**Serratula tinctoria**, a source of natural dye: Flavonoid pattern and histolocalization

P. Guinot\(^a\), A. Gargadennec\(^a\), P. La Fisca\(^a\), A. Fruchier\(^b\), C. Andary\(^a\), L. Mondolot\(^{a,*}\)

\(^a\) Laboratoire de Botanique, Phytochimie et Mycologie, UMR 5175 CEFE, Faculté de Pharmacie, Université Montpellier 1, 15 Avenue Charles Flahault, BP 14491, 34 093 Montpellier Cedex 5, France

\(^b\) Ecole Nationale Supérieure de Chimie, UMR 5253, 8 rue de l’École Normale, 34 296 Montpellier Cedex 5, France

---

**Abstract**

In the context of new alternative crop development in Europe, flavonoids were investigated in saw-wort, *Serratula tinctoria* L., a perennial Asteraceae which was used as a yellow dye until the 19th century. The phytochemical study described in this report indicates that leaves rather than stems should be used, and harvested at the end of the plant growing cycle, when flavonoids are particularly concentrated. Microspectrofluorometry showed a specific distribution of the flavonoid aglycone, luteolin in stomatal cells whereas the corresponding glycoside (luteolin-7-O-glucoside) was observed in palisade parenchyma cells. The flavonoids luteolin-4′-O-glucoside and 3-methylquercetin were isolated for the first time in *S. tinctoria* leaves and identified by NMR spectroscopy. The role of these flavonoids is discussed in this paper. Using a rapid and simple method, i.e. flavonoid histolocalization associated with UV, it was demonstrated that saw-wort contains high concentrations of luteolin derivatives and could be considered for use again as a natural dye.

© 2008 Elsevier B.V. All rights reserved.

---

**1. Introduction**

The worldwide demand for natural raw materials is nowadays of great interest as a result of increased consumer awareness and a popular demand for natural products. Natural dyes are particularly relevant today because of their attractiveness in terms of minimal environmental and health impact versus synthetic dyes, which have been suggested as sources of human diseases as skin cancer or allergic contact dermatitis (Anliker et al., 1988; Moreau and Goossens, 2005). As a result, many studies have focused on well-known dyeing plants that represent high value and quality alternative crops as *Reseda luteola* for yellow dye (Cerrato et al., 2002; Angelini et al., 2003), *Rubia tinctorum* for red dye (Angelini et al., 1997; De Santis and Moresi, 2007) or *Isatis tinctoria* for blue dye (Oberthuer et al., 2004; Vetter, 1997).

The perennial plant *Serratula tinctoria* L. (saw-wort) was also an important dyeing plant well known to the medieval textile dyers in Europe and is quoted in different recipes (Hofenk de Graaff, 2003). The whole plant, except roots, was used until the 19th century as a yellow dye, especially when *R. luteola* was not available.

Despite significant historical and traditional uses, little information is available regarding its dyeing components, as the plant was principally studied for its ecdysteroid content (Báthori et al., 1998, 1999; Corio-Costet et al., 1999). Ecdysteroids have several pharmacological properties (Lafont and Dinan, 2003), but are not dyeing compounds. Flavonoids (plant...
pigments) were previously reported as only phenolic compounds present in a traditional dyeing bath (Guinot et al., 2006). Kaiser (1993) demonstrated the high flavonoid content of saw-wort, especially in leaves. A previous study, by Andary et al. (1996) reported the isolation of luteolin and luteolin-7-O-glucoside as the main dyeing component in this plant. Moreover, a minor component, 3-methylquercetin was proposed but no structural evidence was provided to confirm the structure of this compound.

The present investigation, therefore, studied saw-wort flavonoids in order to obtain useful information for the development of this plant as an original alternative crop. The evolution of flavonoid content during plant growth was first evaluated in leaves in order to determine the optimum period to harvest. Specialized tissues and cells accumulating flavonoids were observed in situ, in cross-sections of fresh leaves. Microspectrofluorometrical analysis of these cells provided further information as the histolocalization of these compounds. Further work was also carried out in term of phytochemical analysis to determine the polyphenolic content of saw-wort.

2. Materials and methods

2.1. Plant material

Leaves and stems of S. tinctoria L. were provided from Le Jardin Conservatoire des Plantes Tintoriales (Lauris, Mediterranean climate, France). Mediterranean climate is characterised by dry, sunny and hot summers, radiation ≥250 h/month.

Seeds came from one wild ecotype harvested in Mont Aigoual, Cévennes, France. Seeds were sown in February in an unheated greenhouse, then planted out at the beginning of May in a limestone soil in Lauris. In order to investigate the evolution of flavonoids during plant growth, samplings were carried out at different growth stages during the same year: after 1 month of growth in March (I), at the beginning of flowering in June (II) and when flowers were fading in August (III). The same experiment was carried in temperate climate conditions in Le Jardin Botanique de la Faculté de Pharmacie de Lille (north of France) from the same batch of seeds, planted out in a silt soil. Lille is in an oceanic temperate climate zone: spring and summer are slightly moist, moderate sunshine, radiation ≥200 h/month.

Plants were directly air-dried after harvesting at 30 °C in a well ventilated room, to avoid degradation of these compounds and then transported to the laboratory to be powdered in a domestic grinder before analysis. For histochemical analysis, saw-wort plantations were established from the same previous batch of original seed material in Le Jardin Botanique de la Faculté de Pharmacie de Montpellier (south of France). Fresh leaves were collected at stage (III) 30 min before analysis.

2.2. Histochemical analysis

This is a qualitative method, used here to determine flavonoid localization in plant cells.

Small fragments of freshly collected S. tinctoria leaves were embedded in 3% agarose (type II EEO, Panreac) before cutting for histochemical examination. Transverse sections (40 μm) were obtained using a Leica VT 1000S vibrating blade microtome (frequency 7, speed 2). Neu’s reagent (1% 2-aminoethyl-diphenylborinate (Fluka) in absolute methanol), a standard reagent for phenolic compounds (Neu, 1957), was used. Transverse sections were immersed (10 s) in Neu’s reagent and then mounted in glycerol-water (10:90; v/v) solution. Specimens were viewed under a light microscope (Nikon Optiphot) with UV light (filter UV-1A: 365 nm excitation filter, 400 nm barrier filter). Under these conditions, flavonoids were detected by a yellowish fluorescence (Mondolot-Cosson et al., 1997). Photographs were taken with a digital Nikon Coolpix 4500 camera.

2.3. Microspectrofluorometry

This method is qualitative but more informative than histochemical analysis because flavonoid emission spectra may be obtained.

A microspectrofluorometer (Jobin-Yvon) equipped with an Olympus BX 60 microscope was used to obtain emission fluorescence spectra from fresh leaf transverse sections previously immersed in Neu’s reagent for 10 s (an area of 5 μm in diameter was selected). Each leaf was analysed in triplicate. Using a xenon lamp and monochromators, 365.5–368.5 nm wavelengths were produced to excite compounds in the sample. The resultant fluorescence was detected with a CCD camera, and the fluorescence emission spectra were produced by the SpectraMax software package. Standards of flavonoids (luteolin, luteolin-7-O-glucoside) were tested at 0.2% concentration (w/v) in Neu’s reagent.

2.4. Phytochemical analysis

2.4.1. Sample preparation

Plant extracts (1:100, w/v) were obtained from dried leaves and stems in ethanol/water (3:7; v/v) with ultrasonication (15 mn, 24 kHz, R.E.U.S.-GEX 180). After passing through filter paper, the filtrate was centrifuged for 10 min (7176 g). The supernatant was then directly used for thin layer chromatography (TLC) analysis. Extractions were carried out in triplicate.

2.4.2. UV spectroscopy

This is a quantitative method.

A Unicam UV–vis spectrophotometer (ATI Unicam) was used. Solutions were diluted in ethanol/water (3:7; v/v) in order to use the spectrophotometer in a linear range (from 0.2 to 0.9 Au). Quantitative analysis was carried out by interpolation on a calibration curve prepared using luteolin standard solutions. Analyses were carried out at 352 nm. Results were expressed in % of luteolin equivalent in the dried plant. Analyses were performed in triplicate (three different plots).

2.4.3. Thin layer chromatography

This method is qualitative.

A 5-μl aliquot of sample was spotted using an ATS3, Camag instrument onto a TLC plate (10 cm × 10 cm) covered with silica gel (Merck F254, 5554) or silica reversed phase gel (Merck F254, 5559). TLC were repeated twice. The former was developed in mobile phase (1) (ethyl acetate/formic
acid/acetic acid/water; 100:11:11:26) and the latter in mobile phase (2) (acetonitrile/water/formic acid; 50:45:15). Cellulose plates (Merck 5552) were also used for two-dimensional TLC: the plate was first developed in mobile phase (3) (dichloromethane/acetic acid/water; 50:45:15) followed by mobile phase (4) (ethyl acetate/acetic acid/water; 10:30:70). After drying, the plates were observed under UV light at 366 nm before and after revelation with Neu’s reagent. Methanol solutions of authentic standards (1:1000, w/v) were used for comparative purposes.

2.5. Isolation and identification of flavonoids

Dried ground leaves (50 g) were extracted twice in 800 mL of ethanol/water (3:7; v/v) (that is equivalent to 3% extract), by ultrasonication for 15 min at 24 kHz (3.1% extract). After filtration through filter paper, the solution was concentrated to 50 mL under vacuum. The resulting crude extract was purified on a cellulose stationary phase using medium pressure liquid chromatography (4–5 bars). Elution was carried out by successively percolating water, water:ethanol (9:1, 8:2, and 7:3; v/v) and pure ethanol through the column. The collected fractions were monitored by TLC (cellulose plate, mobile phase 3). Flavonoids 1 and 2 were purified from those fractions on Sephadex LH20 support with water/ethanol mixture (8:2; v/v).

2.6. Nuclear magnetic resonance (NMR)

1H and 13C NMR spectra of sample solutions in CD$_3$OD or DMSO-$_d_6$ were recorded at 400.13 and 100.61 MHz, respectively with a Bruker Avance-400 spectrometer. Chemical shifts are reported in ppm/TMS. CHD$_2$ and CD$_3$ signals of CD$_3$OD are at 3.300 and 47.84 ppm, respectively and those of DMSO-$_d_6$ are at 2.50 and 39.98 ppm, respectively. Coupling constants are expressed in Hz. Standard Bruker sequences were used for correlation spectra (COSYGP, HMQCGP and HMBCGP).

3. Results and discussion

3.1. Flavonoid accumulation

Fig. 1 presents plant flavonoid concentrations expressed as % luteolin equivalent in dry matter. It appears that these compounds are particularly concentrated in leaves in the early and late of the plant’s growth cycle, respectively stage I (1.55%) and stage III (1.45%). The flavonoid content of stems determined at stage III of the growth cycle was very low (0.3%) compared to the flavonoid content of leaves at the same vegetative stage. These findings were previously observed by Kaiser (1993). The same result was also observed with other species (Artemisia vulgaris, R. luteola; Guinot, 2006) and could be attributed to one of the fundamental purposes of flavonoid biosynthesis in plants, which is the accumulation of phenolic compounds, particularly flavonoids in leaves to a greater extent than in stems. The most likely reason for this is that leaves are more exposed to UV radiation, against which polyphenols have been shown to have protective effects in plants (Caldwell et al., 1983; Smith and Markham, 1998). Moreover, Tattini et al. (2000) showed that in sun-exposed leaves of Phyllirea latifolia (a species of the Oleaceae family) there was a marked increase in luteolin-7-O-glucoside, one of the major flavonoid glycosides apart from quercetin-3-O-rutinoside. These authors also demonstrated that these same flavonoids increased sharply in response to solar radiation in Ligustrum vulgare leaves, and they suggested that flavonoids may also have antioxidant properties in response to excessive light and water stress (Tattini et al., 2004). A similar observation was made in this study, in that the flavonoid content of saw-wort leaves harvested after 3 days of rainfall was very low compared to leaves harvested after several dry days (unpublished data). Furthermore, this result is consistent with a possible climate effect on the accumulation of flavonoids. In fact, as is shown in Fig. 2, it appeared that in Mediterranean climate conditions, the concentration of flavonoids after 1 month’s growth was greater than in temperate climate conditions. Thus, a warmer and sunnier climate may be the cause of greater flavonoid accu-

![Fig. 1 – Flavonoids evolution during saw-wort growth in leaves and stems: after 1 month of growth (I), at the beginning of flowering (II) and when flowers were fading (III) (UV analysis at 352 nm, results expressed in luteolin equivalent content, data are given as the mean value of four plots ± standard error).](image1)

![Fig. 2 – Flavonoids content in leaves of saw-wort after 1 month of growth under Mediterranean climate (mc) and under temperate climate (tc) (UV analysis at 352 nm, results expressed in luteolin equivalent content, data are given as the mean value of four plots ± standard error).](image2)
mulation due to higher exposure to UV light. Chaves et al., 1997 confirmed that the UV irradiation is the major inducer of the enhanced flavonoid secretion during summer in Cistus ladanifer. Similar results were obtained in assays carried out with other dyeing plants as Tagetes tinctoria (Guinot et al., 2008) and Artemisia vulgaris (unpublished data).

In agronomic terms, these results indicate that to maximise flavonoid yield, saw-wort leaves should ideally be harvested at the end of the growth cycle, when flavonoids are concentrated and biomass weight is greatest. Moreover, saw-wort leaves should ideally be harvested after a relatively long period of sunny conditions.

3.2. Histolocalization

On the basis of previous results, fresh leaves were subjected to histochemical analysis. Neu’s reagent, a standard reagent for flavonoids, forms complexes with phenolics which then emit a fluorescence emission spectrum of flavonoids in the presence of Neu’s reagent had a maximal emission at 575 nm, which is very close to the value obtained for luteolin standard with the same reagent (574 nm). In palisade parenchyma cells, the fluorescence emission spectrum of flavonoids in the presence of Neu’s reagent had a maximal intensity at 590 nm, close to the value obtained for luteolin-7-O-glucoside standard under the same conditions (595 nm). Differences observed between the λmax Values of standards and leaf samples were attributed to a matrix effect.

Histochemical observations showed a specific distribution of flavonoid aglycones and glycosides at a cellular level. This distribution is in accordance with previous reports which showed that superficial cells (epidermal, stomatal) contained the flavonoid aglycone whereas more internal cells contained flavonoid glycosides (Wollenweber and Mam, 1987; Valant-Vetschera and Wollenweber, 1988, 1989, 1995).

3.3. Phytochemical analysis

On the basis of TLC analysis, a significant flavonoid pattern was detected in the ethanol extract of saw-wort leaves. Comparison of TLC frontal ratio of standard solutions with plant extracts indicated the presence of luteolin and luteolin-7-O-glucoside as previously described in the literature (Andary et al., 1996). Furthermore, the TLC results are consistent with those obtained by histochemical observations on fresh leaves. These two flavonoids are also found in R. luteola, a well-known yellow dyeing plant, and are of importance in terms of the natural dye industry. In fact, these flavonoids are reportedly more lightfast than other flavonoids due to the absence of hydroxyl group on the C3 position (Smith et al., 2000). This observation may explain why saw-wort was much appreciated and widely used for dyeing purposes in the past.

TLC analysis also revealed that kaempferol, luteolin-3′-7-O-diglucoside, luteolin-3′-O-glucoside and luteolin-4′-O-glucoside may possibly be present. After purification of the ethanol extract, two flavonoids were isolated as pure compounds. Results from the 1H and 13C NMR spectra (indicated below) of those compounds were consistent with the structure of luteolin-4′-O-glucoside and of 3-methylquercetin (Fig. 4), previously described in other species, by Kellam et al. (1993) and Bouktaid et al. (2002), respectively.

Luteolin-4′-glucoside: 1H—b: 3.193 (H-4′), 3.317 (H-3′), 3.349 (H-2′), 3.402 (H-5′); 3.492 (H-6′ a); 3.735 (H-6′ b); 4.894 (H-1′); 6.138 (H-6); 6.493 (H-8); 6.825 (H-3); 7.247 (H-5′); 7.500 (H-2′); 7.520 (H-6′); 7.621 (H,8); J(6,8) = 2.1; J(2′,6′) = 2.3; J(5′,6′) = 8.4; J(1′,2′) = 7.6; J(2′,3′) = 8.3; J(3′,4′) = 9.5; J(4′,5′) = 9.4; J(5′,6′ a) = 5.9; J(5′,6′ b) = 1.9; J(6′ a,6′ b) = 11.8. 13C: δ 61.60 (C-6′); 70.24 (C-4′); 73.71 (C-2′); 76.31 (C-3′); 77.78 (C-5′); 94.54 (C-8);
99.46 (C-6); 101.68 (C-1'); 104.43 (C-3); 104.16 (C-4a); 114.05 (C-2'); 116.46 (C-5'); 118.96 (C-6'); 125.19 (C-1'); 147.92 (C-3'); 149.01 (C-4'); 157.84 (C-8a); 115.30 (C-2'); 121.16 (C-6'); 125.19 (C-1'); 138.39 (C-3); 145.32 (C-3'); 148.81 (C-4'); 156.85 (C-8a); 157.28 (C-2'); 161.95 (C-5); 164.80 (C-7); 178.86 (C-1'); 201.77 (C-9); 149.01 (C-4); 160.78 (C-8a); 161.90 (C-5); 165.06 (C-7); 163.60 (C-2'); 182.20 (C-4).

3-Methylquercetin: 1H—δ 3.801 (CH3-3); 6.215 (H-6); 6.413 (H-8); 6.919 (H-5'); 7.551 (H-6'); 7.642 (H-2'); J(6,8)=2.1; J(2,5')=2.2; J(5',6')=8.5. 13C δ: 59.35 (CH3-3); 93.54 (C-8); 98.59 (C-6); 104.70 (C-4a); 115.25 (C-5'); 115.30 (C-2'); 121.16 (C-6'); 121.76 (C-1'); 138.39 (C-3); 145.32 (C-3'); 148.81 (C-4'); 156.85 (C-8a); 157.28 (C-2'); 161.95 (C-5); 164.80 (C-7); 178.86 (C-1').

It was previously supposed that 3-methylquercetin was present, but, to our knowledge no structural evidence has been published to confirm the presence of this compound in this plant. Furthermore, both luteolin-4'-O-glucoside and 3-methylquercetin were isolated from another Serratula species, Serratula corona (Báthori et al., 2004), suggesting that these compounds may be useful as chemotaxonomical agents.

4. Conclusion

Results presented in this study on the flavonoid content of S. tinctoria provided valuable information in terms of the development of new alternative crops such as dyeing plants. The optimum harvesting period was identified and the flavonoid content was specified in terms of structure and tissue localization. The high content of luteolin derivatives evidenced the quality of the yellow dye obtained, especially in terms of saturation. The high content of luteolin derivatives, which suggests that enzymatic degradation does not occur during plant drying. Moreover, histolocalization of interesting compounds is important in terms of plant valorisation. The rapidity and simplicity of the methods employed are particularly relevant and their advantages have been previously demonstrated (Dai et al., 1996; Housti et al., 2002; Hjorth et al., 2006). These observations confirm that the technique of histolocalization could be developed as a useful tool in association with a quantitative UV analysis in order to determine flavonoid distribution in plants and to establish the most appropriate time to harvest in terms of concentration of the target compounds.

Acknowledgments

Authors would like to thank Florent Valentin from Le Jardin des Plantes Tinctoriales (Lauris, France) for his technical assistance in growing and harvesting plants and Mr. Gantz from Reus society for providing us ultrasonicator. The FEDER program is thanked for financial support.

REFERENCES


