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# Hot-Water Extractives of the Leaves of *Populus heterophylla* L.

Irwin A. Pearl* and Stephen F. Darling

Fresh May leaves of swamp cottonwood (*Populus heterophylla* L.) were extracted with ethanol, and the hot water soluble portion was fractionated by means of ethyl acetate extraction and polyamide chromatography employing step-gradient elution with water and dilutions of ethanol. The results obtained for the leaves of this species from the *Leucoides* section of the *Populus* genus were entirely different from those obtained in the past from leaves of species of all other sections of this genus. Thus, salicin and salicortin were not found, but tremulacin was. *C*-Glycosyl flavones such as vitexin and orientin were found for the first time in the Salicaceae family. A new diterpenoid, heterophyllin, is the major component in the water-soluble extractives of these leaves.

In our continuing investigations on the components of the barks and leaves of *Populus* species, fresh leaves of swamp cottonwood (*P. heterophylla* L.) were gathered from a tree in Quitman County, Miss. in May and were covered immediately with ethanol. The wet leaves were extracted with ethanol by the Waring Blendor technique (Pearl and Darling, 1970), and all ethanol extracts were combined. The hot water soluble portion of the ethanol extractives was extracted fractionally with ethyl acetate, and the ethyl acetate soluble fractions were chromatographed on columns of polyamide and eluted with water, followed by 20% ethanol and 50% ethanol as described previously (Pearl and Darling, 1968, 1970). As in previous studies, all eluate fractions were monitored by thin-layer chromatography, concentrated to small volumes, allowed to stand, filtered if crystals separated, and finally freeze-dried. Weights of all fractions and of separated crystals were noted, and elution curves were obtained.

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## RESULTS

Fractional ethyl acetate extraction of the hot water extractives representing 1230 g of original oven-dry leaf solids yielded 2.7% of the first extract (A), 2.7% of the second extract (B), and 1.4% of the third extract (C).

Data for the polyamide chromatograms of the three ethyl acetate fractions are presented in Figures 1–3. The weights noted in these figures are actual weights obtained experimentally from the sample aliquots applied to the polyamide column 50 mm in diameter and 80 cm in length. Major components under the peaks are noted on the figures. Quantitative data for the crystalline components isolated from the three ethyl acetate fractions are given in Table I.

The crystalline products isolated in the polyamide chromatograms of Figures 1, 2, and 3 and noted in Table I were quite different from most of the compounds isolated in previous studies of *Populus* species bark and leaf extractives. The most surprising fact was the absence of

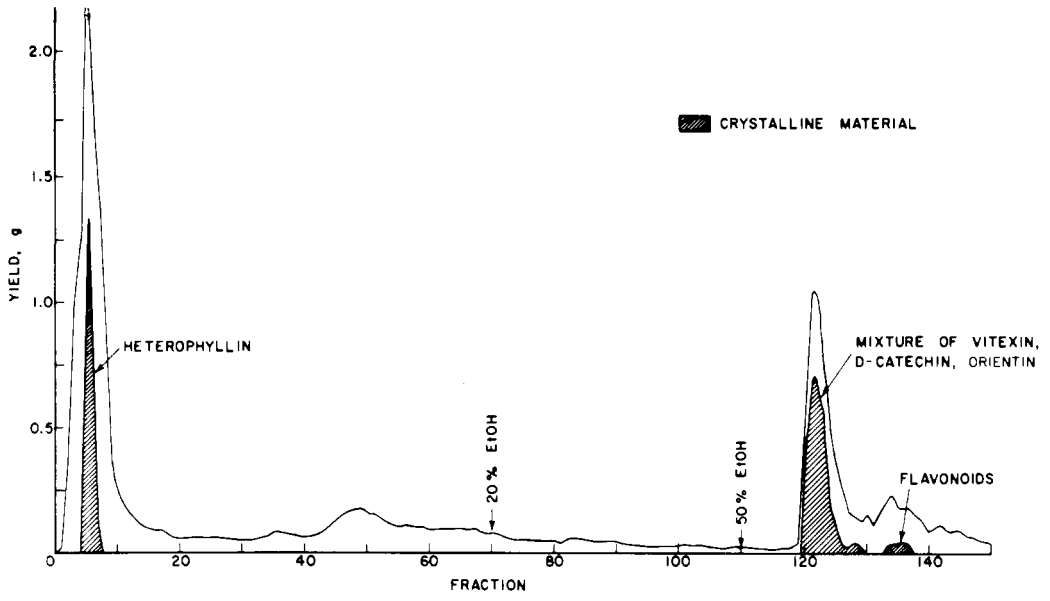


**Figure 1.** Column chromatography of first ethyl acetate extract.

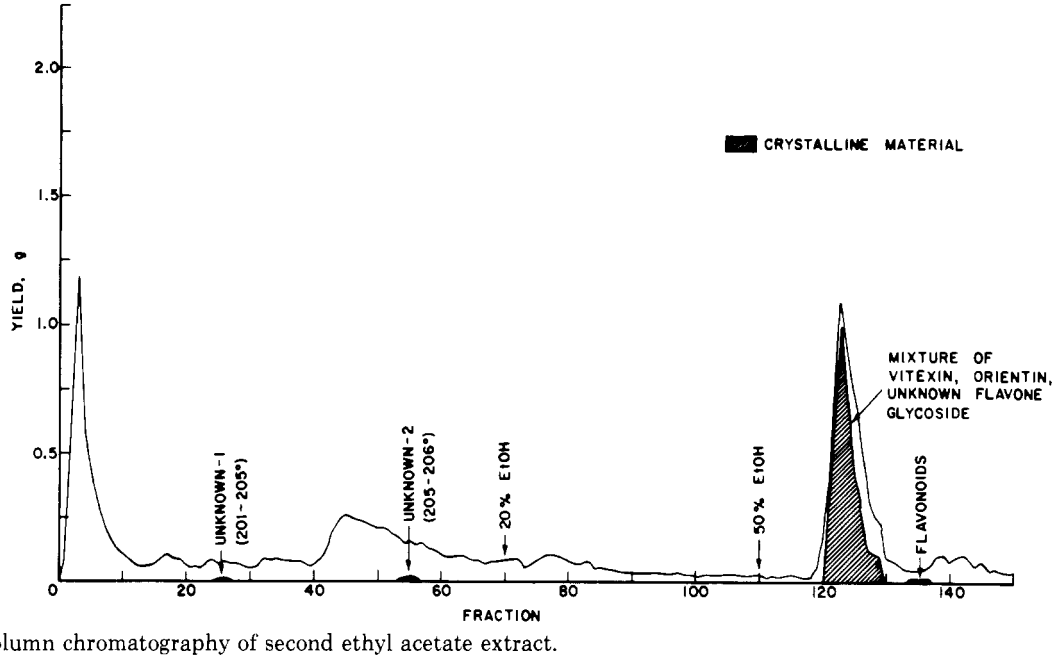


**Figure 2.** Column chromatography of second ethyl acetate extract.

**Table I. Crystalline Components of Ethyl Acetate Soluble Fractions of Hot-Water Extractives of the Leaves of *Populus heterophylla* L.**

| Component | Elution fraction | Extract A yield, g | Extract B yield, g | Extract C yield, g | Total yield g | Total yield %[a] |
|---|---|---|---|---|---|---|
| Crude extract | | 33.5 | 33.5 | 17.2 | 84.2 | 6.85 |
| Heterophyllin | 4-7 | 3.76 | | | 3.76 | 0.31 |
| Unknown 1 (mp 201-205 °C) | 25-27 | | 0.03 | | 0.03 | 0.002 |
| Tremulacin | 51-76 | | | 1.2[b] | 1.2 | 0.10 |
| Unknown 2 (mp 205-206 °C) | 54-56 | | 0.014 | | 0.01 | 0.001 |
| Rutin | 120-124 | | | 0.34 | 0.34 | 0.028 |
| Vitexin, orientin, *d*-catechin | 120-129 | 1.99 | | | 1.99 | 0.16 |
| Vitexin, orientin, flavone glycoside | 121-129 | | 3.44 | | 3.44 | 0.28 |
| Vitexin | 127-129 | | | 0.12 | 0.12 | 0.01 |
| Flavonoids | 134-137 | 0.03 | 0.01 | | 0.04 | 0.003 |
| Total solids recov. from eluate | | 24.17[c] | 21.41[d] | 11.32[e] | 56.90 | 4.63 |

[a] On basis of 1230 g of original g oven-dry leaf solids. [b] Did not crystallize immediately. Identity established by TLC and crystallization of ω-salicyloyltremuloidin on long standing. [c] Represents 72.1% of material applied to column. [d] Represents 63.9% of material applied to column. [e] Represents 63.9% of material applied to column.

salicin and salicortin in the early fractions of the polyamide chromatograms and the presence in these fractions of a new diterpenoid, heterophyllin. Salicin and salicortin have always been found in the early fractions obtained from the

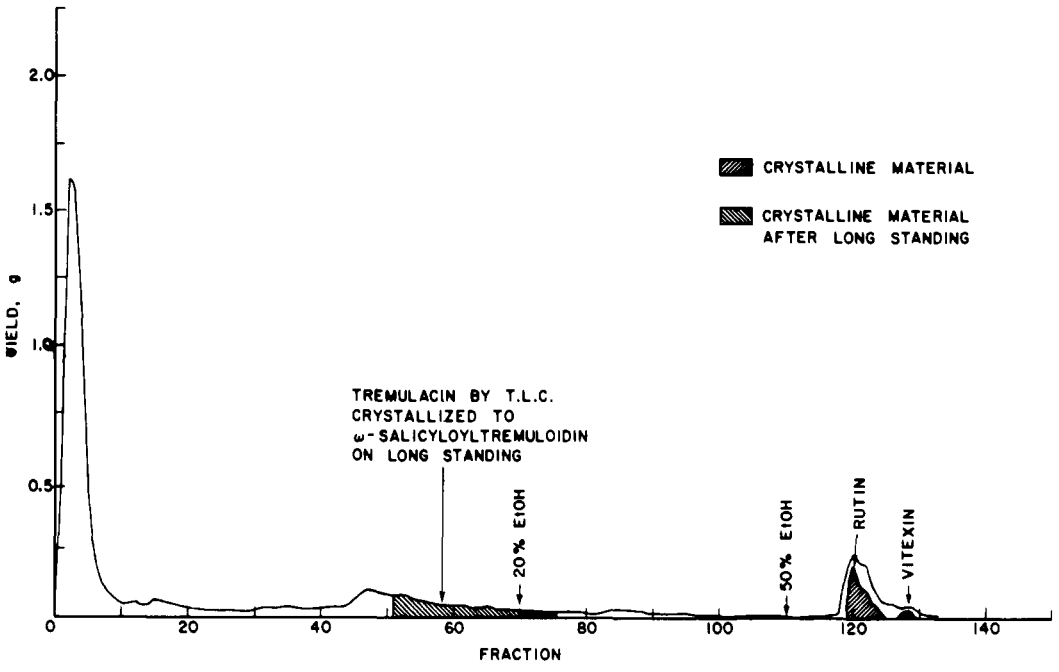


**Figure 3.** Column chromatography of third ethyl acetate extract.

barks and leaves of every species of *Populus* (and *Salix*) studied in our laboratories. This finding suggests a substantial taxonomic difference between *P. heterophylla*, a member of the *Leucoides* section of the *Populus* genus (FAO Forestry and Forest Products Studies, 1958) and the species of all other sections of this genus. The finding of vitexin and orientin in the leaves of *P. heterophylla* was the first finding of these *C*-glycosyl flavones in any *Populus* species and is the first reported finding of *C*-glycosyl flavones in the Salicaceae family.

**Isolation and Properties of Heterophyllin.** The crystals which separated from fractions 4–7 of Figure 1 were recrystallized from water to give large colorless beveled plates melting at 243–244 °C and having optical activity. The material was named "heterophyllin". Attempted hydrolysis with dilute sulfuric acid produced no sugar whatsoever, but yielded two ether-soluble components, one of which appeared to be "unknown 1" of Table I. Elemental analysis and mass spectrum suggested the formula $C_{20}H_{32}O_6$, and infrared spectrophotometry indicates the presence of hydroxyl and aliphatic carbon–hydrogen groups, but no carbonyl groups or aromatic rings. Carbon-13 NMR spectra, including off-resonance and coupled spectra, confirm the molecular formula and indicate five $CH_3$, three $CH_2$, and four CH carbons not bound to oxygen. There are also two quaternary aliphatic carbons not bound to oxygen. One methyl group is probably fairly isolated from protons since its coupled spectrum is a fairly clean quartet. The others are probably next to CH or $CH_2$ carbons. The NMR spectra indicate a total of 32 hydrogens including five hydroxyls and one carbon with two oxygens and are suggestive of a sugar unit. The structure of heterophyllin is still unresolved.

**Identification of Flavonoid Components.** The following flavonoid components were identified in the indicated fractions by comparison with authentic samples using infrared and ultraviolet spectrophotometry, cochromatography, and mixed melting point: vitexin, orientin, *d*-catechin, and rutin.

**Identification of Tremulacin.** In the chromatogram of Figure 3, evaporation of fractions 51–76 to small volume and freeze-drying yielded colorless syrups which gave the characteristic spots of tremulacin on TLC. These fractions were dissolved in tetrahydrofuran, combined, concentrated, and allowed to stand for several years. The entire combined fraction crystallized. The crystals were recrystallized to yield fine white balls of salicyloyltremuloidin melting at 192–193 °C, identified by mixed melting point and identity of infrared spectra with authentic material. Two other tremulacin hydrolysis products, tremuloidin and salicyloylsalicin, were identified in the filtrate and washings from the salicyloyltremuloidin.

## DISCUSSION

The results obtained in this study on *P. heterophylla*, a representative of the *Leucoides* section of the *Populus* genus, were startling because they were so different from those obtained in the past from three other sections of the *Populus* genus, *Leuce*, *Aigeiros*, and *Tacamahaca*. The diterpenoid heterophyllin was the major component under the first large peak (fractions 2–10) of all three polyamide chromatograms shown in Figures 1, 2, and 3 in the case of *P. heterophylla* leaves, whereas salicortin and/or salicin have always been the chief components under peaks of polyamide chromatograms of all other *Populus* species leaves and barks studied in our laboratories. No indication whatsoever of salicortin or salicin was found in any of the fractions of the three chromatograms of the present study.

The absence of salicortin and salicin was even more surprising in view of the finding of substantial amounts of tremulacin, the 2-*O*-benzoyl derivative of salicortin in *P. heterophylla* leaves. Tremulacin has been found in the past in *P. tremuloides* and *P. grandidentata* of the *Leuce* section and in *P. trichocarpa* of the *Tacamahaca* section, and always accompanied by larger amounts of salicortin. Tremulacin has not been found in *P. deltoides* of the *Aigeiros* section or in *P. balsamifera* of the *Tacamahaca* section.

Although noted in Table I as a crystalline compound, tremulacin was actually obtained as a colorless syrup which was identified as tremulacin by TLC and by the fact that on long standing it deposited crystals of salicyloyltremuloidin. Tremulacin always appears in fractions 50–75 in polyamide chromatograms run under the conditions employed in this experiment, whereas salicyloyltremuloidin always appears in fractions 170–180 under these conditions

(Pearl and Darling, 1970). We have demonstrated in the past (Pearl and Darling, 1971a) that tremulacin is converted rapidly in acidic medium to salicyloyltremuloidin, and the latter is hydrolyzed to tremuloidin and/or salicyloylsalicin depending on conditions. Chromatographic evidence for both salicyloylsalicin and tremuloidin was found in the filtrate from the salicyloyltremuloidin obtained after years of standing.

The first major peak in all three chromatograms and the second major peak in Figures 1 and 2 represented components not found in any other *Populus* species studied to date. The first peak is the new diterpenoid, heterophyllin. Although elemental analysis and mass, infrared, and C-13 NMR spectra have identified essentially all of the individual atoms present, the unequivocal structure cannot be assigned without more chemical evidence.

The second major peak in Figures 1 and 2 is due to the *C*-glycosyl flavones, vitexin and orientin. This is the first instance of finding any *C*-glycosyl flavonoid in the Salicacea family.

In the chromatogram of Figure 3, the second major peak comprises rutin with just a trace of vitexin. Rutin has been found previously in *P. tremuloides* barks and leaves (Pearl and Darling, 1971b).

## EXPERIMENTAL SECTION

**Preliminary Processing of Leaves.** Fresh leaves gathered from the crowns of four young female *P. heterophylla* L. trees in Quitman County, Miss. in May were weighed and covered immediately with ethanol. The ethanol was decanted, and the wet leaves were extracted and processed by the Waring Blendor technique (Pearl and Darling, 1970). All ethanol extracts were combined. A total of 3515 g of fresh leaves containing 1230 g of oven-dry solids was processed in five equal batches, and the combined extract was concentrated to a smaller volume in a vacuum circulating evaporator and to dryness in a vacuum rotating evaporator. The residue was covered with 4 L of water, stirred, heated on a steam bath, and allowed to stand overnight. The aqueous extract was filtered, concentrated to 1 L in a vacuum rotatory evaporator, and extracted fractionally with ethyl acetate. The ethyl acetate fractions were applied to large polyamide columns as described previously (Pearl and Darling, 1968), eluting with water, and collecting 200-mL samples in the eluate. After collecting 70 fractions of aqueous eluate, the eluting solvent was changed to 20% ethanol, and after fraction 110, the eluting solvent was changed to 50% ethanol. All eluate fractions were monitored by TLC on silica gel, developed with 4:1 chloroform–methanol containing a little acetic acid, and detected with sulfuric acid and heat. Flavonoid-containing samples were also monitored by TLC on Polygram Cel, developed with 30% acetic acid, and detected with ultraviolet and visible light before and after spraying with sodium carbonate solution. Eluate fractions were concentrated under reduced pressure to approximately 5-mL volume and allowed to stand. If crystals separated, they were separated and weighed. Filtrates and fractions which did not crystallize were freeze-dried and weighed. Data are given in Figures 1–3.

**Isolation and Properties of Heterophyllin.** A total of 3.76 g of crystalline solid separted from eluate fractions 4–7 of the chromatogram of Figure 1. These were recrystallized from water to give large colorless beveled plates melting at 243–244 °C, $[\alpha]^{23}_{D}$ +4.26 (*c* 3.0 in water).

Anal. Calcd for $C_{20}H_{32}O_6$: C, 65.19; H, 8.75; mol wt, 368. Found: C, 64.90, 64.95; H, 8.63, 8.66; mol wt by mass spectrometry, 368.

Its IR spectrum contained bands at 2.95 (broad), 3.37, 3.39, 3.43, 3.48, 6.15, 6.82, 7.10, 7.24, 7.40, 7.52, 7.60, 8.02, 8.25, 8.38, 8.54, 8.91, 9.42, 9.72, 9.81, 10.22, 10.44, 10.57, 10.94, 11.28, 11.52, 11.83, 12.43, 13.46, 13.60, 14.15, 14.80, 16.4, 17.0, 17.6, 19.4, 20.3, 23.3, 24.7, and 27.0 $\mu$. Its mass spectrum contains the following major and important $m/e$ peaks: 107 (25.7), 109 (24.8), 111 (20.2), 121 (26.6), 123 (24.8), 135 (24.8), 139 (28.4), 201 (23.8), 211 (25.7), 229 (49.5), 243 (34.9), 272 (20.2), 289 (32.1), 290 (24.8), 307 (25.7), 308 (21.1), 325 (27.5), 300 (45.0), 301 (27.7), 302 (20.2), 304 (100), 307 (69.7), 308 (45.9), 309 (26.6), 314 (23.8), 315 (28.6), 317 (24.8), 325 (58.6), 326 (45.8), 332 (42.2), 333 (34.0), and $M^+$ 368 (5.5).

Its C-13 NMR in $Me_2SO$-$d_6$ gave the following peaks in ppm from $Me_4Si$: 10.05 (95), 15.18 (72), 17.91 (100), 18.36 (75), 21.80 (64), 33.13 (53), 33.89 (49), 34.88 (52), 36.11 (38), 37.27 (12), 37.33 (12), 38.35 (25), 39.40 (32), 39.93 (8), 40.46 (91), 41.51 (14), 44.31 (31), 52.25 (42), 55.59 (41), 60.59 (59), 74.20 (52), 77.48 (41), 81.20 (29), 81.29 (58), 85.56 (30), 85.63 (54), 89.02 (37), and 104.12 (37). Off-resonance and coupled spectra were also obtained. Proton NMR spectra in $Me_2SO$-$d_6$ before and after deuteration suggested five hydroxyl groups and the absence of aromatic, aldehydic, and carboxyl groups and olefinic protons.

Heterophyllin (10 mg) was covered with 2 mL of 1 *N* sulfuric acid and heated on the steam bath for 1.5 h. The dark solution containing precipitate was chromatographed on paper for sugars, but none was detected.

Heterophyllin (100 mg) was mixed with 5 mL of 0.1 N sulfuric acid, and the mixture was boiled under reflux for 30 min. The solution became clear and then deposited crystalline material. The cooled mixture was filtered, and the crystals were washed with water and dried to give 47 mg of crude unknown 1 which when recrystallized from dilute ethanol melted at 201–204 °C and did not depress a mixed melting point with unknown 1. Infrared spectra of the two compounds were identical, and thin-layer chromatography gave identical spots. Another 55 mg of crude compound 1 was recovered by extraction of the aqueous filtrate and washings with ether.

**Isolation of Unknown 1.** The rosettes of crystals which separated from fractions 25–27 of Figure 2 amounted to 30 mg. TLC on silica gel developed with 4:1 chloroform–methanol and detected with sulfuric acid and heat gave a maroon spot at $R_f$ 0.80 identical with that produced by the acid reaction product of heterophyllin. Its IR spectrum contained bands at 2.95 (broad), 3.28, 3.38, 3.42, 3.48, 3.52, 6.15 (broad), 6.81, 6.90, 7.25, 7.39, 7.50, 7.79, 7.94, 8.05, 8.37, 8.85, 9.01, 9,22, 9.26, 9.36, 10.13, 10.34, 10.60, 10.85, 11.35, 11.70, 11.85, 12.20, 12.50, 13.45, 14.80, 16.50, 17.70, and 18.25 $\mu$.

**Isolation of Unknown 2.** Concentration of fractions 54–56 of Figure 2 yielded 14 mg of colorless crystals melting sharply at 205–206 °C. TLC as with unknown 1 gave a lavender spot almost at the origin. Diazotized *p*-nitroaniline spray followed by sodium carbonate gave a yellow spot.

**Identification of Tremulacin.** Freeze-drying of the evaporated fractions 51–76 of the chromatogram of Figure 3 yielded colorless syrups. These were monitored by TLC on silica gel, developed with 4:1 chloroform–methanol, and sprayed with sulfuric acid, followed by short heating. The major spot in all cases, and the only spot in some, was a brick-red spot at $R_f$ 0.62 identical with that of authentic tremulacin (Pearl and Darling, 1971a). Tremulacin was not crystallized, but all the fractions were dissolved in moist tetrahydrofuran, combined, and concentrated to a small volume. After standing for 5 years, the solvent had evaporated, and the product had crystallized. The crystals

were slurried in cold dilute ethanol, filtered, and recrystallized from dilute ethanol to give fine white balls of salicyloyltremuloidin melting at 192–193 °C identified by mixed melting point and identity of infrared absorption spectrum with authentic material (Pearl and Darling, 1965).

The combined filtrates and washings from the salicyloyltremuloidin were spotted on silica gel plates alongside authentic tremuloidin (Pearl and Darling, 1959) and salicyloylsalicin (Pearl and Darling, 1965), developed in the 4:1 chloroform–methanol developer, and sprayed with sulfuric acid to give spots only for these two compounds.

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# Scanning Electron Microscopy of Kenaf Paper Structures

Gerald F. Touzinsky, Frederick L. Baker, R. Leo Cunningham, and Marvin O. Bagby*

Scanning electron microscopy was used to study papermaking properties of soda pulps prepared identically from whole kenaf and from its separated bark and core. The behavior of the various cellular materials and fiber preparations was examined at different stages of the web-forming process on a laboratory fourdrinier machine. Critical-point drying was used to preserve the configuration of the mat and the fine structure of the individual fibers. Web structure for all pulps remained very open throughout the wet end, and finally coalesced to form a well-bonded sheet in the last stages of machine drying. Kenaf bark pulp responded to beating much the same as did a southern pine kraft pulp. Core pulp does not become highly fibrillated during beating but shows increased flexibility. On drying, core fibers collapsed into intimate contact to give a tightly compacted sheet. Behavior of whole kenaf pulp was a composite of bark and core behaviors.

Kenaf (*Hibiscus cannabinus*) is a promising, annually renewable, papermaking raw material. Recent economic comparisons of kenaf with pulpwood and major annual crops of the southeastern United States place it in a competitive position (Moore et al., 1976). Pulps with desirable papermaking properties have been prepared by commercial chemical processes (Clark et al., 1962; Clark and Wolff, 1965). Papers containing considerable kenaf fiber in the furnish have been made on commercial paper machines (Clark et al., 1971b; Jeyasingam, 1974; Cathirgamu and Manokeran, 1975).

Being a dicotyledon, kenaf has two distinct regions to its stem: an outer bark and an inner, woody core. Relative to the core, the bark contains more cellulose and correspondingly less pentosan and lignin (Clark et al., 1971a). These chemical differences cause higher yields and greater ease of chemical pulping of the bark (Clark et al., 1971a; Touzinsky et al., 1973). Nieschlag et al. (1961) reported average lengths of 2.60 and 0.60 mm for bark and core fibers, respectively. Core fibers have a lower ratio of cell wall thickness to cell diameter than do bark fibers (Clark et al., 1967), and this contributes to their greater flexibility (Touzinsky et al., 1972). These fiber differences produce the greater strength and porosity of bark sheets and the greater smoothness and density of core sheets (Touzinsky et al., 1972; Nagasawa and Yamamoto, 1970). In combination, bark and core pulps show anomalous strengths exceeding those expected for the combination (Touzinsky et al., 1972). Clark and Wolff (1962) reported related synergistic effects for blends of whole kenaf and wood pulps.

To understand better the strength characteristics and papermaking properties of kenaf, we examined the mechanical behavior of bark, core, and whole kenaf pulps during web formation on a fourdrinier, laboratory paper machine and compared their relative responses to those of a commercial softwood pulp. In this report, we discuss

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