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Evaluation of the Genotoxicity of Aqueous Extracts of *Ilex paraguariensis* St. Hil. (*Aquifoliaceae*) Using the *Allium* Test

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Summary Infusions and concoctions of *Ilex paraguariensis* are used as medicinal, nutritional and stimulant beverages in southern South America. Crop production is about 300,000 tons/year in Argentina, where the consumption rate reaches 5–9 kg/capita/year. In this study, we assessed the cytotoxicity of aqueous extracts of *I. paraguariensis* in the *Allium* test and *Artemia salina* microwell test. The extracts were prepared from commercial and "home processed" (laboratory) and were tested at concentrations of 5–40 g/l. Both extracts significantly decreased root growth and the mitotic index (MI). These effects were greater for the commercial material for which concentrations ≥10 g/l virtually abolished mitosis. The disturbance of mitotic behaviour was significant at 5–10 g/l of the "home-processed" product and included c-mitotic phenomena (over-condensed and disorganized metaphases, sticky metaphases, arrested anaphases, binucleated interphases) which could contribute to the increase in metaphase and anaphase indexes. None of the extracts were cytotoxic in the *Artemia salina* test. It is concluded that the *Allium* test is adequate for a preliminary screening of genotoxicity of medicinal plants and that genotoxic effects can be increased by the commercial manipulation of the raw product.

Key words *Allium* test, Antimitotic activity, Bioassay, Genotoxicity, *Ilex paraguariensis.*

Infusions and concoctions of *Ilex paraguariensis* St. Hil. (*Aquifoliaceae*) are widely used as medicinal, nutritional and stimulant beverages in southern South America (Argentina, Brazil, Chile, Paraguay, Uruguay). The crop production of *I. paraguariensis* is about 300,000 tons per year (Navajas Artaza 1995). The species contains xanthines (caffeine, theobromine), vitamins, flavonoids and other phenolic constituents as well as saponins (Alikaridis 1987, Ricco *et al.* 1995, Schenkel *et al.* 1995). The high level of consumption and our limited knowledge of the toxicity of these infusions as well as the potential health risks involved prompted us to study this species. Extracts of other species of the genus such as *I. verticillata*, are known to be cytotoxic (Fang and McLaughlin 1990).

Two bioassays were performed: the *Allium* test for general toxicity and genotoxicity, and the *Artemia salina* microwell test for cytotoxicity. The *Allium* test was first introduced by Levan (1938) and was later proposed as a standard method for testing the genotoxicity of chemicals, drinking water, natural waters, environmental pollutants and complex mixtures (Fiskesjö 1985, 1988, Fiskesjö and Levan 1993, Rank and Nielsen 1993, 1998, Repetto *et al.* 2000). The test is simple and sufficiently sensitive to reveal various toxic and clastogenic effects of directly acting chemicals at the macroscopic (e.g., growth) and microscopic (c-mitosis, chromosome stickiness, chromosome breaks) levels (Kendler and Koritz 1990, Fiskesjö 1993, 1994).

In vivo lethality in a simple animal organism is useful for monitoring bioactive natural prod-

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ucts (McLaughlin *et al.* 1998). Eggs of brine shrimp (*Artemia salina*) hatch within 48 h exposure to sea water producing large numbers of nauplii (larvae) which can be used in a number of bioassay systems (Solís *et al.* 1993, McLaughlin *et al.* 1998, Carballo *et al.* 2002). The brine shrimp lethality assay was proposed by Michael *et al.* (1956) and developed by Vanhaecke *et al.* (1981), and Sleet and Brendel (1983). It is based on the ability to kill *Artemia* nauplii and allows a quick preliminary assessment of cytotoxicity (Solís *et al.* 1993) and it has been used for the detection of fungal and cyanobacteria toxins, plant extract toxicity, heavy metals and pesticides (Carballo *et al.* 2002). Furthermore, the large number of individuals that can be employed, allows more precise statistical analyses (Solís *et al.* 1993).

In this paper we analyzed, using both methods mentioned above, extracts of *I. paraguariensis* samples of natural and commercial origin that were assayed for general toxicity, genotoxicity and cytotoxicity.

Materials and methods

Plant material

Leaves and young stems of *I. paraguariensis* St. Hil. were collected from trees cultivated at Santa Ana and Posadas (Misiones province, Argentina) in March and April 1996; voucher specimens were deposited at the herbarium of the Departamento de Farmacia (FCEQyN, Universidad Nacional de Misiones, Posadas, Argentina).

To obtain a dry product, the fresh material was air-dried, rapidly toasted in an oven at 100–120°C for 15 min and powdered. This product was referred to as "natural". The "commercial" product was obtained from industrial sources from the same geographic origin. This commercial material undergoes a relatively complex industrial process which the "natural" product does not (see Results and discussion section).

Experimental procedure

a) *Allium* test. The procedure generally followed that of Fiskesjö and Levan (1993) with few variations. Onion bulbs were grown in tap water for two days. On the third day tap water was replaced by aqueous extracts of *I. paraguariensis* (5, 10, 20, 40 g/l), which were renewed daily to prevent contamination; five replicates per dose were assayed. In each case, one group of five bulbs maintained in tap water served as controls. After a 48 h exposure, to ensure approximately two cell division cycles, five root tips were excised from each bulb and processed for examination by light microscopy. Root length was measured on the fifth day of the experiment in the rest of the bulb-roots, and the macroscopic abnormalities (tumors, necroses and hooks) were tabulated. Root tips were fixed in absolute ethanol : acetic acid (3 : 1 v/v) for 18–24 h, then transferred to 70% ethanol and stored at 4–6°C. Slide preparations were made from material hydrolized with 1 N HCl at room temperature (5–10 min) and squashed in lacto-propionic orcein.

b) *Artemia salina* microwell test. This test was performed as described by Solís *et al.* (1993). Brine shrimp eggs (from "S & S", Argentina) were incubated in oxigenated artificial sea water ("Red Sea Salt", Israel, 40 g/l) at 27–28°C. Nauplii were collected with an automatic pipette on the second day of incubation. Extracts of "natural" and "commercial" *I. paraguariensis* were prepared to provide stock solutions of 5, 10, 20 and 40 g/l. The mortality of *A. salina* was assessed after a 24 h exposure to the extracts. Artificial sea water was used as a negative control and thymol, as a possitive control. Each experiment was performed six times.

Statistical analysis

The results of the *Allium* test were analyzed using correlation/regression analysis and analysis of variance (ANOVA). ANOVA was also used to evaluate the results of the *Artemia salina* assay

after performing the arcsin transformation of the proportion of dead nauplii in each experiment (Sokal and Rohlf 1969).

Results and discussion

The results of the *Allium* tests are summarized in Figs. 1 and 2 and Tables 1, 2 and 3. Both extracts significantly decreased root length to 24.2–65.1% of the normal control size (Fig. 1a). This effect was more marked with higher concentrations (10, 20 g/l) of the commercial extract although the decrease in root length was significant only at 20 g/l (t=2.38, df=154, p=0.01854). When both sets of experiments were compared by means of two-way ANOVA, no significant differences in root length were observed between sources of *I. paraguariensis* (Natural and Commercial) in their response to the treatments but highly significant differences occurred between treatments within sources (Table 1a). There was a significant negative correlation between extract concentration and root length: Natural, r=−0.55, t=13.45, df=418, p=0.00062; Commercial, r=−0.60, t=15.66, df=446, p=0.00000.

The frequencies of macroscopic abnormalities (tumors, necroses and hooks together) in the root tips on the sixth day after treatment, are shown in Fig. 1b. For the natural product, the frequencies varied from 30.0% (5 g/l) to 87.6% (40 g/l) (r=0.88, t=3.26, df=3, p=0.04713). At 5 g/l the commercial extract produced no abnormalities, whereas at higher concentrations the values were similar to or greater than those seen with the natural extract (r=0.68, t=1.62, df=3, p=0.20367). For both types of extract, differences in the frequencies of macroscopic abnormalities were statistically significant with respect to controls when an independence test was performed (Natural, χ^2=170.5, df=4, p=0.00000; Commercial, χ^2=312.5, df=4, p=0.00000). Tumors and tumor plus necroses were the most common anomalies observed. Again, a two-way ANOVA revealed statistically highly significant differences between tretaments within sources (Table 1b).

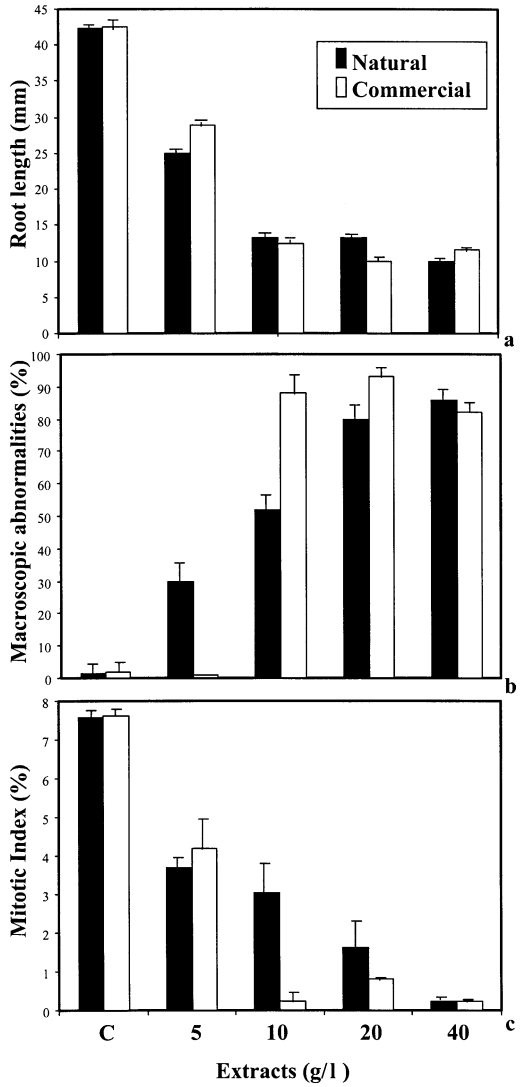

Fig. 1. a) Mean length of treated and control *Allium cepa* roots. The values are expressed as root length in mm, and represent the mean of 59 to 111 roots obtained from five bulbs for each concentration and controls. b) Mean frequencies of macroscopic abnormalities in treated and control *Allium cepa* root tips. The values are expressed as a mean percentage of abnormalities of five bulbs for each concentration and controls. c) Mitotic indices of control and treated *Allium cepa* root tip cells. The values are expressed as a percentage of cells in mitosis with respect to 1000 cells scored in each root tip from five bulbs per concentration and controls. The number of root tips cytologically analyzed varied from 5 to 13 per bulb. Bars express standard error of the mean in all cases.

A significant reduction in the Mitotic Index (MI) was observed at all concentrations of both

types of extract (Fig. 1c). The correlation coefficients were: Natural, $r=-0.68$, $t=6.33$, df=47, $p=0.00000$; Commercial, $r=-0.71$, $t=7.02$, df=48, $p=0.00000$. The antimitotic effect was significantly greater at higher concentrations (≥10 g/l) of the commercial product. Overall however, no significant differences in the reduction of the mitotic index were observed between the natural and the commercial product, while highly significant differences occurred between treatments within sources (Table 1c). Fig. 2 shows that a lower MI led to a lower frequency of abnormalities at high concentrations of the extracts (20, 40 g/l). The reason for the latter is probably that although a higher frequency of cytogenetic abnormalities would be expected as the concentration of the extracts increases, a simultaneous decrease of MI by an inhibition of mitosis, produces very few dividing cells most of which are prophase cells in which anomalies are difficult to detect, while metaphases, anaphases and telophases are very rare or do not exist in some root tips.

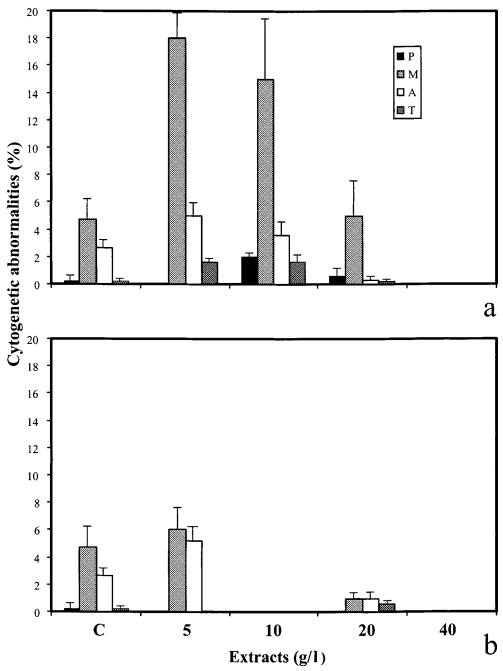

Fig. 2. Mean frequencies of mitotic abnormalities per phase in control and treated *Allium cepa* root tip cells. The values are expressed as a percentage of abnormal cells in mitosis with respect to the total number of mitotic cells scored in each root tip from five bulbs per concentration and controls. The number of root tips cytologically analyzed varied from 5 to 13 per bulb. The first set a) of data correspond to the natural material and the second b) to the commercial product. P=prophase; M=metaphase; A=anaphase; T=telophase. Bars express standard error of the mean in all cases.

Disturbances of the mitotic behaviour of chromosomes were significant at 5 and 10 g/l of the natural extract, with the appearance of highly condensed metaphase chromosomes, sticky chromosomes at metaphase and arrested anaphase cells (Figs. 3, 4). These changes are indicative of c-mitotic phenomena and partially explain the increase in the metaphase and anaphase indices in relation to controls (Table 2). C-mitotic phenomena are the result of mitotic spindle inhibition or disorganization which impedes chromosome segregation and results in typical over-condensed chromosomes at metaphase and arrested anaphases. C-mitotic behaviour in plants is a function of chemical concentration (Grant 1978) and is thus a sensitive indicator of genotoxicity. Stickiness and clumping of chromosomes usually accompany c-mitotic activity and, according to KlásterŠká *et al.* (1976), may arise from improper folding of the chromosome fiber; these changes are probably irreversible (Fiskesjö and Levan 1993).

The interphase cells of treated root-tips showed some distinctive features, including the presence of binucleated cells which were significantly more frequent than in control root-tip cells (Table 3). These cells usually arise as a consequence of the inhibition of cell plate formation (Grant 1978), as seen in Fig. 5. Arrested or delayed anaphase cells such as those found in the present study are a probable cause of the binucleated condition. These observations further support the occurrence of inhibitory processes following exposure to the above extracts.

The decrease in root-growth and the decrease in the mitotic indexes are significantly correlated (Natural, $r=0.96$, $t=33.02$, df=3, $p=0.00006$; Commercial, $r=0.99$, $t=233.62$, df=3, $p=0.00000$). Thus, the depression of mitotic activity was responsible for the growth-inhibitory action of the extracts. This action is not uncommon among plant extracts (Ene-Obong *et al.* 1991, Agarwal *et al.* 1992, Dias and Takahashi 1994, Padmaja *et al.* 1994) and can be regarded, as a turbagenic ac-

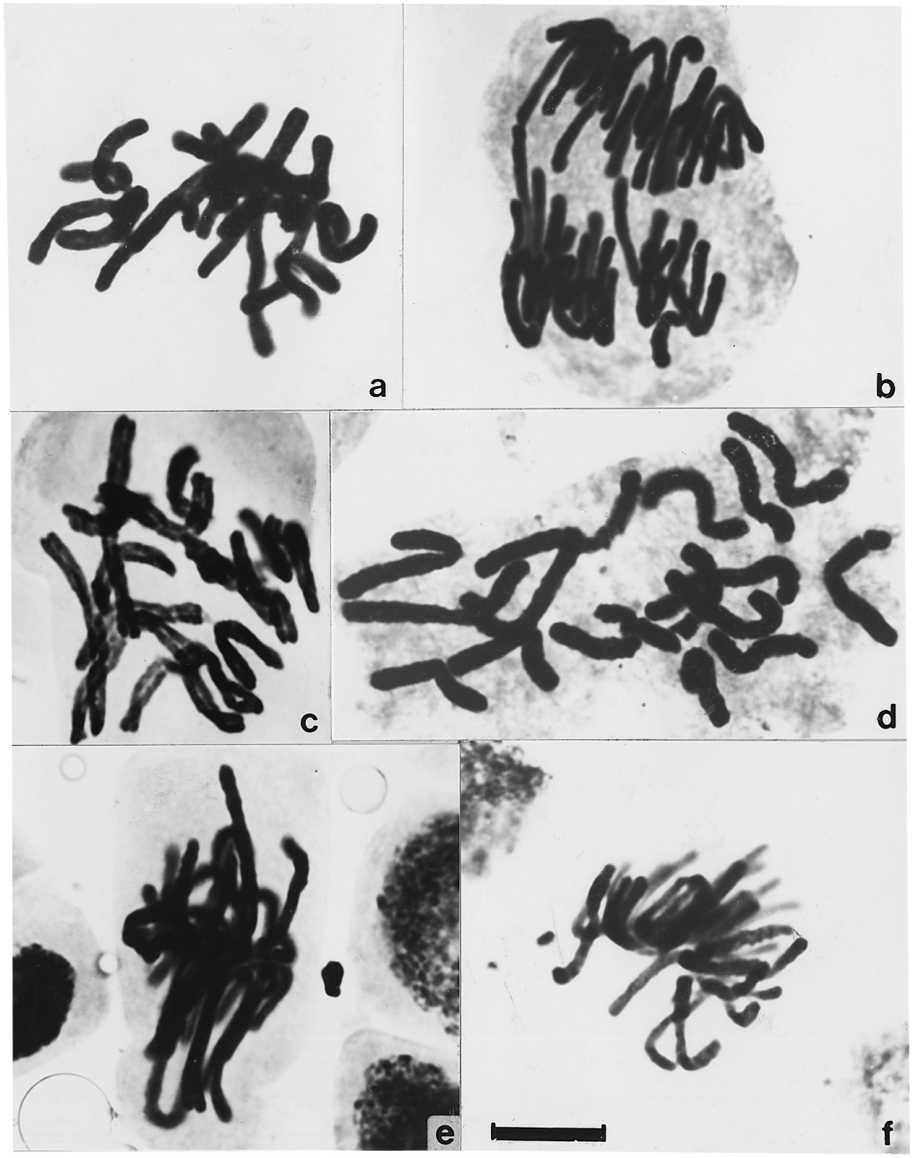

Fig. 3. Cytological effects of *I. paraguariensis* extracts on *Allium cepa* root tip cells. a) normal metaphase. b) normal anaphase. c, d) metaphases: note over-condensed chromosomes and disorganization of the metaphase plate. e, f) sticky and disorganized metaphases. Bar = 10 μm.

tion on the processes of cell division (Grant 1978).

The cause of the apparently divergent behaviour of the two extracts in causing macroscopic and cytogenetic anomalies is unknown. The industrial procedures used to transform the raw material (fresh leaves and stems of *I. paraguariensis*) into the manufactured product known as "yerba mate" involves fast dessication followed by pulverization and storage for 6 to 24 months before comsumption. It is possible that the industrial procedures of drying up to 400°C and blending, may alter the chemical composition of the raw material. For example, antioxidant systems with antimutagenic activity may be destroyed or altered, thereby changing the ratio of mutagenic/antimutagenic activity of the total extract. The antimutagenic activity of such antioxidant systems may involve pigments, vitamins, polyphenols and flavonoids (Agarwal *et al.* 1992), some of which are known to be

Table 1. Two-way ANOVAs comparing Root Length (a), frequency of Macroscopic Abnormalities (b) and Mitotic Index (c) obtained from the appliccation of the *Allium* test to aqueous extracts of *Ilex paraguariensis* from two different sources. Sources: Natural, Commercial; Treatments: 0, 5, 10, 20 and 40 g/l of extract

a) Mean Root Length

Source of Variation	df	SS	MS	Fs	*p*
Between sources	1	0.00100	0.00100	0.03065	0.86953 NS
Between treatments	4	17.77606	4.44402	136.21502	0.00016***
Error	4	0.13050	0.03263		
Total	9	17.90756			

b) % Macroscopic abnormalities

Source of Variation	df	SS	MS	Fs	*p*
Between sources	1	0.01000	0.01000	0.00005	0.99470 NS
Between treatments	4	13146.22	3286.56	16.08142	0.00988**
Error	4	817.41	204.37		
Total	9	13963.64			

c) Mitotic Index

Source of Variation	df	SS	MS	Fs	*p*
Between sources	1	1.03690	1.03690	1.21096	0.33292 NS
Between treatments	4	69.31020	17.32755	20.23620	0.00644**
Error	4	3.42506	0.85627		
Total	9	73.77216			

** Highly significant at the 0.01% level.
*** Highly significant at the 0.001% level.

Table 2. Mean phase indices of *Allium cepa* root tip cells treated with aqueous extracts of *Ilex paraguariensis*

Extract (g/L)	Mean Phase Index (%)±SE				
	Prophase	Metaphase	Anaphase	Telophase	N
Natural					
0	65.8±3.04	13.6±1.63	9.0±1.28	11.6±1.19	12
5	37.8±3.60	27.8±1.78	12.1±2.24	22.3±1.17	6
10	43.4±5.98	27.5±3.73	11.0±3.37	18.1±2.31	13
20	69.6±7.19	13.9±3.55	4.4±1.42	12.1±4.36	6
40	100*	0	0	0	11
Commercial					
0	65.8±3.04	13.6±1.63	9.0±1.28	11.6±1.19	12
5	61.5±6.34	11.5±2.29	8.3±1.94	18.7±3.56	9
10	96.4±4.29	1.1±1.59	0±	1.9±1.59	7
20	80.8±7.95	5.9±2.34	2.5±1.23	10.8±4.41	8
40	100*	0	0	0	11

Phase indexes are the relative frequencies of dividing cells at prophase, metaphase, anaphase and telophase calculated for each extract. N=number of root tips analyzed.

* Considering only those root tips for which the mitotic index was different from zero.

Table 3. Mean frequencies of binucleated interphase cells in *Allium cepa* root tips treated with aqueous extracts of *Ilex paraguariensis*

Source	Binucleated interphases (%)*±SE Extracts (g/l)				
	0	5	10	20	40
Natural	0.13±0.05	0	0.88±0.22	1.11±0.53	1.05±0.20
Commercial	0.13±0.05	0.32±0.21	0.46±0.45	0.24±0.05	0.18±0.04

* Percentage of binucleated interphase cells relative to total number of interphase cells based on approximately 5000 cells (5 root tips) for each extract and source.

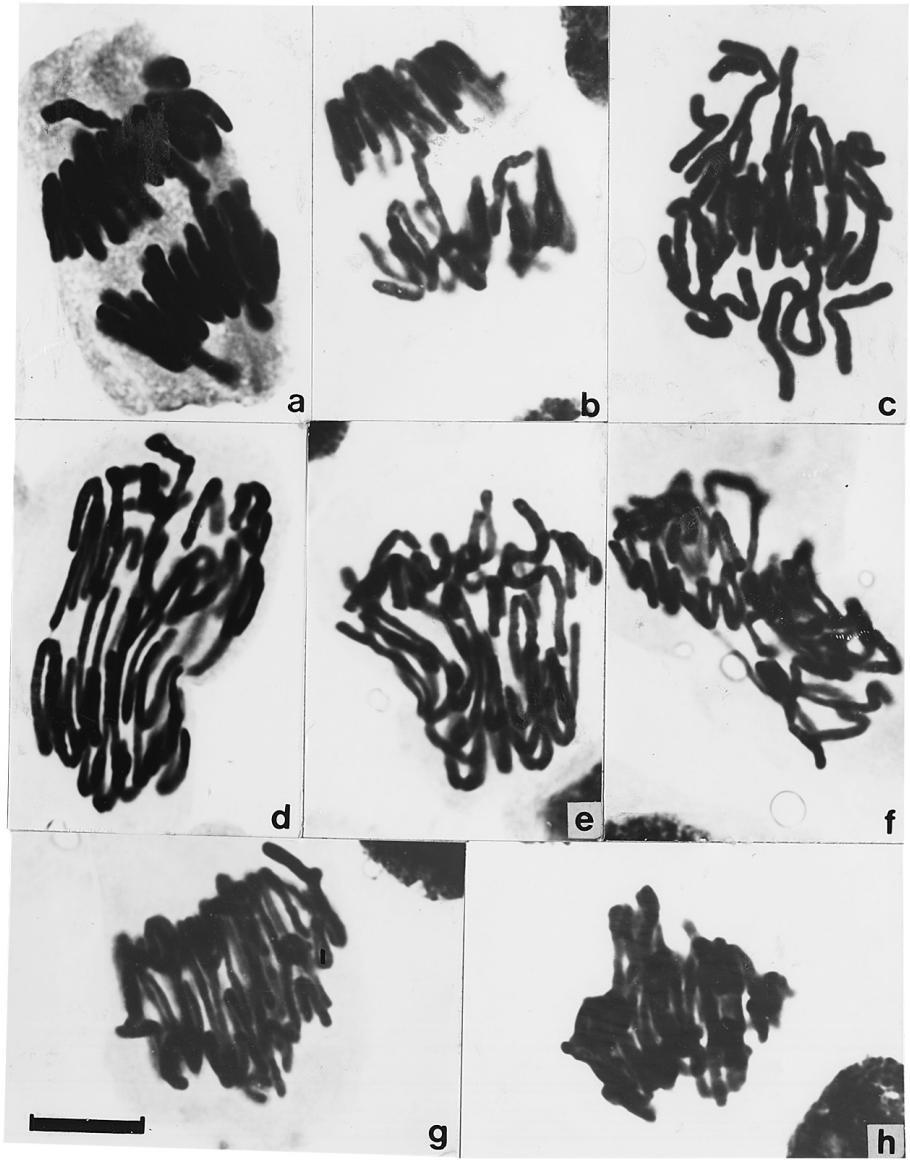

Fig. 4. Cytological effects of *I. paraguariensis* extracts on *Allium cepa* root tip cells. a, b) late anaphase nuclei with sticky chromatid bridges. c–h) anaphase nuclei showing different degrees of anaphase arrest. Bar=10 μm.

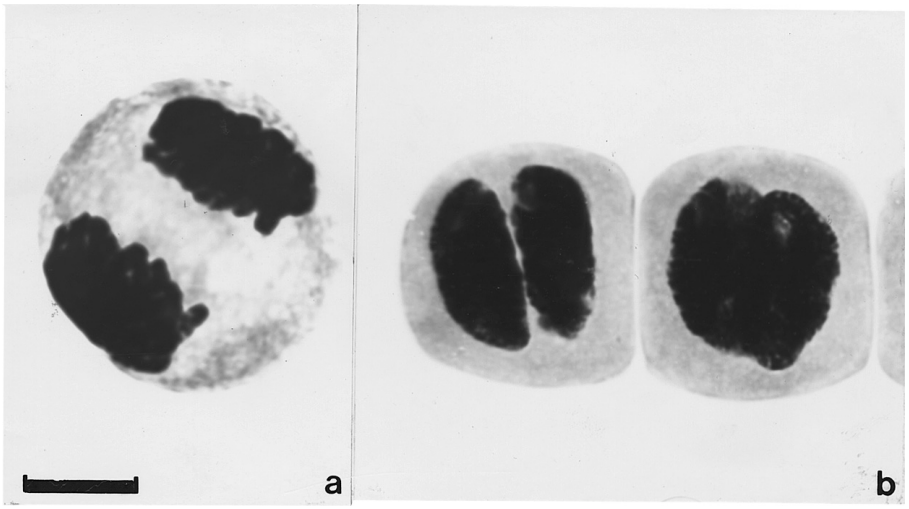

Fig. 5. Cytological effects of *I. paraguariensis* extracts on *Allium cepa* root tip cells. a) telophase without cytokinesis. b) two binucleated interphase cells. Bar=10 μm.

present in *I. paraguariensis*. Caffeine (a well-known co-mutagen) is a common component (ca. 5%) of *I. paraguariensis* and 10 triterpenic saponins (based mainly on ursolic acid) have been recorded in the species; ursolic acid is a cytotoxic compound (Fang and McLaughlin 1990). The enrichment of processed material in this substances could explain the greater genotoxic action of the commercial product.

Neither of the extracts exhibited cytotoxicity in the *Artemia salina* test at the concentrations assayed. Analysis of variance showed no significant differences between the control responses and the four concentrations of commercial extract tested ($F=2.35$, df=4 and 25, $p=0.08171$). The F statistic for the natural extract showed statistical significance ($F=3.20$, df=4 and 25, $p=0.02981$). However, a Keuls test (Snedecor 1956) revealed that this was attributable to the difference between the control and the third highest concentration tested (20 g/L); no other difference was detected.

It is concluded that the *Allium* test is useful as a preliminary means for the screening of genotoxic effects of plants of wide use in folk medicine and could serve to prevent potential negative effects on human health. The test can also increase our knwoledge as to what extent, industrial processing of the raw materials, could modify the genotoxic properties of the plants.

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