1970

The coumarin compounds present in Artemisia tridentata subspecies vaseyana

Arnold Boyd Melnikoff

The University of Montana

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THE COUMARIN COMPOUNDS PRESENT IN Artemisia tridentata
SUBSPECIES vaseyana

By
Arnold Melnikoff
B.S., Northern Illinois University, 1966
Presented in partial fulfillment of the requirements
for the degree of Master of Science

UNIVERSITY OF MONTANA

1970

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

Date May 6, 1970
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CHAPTER I. INTRODUCTION

A group of closely related plants, commonly called sagebrush, occur over a large geographic area from Northern Mexico, through Western United States and into Southwestern Canada. The plants belong to the Genus *Artemisia Section Tridentatae* and are more closely related as a group than any of the Old World species of that Genus. It was proposed by Beetle that the Section *Tridentatae* has come into existence as a result of differentiation of a common genetic ancestor, in response to environmental and climatic changes.¹

Four monographs have been published in the last half century in which the authors have attempted to classify the Section *Tridentatae* into its respective species and subspecies. Rydberg first proposed in 1916 that the Section *Tridentatae* contained eleven species.² Clemens and Hall in 1923 proposed that the Section contained five species.³ They used an analogous Section with the name of *Seriphidium* in place of *Tridentatae*. The Section name of *Seriphidium* was retained by Ward who published a monograph in 1953 in which he recognized eight species and six subspecies.⁴ He also postulated that two parallel lines of development existed in the Section *Tridentatae*. One of the lines of development was centered about *A. tridentata* and the other was centered about *A. cana*. Moss, upon completing a study of the interxylary cork of the Genus *Artemisia*, published a monograph in 1940 in which he also placed eight species in the Section *Tridentatae*.⁵ However, he proposed that one of Ward's later selections in the Section *Tridentatae*, *A. palmeri* should in
fact be excluded, while *A. bigelovii* should be included in the Section. Beetle published a monograph in 1960 in which he included eleven species and nine subspecies in the Section *Tridentatae*.\(^1\)

Several investigators have resorted to the use of chemotaxonomy in order to solve the confusion that has resulted from the consideration of the morphological characteristics.

Young and his co-workers published several papers in *Wyoming Range Management* dealing with the chemical taxonomy of the Section *Tridentatae*.\(^6,7\) Leaf specimens were extracted with 95% ethanol and a small quantity of the concentrated extract was used for thin layer chromatography. No chemical characterization was made. They did speculate, however, that the compounds which they had separated by thin layer chromatography were fluorescent lactones related to santonin. Through the use of thin layer chromatography they were able to differentiate seventeen *Tridentatae* species and subspecies.

H. R. Holbo and H. N. Mozingo published a paper in 1965 in which two-dimensional paper chromatography was employed as the chemotaxonomical method.\(^8\) Sagebrush leaves were extracted with an acidic methanol solution. A small amount of the concentrated extract solution was placed upon Whatman No. 1 paper and developed with a two-solvent system. The compounds which they separated were detected by simultaneous exposure of the chromatogram to ammonia vapor and UV light. The compounds were chemically characterized as phenolics due to a positive blue color reaction with either FeCl\(_3\) or K\(_2\)Fe(CN)\(_6\). The
**THIN LAYER CHROMATOGRAM OF ARTEMISIA SPECIES**

<table>
<thead>
<tr>
<th>A. tri-dentata ssp.</th>
<th>A. tri-dentata var. raseyana</th>
<th>A. arbucula ssp. arbuscula</th>
<th>A. partitita ssp. longiloba</th>
<th>A. cana ssp. longiloba</th>
</tr>
</thead>
<tbody>
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<td>A. nova tridentata</td>
<td>A. tri-dentata var. raseyana</td>
<td>A. arbucula ssp. arbuscula</td>
<td>A. partitita ssp. longiloba</td>
<td>A. cana ssp. cana</td>
</tr>
</tbody>
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Florescent spots developed in solvent system A:
- **Purple 0.95**
- **Purple 0.93**
- **Yellow 0.89**
- **Purple 0.89**
- **Green 0.87**
- **Blue 0.86**
- **Purple 0.86**
- **Blue 0.84**
- **Green 0.81**
- **Blue 0.80**
- **Blue 0.78**
- **Green 0.75**
- **Blue 0.72**
- **Green 0.72**
- **Green 0.67**
- **Blue 0.67**
- **Green 0.65**
- **Green 0.62**
- **Orange 0.61**
- **Orange 0.56**
- **Orange 0.50**
- **Blue 0.48**
- **Blue 0.44**
- **Orange 0.37**
- **Orange 0.34**
- **Orange 0.30**

Florescent spots developed in solvent system B:
- **Blue 0.98**
- **Blue 0.82**
- **Blue 0.60**
- **Blue 0.34**
- **Blue 0.24**
- **Blue 0.21**

*Color and Rf of the spots.*

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more polar compounds present were thought to be glycosides due to the fact that they were not present on the chromatograms after the methanol leaf extracts were refluxed with 2 N HCl. The less polar compounds were believed to be the phenolic aglycones of the glycosides. No further chemical characterization was made. They concluded that their chromatography data supported Rydberg's old paper in which *A. pygmaea* and *A. rigida* were excluded from Section Tridentatae. Their data also supported Ward's contention that *A. bigelovii* should also be excluded from the Section Tridentatae. Their chemotaxonomical data, consequently, is in direct contrast to Beetle's morphological data. He contends that all three species should be included in the Section Tridentatae.

In an earlier study on the nutritional components of *A. Tridentata*, Kinney and Sugihara (1948) extracted a brown, amorphous powder from the leaves with hot water. They did not succeed in their attempts to crystallize the amorphous material. However, they were able to form D-glucose phenylosazone from the hydrolysis of the powder, demonstrating that it was some type of glycoside. They referred to this powder as the bitter principle of sagebrush due to the exceedingly bitter taste of the hot water extract.

In this work on instant thin layer chromatography (i TLC) a method was devised to separate the major phenolic constituents present in methanolic leaf extracts of sagebrush. The reproducibility of the i TLC system employed was tested by extracting three
different samples of eight different varieties of sagebrush (Table 2). Reproducible results were obtained for each of the eight different varieties tested. The chromatograms contained a variety of coumarin and flavonoid compounds (Table 1). The coumarin compounds were detected on the chromatogram by their blue fluorescence when exposed to UV light. The flavonoids were detected by their fluorescence in the presence of UV light after spraying the chromatogram with a solution of diphenylboric acid-ethanolamine (Table 1).

The validity of using chemical constituents as reliable expressions of the genetic uniqueness of a plant species is always questionable if the chemical constituents are not positively identified. It has been established that certain phenolic constituents of plants are under direct genetic (enzymatic) control (coumarins and flavonoids, etc.) while other plant constituents (lignin, etc.) may be formed through the random action of free radicals. A knowledge of the phenolic constituents present in a taxonomical section may provide the information necessary to elucidate the phylogeny of the section. The biosynthetic pathway of most plant constituents is now known, while those which are not known may be determined. The different constituents can then be arranged according to significance of the biosynthetic pathway. This could provide a clue to the evolution of the plants in question. Consequently, biochemical comparison of the identified constituents of a taxonomical section may elucidate its phylogeny, provided that the chemical differences among members of the section are sufficient for a valid comparison.
Table 2. Location of *Artemisia* Species

<table>
<thead>
<tr>
<th>Variety</th>
<th>Town</th>
<th>Range</th>
<th>Township</th>
<th>Section</th>
<th>Quadrant</th>
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<td></td>
<td>S of Virginia City</td>
<td>R.38.E.</td>
<td>T.9.S.</td>
<td>20</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>S of Dillon</td>
<td>R.36.E.</td>
<td>T.9.S.</td>
<td>8</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>S of Dillon</td>
<td>R.36.E.</td>
<td>T.9.S.</td>
<td>8</td>
<td>NE</td>
</tr>
<tr>
<td><em>A. longiloba</em></td>
<td>S of Virginia City</td>
<td>R.38.E.</td>
<td>T.9.S.</td>
<td>2</td>
<td>SW</td>
</tr>
<tr>
<td><em>A. tripartita</em> ssp. <em>tripartita</em></td>
<td>S of Virginia City</td>
<td>R.38.E.</td>
<td>T.9.S.</td>
<td>2</td>
<td>SW</td>
</tr>
<tr>
<td><em>A. cana</em> ssp. <em>cana</em></td>
<td>N of Malta</td>
<td>R.30.E.</td>
<td>T.32.N.</td>
<td>5</td>
<td>NW</td>
</tr>
</tbody>
</table>
CHAPTER II. RESULTS

This work was initiated to determine the major phenolic components of A. tridentata ssp. vaseyana. The species A. tridentata grows over a larger geographical area than any other species of sagebrush, and A. tridentata ssp. vaseyana grows abundantly in several areas close to Missoula, Montana.

Isolation of the coumarin components contained in the leaves of A. tridentata ssp. vaseyana was attempted first because they could easily be located on a silicic acid column as a blue fluorescent band under UV light. The first blue fluorescent spot (R_f = 0.950, Table 1) could not be isolated in sufficient quantities for chemical investigation. Later on when it became apparent that the major phenolic extractives were 6,7-dioxygenated coumarin compounds, 6,7-di-methoxy coumarin was synthesized, and compared with this spot, but was found to have a different R_f value. The dimethoxy compound was considered as a likely candidate because it was originally isolated from the flower buds and seeds of A. scoparia (an Old world species).

\[
\begin{align*}
&\text{Coumarin}
\end{align*}
\]
Figure 1. The UV, NMR, and Mass Spectra of Isoscopecolin.

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The second blue fluorescent spot on the chromatogram ($R_f = 0.838$) was found to be isoscopoletin ($R_1 = \text{OH}$, $R_2 = \text{OMe}$). The UV spectrum of this compound is typical of 6,7-dioxygenated coumarin compounds (Fig. 1). Two major bands appear in the spectrum at 227 and 349 $\text{m} \mu$, while two minor bands appear at 258 and 292 $\text{m} \mu$. The mass spectrum of isoscopoletin had a base and parent peak of 192, and major peaks of 177, 164 and 149 $\text{m/e}$ resulting from the loss of methyl and carbon monoxide radicals. The NMR spectrum contained two doublets, two singlets and a broad signal at 9.52 ppm which was due to the presence of the phenoxy proton. The two doublets at 6.10 and 7.80 ppm ($H_a$ and $H_b$) are coupled. The coupling constant of 10 c/s is rather large for cis coupling and can be explained by the formation of a dihedral angle of near zero degrees due to the presence of the double bond and planar configuration of the benzo-α-pyrone ring system. The partial positive charge at carbon $C_4$ results in the large downfield shift of the doublet $H_b$ and is due to the electron withdrawing effect of the carbonyl oxygen. The two singlets at 3.80 and 6.92 ppm represent the methoxy protons and the two aromatic protons $H_c$ and $H_d$ respectively. The relative integration for the methoxy protons, $H_a$, $H_b$, and $H_c + H_d$ is 3:1:1:2. Isoscopoletin has been also identified in another Old World Artemisia species, $A. \text{messerschmidiana}$. This is the only reference that could be found for identification of isoscopoletin as a natural product. It is possible that isoscopoletin occurs more often as a natural product.
but has been overlooked due to the common occurrence of its geometric isomer scopoletin, which has similar chemical and physical properties.

The third blue fluorescent spot \((R_f = 0.776)\) on the chromatogram (Table 1) was scopoletin \((R_1 = \text{OMe}, R_2 = \text{OH})\). This compound has been isolated from the roots of \textit{Scopolia japonica}.\textsuperscript{18} Although the spectroscopic characteristics of scopoletin is similar to isoscopoletin, there are some distinct differences between them.

The UV spectrum shows slight differences in adsorption frequencies of the major and minor bands of the two compounds (Fig. 2).

Addition of NaOAc, however, increases the long wavelength absorption band of scopoletin due to a bathochromic shift of 46 nm, while there is no major shift of the corresponding band of isoscopoletin.\textsuperscript{19}

The mass spectrum contained the same major peaks with one exception. The base peak occurred at 89 m/e in contrast to isoscopoletin which has no peak at 89 m/e.

An interesting difference in the NMR spectrum of scopoletin was that \(H_c\) and \(H_d\) had become two distinct singlets at 7.13 and 6.72 ppm. The identification of \(H_c\) and \(H_d\) was made empirically by the comparison of the chemical shifts of \(H_c\) and \(H_d\) in esculetin with that of scopoletin and isoscopoletin. The probable reason for the difference in chemical shifts of \(H_c\) and \(H_d\) in scopoletin is that the partial positive charge at \(C_4\) (due to resonance) is not transmitted through the ring system to \(C_8\) as effectively as to \(C_5\). Furthermore, the methoxyl group ortho to \(H_c\) in scopoletin results in an even greater downfield
Figure 2. The UV, NMR, and Mass Spectra of Scopoletin.
shift of $H_c$. This results in $H_d$ having a chemical shift equal to $H_c$, consequently $H_c$ and $H_d$ are superimposed in isoscopoletin to form an apparent singlet.

The fourth spot ($R_f = 0.444$) which had a blue fluorescence when examined under UV light and a green fluorescence when sprayed with diphenylboric acid-ethanolamine reagents, proved to be esculetin ($R_1 = R_2 = \text{OH}$). Esculetin is a common coumarin compound found in many plants and has been isolated from the seeds of Euphorbia lathyris. The UV spectrum was again quite characteristic of 6,7-di-oxygenated coumarin compounds (Fig. 3). The mass spectrum of this compound had a base peak of 150 m/e, plus major peaks of 149, 131, 100, 76, 75 m/e and a parent peak of 178 m/e which can be explained by the loss of CO, H, and $H_2O$ in the spectrum. The NMR spectrum contained two phenoxy protons at 9.25 and 10.05 ppm. The coupled doublets of $H_a$ and $H_b$ are at 5.97 and 7.70 ppm and $H_c$ and $H_d$ are at 6.56 and 6.82 ppm. The spectrum of the silated esculetin is identical to the one reported by Malbry, Kagan and Rösl. The glucosides present in the leaf extracts could not be resolved on ITLC plates with the low polarity solvent system employed for the non-conjugated coumarins. Another solvent system was used to separate the coumarin glucosides (Table 2). This showed the presence of two glucosides which were positively identified as esculin ($R_1 = \beta-D$-glycosyl, $R_2 = \text{OH}$) and methyl esculin ($R_1 = \beta-D$-glucosyl, $R_2 = \text{OMe}$). The latter glycoside was first isolated from the water extract of the leaves. Pure samples of methyl esculin and esculin were obtained by chromatography of methanolic extract of the leaves.
Figure 3. The UV, NMR and Mass Spectra of Esculetin.

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on a Woelm polyamide column. A mixture of the two glycosides did not possess a bitter taste, therefore, they could not be the bitter principle of sagebrush reported by Kinney and Sugihara. Methyl esculin ($R_f = 0.344$) which has not been isolated from a natural source before, was present in a relatively larger quantity than any other coumarin compound occurring in *A. tridentata* ssp. *vaseyana* (0.67%). The structure of this compound was proven by spectroscopic data, synthesis from esculin and hydrolysis to $D$-glucose and isoscopoletin.

The spectroscopic data for methyl esculin is shown in Fig. 4. The UV spectrum shows major absorption bands at 227 and 337 $\text{nm}$ and minor absorption band at 259 and 296 $\text{nm}$. The mass spectrum was almost identical to that of isoscopoletin with the same major peaks, having slightly differing intensities, of $m/e$ 177, 164 and 149. Apparently immediate decomposition of the sugar moiety of the glycoside occurs as a result of the elevated temperatures used in the mass spectrometer. Consequently, the mass spectrum contains only major peaks characteristic of the isoscopoletin aglycone. The NMR spectrum of the silylated derivative of the glycoside indicates the presence of a single hexose sugar with an anomeric proton at 4.67 ppm and CH and $\text{CH}_2$ protons present in a broad signal centered at 3.30 ppm. The two doublets due to $H_a$ and $H_b$ are at 6.01 ppm and 7.35 ppm, $H_c$ and $H_d$ are at 6.64 ppm and 6.85 ppm with the methoxy group at 3.73 ppm. The integration of the anomeric proton, methoxy group and the CH and $\text{CH}_2$ protons compared to $H_a$, $H_b$, $H_c$ and $H_d$ is 1:3:6. The hydrolysis of the glycoside with $2\text{N CF}_3\text{COOH}$ at 124°C confirmed that the aglycone was in fact isoscopoletin.
Figure 4. The UV, NMR, and Mass spectra of 6-β-D-Glucosyl-7-Methoxy Coumarin.
The aglycone was crystallized from the hydrolysis product of the glycoside. It had the same melting point, mass spectrum and $R_f$ value as isoscopoletin. The sugar moiety was identified as glucose by GLC analysis of the silated product. The anomeric configuration of the glycoside was shown to be $\beta-D$ by the optical rotation of $[\alpha]_{D}^{26} = -35.1^\circ$ (in pyridine, $c$, 0.304). The same optical rotation was obtained for esculin of $[\alpha]_{D}^{26} = -35.1^\circ$ (in pyridine, $c$, 0.942). The NMR spectrum and optical rotation was identical to that of $6-\beta-D$-glycosyl-7-methoxy coumarin synthesized from esculin with methyl iodide.

Esculin ($R_f = 0.238$) was separated from the Woelm polyamide column and had the same M.P. alone or in a mixture with known materials. The UV and infrared spectra (Fig. 5) were identical to that of a known sample of esculin as were the $R_f$ values in three different solvent systems. Esculin is a rather common glycoside and was first separated from the bark of Aesculus hippocastanum.

Several other phenolic compounds present in the water and methanolic extracts of A. Tridentata ssp. vaseyana (Table 1) appear to be flavanoids due to their $R_f$ values on ITLC and color reactions with ammonia and diphenylboric acid-ethanol amine reagent. One of the compounds has the same $R_f$ value (0.562) and color reactions as quercitin.

In summary, the major phenolic extractives of A. tridentata ssp. vaseyana appear to be $6-\beta-D$-glycosyl-7-methoxy coumarin (0.67%), its aglycone isoscopoletin (0.037%), minor quantities of the
Figure 5. The UV and IR Spectra of Esculin. A = isolated esculin; B = commercially obtained esculin.
corresponding unmethylated compounds—esculin (0.11%), esculetin (0.016%) and trace amounts of scopoletin and other compounds (Table 1).

The phylogeny of the Section *Tridentatae* based on the species and subspecies examined by chemotaxonomical means suggests that *A. tridentata* ssp. *tridentata*, *A. nova* and *A. longiloba*, which contain only esculin and not methyl esculin, may be more primitive than the other species and subspecies of *Artemisia*. This is due to the lack of enzymes necessary for methylation of the hydroxyl group at position C\(_7\) of the benzo-\(\alpha\)-pyrone moiety of esculin. The chromosome data for the two subspecies of *A. tridentata* is in direct disagreement with the lack of C\(_7\) methylation as a basis for determining the relative evolutionary advancement, or the phylogeny, of the two subspecies. *A. tridentata* ssp. *tridentata* has a tetraploid number while *A. tridentata* ssp. *vaseyana* has a diploid number of chromosomes. Botanists believe that *A. tridentata* ssp. *tridentata* is more advanced than *A. tridentata* ssp. *vaseyana* because of this fact.\(^1\),\(^2\),\(^3\)
CHAPTER III. EXPERIMENTAL

Plant Materials

The samples of *Artemisia* species were obtained from different locations within the state of Montana as shown in Table 2. Each sample gave reproducible chromatographic analysis.

Thin Layer Chromatography

Two grams of the leaves were refluxed in 100 ml of methanol for thirty minutes and the extract concentrated on a rotary evaporator to 10 ml. The concentrate was extracted with 2 volumes of pet ether to remove chlorophyll and other plant pigments. Acetone was then added to precipitate the waxy materials and remaining acetone-methanol solution was then concentrated to about 10 ml.

The concentrated solution was spotted on Gelman type SG instant thin layer chromatographic sheets with a capillary tube and developed with a liquid phase system composed of 89:10:1 benzene-ether-acetic acid (see Table 1, solvent system A). The chromatogram was then air dried and sprayed with diphenylboric acid ethanolamine complex and examined under an ultraviolet light. The coumarin compounds showed blue fluorescent without the complexing reagent.

For the separation of the glycosides the solvent system was changed to 94:5:1 chloroform-methanol-acetic acid (see Table 1, solvent system B).
**Instrumental Analysis**

All m.p.'s are uncorrected; the UV and mass spectra were obtained with Coleman-Hitachi EPS-3T and Consolidated Electrody­namics Corporation type 21-103C mass spectrometer. The NMR spectra were recorded with a Varian HA-60 instrument in deuterated DMSO with TMS as internal standard. The glycosides (50 mg) were treated with TRI-SIL (5 ml). The excess reagent was removed under reduced pressure. The trimethylsilyl ether derivative of the glycoside was extracted with CCl₄. The extract was filtered, concentrated (0.25 ml) and used for NMR studies.

**6,7-Di-Methoxy-Coumarin**

The compound was synthesized from a solution of 0.150 g of esculetin and 0.064 g of diazomethane in 100 ml of ether. The reaction mixture was allowed to stand overnight. The solvent was removed with an aspirator. A precipitate that was formed from the concentrate, was removed by filtration. ITLC analysis of the product showed the presence of esculetin, isoscopoletin, scopoletin and a more concentrated spot of less polarity which was 6,7-di-methoxy coumarin, $R_f = 0.910$. The first crystallization from acetone yielded 65 mg, m.p. 138-142°C. The third crystallization from a dilute acetone solution yielded 9 mg of pure material m.p. 143-144°C (lit. 144-146°C).-mass spectra:m/e 207 (21.5), 206 (100.0), 192 (26.0), 191 (62.1), 179 (4.8), 178 (26.0), 164 (21.5), 163 (62.0), 149 (16.3), 148 (5.0), 135 (30.6), 120 (10.5), 119 (17.0), 107 (26.0), 105 (14.2), 91 (21.4), 89 (33.1), 77 (15.0), and 76 (12.2).
Isocopoletin

Fourteen hundred grams of air dried leaves of *A. tridentata* ssp. *vaseyana* were extracted with 10 l of methanol. The methanolic solution was concentrated, dissolved in hot benzene and placed on 20 silicic acid columns (4.3 x 10 cm). The columns were developed with benzene-ether (6:4) to give three UV fluorescent bands. Elution of the first band and subsequent concentration and crystallization from acetone gave crude isocopoletin (220 mg). Recrystallization from acetone yielded pure material (54 mg) m.p. 185° (lit. 25 m.p. 183-185°), Rf 0.838, λ_{max}; 227 and 349 (major bands), and 258 and 292 μ (minor bands). Mass spectrum:m/e 192 (100), 177 (10.5), 164 (25.5), 149 (39.7), 121 (6.0). NMR: ppm doublets at 6.10 (1H) and 7.80 (1H) (J=10 c/s) and singlets at 3.80 (3H), 6.92 (2H) and 9.52 (1H). Found: C, 62.11; H, 4.27. Calc. for C_{10}H_{8}O_{4}: C, 62.50; H, 4.17 percent.

This compound was also obtained by water extraction of dried leaves (340 g), concentration of the aqueous extract, re-extraction of the concentrate with benzene and evaporation of the solvent. This gave a crude material (126 mg) that was purified by recrystallization from acetone. Alternatively the methanolic leaf extract was re-extracted with chloroform and the chloroform extract was processed as before.
Scopoletin

Elution of the second band from silicic acid columns, concentration and recrystallization of the resulting material from acetone gave scopoletin (40 mg). This was recrystallized from acetone (yield 8 mg), m.p. 203-204°C, alone or in a mixture with a known sample (lit. 26 m.p. 204°C, Rf 0.776, λmax: 225 and 350 (major bands), 260 and 292 μm (minor bands). Mass spectrum: m/e 192 (29.3), 177 (27.5), 164 (24.0), 149 (42.4), 121 (18.2), 117 (7.0), 115 (17.3), 103 (13.0), 105 (8.9), 102 (9.0), 91 (40.1), 89 (100), 87 (28.9). NMR: ppm doublets 6.10 (1H) and 7.80 (1H) (J=10 c/s) and singlets 3.80 (3H), 6.72 (1H) and 7.13 (1H), 9.52 (1H).

Esculetin

Elution of the third band from the silicic acid column and subsequent processing gave crude esculetin that could not be purified by fraction recrystallization. The alcoholic extract of the dried leaves (1.4 kg) was concentrated and re-extracted with ethyl acetate. Concentration and crystallization of this extract gave crude esculetin (226 mg, m.p. 234-297°C decomp.). Recrystallization from acetone gave pure material (40 mg) m.p. 263-263.5°C (decomp.), alone or in a mixture with a known sample (lit. 27 m.p. 268°C), Rf 0.444, λmax: 231 and 354 (major bands), 250 and 302 μm (minor bands). Mass spectrum: m/e 178 (60.5), 150 (100), 149 (54.1), 131 (31.0), 121 (10.9), 119 (8.8), 104 (9.2), 100 (16.7), 76 (26.0), 75 (21.6). NMR: ppm doublets 7.70 (1H) and 5.97 (1H) (J=10 c/s) and singlets
6.56 (1H), 6.82 (1H), 9.25 (1H) and 10.05 (1H). Found C, 60.60; H, 3.60. Calc. for C_9H_6O_4; C, 60.67; H, 3.37 percent.

**Esculin**

Methanolic extract of air dried leaves (610 g) was concentrated and extracted with petroleum ether, and the remaining methanolic extract was re-extracted with chloroform and then ethyl acetate. The ethyl acetate extract when concentrated gave a crude mixture of esculin and methyl esculin as determined by TLC.

The concentrated material was placed on a column (4.3 x 12 cm) of Woelm polyamide mixed with Celite 545 (7:3) and developed with water. This gave two UV fluorescent bands. The first band was eluted from the column, concentrated and crystallized from methanol to yield esculin (65 mg), m.p. 208° alone or in a mixture with a known compound (lit. m.p. 205° decomp.), R_f 0.238 (see Table I). The UV and IR spectra of the product were also identical with the spectra of the known compound.

**6-β-D-Glucosyl-7-methoxy Coumarin**

Elution and processing of the second band provided crude methyl esculin. The pet ether extract (41) in the previous experiment gave a strongly fluorescent aqueous layer that settled out on standing for 3 days. This was evaporated and the dry residue (5.23 g) extracted with methanol. The methanolic extract was concentrated to provide crude 6-β-D-glucosyl-7-methoxy coumarin (3.68 g), m.p. 224-228°. Recrystallization from dilute methanolic
solutions gave pure material (137 mg) m.p. 234-234.8° (lit. 28 m.p. 229°), [α]$_D^{260}$ -35.1° (in pyridine c, 0.304) same as esculin. R$_f$ 0.344, λ$_{max}$: 227 and 337 (major bands), 259 and 296 m$_u$ (minor bands). Mass spectrum: m/e 192 (100), 177 (16.4), 164 (33.8), 149 (27.2), 115 (15.0). NMR of trimethylsilyl ether derivative: ppm doublets 6.0 (1H) and 7.35 (1H) (J=10 c/s), singlets 3.73 (3H), 6.64 (1H) and 6.85 (1H), and broad bands 3.40 (6H) and 4.67 (1H).

The above product was also prepared from ethyl acetate extraction of a concentrated aqueous leaf extract.

Methyl esculin (8 mg) was hydrolyzed in a sealed tube with 3 ml of 2 N trifluoracetic acid at 124° for 1½ hr. Isoscopoletin crystallized on evaporation of the hydrolyzate. This had the same m.p., R$_f$ and mass spectrum as the authentic compound. The mother liquor was evaporated to dryness. The residue was treated with TRI-SIL and found to contain D-glucose by GLC analysis. For the synthesis of methyl esculin _28_ 0.6046 g of esculin and 0.6138 g KOH were dissolved in 100 ml methanol and the pH brought to 6.2 by adding Mel. Refluxing for 16 hrs gave crude methyl esculin which was purified by recrystallization; yield 168 mg, m.p. 234-234.8°.

The synthetic product had the same m.p., specific optical rotation, R$_f$ and NMR spectrum as the natural product.
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