Studia Geobotanica. Vol. 16: 45-49 (1997)

CHEMOSYSTEMATIC VALUE OF ARISTOLOCHIC ACIDS IN THE GENUS *ARISTOLOCHIA* L. IN FRIULI-VENEZIA GIULIA (NE ITALY)

Francesca CATENI[1)], Laura COASSINI LOKAR[2)], and Fabrizio MARTINI[2)]

[1)] Dipartimento di Scienze Farmaceutiche, Università di Trieste, P.le Europa 1, I-34127 Trieste – [2)] Dipartimento di Biologia, Università di Trieste, Via L. Giorgieri 10, I-34127 Trieste

Keywords: *Aristolochia* L., aristolochic acids, chemotaxonomy, discriminant analysis.

Abstract: The composition of aristolochic acids from different populations of four *Aristolochia* species was analysed by HPLC; quantitative data were submitted to discriminant and statistical analyses. The interspecific variability was confirmed, and significant differences were detected by canonical discriminant functions. The aristolochic acid II and the ratio AAII/AAI are significant in the discrimination of the examined species.

Introduction

Aristolochia L. (*Aristolochiaceae)* is a well-known genus since the ancient times for its officinal properties. In Italy, several *Aristolochia* species have been widely used as medicinal plants since Plinius up to the last century (Savi 1805, Targioni-Tozzetti 1824, Cassone 1850). Ten species occur in Italy (Nardi 1984), four of which are present in the Friuli-Venezia Giulia Region: *A. clematitis* L., *A. rotunda* L., *A. pallida* Willd. and *A. lutea* Desf. (Gortani 1906, Poldini 1980, 1991, Martini 1990).

Characters related to the hypogean apparatus and the flowers have been considered as the most discriminant taxonomically (Nardi 1984). Nardi's revision was mainly based both on the frequently disregarded flower morphological characters and on chromosome numbers. Some species, i.e. *A. pallida* and *A. lutea*, are morphologically similar, and their determination on morphological evidence alone is difficult.

A previous study (Cateni *et al.* 1993), reported the chemical composition of various populations of *A. clematitis,* and the distribution of aristolochic acids in different parts of the plants, also with regard to the seasonal stages.

In this paper we examine the aristolochic acid composition of the underground apparatus of the *Aristolochia* species occurring in the Friuli-Venezia Giulia Region, which were not yet phytochemically investigated. The distinct properties of aristolochic acids among the species, and the evidence they provide on their mutual relationship were also considered.

The four species differ in their ecology. *A. clematitis* is the most autoapophytic, and grows in waste grounds along streams, ditches and sandy places near seashores, edges of cultivated fields, vineyards, hedges, rubble, margins of woods, coppices, on damp and aerated soils, rich in nitrates, up to 400 m. *Aristolochia rotunda* prefers damp grassy places along field margins, near ditches and streams, hedges, woods, shrubs, up to 400 m. *Aristolochia pallida* grows in woods, hedges, grassy places, mainly on plains and hills (up to 600 m). *Aristolochia lutea* is the taxon of the *A. pallida* complex with the widest distribution, and is also the most euritermic, growing mainly in *Ostrya carpinifolia*-woods (but also in beech woods), up to 1000 m. The distribution area of *A. pallida* includes the western part of the Region, while *A. lutea* is distributed in the eastern part; the northern populations of *A. lutea* co-exist with those of *A. pallida* and are often hardly distinguishable by morphological characters (Martini 1990).

The purpose of this study is to ascertain whether the four species can be distinguished on the basis of their aristolochic acids content, and to contribute further to the knowledge of aristolochic acids variation within the genus.

Materials and methods

Sampling concerned four populations of each species of *Aristolochia* in the following localities:
Aristolochia clematitis: 1) Gorizia, loc. Mainizza, 40 m; 2) Sgonico (TS), 255 m; 3) Polcenigo (PN), loc. S. Giovanni, 60 m; 4) Monfalcone (GO), loc. Alberoni, 2 m.
Aristolochia rotunda: 5) Muggia (TS), loc. Noghere, 5 m; 6) Muzzana del Turgnano (UD), loc. Bosco Coda di Manin, 6 m; 7) Latisana, Precenicco (UD), 3 m; 8) Monfalcone (GO), loc. Alberoni, 2 m.
Aristolochia pallida: 9) Polcenigo (PN), loc. Rui Bragam, 70 m; 10)Tramonti di Sotto (PN), loc. Stalle Spinespes, 590 m; 11) Peonis (UD), loc. Rio Sech, 400 m; 12) Jouf di Maniago (PN), 450 m.
Aristolochia lutea: 13) M. S. Leonardo (TS), 350 m; 14) Fernetti (TS), 360 m; 15) Ajdovščina (Slovenia), Trnovski Gozd, M. Kucelj, 650 m; 16) Maniago (PN), 400 m.

The plants were collected at full anthesis to examine the quantitative variability in the hypogean apparatus. In each population, five samples of ten wild plants were randomly collected and voucher specimens were deposited in the Herbarium Universitatis Tergestinae (TSB).

The hypogean parts were treated with liquid nitrogen, finely grounded (60 mesh) and weighed (1 g dry weight estimated on another sample). The extraction was carried out with 150 ml of methanol in Soxhlet to exhaustion. The extracts were evaporated in vacuum to small volume. The residue was redissolved in 10 ml methanol. The extracts were analysed by high performance liquid chromatography (HPLC) as described in a previous paper (Cateni *et al.* 1992). HPLC analyses were performed using a Perkin Elmer ODS RP-column filled with bonded octadecylsilane on silica (10 μm), flow rate 1,8 ml/min, detection 310 nm (detector sensitivity 0,01 a.u.f.s.) and methanol/water/acetic acid (80:20:1) as mobile phase. All the determinations were performed at 22° C and at isocratic condition of elution.

The analytical data were submitted to elaboration with statistical methods (Zar 1984, Lagonegro & Feoli 1985a, 1985b).

Results and discussion

The study and the experiment were considered a good test for application of aristolochic acid data to taxonomic problems in the genus *Aristolochia* which is currently under revision. All the populations of the four examined species contain in the underground apparatus the same aristolochic acids (AAI and AAII) in different quantities. The quantitative composition in AAI and AAII and their ratio value in the various populations and species are reported in Tab. 1.

Tab. 1 - Variation and discriminant analysis in aristolochic acids I and II (% dry weight) in the examined populations of Aristolochia.

Species	Populations	Aristolochic acids I (%)	Aristolochic acids II (%)	Ratio AAII/ AAI
A. clematitis	1	0.54	0.58	1.1
	2	0.25	0.24	1.0
	3	0.26	0.28	1.1
	4	0.42	0.54	1.3
	Average	0.37	0.41	1.1
	St. dev.	0.14	0.17	0.13
A. rotunda	5	0.09	0.09	1.0
	6	0.05	0.07	1.4
	7	0.07	0.09	1.2
	8	0.07	0.09	1.2
	Average	0.07	0.08	1.2
	St. dev.	0.02	0.01	0.17
A. pallida	9	0.05	0.16	3.1
	10	0.05	0.17	3.5
	11	0.06	0.19	3.4
	12	0.06	0.18	3.2
	Average	0.05	0.18	3.3
	St. dev.	0.003	0.02	0.2
A. lutea	13	0.04	0.1	2.4
	14	0.05	0.1	2.0
	15	0.04	0.1	2.2
	16	0.08	0.18	2.2
	Average	0.05	0.12	2.2
	St. dev.	0.017	0.04	0.16
Variable	Wilkslambda	F		Significance
AAI	0.1766	118.1		0
AAII	0.2697	68.6		0

The quantitative acid composition of *A. clematitis* L. is clearly distinct from those of the other investigated species, and all its populations are more productive, about two to ten times higher; furthermore, in all the populations (except n. 2) AAII prevails. The quantity of AAII in the population n. 16 (*A. lutea*) is similar to that of *A. pallida.* These samples were derived from an isolated locality inside the distribution area of *A. pallida*. The ratio between the two aristolochic acids in the four species is very different for *A. pallida*, *A. lutea* and the remaining species: for *A. clematitis* the mean value is 1.1, for *A. rotunda* 1.2, for *A. pallida* 3.3 and for *A. lutea* 2.2.

The analysis of variance was used to display

Tab. 2.- Analysis of variance of aristolochic acid I (left) and II (right) based on % dry weight in the populations of the four species of *Aristolochia* L. (*Ac = Aristolochia clematitis; Ar = A. rotunda; Ap = A. pallida; Al = A. lutea*).($F_{0.05.(3.16)}$ =3.24; DF=degree of freedom; F= variance ratio test; Q= statistics for Cohrans's Q test).

AA I

Species	Source of variation	Sum of squares	DF	Mean squares	F	Q
	Treatment	0.293	3	0.098	285.691	
	Error	0.005	16	0		
	Total	0.299	19			
	Ac-Ar				208.015	24.981
A. clematitis	*Ac-Ap*				192.511	24.032
	Ac-Al				35.083	10.259
	Ar-Ap				0.300	0.949
	Ar-Al				72.244	14.722
	Ap-Al				63.230	13.773
	Treatment	0.004	3	0.001	326.077	
	Error	0.001	16	0		
	Total	0.005	19			
	Ac-Ar				325.167	31.233
A.rotunda	*Ac-Ap*				75.288	15.023
	Ac-Al				67.132	14.191
	Ar-Ap				87.591	16.210
	Ar-Al				96.805	17.024
	Ap-Al				0.230	0.831
	Treatment	0	3	0	18.368	
	Error	0	16	0		
	Total	0	19			
	Ac-Ar				0.462	1.177
A.pallida	*Ac-Ap*				8.297	4.989
	Ac-Al				5.678	4.127
	Ar-Ap				12.673	6.166
	Ar-Al				9.377	5.304
	Ap-Al				0.248	0.862
	Treatment	0.004	3	0.001	342.908	
	Error	0	16	0		
	Total	0.004	19			
	Ac-Ar				11.837	5.959
A.lutea	*Ac-Ap*				1.606	2.195
	Ac-Al				269.599	28.439
	Ar-Ap				4.724	3.746
	Ar-Al				168.453	22.480
	Ap-Al				229.595	26.245

AA II

Species	Source of variation	Sum of squares	DF	Mean squares	F	Q
	Treatment	0.453	3	0.151	487.210	
	Error	0.005	16	0		
	Total	0.458	19			
	Ac-Ar				308.661	30.430
A. clematitis	*Ac-Ap*				242.963	26.998
	Ac-Al				4.956	3.856
	Ar-Ap				3.926	3.432
	Ar-Al				235.395	26.574
	Ap-Al				178.519	23.142
	Treatment	0.002	3	0.001	23.966	
	Error	0	16	0		
	Total	0.002	19			
	Ac-Ar				20.363	7.816
A.rotunda	*Ac-Ap*				1.006	1.738
	Ac-Al				0.698	1.447
	Ar-Ap				12.315	6.078
	Ar-Al				13.521	6.369
	Ap-Al				0.028	0.291
	Treatment	0	3	0.001	68.335	
	Error	0	16	0		
	Total	0	19			
	Ac-Ar				12.782	6.193
A.pallida	*Ac-Ap*				67.065	14.184
	Ac-Al				22.086	8.140
	Ar-Ap				21.290	7.992
	Ar-Al				1.264	1.947
	Ap-Al				12.179	6.045
	Treatment	0	3	0.008	920.049	
	Error	0	16	0		
	Total	0	19			
	Ac-Ar				0.043	0.360
A.lutea	*Ac-Ap*				0.043	0.360
	Ac-Al				613.250	42.892
	Ar-Ap				0.173	0.721
	Ar-Al				602.990	42.532
	Ap-Al				623.598	42.253

Tab. 3.- T Student's results for regression lines: centroids slopes and intercepts values. Df= 5, $\alpha < 0.05$. (*Ac= Aristolochia clematitis; Ar= A.. rotunda; Ap= A. pallida; Al= A. lutea).*

Species	Centroids		Slopes		Intercepts	
	T Student	Prob (%)	T Student	Prob (%)	T Student	Prob (%)
Ac - Ar	6.49	0.17	2.29	8.37	12.29	0.004
Ac- Ap	6.79	0.25	4.92	0.8	13.39	0.004
Ac- Al	7.09	0.21	2.78	4.97	13.36	0.004
Ar - Ap	11.51	0.03	13.66	0.02	9.24	0.02
Ar - Al	2.71	5.38	9.33	0.08	9.47	0.02
Ap - Al	3.28	3.04	6.36	0.31	11.20	0.001

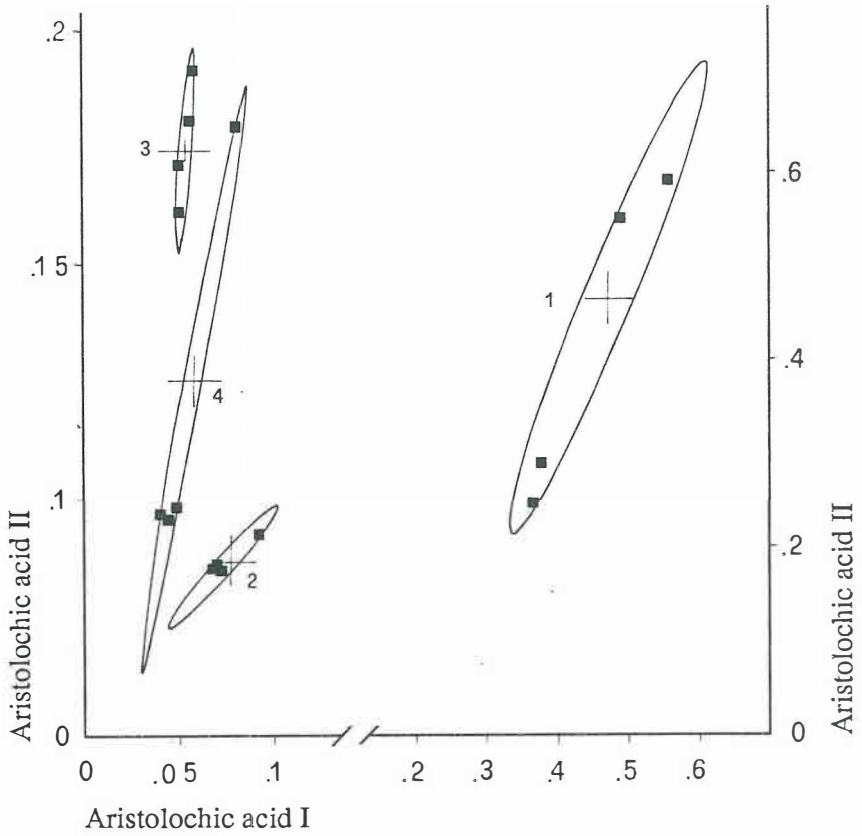

Fig. 1. - Ellipses of equal concentration, regression lines and centroids for the examinated *Aristolochia* species at the 5% level of confidence. The slopes and intercepts of the regression lines are significantly different ($p < 0.05$). 1: *A. clematitis*; 2: *A. rotunda*; 3: *A. pallida*; 4: *A. lutea*. The quantities are expressed as % dry weight.

the source of noticed variation among the four species. Statistically significant variation occurred for AAI and AAII. The most significant is AAI (F = 118.1) (Tab. 1). The results of the analysis of variance among populations are reported in Table 2. Because the critical value F is 3.14 at the 0.05 level of significance, all the values, which are smaller of 3.14, are not rejected. Therefore, *A. clematitis* populations are significatively distinct from those of the other species, in terms of both AAI and AAII. Statistically significant variations in the quantities of AAI occur among all the other populations of the various species. The populations of *A. lutea* significantly differ from those of *A. pallida*, although the two species are hardly distinguishable morphologically.

The four species can be discriminated by regression lines and centroid values of the aristolochic acids (Fig. 1). The values of centroid, slope and intercept are reported in Tab. 3. Most discriminant are the intercept and centroid values. The greatest disjunction is between *A. clematitis* and the other species.

Conclusions

The results demonstrate that aristolochic acids may be used for chemotaxonomical purposes in *Aristolochia*. The co-occurrence of structurally closely related compounds points to the chemical uniformity of the investigated species, differences being only quantitative. Aristolochic acid I and the ratio AAII/ AAI are the more significant taxonomically: chemical and morphological data are highly congruent.

Several questions regarding systematic relationships can be addressed using chemical data. The four *Aristolochia* species may be distinguishable from one another, based on aristolochic acid II data. Quantitative differences were compared: (a) among populations of different species, and (b) by combining the populations of each species to increase the sample size. The two closely related species, *A. pallida* and *A. lutea*, can be also distinguished on a chemical basis. It is confirmed that, chemically, *A. clematitis* is more distant one from the other species.

These results have implications both for basic research on plant populations and for applied studies on plant secondary products as defensive or attractive agents. The different concentrations of the same compounds in the populations and in the species suggest that they may have a specific biological role, and could be related both to their possible function as allelopathic agents (Nishida & Fukami 1989) and to their ecological role, since the plant toxins can be sequestred from the diet and stored by insects for defence. Aristolochic acids are actually used for this purpose by the butterfly *Battus archidamas* Boisd. (larval stage, Harborne 1987, 1988). It is interesting to note that in Friuli-Venezia Giulia, another butterfly, *Zerynthia polyxena* Schiff. (= *Z. hypsipyle* Schulz., *Papilionidae*) also feeds on *Aristolochia* (Morandini, *ex verbis*).

Riassunto

VALORE CHEMOSISTEMATICO DEGLI ACIDI ARISTOLOCHICI NEL GEN. *ARISTOLOCHIA* L. NEL FRIULI-VENEZIA GIULIA. La composizione in acidi aristolochici di differenti popolazioni appartenenti alle quattro specie di *Aristolochia* L. presenti nella regione Friuli - Venezia Giulia è stata analizzata tramite HPLC, analisi discriminanti e statistiche. La variabilità interspecifica è stata confermata e differenze significative sono state evidenziate tramite le funzioni canoniche discriminanti. Gli acidi aristolochici I e II e in particolare il rapporto AAII/AAI hanno valore di marcatori chemotassonomici data la congruenza fra dati chimici e morfologici.

Acknowledgments

The Authors would like to thank the Director of the Museo Friulano di Storia Naturale (Udine) Dr. C. Morandini, for information on the butterfly *Zerinthia polyxena*. This project was carried out thanks to the financial support of the Italian Ministry of Public Instruction (M.P.I. 40%).

References

Cassone F., 1850. *Flora medico farmaceutica*. 5. Torino.

Cateni F., Mamolo M.G. & Coassini Lokar L., 1992. *Rapid HPLC analysis for quantitative determination of aristolochic acids*. Il Farmaco, 47 (10): 1335-1342.

Cateni F., Coassini Lokar L., Martini F. & Vrech E., 1993. *Aristolochic acids variation and distribution in some* Aristolochia clematitis *L. populations*. Studia Geobot.,13: 299-312.

Gortani L. & M., 1906. *Flora friulana con speciale riguardo alla Carnia*. 2. Udine.

Harborne J.B., 1987. *Chemical signals in the ecosystem*. In: Dodge J.E. (ed.), *New Perspectives in Plant Science*. London, New York

Harborne J.B., 1988. *Introduction to Ecological Biochemistry*. New York.

Lagonegro M. & Feoli E., 1985a. *The use of ellypses of equal concentration to analyse ordination vegetation patterns*. Studia Geobot., 5: 143-165.

Lagonegro M. & Feoli E., 1985b. *Analisi multivariata di dati. Manuale d'uso di programmi BASIC per personal computer*. Libreria Goliardica, Trieste.

Martini F., 1990. *Il gruppo* Aristolochia pallida *nell'Italia nordorientale*. Giorn. Bot. Ital., 124: 731-743.

Nardi E., 1984. *The genus* "Aristolochia" *L. (*Aristolochiaceae*) in Italy*. Webbia, 38: 221-300.

Nishida R. & Fukami H., 1989. *Ecological adaptation of an* Aristolochiaceae*-feeding swallowtail butterfly,* Atrophaneura alcinous *to aristolochic acid*. J Chem. Ecol., 15: 2549-2563.

Poldini L., 1980. *Catalogo floristico del Friuli-Venezia Giulia e dei territori adiacenti*. Studia Geobot., 1: 313-474.

Poldini L., 1991. *Atlante corologico delle piante vascolari nel Friuli-Venezia Giulia*. Regione Autonoma Friuli-Venezia Giulia, Direzione Regionale Foreste e Parchi, Udine, & Dipartimento di Biologia, Università di Trieste..

Savi G., 1805. *Materia medica vegetabile toscana*. Firenze.

Targioni-Tozzetti A., 1824 *Scelta di piante officinali più necessarie a conoscersi*. Firenze.

Zar J.H., 1984. *Biostatistical analysis,* 2nd ed. Prentice- Hall Inc., New Jersey.

Received November 11, 1996
Accepted November 10, 1997