Phenolic Acids and Depsides from Some Species of the *Erodium* Genera

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*Erodium*, Phenolic Acids, Plant Polyphenols

Six natural polyphenolic compounds, brevifolin carboxylic acid, brevifolin, ellagic acid, methyl gallate, gallic acid and protocatechuic acid have been isolated from the methanol extract of the whole plant of *Erodium cicutarium* (L.) L’Hérit. (*Geraniaceae*). Structures were determined by conventional methods of analysis and confirmed by MS and NMR spectral analysis. The distribution of these compounds in the other species of the *Erodium* genera (*E. botrys, E. chium, E. ciconium, E. cicutarium, E. glutinosum* subsp. *dunense, E. gruinum, E. manescavi, E. pelargoniflorum, E. petraeum*) were examined by HPLC with a RP-18 column, and MGD-TLC methods on unmodified silica gel and silica gel chemically modified with polar and nonpolar groups (HPTLC-Si 60 LiChrospher, HPTLC-NH$_2$, HPTLC-DIOL, HPTLC-RP-18W).

Introduction

*E. cicutarium* (Common stork’s bill) is a one- or two-year herb growing up to about 50 cm and indigenous to Europe, Asia and North America. Other species of the *Erodium* genera are Mediterranean plants. Decoction from the aerial parts of *E. cicutarium* plant has been recommended for treatment of dysentery, fever, wounds and worm infections as a traditional medicine (Lis-Balchin, 1993; 1994). In Middle Europe it was employed as antihaemorrhagic drug in gynecology to stop the uterine bleeding and as general haemostipticum. Ellagitannin-containing plants, such as these from the genera *Erodium* are used for preparing astringent and antiseptic teas announced in stomatitis (Klocke et al., 1986). Both water and methanol extract from *E. cicutarium* demonstrated antiviral effect in relation to Herpes virus type 1, Vesicular stomatitis, Vaccinia virus and myxoviruses (Zielinska-Janczylik et al., 1987). Non of these extracts did induce interferon in suspension of human leukocytes. However, the methanol extract exerted a stimulatory effect on the synthesis of interferon induced with Newcastle disease virus in cell cultures. The strongest viridical fractions contain some polyphenols, excluding flavonoid glycosides (Zielinska-Janczylik et al., 1988). In immunological tests the low concentrations of methanol extract from *E. cicutarium* stimulated whereas the high concentrations inhibited free radicals activity of human granulocytes in vitro (Fecka et al., 1997). This observation was in agreement with previously described antioxidant properties of several species from *Erodium*. The polyphenolic fraction from flowering aerial parts of these species exerted in vitro a free radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Lamaison et al., 1993) and an inhibitory effect on the Fe$^{2+}$- induced lipid oxidation (Sroka et al., 1994). Previous papers reported the presence of ellagitannins, flavonoid glycosides, phenolic acids and essential oil (Lis-Balchin, 1993). Salech et al. (1983) studied the flavonoids of fourteen *Erodium* from two sections: *Pulmosa* Boiss. and *Erodium* (=Barbata Boiss.). Ellagic acid (0.77%) was detected in hot methanol extract of *E. cicutarium* by GC (Klocke et al., 1986). The total concentration of flavonoids, tannins and geraniin in *E. chium, E. ciconium* and *E. cicutarium* were determined using both colorimetric and chromatographic methods (Lamaison et al., 1993). In this work we have defined the polyphenols content of methanol extracts from nine species of *Erodium*. Phenolic acids and depsides have been examined with help of qualitative and quantitative analytical techniques.
Material and Methods

Plant material

The following species from the Erodium genus (Geraniaceae) were analyzed: E. botrys (Cav.) Bertol., E. ciconium (L.) L.’Hér., E. cicutarium (L.) L.’Hér., E. chium Sibth. et Sm. non Willd., E. glutinosum subsp. dunense (Andreas) Rothm., E. gruinum (L.) L.’Hér., E. manescavi Cosson, E. pelargoniiflorum Boiss. et Heldreich, E. petraeum (Gouan) Willd.. All herbs at the flowering stage were collected in Poland in September 1996–1998. E. cicutarium (L.) L.’Hér. was obtained from a natural habitat (a voucher specimen is deposited in our Department). Another species were cultivated from selected seeds (Jardin Botanique Nantes, France) at the herbarium of the University of Medicine in Wroclaw.

Preparation of extracts

Phenolic compounds, derivatives of phenol carboxylic acid and hydroxycinnamic acids were prepared from 5 g dried and powdered herbs of each species from the Erodium genus according to the method described by Van Sumere et al., (1972). Free phenolic acids and phenolic acids obtained after the hydrolytic cleavage of O-glycosides or esters were designated in diethyl ether extracts. Enzyme hydrolysis: ß-glucosidase (Koch-Light, Colnbrook Bucks, England); the acetic buffer; pH 4.5; 37 °C; 10 h. Alkaline hydrolysis: 2 M NaOH; pH 12; room temperature; 120 min. Methanol extracts were prepared by a well-known procedure. The second portions (5 g) of powdered row materials were first exhaustively extracted (Soxhlet) with CH₂Cl₂ and next with methanol on the waterbath. After filtration crude methanol extracts were concentrated under reduce pressure at 40 °C, and the 0.1 g from each obtained residues dissolved in 10 ml of HPLC grade methanol (Merck, Darmstadt, Germany).

Isolation of polyphenols

The isolation of compounds A-F (Fig. 1) was performed only for Erodium cicutarium herb. The other species were analyzed with help of chromatographic techniques. Powdered and dried aerial parts (800 g) of E. cicutarium were extracted successively with n-hexane, CH₂Cl₂ and methanol in a Soxhlet apparatus. The crude methanol extract was filtered, dryness under reduce pressure at 40 °C, dissolved in water (300 ml) and partitioned with diethyl ether (liquid-liquid extractor). The diethyl ether fraction of the methanol extract from E. cicutarium (6 g from total ratio 22.5 g) was separated on silica gel column (Si 60, 0.15–0.30 mm; Macherey-Nagel, Düren, Germany) using stepwise gradient elution with CH₂Cl₂ containing increasing concentration of methanol –10%, 20%, 30% to 60%. Seven fractions were collected (Fr-1→Fr-7). The fractions consisting of polyphenols were finally purified on octadecyl (RP-18, 40 µm, 60 A, LC J. T.Baker, Phillipsburg, USA) with solvent systems consisted of methanol and water. Subsequent purification by a combination of silica gel and octadecyl chromatographies afforded 4 individual compounds: compound A (115 mg) from Fr-2 using 10% methanol as the eluent, compound B (85 mg) from Fr-3 using 10% methanol, compound C (800 mg) from Fr-5 using 10% methanol, compound D (30 mg) from Fr-6 using 20% methanol. Compound E (65 mg) was separated from compound F (31 mg) on the NH₂ microcolumn (500 mg, 3 ml J. T.Baker, Phillipsburg, USA). In these conditions constituent F was selectively absorbed from the methanol solution of Fr-7 on a propylamine bad and washed with methanol-formic acid (99.5:0.5; v/v). Compound E was crystallized from the methanol eluate. Structure of isolated constituents were elucidated based on chemical evidences and spectral analyses.

Standard solutions

Polyphenolic stock standard solutions (1 mg/ml) were prepared by dissolving 5 mg of each standards in 5 ml of HPLC grade methanol (Merck, Darmstadt, Germany). Working standard solutions were prepared by dilution with methanol and filtered through membrane filters (Millipore, 0.22 µm). All standards of phenolic acids were purchased from Extrasynthese (Lyon, France).

Apparatus and conditions

IR and UV spectra were measurement on a Unicam SP 1000 spectrometer and a Perkin Elmer UV/VIS Lambda 20 spectrophotometer. ESI mass were recorded on a AMD 604 mass spectrometer and ESMS on a AMD 404 mass spectrometer.
13C-NMR spectra were obtained on a Bruker WM 52 spectrometer.

In TLC were used following HPTLC precoated plates (Merck, Darmstadt, Germany), 20 × 10 cm: silica gel Si 60, LiChrospher Si 60, aminopropyl silica, octadeyl RP-18W and HPTLC DIOL 10 × 10 cm. They were spotted with 10–20 μl samples of diethyl ether extracts, 5–15 μl solutions of individual standards and their mixture as 8–10 mm bands. Chromatograms were developed in horizontal teflon DS-chambers (Chromdes, Lublin, Poland) with the gradient mobile phase program I, II, III (Table I) or two time with eluent IV - water-methanol-formic acid (89:10:1; v/v/v), over a path of 9 cm. Plates were dried in a stream of warm air for 5 min after each step. Colored compounds were detected under UV light (254, 365 nm) and by spraying with 1% methanol FeCl3 (Fig. 2), or with bis-diazotized sulfanilamide (Fig. 3).

The HPLC system consisted of two pumps (Knauer, Berlin, Germany), a sample injector and a variable wavelength UV detector (Knauer variable wavelength monitor type 87.00) connected to a personal computer (HPLC Software/Hardware Package Version 2.21A). Separations were achieved using a LiChroCART® 250–4 100 RP-18 (5 μm) column with a LiChroCART® 4–4 100 RP-18 (5 μm) pre-column (Merck, Darmstadt, Germany). Detection was carried out by on-column measurement of UV absorption at 280 nm. The sensitivity was set at 0.04 a.u.f.s. The flow rate was adjusted to 1.0 ml/min. Phenolic acids and depsides were separated using an acetonitrile-water gradient with formic acid addition according to program V (Table II). A 20 μl volume of analyzed samples was injected. The calibration graphs of polyphenols were prepared by measuring the peak area and they were linear in the examined range (0.02–0.10 mg/ml). For the HPLC analyses

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### Table I. Gradient elution programs for the MGD-TLC method when using various plates. Plates were dried after each step.

**Program I. Four-step gradient program for silica gel plates.**

<table>
<thead>
<tr>
<th>Step No</th>
<th>Cyclohexane</th>
<th>Diisopropyl ether</th>
<th>Formic acid</th>
<th>Volume [ml]</th>
<th>Development distance [mm]</th>
<th>Time [min]</th>
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<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>20</td>
<td>1</td>
<td>20</td>
<td>20</td>
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<td>2</td>
<td>78</td>
<td>20</td>
<td>2</td>
<td>3</td>
<td>5</td>
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<tr>
<td>3</td>
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<td>2</td>
<td>3</td>
<td>90</td>
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<td>4</td>
<td>79</td>
<td>79</td>
<td>1</td>
<td>3</td>
<td>90</td>
<td>20</td>
</tr>
</tbody>
</table>

**Program II. Two-step gradient program for propylamine plates.**

<table>
<thead>
<tr>
<th>Step No</th>
<th>Acetone</th>
<th>Acetic acid</th>
<th>Volume [ml]</th>
<th>Development distance [mm]</th>
<th>Time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>15</td>
<td>5</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>10</td>
<td>5</td>
<td>90</td>
<td>15</td>
</tr>
</tbody>
</table>

**Program III. Four-step gradient program for diol plates.**

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>70</td>
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<td>2</td>
<td>60</td>
<td>40</td>
<td>2</td>
<td>90</td>
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<tr>
<td>4</td>
<td>80</td>
<td>20</td>
<td>2</td>
<td>90</td>
<td>15</td>
</tr>
</tbody>
</table>
gradient grade acetonitrile was used. Water was glass distilled and deionized. Solvent solutions were vacuum degassed with sonication prior to usage. All experiments were performed at room temperature (20 °C).

Results and Discussion

Phenolic acids and depsides in the *Erodium* genus were investigated by chromatographic techniques. We carried out an isolation and an identification of main constituents of diethyl ether fraction from the methanol extract of *E. cicutaarium*. The composition of polyphenols from the other species of *Erodium* was established using HPLC and MGD-TLC methods. Dried and powdered aerial parts of plants were extracted with methanol under reflux. Polyphenolic compounds were partitioned between *Et*₂*O* and water. Phenolic acids and depsides were examined before and after hydrolysis of tannins (Van Sumere et al., 1972). The diethyl ether fraction from *E. cicutaarium* was chromatographed on silica gel by the CC method. Elution with *CH*₂*Cl*₂ containing increasing proportions of methanol separated polyphenols according to their polarity. Eluates consisting of phenolic acid or depsides were finally separated on octadecyl with a solvent system containing methanol and water. Subsequent purification by combination of octadecyl and silica gel chromatographies afforded 6 individual compounds (A-F).

Fig. 1. Structures of phenolic acids and depsides identified in the *Erodium* species.

Fig. 2. Chromatogram of methanol extracts from analyzed *Erodium* species and standards developed according with program I on the HPTLC LiChrospher silica gel plate. Polyphenols were visualized by spraying with *FeCl*₃ reagent. Tracks: A methyl gallate, B protocatechuic acid, C gallic acid, F ellagic acid, Eb *E. botrys*, Ech *E.chium*, Eo *E. ciconium*, Eu *E. cicutaarium*, Ed *E. glutinosum* subsp. *dunense*, Eg *E. gruinum*, Em *E. manescavi*, Ept *E. petraeum*, Epl *E. pelargoniflorum*. 

Table II. The gradient elution program V for the HPLC method with the RP-18 column. The composition of mobile phase (v/v); solvent A: acetonitrile-formic acid (95:5), and solvent B: water-formic acid (95:5).

<table>
<thead>
<tr>
<th>Step No (min)</th>
<th>Eluent (v/v)</th>
<th>Type of elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-2 10% A in B</td>
<td>isocratic elution</td>
</tr>
<tr>
<td>2</td>
<td>2-17 10%-30% A in B</td>
<td>linear gradient</td>
</tr>
<tr>
<td>3</td>
<td>17-20 30%-70% A in B</td>
<td>linear gradient</td>
</tr>
</tbody>
</table>

Table II. The gradient elution program V for the HPLC method with the RP-18 column. The composition of mobile phase (v/v); solvent A: acetonitrile-formic acid (95:5), and solvent B: water-formic acid (95:5).
LiChrospher and octadecyl plates using program I (Fig. 1) and mobile phase IV relatively. Satisfied results were observed for diol and program III. Depsides were determined on propylamine plates at program II (Fig. 2). HPLC-RP 18 separation with acetonitrile-water gradient was characterized good resolution for both phenolic acids and depsides. The optimal separation of the standard mixture was recorded for 5% concentration of formic acid as pH modifier (Fig. 4). The described MGD-TLC method on the silica gel and chemically modified stationary phases is selective for separation of phenolic acids and depsides compared with commonly used unmodified layers. Because of, analyzed compounds are well separated from a complex mixture, the MGD-TLC method can be used for densitometric evaluations. The HPLC gradient for the characterization of polyphenolic compounds in an analyzed material can be applied for a chemosystematic study of other species from the Erodium genera in the polyphenols range.

Phenolic acids and depsides are secondary metabolites derived by hydrolysis of tannins. The Geraniaceae family afford ellagitanins with geraniin as a main constituent (Okuda et al., 1983; 1989b). Geraniin are hydrolyzed with dilute HCl yielding ellagic acid, brevifolin carboxylic acid, brevifolin, gallic acid and glucose (Okuda et al., 1989a). Investigated methanol extracts and diethyl ether fractions yield phenolic compounds in high concentration (Table III). Obtained results indicate that the major constituents of free phenolic acids are gallic acid, gallic acid methyl ester and protocatechuic acid. The second polyphenolic group are depsides: ellagic acid, brevifolin and its precursor brevifolin carboxylic acid. In low concentration appear following phenol carboxylic acids: salicylic; m- and p-hydroxybenzoic; 2,3-dihydroxybenzoic; gentisin; vanillic and hydroxycinnamic acid derivatives: caffeic; p-coumaric; ferulic. Gallic acid and gentisin acid are present mainly as free or as β-glucosides. Protocatechuic acid and depsides form ester bonds. E. botrys, E. chium, E. cicutarium, E. glutinosum subsp. dunense, E. manescavi and E. petraeum have the similar qualitative composition of non-glycosylated polyphenols but they reveal quantitative differences. The highest content of depsides have been observed in E. manescavi and E. petraeum. The mean value for gallic acid is about 0.48% of dried weight of extracts and for ellagic acids 2.0% respectively. Methyl gallate has been detected in a high concentration in E. chium and E. petraeum. E. ciconium does not synthesize the methyl ester of gallic acid. It is interesting that E. gruinitum and E. pelargoniiiflorum possess only traces of phenolic acids with no presence of methyl gallate. The presence of gallic acid, protocatechuic acid, brevifolin carboxylic acid and caffeic acid were also described in E. moschatum (L.) L’Hérét by Jer-Huei Lin and Mei-Fan Lin (1997). Because, phenolic acids and depsides have been detected in the majority of tested methanol extracts their biologic significance may be discussed. These identified polyphenols possess antioxidant (Kimura et al., 1983a, 1983b; Hatano et al., 1989, 1990), antibacterial (Kolodziej et al., 1999), antiviral (Kane et al., 1988a; 1988b) and antiphlogistic actions (Okuda et al., 1983; 1989b).
Table III. The contents of major phenolic acids and depsides in methanol extracts (mg per 1 g of dry weight) from nine species of the *Erodium* genera.

<table>
<thead>
<tr>
<th>POLYPHENOLS</th>
<th><em>E. botrys</em></th>
<th><em>E. ciconium</em></th>
<th><em>E. cicaturn</em></th>
<th><em>E. chium</em></th>
<th><em>E. glutinosum</em> subsp. dunense</th>
<th><em>E. gruinum</em></th>
<th><em>E. manescavi</em></th>
<th><em>E. pelargoniiiflorum</em></th>
<th><em>E. petraeum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>2.00</td>
<td>7.39</td>
<td>12.40</td>
<td>7.07</td>
<td>0.92</td>
<td>0.10</td>
<td>8.29</td>
<td>0.45</td>
<td>10.76</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>-</td>
<td>0.65</td>
<td>3.93</td>
<td>0.67</td>
<td>2.28</td>
<td>0.39</td>
<td>0.08</td>
<td>0.98</td>
<td>1.46</td>
</tr>
<tr>
<td>Gallic acid methyl ester</td>
<td>5.30</td>
<td>-</td>
<td>18.38</td>
<td>27.95</td>
<td>14.73</td>
<td>-</td>
<td>11.47</td>
<td>-</td>
<td>37.87</td>
</tr>
<tr>
<td>Brevifolin</td>
<td>3.62</td>
<td>8.41</td>
<td>25.95</td>
<td>7.12</td>
<td>6.79</td>
<td>0.45</td>
<td>50.74</td>
<td>-</td>
<td>13.99</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>17.49</td>
<td>5.19</td>
<td>11.88</td>
<td>8.33</td>
<td>23.43</td>
<td>6.81</td>
<td>26.41</td>
<td>0.51</td>
<td>80.28</td>
</tr>
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</table>

(*) absent.

Ellagic acid, gallic acid and methyl gallate are also known as antimutagenic and anticarcinogenic plant agents. They prevent of a carcinogenesis process by inhibition of initiation or promotion stages (Perchellet et al., 1992). Additionally, gallic acid and its metabolites induce the death signal (apoptosis) via their prooxidant action in some cancer cell lines (Nogaki et al., 1998; Sakagami et al., 1997a, 1997b; Sakaguchi et al., 1998, 1999). The other studies show that ellagic acid significantly reduces acid secretion and H⁺.K⁺ATPase activity on gastric ulcers (Murakami et al., 1991). To sum up, we can conclude that isolated polyphenols might be considered as a potential substances of promising previously detected properties of methanol extracts from *E. cicaturn* and other species of *Erodium*. Also, one might speculate that dietary intake of such polyphenols reduces the risk of some forms of cancers and other various degenerative diseases.

**Compound A: Methyl gallate**
- Pale needles, recrystallized from MeOH-H₂O, mp. >202 °C;
- M₀, 184.14, EI-MS m/z: 184, 153, 125, 107, 97, 79, 51, 39;
- TLC Rₖ: 0.51 (HPTLC Si 60 L, program I), 0.55 (HPTLC DIOL, program III), 0.57 (HPTLC RP-18, eluent IV); brawn under UV₃₆₅, dark blue after spraying with FeCl₃ reagent, pink-gray with bis-diazotized sulfanilamide; HPLC Rₖ: 4:14 (program V).

**Compound B: Protocatechuic acid**
- Colorless needles, recrystallized from MeOH-H₂O, mp 214–216 °C;
- TLC Rₖ: 0.58 (HPTLC Si 60 L, program I), 0.76 (HPTLC NH₂, program II), 0.41 (HPTLC DIOL, program III), 0.74 (HPTLC RP-18, eluent IV); brawn under UV₃₆₅, dark blue after spraying with FeCl₃ reagent, pink-gray with bis-diazotized sulfanilamide; HPLC Rₖ: 7:17 (program V).

**Compound C: Gallic acid**
- Colorless needles, recrystallized from MeOH-H₂O, mp 240 °C;
- TLC Rₖ: 0.44 (HPTLC Si 60 L, program I), 0.46 (HPTLC NH₂, program II), 0.37 (HPTLC DIOL, program III), 0.89 (HPTLC RP-18, eluent IV); brawn under UV₃₆₅, dark blue after spraying with FeCl₃ reagent, yellow-brown with bis-diazotized sulfanilamide; HPLC Rₖ: 4:14 (program V).

**Compound D: Brevifolin carboxylic acid**
- Yellow powder, recrystallized from MeOH, mp > 310 °C;
- M₀, 292.18, ESI-MS m/z: neg. ion. 291.1 [M-H]⁻, pos. ion 293[M+H]⁺;
- TLC Rₖ: 0.20 (HPTLC NH₂, program II), 0.36 (HPTLC RP-18, eluent IV); violet under UV₃₆₅, yellow-green after spraying with FeCl₃ reagent, pink with bis-diazotized sulfanilamide; HPLC Rₖ: 15:25 (program V); a solution of D (5 mg) in distilled H₂O (3 ml) was heated in a fused glass tube at 140 °C for 1h, and the precipitate was recrystal-
lized from MeOH to afford brevifolin, which was identified by comparison (TLC, HPLC) with compound E; UV λ<sub>max</sub> (MeOH): 279, 350, 362 nm; IR ν<sub>max</sub> (KBr): 3200, 1600, 1598, 1550, 1526, 1484, 1390, 1260, 1204, 1142, 1096, 930 cm<sup>-1</sup>; <sup>1</sup>H NMR (CD<sub>3</sub>OD-DCl<sub>2</sub>) δ: 7.76 (s, 1H, H-6), 4.88–4.86 (t, 1H, H-1), 3.01 (s, 2H, H-2); <sup>13</sup>C NMR (CD<sub>3</sub>COCD<sub>2</sub>DCl<sub>2</sub>) δ: 195.68 (C-3), 174.73 (CO<sub>2</sub>H) 161.68 (C-5), 149.54 (C-3a), 145.62 (C-9), 143.94 (C-7), 141.18 (C-8), 140.59 (C-9a), 115.98 (C-9b), 113.89 (C-5a), 109.13 (C-6), 42.06 (C-1), 37.67 (C-2).

**Compound E: Brevifolin**

Yellowish powder, recrystallized from MeOH, mp. > 310 °C; M<sub>r</sub> 248.18, LSI-MS m/z: pos. ion. 249 [M+H]+; TLC R<sub>t</sub>: 0.60 (HPTLC NH<sub>2</sub>, program II), 0.22 (HPTLC DIOL, program III), 0.16 (HPTLC RP-18, eluent IV); w UV<sub>365</sub> violet under UV<sub>365</sub>, gray after spraying with FeCl<sub>3</sub> reagent, pink with bis-diazotized sulfanilamide; HPLC R<sub>t</sub>: 13:43 (program V);


