, Ulmus*, and *Zelkova*) make it possible to draw valid chemotaxonomic conclusions. The association of lava flows with lake-bed sediments in the Succor Creek locality suggests that the area had many lakes and streams caused by lava-blocked rivers (*5*). Vegetation that grew peripherally to these lakes caused accumulation of debris in lake bottoms or was buried under volcanic ash. As a result, two very different states of preservation may be found in relatively close proximity. Leaves preserved in shales from Succor Creek do not show the flavonoid profiles reported here.

The possibility of comparing the flavonoid profiles (and other constituents) of known fossil angiosperms with those of extant North American and Asian taxa is suggested by this research and may provide an opportunity for chemophytogeographic interpretations of Tertiary flora.

DAVID E. GIANNASI
KARL J. NIKLAS
Harding Research Laboratory, New York Botanical Garden, Bronx 10458

References and Notes

1. K. J. Niklas and D. E. Giannasi, *Science* **196**, 877 (1977).
2. ———, *ibid.* **197**, 767 (1977).
3. V. R. D. Kirkham, *J. Geol.* **39**, 193 (1931).
4. R. W. Chaney, *Carnegie Inst. Washington Publ. 617* (1959), part 1.
5. A. Graham, *Am. J. Bot.* **50**, 921 (1963).
6. T. J. Mabry, K. R. Mankham, M. B. Thomas, *The Systematic Identification of Flavonoids* (Springer-Verlag, New York, 1970).
7. J. B. Harborne, *Phytochemistry* **4**, 107 (1965).
8. J. Chopin and M. L. Bouillant, in *The Flavonoids*, J. B. Harborne, T. J. Mabry, H. Mabry, Eds. (Chapman & Hall, London, 1975), p. 632.
9. J. W. Wallace and T. J. Mabry, *Phytochemistry* **9**, 2133 (1970).
10. F. S. Santamori, *Bull. Torrey Bot. Club* **99**, 127 (1972).
11. E. C. Bate-Smith, *J. Linn. Soc. London Bot.* **58**, 95 (1962).
12. L. H. Santa-Cruz, C. E. Turner, J. E. Knapp, P. L. Schiff, Jr., D. J. Slatkin, *Phytochemistry* **14**, 2532 (1975).
13. F.-C. Chen, Y.-M. Lin, A.-H. Chem, *ibid.* **11**, 1190 (1972); N. Tanaka, M. Yasue, H. Imamura, *Tetrahedron Lett.* (1966), p. 2767.
14. J. W. Rowe, M. K. Seikel, D. N. Roy, E. Jorgensen, *Phytochemistry* **11**, 2513 (1972).
15. D. L. Dilcher, R. J. Pavlick, J. Mitchell, *Science* **168**, 1447 (1970).
16. Supported by NSF grants BMS-74-18294 (D.E.G.) and DEB-76-82573 (K.J.N.).

17 February 1977; revised 8 April 1977

Geochemistry and Thermolysis of Flavonoids

Abstract. *The thermal and* pH *stability of selected flavonoids has been determined under simulated geologic conditions. Thermolytic rates and products for various regimes, as determined by gas chromatography–mass spectroscopy, indicate the potential usefulness of flavonoids as thermometric indicators in sediments. The parametric factors affecting flavonoid stability are used to geochemically characterize angiosperm "green leaves" (36 to 25 × 10⁶ years old) from Succor Creek and indicate that these sediments have not experienced temperatures higher than 80°C or extreme* pH *shifts (beyond the range 6.3 to 7.2) during postdepositional maturation.*

The isolation and identification of flavonols, dihydroflavonols, and glycoflavones from Oligocene to Miocene angiosperm leaf fossils (36 to 25 × 10^6 years old) (*1*) suggest that very mild geologic factors were associated with postdepositional maturation of these sediments. Various geophysical and geochemical factors may affect the diagenesis of organic compounds (*2*) and severely alter more labile functional groups (such as OH and COOH) or individual organic constituents. Through model experiments with pure compounds geologic conditions may be simulated and diagenetic pathways of organic compounds may be assessed. (*3*). In an attempt to determine the relative thermal and *p*H stability of various flavonoids, pure standards of dihydrokaempferol, kaempferol, quercetin, and robinin were packed in sealed glass tubes with extracted bentonite clay (*4*) and subjected to various regimes of heat and *p*H (*5*). Bentonite clay was used as the inorganic matrix partly because previous investigators have used this material and it would thus be possible to directly compare the results with published data, but more importantly because this clay is formed from the in situ alteration of volcanic ash. The fossil material that we analyzed from the Succor Creek Flora (*1*) is preserved in a pyroclastic deposit (tephra) similar to bentonite. At various time intervals, the tubes containing organic constituents were opened in a hydrogen atmosphere, and the volatile reaction products were stripped off with a 150°C stream of hydrogen and collected in a liquid nitrogen–cooled vessel. The low-molecular-weight fraction was analyzed by gas chromatography, and nonvolatile reaction products were extracted from the clay and identified by gas chromatography–mass spectroscopy (*6*).

The thermolytic products of a flavanone (dihydrokaempferol) and a flavonol (kaempferol) under alkaline conditions are shown in Fig. 1. Dihydrokaempferol (aromadendrin) occurs widely as the phenol, the 3-*O*-rhamnoside engelitin, the 7-*O*-rhamnoside, and the 7-*O*-glucoside sinensin (*7*), and is thus a useful compound from which to derive a preliminary scheme of flavonoid diagenesis. Under alkaline conditions (*p*H 8.5) the flavanone (A in Fig. 1) undergoes thermal hydrolysis (B → C), with subsequent reduction producing the *p*-hydroxyphenylethanol (G) from *p*-hydroxyacetophenone, and *p*-hydroxybenzyl alcohol (F′) from the hydroxybenzoic acid (D′). The major products of this reaction are phloroglucinol (F) and 2,4,6-trihydroxyacetophenyl (E′). Basic hydrolysis and subsequent oxidation (A and I to L) yield the tetrahydroxyacetophenyl (K) and *p*-hydroxybenzoic acid (L). On the basis of the relative amounts of the products detected, the reaction A to F (or F′) seems preferred under these conditions. Thermal degradation products from the flavanone were not detected until sustained (5 days) temperatures at 100°C were achieved. Dihydrokaempferol yields kaempferol with peroxides and under alkaline conditions (A → H); subsequent thermolysis produces a tetrahydroxyacetophenyl and hydroxybenzoic acid as the major products. Under simulated geologic conditions the reaction A to H was not observed unless peroxide was added to the bentonite clay. The reaction H to L proceeded at 80°C after 4 days; before