Phytochemical Investigation of the Bark of *Salix petiolaris* Sm. (*Salix gracilis* Anderss. var. *textoris* Fern.)

A Thesis Presented to the Faculty of Graduate Studies
University of Manitoba

In Partial Fulfilment of the Requirements for the
Degree of Master of Science

Peter F. Weitzel

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The author also acknowledges the receipt of a Medical Research Council Studentship for the term of studies.

ABSTRACT

The November bark of Salix Petiolaris Sm. was extracted with acetone followed by ethanol. The acetone residue was submitted to continuous ethyl acetate extraction and the fractions from the ethyl acetate extraction as well as the initial ethanol extraction residue were chromatographed on polyamide columns.

Two unknown components were isolated, purified and identified as β-sitosterol and (+)-catechin.

The bulked polyamide column chromatography eluate fractions were examined for phenolic glycoside content using two thin-layer chromatographic systems and a gas-liquid chromatographic system. The presence of salicin, picein, vimalin, salicyloylsalicin, grandidentatin, salireposide, populin, salicyloylsalicin-2-O-benzoate and tremuloidin and/or tremulacin was shown.

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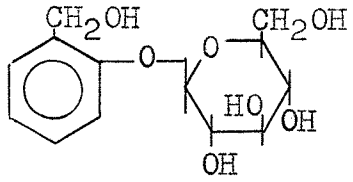
I. INTRODUCTION

General

The phytochemical investigation of the leaves and barks of *Salix* sp. has a history of at least one hundred and forty years. In 1828, Buchner (1) isolated the phenolic glycoside, salicin, from the barks of *Salix alba* and *Salix incana*. Salicin has since been found in the sap, bark and leaves of most *Salix* sp. and is the only simple phenolic glycoside to have been used medicinally, as an analgesic.



Salicin

Apart from other simple phenolic glycosides, a number of imino acids, carbohydrates, phenols, sterols and flavonoid compounds have also been isolated and the tannin and lignan content has been studied.

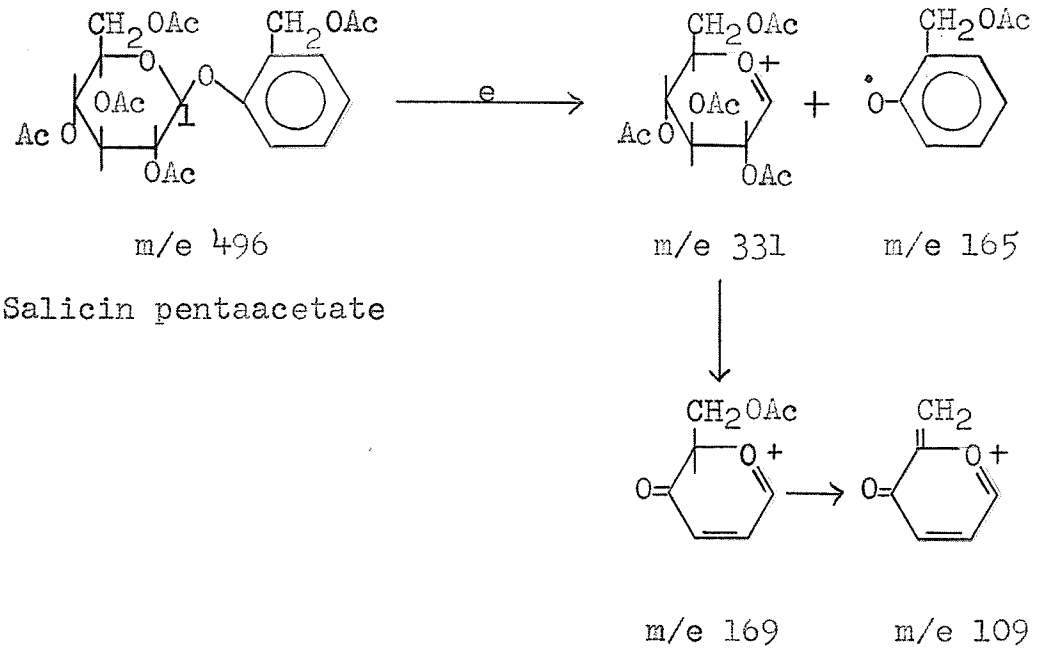
It is the purpose of this introduction to relate some of the more important discoveries, giving structures and methods of isolation and identification of these compounds where possible. It should be kept in mind that collection and isolation techniques can effect gross changes in the nature of the chemicals eventually isolated. However, the importance of this depends on whether the researcher wishes to find what can be obtained from a given plant or whether he wishes to reflect the chemical

content of the intact plant.

Simple Phenolic Glycosides

Much of the work to date on the leaves and barks of Salix sp. concerns simple phenolic glycosides. These compounds have also been found in the leaves and barks of Populus sp., the only other well defined genus in the family Salicaceae. The structurally simple glycosides such as salicin or picein are readily soluble in water and all are readily soluble in polar organic solvents such as ethanol, methanol or acetone. Isolation is generally effected by selective solvent extraction using a polar organic solvent for the initial extraction, followed by ethyl acetate to remove polysaccharides and chloroform to remove the chlorophylls. This is then followed by polyamide column chromatography to remove the tannins and high molecular weight non-polar compounds. The final step can be replaced by treatment with lead subacetate; however, this reagent can cause rearrangement or decomposition of some glycosides and is not recommended. The fractions thus obtained are best recrystallized from water to a constant melting point. Gas-liquid chromatography (GLC) (2), thin-layer chromatography (TLC) (3) and paper chromatography (4) have all been used to identify isolated phenolic glycosides. Optical rotation (4) and infra-red, ultra-violet and mass spectroscopy (5) have also been found to be invaluable aids to identification.

Pearl and Darling (5) have proposed a degradative scheme for phenolic glycosides undergoing mass spectral analysis. It was found that unsubstituted phenolic glycosides did not show a molecular ion. Since glucose does not give a satisfactory mass spectrum unless acetylated, the phenolic glycosides were acetylated prior to analysis. Salicin pentaacetate displayed primary fragmentation at the number one carbon atom (C-1). Acetates of phenolic glucosides lost acetyl as ketene before or at the same time as the C-1 rupture. The primary and secondary fragmentation for salicin pentaacetate was proposed to be as follows:



m/e 496

Salicin pentaacetate

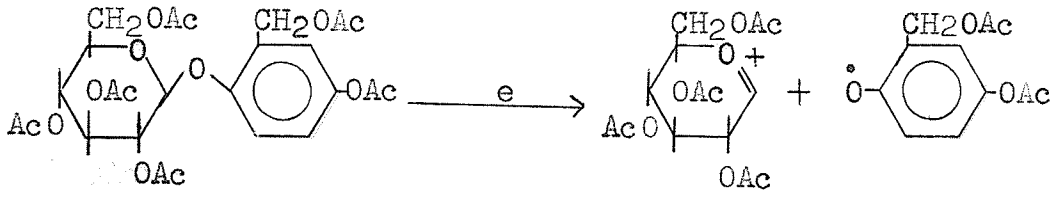
m/e 331

m/e 165

m/e 169

m/e 109

Similarly for salirepin the scheme was:



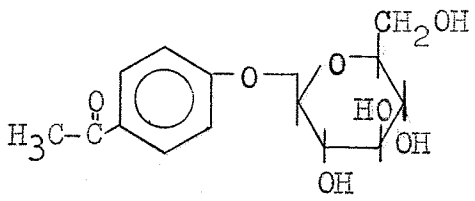
m/e 554 m/e 331 m/e 223

Salirepin hexaacetate

Mass spectroscopy can be used to differentiate glycosides whose glucose moieties are mono or disubstituted. Benzoyl substituted glycosides such as tremuloidin would yield a benzoyl glucose fragment ion of m/e 393 and not the glucose fragment ion of m/e 331 as noted with salicin and salirepin.

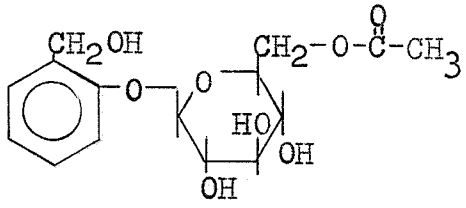
Another important technique used in glycoside identification is hydrolysis (6). Weak acid, weak alkali and enzymatic hydrolyses have been used to cleave various phenolic glycosides and through identification of the hydrolysis products, useful information for structural elucidation is obtained. Specific examples of this method will be given later.

Picein (6) has been found in the bark of Salix cinerea and is very widespread in Salicaceae.



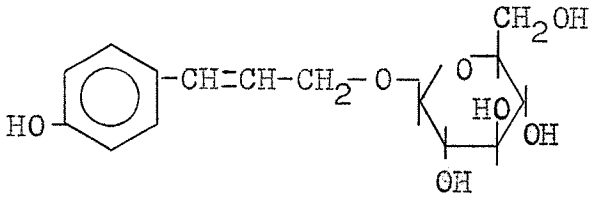
Picein

Fragilin (7) has been found in the bark of Salix fragilis and is 6-acetylsalicin. Barium hydroxide treatment (2%) for one hour on a water bath leads to 87% conversion to salicin and acetic acid (8). Thus, it can be seen how alkali treatment can aid in the identification of phenolic glycosides and why alkali treatment during the extraction procedure should be avoided.



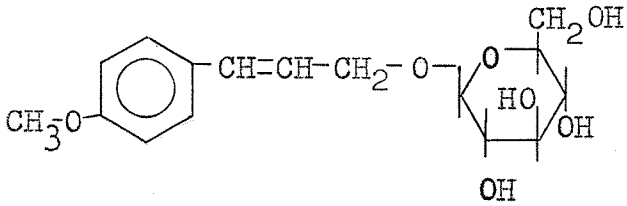
Fragilin

Triandrin has been isolated from the bark of Salix triandra (6). Emulsin hydrolysis yielded glucose and 4-hydroxycinnamyl alcohol.



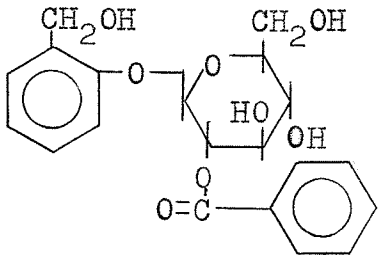
Triandrin

Vimalin (methyl triandrin) has been isolated from the bark of Salix viminalis (7). Alkaline hydrolysis yielded glucose and 4-methoxycinnamyl alcohol.



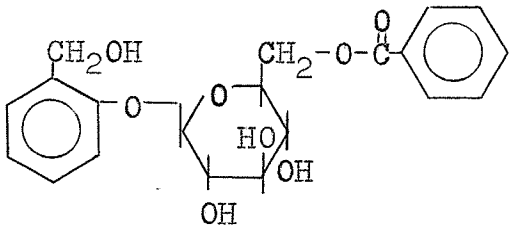
Vimalin

Tremuloidin (2-benzoylsalicin) has been isolated from the leaves of Salix purpurea and Salix fragilis (6). Ammonium hydroxide treatment was found to cause the benzoyl group to migrate to the number six position of the glucose moiety giving populin (9).



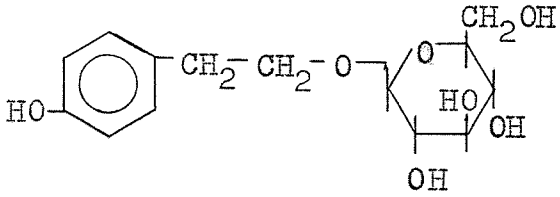
Tremuloidin

Populin (6-benzoylsalicin) is an isomer of tremuloidin and was originally isolated from Populus tremula but has since been isolated from the leaves of Salix fragilis (6).



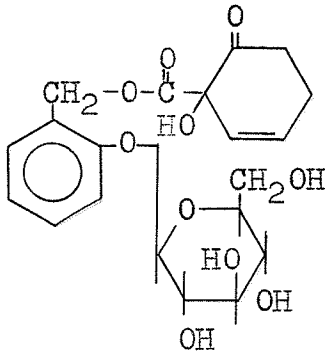
Populin

Salidroside has been isolated from the bark of Salix triandra and was also identified with the aid of hydrolysis (7).



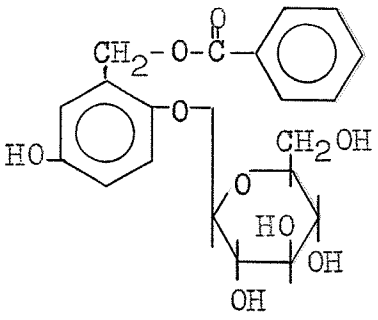
Salidroside

Salicortin has been found to be the main phenolic glycoside of the bark and leaves of Salix purpurea (7). It has also been found in many other Salix and Populus sp. Upon treatment with 0.1 M barium hydroxide at room temperature, 56% was converted to salicin (10). Pearl and Darling have assigned the following structure (11).



Salicortin

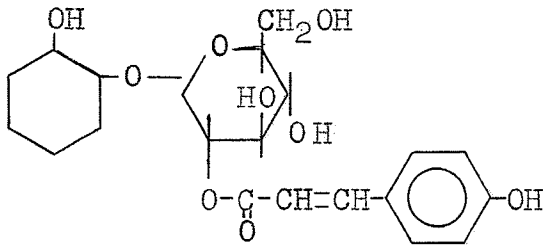
Salireposide has been found in the bark of Salix repens, Salix purpurea and many other Salix and Populus sp., but never in the leaves (7).



Salireposide

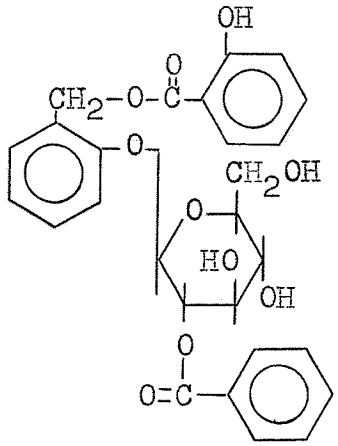
Grandidentatin was first found in the bark of Populus grandidentata by Pearl and Darling (12) and has

since been found in the bark of Salix fragilis, Salix purpurea and Salix triandra (6). Treatment of grandidentatin with barium hydroxide yields grandidentin and p-coumaric acid.

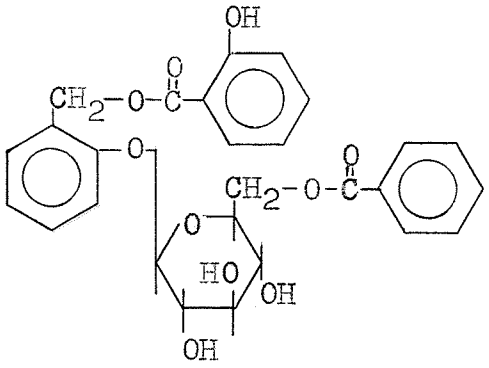


Grandidentatin

Salicyloyltremuloidin has been found in the leaves of Salix fragilis and is the 2-O-benzoate of salicyloylsalicin (13). It has been reported that under mild alkaline conditions the 2-benzoate moiety migrates to the number six position yielding salicyloylpopulin (14). This is a similar rearrangement to the one which yields populin from tremuloidin. Therefore, either salicyloyltremuloidin or salicyloylpopulin may be obtained depending on the conditions of the isolation.



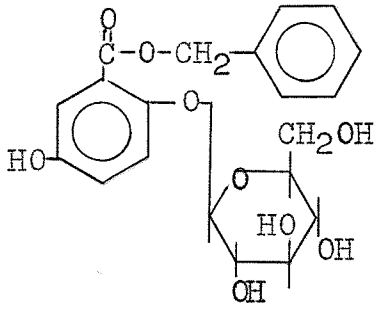
Salicyloyltremuloidin



Salicyloylpopulin

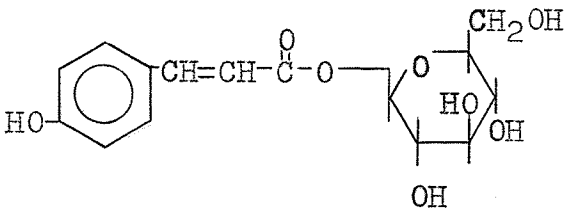
Recently, purpurein, a diasterioisomer of grandidentatin, has been isolated from the bark of Salix purpurea (15).

A number of other phenolic glycosides have been discovered in Populus sp. and this is not surprising considering their close phylogenetic relationship to the Salix genus. Trichocarpin has been isolated from the bark of Populus trichocarpa with the aid of cellulose column chromatography (7). It is the glucoside of the benzyl ester of gentisic acid and is isomeric with salireposide which is the glucoside of the benzoate of gentisyl alcohol.



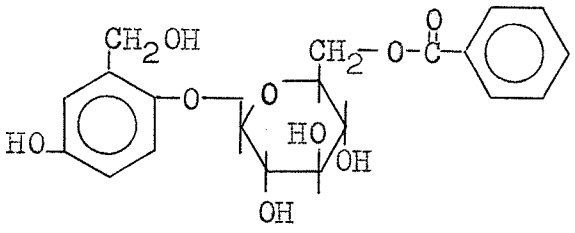
Trichocarpin

1-p-coumaroyl-β-D-glucose has been isolated from the leaves of Populus candicans (16) and from the bark of Populus tremuloides and Populus grandidentatin (17). In the latter study, it was isolated using a hot water extraction followed by ethyl acetate extraction and polyamide column chromatography.



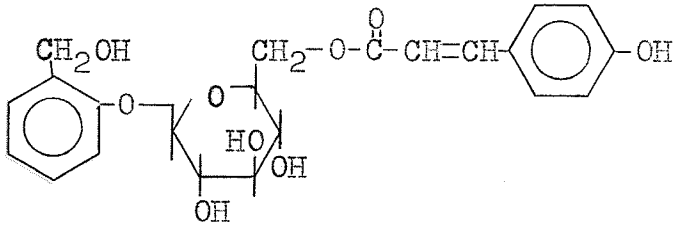
1-p-coumaroyl-β-D-glucose

Nigracin, an isomer of salireposide, has been isolated from the bark and leaves of Populus nigra (18).



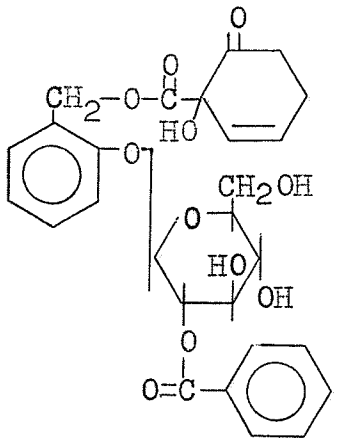
Nigracin

Trichocarposide was isolated by Estes and Pearl (19) from the bark of Populus trichocarpa.



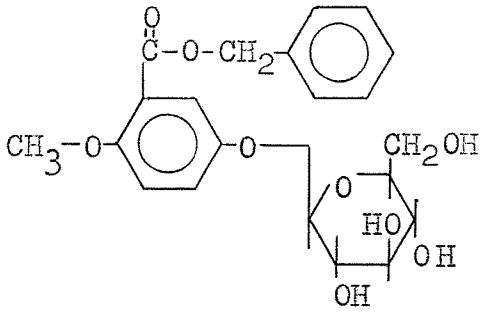
Trichocarposide

Tremulacin (2-benzoylsalicortin) from the bark of Populus tremula has been assigned the following structure on the basis of mass spectral work by Pearl and Darling (11).



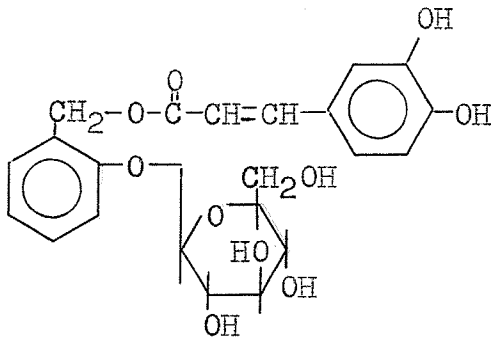
Tremulacin

Trichoside has been isolated from the hot water extractives of Populus trichocarpa (20).



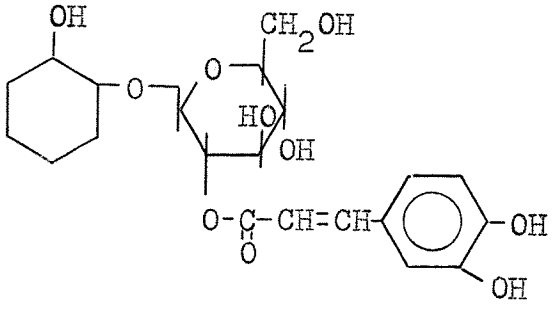
Trichoside

Two new phenolic glycosides have been isolated from the bark of Populus grandidentata (21). Populoside, which is a caffeic acid ester of salicin, has been assigned the following structure.



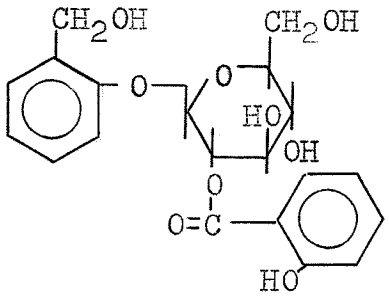
Populoside

The structure of the other glycoside, grandidentoside, has not been fully elucidated but NMR and mass spectral studies indicate the following structure.

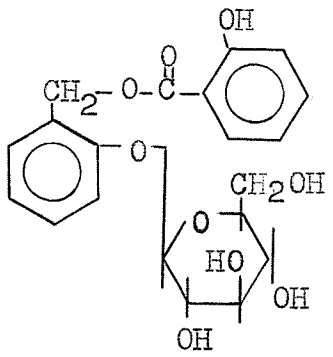


Grandidentoside

From the leaves of Populus deltoides, Pearl and Darling have isolated deltoidin (2-O-salicyloylsalicin) and ω-salicyloyl salicin (22).

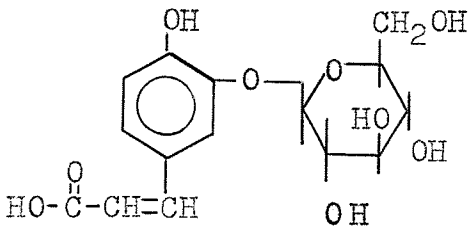


Deltoidin



ω-salicyloylsalicin

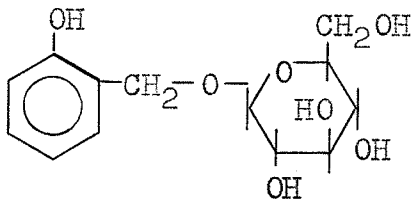
Thieme and Benecke have isolated caffeic acid -3-β-D-glucose from the leaves of Populus nigra (23).



Caffeic acid-3-β-D-glucose

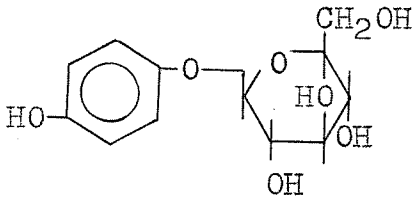
Finally, a monoacetate of salicyloylsalicin, triploside, has been isolated from the leaves of Populus tremuloides but no further clarification of its structure has been made (24).

It is interesting to note that very closely related glucosides are found in other families, as the isolation of isosalicin by Thieme from the leaves of Filipendula ulmaria illustrates (25). This compound has never been isolated from any member of the Salicaceae.



Isosalicin

Arbutin, which was used as a standard in part of the present study, is another phenolic glycoside which has never been found in members of the Salicaceae.



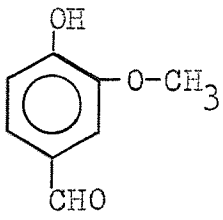
Arbutin

Phenols

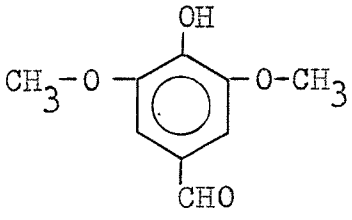
Work has also been carried out on the phenolic content of Salicaceae. They are generally isolated by extraction

with polar solvents followed by alkaline hydrolysis of the extraction residue. Modern detection techniques include the chromatographic and spectral methods mentioned earlier for phenolic glycosides. The free phenols found in Salix and Populus sp. are very likely derived from the aglycones of various phenolic glycosides and seem to be an integral part of the shikimic acid pathway in the biosynthesis of flavonoids and other phenolic natural products such as lignan and possibly tannins (26). Free phenols have been reported to occur only in dead plant tissue (26).

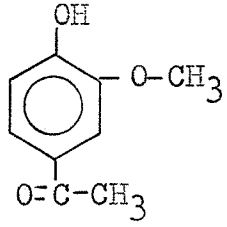
Pearl et al. have identified the following phenolic substances from the barks of various Populus sp. using diazotized p-nitroaniline on paper for detection (27). The phenols were obtained by alkaline hydrolysis of the hot water extractives of the barks.



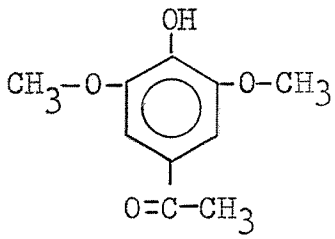
Vanillin



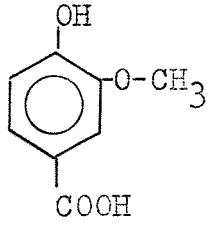
Syringaldehyde



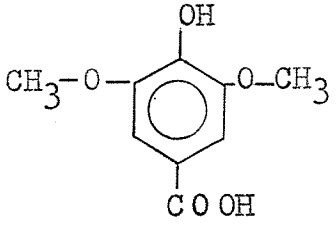
Acetovanillone



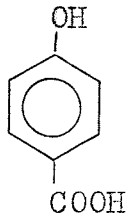
Acetosyringone



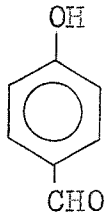
Vanillic acid



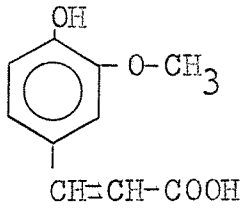
Syringic acid



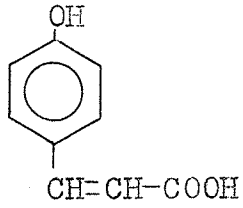
p-Hydroxybenzoic acid



p-Hydroxybenzaldehyde

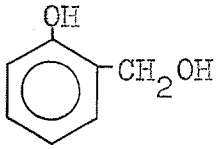


Ferulic acid

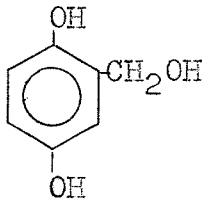


p-Coumaric acid

Pearl et al. also isolated salicyl alcohol and gentisyl alcohol from the bark of Populus trichocarpa (28).

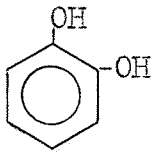


Salicyl alcohol

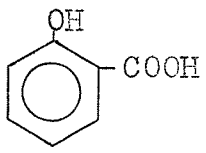


Gentisyl alcohol

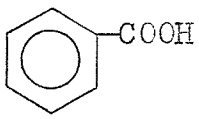
Pyrocatechol has been isolated from the bark of Populus grandidentata (29) and benzoic and salicylic acids have been isolated from the bark of Populus tremuloides (14).



Pyrocatechol

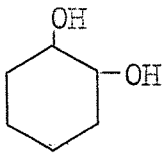


Salicylic acid

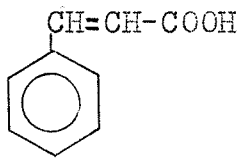


Benzoic acid

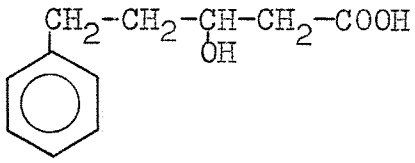
Four related substances isolated from the bark of Populus sp. are cis-1,2-cyclohexanediol (14) (part of the aglycone of grandidentatin), cinnamic acid (30), 3-hydroxy-5-phenylvaleric acid (30) and 2,6-dimethoxy-p-benzoquinone (31).



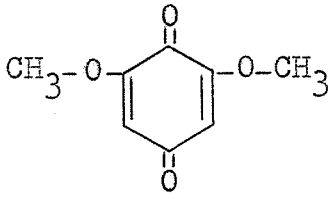
cis-1,2-Cyclohexane diol



Cinnamic acid



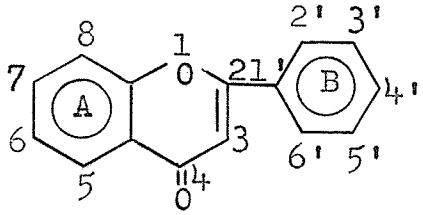
3-Hydroxy-5-phenylvaleric acid



2,6-Dimethoxy-p-benzoquinone

Flavonoids

Several flavonoid compounds have also been isolated from the barks and leaves of Salix and Populus sp. The basic skeleton consists of a six carbon unit linked to a three carbon unit which is in turn linked to another six carbon unit. The following structure illustrates the conventional numbering system of flavonoids.



Flavone

The state of oxidation of the three carbon unit determines the class of flavonoid to which each individual compound belongs (32a). Flavonoids may be extracted from the plant

using a polar solvent and further purified by precipitation using lead subacetate (33). Identification may be carried out using various chromatographic and spectral methods (32a). Hydrolytic studies are often used in the structure elucidation (32a). Flavonoid pigments may be present in plants to make the plant conspicuous to insects. It has been postulated that some flavonoids act as growth control factors in plants (32a). Flavonoids generally occur as glycosides with one or more of the phenolic groups bound to a sugar moiety (32a).

Several flavonoid compounds have been isolated from Salicaceae. It should be kept in mind that isomeric flavonoids such as chalcones and flavanones can be readily interconverted during extraction or identification (32a). The flavonoids found in the barks and leaves of the Salicaceae to date are listed in TABLE - 1 along with their structure and source.

Carbohydrates

Work has also been done on the isolation of sugars from Salicaceae. Their discovery is not surprising considering the high glycoside content of the plant. Pearl and Larsen (45) used ethyl acetate to extract the polysaccharides from the original aqueous extraction residue. This was followed by a chloroform extraction and lead subacetate treatment. The resultant polysaccharide breakdown was responsible for the oligosaccharides and monosaccharides subsequently isolated and identified. From the

TABLE - 1 - Flavonoid constituents of Salicaceae

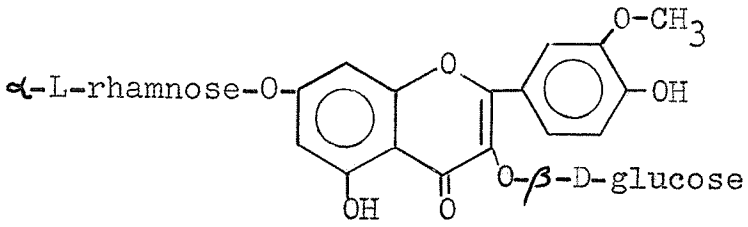
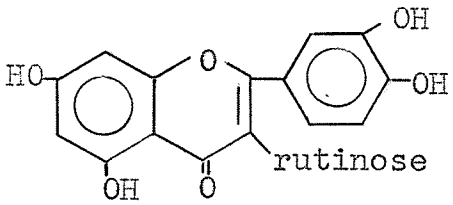
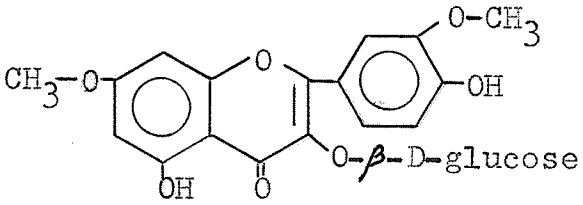
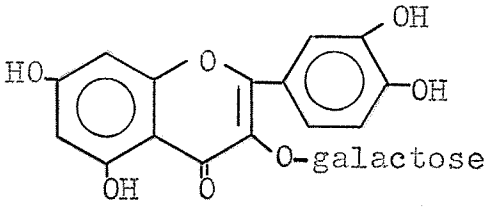
Compound	Source
A. Flavonols	
1. brassidin 	leaves of Salix fragilis (34)
2. rutin 	leaves of Salix triandra (34)
3. alboside 	leaves of Salix alba (34)
4. hyperoside 	leaves of Populus tremuloides (33)

TABLE - 1 - cont'd.

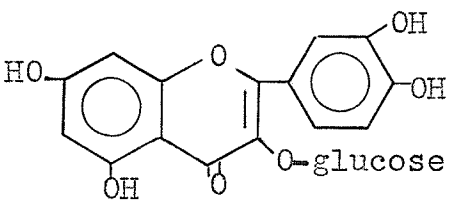
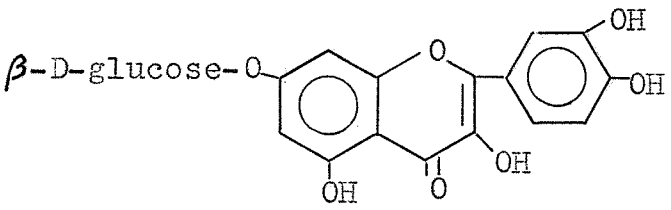
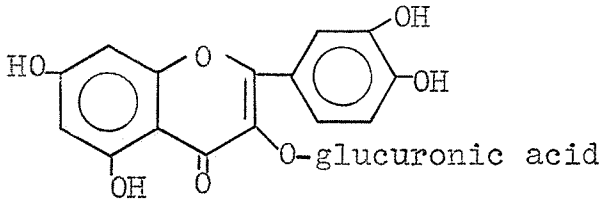
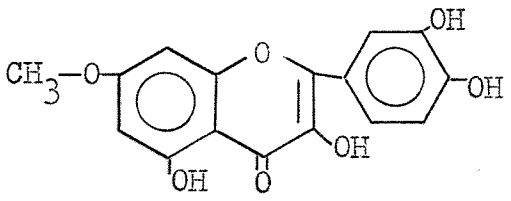
Compound	Source
5. isoquercitin 	leaves of <u>Populus tremuloides</u> (33)
6. quercimeritin 	leaves of <u>Salix purpurea</u> (35)
7. quercitin-3-glucuronide 	leaves of <u>Populus grandidentata</u> (36)
8. rhamnetin 	leaves of <u>Populus tremuloides</u> (33)

TABLE - 1 - cont'd.

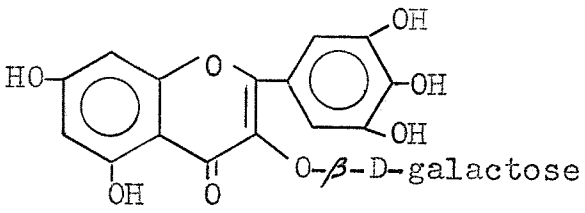
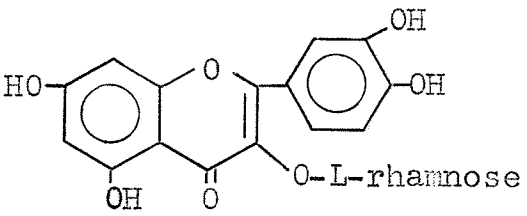
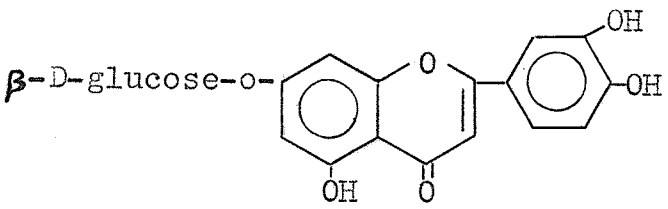
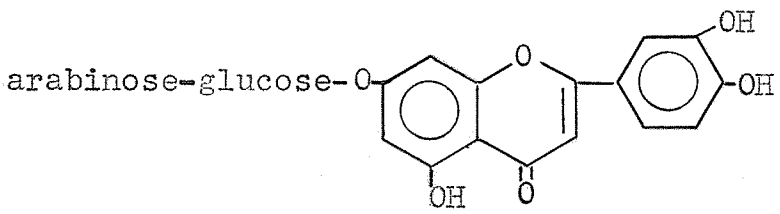
Compound	Source
9. myricetin-3-β-D-galactose 	leaves of *Populus candicans* (37)
10. quercitrin 	leaves of *Salix* sp. (38)
B. Flavones	
1. luteolin-7-β-D-glucoside 	leaves of *Salix purpurea* (34)
2. luteolin-7-glucosylarabinose 	leaves of *Salix bakko* (39a)

TABLE - 1 - cont'd.

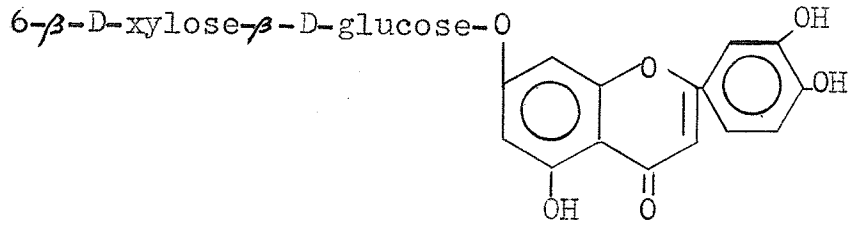
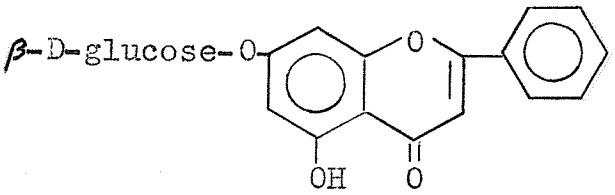
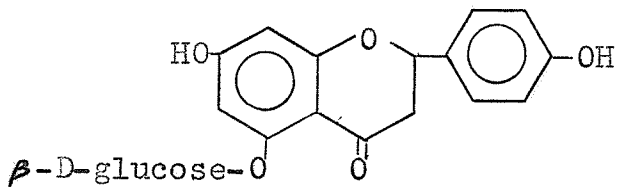
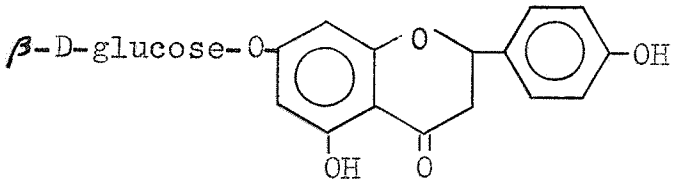
Compound	Source
3. caesioside 	leaves of *Salix repens* (34)
4. chrysin-7-glucoside 	leaves of *Populus deltoides* (22)
C. Flavanones	
1. naringenin-5-β-D-glucoside 	bark of *Salix purpurea* (40)
2. prunin 	leaves of *Salix purpurea* (34)

TABLE - 1 - cont'd.

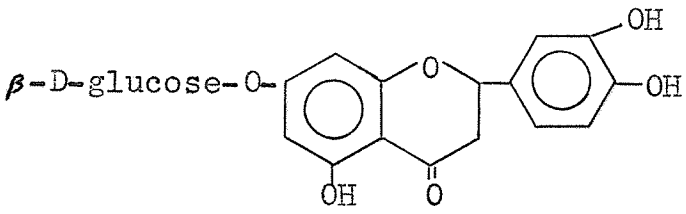
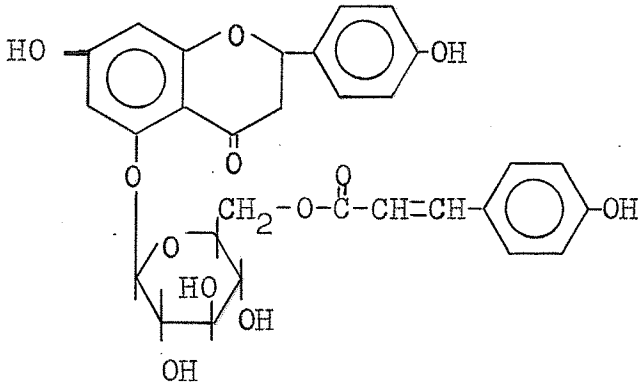
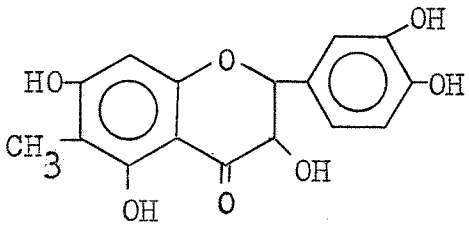
Compound	Source
3. eriodictyol-7-β-D-glucose 	leaves of *Salix purpurea* (34)
4. 4',5,7-trihydroxyflavanone-5-[(6-O-p-coumaroyl)-β-D-glucose] 	bark of *Salix* sp. (41)
D. Flavanonol	
1. 6-methyldihydroquercitin 	bark of *Populus deltoides* (22)

TABLE - 1 - cont'd.

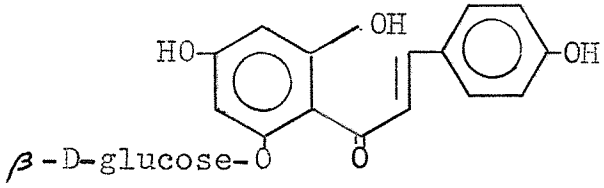
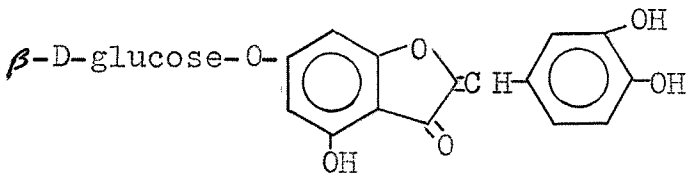
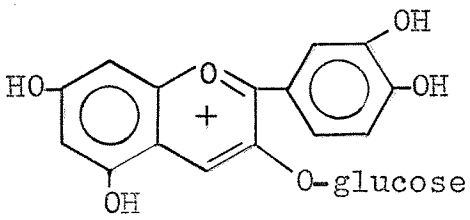
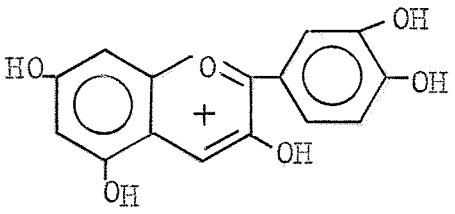
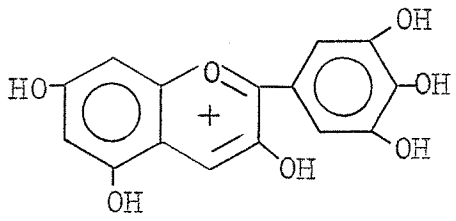
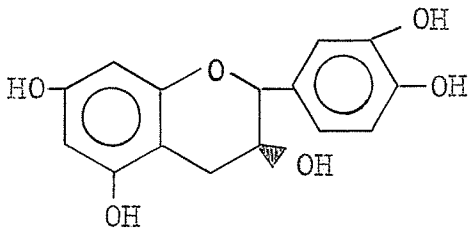
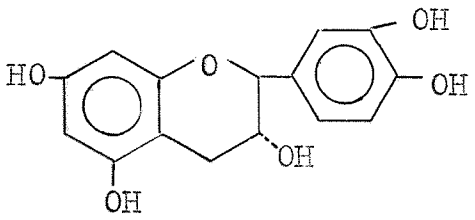
Compound	Source
E. Chalcone	
1. isosalipurposide 	leaves of Salix purpurea (34)
F. Aurone	
1. aureusin 	leaves of Salix purpurea (34)
G. Anthocyanidins	
1. cyanidin-3-glucoside 	leaf galls of Salix fragilis (39b)
2. cyanidin-by acid hydrolysis of leucoanthocyanidins 	leaf galls of Salix fragilis (42)

TABLE - 1 - cont'd.

Compound	Source
3. delphinidin-by acid hydrolysis of leucoanthocyanidins 	leaf galls of <u>Salix fragilis</u> (42)
H. Catechins	
1. Catechin 	leaves and galls of <u>Salix fragilis</u> (43) (44)
2. epicatechin 	leaves and galls of <u>Salix fragilis</u> (43)

barks of various Populus sp., Pearl et al. (28, 29, 45) have isolated glucose, fructose, mannose, galactose, xylose, arabinose, rhamnose and sucrose as well as two unidentified oligosaccharides. The cellulose (46) and hemicellulose (47) content of some Salix sp. has also been studied. Finally, from the lead subacetate treated leaves of Populus tremuloides Kinsley and Pearl (33) have isolated the polyuronide, pectin, and inositol, a compound which is isomeric with the hexoses.

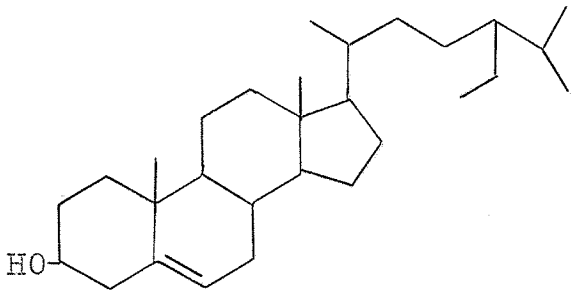
Miscellaneous constituents

A number of amino acids have been isolated from the leaves and leaf galls of Salix fragilis (48). These were lysine, arginine, aspartic acid, glutamic acid, serine, asparagine, γ-amino butyric acid, ethanolamine, histidine, proline, α-alanine, threonine, valine, tyrosine, isoleucine, leucine, tryptophan and phenylalanine. Binns et al. (42) have isolated three piperidine based imino acids, two of which were identified as pipecolic acid and 5-hydroxy-pipecolic acid.

The tannin content of the May barks of several Salix sp. has been studied by Thieme (49). The percentages of tannin present for the ten species studied ranged from 8.7% to 20.5% with an average value of 13.6%.

Thieme and Benecke (50) studied cyclolignans found in Populus nigra bark and isolated and identified (+)-isolariciresinolmono-β-D-glucoside, another phenolic type glucoside found in Salicaceae.

β-Sitosterol has been isolated from the bark of Populus tremuloides (51) and the bark of Populus grandidentata (9).



β-Sitosterol

A triterpenoid, epifriedelinol, has been isolated from Salix japonica (52). Other miscellaneous substances isolated from Salicaceae include azelaic acid (31), succinic acid (33), lignoceric acid (51), linoleic acid (51), ceryl alcohol (51) and a plant growth inhibitor, abscisic acid (53)(54).

It can be seen from the preceding pages that the phytochemical investigation of the leaves and barks of Salicaceae has yielded a diverse collection of constituents but those which have aroused the greatest interest are the secondary metabolites which are derived from phenolic type compounds such as lignans, flavonoids or the simple phenolic glycosides.

Quantitative Studies of Salicaceae

Quantitative determination of the phenolic constituents of Salicaceae has traditionally involved the isolation of the crystalline products and the subsequent determination of the physical characteristics of these

components. Attempts at identification without final purification have generally yielded only qualitative results or at best semi-quantitative results except when quantitative GLC has been used.

In a review, Thieme (55) outlined some of the semi-quantitative techniques employed. One method involved the titration with potassium permanganate of the glucose liberated from salicin by hydrolysis with sulfuric acid. Another involved the polarimetric determination of glucose after hydrolysis of the glucosides using emulsin. Attempts have also been made at determining colorimetrically the 2-hydroxylbenzyl alcohol freed upon treating salicin with 4-aminophenasone. Pearl and Estes (56) measured phenolic acids quantitatively after acid hydrolysis of glycosides using a paper chromatographic and spectrophotometric analysis. It can be seen that the above methods are non-specific and give little insight into the glycosidic makeup of the plant. Thieme (55) proposed a quantitative paper chromatographic scheme whereby spots sprayed with Millon's reagent were monitored colorimetrically after extraction from the paper. This allowed determination of amounts as low as 300 μg with a precision of ± 5%. The method suggested by Persson (57) for the determination of salicin involved a densiometric determination of the spot while still on the paper. By applying these methods to as many glycosidic standards as desired, these could prove to be successful methods of indicating the amounts of various phenolic glycosides in

botanical samples.

Quantitative GLC may also be used. Kripiakevich (58a) studied the amounts of salicin, picein and salireposide in Salix petiolaris Sm. quantitatively and found picein to be present in the greatest amount. The study involved the preparation of calibration curves for each standard and peak area determination of each component. The time required for routine study using 20-30 standards would be prohibitive but this method still provides an accurate means of studying the phenolic glycoside content of botanical samples.

Another method used is illustrated in the work of Pearl and Darling (22). Initially, samples were extracted with water, followed by an ethyl acetate extraction. The ethyl acetate residues were then chromatographed on polyamide columns and the results displayed as elution curves. That is, the weight in grams of each fraction was plotted against the eluate volume from the polyamide column noting the chief components which have been identified in each fraction. Distinction was made between components which were isolated in the crystalline form and those which were identified by TLC alone. In this way, values were obtained for the amounts of particular components present in the samples without necessarily obtaining crystalline materials, although these values were only of a semi-quantitative nature.

II. EXPERIMENTAL AND RESULTS

Materials and Equipment

All chemicals and solvents used were of the highest grade available. The following lists include the major items used.

Materials

Polyamide, Woelm (M. Woelm, Eschwege, Germany)

Silica Gel GF-254, Merck (E. Merck A.G., Darmstadt, Germany)

Tri-Sil, Pierce Chemical Co.

β-Sitosterol, Aldrich Chemical Co.

(+)-Catechin, Nutritional Biochemical Corp.

Equipment

Hamilton microsyringe

Quickfit thin-layer spreader

Fitzmill, Model D, Comminuting Machine

Quickfit steam-heated Soxhlet extractor

Quickfit steam-heated flash evaporator

Towers, Model A, Automatic Fraction Collector

Rotavapor-R, Büchi, Switzerland, rotary evaporator

Beckman DU Spectrophotometer

Hilger-Watts Ultrascan, scanning ultra-violet spectrophotometer

Bellingham and Stanley Polarimeter

Beckman IR-8 Infra-red Spectrophotometer

Thomas Hoover Capillary Melting Point Apparatus

Beckman GC-4 Gas Chromatograph

Analyses

Mass spectra were determined by the direct insertion technique with an A.E.I. MS2 or MS9 mass spectrometer operating at 70 eV. The analyses were performed by Dr. A.M. Hogg of the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta.

The carbon-hydrogen analysis was carried out by Drs. F. Pascher and E. Pascher, Germany.

Authentication of the sample

Salix petiolaris Sm. (Salix gracilis Anderss. var. textoris Fern.) was authenticated by Dr. B. Boivin of the Plant Research Institute, Ottawa.

Thin-layer chromatography (TLC)

After standing overnight in alcoholic potassium hydroxide and after thorough rinsing, 20 cm by 20 cm and 20 cm by 5 cm glass plates were coated with 0.25 mm of silica gel GF-254 by applying a slurry of 23 g of the silica gel in 46 ml of distilled water to a TLC spreader holding the equivalent of five of the larger plates. The plates were air dried and then activated immediately before use by heating in an oven at 110° for one hour. The samples were applied 1.5 cm from the bottom edge of the plate and the plates were developed for a distance of 15 cm from the origin in the stated solvents. The plates were divided into 1 cm wide segments prior to application of the samples. The super-saturated method of Stahl was used in the development (59). The spots were located by heating the plate at

110° for ten minutes after spraying with 4% sulfuric acid in anhydrous ethanol (3). Compounds gave a characteristic color, most appearing after the initial ten minutes, some only after standing at room temperature for several hours.

Suitable reference standards were chromatographed side by side with the experimental fractions. The standards available were salirepin, salicin, picein, salidroside, triandrin, grandidentatin, 1-p-coumaroyl-β-D-glucose, vimalin, salicortin, trichoside, fragilin, nigracin, salireposide, trichocarpin, trichocarposide, populin, salicyloylsalicin, tremuloidin, tremulacin, salicyloylsalicin-2-O-benzoate and salicyloylsalicin-6-O-benzoate.

The solvent systems used were ethyl acetate-xylene-formic acid-water (35-1-2-2) (3), ethyl acetate-methanol (9-1) (3) and n-butanol-acetic acid-ethyl ether-water (9-6-3-1) (32b). The latter system employed a different means of visualizing the spots which is described later (p. 50). These systems were abbreviated to EXFW, EM and BAEW respectively for future reference.

Gas-liquid chromatography (GLC)

A Beckman model GC-4 gas chromatograph, equipped with a flame ionization detector and a temperature programmer, was used. The signal was recorded on a 10 inch Beckman linear, potentiometric recorder set to a range of 1 mV. Helium was used as the carrier gas.

The column used was 0.3% OV-1 on Chromosorb G AW/DMCS 60/80 mesh in a 6 foot long, 1/8 inch O.D., grade 304

stainless steel tube prepared according to Bolan and Steele (2).

The temperature program was started at 190° and the temperature was held for ten minutes. The temperature then rose 6° per minute to 250° and was held until all components were eluted.

Samples were injected into the chromatograph as their trimethylsilyl (TMS) ethers, prepared by dissolving 0.1-0.5 mg of the sample in 50 µl of Tri-Sil (Pierce Chemical Co.) and, after shaking, allowing the mixture to stand at room temperature for 15 minutes. A Hamilton 0-10 µl syringe was used to inject 1-6 µl of the solution into the gas chromatograph.

Retention times were recorded relative to TMS arbutin which was injected every 4-5 runs to allow for minor variations in carrier gas flow rate.

The flow rates for the helium, hydrogen and air were 80 ml/min, 50 ml/min and 300 ml/min respectively.

The temperatures of the detector, detector line and inlet line were 300°, 250° and 200° respectively.

Collection and preparation of the sample

Salix petiolaris Sm. bark was obtained from young shrubs up to five years old cut on November 6, 1969 near Stonewall, Manitoba. The leaves had fallen from the shrubs. The bark was smooth and red-brown. The bark was peeled from the shrubs within 48 hours, air dried and reduced to 2,550 g of dry powder in a Fitzmill comminuting machine.

Extraction of the sample

The entire sample (2,550 g) was extracted for 20 hours in a Soxhlet with 25 l of acetone. This was evaporated to 6 l using a steam-heated flash evaporator. Further concentration at 40° on a vacuum rotary evaporator yielded 655.7 g of a viscous residue. The Soxhlet extraction was continued for a further five hours with 25 l of 95% ethanol. The extract, concentrated to 1½ l on the flash evaporator and further concentrated on the rotary evaporator at 40°, yielded 296.1 g of a viscous residue (fraction E).

The acetone extraction residue was transferred to a 3 l flask, suspended in 1 l of water and extracted continuously with 17 x 2 l lots of ethyl acetate. The ethyl acetate soluble portions were monitored using the EXFW TLC system. Similar fractions were bulked and taken to dryness at 40° on the rotary evaporator yielding fraction F-1 after 49 hours (134.2 g), fraction F-2 after a further 55 hours (135.4 g) and fraction F-3 after a further 37 hours (57.7 g). At this point, the aqueous/ethyl acetate suspension was filtered because of problems with emulsification. The dried filtered solid was set aside as fraction B (177.9 g) and the ethyl acetate portion was combined with fraction F-3. The aqueous portion of the filtrate was extracted with ethyl acetate for a further 365 hours to yield fraction F-4 (74.3 g). The remaining aqueous portion was evaporated to dryness at 40° on the rotary evaporator to yield fraction W (64.5 g). The extraction flow scheme is summarized in

FIG. 1. The results of the TLC analysis of the various fractions are summarized in TABLE 2. Attempts were made to fractionate B further by ethyl acetate extraction but this was unsuccessful. The above fractions were purified further through the use of polyamide column chromatography.

Preparation of the polyamide chromatography columns

The polyamide powder was pretreated by extraction with boiling methanol (twice) and with cold water (once) to remove low molecular weight material (60). The resulting powder was poured into a column filled with distilled water and 2 l of distilled water were run through the column.

The sample to be fractionated was taken up in a minimum volume of tetrahydrofuran and this was triturated with dry polyamide powder until a semi-dry granular solid was obtained. The tetrahydrofuran was allowed to evaporate and the material was placed on top of the column after being passed through a number 20 sieve. The packing was just covered with distilled water and elution was begun (17).

Preparation and development of the polyamide chromatography columns for fractions F 1-4, W, B and E

Pretreated polyamide (150 g) was poured into a glass column 2 inches in diameter to form a column 18-20 inches in height. The residue (30 g) was adsorbed onto 15 g of pretreated polyamide as described above and applied as a band of 1-1½ inches to the top of the column. Fractions of 50 ml were collected. Elution was begun with distilled

FIG. 1 - Extraction flow scheme of *Salix petiolaris* Sm. bark prior to polyamide column chromatography

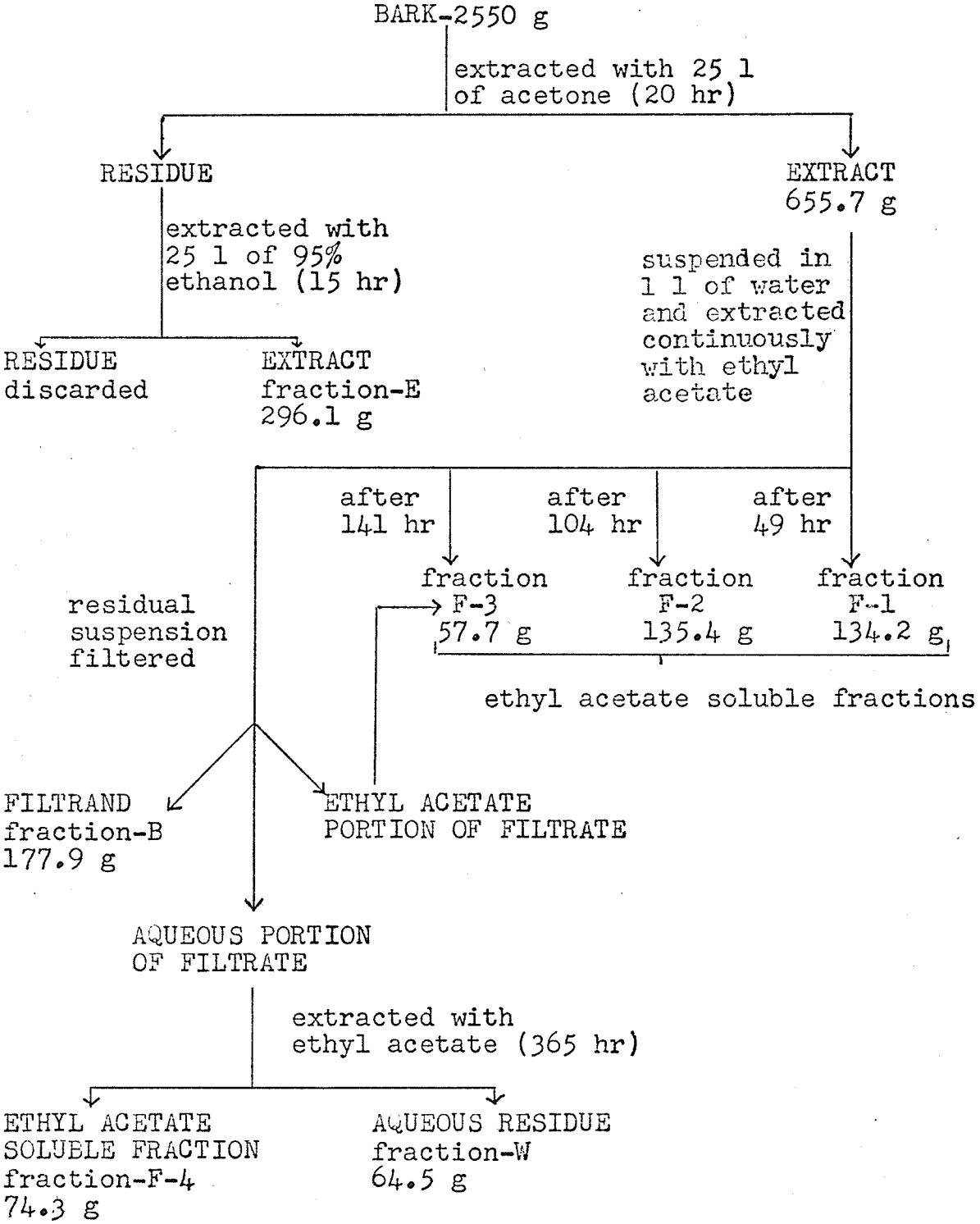


TABLE 2 - TLC results of the ethyl acetate extraction fractions

FRACTION	SPOT COLOR	R_f	STD. R_f	POSSIBLE IDENTITY
F-1	red	0.11	0.11	salicin
	red-brown	0.14	0.15	picein
	yellow	0.30	0.31	salireposide
	purple	0.84		
F-2	red	0.22	0.20	salicin
	red-brown	0.26	0.24	picein
	yellow	0.36	0.39	salireposide
	red	0.52	0.47	populin
			0.52	tremuloidin
	yellow-red	0.69		
	purple	0.87		
F-3	red	0.25	0.24	salicin
	red-brown	0.28	0.26	picein
	yellow	0.39	0.41	salireposide
	red	0.54	0.49	populin
			0.53	tremuloidin
	orange	0.72		
F-4	red	0.21	0.21	salicin
	yellow	0.36	0.37	salireposide
W	red	0.21	0.21	salicin
	red-brown	0.25	0.24	picein
B	red	0.20	0.20	salicin
	red-brown	0.25	0.23	picein
	yellow	0.40	0.37	salireposide

water followed by 25%, 50%, 75% and 95% ethanols. Every second fraction of 50 ml was monitored by thin-layer chromatography using approximately 0.05 ml of the column eluate and the EXFW system. The fractions were bulked according to the TLC results and evaporated to dryness at 40° using a vacuum rotary evaporator. The results of the TLC analysis are summarized in TABLE 3 and the solvent schemes for the seven large columns are summarized in TABLE 4.

From fractions U-3, T-7, D-5 and Z-4, 0.3945 g, 1.3422 g, 1.2062 g and 0.0972 g of salireposide crystallized out in the column eluate. The crystals were identified by co-chromatography on the EXFW and the EM TLC systems. The crystals from D-5 were colorless while those from the other fractions were discolored (greyish). No further purification was attempted since salireposide is a well known phenolic glycoside.

A compound which gave an orange spot with an R_f of 0.76-0.79 on the EXFW TLC system was noticed in fractions U-5, T-9, D-7, P-6, Z-6 and H-7. A compound which gave a purple spot with an R_f of 0.83-0.88 in the same system was noticed in fractions T-10, T-11 and H-8. These two components did not correspond with any of the standards. They were labelled N and S respectively and subjected to further study. In order to collect more of these two components, a further 30 g of F-2 was subjected to polyamide chromatography.

TABLE 3 - TLC monitoring results of the column eluate fractions of F 1-4, W, B and E*

ETHYL ACETATE FRACTION	COLUMN ELUATE FRACTION	WEIGHT OF ELUATE FRACTION (g)	VOLUME OF ELUATE FRACTIONS (50 ml units)	TOTAL WEIGHT (g)	% RECOVERY	TLC OF COLUMN ELUATES-EXFW SPOT COLOR	R_f	STD. R_f	POSSIBLE IDENTITY
F-1	U-1	9.8329	11-44	15.63	52.1				
						red	0.20	0.21	salicin
	U-2	0.4067	45-64			red-brown	0.23	0.25	picein
	U-3	3.0620	65-215			red-brown	0.23	0.25	picein
	U-4	0.3780	216-380			yellow	0.44	0.43	salireposide
	U-5	1.9543	381-432			blank			
						orange	0.78		
F-2	T-1	0.0984	1-14	21.25	70.8	blank			
	T-2	10.6672	15-38			red	0.22	0.21	salicin
						red-brown	0.27	0.25	picein
	T-3	0.8930	39-65			red-brown	0.27	0.25	picein
	T-4	0.3725	66-90			blank			
	T-5	0.3029	91-104			red	0.55	0.52	tremuloidin
								0.48	populin
	T-6	0.6342	105-120			blank			
	T-7	1.5810	121-230			yellow	0.39	0.42	salireposide
	T-8	0.4670	231-432			blank			
	T-9	0.7719	433-450			orange	0.78		
	T-10	1.6361	451-455			purple	0.86		
	T-11	0.2416	456-458			purple	0.88		
	T-12	3.5901	459-493			blank			
F-3	D-1	0.1583	1-18	20.25	68.3	blank			
	D-2	14.2548	19-28			red	0.20	0.21	salicin
	D-3	1.2285	29-83			red-brown	0.25	0.24	picein
	D-4	0.3117	84-104			red		0.48	populin
	D-5	1.4419	105-200			yellow	0.39	0.42	salireposide
	D-6	0.4098	201-388			blank			
	D-7	0.5584	389-402			orange	0.79		
	D-8	2.3273	403-600			blank			
F-4	P-1	0.1495	1-16	27.89	92.9	blank			
	P-2	21.3884	17-38			red-brown	0.23	0.25	picein
	P-3	0.9747	39-106			yellow	0.39	0.39	salireposide
						red	0.49	0.52	tremuloidin
								0.47	populin
	P-4	3.9710	107-200			yellow	0.39	0.39	salireposide
	P-5	0.2865	201-364			blank			
	P-6	0.3605	365-386			orange	0.78		
	P-7	0.7639	387-600			blank			
W	K-1	0.1476	1-12	17.80	59.3	blank			
	K-2	16.7717	13-36			red	0.20	0.21	salicin
						red-brown	0.24	0.24	picein
	K-3	0.8816	37-462			blank			
B	Z-1	0.0183	1-12	11.71	39.0	blank			
	Z-2	9.0396	13-36			red	0.20	0.21	salicin
						red-brown	0.25	0.24	picein
	Z-3	0.6755	37-236			yellow	0.41	0.39	salireposide
						red	0.48	0.52	tremuloidin
								0.47	populin
	Z-4	0.1464	237-252			yellow	0.39	0.39	salireposide
	Z-5	0.2093	253-317			blank			
	Z-6	0.0879	318-332			orange	0.75		
	Z-7	1.5303	333-600			blank			
E	H-1	0.0165	1-12	18.02	60.1	blank			
	H-2	14.4275	13-40			black	0.10		
						red-brown	0.24	0.24	picein
	H-3	0.5017	41-64			red-brown	0.24	0.24	picein
	H-4	0.2686	65-104			red	0.57	0.52	tremuloidin
	H-5	0.5488	105-190			yellow	0.39	0.39	salireposide
	H-6	0.3306	191-252			blank			
	H-7	0.5690	253-270			orange	0.76		
	H-8	0.5069	271-323			orange	0.76		
						purple	0.86		
	H-9	0.8530	324-418			blank			

*The results on this table were obtained from the monitoring of the unconcentrated column eluates. For a detailed portrayal of the glycoside content of the concentrated fractions, see the examination of the dried eluate fractions (TABLE 6).

TABLE 4 - Elution solvent schemes for the polyamide chromatography of fractions F 1-4, W, B and E

FRACTION APPLIED	DISTILLED WATER	25% ETHANOL	50% ETHANOL	75% ETHANOL	95% ETHANOL
F-1	1-318*	319-366	367-406	---	407-432
F-2	1-361	362-409	410-435	---	436-493
F-3	1-290	291-369	370-459	460-512	513-600
F-4	1-280	281-348	349-436	437-519	520-600
W	1-280	281-322	323-372	---	373-462
B	1-219	220-286	287-367	368-441	442-600
E	1-228	---	229-319	320-386	387-418

*Each unit represents a 50 ml fraction.

Isolation, purification and identification of compound S

Fraction T-10(1.6361 g) and the corresponding fraction from the duplicate column of F-2(1.7183 g) were concentrated in turn to yield 70.3 mg and 151.5 mg of precipitate respectively which were combined. The precipitate (compound S) recrystallized three times from 95% ethanol, yielded colorless crystals (51.5 mg); m.p. 135.5-136°; R_f = 0.90 (EXFW); $[\alpha]_D^{23}$ = -34.6° (c = 2 in chloroform). A mass spectrum (A.E.I.-MS9) was obtained with a source temperature of 150°. All fragment ions of over 5% abundance are reported in FIG. 2. An accurate mass determination gave a measured molecular weight of 414.3873; the calculated mass for $C_{29}H_{50}O$ is 414.3862. The infra-red spectrum (KBr) contained bands at 2.91, 3.41, 3.50, 6.95, 7.35, 9.51, 9.85 and 10.50 μ (FIG. 3).

Compound S (25 mg) was acetylated by allowing it to stand at room temperature for 16 hours in acetic anhydride (1 ml) and pyridine (2 ml). Addition of the reaction mixture to ice water (5 ml) yielded a colorless precipitate which recrystallized from ether-methanol as colorless crystals (12.3 mg), m.p. 127-128°.

Authentic β-sitosterol (Aldrich Chemical Co.), recrystallized three times from 95% ethanol, yielded colorless crystals, m.p. 138-139°; R_f = 0.90(EXFW); $[\alpha]_D^{23}$ = -34.1° (C = 2 in chloroform); the infra-red spectrum (KBr) contained bands at 2.91, 3.41, 3.50, 6.95, 7.35, 9.51, 9.85 and 10.50 μ (FIG. 4). β-Sitosterol (20 mg) was acetylated

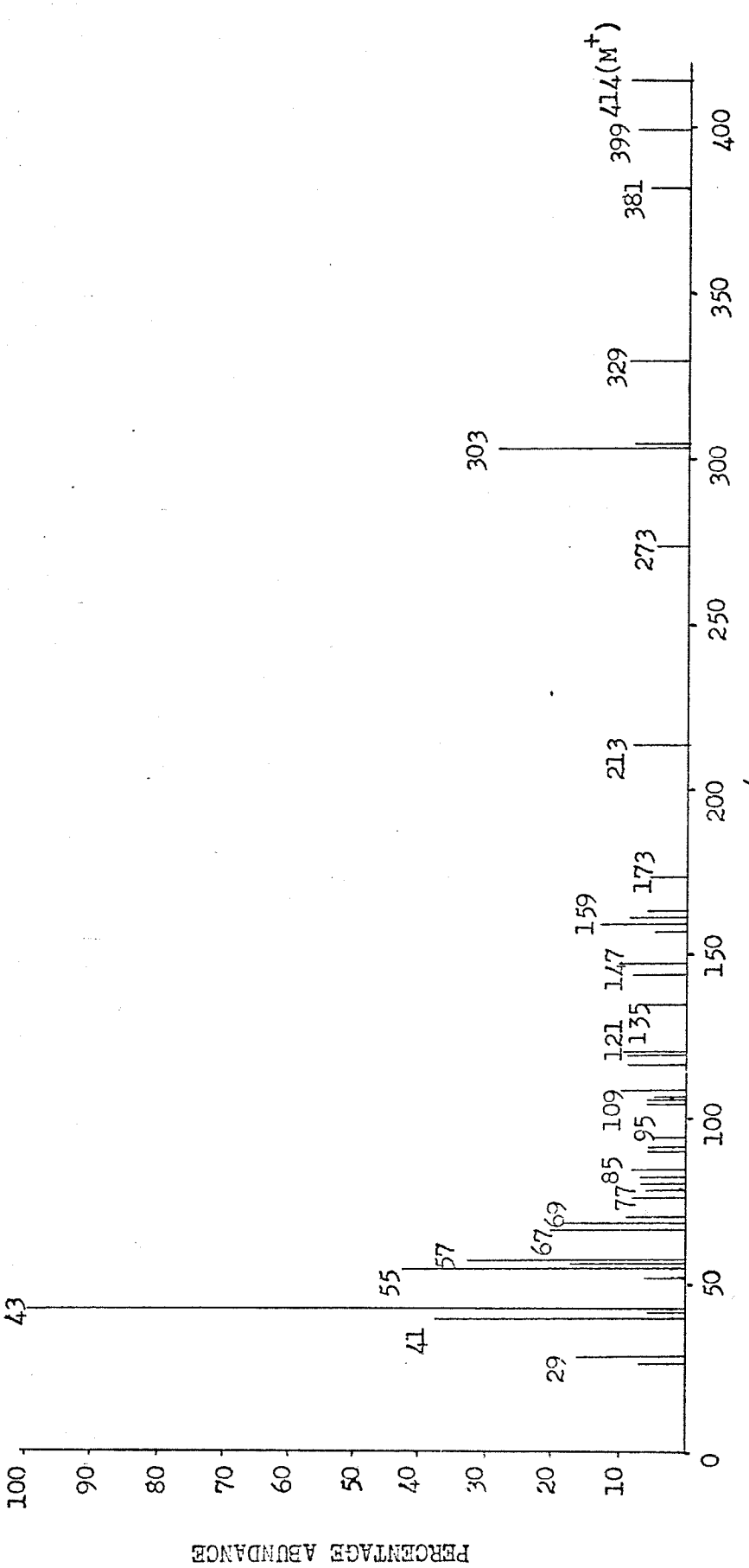


FIG. 2 - MASS SPECTRUM OF COMPOUND S

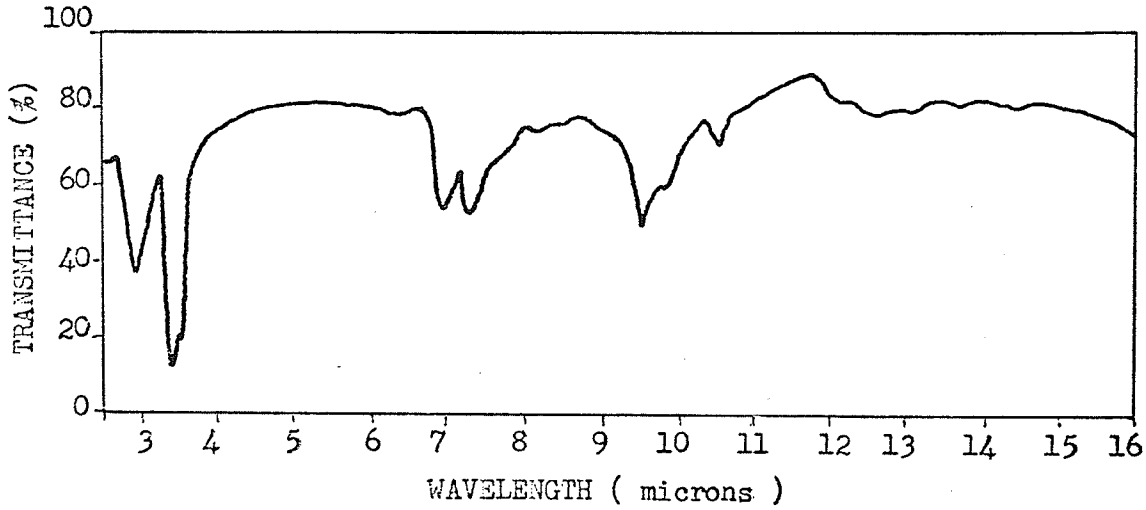


FIG. 3 - INFRA-RED SPECTRUM OF COMPOUND S (KBr)

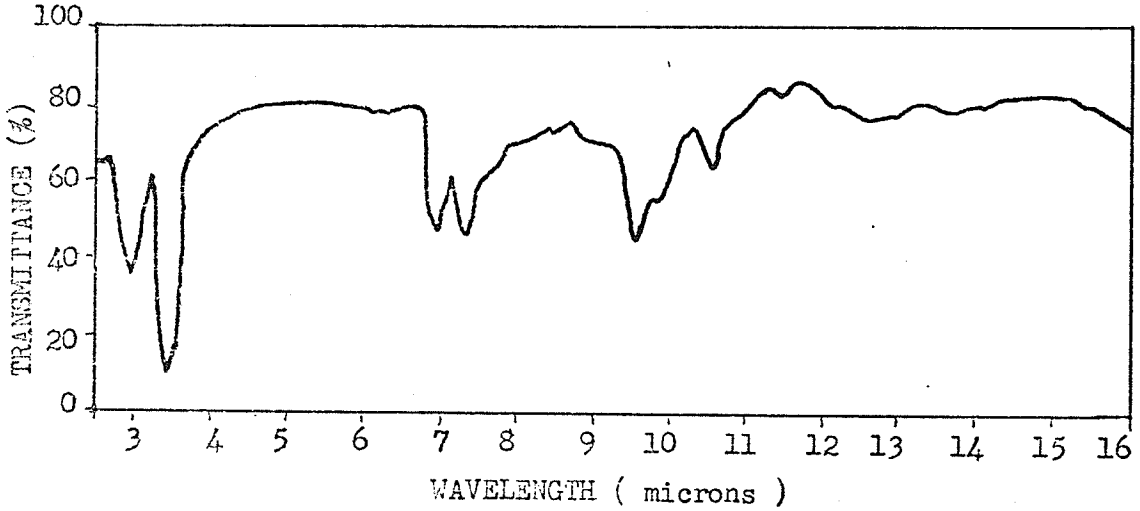


FIG. 4 - INFRA-RED SPECTRUM OF β-SITOSTEROL (KBr)

as described for compound S, yielding 10.9 mg of the acetate, m.p. 127-128°.

Compound S and authentic β-sitosterol were found to co-chromatograph on the EXFW TLC system (R_f = 0.90). When mixed, they melted at 137-137.5° and when mixed the acetates melted at 127-128°.

Isolation, purification and identification of compound N

Fraction T-9 (0.7719 g) and the corresponding fraction of the duplicate column of F-2 (0.8156 g) were combined to give 1.5875 g of a dark brown solid which would not recrystallize from acetone-petroleum ether, ethanol or water saturated ethyl acetate. An infra-red spectrum (KBr) showed absorption bands at 2.95, 3.41, 6.21, 6.59, 6.85, 7.39, 7.80, 8.05, 8.78, 9.12, 9.71, 10.35, 11.60, 12.30, 13.12 and 15.10 μ (FIG. 5).

A portion of the above fraction (0.7541 g) was taken up with 0.5 g of pretreated polyamide in tetrahydrofuran as described earlier and applied to a column 2.5 cm wide filled with 30 g of pretreated polyamide. The column was eluted with 25% ethanol and 50 ml fractions were collected. In the fractions ranging from 450 ml to 1,450 ml, an orange spot (R_f = 0.70) was observed with the EXFW TLC system. This fraction was evaporated to dryness at 40° to yield 430.2 mg of a brown solid.

The remaining portion of this fraction was processed in a similar manner to yield a further 462.1 mg of the brown solid.

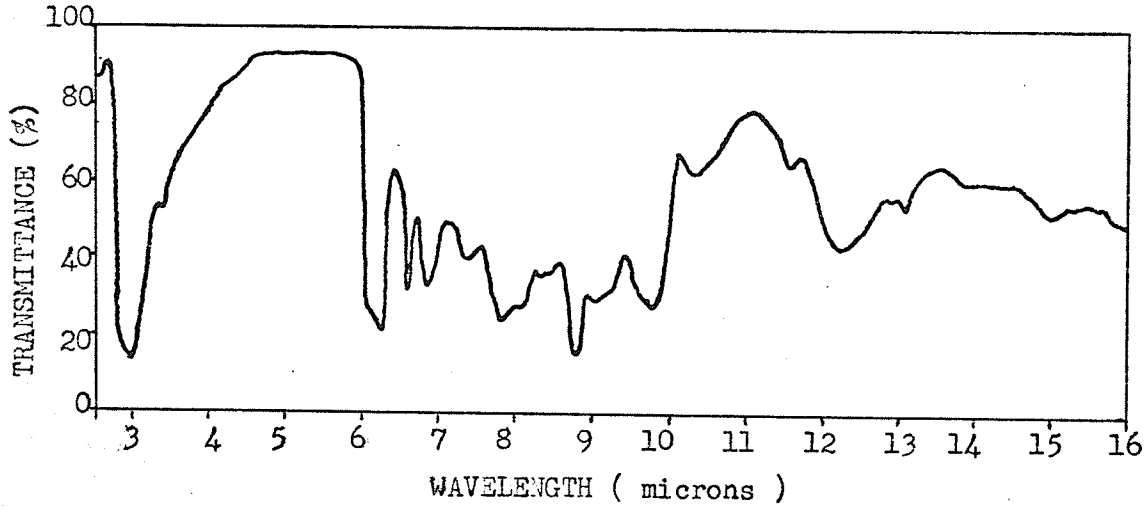


FIG. 5 - INFRA-RED SPECTRUM OF COMPOUND N (KBr)

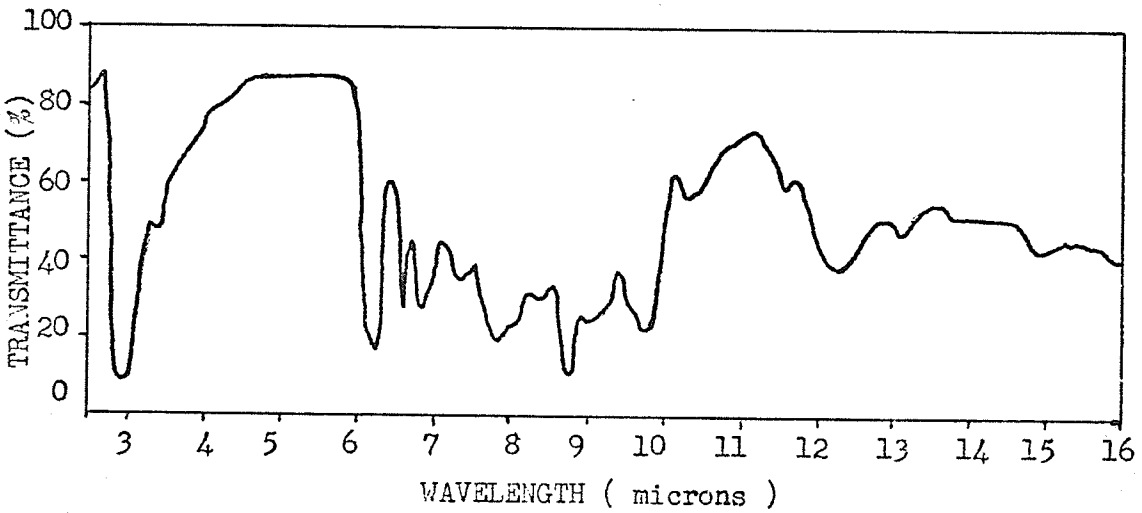


FIG. 6 - INFRA-RED SPECTRUM OF (+)-CATECHIN (KBr)

Fraction U-5 (1.9543 g) was chromatographed in a similar manner using 60 g of pretreated polyamide and a 4 cm wide column. The fractions ranging from 950 ml to 6,500 ml were bulked and evaporated to dryness at 40° to yield 1.0061 g of the same brown solid.

These three fractions were bulked as fraction N and recrystallized from water to yield 727.6 mg of brownish crystals and a brown residue. The crystals were re-crystallized twice more from water to yield 316.3 mg of pale brown crystals.

The 316.3 mg of purified crystals were applied directly to the top of a 2 cm wide column made up of 20 g of pretreated polyamide. The column was eluted with water and 50 ml fractions were collected. The fractions ranging from 1,300 ml to 6,000 ml showed an orange spot (R_f = 0.72) on the EXFW TLC system. Concentration of the column eluate yielded a buff colored solid (198.1 mg), m.p. 171-173° with sintering at 140°; $[\alpha]_D^{23}$ = +14.4° (c = 2 in acetone/water 1-1); (Found: C, 61.97; H, 4.73. $C_{15}H_{14}O_6$ requires C, 62.06; H, 4.86); λ max. = 280.5 mμ (c = 0.00098 in 95% ethanol) ($E_{1cm}^{1\%}$ = 230.6), shifting to 296 mμ immediately after the addition of 4 drops of N/1 NaOH to the spectrophotometer cell (FIG. 7). The infra-red spectrum was identical with the one previously determined (FIG. 5). A mass spectrum (A.E.I.-MS2) was obtained with a source temperature of 220°. All fragment ions of over 5% abundance are shown in FIG. 8. The compound (N-1) was found to be

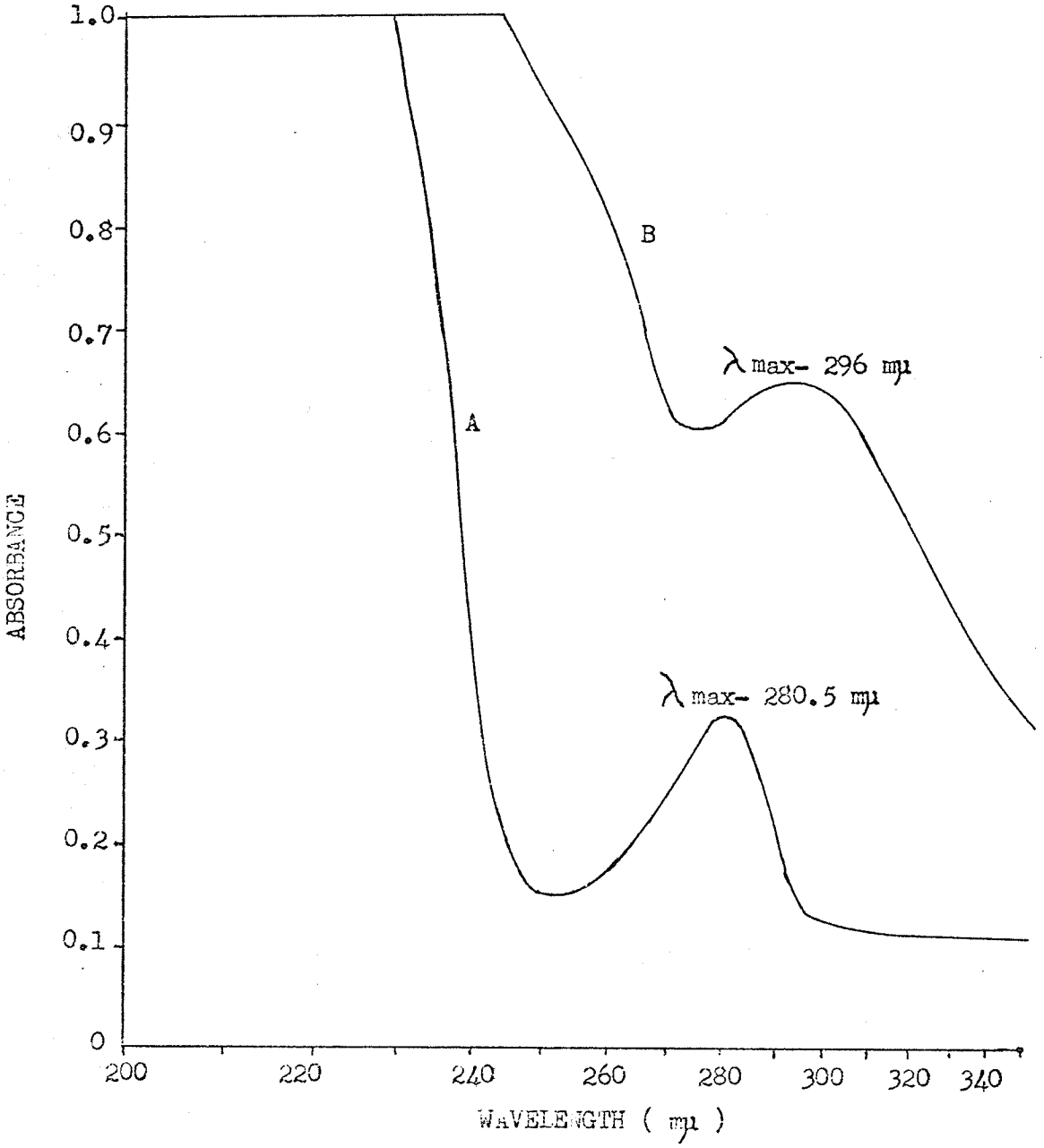


FIG. 7 - ULTRA-VIOLET SPECTRUM OF COMPOUND N

'A' - 0.00098 % w/v of N in 95 % ethanol
'B' - 'A' plus 4 drops N/1 sodium hydroxide added directly to the spectrophotometer cell.

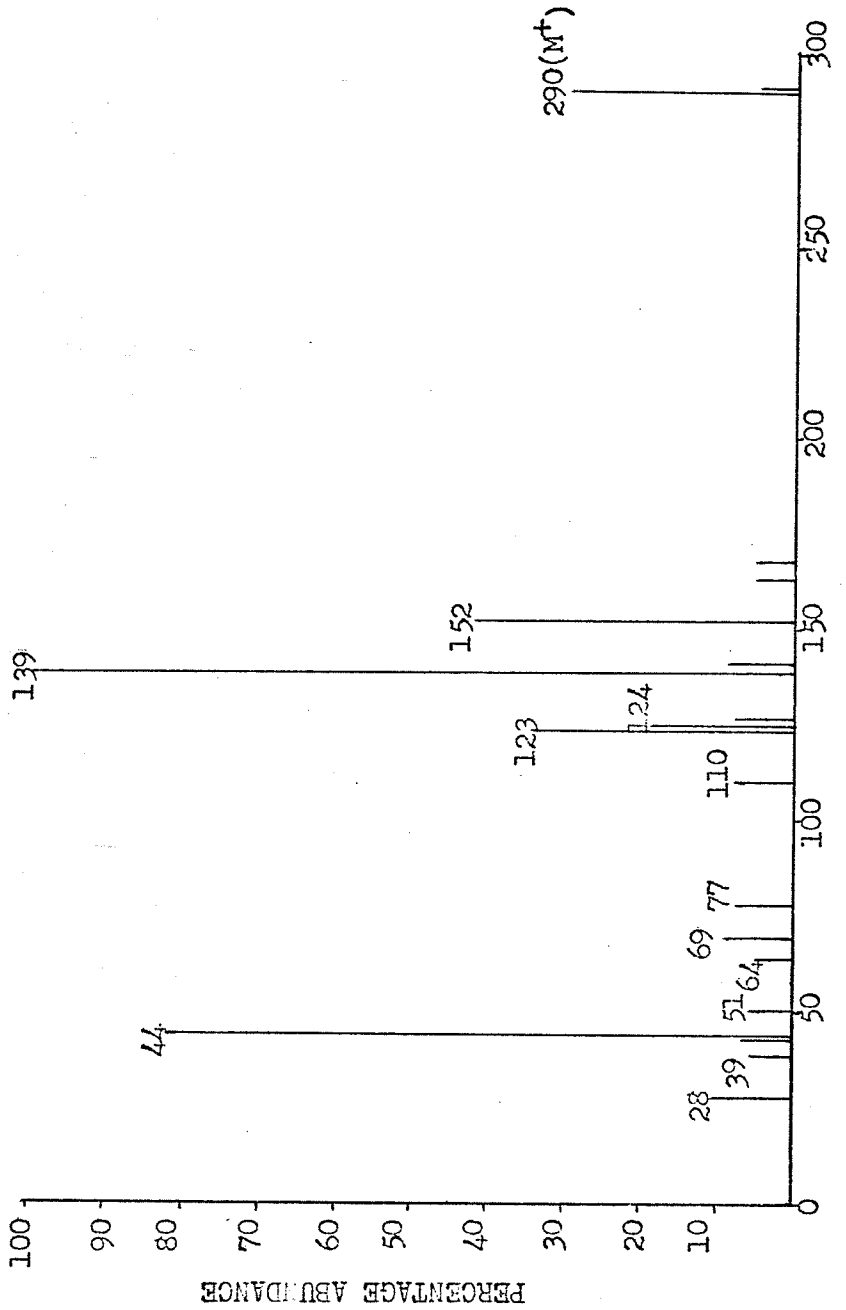


FIG. 8 - MASS SPECTRUM OF COMPOUND N

soluble in hot water, ethanol, acetone and methanol, slightly soluble in cold water and petroleum ether and insoluble in benzene and chloroform.

Fraction F-1 (35 g) was taken up with pretreated polyamide (17 g) and tetrahydrofuran and chromatographed as described for fractions F 1-4, W, B and E. The fraction which corresponded with U-5 of the first column for F-1 and showed an orange spot (R_f = 0.72) on the EXFW TLC system was taken to dryness to yield 1.8712 g of a tan colored material. The elution solvent was distilled water up to fraction 306 and then 25% ethanol up to fraction 460. The tan fraction was chromatographed on a 60 g polyamide column as described for fraction U-5 except that elution was with distilled water. Upon concentration, the eluate yielded two successive buff colored precipitates of 290.1 mg and 227.5 mg respectively and a final residue of 188.4 mg. These were set aside as N-2-1, N-2-2 and N-2-3 respectively for future reference.

Two successive attempts at acetylating N-1 (25 mg) using pyridine (2 ml) and acetic anhydride (1 ml) at room temperature yielded only a tan colored amorphous solid (12-15 mg),(R_f = 0.85, EXFW) which failed to recrystallize.

Normal sodium hydroxide (0.1 ml) was added to N-1 (4.3 mg) in distilled water (1 ml) yielding an orange color. When chromatographed on the EXFW TLC system, no components appeared to migrate up the plate and the orange color remained at the origin.

Fraction N-1 (3 mg) was added to 2 ml of a solution prepared from concentrated hydrochloric acid (20 ml), n-butanol (30 ml) and ferrous sulfate heptahydrate (7.7 mg) and was heated at 100° for 45 minutes (61). The hydrolysate was chromatographed on the BAEW TLC system and sprayed with a mixture of anisaldehyde (0.5 ml), sulfuric acid (0.5 ml), acetic acid (0.1 ml) and 95% ethanol (9 ml). After heating for ten minutes, any sugars present should have shown as blue to green spots (32b). Rhamnose, xylose, glucose, fructose and sucrose were chromatographed as reference standards. No sugar was detected in the hydrolysate.

When applied to Whatman No. 1 filter paper, compound N turned yellow upon exposure to ammonia vapours. Before and after exposure to ammonia vapours the compound showed a blue fluorescence under short wavelength ultra-violet light.

The four following spray reagents were prepared and applied to compound N on Whatman No. 1 filter paper (62). A ferric reagent was prepared by dissolving 3 g of AnalaR ammonium ferric sulfate in 100 ml of water and was used immediately. The second spray was a 3% solution of toluene-p-sulfonic acid in absolute ethanol. The third spray was a solution of vanillin (2 g) and toluene-p-sulfonic acid (1 g) in absolute ethanol (100 ml). The latter two reagents required heating at 110° for five minutes to bring out the colors. The final spray was bis-diazotized benzidine. This was prepared by stirring benzidine (5 g) with con-

centrated hydrochloric acid (14 ml) and dissolving this in water (980 ml). Three parts of this solution were added to two parts 10% sodium nitrite solution and the spray was used immediately.

The colors produced were a grey-green with the ferric reagent, a red with the toluene-p-sulfonic acid reagent, a deep red with the toluene-p-sulfonic acid/ vanillin reagent and a claret maroon with the bis-diazotized benzidine reagent.

(+)-Catechin (Nutritional Biochemical Corp.) was recrystallized three times from water yielding buff colored crystals, m.p. 174.5-175.5°; R_f = 0.70(EXFW); R_f = 0.72 (BAEW); the infra-red spectrum (KBr) (FIG. 6) was identical with that for compound N. A mass spectrum was obtained under the same conditions as that for compound N and all fragment ions of over 5% abundance are shown in FIG. 9.

Compound N and (+)-catechin co-chromatographed on the EXFW and BAEW TLC systems and when mixed in equal proportions, melted at 174-175°.

Examination of fractions F 1-4, W, B and E for phenolic glycosides

The eluate fractions obtained from the polyamide column chromatography of the ethyl acetate soluble fractions and the initial ethanol extraction residue were chromatographed on the EXFW and EM TLC systems. The plates were left for 24 hours after spraying with 4% sulfuric acid in absolute ethanol to ensure that all spots developed color.

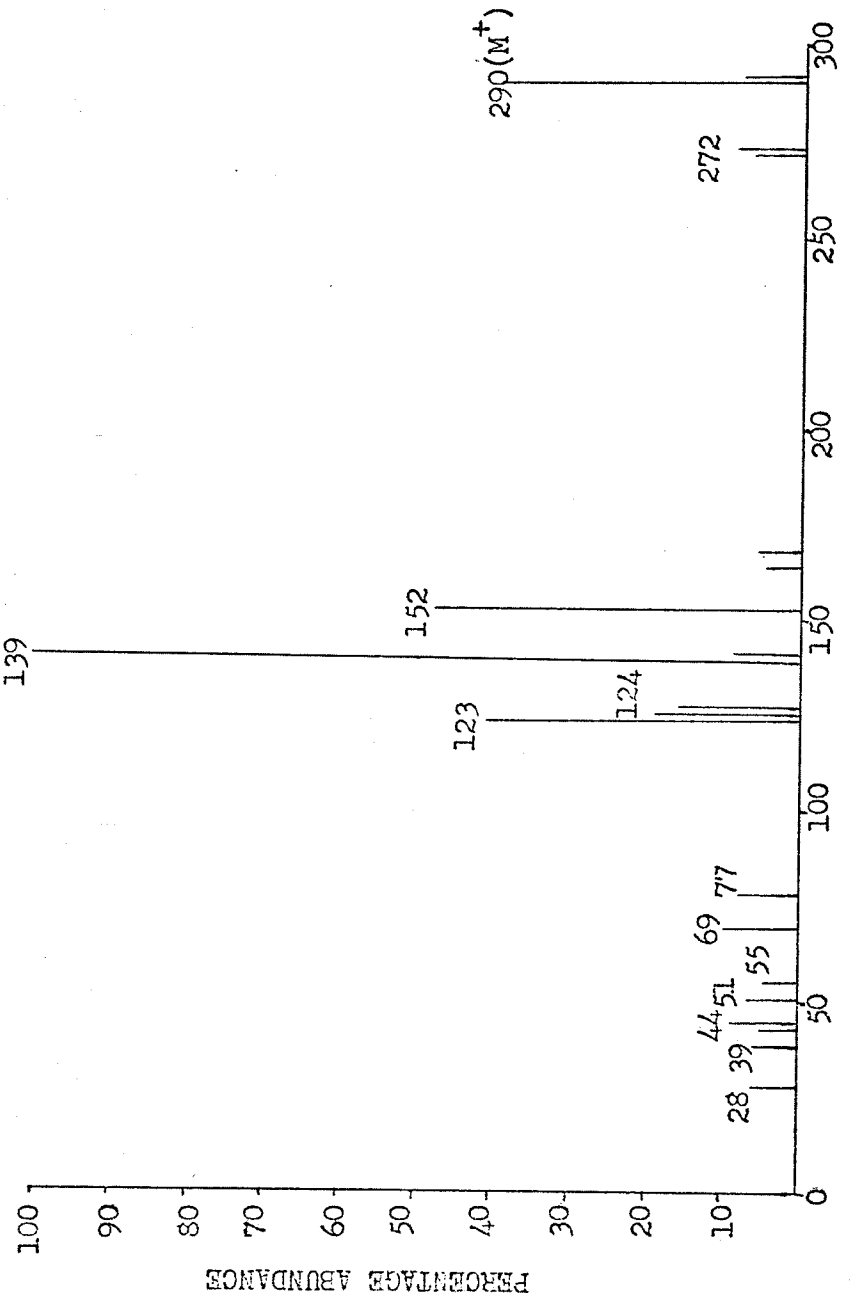


FIG. 9 - MASS SPECTRUM OF (+)-CATECHIN

(+)-Catechin and β-sitosterol were run as standards as well as the 21 phenolic glycosides listed earlier. The R_f values of the 23 standards in both TLC systems are listed in TABLE 5. The results of this analysis are summarized in TABLE 6. Three brown spots were noted which had R_f values lower than that for salicin and it was suspected that these could be monosaccharides or disaccharides. These spots were particularly evident in fractions U-1, T-2, D-2, P-2, K-2, Z-2 and H-2. Therefore, fractions K-2 and H-2 were chromatographed on the BAEW TLC system using the anisaldehyde spray reagent previously described for this system (p. 50). The major spot of the three observed showed a bluish color (R_f = 0.28) similar to glucose (R_f = 0.27) and fructose (R_f = 0.27). The other two spots did not correspond with any of the reference standards (glucose, fructose, arabinose, galactose, rhamnose, xylose and sucrose).

The same fractions used in the TLC examination were chromatographed on the GLC system described previously using the TMS derivatives of the same 23 standards. The retention times of these standards relative to TMS arbutin are listed in TABLE 7. The results from this study are recorded in TABLE 8.

The data from the screening of the seven fractions along with the elution patterns and solvent schemes of the polyamide columns on which they were run are summarized as FIGS. 10-16.

TABLE 5 - Standards used in the TLC examination of fractions F 1-4, W, B and E

COMPOUND	COLOR	R_f-EXFW	R_f-EM
salirepin	yellow	0.19	0.14
salicin	red	0.22	0.18
picein	red-brown	0.24	0.22
salidroside	yellow-brown	0.26	0.20
triandrin	dark green	0.28	0.20
grandidentatin	yellow	0.29	0.21
1-p-coumaroyl-β-D-glucose	yellow-brown	0.32	0.25
vimalin	dark green	0.33	0.20
salicortin	red	0.33	0.23
trichoside	yellow-brown	0.33	0.18
fragilin	red	0.37	0.32
nigracin	yellow	0.43	0.37
salireposide	yellow	0.43	0.36
trichocarpin	dark green	0.43	0.39
trichocarposide	orange-brown	0.45	0.40
populin	red	0.47	0.30
salicyloylsalicin	red	0.49	0.41
tremuloidin	red	0.52	0.48
tremulacin	red	0.55	0.51
salicyloylsalicin-2-O-benzoate	red	0.67	0.60
salicyloylsalicin-6-O-benzoate	red	0.73	0.70
β-sitosterol	purple	0.84	0.80
(+)-catechin	orange	0.72	0.72

TABLE 6 - TLC results of the examination of fractions F 1-4, W, B and E

FRACTION AND WEIGHT (g)	SPOT COLOR	POSSIBLE IDENTITY	EXFW R_f	EXFW STD. R_f	EM R_f	EM STD. R_f
U-1	red	salicin	0.21	0.22	0.19	0.18
9.8329	red-brown	picein	0.25	0.24	0.24	0.22
	red	salicyloylsalicin	0.49	0.47	0.38	0.41
U-2	red	salicin	0.22	0.22	0.20	0.18
0.4067	red-brown	picein	0.24	0.24	0.23	0.22
	dark green	vimalin	0.32	0.33	0.21	0.20
	red	populin	0.49	0.47	0.33	0.30
	red	salicyloylsalicin	0.49	0.49	0.40	0.41
U-3	yellow	salireposide	0.41	0.43	0.39	0.36
3.0620	red	tremulacin and/or	0.58	0.55	0.51	0.51
		tremuloidin	0.49	0.52	0.49	0.48
U-4	yellow	salireposide	0.41	0.43	0.39	0.36
0.3780						
U-5	orange	(+)-catechin	0.73	0.72	0.73	0.72
1.9543						
T-1	blank (indicates no color was evident)					
0.0984						
T-2	red	salicin	0.21	0.22	0.20	0.18
10.6672	red-brown	picein	0.25	0.24	0.24	0.22
	red	salicyloylsalicin	0.49	0.47	0.37	0.41
T-3	red	salicin	0.21	0.22	0.20	0.18
0.8930	red-brown	picein	0.25	0.24	0.24	0.22
	red	populin	0.49	0.47	0.31	0.30
	red	salicyloylsalicin	0.49	0.49	0.37	0.41
T-4	blank					
0.3725						
T-5	red	salicyloylsalicin	0.49	0.49	0.38	0.41
0.3029	red	tremulacin and/or	0.56	0.55	0.55	0.51
		tremuloidin	0.56	0.52	0.55	0.48
	yellow	salireposide	0.41	0.43	0.35	0.36
	yellow	grandidentatin	0.31	0.29	0.22	0.21

TABLE 6 - cont'd.

FRACTION AND WEIGHT (g)	SPOT COLOR	POSSIBLE IDENTITY	EXFW R_f	EXFW STD. R_f	EM R_f	EM STD. R_f
T-6	red	salicyloylsalicin	0.48	0.49	0.40	0.41
0.6342	yellow	salireposide	0.41	0.43	0.37	0.36
	yellow	grandidentatin	0.30	0.29	0.21	0.21
T-7	red	salicin	0.21	0.22	0.20	0.18
1.5810	red	populin	0.49	0.49	0.30	0.30
	yellow	salireposide	0.41	0.43	0.37	0.36
	yellow	grandidentatin	0.30	0.29	0.22	0.21
T-8	yellow	salireposide	0.41	0.43	0.40	0.36
0.4670						
T-9	orange	(+)-catechin	0.73	0.72	0.73	0.72
0.7719						
T-10	purple	β-sitosterol	0.86	0.84	0.81	0.80
1.6361						
T-11	purple	β-sitosterol	0.86	0.84	0.81	0.80
0.2416						
T-12	blank					
3.5901						
D-1	blank					
0.1583						
D-2	red	salicin	0.21	0.21	0.20	0.18
14.2548	red-brown	picein	0.25	0.24	0.25	0.22
	red	salicyloylsalicin	0.49	0.47	0.38	0.41
D-3	blank					
1.0285						
D-4	red	salicin	0.21	0.22	0.19	0.18
0.3117	red-brown	picein	0.25	0.24	0.21	0.20
	red	salicyloylsalicin	0.48	0.49	0.43	0.41
	yellow	grandidentatin	0.29	0.29	0.23	0.21
	red	tremulacin and/or	0.55	0.55	0.53	0.51
		tremuloidin	0.55	0.52	0.53	0.48
	yellow	salireposide	0.41	0.43	0.37	0.36
	red	salicyloylsalicin-2-O-benzoate	0.66	0.67	0.61	0.60
D-5	yellow	salireposide	0.40	0.43	0.39	0.36
1.4419						

TABLE 6 - cont'd.

FRACTION AND WEIGHT (g)	SPOT COLOR	POSSIBLE IDENTITY	EXFW R_f	EXFW STD. R_f	EM R_f	EM STD. R_f
D-6 0.4098	yellow	salireposide	0.40	0.43	0.39	0.36
D-7 0.5584	orange	(+)-catechin	0.73	0.72	0.73	0.72
D-8 2.3273	purple	β-sitosterol	0.87	0.84	0.81	0.80
P-1 0.1495	red	salicin	0.22	0.21	0.19	0.18
	red-brown	picein	0.25	0.24	0.21	0.22
P-2 21.3884	red	salicin	0.21	0.21	0.20	0.18
	red-brown	picein	0.25	0.24	0.21	0.22
	yellow	salireposide	0.41	0.43	0.37	0.36
P-3 0.9747	red	salicin	0.22	0.21	0.20	0.18
	red-brown	picein	0.25	0.24	0.22	0.22
	yellow	salireposide	0.44	0.43	0.39	0.36
	red	salicyloylsalicin	0.53	0.49	0.43	0.41
	red	tremulacin and/or	0.53	0.55	0.52	0.51
		tremuloidin	0.53	0.52	0.52	0.48
	yellow	grandidentatin	0.31	0.29	0.22	0.21
P-4 3.9710	yellow	salireposide	0.40	0.43	0.39	0.36
P-5 0.2865	yellow	salireposide	0.40	0.43	0.39	0.36
P-6 0.3605	orange	(+)-catechin	0.74	0.72	0.73	0.72
P-7 0.7639	blank					
K-1 0.1476	blank					
K-2 16.7717	red	salicin	0.22	0.21	0.18	0.18
	red-brown	picein	0.24	0.24	0.24	0.22
K-3 0.8816	blank					
Z-1 0.0183	blank					

TABLE 6 - cont'd.

FRACTION AND WEIGHT (g)	SPOT COLOR	POSSIBLE IDENTITY	EXFW R_f	EXFW STD. R_f	EM R_f	EM STD. R_f
Z-2 9.0396	red	salicin	0.21	0.21	0.19	0.18
	red-brown	picein	0.25	0.24	0.23	0.22
	red	salicyloylsalicin	0.48	0.47	0.39	0.41
	yellow	grandidentatin	0.31	0.29	0.23	0.21
Z-3 0.6755	red	salicin	0.22	0.22	0.18	0.18
	red-brown	picein	0.24	0.24	0.24	0.22
	dark green	vimalin	0.32	0.33	0.21	0.20
	yellow	grandidentatin	0.28	0.29	0.22	0.21
	yellow	salireposide	0.44	0.43	0.34	0.36
	red	salicyloylsalicin	0.48	0.47	0.39	0.41
Z-4 0.1464	yellow	salireposide	0.41	0.43	0.35	0.36
Z-5 0.2093	yellow	salireposide	0.41	0.43	0.35	0.36
Z-6 0.0879	orange	(+)-catechin	0.74	0.72	0.73	0.72
Z-7 1.5303	blank					
H-1 0.0165	blank					
H-2 14.4275	red-brown	picein	0.24	0.24	0.23	0.22
	yellow	grandidentatin	0.28	0.29	0.23	0.21
H-3 0.5017	red-brown	picein	0.24	0.24	0.23	0.22
	yellow	grandidentatin	0.30	0.29	0.23	0.21
	red	tremulacin and/or	0.56	0.55	0.50	0.51
		tremuloidin	0.56	0.52	0.50	0.48
H-4 0.2686	red	tremulacin and/or	0.56	0.55	0.50	0.51
		tremuloidin	0.56	0.52	0.50	0.48
H-5 0.5488	yellow	salireposide	0.40	0.43	0.39	0.36
H-6 0.3306	blank					

TABLE 6 - cont'd.

FRACTION AND WEIGHT (g)	SPOT COLOR	POSSIBLE IDENTITY	EXFW R_f	EXFW STD. R_f	EM R_f	EM STD. R_f
H-7 0.5690	orange	(+)-catechin	0.74	0.72	0.73	0.72
H-8 0.5069	orange	(+)-catechin	0.73	0.72	0.73	0.72
	purple	β-sitosterol	0.87	0.86	0.81	0.80
H-9 0.8530	blank					

TABLE 7 - Standards used in the GLC examination of fractions F 1-4, W, B and E

COMPOUND (TMS derivative)	RELATIVE RETENTION TIME TO TMS ARBUTIN
salicin	0.79
fragilin	1.07
picein	1.38
salirepin	1.73
salidroside	2.07
(+)-catechin	2.17
vimalin	2.31
triandrin	2.56
tremuloidin	2.65
populin	2.65
tremulacin	2.65
1-p-coumaroyl-β-D-glucose	2.83
salicortin	2.99
β-sitosterol	3.03
salicyloylsalicin	3.07
salireposide	3.18
trichocarpin	3.23
nigracin	3.31
trichoside	3.33
grandidentatin	3.79
salicyloylsalicin-2-O-benzoate	4.10
trichocarposide	4.20

TABLE 8 - GLC results of the examination of fractions F 1-4, W, B and E

FRACTION	RELATIVE RETENTION TIME	REFERENCE STANDARD	RELATIVE RETENTION TIME OF STANDARD
U-1	0.79	salicin	0.79
	1.45	picein	1.38
	3.00	salicyloylsalicin	3.07
U-2	0.78	salicin	0.79
	1.40	picein	1.38
	2.30	vimalin	2.31
	2.65	populin	2.65
	2.96	salicyloylsalicin	3.07
U-3	2.62	tremuloidin and/or tremulacin	2.65
	3.26	salireposide	3.18
U-4	3.12	salireposide	3.18
U-5	2.15	(+)-catechin	2.17
T-2	0.77	salicin	0.79
	1.39	picein	1.38
	2.97	salicyloylsalicin	3.07
T-3	0.76	salicin	0.79
	1.37	picein	1.38
	2.66	populin	2.65
	2.95	salicyloylsalicin	3.07
T-5	2.64	tremuloidin and/or tremulacin	2.65
	2.99	salicyloylsalicin	3.07
	3.21	salireposide	3.18
	3.65	grandidentatin	3.79
T-6	2.97	salicyloylsalicin	3.07
	3.14	salireposide	3.18
	3.66	grandidentatin	3.79
T-7	0.79	salicin	0.79
	2.68	populin	2.65
	3.18	salireposide	3.18
	3.73	grandidentatin	3.79
T-8	3.18	salireposide	3.18
T-10	2.95	β-sitosterol	3.03

TABLE 8 - cont'd.

FRACTION	RELATIVE RETENTION TIME	REFERENCE STANDARD	RELATIVE RETENTION TIME OF STANDARD
T-11	2.93	β-sitosterol	3.03
D-2	0.77	salicin	0.79
	1.40	picein	1.38
	3.02	salicyloylsalicin	3.07
D-4	0.77	salicin	0.79
	1.38	picein	1.38
	3.06	salicyloylsalicin	3.07
	3.71	grandidentatin	3.79
	2.64	tremuloidin and/or tremulacin	2.65
	3.26	salireposide	3.18
	4.17	salicyloylsalicin-2-O-benzoate	4.10
D-5	3.17	salireposide	3.18
D-6	3.17	salireposide	3.18
D-7	2.09	(+)-catechin	2.17
D-8	3.02	β-sitosterol	3.03
P-1	0.79	salicin	0.79
	1.42	picein	1.38
P-2	0.78	salicin	0.79
	1.42	picein	1.38
	3.13	salireposide	3.18
P-3	0.78	salicin	0.79
	1.42	picein	1.38
	3.12	salireposide	3.18
	3.02	salicyloylsalicin	3.07
	2.73	tremuloidin and/or tremulacin	2.65
	3.68	grandidentatin	3.79
P-4	3.18	salireposide	3.18
P-5	3.18	salireposide	3.18
P-6	2.13	(+)-catechin	2.17
K-2	0.79	salicin	0.79
	1.48	picein	1.38

TABLE 8 - cont'd.

FRACTION	RELATIVE RETENTION TIME	REFERENCE STANDARD	RELATIVE RETENTION TIME OF STANDARD
Z-2	0.79	salicin	0.79
	1.38	picein	1.38
	3.07	salicyloylsalicin	3.07
	3.68	grandidentatin	3.79
Z-3	0.76	salicin	0.79
	1.39	picein	1.38
	2.31	vimalin	2.31
	3.71	grandidentatin	3.79
	3.25	salireposide	3.18
	3.06	salicyloylsalicin	3.07
Z-4	3.27	salireposide	3.18
Z-5	3.27	salireposide	3.18
Z-6	2.25	(+)-catechin	2.17
H-2	1.40	picein	1.38
	3.69	grandidentatin	3.79
H-3	1.38	picein	1.38
	3.69	grandidentatin	3.79
	2.73	tremuloidin and/or tremulacin	2.65
H-4	2.68	tremulacin and/or tremuloidin	2.65
H-5	3.22	salireposide	3.18
H-7	2.20	(+)-catechin	2.17
H-8	2.20	(+)-catechin	2.17
	3.03	β-sitosterol	3.03

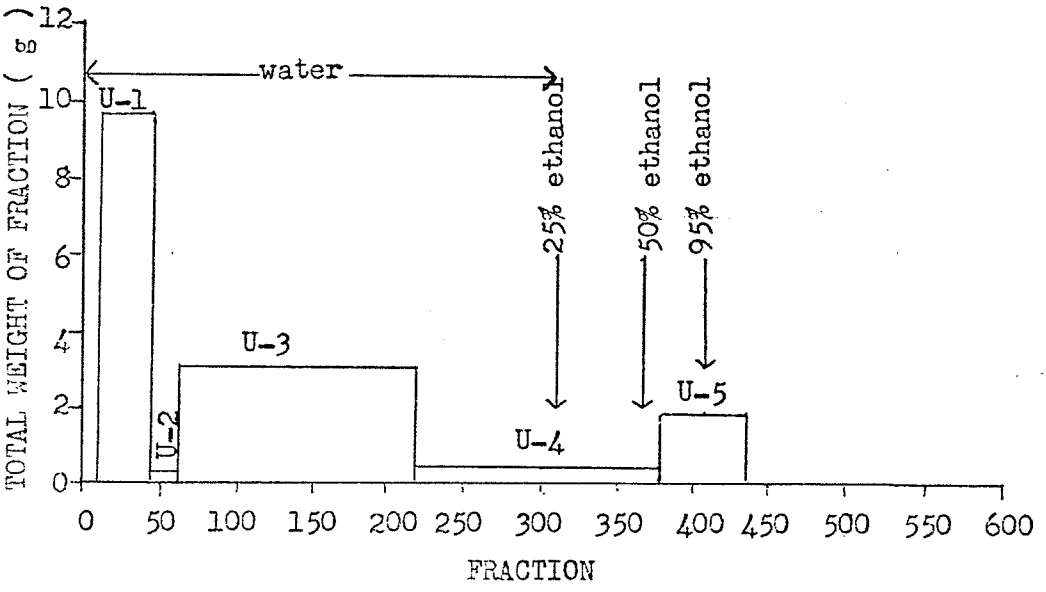


FIG. 10 - Polyamide column chromatography of the ethyl acetate fraction F-1

ELUTION FRACTION	COMPOUNDS IDENTIFIED
U-1	salicin , picein , salicyloylsalicin
U-2	salicin , picein , salicyloylsalicin , vimalin , populin
U-3	salireposide , tremulacin and/or tremuloidin
U-4	salireposide
U-5	(+)-catechin

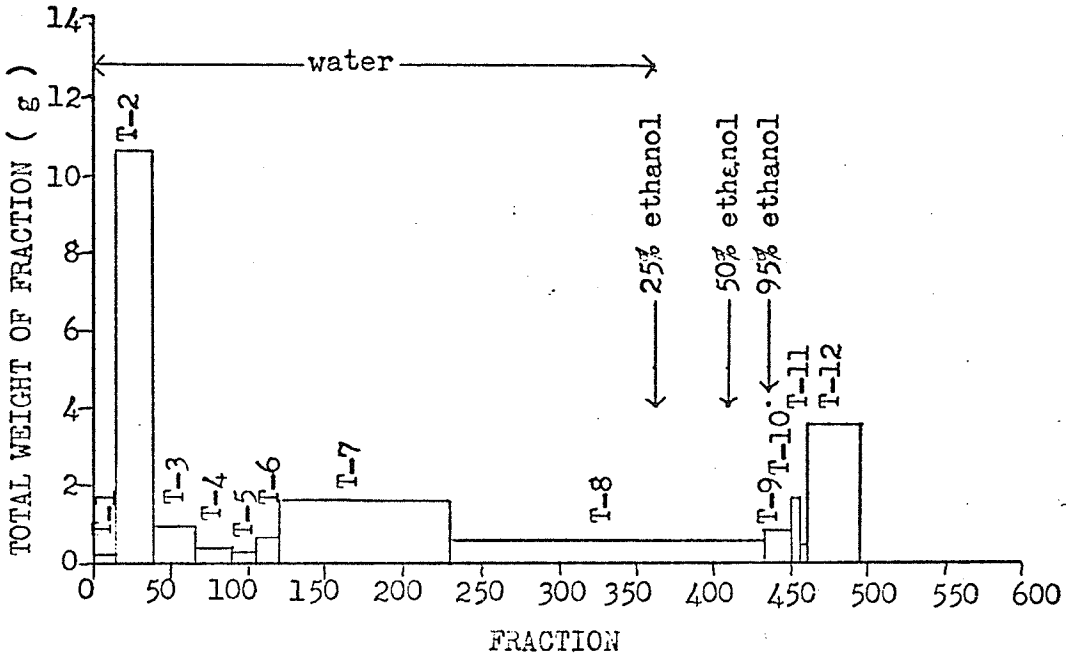


FIG. 11 - Polyamide column chromatography of the ethyl acetate fraction F-2

ELUTION FRACTION	COMPOUNDS IDENTIFIED
T-1	blank(indicates no compound identified)
T-2	salicin , picein , salicyloylsalicin
T-3	salicin , picein , salicyloylsalicin , populin
T-4	blank
T-5	salicyloylsalicin , tremulacin and/or tremuloidin , salireposide , grandidentatin
T-6	salicyloylsalicin , salireposide , grandidentatin
T-7	salicin , populin , salireposide , grandidentatin
T-8	salireposide
T-9	(+)-catechin
T-10	β-sitosterol
T-11	β-sitosterol
T-12	blank

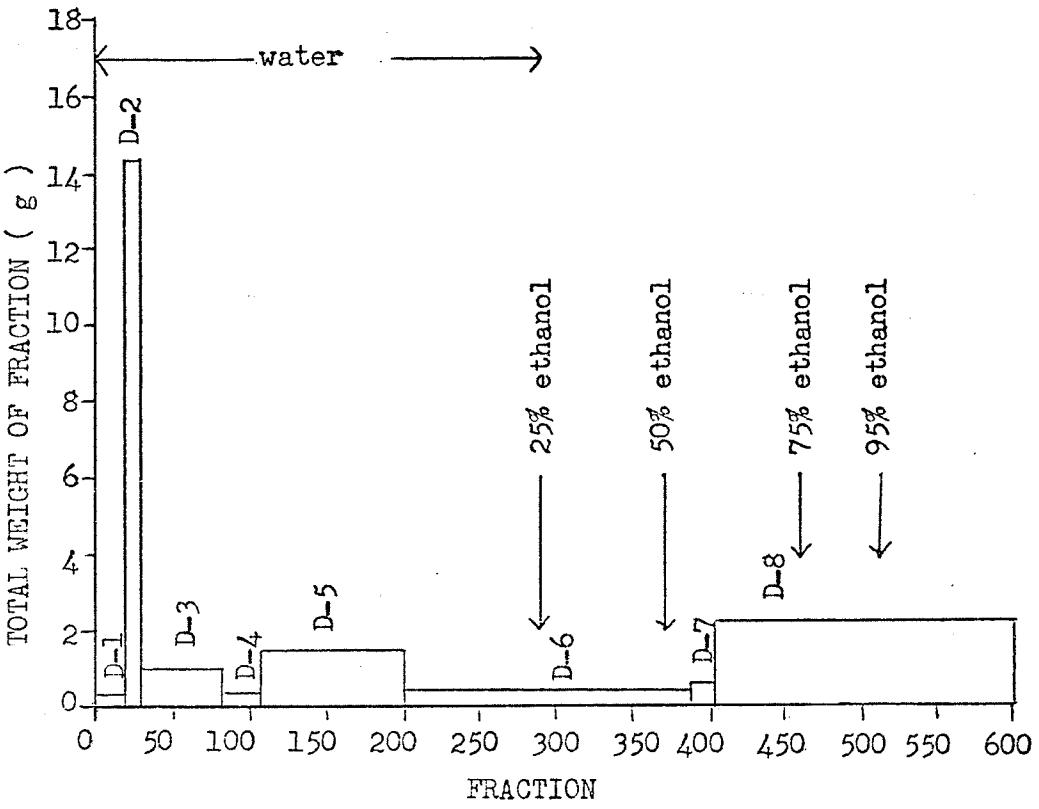


FIG. 12 - Polyamide column chromatography of the ethyl acetate fraction F-3

ELUTION FRACTION	COMPOUNDS IDENTIFIED
D-1	blank
D-2	salicin , picein , salicyloylsalicin
D-3	blank
D-4	salicin , picein , salicyloylsalicin , grandidentatin , tremulacin and/or tremuloidin , salireposide , salicyloylsalicin-2-O-benzoate
D-5	salireposide
D-6	salireposide
D-7	(+)-catechin
D-8	β-sitosterol

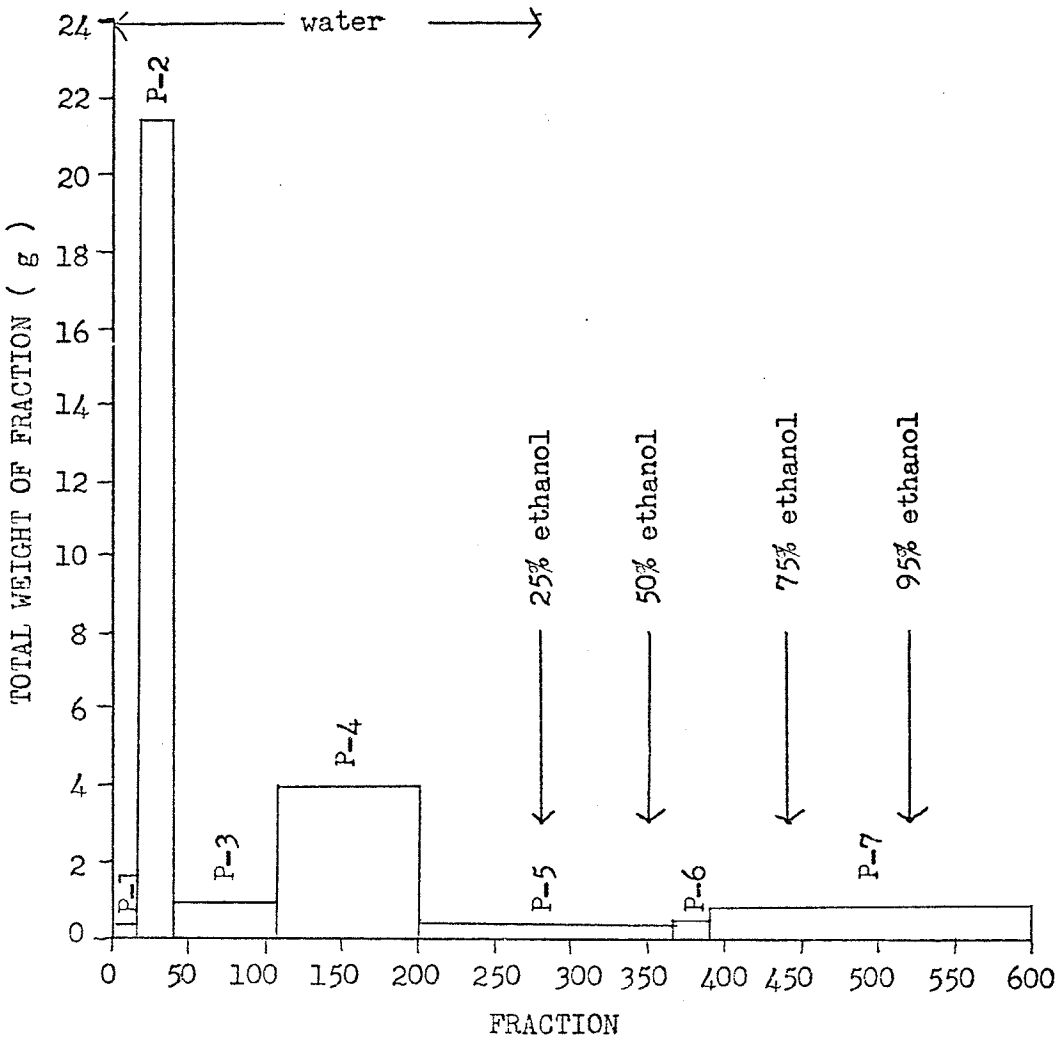


FIG. 13 - Polyamide column chromatography of the ethyl acetate fraction F-4

ELUTION FRACTION	COMPOUNDS IDENTIFIED
P-1	salicin , picein
P-2	salicin , picein , salireposide
P-3	salicin , picein , salicyloylsalicin , salireposide , tremulacin and/or tremuloidin , grandidentatin
P-4	salireposide
P-5	salireposide
P-6	(+)-catechin
P-7	blank

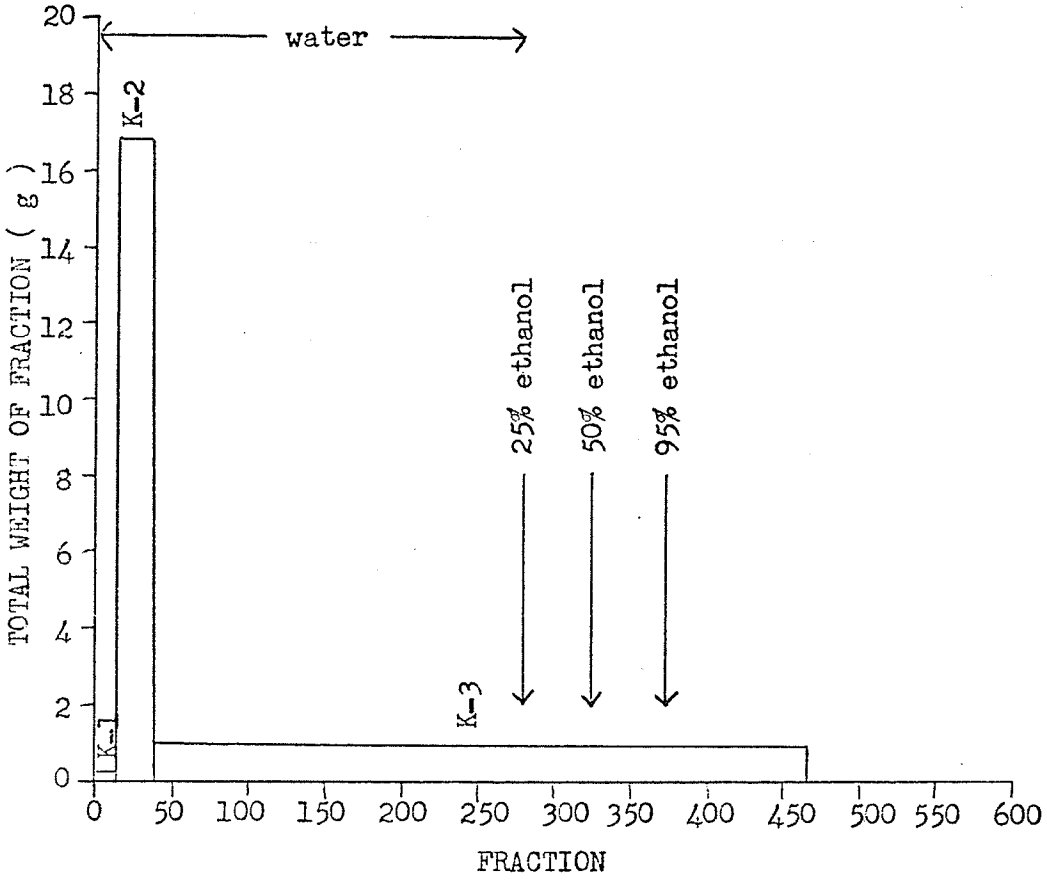


FIG. 14 - Polyamide column chromatography of the ethyl acetate fraction W

ELUTION FRACTION	COMPOUNDS IDENTIFIED
K-1	blank
K-2	salicin , picein
K-3	blank

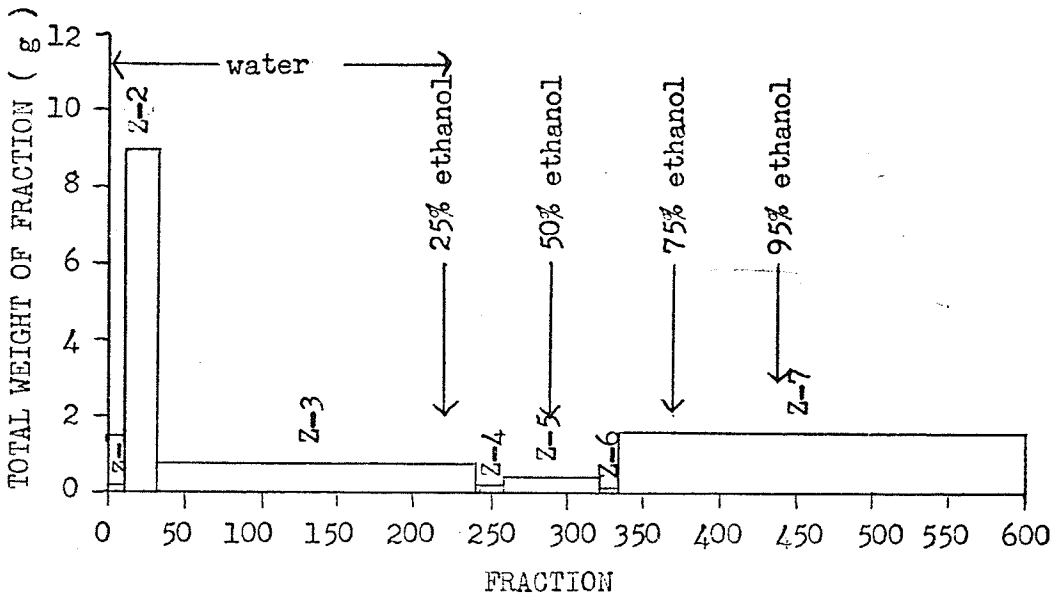


FIG. 15 - Polyamide column chromatography of the ethyl acetate fraction B

ELUTION FRACTION	COMPOUNDS IDENTIFIED
Z-1	blank
Z-2	salicin , picein , salicyloylsalicin , grandidentatin
Z-3	salicin , picein , salicyloylsalicin , grandidentatin , salireposide , vimalin
Z-4	salireposide
Z-5	salireposide
Z-6	(+)-catechin
Z-7	blank

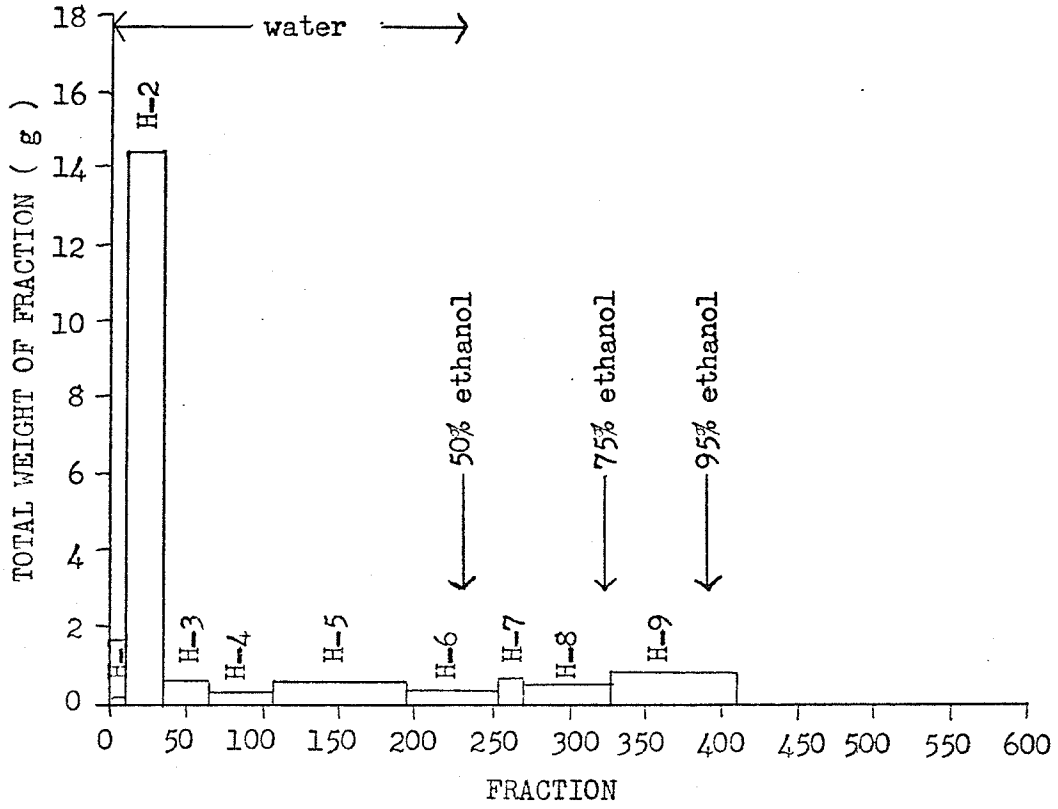


FIG. 16 - Polyamide column chromatography of the ethanol extract , fraction E

ELUTION FRACTION	COMPOUNDS IDENTIFIED
H-1	blank
H-2	picein , grandidentatin
H-3	picein , grandidentatin , tremulacin and/or tremuloidin
H-4	tremulacin and/or tremuloidin
H-5	salireposide
H-6	blank
H-7	(+)-catechin
H-8	(+)-catechin , β-sitosterol
H-9	blank

III. DISCUSSION

Collection and preparation of the sample

The bark was collected in November because a previous study on the seasonal variation of the phenolic glycosides of Salix petiolaris Sm. bark (58b) indicated that the greatest amount of extractable material was found in the November bark and the highest glycoside content was found in the October bark.

The bark was extracted with acetone because a study by Steele et al. (63) indicated that this was a non-destructive solvent when tested with a few selected phenolic glycosides. This was followed by ethanol to ensure complete extraction. It appeared that the acetone had extracted all the salicin, salicyloylsalicin, salicyloylsalicin-2-O-benzoate, populin and vimalin because none of these compounds were identified in the ethanol extract (FIGS. 10-16). Acetone did not seem to be as good a solvent as 95% ethanol for the extraction of grandidentatin since a major part of the ethanol extract (FIG. 16) appeared to be grandidentatin while examination of fraction F-2 (FIG. 11), fraction F-3 (FIG. 12), fraction F-4 (FIG. 13) and fraction B (FIG. 15) indicated that grandidentatin was one of many components in generally smaller fractions of the acetone extract.

Apart from the Soxhlet extraction and the flash evaporation, which utilized a steam coil, temperatures for operations throughout this study were kept below 40° to safeguard against decomposition of the glycosides.

The continuous ethyl acetate extraction was utilized

to effect an initial purification and fractionation of the acetone extract. It was found that even after 659 hours of continuous ethyl acetate extraction, glycosides could still be detected in the aqueous phase. This agreed with the findings of Steele et al. (63) who claimed that for this reason the traditional ethyl acetate extraction was useless as a purification step. Although it still served some function as an initial fractionation step, only the final aqueous fraction, fraction W, was significantly simplified. The other fractions still contained 7-10 of the 12 components identified (FIGS. 10-16). The possible decomposition which could have resulted from such prolonged heating outweighed the gain that this extraction gave as a means of fractionation. Attempts to further fractionate B prior to polyamide column chromatography were unsuccessful but since this fraction was not unduly complex, this was not important. After this fractionation of the acetone extract, salicin, picein, salireposide, and populin and/or tremuloidin appeared to be present along with a purple spot with R_f = 0.84-0.87 and an orange spot with R_f = 0.72 on the EXFW TLC system.

Thin-layer chromatography

The thin-layer chromatographic systems of Audette et al. (3) were employed as a monitoring system because they were simple, quick and gave a satisfactory initial identification. Humidity was not controlled but salicin was chromatographed with each plate to check the

reproducability of the R_f values.

Polyamide column chromatography

The eluate fractions of the polyamide columns were of sufficient purity to allow a detailed identification of the compounds therein. Waxes and high molecular weight material of a non-polar nature were retained on the column. Fractions U-1, T-2, D-2, P-2, K-2, Z-2 and H-2 were the largest eluate fractions from their respective polyamide columns, containing approximately 30% to 70% of the material applied to the column. These fractions all contained picein and most contained salicin and salicyloylsalicin as well, establishing these as major components. The only other fractions which weighed over 1 g were U-3, T-7, D-5 and P-4 which were associated with the elution of salireposide and U-5, T-10 and D-8 which were associated with the elution of chlorophylls and less polar compounds. The total column eluate varied from 20 l to 30 l. The percentage recovery varied from 39.0% to 92.9% and probably reflected the percentage of non-polar contaminants in each fraction.

As a column adsorbent for separating polar compounds, polyamide has the advantage that columns are easily prepared and can be run for several days without compacting.

The TLC monitoring system seemed to be the best system for the initial identification of eluate components since there were 200-300 fractions to monitor for each column and speed and simplicity were essential. Since the R_f values of the 21 available glycoside standards ranged

between 0.19 and 0.73 on the EXFW TLC system, only spots within this range were considered. The spot which corresponded to β-sitosterol ran somewhat higher but attention was focused on this compound because it crystallized out in the eluate. The only other component which aroused interest was the one which gave an orange spot on the EXFW TLC system at R_f = 0.76-0.79.

Compound S

Once recrystallized from 95% ethanol to a constant melting point of 135.5-136°, 3 mg of S were sent for an accurate mass determination and the molecular weight determined was 414.3873. For $C_{29}H_{50}O$, the calculated molecular weight is 414.3862. Since this corresponded with β-sitosterol, a common plant sterol, an attempt was made to confirm this structure. The isolated product was optically active, $[\alpha]_D^{23}$ = -34.6° (C = 2 in chloroform) while for the authentic β-sitosterol, $[\alpha]_D^{23}$ = -34.1° (C = 2 in chloroform). The reported value was $[\alpha]_D^{25}$ = -32.8° in chloroform (51).

Authentic β-sitosterol melted at 138-139°. The isolated material mixed with authentic β-sitosterol did not depress this melting point. The two compounds co-chromatographed on the EXFW TLC system and had identical infra-red spectra (FIGS. 3 and 4). The acetates prepared from the isolated product and the authentic β-sitosterol melted at the same temperature and when mixed, this melting point was not depressed.

The mass spectrum of β-sitosterol reported by Knights (64) had the same base ion as the mass spectrum of the isolated product, at m/e 43. Knights also reported 13 major fragment ions over m/e 210. All these fragment ions were present in the mass spectrum of the isolated product but with different relative abundances and six were below 5% abundance. These differences could be explained by differences in instrumentation and the technique of insertion of the sample. The above evidence confirmed the identity of the isolated product as β-sitosterol, without further clarification of the mass spectrum.

β-Sitosterol has been found previously in the pollen of unspecified Salix sp (65), the bark of Populus grandidentata (9), the heartwood of Populus tremuloides (66) (67) and the bark of Populus tremuloides (51) but not in the bark of Salix sp.

Compound N

Fraction T-9 was dark brown but gave a single orange spot (R_f = 0.72) on the EXFW TLC system, indicating a relatively pure fraction. An infra-red spectrum (FIG. 5) indicated a polyhydroxylated compound with no carbonyl moiety. Since this fraction could not be purified by recrystallization, it was chromatographed on a further polyamide column. This step appeared to be successful since much of the color of the crude fraction was eliminated. Fraction U-5 was processed in a similar manner. The eluate fractions were dried, combined and recrystallized three

times from water. However, the crystals obtained were still brown in color and the recrystallizations appeared to cause large losses. As a result, these crystals were again chromatographed on a polyamide column which was eluted with distilled water. This yielded a buff colored solid which was submitted to further study.

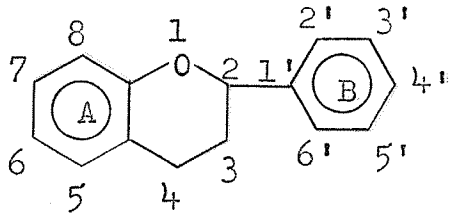
The addition of normal sodium hydroxide to the compound produced an orange color. This marked color reaction suggested the possibility of a flavonoid compound. The infra-red spectrum indicated that there was no carbonyl group, so a catechin (flavan-3-ol) or leucoanthocyanidin (flavan-3,4-diol) was indicated. Acid hydrolysis indicated that there was no sugar present. The carbon-hydrogen analysis was reported as 61.97% carbon and 4.73% hydrogen. The carbon-hydrogen analysis of a pentahydroxyflavan was calculated to be 62.06% carbon and 4.86% hydrogen. Therefore, it became necessary to determine the pattern of hydroxylation on the basic flavan nucleus.

The ultra-violet spectrum showed a maximum at 280.5 mμ which shifted to 296 mμ immediately upon addition of alkali (FIG. 7). This is typical of polyhydric phenols in which no carbonyl conjugation is present, such as catechins and leucoanthocyanidins (68a).

The acetylation was attempted in order to produce a derivative to aid in the screening of known compounds. However, two attempts only produced amorphous solids from which no reliable information could be gathered.

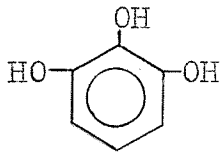
The acid hydrolysis produced a brown color which is typical of catechins. Leucoanthocyanidins, on the other hand, produce red colors upon acid treatment (69a).

The four color tests carried out aided in the rationalization of the pattern of hydroxylation on the basic flavan nucleus of compound N (62). The green color

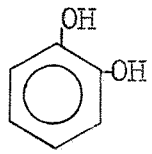


Flavan Nucleus

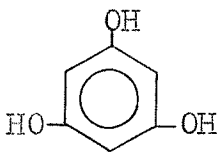
produced by the ferric reagent was indicative of a B ring with a pattern of hydroxylation as in catechol. The red color pro-



Pyrogallol



Catechol



Phloroglucinol

duced by the vanillin-toluene-p-sulfonic acid reagent was indicative of a phloroglucinol hydroxylation pattern in the A ring. The claret maroon color produced by the bis-diazotized benzidine reagent was indicative of a phloroglucinol pattern of hydroxylation in the A ring and a catechol or pyrogallol pattern of hydroxylation in the B ring. From this it was

concluded that the A ring was hydroxylated at the 5 and 7 positions giving a phloroglucinol pattern of hydroxylation (the number 1 oxygen atom is considered as the third hydroxyl group). Position 2' is less likely to be substituted than positions 3' or 4' because of steric hinderance. Therefore, the evidence indicated that the B ring was hydroxylated at the 3' and 4' positions giving a catechol pattern of hydroxylation. Thus, four aromatic hydroxyl groups were indicated, leaving only one hydroxyl group of the five available for aliphatic substitution at the 3 or 4 positions. Thus a catechin was favored over a leucoanthocyanidin. The toluene-p-sulfonic acid reagent gave a pink color which was supposed to be typical of leucoanthocyanidins while catechins were supposed to give a yellow color. However, it was found that authentic (+)-catechin sprayed with this reagent gave a pink color and the interpretation of this test by Roux and Maihs (62) is therefore questionable.

Upon search of the literature, it was found that (+)-catechin had a melting point close to that of compound N, similar solubility and fitted the color tests and ultraviolet spectrum.

Therefore, a sample of (+)-catechin was obtained and recrystallized from water. (+)-Catechin and compound N co-chromatographed on the EXFW and BAEW TLC systems. Compound N did not depress the melting point of (+)-catechin when mixed with it and the infra-red spectra of compound N

and (+)-catechin were found to be identical (FIGS. 5 and 6). The optical rotation of compound N ($+14.4^{o}$) corresponded closely to that reported in the literature for (+)-catechin ($+16.5^{o}$) (70). There was not enough authentic (+)-catechin on hand to determine an optical rotation.

Compound N and (+)-catechin were sent for mass spectral analysis (FIGS. 8 and 9). Because both samples had been dried to different degrees before analysis, the fragment ion of m/e 18, which corresponded to the loss of water, was ignored. The ion of m/e 139 became the base peak in both cases. The only major difference between the two spectra was the ion of m/e 44. The percentage abundance of this ion in the spectrum of compound N was 82% while in the spectrum of (+)-catechin it was 9%. This difference could be explained if there was a greater amount of carbon dioxide introduced into the instrument when compound N was analyzed. It should be noted that the ions of m/e 55, 271 and 272 shown in the spectrum of (+)-catechin were also present in the spectrum of compound N but had a percentage abundance of less than 5%. This was also true for the ions of m/e 64 and 110 seen in the spectrum of compound N but not in the spectrum of (+)-catechin. These spectra agreed closely with that for (+)-catechin reported by Clark-Lewis (71).

The above evidence was considered adequate to identify compound N as (+)-catechin.

The identification of compound N as (+)-catechin

accounted for several observations made during the isolation. Catechins polymerize or condense with heat in aqueous media or with mineral acids to produce phlobophenes (68b). This colored polymeric substance could have accounted for the original brown color of the extract as well as the color produced upon acid hydrolysis and recrystallization from hot water. The marked loss of material during recrystallization from water was almost certainly due to polymerization. Polyamide column chromatography with water was found to be an effective method of decolorising the extract by retaining the water insoluble polymer at the top of the column as a red-brown colored band, while allowing the monomer to pass through freely.

Catechin is one of the most widely occuring flavonoids (69a) and along with the leucoanthocyanidins, forms the economically important condensed tannins (69b). Thieme (49) found a considerable amount of tannin material in the barks of several Salix sp. Because of the nature of the polymeric condensed tannins, it would have been likely that monomeric catechin was also present. (+)-Catechin was found by Pearl and Darling (40) in the bark of Salix purpurea. It was claimed that this was the first time that it had been found in Salicaceae. However, this is incorrect since catechin was reported to have been found in the leaves and leaf galls of Salix fragilis in 1929 (44) as well as 1959 (43). Tsiskarishvili (38) reported finding (+)-catechin in the leaves of unspecified Salix sp. and (±)-catechin in

the wood of these same species in 1956. Jaggi and Haslam (72) have provisionally identified (+)-catechin in the leaf extracts of several Salix sp. in 1969.

Examination of fractions F 1-4, W, B and E for phenolic glycosides

Apart from looking for new phenolic compounds, the aim of this study was to examine fractions F 1-4, W, B and E for phenolic glycosides. Initially, the dried eluate fractions from the polyamide chromatography of these fractions were run on the EXFW and EM TLC systems using the 21 available phenolic glycosides as standards. All the standards are naturally occuring glycosides except salirepin which is a synthetic compound (73). (+)-Catechin and β-sitosterol were also used as standards since they were isolated in this study.

The initial TLC screening of the ethyl acetate fractions on the EXFW TLC system (TABLE 2) indicated salicin, picein, salireposide and tremuloidin and/or populin to be present. The TLC monitoring of the individual 50 ml eluate fractions on the same system indicated the presence of the same phenolic glycosides. The final TLC examination of the dried bulked column eluates on both the EXFW and EM TLC systems (TABLE 6) indicated the presence of salicin, picein, salicyloylsalicin, vimalin, populin, salireposide, grandidentatin, salicyloylsalicin-2-O-benzoate and tremuloidin and/or tremulacin. (+)-Catechin and β-sitosterol were evident in all three analyses.

The polyamide column chromatography purified the crude extracts sufficiently well to allow a detailed analysis of the components. It was found necessary to dry the bulked column eluate fractions to give the best analysis. No components were noted in fractions U-4, T-6, T-8, D-6, D-8, P-1, P-5 and Z-5 in the initial monitoring of the unconcentrated polyamide column eluates. In the examination of the dried bulked eluate fractions, they were found to contain components of the preceding or following fractions. This was because the concentration in the solution was insufficient to register during the monitoring of the eluates themselves. However, concentrating each of the 3600 individual 50 ml eluate fractions prior to bulking would have proved much too time consuming. The chromatographing of 0.05 ml of every second 50 ml fraction of the column eluate on the EXFW TLC system proved to be an effective means of monitoring the column eluate prior to bulking. Wong (74a) found that 4% sulfuric acid spray detected as little as 0.2-0.4 µg of phenolic glycoside.

The GLC examination of the bulked eluate fractions confirmed the TLC results indicating the same 12 compounds to be present. In addition, two unknown peaks were apparent but these were very likely non-glycosidic since they were not evident in the TLC examination. Fraction T-9 was not chromatographed in the GLC examination because the entire fraction was utilized in the isolation of (+)-catechin before this analysis was performed. However, TLC indicated

that (+)-catechin was the only component present in fraction T-9. Of the 23 reference compounds used, only TMS salicyloylsalicin-6-O-benzoate did not elute from the column. Trace components were noticed in the GLC examination which corresponded to compounds in previous or following fractions. However, since they were not detectable using TLC, they were not included in the results of this analysis. If the amount of substance applied to the TLC plate was increased in an attempt to locate these trace components, the major components streaked and the TLC chromatogram became useless.

Some difficulty was encountered in distinguishing certain glycosides in single chromatographic systems. On the EXFW TLC system populin, salicyloylsalicin, tremuloidin and tremulacin displayed red spots with R_f values of 0.47, 0.49, 0.52 and 0.55 respectively. However, on the EM TLC system the R_f values were 0.30, 0.41, 0.48 and 0.51 respectively. Therefore, populin and salicyloylsalicin could be distinguished from each other and from tremuloidin and tremulacin but tremuloidin and tremulacin could not be distinguished from one another. On the GLC system used, the populin, tremuloidin and tremulacin derivatives could not be distinguished from one another but the salicyloyl-salicin derivative was readily distinguished from these three glycosides. It remained impossible to resolve tremuloidin and tremulacin. When monitoring for phenolic glycosides previous to this examination using only the EXFW

system, this TLC spot was reported as tremuloidin or tremuloidin and/or populin because this ambiguity was not apparent at the time.

On the EM TLC system, picein, vimalin and grandidentatin had R_f values of 0.22, 0.21 and 0.20 respectively. Therefore, when two or more of these compounds were present in the same fraction difficulty was encountered in distinguishing them despite the fact that individually they developed different colors. Generally, a long dark spot was observed. On the EXFW TLC system picein, vimalin and grandidentatin had R_f values of 0.24, 0.29 and 0.33 respectively and some difficulty was still encountered in distinguishing them in fractions where two or more were present. However, on the GLC system, the picein, vimalin and grandidentatin derivatives had relative retention times of 1.38, 2.31 and 3.79 respectively and were readily distinguished from each other.

Finally, the salicortin, β-sitosterol and salicyloylsalicin derivatives had similar retention times on the GLC system but were readily distinguished by color and R_f value on the two TLC systems.

Kripiakevich (58c) examined the December bark of Salix petiolaris Sm. for salicin, picein, tremuloidin, salireposide, fragilin, triandrin, populin, grandidentatin, vimalin, salicortin and salidroside and found that the first six compounds were present. Wong (74b) examined the June bark of the same species for the same eleven compounds

and found that the first nine compounds were present. Neither of the studies used tremulacin as a standard so that the tremuloidin identified could have included tremulacin. The present study agrees with these two studies that salicin, picein, tremuloidin and salireposide are present and that salicortin and salidroside are absent in the bark of this species. However, both Kripiakevich and Wong found triandrin and fragilin present but these were not found in the present study. Also, Kripiakevich did not find grandidentatin, vimalin and populin while these were found in the present study and the study by Wong. These differences may be due to seasonal variation which can cause even qualitative differences in the glycosides found (30). Wong used a hot lead subacetate treatment in his extraction and this will definitely cause differences in phenolic glycoside content (75). As noted earlier acetone is a poor solvent for extracting grandidentatin. Since Kripiakevich extracted a rather small sample with acetone for only two hours, grandidentatin may not have been extracted. Also, the absence of populin in the study by Kripiakevich may be accounted for by the fact that both populin and tremuloidin have very similar retention times in both GLC systems used in the analysis. Vimalin, which occured in rather small quantities in the present study and the study of Wong, may have been missed by Kripiakevich because of incomplete extraction. The present study utilized ten more standards than the previous two studies and this

accounts for the reporting of salicyloylsalicin, salicyloyl-salicin-2-O-benzoate and the possibility of tremulacin in the present study. It is likely that β-sitosterol and (+)-catechin were two of the unknown components mentioned by Wong and Kripiakevich. In the present study, salicyloyl-salicin-2-O-benzoate was found in only one small fraction (D-4) as one of seven components and was present only in trace amounts.

It was originally hoped that the purification scheme used in this study would yield eluate fractions which were virtually free from non-glycosidic material. In this way, it would have been possible to make quantitative evaluations of the individual glycosides isolated from the bark. However, examination of the largest fractions from two of the polyamide columns indicated the presence of considerable amounts of monosaccharide material. Quantitative evaluation was therefore impossible.

IV. SUMMARY

1. Acetone followed by 95% ethanol was found to be a suitable means for extracting phenolic glycosides from Salix petiolaris Sm. bark.
2. Ethyl acetate was found to be unsuitable for the purification of the extraction residue and afforded only a minimal fractionation of the residue.
3. Polyamide column chromatography was found to be an effective means of purifying and fractionating extraction residues using water, 25% ethanol, 50% ethanol, 75% ethanol and 95% ethanol as successive eluting solvents. Less polar eluate components could be purified by further polyamide chromatography, using water as the elution solvent.
4. β-Sitosterol and (+)-catechin were isolated from the bark of Salix petiolaris Sm. and identified by comparison with authentic samples.
5. Gas-liquid chromatography gave the best analysis of the bark extracts but for large numbers of samples, the relative speed of thin-layer chromatography made it the method of choice.
6. The extracts of the bark of Salix petiolaris Sm. were examined for known phenolic glycosides using two thin-layer chromatographic systems and one gas-liquid chromatographic system. Salicin, picein, salicyloylsalicin, vimalin, populin, salireposide, grandidentatin, salicyloylsalicin-2-O-benzoate and tremuloidin and/or tremulacin were shown to be present.

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VI. APPENDIX

TABLE 9 - List of symbols

SYMBOL	EXPLANATION
F 1-4	The four dried ethyl acetate fractions from the ethyl acetate extraction of the acetone extraction residue.
W	The dried aqueous residue from the ethyl acetate extraction of the acetone extraction residue.
B	The dried suspension filtered from the aqueous fraction of the ethyl acetate extraction of the acetone extraction residue.
E	The dried ethanol extraction residue.
U 1-5	Polyamide column eluate fractions from fraction F-1.
T 1-12	Polyamide column eluate fractions from fraction F-2.
D 1-8	Polyamide column eluate fractions from fraction F-3.
P 1-7	Polyamide column eluate fractions from fraction F-4.
K 1-3	Polyamide column eluate fractions from fraction W.
Z 1-7	Polyamide column eluate fractions from fraction B.
H 1-8	Polyamide column eluate fractions from fraction E.
S	The isolated compound identified as β-sitosterol.
N	The isolated compound identified as (+)-catechin.