Acta Farm. Bonaerense **25** (4): 555-9 (2006)
Recibido el 4 de febrero de 2006
Aceptado el 9 de julio de 2006

Evaluation of Cytotoxic and Mitodepressive Activity of Aqueous Extracts from Thirteen Argentine Medicinal Plants

Claudio J. BIDAU [1,2]*, Aníbal G. AMAT [3], Marta E. YAJIA [3], Dardo A. MARTI [2,3], Mabel D. GIMENEZ [3], Ana G. RIGLOS [3], Aurelio SILVESTRONI [3], Guillermo M. TORRES [3] & Emiliano FUMAGALLI [3]

[1] *Laboratório de Biologia e Controle da Esquistossomose, Departamento de Medicina Tropical, FIOCRUZ, Rio de Janeiro, Brazil-CNPq.*

[2] *CONICET*

[3] *Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, CP 3300 Posadas, Misiones, Argentina.*

SUMMARY. Thirteen vascular plant species used in argentine folk medicine were studied in order to evaluate the cytotoxic and mitodepressive activity of their aqueous extracts at different concentrations, using both the *Artemia salina* Test and the *Allium* Test. No cytotoxic activity was observed for analyzed extracts with the *Artemia salina* Test, and in relation to macroscopic parameters of general toxicity, all species exhibited negative albeit non-significant correlations between average root length and extract concentration in the *Allium* Test. Frequency of root macroscopic abnormalities and average root length showed significant or highly significant negative correlations in all but two of the analyzed species which nevertheless exhibited comparable negative trends, indicating a concentration effect. In almost all assayed samples a C-mitotic effects of the extracts was observed since the mitotic index decreased and mitotic abnormalities increased with increasing extract concentrations. Clastogenic activity was not found in the analyzed extracts.

RESUMEN. "Evaluación de la Actividad Citotóxica y Mitodepresiva de Extractos Acuosos de Trece Plantas Medicinales Argentinas". Trece especies de plantas vasculares usadas en la medicina popular argentina, fueron estudiadas para evaluar las actividades citotóxicas y mitodepresivas de sus extractos acuosos a diferentes concentraciones usando los tests de *Artemia salina* y *Allium*. No se observó actividad citotóxica de los extractos analizados con el test de *Artemia salina* y, en relación a los parámetros macroscópicos de toxicidad general, todas las especies mostraron correlaciones negativas aunque no significativas entre la longitud media de las raíces y la concentración del extracto y el test de *Allium*. La frecuencia de las anormalidades macroscópicas de las raíces mostró correlaciones negativas en todas las especies analizadas siendo la mayoría, estadísticamente significativas, indicando un efecto de la concentración. En casi todas las muestras ensayadas, se observaron efectos de tipo C-mitótico ya que el Indice Mitótico disminuyó en tanto que las anormalidades mitóticas se incrementaron con las concentraciones crecientes de los extractos. En ningún caso se observó actividad clastogénica significativa.

INTRODUCTION

A diverse array of bioassays is now available for testing heterogeneous botanical products [1] some of which are useful for the analysis of cytotoxicity and genotoxicity of aqueous extracts of medicinal plants. A large number of vascular plants are used in folk medicine in the province of Misiones, Argentina, mainly as infusions or decoctions [2,3]. Nevertheless, despite their generalised use, most of their effects on human health have not been evaluated even by the simplest methods. Uses of plants in folk medicine of Misiones are diverse. For example, infusions or decoctions of *Ilex paraguariensis,* alone or in combination with *Alophyllus edulis, Aloysia polystachya, Achyrocline flaccida, Baccharis ariculata* and *Peumus boldus,* are used in treatments of stomach disorders or as digestive

KEY WORDS: *Allium* Test, Aqueous extracts, Argentina, *Artemia salina* Test, C-mitotic activity, Cytotoxic activity, Folk medicine, Genotoxic activity, Medicinal plants, Misiones.
PALABRAS CLAVE: Actividad citotóxica, Actividad C-mitótica, Actividad genotóxica, Argentina, Extractos acuosos, Medicina popular, Misiones, Plantas medicinales, Test de *Allium,* Test de *Artemia salina*.

* Author to whom correspondence should be addressed. *E-mail:* bidau50@gmail.com.

* CJB is a memeber of the CONICET Researcher Career (Argentina) and is grateful the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil and Fundação Oswaldo Cruz (FIOCRUZ) of Rio de Janeiro for a visiting Professorship that allowed the completion of this paper.

stimulants. Other plants are employed as polyvalent therapeuthic agents in general dermatopathies and cancer *(Aloë arborescens),* while some have highly specific applications (e.g. *Phyllantus tenellus,* kidney ailments; *Heteropteris glabra,* ansiolytic and sedative). Finally, some species are used as abortives or contraceptives *(Ruta chalepensis, Origanum vulgare, Melia azedarach, Hemionitis tomentosa)* [2,3]. Additionally, *B. articulata* and *P. boldus* are official drugs in the argentine Pharmacopoeia VI [4].

Previously isolated classes of constituents in the former species include,. acid polysaccharides in *A. arborsecens* [5], primary and secondary amines, free phenols, tannins, flavonoids, triterpenoids and/or steroids, leucoanthocyanidins, saponins, cyanosides, naftoquinones, anthraquinones and alkaloids in *A. edulis* [6,7], flavonoids in *A. flaccida* [8], essential oils in *A. polystachya* [9,10], leucoanthocyanidins, steroids, triterpenoids, phenols, flavonoids, tannins, cardenolides, saponins and alkaloids in *B. articulata* [7,11], alkaloids, tannins, glicosides, flavonoids and essential oils in *P. boldus* [12,13], free phenols, tannins, flavonoids, triterpenoids and steroids, leucoanthocianydins, an aliphatic nitro-compound (hiptagin) in *H. glabra* [14,15]; alkaloids, xantines (caffein, teobromine), alpha-amyrin, ursolic acid, 3-caffeoilquinic acid and saponins in *I. paraguariensis* [12,13,16], flavonoids, triterpenoids and alkaloids in *M. azedarach* [17-19], volatile oils (carvacrol, thymol, and borneol), flavonoids, rosmarinic acid, triterpenoids (ursolic and oleanolic acids), sterols, and vitamins A and C, tannins, caffeic acid and erodictyol in *O. vulgare* [20, 21], and alkaloids, coumarins, essential oil, flavonoids and tannins in *R. chalepensis* [22-24].

In this work, we evaluated the cytotoxic and mitodepressive activity of the aqueous extracts of the former 13 medicinal plants using two standard bioasays: the *Artemia salina* (brine shrimp) Test and the *Allium* Test.

MATERIALS AND METHODS

Plant materials were collected at natural habitats, obtained from cultivation and/or acquired at popular markets of the Capital and Candelaria Departments, Misiones Province, Argentina Voucher specimens were deposited at the Herbarium, Department of Pharmacy, FCE-QyN-UNaM. The following species were analyzed (family, most commonly used parts and species name abbreviation in parentheses): *Aloë arborescens* Mill. (Liliaceae, leaves; *A.a.), Allophyllus edulis* (Camb.) Radlk. *(Sapindaceae,* leaves; *A.e.), Achyrocline flaccida* (Weinm.) DC. *(Asteraceae,* aerial parts; *A.f.), Aloysia polystachya* (Gris.) Mold. (Verbenaceae, stems and leaves; *A.p.), Baccharis articulata* (Lam.) Pers. *(Asteraceae,* aerial parts; *B.a.), Hemionitis tomentosa (Adiantaceae,* aerial parts; *H.t.), Heteropteris glabra* Hook. et Arn. *(Malpighiaceae,* flowering branches and fruits; *H.g.), Ilex paraguariensis* St. Hil. *(Aquifoliaceae,* young stems and leaves in natural and commercial forms; *I.p.), Melia azedarach* L. *(Meliaceae,* leaves; M.p), *Origanum vulgare* L. *(Lamiaceae,* young stems and leaves; *O.v.), Peumus boldus* Mol. *(Monimiaceae,* leaves; *P.b.), Phyllanthus tenellus* Roxb. *(Euphorbiaceae,* entire plants; P.t.), *Ruta chalepensis* L. *(Rutaceae,* young stems and leaves; *R.c.).*

Aqueous extracts of the above cited parts, were obtained according to ethnotherapeutic procedures at different concentrations that include the ethnotherapeutic ones as follows: *A. arborsecens,* 1.875, 3.75, 7.5, 15, 30 g/L; *A. edulis,* 0.75, 1.5, 3, 6, 12 g/L; *A. flaccida,* 3.75, 7.5, 15, 30, 60 g/L; *A. polystachya,* 2.5, 5, 10, 20 g/L; *B. articulata,* 7.5, 15, 30, 60 g/L; *H. tomentosa,* 0.5, 1, 2, 4, 8 g/L; *H. glabra,* 3.75, 7.5, 15, 30, 60 g/L; *I. paraguariensis* (natural and commercial products), 5, 10, 20, 40 g/L; *M. azedarach,* 1.25, 2.5, 5, 10, 20 g/L; *O. vulgare,* 0.625, 1.25, 2.5, 5, 10 g/L; *P. boldus,* 6.25, 12.5,

	***Artemia salina* Test**	
Species	**Maximum action peak (µg/L)**	**LC_{50} & confidence intervals (g/L)**
A.a.	1875-7500	>1000
A.e.	1500-3000	>1000
A.f.	3750	>1000
A.p.	2500	>1000
B.a.	25000	>1000
H.g.	15000	>1000
H.t.	4000	>1000
I.p. (C)	40000	380 (83-1150)
I.p. (N)	ND	ND
M.a	5000	145 (138.446-151.860)
O.v.	1250	>1000
P.b.	50000	100 (30.3-330)
P.t.	12000	83.2 (21.63-320)
R.c.	1250	38 (10.26-140.76)

Table 1. Results of the *Artemia salina* microwell Test as applied to all assayed extracts. Abbreviations of species names as in Materials and Methods. *I.p.*(C.), *I.p.*(N): *Ilex paraguariensis* leaves of commercial (C.) or natural (N) origin.

Species	Control		A		B		C		D		E		r	r
	RL	%AN	RL	%AN	RL	%AN	RL	%AN	RL	%AN	RL	%AN	RL/Conc	FA/L
A.a.	37.98	4.06	44.53	12.22	41.03	0.74	33.52	13.46	26.6	17.44	30.67	12.05	-0.712	-0.612
A.e.	30.87	2.26	32.62	33.01	26.54	30.58	25.94	78.87	21.09	84.78	23.20	94.82	-0.746	-0.921*
A.f.	58.89	0.00	39.01	2.08	26.39	86.25	16.02	95.74	17.6	100	27.59	100	-0.458	-0.883*
A.p.	40.59	0.00	26.59	57.00	21.57	95.71	23.49	96.42	17.86	100	ND	ND	-0.758	-0.972*
B.a.	50.26	1.19	24.56	86.95	19.07	92.31	26.79	100	26.62	100	ND	ND	-0.388	-0.940*
H.g.	42.99	0.00	17.67	37.50	19.30	52.54	18.71	100	23.07	94.83	16.91	91.95	-0.434	-0.713
H.t.	50.26	1.19	46.91	14.81	26.00	43.69	23.29	54.54	31.80	57.57	27.22	65.55	-0.520	-0.982**
I.p (C.)	42.61	2.00	28.80	0.00	12.20	87.50	10.24	92.86	11.35	81.65	ND	ND	-0.724	-0.932*
I.p.(N.)	42.61	2.00	25.10	30.00	13.44	51.35	13.46	79.66	10.14	81.69	ND	ND	-0.871	-0.926*
M.a.	20.81	0.00	9.44	62.50	8.06	52.17	7.61	61.02	4.20	58.33	3.35	91.52	-0.704	-0.934*
O.v.	33.5	2.38	19.68	100	13.05	100	10.75	100	16.10	95.38	9.59	100	-0.601	-0.914*
P.b.	49.13	ND	19.96	ND	25.96	ND	32.06	ND	30.70	ND	23.98	ND	-0.357	ND
P.t.	84.65	0.00	37.21	92.93	54.91	90.6	40.47	100	43.81	100	43.81	100	-0.405	-0.889*
R.c.	58.89	0.00	37.92	26.55	31.79	23.01	30.01	21.87	27.94	30.95	22.51	58.57	-0.600	-0.852*

Table 2. Mean root length and percentage of macroscopic abnormalities for all extracts and concentrations assayed with the *Allium* Test. The last two columns indicate Pearson's correlation coefficients and their statistical significance. Abbreviations of species names as in Materials and Methods. *I.p.*(C.), *I.p.*(N): *Ilex paraguariensis* leaves of commercial (C.) or natural (N) origin. A-E: increasing concentrations as described in Materials and Methods. RL: mean root length. %NA: percent macroscopic abnormalities. r= Pearson's correlation coefficient. RL/Conc: correlation between root length and extract concentration. FA/RL: correlation between frequency of macroscopic abnormalities and mean root length. *: significant at the 0.05% level; **: significant at the 0.001% level.

	Control			A			B			C			D			E			r	r
	MI	CA	MA	MI	CA	MA	MI	CA	MA	MI	CA	MA	MI	CA	MA	MI	CA	MA	MI/ Conc.	TA/ Conc.
A.a.	5.3	0.3	5.0	4.0	0.4	7.8	6.1	0.8	10.8	2.5	0.4	15.6	3.7	0.6	15.7	-	-	-	-0.440	0.860
A.e.	4.7	0.6	5.2	3.5	1.3	22.9	1.5	0.9	17.0	1.3	0.5	9.7	0.2	0.4	37.5	0.2	0.6	20.00	-0.776*	0.397
A.f.	3.0	5.1	20.9	4.6	1.1	21.1	3.9	0.9	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.548	0.980**
A.p.	3.6	1.6	16.3	3.3	4.1	36.7	1.3	2.2	30.5	0.0	0.3	-	0.0	0.7	-	-	-	-	-0.851*	ND
B.a.	2.3	0.3	8.6	1.5	1.8	6.0	0.2	0.1	7.1	0.6	0.8	10..4	2.2	1.3	18.6	-	-	-	-0.155	0.920*
H.g.	6.5	0.2	3.2	2.2	0.4	17.2	1.5	0.4	21.7	0.2	0.0	0.0	0.5	4.2	0.0	-	-	-	-0.705	0.960**
H.t.	2.3	0.3	8.6	1.9	0.4	13.9	1.1	0.8	23.7	1.7	0.4	11.0	3.7	0.6	11.8	0.9	0.2	9.6	-0.337	0.316
I.p. (c)	7.6	ND	7.9	4.2	ND	11.5	0.3	ND	0.00	0.8	ND	5.6	0.2	ND	0.00	-	-	-	-0.710*	ND
I.p. (n)	7.6	ND	7.9	3.7	ND	23.9	3.1	ND	19.4	1.5	ND	13.4	0.3	ND	3.22	-	-	-	-0.680*	ND
M.a.	7.3	1.2	5.9	5.2	4.8	33.7	3.8	1.9	28.9	1.4	2.2	44.7	0.1	0.1	21.4	-	-	-	-0.937*	0.205
O.v.	3.4	3.2	20.0	2.4	2.9	24.4	1.6	3.1	28.8	1.4	4.2	40.9	2.4	5.0	36.3	1.6	4.3	38.2	-0.446	0.992*
P.b.	5.4	0.4	5.5	2.3	0.6	9.9	0.7	0.3	3.6	0.4	0.3	ND	1.1	1.1	32.6	-	-	-	-0.607	0.942*
P.t.	4.2	0.2	4.2	2.6	0.6	14.5	0.8	0.5	4.4	0.4	0.2	100	0.3	0.1	0.0	-	-	-	-0.778	0.870
R.c.	3.0	5.1	20.9	2.1	5.0	33.3	2.0	5.0	37.3	1.8	5.1	38.4	1.38	3.9	41.5	0.1	0.2	44.4	-0.961**	0.760

Table 3. Mitodepressive activity using the *Allium* Test. Abbreviations of species names as in Materials and Methods. *I.p.*(C.), *I.p.*(N): *Ilex paraguariensis* leaves of commercial (C.) or natural (N) origin. A-E: increasing concentrations as described in Materials and Methods. MI: Mitotic Index. CA: frequency of chromosomal abnormalities. MA: frequency of mitotic abnormalities. r = Pearson's correlation coefficient. MI/Conc: correlation between mitotic index and extract concentration. TA/Conc.: correlation between frequency of total abnormalities and extract concentration. *: significant at the 0.05% level; **: significant at the 0.001% level.

25, 50, 100 g/L; *P. tenellus,* 3.125, 6.25, 12.5, 25, 50 g/L; *R. chalepensis,* 0.625, 1.25, 2.5, 5, 10 g/L.

Cytotoxicity and general toxicity were evaluated by the *Artemia salina* Test [25-27]. *Artemia salina* eggs (S&S, Argentina) were incubated in artificial sea water (Red Sea Salt, Israel, 40 g/L at 27-28 °C). On the second day of incubation, *nauplii* (10-15) were transferred to a microwell plate, and exposed to the extracts for 24 h. Artificial sea water and thymol (60 µM) were used as negative and positive controls, respectively. Mitodepressive activity was assessed by the *Allium* Test [26,28-30], in which morphological parameters analyzed were root length (RL) and macroscopic abnormalities (AN%). Cytological parameters studied were Mitotic Index (MI), Prophase (PI), Metaphase (MeI), Anaphase (AI) and Telophase (TI) Indexes, total chromosomal abnormalities (CA%) and total mitotic abnormalities (MA%). For the *Allium* Test, common onion bulbs were used. The roots were exposed to the extracts for 48 h and sampled for cytogenetic studies as follows: root tips were excised, fixed in ethanol-acetic acid (3:1) for 12-20 h, squashed in lacto-propionic orcein and mounted for examination. Samples of at least five onion bulbs were analyzed for each extract concentration and respective controls. Tap water was used as negative control. For macroscopic studies roots were exposed for 5 days, after which they were measured and the abnormalities present, recorded.

The Litchfield-Wilcoxon method [31] was used for statistical treatment of the *Artemia salina* Test results. Correlation/regression analysis [32] was used for the evaluation of the *Allium* Test data.

RESULTS AND DISCUSSION

None of the extracts of analyzed species exhibits significant cytotoxic activity in the *Artemia salina* microwell Test according Solís *et al.* [25] (LC50 ≤ 1000 ppm) nor according Mongelli et al. 33 criteria for aqueous solutions (LC50 ≤ 10000, Table 1). Comparatively, the species *Melia azedarach, Phyllantus tenellus, Peumus boldus, Ruta chalepensis* and *Ilex paraguariensis* (commercial product) were responsible of the largest effects in the *Artemia salina* assays.

Macroscopic parameters of general toxicity analysed by the *Allium* Test showed that for all assayed species, negative correlations occur between average mean root length and increasing extract concentration. None of these correlations was statistically significant but this probably obeyed to the reduced degrees of freedom available in the study (Table 2). Nevertheless, all these negative correlations become significant when individual root length values are used instead of means (not shown). Furthermore, the frequency of root macroscopic abnormalities and average root length showed significant negative correlations in all but one *(Aloë arborescens)* of the assayed species, indicating a concentration effect on both root growth and the production of macroscopic abnoramlities such as necroses.

The comparison of the results obtained in the *Allium* Test for general toxicity (macroscopic parameters), and considering that apex necrosis production also indicates a cytotoxic effect, it was evident a major sensibility of this bioassay in relation to *Artemia salina* Test for this type of activity.

Mitodepressive activity was verified for most assayed samples using the *Allium* Test (Table 3) with the exception of A flaccida where only the controls and two concentrations could be assayed. Of the negative trends observed in the rest of samples, 6 were statistically significant (Table 3). Also, of the 11 samples in which mitotic anomalies where scored, 6 showed statistically significant positive correlations between mitotic abnormalities and extract concentration (Table 3). Again, in some of the cases where the correlation was not significant, the reduced number of degrees of freedom is responsible for the statistical non-significance. The generalised effect of the assayed aqueous extracts is thus an increasing c-mitotic activity. In contrast, clastogenic effects were not observed.

Acknowledgments. CJB is grateful the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil and Fundação Oswaldo Cruz (FIOCRUZ) of Rio de Janeiro for a visiting Professorship that allowed the completion of this paper.

REFERENCES

1. McLaughlin, J.L., L.L. Rogers & J.E. Anderson (1998) *Drug Inform. J.* **32**: 513-24.
2. Amat, A G. & M.E. Yajía (1986) *Acta Farm. Bonaerense* **125**: 153-9.
3. Amat, A.G. & M.E. Yajía (2001) *"Plantas Vasculares utilizadas en la Fitoterapia popular de la Provincia de Misiones (Argentina)",* en "Farmacobotánica y Farmacognosia en Argentina 1980-1998" (A.G. Amat, ed.). Ediciones Científicas Americanas, La Plata, 119-52.
4. Farmacopea Nacional Argentina VIª Edición

(1976) Ministerio de Salud de la Nación, Argentina.
5. Mateu Amengual, B. (1983) *"Catálogo Bibli - ográfico Fitoquímico Argentino VI".* Miscelánea Nro. 76, Fundación Lillo, Tucumán.
6. Rondina, R.V.D., M. Mendiondo & J.D. Coussio (1970) *Rev. Invest.Agrop., INTA,* Serie 2 (**7**) : 271-6.
7. Bandoni, A, M. Mendiondo, R.V.D. Rondina & J.D. Coussio (1984) *Lloydia* **72**: 35-69.
8. Norbedo, C, G. Ferraro & J.D. Coussio (1984) *Phytochemistry* **23**: 2698-700.
9. Huergo, H.H. & J.A. Retamar (1973) *Arch. Bioq. Quím. Farm. Univ. Nac. Tucumán* **18**: 15-9.
10. Gatto, Z.H., J.A. Retamar & C. Catalán (1981) *Essenze. Derivati Agrumari* **51**: 109.
11. Rondina, R.V.D. & J. D. Coussio J.D. (1969) *Rev. Invest. Agrop. INTA* **6**: 351-66.
12. Willaman, J. J. & H.L. Li. (1970) *Lloydia,* Supplement **33** (3A): 1-286
13. Gupta, M.P. (1995) *"270 Plantas Medicinales Iberoamericanas"* CYTED-SECAB, Santa Fe de Bogotá pags. 1-617.
14. Stermitz, F, O. Hnatyszyn, A.L. Bandoni, R.V.D. Rondina & J.D. Coussio (1975) *Phyto - chemistry* **14**: 1341-5.
15. Bandoni, A.L., R.V.D. Rondina & J.D. Coussio (1972) *Rev. Invest. Agrop. INTA,* **9**: 49-53.
16. Franck, G. (1972) *Rev. Fac. Agron. La Plata* **48**: 1-14.
17. Smolenski, S.J,. Silinis, H. & Farnsworth, N.R. (1975) *Lloydia* **38**: 225-55
18. Banerji, R. (1977) *Fitoterapia* **48**: 166.
19. Marco, J.A., O. Barbera, J.F. Sanz & J. Sánchez-Parareda (1986) *J. Nat. Prod.* **49**: 170-170
20 Beckstrom-Sternberg, S.M. & J.A. Duke (1994) "The Phytochemical Database" (http://ars-genome.cornell.edu/cgi-bin/WebAce/webace?db=phytochemdb).
21. Wren, R.C. (1988) *"Potter's New Cyclopedia of Botanical Drugs and Preparations"* C.W. Daniel, Essex. pags. 1-362.
22. Ulubelen, A., B. Terem, E. Tuzlac, K.F. Cheng & Y.C. Kong (1986) *Phytochemistry* **25**: 2692-3.
23. Gastaldo, P. (1973) *Fitoterapia* **44**: 61-7.
24. Baper, K.H.C., T. Özek & S.H. Beis (1996) *J. Essent. Oil Res.* **8**: 413-4.
25. Solís, P.N., C.W. Wright, M.M. Anderson, M.P. Gupta & J.D. Phillipson (1993) *Planta Med.* **59**: 250-2.
26. Bidau, C.J., A.G. Amat, M. Yajia, D.A. Marti, A.G. Riglos & A. Silvestroni (2004) *Cytologia* **69**: 109-17.
27. Carballo, J.L., Z.I. Hernández-Inda, P. Pérez & M.D. García-Grávalos (2002) *BMC Biotechnolo - gy* **2**: 17-21.
28. Fiskesjö, G. & A. Levan (1993) *ATLA* **21**: 139-49.
29. Fiskesjö, G. (1997) *Allium "Test for screening chemicals; evaluation of cytological parame - ters",* in: "Plants for Environmental Studies" (W. Wang, J.W. Gorsuch & J.S. Hughes, eds.) CRC Press- Lewis Publishers, Boca Raton, New York. págs. 313-33.
30. Yajia, M, D.A. Marti, C.J. Bidau, A.G. Amat, A.G. Riglos & A. Silvestroni (1999) *Acta Horti - culturae* **501**: 31-6.
31. Litchfield, J. & F.J. Wilcoxon (1949) *Pharm. Exp. Ther.* **96**: 99-113.
32. Sokal, R.R. & F.J. Rohlf (1969) *"Biometry"* W.H. Freeman Co, San Francisco.
33. Mongelli, E.R., J.D. Coussio & G.N. Ciccia (1995) *Dominguezia* **12**: 35-42.