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AN HPLC SCREENING OF SOME ITALIAN RANUNCULACEAE FOR THE LACTONE PROTOANEMONIN

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Key Word Index—Ranunculaceae; protoanemonin; taxonomic marker; chemotaxonomy; HPLC.

Abstract—An HPLC study of the distribution and quantitation of protoanemonin was carried out on several Italian Ranunculaceae species. The results indicated that protoanemonin may be a useful chemical marker in elucidating systematic relationships within this family.

INTRODUCTION

Among the Angiospermae, few families show wider morphological variation than the Ranunculaceae [1]. Systematic research has been carried out on the karyotype [2], serology [3], and the presence of phenolics, alkaloids, cyanogenic compounds and acrid principles [4–6]. In the recent phylogenetic schemes [7, 8], particular significance has been given to protoanemonin, an acrid principle that is considered to be basic to the family. Protoanemonin, the lactone of γ-hydroxyvinylacrylic acid, derives from the glucoside ranunculin and may dimerize producing anemonin, a water-insoluble derivative. Several methods of analysis have been employed such as tritiation of plant extracts, weighing of crystalline products, paper and thin-layer chromatography, colorimetry and spectrophotometry [9–11]. Recently, we have used the more sensitive high pressure liquid chromatography analysis [12].

This paper presents the results of studying protoanemonin distribution in 29 representative species of the Ranunculaceae.

RESULTS AND DISCUSSION

Both the reversed phase (RP) and normal phase (NP)-high pressure liquid chromatography (HPLC) techniques were employed to separate protoanemonin. The RP system with a Lichrosorb RP18 column and a binary solvent system was used for samples with a protoanemonin content greater than 10 μg/g wet wt. For samples with a lower lactone content, a NP system with Lichrosorb Si60 and quaternary solvent system elution procedure was employed. Protoanemonin was identified and calibrated following a previously reported procedure [12]. It is well known that the quantitation of protoanemonin is critical because of the tendency of this compound to dimerize to anemonin [13]. This HPLC procedure detects 0.7 ng of protoanemonin per 10 μl injected sample (0.005 a.u.f.s.) and has a good correlation coefficient of regression analysis. In this context, it is interesting to compare our HPLC data with earlier results obtained using spectrophotometry. Table 1 lists the protoanemonin content for 29 members of Italian Ranunculaceae and the spectrophotometric data of Ruijgrok [4].

Since the HPLC-evaluated protoanemonin content varies from 0.17 to 5820.50 μg/g wet wt among the species studied, some difficulties may arise as to the choice of the appropriate NP- or RP-HPLC method. For these reasons, after extraction by micro steam distillation, the extracts were first analysed by spectrophotometry in order to select the appropriate HPLC procedure.

As expected, the species showing a high protoanemonin content (> 500 μg/g wet wt) are *Helleborus foetidus*, *H. niger*, *Ranunculus acris* and *R. bulbosus*. Other species, not previously tested, also contain an appreciable amount of protoanemonin: *Clematis jubata*, *Ranunculus arvensis*, *R. illyricus*, *R. serbicus* and *R. velutinus*. Therefore, these species must be regarded as potentially dangerous for livestock and humans. Except for *R. bulbosus*, HPLC determinations show a protoanemonin content lower than that reported in the literature (see Table 1). This is not surprising because it is known that many factors may influence the evaluation of this compound, especially when data collected by different analytical procedures are compared. The HPLC method is faster and more accurate than spectrophotometric procedures of Ruijgrok [4]. Furthermore, HPLC methods furnish very homogeneous protoanemonin values when several specimens of the same species and habitat are compared. However, as previously reported, some difficulties may arise when specimens of the same species but of different provenance are examined. For example, *Clematis vitalba*, growing in different geographic areas, varies considerably in protoanemonin content, using spectrophotometric analysis [4]. For the same species, HPLC data do not show such a marked difference and fluctuate only within 10%.

HPLC data indicated that, in a large group of species (*Anemone nemorosa*, *A. trifolia alba*, *Clematis flammula*, *C. montana*, *C. vitalba*, *Ranunculus nemorosus*, *R. repens*), the protoanemonin content fluctuated between 500 and 100 μg/g wet wt. Among the tested species, the largest group is formed by members which produce only a small amount of protoanemonin (< 100 μg/g wet wt).

Interestingly, it was previously thought that *Aquilegia vulgaris*, *Caltha palustris*, *Paeonia officinalis* and *Thalictrum aquilegifolium* contained no protoanemonin and some authors [4, 7] thought this was systematically significant. However, among the 29 species studied here, only two lack protoanemonin.

Table 1. Comparison of the protoanemonin content evaluated by HPLC and that determined by other methods reported in literature

	Ruijgrok 1963	Ruijgrok 1966		This work
Trollieae	—			
Caltha palustris	—	0.0		0.26
Helleboreae				
Helleborus foetidus	—	4827–5827	m	672.00
H. niger	—	10137–11758	m	5820.50
H. odorus	—	—		4.60
H. viridis	—	—		28.40
Nigella damascena	—	—		0.0
Anemoneae				
Anemone nemorosa	586.2	5172.4		333.30
A. trifolia albida	—	—		169.40
Clematis flammula	—	—		494.10
C. montana	—	896.5–931	m	417.70
C. jubata	—	—		626.14
C. recta	—	—		95.60
C. vitalba	—	931–3137.9	p	150.00
Ranunculeae				
Runnunculus aconitifolius	—	413.7		18.70
R. acris	1034.5	4482–5724	p	1372.50
R. arvensis	—	—		1646.20
R. bulbosus	—	8965–10931	m	7765.60
R. illyricus	—	—		5127.80
R. nemorosus	—	—		75.04
R. repens	—	207–276	p	125.70
R. serbicus	—	—		3066.00
R. velutinus	—	—		787.30
Isopyreae				
Aquilegia atrata	—	—		0.30
A. oxisepala	—	—		1.08
A. vulgaris	—	0.0		0.45
Paeonieae				
Paeonia moutan	—	—		0.17
P. officinalis	—	0.0		0.45
Thalictreae				
Thalictrum aquilegifolium	—	0.0		1.48
T. flavum	—	0.0		0.0

All data are referred in μg/g w.w. When two numbers are reported for the same species, they refer to different analytical methods (m) or provenances (p).

In conclusion, our results suggest: (a) The occurrence of protoanemonin in *Thalictrum* indicates a close relationship between the Thalictreae and the Ranunculeae, a fact which contrasts with other systematic parameters but confirms the trend suggested by multifactorial analysis [14, 15]; (b) The constant presence of protoanemonin confirms the centrality of the Ranunculeae in the family and the homogeneity of the tribe Helleboreae.

EXPERIMENTAL

Plant material. Leaf samples of the following species of Ranunculaceae were collected from naturally growing or cultivated plants and tested for evaluating their protoanemonin content: *Anemone nemorosa* L.; *A. trifolia*, subsp. *albida* (Mariz) Tutin; *Clematis flammula* L.; *C. montana* Buch.; *C. jubata* Bisch.; *C. recta* L.; *C. vitalba* L.; *Helleborus foetidus* L.; *H. niger* L. subsp. *niger*; *H. odorus* Waldst. and Kit. subsp. *laxus* (Host) Merxm and Podl.; *H. viridis* L. subsp. *viridis*; *Ranunculus aconitifolius* L.; *R. acris* L. subsp. *acris*; *R. arvensis* L.; *R. bulbosus* L.; *R. illyricus* L.; *R. nemorosus* DC.; *R. repens* L.; *R. serbicus* Vis.; *R. velutinus* Ten.; *Aquilegia atrata* Koch; *A. oxysepala* Trantv. and Mey.; *A. vulgaris* L.; *Caltha palustris* L. subsp. *palustris*; *Nigella damascena* L.; *Paeonia Moutan* Sims; *P. officinalis* L. subsp. *officinalis*; *Thalictrum aquilegifolium* L.; *T. flavum* L. subsp. *flavum*. *Clematis montana*, *C. jubata*, *Aquilegia oxysepala* and *Paeonia Moutan* are not included in the Italian Flora, but are considered here due to their wide distribution in the Italian gardens.

Voucher specimens have been deposited in the Herbarium of the Institute of Botany, University of Ferrara (Italy). Leaf samples, only of plants in flower, were put immediately into plastic bags, weighed, and frozen within 2 hr of collection.

Apparatus and operating conditions. All chromatographic experiments were carried out using a Varian HPLC-system (Model 5010) with a 10 μl sample loop for the analytical scale and 100 μl sample loop for the microprep. scale, a Model UV50 detector and a Model 9176 recorder. For separation, Hibar Lichrosorb RP18 (10 μm) 300 × 4.5 mm I.D. and Si60 (5 μm) 250 × 4.0 mm I.D. columns were employed. The mobile phase was CH_3CN–H_2O (1:4) in a reverse phase system and *n*-hexane –Et_2O–CH_2Cl_2–$CHCl_3$ (7:1:1:1) in normal phase mode. The flow-rate was 2.5 ml/min (119 atm) for RP-HPLC and 2 ml/min (90 atm) for NP-HPLC, the chart speed was 1 cm/min, the detector sensitivity was 0.005 a.u.f.s., and the absorbance was monitored at 258 nm. The analyses were carried out at room temp. All solvents were HPLC grade.

Analysis procedure. 0.5–10 g of frozen leaf samples were steam distilled and the distillates directly examined by RP-HPLC mode whenever the protoanemonin content was higher than or equal to 0.5 μg/ml. Otherwise, after saturation with NaCl and exhaustive extraction by *n*-hexane, extracts were dryed and condensed. After elution with Et_2O–CH_2Cl_2–$CHCl_3$ (1:1:1), extracts were reduced to a small vol., filtered through Millipore filters (FH 0.5 μm), and examined in NP-HPLC mode. For details see [12].

Standards and calibration graphs. The protoanemonin standard was isolated, identified and quantitated in our laboratory from *Helleborus niger* following the procedure already described [12].

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