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FLAVONOIDS FROM THE WOOD OF *SALIX CAPREA* AS INHIBITORS OF WOOD-DESTROYING FUNGI

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ABSTRACT.—The flavonoids dihydrokaempferide (**1**), naringenin (**2**), aromadendrin (**3**), taxifolin (**4**), prunin (**5**), and (+)-catechin (**6**) have been isolated from the wood of *Salix caprea*. Their inhibitory effect on some microorganisms has been tested.

Out of five rot-producing fungi tested, *Coniophora puteana*, *Sporotrichum pulverulentum*, and *Trichoderma viride* are inhibited by some of the flavonoids, while *Chaetomium globosum* and *Myrothecium verrucaria* are not inhibited. Ten bacteria and nonrot-producing fungi tested show slight inhibition. Thus, the flavonoids form part of the plant's defense against attack by rot fungi, but the effect is not a general antimicrobial one.

Salix caprea L. is a small tree common throughout northern Europe. Among its varied uses is the making of fence poles for use in wet and marshy areas because the *S. caprea* wood shows greater resistance to rotting than wood from other common trees (1,2).

Several workers have studied the composition of the flowers, leaves, and bark of this plant. Nasudari (3) isolated diosmetin, isorhamnetin, and glycosides of these flavonoids from the flowers, as well as luteolin-7-glucoside from the leaves (4). The presence of (+)-catechin, (+)-gallocatechin, and leucocyanidin in the leaves was demonstrated by Jaggi and Haslam (5).

Phenol glycosides such as salicin and salicortin, which are common in the bark of other *Salix* species, are absent or present in only low concentrations in *S. caprea* bark (5,6). The main glycoside is reported to be triandrin, a glucoside of 4-hydroxycinnamyl alcohol (6). Tannins are present in the bark in an amount of 8-13% (7).

To our knowledge, no reports have appeared on the composition of the wood of this species. Due to the reputed resistance of the wood against rot-producing fungi, we have investigated the constituents of an extract from the wood of *S. caprea*.

RESULTS AND DISCUSSION

On extraction of *S. caprea* wood with EtOAc, a 5% yield was obtained. The nmr spectrum of the crude extract showed the presence of lipids (mostly glycerides) and aromatic substances. Column chromatography on silica gel with toluene yielded lipids (49% of the crude extract). The composition of the lipid fraction is under investigation and will be reported elsewhere.

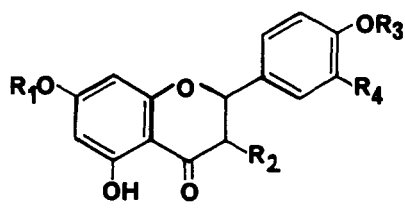
Elution with toluene/EtOAc mixtures, followed by EtOAc, Me_2CO, and MeOH gave several flavonoid-containing fractions. Preparative tlc and recrystallization furnished chromatographically and spectroscopically pure substances.

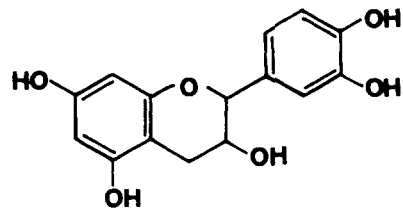
From the fractions eluted with 5% EtOAc in toluene, 3,5,7-trihydroxy-4′-methoxyflavanone (dihydrokaempferide, **1**) was isolated. This is a rare flavonoid, hitherto only known from the wood of *Prunus cerasus* L. (8). Its 3-glucuronide has been isolated from the roots of *Cleome viscosa* L. (9).

With 20% EtOAc in toluene, the flavanone naringenin (**2**) was eluted (identification: spectra, comparison with authentic sample). Naringenin is a common flavonoid, but to our knowledge, this is the first report of this substance within the Salicaceae (10).

From the fractions eluted with 50% EtOAc in toluene, two flavonoids were isolated. From spectroscopical data and mp's, these were identified as the flavanonols aromadendrin (**3**) and taxifolin (**4**). Both are fairly common in nature, but neither substance seems to be known as a constituent of the Salicaceae (10).

With pure EtOAc, two flavonoids were eluted, which, after purification were identified as (+)-catechin (**6**) and 7-glucosyloxy-5,4′-dihydroxyflavanone (prunin, **5**) (spectra, comparison with authentic samples). Prunin has been reported as a constituent of the leaves of *Salix purpurea* L. (11). (+)-Catechin is known as a component of the leaves of several *Salix* species (5), one of which is *S. caprea*.





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1 R_1 and R_4=H, R_2=OH, R_3=CH_3
2 R_1, R_2, R_3, R_4=H
3 R_1, R_3, R_4=H, R_2=OH
4 R_1 and R_3=H, R_2 and R_4=OH
5 R_1=glucose, R_2, R_3, R_4=H

Bacteriostatic and fungistatic effects of the substances isolated were studied using an agar diffusion method as described by Malterud and Faegri (12). Initially, the flavonoid solutions to be tested were made by dissolving the substances in 0.1M aqueous NaOH and immediately adjusting the pH to between 9 and 9.5 with 0.1M HCl. In later work, it was found easier to use Macrogol 400 (a polyethylene glycol) as solvent. The results obtained seemed independent of the solvent used. The rot-producing microorganisms used in the test were the brown rot fungi *Coniophora puteana* and *Myrothecium verrucaria*, the white rot fungus *Sporotrichum pulverulentum*, and the soft rot fungi *Chaetomium globosum* and *Trichoderma viride*. In addition, some bacteria and fungi that do not cause rotting were tested: the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus faecalis*, and the fungi *Aspergillus fumigatus, Aspergillus niger, Candida albicans, Penicillium notatum, Rhizopus nigricans*, and *Trichophyton mentagrophytes*. The commercial fungicides Captan (*N*-trichloromethylmercapto-4-cyclohexene-1,2-carboximide) and Quintozene (pentachloronitrobenzene) were used as controls.

The inhibitory effects toward rot-producing fungi are shown in Table 1. Out of five fungi tested, three showed inhibited growth when treated with the flavonoids from *S. caprea* wood. One soft rot fungus, *C. globosum*, and one brown rot fungus, *M. verrucaria*, were unaffected and have been left out of the table. Naringenin (**2**) is the only substance tested that inhibits all the fungi that respond to flavonoid treatment. Dihydrokaempferide (**1**) inhibits *C. puteana*, as does aromadendrin (**3**), while taxifolin (**4**) and (+)-catechin (**6**) show an effect against *T. viride*, and prunin (**5**), the only glycoside tested, an effect against *S. pulverulentum*, the only white rot fungus tested. From these results, it would seem that the effect of flavonoids on rot-producing fungi is a selective one: All of the flavonoids tested showed some antifungal activity, but most of them were active against only one of the fungi tested. At present, it seems difficult to point out any correlation between structure and fungistatic activity. It seems, however, reasonable to infer that the flavonoids present in the wood do, in fact, contribute to the resistance to rotting shown by *S. caprea* wood.

Taxifolin from the wood of *Pseudotsuga menziesii* (Mirb.) Franco has been demon-

TABLE 1. Inhibition of Rot Producing Fungi by *Salix caprea* Flavonoids and by Two Commercial Antifungal Substances

	Coniophora puteana	*Sporotrichum pulverulentum*	*Trichoderma viride*
Dihydrokaempferide[a]	(+)[b]	−	−
Naringenin	+	+	+
Aromadendrin	+	−	−
Taxifolin	−	−	+
(+)-Catechin	−	−	+
Prunin	−	++	−
Captan (1%)	+++	++	+++
Captan (0.01%)	+	−	++
Quintozene (1%)	+	(+)	+

[a]Flavonoids are in 1% solution. Macrogol 400 was used as solvent.

[b]Since the inhibition zones often are irregular, the following symbols have been used: (+), estimated average zone of inhibition less than 10 mm; +, 10 to 15 mm; ++, 15 to 25 mm; +++, above 25 mm. Medium used: malt extract agar.

strated to inhibit the growth of the wood-destroying fungi *Fomes annosus* and *Lentinus lepideus* (13), while both taxifolin and (+)-catechin are known to act as inhibitors of *F. annosus* in the bast of *Picea abies* Karst. (14). To our knowledge, no work has been published on the antifungal effects of the other flavonoids reported here.

In Table 2, the effects toward some other microorganisms are shown. Most of the bacteria and fungi tested are unaffected by the flavonoids and have been left out of the table. Here, as well, naringenin shows the strongest inhibitory activity. However, no general antimicrobial effect is evident.

TABLE 2. Inhibition of Bacteria and Non-rot Fungi by Flavonoids from *Salix caprea* Wood

	Escherichia coli	*Streptococcus faecalis*	*Staphylococcus aureus*	*Trichophyton mentagrophytes*
Dihydrokaempferide, PDM[a]	−	−	8(13.4)[b]	−
PDM-blood	−	−	−(10.8)	−
Naringenin, PDM	8	10	17.2(20.7)	11
PDM-blood	−	−(13.4)	−(17.9)	−

[a]Flavonoids are in 1% aqueous solution, pH 9-9.5.

[b]Absolute inhibition zones are totally devoid of growth, relative inhibition zones (in parentheses) show distinctly inhibited growth. Values are in mm. Media used are PDM Antibiotic Sensitivity Medium or PDM with 5% defibrinated horse blood added.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Spectra were recorded with the following instruments: uv, Beckman 26; ir, Beckmann Acculab 2; ^{1}H nmr, JEOL PMX60Si; eims, Micromass 7070E, direct inlet, 70 eV ionization potential. Tlc, both analytical and preparative, was carried out on Merck Dünnschichtfolien, Si gel 60F254, thickness 0.2 mm, where no other indication is given. In preparative tlc, sample amounts of 10-20 mg/plate were applied. Detection was by uv irradiation (254 nm), fractions were recovered by extraction with Me_2CO. Mp's were obtained with a Reichert melting point microscope and are uncorrected. Evaporation of solvent was carried out on a rotary evaporator at a temperature of 40-50°. Commercial (+)-catechin and taxifolin were used for purposes of comparison. Naringenin and prunin standards were obtained by hydrolysis of naringin, while aromadendrin was obtained by reduction of kaempferol with sodium dithionite.

Microorganisms used were from the collection of the Department of Microbiology, Institute of Pharmacy, The University of Oslo (strain designation Sc), or from the collection of the Institute of Microbiology and Plant Physiology, The University of Bergen, Norway (other strain designations).

PLANT MATERIAL.—*S. caprea* stems were collected in Nordre Land community during the summers of 1981 and 1982. Samples have been deposited in the herbarium at the Institute of Pharmacy, Department of Pharmacognosy.

After removal of the bark and cambium, the wood was cut to pass through a 4-mm sieve and extracted with EtOAc, 5 liters per kilo wood, in a Soxhlet extractor for 5 h. Extract yield was ca. 5% (49 g from 986 g of fresh wood).

ISOLATION OF SUBSTANCES.—Chromatography of the wood extract on a Si gel column (53×7 cm, 70-230 mesh) with toluene yielded 23.8 g of nonpolar material. The fractions eluted with 5% EtOAc in toluene contained 0.9 g of substance, the main constituent of which was isolated by preparative tlc (5% EtOAc in toluene) and recrystallized from aqueous MeOH. Mp 210-211° [lit. for dihydrokaempferide (**1**) 204-205° (8), 220-222° (9)], uv and ir spectra in accordance with lit. for **1**. ^{1}H nmr (60 MHz, CD_3COCD_3) δ 3.79 (3H, s, OCH_3-4′), 4.57 (1H, d, *J* 11 Hz, H-3), 5.01 (1H, d, *J* 11 Hz, H-2), 5.88 (2H, narrow m, H-6 and H-8), 6.80-7.48 (4H, AA′BB′ system, H-2′, 3′, 5′, 6′), 12.5 (1H, broad s, 5-OH). Eims *m/z* (%) 302 (M, 7), 273 (M-CHO, 10), 153 [A-ring fragment, retro Diels-Alder reaction with hydrogen transfer (splitting between O_1-C_2 and between C_3-C_4),23], 150 [B-ring fragment, RDA reaction (splitting as above),13], 135 (O=C_6H_4=CH-CHOH, 5), 121 (CH_3O-C_6H_4-CH_2, 17).

The column fractions eluted with 20% EtOAc in toluene (0.8 g) gave, besides **1**, naringenin (**2**) as the main product by preparative tlc (50% EtOAc in toluene). Mp 251-254° [lit. (15) 252-253°]. Identification was made by mp, mixed mp, spectra (uv, ir, nmr, ms) and co-tlc with authentic sample.

From the column fractions eluted with 50% EtOAc in toluene (0.7 g), two substances were isolated by preparative tlc (40% Me_2CO in toluene) and purification by recrystallization from aqueous MeOH. Of these, one had mp 220-221° [lit. for aromadendrin (**3**) 222-224°, 246° (16), depending on the type of glass in which the mp was determined]. Spectra in accordance with literature for **3**. The second substance had mp 228-232° [lit. (15) for taxifolin (**4**) 238-240°], but had spectra in accordance with literature for **4**. Substances **3** and **4** co-chromatographed with authentic aromadendrin and taxifolin, respectively.

The main substance of the EtOAc fractions (6.8 g), isolated by crystallization from aqueous MeOH, then from H_2O, mp 175-177°, was identified as (+)-catechin (**6**), mp (15) 173-176°, by comparison with authentic substance (spectra, co-tlc, no mp depression). From these fractions, another substance was isolated by preparative tlc on polyamide (Merck 11F254, 0.15 mm), eluent $CHCl_3$-MeOH-2-butanone-2,4-pentanedione, 45:7:7:3. This substance had mp 220-223° after recrystallization from MeOH [lit. for prunin (**5**) 221-223° (18)], identification as for catechin.

TESTING OF ANTIMICROBIAL ACTIVITY.—The agar diffusion plates used in tests with bacteria, *A. fumigatus*, *C. albicans*, and *T. mentagrophytes*, were made by pouring 60-ml portions of PDM Antibiotic Sensitivity Medium (AB Biodisk, Solna, Sweden) or PDM with 5% defibrinated horse blood added, into 14-cm Petri dishes. For the rest of the fungi, malt extract agar (12 g malt extract, Oxoid L39, and 20 g Difco Bacto-Agar per liter) was used. The test inocula were prepared as follows: Bacterial strains (*E. coli* Sc, *P. aeruginosa* Sc, *S. faecalis* Sc, *S. aureus* Sc) were grown on blood-agar plates for 20 h at 37° and harvested by meat-peptone broth. The suspensions were diluted with sterile, distilled H_2O to a concentration predetermined to give individual but distinctly confluent colonies when inoculated on agar plates. The fungus *C. albicans* Sc was grown on Sabouraud agar at 25° for 2-3 days, harvested and diluted as above. The fungi *A. fumigatus* Sc, *A. niger* F508-1, *C. globosum* F540-1, *C. puteana* 588-1, *M. verrucaria* F559-1, *P. notatum* F506-1, *R. nigricans* F503-1, *S. pulverulentum* 619-1, *T. viride* F529-1, and *T. mentagrophytes* Sc were cultivated as *C. albicans* but with an incubation period of 8 days. Spore suspensions were made by homogenization of the mycelium with meat-peptone broth in a mortar and dilution to a concentration of $1\text{-}5\times10^6$ spores/ml (determined by counting in a Bürker counting chamber) with Winogradsky's solution (19).

The solutions to be tested (concentration ca. 1%) were made by dissolving either the test flavonoids in 0.1 M aqueous NaOH and immediately adjusting the pH to between 9 and 10 with 0.1 M HCl, or in Macrogol 400. As controls, 1% Captan, 0.01% Captan, and 1% Quintozene solutions in Macrogol 400 were used. The agar diffusion plates were inoculated with the suspensions of the test microorganisms, and 5-mm wells were punched in the agar. After a period of 30 min (bacteria, *C. albicans*) or 60 min (other fungi), the freshly prepared test solutions (50 μl aliquots) were applied in the wells.

After 30 min (bacteria, *C. albicans*) or 120 min (other fungi), the test systems were incubated as described for incubation of the microorganisms (bacteria, *C. albicans*) or for 3 days (*C. puteana*) or 2 days (other fungi). The diameters of the inhibition zones were then measured.

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