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# Methoxylated Xanthones in the Quality Control of Small Centaury (*Centaurium erythraea*) Flowering Tops

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In the course of a phytochemical study of the bitter tonic plant, small centaury (*Centaurium erythraea*), six methoxylated xanthones (1,5-hydroxy-3-methoxyxanthone, 1-hydroxy-3,5,6-trimethoxyxanthone, 1-hydroxy-3,5,6,7-tetramethoxyxanthone, 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone, 1-hydroxy-3,7,8-trimethoxyxanthone and 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone) were isolated and identified by spectroscopic means (nuclear magnetic resonance, mass spectroscopy, and UV). Subsequently, a high-performance liquid chromatography/diode array detection method was developed for the determination of these and other methoxylated xanthones occurring in the chloroform extract of small centaury aerial parts. The methodology developed was applied to twelve samples, and in all of them, nine xanthones were identified and quantified. This methodology can be considered complimentary to the one proposed by the European Pharmacopoeia.

**KEYWORDS: Small centaury; *Centaurium erythraea*; methoxylated xanthones; HPLC/DAD**

## INTRODUCTION

Small centaury (*Centaurium erythraea* Rafn.) (Gentianaceae) is an herbaceous plant that is listed by the Council of Europe as a natural source of food flavoring, in category N2, which allows it to be added to foodstuffs in small quantities. In the U.S., its bitter properties are utilized in alcoholic and nonalcoholic beverages with maximum permitted doses between 0.0002 and 0.0008% (*1*). Its aerial parts are reputed to possess digestive, stomachic, tonic, depurative, sedative, and antipyretic properties (*1*, *2*). Recently, we have reported the antioxidant activity of the infusion obtained from this species (*3*). Compounds reported to exist in this species include xanthones (*4*−*7*), phenolic acids (*1*, *8*), and iridoids (*1*, *2*).

The quality control of the drug is usually carried out by macroscopic and microscopic characterization that is described, for instance, in the European Pharmacopoeia (*9*). This Pharmacopoeia also proposes a chemical characterization by checking for the presence of swertiamarin. However, when the drug is reduced to a powder or when it is marketed as an extract, the chemical characterization proposed is, obviously, insufficient since swertiamarin cannot be considered an exclusive chemical marker for the drug. Because xanthones are known to occur in small centaury, the objective of this study was to thoroughly investigate this class of compounds that can be found in its aerial parts and that, therefore, could be used for the chemical characterization of the plant. A method for quality control, based on the qualitative and quantitative analysis of these compounds by high-performance liquid chromatography (HPLC) coupled to diode array detection (DAD), is also proposed.

## MATERIALS AND METHODS

**General Procedures.** $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AMX-300 instrument, operating at 300.13 and 75.47 MHz, respectively. UV spectra were recorded on a Unicam Heλios α spectrophotometer. Silica gel (230−400 mesh) (Merck, Darmstadt, Germany), RP18 (Lobar and Spherisorb ODS2 (10 μm, 25 × 1 cm)), and Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO) were used for column chromatography. Silica gel (60 G; Merck, Darmstadt, Germany) was used for thin-layer chromatography (TLC). HPLC was performed on a Gilson unit. All solvents were of analytical grade.

**Plant Material.** Plants were collected from 1997 to 2000 in the north of Portugal (Table 1). Seeds of samples 9−11 were kindly supplied by the Botanic Garden of Lisbon University (Portugal), were germinated and propagated at the University of Minho, and were further cultivated in an experimental field (Arouca) with and without fertilization. The other samples were grown spontaneously in the north of Portugal.

Identification was established by the Botanical Institute of Oporto (Portugal). Flowering tops were separated, air-dried, and grounded in a hammer mill to pass 1600 μm.

**Isolation Procedures.** The dried plant material (ca. 600 g) was extracted with chloroform by maceration. The chloroform extract was

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**Table 1.** Small Centaury Xanthones Separated by HPLC

| no. | xanthone | Rt (min) | $\lambda_{max}$ (nm) |
|---|---|---|---|
| | xanthone | 43.26 | 262, 279sh, 288, 329, 344 |
| **1** | 3-OH-1,5,6-triMeO-xanthone | 32.34 | 244, 284, 306, 334sh |
| **2** | 1,3,5-triOH-2-MeO-xanthone | 34.53 | 244, 273sh, 312, 356 |
| **3** | 1,5-diOH-3-MeO-xanthone | 42.48 | 255, 310, 349 |
| **4** | 1,6-diOH-3,5-diMeO-xanthone | 44.14 | 244, 277, 314, 352sh |
| **5** | 1-OH-3,7,8-triMeO-xanthone | 48.38 | 257, 277sh, 313, 348sh, 367 |
| **6** | 1-OH-3,5,6-triMeO-xanthone | 50.01 | 251, 282, 316 |
| **7** | 1-OH-3,5,6,7,8-pentaMeO-xanthone | 50.24 | 258, 310, 369 |
| **8** | 1-OH-3,5,6,7-tetraMeO-xanthone | 52.53 | 258, 310, 352sh, 366 |
| **9** | 1,8-diOH-3,5,6,7-tetraMeO-xanthone | 56.08 | 258, 327 |

passed through a Sephadex LH-20 column with MeOH, giving four fractions. The two first fractions were dark colored and were discarded after HPLC analysis. Fractions 3 and 4 presented a yellow-blue fluorescence. Compound **1** was obtained from the third fraction while compounds **2** and **4** were isolated from the fourth fraction as previously reported (*7*).

The third fraction was further chromatographed on silica gel, using petroleum ether:$CHCl_3$ (from 90:10 to 60:40). The second UV-fluorescent fraction, eluted with 60% $CHCl_3$, was subjected to further silica gel chromatography, using petroleum ether:$CH_2Cl_2$ (from 90:10 to 20:80). The second fraction obtained was dissolved in $CHCl_3$ and separated by means of preparative TLC (toluene:$CHCl_3$, 25:75), yielding four fluorescent zones.

The fraction with the higher $R_f$ was further purified by repeated liquid chromatography over a Lobar column in MeOH. This procedure allowed the isolation of 1-hydroxy-3,5,6-trimethoxyxanthone (**6**), 1-hydroxy-3,5,6,7-tetramethoxyxanthone (**8**), and 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone (**9**).

The second fluorescent zone, in order of decreasing $R_f$ values, was subjected to semipreparative HPLC with $H_2O$:MeOH (30:100) at a flow rate of 2 mL/min to give 1-hydroxy-3,7,8-trimethoxyxanthone (**5**) and 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone (**7**).

The fourth fraction obtained from Sephadex was subjected to repeated silica gel column chromatography, using petroleum ether: $CHCl_3$ (from 90:10 to 40:60). The fraction eluted with 60% $CHCl_3$ was further subjected to a second chromatography, using a gradient from petroleum ether to $CH_2Cl_2$, which allowed the isolation of 1,5-hydroxy-3-methoxyxanthone (**3**).

**Spectrometric Data of Isolated Compounds.** Spectrometric data of compounds **1**, **2**, and **4** was previously reported (*7*).

*1,5-Dihydroxy-3-methoxyxanthone (**3**).* UV (MeOH): see Table 1. $^1$H NMR (DMSO-$d_6$): $\delta$ 3.90 (3H, s, $OCH_3$), 12.87 (1H, s, OH-1), 6.40 (1H, d, $J = 2.2$ Hz, H-2), 6.64 (1H, d, $J = 2.2$ Hz, H-4), 7.33 (1H, dd, $J = 7.8$, 1.8 Hz, H-6), 7.27 (1H, t, $J = 1.8$ Hz, H-7), 7.56 (1H, dd, $J = 7.8$, 1.8 Hz, H-8). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 56.2 ($OCH_3$), 162.6 (C-1), 97.1 (C-2), 166.1 (C-3), 92.8 (C-4), 156.8 (C-4a), 146.3 (C-5), 120.9 (C-6), 124.3 (C-7), 114.5 (C-8), 103.0 (C-9), in agreement with data reported in the literature (*6*).

*1-Hydroxy-3,7,8-trimethoxyxanthone (Decussatin) (**5**).* UV (MeOH): see Table 1. $^1$H NMR ($CDCl_3$): $\delta$ 3.93 (3H, s, $OCH_3$-3), 3.87 (3H, s, $OCH_3$-7),[a] 3.99 (3H, s, $OCH_3$-8),[a] 13.26 (1H, s, OH-1), 6.33 (1H, d, $J = 2.1$ Hz, H-2), 6.30 (1H, d, $J = 2.1$ Hz, H-4), 7.33 (1H, d, $J = 9.2$ Hz, H-5), 7.16 (1H, d, $J = 9.2$ Hz, H-6). $^{13}$C NMR ($CdCl_3$): $\delta$ 55.8 ($OCH_3$-3),[b] 57.1 ($OCH_3$-7),[b] 61.8 ($OCH_3$-8),[b] 163.8 (C-1), 96.8 (C-2), 166.4 (C-3), 92.0 (C-4), 157.1 (C-4a), 150.9 (C-4b), 112.8 (C-5), 120.3 (C-6), 149.2 (C-7), 148.7 (C-8), 115.7 (C-8a), 104.0 (C-8b), 181.2 (C-9).[a,b] Assignments are interchangeable in agreement with data reported in the literature (*5*, *11*).

*1-Hydroxy-3,5,6-trimethoxyxanthone (**6**).* UV (MeOH): see Table 1. $^1$H NMR ($CDCl_3$): $\delta$ 3.90 (3H, s, $OCH_3$-3), 4.02 (3H, s, $OCH_3$-5), 4.02 (3H, s, $OCH_3$-6), 12.93 (1H, s, OH-1), 6.36 (1H, d, $J = 2.6$ Hz, H-2), 6.53 (1H, d, $J = 2.6$ Hz, H-4), 7.00 (1H, d, $J = 8.9$ Hz, H-7), 8.00 (1H, d, $J = 8.9$ Hz, H-8), in agreement with data reported in the literature (*5*).

*1-Hydroxy-3,5,6,7,8-pentamethoxyxanthone (Eustomin) (**7**).* UV (MeOH): see Table 1. $^1$H NMR ($CDCl_3$): $\delta$ 3.93 (3H, s, $OCH_3$-3), 3.87 (3H, s, $OCH_3$-5),[a] 3.98 (3H, s, $OCH_3$-6),[a] 3.99 (3H, s, $OCH_3$-7),[a] 4.13 (3H, s, $OCH_3$-8),[a] 13.26 (1H, s, OH-1), 6.31 (1H, d, $J = 2.1$ Hz, H-2), 6.44 (1H, d, $J = 2.1$ Hz, H-4). $^{13}$C NMR ($CdCl_3$): $\delta$ 55.7 ($OCH_3$-3), 61.6 ($OCH_3$-5),[b] 62.0 ($OCH_3$-6),[b] 60.1 ($OCH_3$-7),[b] 62.1 ($OCH_3$-8),[b] 163.6 (C-1), 97.3 (C-2), 166.2 (C-3), 92.2 (C-4), 156.8 (C-4a), 136.8 (C-5), 150.4 (C-6), 148.8 (C-7), 142.4 (C-8), 181.6 (C-9).[a,b] Assignments are interchangeable in agreement with data reported in the literature (*5*, *11*).

*1-Hydroxy-3,5,6,7-tetramethoxyxanthone (**8**).* UV (MeOH): see Table 1. $^1$H NMR ($CDCl_3$): $\delta$ 3.90 (3H, s, $OCH_3$-3), 3.97 (3H, s, $OCH_3$-5),[a] 4.06 (3H, s, $OCH_3$-6),[a] 4.07 (3H, s, $OCH_3$-7),[a] 12.88 (1H, s, OH-1), 6.37 (1H, d, $J = 2.3$ Hz, H-2), 6.52 (1H, d, $J = 2.3$ Hz, H-4), 7.42 (1H, s, H-8).[a] Assignments are interchangeable in agreement with data reported in the literature (*5*).

*1,8-Dihydroxy-3,5,6,7-tetramethoxyxanthone (Demethyleustomin) (**9**).* UV (MeOH): see Table 1. $^1$H NMR ($CDCl_3$): $\delta$ 3.93 (3H, s, $OCH_3$-3), 3.90 (3H, s, $OCH_3$-5),[a] 3.94 (3H, s, $OCH_3$-6),[a] 4.14 (3H, s, $OCH_3$-7),[a] 11.98 (1H, s, OH-1),[b] 11.90 (1H, s, OH-8),[b] 6.35 (1H, d, $J = 2.3$ Hz, H-2), 6.50 (1H, d, $J = 2.3$ Hz, H-4).[a,b] Assignments are interchangeable in agreement with data reported in the literature (*5*).

**Extraction of Xanthones for Quantification Purposes.** Each powdered sample (ca. 1 g) was extracted with 100 mL of chloroform for 10 min with agitation, followed by extraction with $2 \times 100$ mL of chloroform for 15 min. The extracts were combined and filtered, and the chloroform was removed under reduced pressure (30 °C) to dryness. The residue was dissolved in 2 mL of methanol, and 20 $\mu$L was analyzed by HPLC.

**HPLC Analysis.** Separation of xanthones was achieved with an analytical HPLC unit, using a reversed-phase Hypersil ODS ($20 \times 0.4$ cm i.d.; 5 $\mu$m) column. The solvent system was acetonitrile/water/acetic acid (15:84:0.85) (A) and methanol (B), starting with 0% methanol, with a gradient to obtain 100% B at 70 min. Elution was performed at a solvent flow rate of 0.8 mL/min. Detection was accomplished with a diode array detector, and chromatograms were recorded at 320 nm.

*Identification and Quantification of Compounds.* The compounds in each sample were identified by comparing their retention times and UV−vis spectra in the 200−400 nm range with those of the previously isolated compounds. Peak purity was checked by means of the Gilson 160 spectra viewer software contrast facilities.

Xanthone quantification was achieved by the absorbance recorded in the chromatograms relative to external standard, with detection at 320 nm. All of the compounds were quantified as xanthone (Sigma Chemical Co., St. Louis, MO).

## RESULTS AND DISCUSSION

The plant family Gentianaceae is characterized by the presence of xanthones, and several methoxylated derivatives have been found to occur in small centaury aerial parts, whole plants, roots, and even cell cultures (*4*−*7*). However, the unavailability of such compounds commercially and the fact that their chromatographic characteristics are not always reported make the phytochemical data useless for routine quality control. If small centaury xanthones are intended to be used for its quality control, a different approach is needed. First, it was necessary to know what compounds, among those described in the species, really occur in the aerial parts since only this part is used as a bitter tonic. Second, it was necessary to obtain data that can be easily used in routine quality control, namely, HPLC/DAD: retention times (Rt) and UV−vis spectra.

A chloroform extract of the flowering tops was investigated, and nine compounds were isolated and identified. Recently, we reported (*7*) the isolation and chemical characterization of 3-hydroxy-1,5,6-trimethoxyxanthone (**1**), 1,3,5-trihydroxy-2-methoxyxanthone (**2**), and 1,6-dihydroxy-3,5-dimethoxyxanthone (**4**), and six more compounds have now been identified. Compound **3** (see Figure 1) had spectral properties identical to those published for 1,5-dihydroxy-3-methoxyxanthone (*10*). This compound was previously described only in cell cultures of *C. erythraea* (*12*) and is now reported from natural plant material.

**Table 2.** Xanthone Contents of Small Centaury Samples (mg/kg, Dry Basis)[a]

| | | xanthone compounds[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| smpl | origin | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | Penacova August, 1997 | nq | 17.04 (0.498) | 18.60 (0.148) | 212.78 (3.426) | 11.75 (0.218) | 11.28 (0.531) | 27.28 (0.807) | 8.96 (0.366) | 157.68 (9.361) |
| 2 | Monção August, 1997 | 12.57 (4.899) | 91.12 (2.513) | 70.43 (2.608) | 502.71 (45.321) | 40.15 (11.716) | 37.83 (7.259) | 68.43 (11.809) | 13.76 (2.691) | 187.94 (17.079) |
| 3 | Monção June, 1998 | 21.79 (4.754) | 47.61 (0.617) | 29.49 (0.522) | 661.13 (15.891) | 114.96 (0.509) | 79.84 (0.056) | 168.33 (7.389) | 36.56 (11.63) | 321.25 (11.451) |
| 4 | Gerês July, 1998 | 6.32 (0.961) | 29.75 (1.395) | 40.95 (0.279) | 161.50 (19.458) | 132.46 (2.594) | 37.91 (2.837) | 100.33 (0.830) | 19.55 (0.181) | 317.57 (0.210) |
| 5 | Ancede July, 1998 | 12.21 (0.282) | 287.38 (6.156) | 56.94 (4.596) | 448.01 (13.091) | 54.28 (5.146) | 38.44 (5.783) | 41.39 (3.995) | 13.01 (3.112) | 107.85 (27.880) |
| 6 | Penacova June, 1999 | 20.82 (0.159) | 37.68 (0.338) | 29.24 (0.593) | 103.29 (0.882) | 16.38 (0.221) | 17.23 (0.503) | 22.16 (1.200) | 10.29 (0.178) | 182.38 (4.751) |
| 7 | Penacova June, 1999 | 9.13 (0.518) | nq | 30.04 (1.275) | 74.05 (1.288) | 19.17 (0.796) | 12.58 (0.507) | 34.57 (0.265) | 11.97 (0.985) | 210.91 (17.642) |
| 8 | Arada June, 1999 | nq | 12.70 (0.073) | 27.09 (4.758) | 86.30 (11.545) | 46.39 (6.841) | 27.57 (5.701) | 49.08 (9.057) | 22.98 (3.883) | 399.64 (58.626) |
| 9 | Arouca July, 1999 | nq | 20.69 (0.088) | 12.06 (0.131) | 171.00 (8.690) | nq | 14.67 (0.364) | 31.72 (0.227) | 5.99 (0.464) | 125.02 (8.375) |
| 10 | Arouca[c] July, 1999 | nq | 41.14 (7.088) | 25.22 (1.302) | 232.23 (10.878) | nq | 11.00 (1.206) | 21.46 (5.817) | nq | 87.00 (4.600) |
| 11 | Arouca[d] July, 1999 | nq | 13.70 (1.132) | 19.19 (0.841) | 93.83 (2.019) | nq | 11.02 (1.767) | 31.06 (3.196) | nq | 96.82 (4.174) |
| 12 | Penacova June, 2000 | 31.12 (6.029) | 35.79 (6.386) | 11.00 (0.597) | 54.60 (1.319) | 15.64 (2.708) | 7.50 (1.365) | 20.28 (1.520) | 11.90 (1.063) | 349.11 (15.537) |

[a] Values are expressed as mean (standard deviation) of two assays; nq = not quantified. [b] Identity of compounds as in Table 1. [c,d] Represent fertilization with 120 and 60 kg/ha/year of nitrogen, respectively.

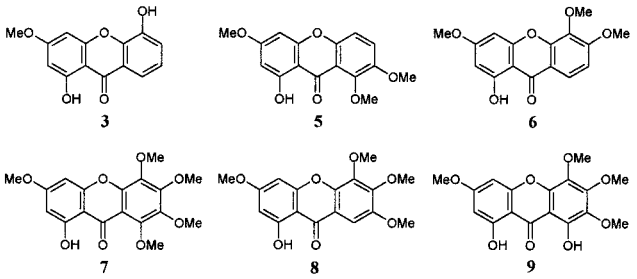


**Figure 1.** Chemical structures of the isolated xanthones from small centaury. 1,5-Hydroxy-3-methoxyxanthone (**3**); 1-hydroxy-3,7,8-trimethoxyxanthone (**5**); 1-hydroxy-3,5,6-trimethoxyxanthone (**6**); 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone (**7**); 1-hydroxy-3,5,6,7-tetramethoxyxanthone (**8**); and 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone (**9**).

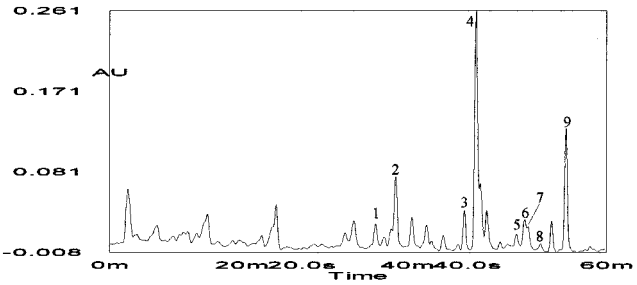


**Figure 2.** HPLC profile of a small centaury sample extracted with chloroform. Detection at 320 nm. 3-Hydroxy-1,5,6-trimethoxyxanthone (**1**); 1,3,5-trihydroxy-2-methoxyxanthone (**2**); 1,5-dihydroxy-3-methoxyxanthone (**3**); 1,6-dihydroxy-3,5-dimethoxyxanthone (**4**); 1-hydroxy-3,7,8-trimethoxyxanthone (**5**); 1-hydroxy-3,5,6-trimethoxyxanthone (**6**); 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone (**7**); 1-hydroxy-3,5,6,7-tetramethoxyxanthone (**8**); and 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone (**9**).

Compounds **5**, **6**, and **8** were described in roots (*4*, *5*) and are now reported from the flowering tops. Compounds **7** and **9** were previously described in whole plants (*4*, *6*), and its existence is now confirmed in flowering tops. The structural identification of the compounds was made by spectroscopic techniques, and data obtained are in good agreement with those published for the respective compounds.

The HPLC analysis of the chloroform extract (Figure 2) revealed the presence of the nine identified compounds. The retention times under the analysis conditions and UV−vis spectra data, as recorded from DAD, are listed in Table 1. Other minor compounds were also detected but not identified.

All analyzed samples exhibited the nine identified compounds, although in different amounts. The amounts of the various xanthones present are shown in Table 2. Despite the differences observed in the xanthone contents, there are some common characteristics in the phenolic fingerprint obtained. All of the samples (except sample 5, see below) showed a common pattern in which **4** and **9** were the major compounds. Samples 1−3 and 9−11 exhibited **4** as the compound present in highest amounts, ranging from 36 to 56% of the total quantified compounds, followed by **9**, while in samples 4, 6−8, and 12 there is an inversion in the content of these compounds, with compound **9** ranging from 36 to 65%.

Sample 5 is a unique case that showed **4** and **2** as the major compounds. In fact, this sample exhibited a very high amount of compound **2** (27%) when compared with the other samples where it ranged from 2 to 10%. This phytochemical variability may be related to the existence of different chemotypes that some authors consider as being subspecies or varieties. In this study, the botanical classification was not carried out at the subspecies level. However, this is not always possible because transitional forms can exist that do not allow a correct classification (*13*).

Samples from Arouca (samples 9−11) have the same genetic origin and were cultivated in the same field but under different conditions. Sample 9 received no treatment, while samples 10 and 11 received 120 and 60 kg/ha/year of nitrogen fertilizer, respectively. It seems that fertilization with nitrogen leads to an increase in xanthone production, especially in compounds **2**−**4**.

The technique described herein may be considered a complimentary approach for the characterization of small centaury, since a set of nine compounds can be unequivocally identified. The quantification of individual compounds, besides being useful in the quality control, can also be explored as a tool for the classification at a subspecies or varietal level.

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