Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Genotoxicity testing of *Ambelania occidentalis* (Apocynaceae) leaf extract *in vivo*

L.S. Castro[1], F.F. Perazzo[2] and E.L. Maistro[1]

[1]Departamento de Fonoaudiologia,
Faculdade de Filosofia e Ciências,
Universidade Estadual Paulista, Marília, SP, Brasil
[2]Laboratório de Pesquisa em Fármacos,
Universidade Federal do Amapá, Macapá, AM, Brasil

Corresponding author: E.L. Maistro
E-mail: edson.maistro@marilia.unesp.br

Genet. Mol. Res. 8 (2): 440-447 (2009)
Received February 20, 2009
Accepted March 9, 2009
Published April 22, 2009

ABSTRACT. *Ambelania occidentalis* is routinely used in folk medicine for treating gastrointestinal disorders, even though there have been no safety trials. We evaluated the genotoxic potential of hydro-alcoholic extracts of this plant in mice; induced DNA damage was assessed in peripheral blood leukocytes and micronucleus induction was assessed in polychromatic erythrocytes from bone marrow. The extract was administered by an oral route at single doses of 1000, 1500 and 2000 mg/kg body weight. N-nitroso-N-ethylurea was used as a positive control. The comet assay was performed on peripheral blood leukocytes at 4 and 24 h after treatment, and the micronucleus test was carried out on bone marrow cells collected at 24 and 48 h after treatment. The ratio of polychromatic/normochromatic erythrocytes was scored for cytotoxicity assessment. No increase in the number of micronucleated polychromatic erythrocytes from bone marrow or in leukocyte DNA damage was observed. The hydro-alcoholic extracts of *A. occidentalis* had no mutagenic or cytotoxic effects in the mouse cells.

Key words: *Ambelania occidentalis*; Comet assay; Micronucleus test; Single-cell gel electrophoresis; Mutagenicity assay

 ©FUNPEC-RP www.funpecrp.com.br

INTRODUCTION

The use of medicinal plants in therapeutics or as dietary supplements goes back beyond recorded history, but has increased substantially in the last decades (Woods, 1999; WHO, 2002). The popularity of herbal medicines is connected with their easy access, therapeutic efficacy, relatively low cost, and assumed absence of toxic side effects. Widespread public opinion is that being a natural product, herbal medicines are harmless and free from adverse effects, and it is believed that even if the expected medical effect is not achieved, their consumption is not dangerous. However, the safety of their use has recently been questioned due to the reports of illness and fatalities (Stewart et al., 1999; Ernst, 2002; Veiga-Junior et al., 2005). Considering the complexity of herbals in general and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality (WHO, 2002). Thus, an assessment of their mutagenic and cytotoxic potential is necessary to ensure the relatively safe use of plant-derived medicines.

The Apocynaceae family has been studied extensively over the years with regard to the cytotoxicity of its secondary metabolites, mainly alkaloids. The first well-known plants of this family were *Rauwolfia serpentine*, containing reserpine, used for hypertension, and *Catharanteus roseus*, containing vincristine and vimblastine, used in the treatment of uterus and breast cancer (Ram and Kumari, 2001). Also, another genus, *Tabernaemontana*, has been studied as a potential herbal medicine against Leishmania (Estevez et al., 2007), Chagas disease and malaria (Ndjakou et al., 2007)

Ambelania occidentalis belongs to the Apocynaceae family. This botanical family includes 4555 species, distributed in 415 genera. In Brazil, there are 376 species from 41 genera. There is no available detailed phytochemical screen of *A. occidentalis* leaf extract, which is under study by our group. Thus, the Apocynaceae family has shown over the decades high cytotoxic effects because of alkaloids in its composition, where they are common secondary metabolites (Endress and Bruyns, 2000). Therefore, this study was undertaken to determine the *in vivo* genotoxic/mutagenic effect, if any, of acute administration of *A. occidentalis* leaf extract in mice, using the comet and micronucleus assays.

MATERIAL AND METHODS

Plant material

Dried leaves of *A. occidentalis* (300 g) were submitted to dynamic maceration with ethanol (2000 mL, 99.0%) for 4 h. The extract of the macerated leaves was filtered and the resulting filtrate concentrated under reduced pressure, yielding 23.46 g (7.82%) of crude hydro-alcoholic extract.

Chemicals

N-nitroso-N-ethylurea (ENU, CAS No. 759-73-9) was used as the DNA damaging agent in the comet and chromosome aberration assays. It was dissolved in phosphate buffer, pH 6. The other main chemicals were obtained from the following suppliers: normal melting point agarose (Cat. No. 15510-019; Invitrogen); low melting point agarose (Cat. No. 15517-

014; Invitrogen); *N*-lauroyl sarcosine sodium salt (L-5125; Sigma), and ethylenediaminetetraacetic acid (Merck).

Animals and dosing

Experiments were carried out on 12-week-old male Swiss albino mice (*Mus musculus*), weighing 25-30 g. The animals were acquired from the animal house of the Universidade Estadual Paulista (UNESP), Botucatu, São Paulo State, Brazil, and kept in polyethylene boxes (N = 11), in a climate-controlled environment (25 ± 4°C, 55 ± 5% relative humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (NUVILAB CR1 - NUVITAL) and water were available *ad libitum*. The mice were divided into experimental groups of 11 animals. An extract of *Ambelania occidentalis* leaves was administered in a single dose of 0.5 mL by gavage, at concentrations of 1000, 1500, and 2000 mg/kg body weight, chosen on the basis of our acute toxicity studies in mice, which was higher than 2000 mg/kg. The negative control group received distilled water. The positive control group received an intraperitoneal injection of ENU at 50 mg/kg. The animals used in this study were sacrificed by cervical dislocation. The Animal Bioethics Committee of the UNESP, Botucatu, Brazil, approved the present study on September 28, 2006 (protocol number 47/06), in accordance with Brazilian regulations on animal care.

Micronucleus assay

The assay was carried out following standard protocols as recommended by Schmid (1975) and Krishna and Hayashi (2000). Ten mice were used per group (five for each gender) in the test. Five animals of each group were sacrificed 24 h after the treatment and the other five 48 h after the treatment. This protocol was chosen considering the observation that the time course of micronucleus production in polychromatic erythrocytes can be different for each of the chemicals studied (Salamone et al., 1980). The bone marrow from both femurs was flushed out using 2 mL saline (0.9% NaCl) and centrifuged for 7 min. The supernatant was discarded and smears were made on slides. The slides were coded for a "blind" analysis, fixed with methanol and stained with Giemsa. For the analysis of the micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored to determine the mutagenic property of the extract. To detect possible cytotoxic effects, the PCE:NCE (normochromatic erythrocytes) ratio in 200 erythrocytes/animal was calculated (Gollapudi and McFadden, 1995). The cells were blindly scored using a light microscope at 1000X magnification. The mean number of micronucleated PCE in individual mice was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

Comet assay

The comet assay (single-cell gel electrophoresis, SCGE) was carried out by the method described by Speit and Hartmann (1999), which is based on the original work of Singh et al. (1988) and includes modifications introduced by Klaude et al. (1996) as well as additional modifications. Peripheral blood samples were obtained from six Swiss mice from each group at 4 and 24 h after treatment. An aliquot was removed from the peripheral blood cell suspension to determine cell viability. Cell counting was performed using a hemocytometer. Cell viability was

©FUNPEC-RP www.funpecrp.com.br

determined by Trypan blue dye exclusion. The number of Trypan blue-negative cells was considered the number of viable cells and was greater than 90%. A 10-µL aliquot of cells from each animal was mixed with 120 µL 0.5% low melting point agarose at 37°C, and rapidly spread onto two microscope slides per animal, pre-coated with 1.5% normal melting point agarose. The slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL distilled water and 1% sodium lauryl sarcosine), plus 1 mL Triton X-100 (Merck) and 10 mL dimethylsulfoxide (Merck). The slides, which were protected from light, were allowed to stand at 4°C for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH-1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4°C for 20 min prior to electrophoresis, to allow DNA unwinding. The electrophoresis run was carried out in an ice bath (4°C) for 20 min at 300 mA and 25 V (0.722 V/cm). The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer, before staining. For the staining process, the slides were briefly rinsed in distilled water, covered with 30 µL 1X ethidium bromide-staining solution prepared from a 10X stock (200 µg/mL) and coverslipped. The material was evaluated immediately at 400X magnification, using a fluorescence microscope (Olympus BX 50) with a 515-560-nm excitation filter and a 590-nm barrier filter.

The extent and distribution of DNA damage indicated by the SCGE assay were evaluated by examining at least 100 randomly selected and non-overlapping cells on the slides (50 cells per slide), per animal. These cells were scored visually, according to tail size, into four classes, as follows: class 0: no tail; class 1: tail shorter than the diameter of the head (nucleus); class 2: tail length 1 to 2 times the diameter of the head, and class 3: tail longer than 2 times the diameter of the head. Comets with no heads and images with nearly all DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represent dead cells (Hartmann and Speit, 1997). The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

Statistical analysis

After verifying if the data were normally distributed, the data obtained on micronucleus and SCGE assays were submitted to one-way analysis of variance (ANOVA) followed by the Tukey test (Sokal and Rohlf, 1995) comparing all groups with the negative control group. GraphPad Instat® software (version 3.01) was used. The results were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

From a phytomedicinal point of view, the evaluation of genotoxicity is of particular importance due to the fact that genotoxic effects of chemicals or complex mixtures may be crucially important at the population level. Of the variety of *in vivo* assays used to detect genotoxic chemicals, the most common is the *in vivo* rodent erythrocyte micronucleated assay.

This assay has been used routinely for decades and has typically been used to detect damage to the chromosomes or the mitotic apparatus. Substances that cause cytogenetic damage may give rise to cells with micronuclei, which consist of lagging chromosome fragments (a result of clastogenic activity) or whole chromosomes (due to aneugenic activity) (MacGregor et al., 1990; Heddle et al., 1991; Hayashi et al., 1994).

The micronucleated data for *A. occidentalis* extract are summarized in Table 1. All results regarding micronucleated cells from extract-treated groups were not statistically different from the negative control. Also, no significant difference between the test groups was observed.

Table 1. Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of male (M) Swiss mice treated with *Ambelania occidentalis* extract, and respective controls.

Treatment	Times of cell collection	Number of MNPCE per animal					MNPCE (mean ± SD)	PCE/NCE (mean ± SD)
		M_1	M_2	M_3	M_4	M_5		
Negative control	24 h	1	3	2	2	2	2.0 ± 0.70	0.84 ± 0.11
(water)	48 h	1	1	0	0	2	0.8 ± 0.83	0.84 ± 0.12
A. occidentalis extract	24 h	0	1	1	1	0	0.6 ± 0.54	0.97 ± 0.11
(1000 mg/kg)	48 h	1	0	1	1	1	0.8 ± 0.44	0.88 ± 0.05
A. occidentalis extract	24 h	1	0	1	2	1	1.0 ± 0.70	0.83 ± 0.10
(1500 mg/kg)	48 h	1	0	1	1	1	0.8 ± 0.44	0.90 ± 0.03
A. occidentalis extract	24 h	2	1	1	1	2	1.4 ± 0.54	0.87 ± 0.06
(2000 mg/kg)	48 h	0	1	0	1	1	0.6 ± 0.54	0.84 ± 0.07
N-nitroso-N-ethylurea	24 h	13	14	14	13	14	13.6 ± 0.54[a]	0.91 ± 0.05
(50 mg/kg)	48 h	11	14	12	9	12	11.6 ± 1.81[b]	0.84 ± 0.06

For each period (24 and 48 h), 2000 cells were analyzed. SD = standard deviation; NCE = normochromatic erythrocytes. [a]Significantly different from 24-h negative control (P < 0.001). [b]Significantly different from 48-h negative control (P < 0.001).

An evaluation in the bone marrow cells of the percentage of PCE among total erythrocytes was included to provide an assessment of the rate of erythropoiesis and thus a measure of cytotoxicity. The estimated ratio of PCE:NCE in bone marrow preparations showed no statistically significant alterations in hematopoiesis as a result of extract treatment, indicating no cytotoxic effects.

The SCGE assay, commonly called the comet assay, is an assay for genotoxicity that is gaining widespread use. This assay has the advantages of requiring a small number of cells per sample, being amenable to almost all cell types whether actively dividing or quiescent, and being relatively inexpensive, since only basic equipment is required (Tice et al., 2000; Hartmann et al., 2001, 2003; Brendler-Schwaab et al., 2005). In the comet assay, cells are embedded on agarose gel on a glass slide, lysed to break cell and nuclear membranes, and exposed to an alkaline solution to yield single-stranded DNA from released double-stranded DNA. Following electrophoresis, the sample is stained with a DNA-binding dye and viewed under a microscope. Short strands of DNA generated from DNA strand breaks and/or alkaline labile sites (depending on pH of the electrophoresis solution) migrate farther than intact DNA during electrophoresis and form the "tail" of the "comet".

 ©FUNPEC-RP www.funpecrp.com.br

The results of the comet assay in evaluating the *A. occidentalis* extract, namely data on the total number of cells with damage and scores for mice treated with 1000, 1500, and 2000 mg/kg, besides negative and positive control (50 mg/kg ENU) are presented in Tables 2 and 3.

Table 2. DNA migration in the comet assay for the assessment of genotoxicity of *Ambelania occidentalis* extract in peripheral blood cells (collected 4 h after treatment) from male Swiss mice (M) *in vivo*.

Treatment	Animals	Total[1]	Comet class				Score
			0	1	2	3	
Control	M_1	1	99	1	0	0	2
	M_2	2	98	2	0	0	2
	M_3	1	99	1	0	0	1
	M_4	4	96	4	0	0	4
	M_5	2	98	2	0	0	2
	M_6	3	97	3	0	0	3
	Mean ± SD	2.1 ± 1.1					2.3 ± 1.0
A. occidentalis extract (1000 mg/kg)	M_1	1	99	1	0	0	1
	M_2	1	99	1	0	0	1
	M_3	9	91	9	0	0	9
	M_4	6	94	6	0	0	6
	M_5	2	98	2	0	0	2
	M_6	3	97	3	0	0	3
	Mean ± SD	3.6 ± 3.2					3.6 ± 3.2
A. occidentalis extract (1500 mg/kg)	M_1	2	98	2	0	0	2
	M_2	1	99	1	0	0	1
	M_3	2	98	2	0	0	2
	M_4	3	97	3	0	0	3
	M_5	1	99	1	0	0	1
	M_6	1	99	1	0	0	1
	Mean ± SD	1.6 ± 0.8					1.6 ± 0.8
A. occidentalis extract (2000 mg/kg)	M_1	7	93	7	0	0	7
	M_2	3	97	3	0	0	3
	M_3	4	96	4	0	0	4
	M_4	4	96	4	0	0	4
	M_5	5	95	5	0	0	5
	M_6	6	94	6	0	0	6
	Mean ± SD	4.8 ± 1.4					4.8 ± 1.4
N-nitroso-N-ethylurea (50 mg/kg)	M_1	70	30	67	3	0	73
	M_2	75	25	74	1	0	76
	M_3	66	34	66	0	0	66
	M_4	76	24	75	1	0	77
	M_5	74	26	72	2	0	76
	M_6	75	25	75	0	0	75
	Mean ± SD	72.6 ± 3.8*					73.8 ± 4.0*

[1]Total number of damaged cells (classes 1 + 2 + 3). *Significantly different from the negative control (P < 0.001).

As expected, N-nitroso-N-ethylurea, the positive control, induced a significant increase in DNA migration in leukocytes (P < 0.001). Despite some increase in damaged cells in animals treated with high doses of the test extract, no statistically significant difference between treated and untreated animals was observed for *A. occidentalis* extract at all doses tested, in both leukocyte samples (4 h and 24 h) (P > 0.05). When cells were exposed to three concentrations of the extract, most of the cells examined on slides were undamaged, few cells showed minor damage (class 1) and very few showed a large amount

Table 3. DNA migration in the comet assay for the assessment of genotoxicity of *Ambelania occidentalis* extract in peripheral blood cells (collected 24 h after treatment) from male Swiss mice (M) *in vivo.*

Treatment	Animals	Total[1]	Comet class				Score
			0	1	2	3	
Control	M_1	3	97	3	0	0	3
	M_2	5	95	5	0	0	5
	M_3	3	97	3	0	0	3
	M_4	4	96	4	0	0	4
	M_5	4	96	4	0	0	4
	M_6	5	95	5	0	0	5
	Mean ± SD	4.0 ± 0.8					4.0 ± 0.8
A. occidentalis extract (1000 mg/kg)	M_1	10	90	10	0	0	10
	M_2	2	98	2	0	0	2
	M_3	1	99	1	0	0	1
	M_4	1	99	1	0	0	1
	M_5	5	95	5	0	0	5
	M_6	11	89	11	0	0	11
	Mean ± SD	5.0 ± 4.5					5.0 ± 4.5
A. occidentalis extract (1500 mg/kg)	M_1	3	97	3	0	0	3
	M_2	2	98	2	0	0	2
	M_3	1	99	1	0	0	1
	M_4	1	99	1	0	0	1
	M_5	2	98	2	0	0	2
	M_6	1	99	1	0	0	1
	Mean ± SD	1.6 ± 0.8					1.6 ± 0.8
A. occidentalis extract (2000 mg/kg)	M_1	1	99	1	0	0	1
	M_2	4	96	4	0	0	4
	M_3	4	96	4	0	0	4
	M_4	2	98	2	0	0	2
	M_5	5	95	5	0	0	5
	M_6	4	96	4	0	0	4
	Mean ± SD	3.3 ± 1.5					3.3 ± 1.5
N-nitroso-N-ethylurea (50 mg/kg)	M_1	78	22	71	6	1	86
	M_2	77	23	70	7	0	84
	M_3	77	23	75	2	0	79
	M_4	63	37	60	3	0	66
	M_5	62	38	61	1	0	63
	M_6	69	31	66	3	0	72
	Mean ± SD	71.0 ± 7.3*					75.0 ± 9.5*

[1]Total number of damaged cells (classes 1 + 2 + 3). *Significantly different from the negative control (P < 0.001).

of damage (classes 2 and 3). Furthermore, there were no significant differences in DNA migration between the three extract concentrations tested and between the two leukocyte time samples, indicating no significant differences in DNA damage before and after the DNA cell repair process. The cell viability for leukocytes was greater than 90% using Trypan blue staining, confirming the absence of cytotoxicity observed by the PCE:NCE ratio in the micronucleus test.

Thus, under the conditions of the assays employed here, the data obtained in the present study permit us to conclude that acute oral administration of *A. occidentalis* leaf extract in mice, at the doses of 1000, 1500, and 2000 mg/kg, showed no evidence of clastogenicity/genotoxicity (i.e., no increase in micronucleus PCE or DNA damage) or cytotoxicity *in vivo*.

ACKNOWLEDGMENTS

Research supported by the Brazilian agencies CNPq (#306544/2006-7) and FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (#2006/57514-2). We thank Patrícia C. Martins Mello for technical assistance.

REFERENCES

Brendler-Schwaab S, Hartmann A, Pfuhler S and Speit G (2005). The *in vivo* comet assay: use and status in genotoxicity testing. *Mutagenesis* 20: 245-254.

Endress ME and Bruyns PV (2000). A revised classification of Apocynaceae. *Bot. Rev.* 66: 1-56.

Ernst E (2002). Toxic heavy metals and undeclared drugs in Asian herbal medicines. *Trends Pharmacol. Sci.* 23: 136-139.

Estevez Y, Castillo D, Pisango MT, Arevalo J, et al. (2007). Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group. *J. Ethnopharmacol.* 114: 254-259.

Gollapudi BB and McFadden LG (1995). Sample size for the estimation of polychromatic to normochromatic erythrocyte ratio in the bone marrow micronucleus test. *Mutat. Res.* 347: 97-99.

Hartmann A and Speit G (1997). The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). *Toxicol. Lett.* 90: 183-188.

Hartmann A, Elhajouji A, Kiskinis E, Poetter F, et al. (2001). Use of the alkaline comet assay for industrial genotoxicity screening: comparative investigation with the micronucleus test. *Food Chem. Toxicol.* 39: 843-858.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, et al. (2003). Recommendations for conducting the *in vivo* alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis* 18: 45-51.

Hayashi M, Tice RR, MacGregor JT, Anderson D, et al. (1994). *In vivo* rodent erythrocyte micronucleus assay. *Mutat. Res.* 312: 293-304.

Heddle JA, Cimino MC, Hayashi M, Romagna F, et al. (1991). Micronuclei as an index of cytogenetic damage: past, present, and future. *Environ. Mol. Mutagen.* 18: 277-291.

Klaude M, Eriksson S, Nygren J and Ahnstrom G (1996). The comet assay: mechanisms and technical considerations. *Mutat. Res.* 363: 89-96.

Krishna G and Hayashi M (2000). *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. *Mutat. Res.* 455: 155-166.

MacGregor JT, Wehr CM, Henika PR and Shelby MD (1990). The *in vivo* erythrocyte micronucleus test: measurement at steady state increases assay efficiency and permits integration with toxicity studies. *Fundam. Appl. Toxicol.* 14: 513-522.

Ndjakou LB, Vonthron-Senecheau C, Fongang SR, Tantangmo F, et al. (2007). *In vitro* antiprotozoal activities and cytotoxicity of some selected Cameroonian medicinal plants. *J. Ethnopharmacol.* 111: 8-12.

Ram VJ and Kumari S (2001). Natural products of plant origin as anticancer agents. *Drug News Perspect.* 14: 465-482.

Salamone M, Heddle J, Stuart E and Katz M (1980). Towards an improved micronucleus test: studies on 3 model agents, mitomycin C, cyclophosphamide and dimethylbenzanthracene. *Mutat. Res.* 74: 347-356.

Schmid W (1975). The micronucleus test. *Mutat. Res.* 31: 9-15.

Singh NP, McCoy MT, Tice RR and Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184-191.

Sokal RR and Rohlf FJ (1995). Biometry. W.H. Freeman, San Francisco.

Speit G and Hartmann A (1999). The Comet Assay (Single-Cell Gel Test). In: Methods in Molecular Biology, Vol. 113, DNA Repair Protocols: Eukaryotic Systems (Henderson DS, ed.). Humana Press Inc., Totowa, 203-212.

Stewart MJ, Moar JJ, Steenkamp P and Kokot M (1999). Findings in fatal cases of poisoning attributed to traditional remedies in South Africa. *Forensic Sci. Int.* 101: 177-183.

Tice RR, Agurell E, Anderson D, Burlinson B, et al. (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* 35: 206-221.

Veiga-Junior VF, Pinto AC and Maciel MAM (2005). Medicinal plants: safe cure? *Quim. Nova* 28: 519-528.

WHO (World Health Organization) (2002). Drug Information Herbal Medicines. Vol. 16. World Health Organization, Geneva.

Woods PW (1999). Herbal healing. *Essence* 30: 42-46.