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Genotoxicity testing of *Cecropia obtusifolia* extracts in two in vivo assays: The wing somatic mutation and recombination test of *Drosophila* and the human cytokinesis-block micronucleus test

Varenka Martínez Toledo [a], María Guadalupe Ordáz Tellez [a], América Nitxin Castañeda Sortibrán [a], Adolfo Andrade-Cetto [b], Rosario Rodríguez-Arnaiz [a,*]

[a] *Laboratorio de Genética, Facultad de Ciencias, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D.F., Mexico*
[b] *Laboratorio de Etnofarmacología, Facultad de Ciencias, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D.F., Mexico*

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Abstract

Cecropia obtusifolia Bertol. (Cecropiaceae) is a tree that grows in secondary vegetation in the tropical rain forest along both coasts of Mexico. Its leaves are used in folk medicine for the treatment of diabetes mellitus type 2. The aim of the present studies was the evaluation of possible genotoxic effects of the aqueous extract from the leaves of *Cecropia obtusifolia* by means of two different experimental assay models: the wing somatic mutation and recombination test in flies and the micronucleus test from lymphocytes obtained from patients treated with the extract. No toxicity was found to be induced by the leaves of *Cecropia obtusifolia*. The *Drosophila* wing somatic mutation and recombination test (SMART) was applied in the standard version with basal biotransformation activity as well as in a variant version with increased cytochrome P450-dependent bioactivation capacity. The ranges of exposure concentrations for these genotoxicity experiments were between 0.82 and 13.32 mg/ml. The extract did not produce any genotoxic effect; however it showed a non significant antigenotoxic effect. The human micronucleus assay in vivo was performed with cultured lymphocytes obtained from six diagnosed type 2 diabetic patients treated daily with 13.5 g of the aqueous extract between 32 and 85 days. No statistically significant increases in cytotoxicity and/or genotoxicity between control and diabetic blood samples were observed.

Keywords: *Cecropia obtusifolia* Bertol. (Cecropiaceae); Folk medicinal plant; Genotoxicity; SMART wing assay; MN assay; Type 2 diabetic patients

1. Introduction

*Cecropia obtusifoli*a Bertol. (Cecropiaceae) known popularly as "Guarumbo", "Chancarro", "Hormiguillo", "Chiflón" and "Koochlé" (Andrade-Cetto, 1999) is a monopodic tree 20 m tall that grows in secondary vegetation in the tropical rain forest along both coasts of Mexico. It is a fast-growing pioneer tree from tropical America and the hollow septate twigs are inhabited by ants (Pennington and Sarukhán, 1998). The plant is used by traditional healers for the treatment of diabetes. Ethnobotanical studies have shown that in folk medicine, the dry leaves (15 g) are boiled in water (500 ml), the resulting infusion is cooled in the pot, then filtrated and drunk several times a day as "agua de uso". Phytochemical analysis of the extracts showed that the main constituents are chlorogenic acid, isoorientin and several phytosterols (β-sitosterol and stigmasterol) (Andrade-Cetto and Wiedenfeld, 2001). Pharmacological studies have shown a hypoglycemic effect of the water extract of *Cecropia obtusifolia* in alloxan-induced diabetic mice and rabbits (Pérez et al., 1984; Román Ramos et al., 1991). A significant hypoglycemic effect was observed in diabetic patients treated over several weeks with daily administration of aqueous extracts

* Corresponding author. Tel.: +52 55 5622 4906; fax: +52 55 5622 4828.
E-mail address: rra@hp.fciencias.unam.mx (R. Rodríguez-Arnaiz).

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of the leaves of this plant (Herrera-Arellano et al., 2004) while no significant changes in cholesterol, triglycerides or insulin could be detected (Revilla-Monsalve et al., 2007). Chlorogenic acid was identified as a specific inhibitor of the glucose-6-phosphate translocase component in microsomes of rat liver (Hemmerle et al., 1997). Simultaneous targeting of gluconeogenesis and glycogenolysis with an inhibitor of glucose-6-phosphate translocase would result in a reduction in hepatic glucose production (Andrade-Cetto and Heinrich, 2005).

Due to the wide use of extracts of *Cecropia obtusifolia* in traditional folk medicine to treat diabetic patients, the fact that a pilot study with diabetic type 2 diagnosed patients was running at the Centro Médico Nacional Siglo XXI and to the lack of published data on the possible genotoxic effects induced by this complex mixture we performed the experiments detailed in this study. Thus the aim of the present study was to establish whether water phytoextracts of the plant are able to induce mutations and recombination in somatic cells of flies by means of the in vivo wing spot assay and clastogenicity and chromosome breakage and loss by means of the micronucleus assay with human lymphocytes obtained from diabetic patients treated daily with aqueous extracts of the leaves of the plant from 32 to 85 days.

2. Materials and methods

2.1. Plant material, preparation of the aqueous extract and concentrations employed

Extracts of the leaves of *Cecropia obtusifolia* Bertol. (Cecropiaceae) were obtained in the form of a lyophilized powder. The aqueous extracts were prepared according to Andrade-Cetto and Wiedenfeld (2001). The extracts were monitored by HPLC for the chemical constituents and lyophilized. The main components of the extracts were chlorogenic acid, isoorientin and phytosterols. In preliminary experiments, we tried to obtain the LD_{50} of the extract and found no toxicity even at 13.32 mg/ml in adult flies. The concentrations of the powder extract employed for genotoxicity testing were 0.83, 1.66, 3.33, 6.66 and 13.32 mg/ml for the treatments in the wing assay. Patients received a prepared infusion of the dry leaves of the plant (13.5 g/day) for between 32 and 85 days.

2.2. Somatic mutation and recombination test (SMART)

The assay was essentially performed as described by Graf et al. (1984) and Graf and van Schaik (1992), using two different crosses of flies: the standard (ST) and the high-bioactivation (HB) crosses, both carrying visible wing genetic markers on the left arm of chromosome 3: multiple wing hair (mwh, 3–0.3) and flare (flr^3, 3–38). The ORR strain has chromosomes 1 and 2 from a DDT-resistant Oregon line (OR-R) which constitutively overexpressed CYP450 genes. Larvae of 72 ± 3 h were washed out of the culture bottles with a solution of 20% sucrose and seeded in plastic vials (200 larvae/vial) containing 50 mg of cellulose wetted with 0.3 ml of the test solution. Concurrent negative controls treated with the solvent alone (distilled water) were run. The larvae were fed on the above medium for 6 h and transferred to fresh medium for the rest of their development. For evaluation of the genotoxic effects recorded, the frequencies of spots per fly of a treated series were compared to its concurrent negative control series (Frei and Würgler, 1988, 1995). Statistical analyses were done for single, large, twin and total number of spots recovered.

2.3. In vivo cytokinesis-block micronucleus assay

Two healthy non-smoking donors (one female and one male) aged between 22 and 23 years, six recently diagnosed patients without treatment (two females and four males aged between 41and 74 years) and six diagnosed (2 ± 0.8 years) type 2 diabetic patients (four females and two males aged between 42 and 59 years) were recruited after giving informed consent. At the beginning of the treatment none of the patients had taken or was taking any hypoglycemic drug treatments. Blood samples were obtained after administration of the *Cecropia obtusifolia* extract (13.5 g daily) to diabetic patients on two occasions between 32 and 85 days of treatment.

Between 5 and 7 ml of blood was collected by venipuncture into vacutainer tubes containing 0.3 ml of heparin as anticoagulant. Blood was taken early in the morning before breakfast and the blood sample was centrifuged at $302 \times g$ during 30 min at room temperature. Approximately 0.5 ml of the isolated lymphocytes were added to 5 ml McCoy's 5 A culture media (Gibco) supplemented with 20% fetal bovine serum (Gibco), 2.5% phytohaemagglutinin (Gibco) and 1% streptomycin-penicillin (Gibco). All cultures were prepared in duplicate. Cultures were incubated at 37° C for 44 h and 0.3 ml of 3 mg/ml of cytochalasin B were added to each tube. Cultures were incubated at 37 °C for another 28 h. Cells were harvested at 72 h after the initiation of the culture. The cell suspensions were centrifuged at $302 \times g$ for 10 min, the supernatant was aspirated to a minimal level, cells were resuspended and a hypotonic solution at 37 °C was added for 5 min. The cell suspensions were centrifuged at $302 \times g$ for 10 min, the supernatant was aspirated to a minimal level and fixed in a 1 ml mixture of 3 methanol:1 glacial acetic acid. Resuspended fixed cells were centrifuged for 5 min at $100.66 \times g$. One additional change of the fixative was done. The slides were air dried for 10 min and stained with 2.5% Giemsa (45 ml Sorensen solution and 5 ml Giemsa) for 3 min. Excess of stain was eliminated with water. All slides were coded prior to scoring. Scoring was carried out in a light microscope at 1000× (Fenech, 1997; Fenech et al., 1999, 2003; Kirsh-Volders et al., 2003). For evaluation of cytotoxicity (CBPI), a Student's *t*-test was employed (Fenech, 1977) and for genotoxicity (MN and bridges) the Kruskall–Wallis test was used (statistical Program version 6.0).

3. Results

3.1. In vivo somatic mutation and recombination test (SMART)

In the wing somatic assay, the phytotherapeutic extract was assayed in three acute and independent experiments. The data from each experiment were not heterogeneous, as was shown

Table 1
Fly spot data obtained after acute exposure of trans-heterozygous larvae of *Drosophila melanogaster* to different concentrations of an aqueous extract from *Cecopia obtusifolia*

Cross/concentration (mg/ml)	Number of flies	Spots per fly (number of spots) statistical diagnoses[a]				Spots with	Mean number frequency of cell for-of cell division mation per 10^5cells		
		Small single (1–2 cells)	arge single (>2 cells)	Twin spots	Total spots	mwh clone	Cycles	Observed	Corrected
Standard cross									
Control	60	0.26 (16)	0.05 (3)	0	0.31 (19)	19	1.53	0.6	
0.83	60	0.62 (37)+	0.1 (6)i	0	0.72 (43)+	43	1.95	1.5	0.8
1.66	60	0.16 (10)−	0.04 (2)	0	0.20 (12)−	12	1.42	0.4	−0.2
3.33	60	0.30 (18)i	0.08 (5)i	0	0.38 (23)i	23	1.7	0.8	0.1
6.66	60	0.32 (19)i	0.02 (1)−	0	0.34 (20)−	20	1.7	0.7	0
13.32	60	0.16 (9)−	0.05 (3)i	0	0.21 (12)−	12	2.33	0.4	−0.2
High-bioactivation cross									
Control	60	0.23 (14)	0.1 (6)	0	0.33 (20)	20	1.95	0.7	
0.83	60	0.34 (20)i	0.06 (4)−	0.04 (2)i	0.44 (26)i	26	1.96	0.9	0.2
1.66	40	0.16 (6)−	0.05 (2)−	0.05 (2)−	0.2 (20)−	10	2.8	0.5	−0.2
3.33	60	0.24 (14)i	0	0.02 (1)i	0.24 (30)−	15	1.47	0.5	−0.2
6.66	60	0.34 (20)i	0.06 (3)−	0.02 (1)i	0.4 (48)i	24	1.54	0.8	0.1
13.32	60	0.12 (7)−	0.06 (3)−	0.02 (1)i	0.18 (11)−	11	2.27	0.4	−0.3

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995): + (positive); − (negative); i (inconclusive); w+ (weakly positive).

clearly by a Kruskall and Wallis test at $P < 0.05$ (statistical Program version 6.0), so the data were pooled for statistical analysis. Table 1 shows the pooled data recorded in the marker transheterozygous flies. The frequency of total spots per fly in the negative control of the ST cross was 0.31, and in the HB cross it was 0.33. These data are in accordance with the reported frequency of basal spots obtained in the ST cross and those observed in the HB cross (Graf and van Schaik, 1992). The size distributions of single, large, twin and total spots for the negative control and the concentrations of the extract assayed after acute treatment of larvae are shown in Fig. 1A and B. It should be noted that there are very few spots, and that those obtained for both crosses are principally small. Overall, no statistically significant differences between concurrent negative controls and the different concentrations of the phytotherapeutic extract was found in the ST cross, although at the lowest concentration assayed (0.83 mg/ml) a clearly positive effect was observed (Fig. 1A), in the HB cross the results are negative (Fig. 1B). Fig. 2 shows the number of mwh clones obtained. The induced frequencies of all types of spots are shown in Fig. 3, where a non-significant reduction in spot frequencies at several concentrations can be observed.

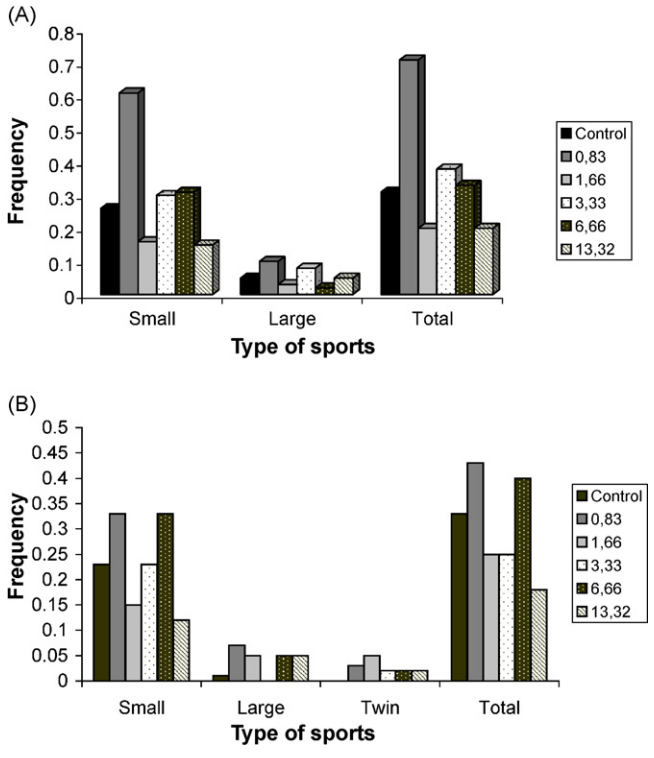

Fig. 1. (A) Frequency of small, large and total spots obtained after acute treatment of ST larvae with an extract of *Cecropia obtusifolia*. (B) Frequency of small, large, twin and total spots obtained after acute treatment of HB larvae with an extract of *Cecropia obtusifolia*.

3.2. *In vivo* cytokinesis-block micronucleus assay

The results obtained for the micronucleus assay are shown in Table 2. Blood samples of the diabetic patients were obtained at

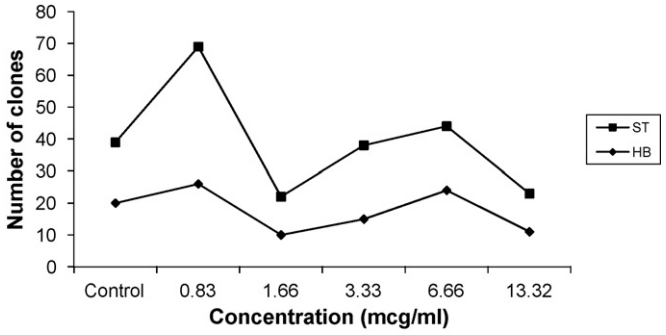

Fig. 2. Number of mwh clones obtained after acute treatment to *Drosophila melanogaster* larvae with different concentrations of an extract of *Cecropia obtusifolia*.

Table 2
Number and distribution of micronuclei in binucleated cells, frequency of MN, distribution and frequency of cells according to the number of nuclei, percentage of binucleated cells and cytochalasin-block proliferation index obtained in the in vivo micronucleus assay

Sex/age	Distribution of MN in binucleated cells				Total of binucleated cells	Frequency (no.) of MN and statistical diagnoses[a]	Distribution (frequency) of cells according to the number of nuclei					Total number of cells	Percentage of binucleated cells	CBPI[b]
	0	1	2	3			1	2	3	4	5			
Healthy controls														
F/23	1985	13	2	0	2000	0.008 (17)	208	2000	279	144	0	2631	76.02	2.14
M/22	1983	16	1	0	2000	0.009 (18)	805	2000	132	85	0	3022	66.18	1.83
Diabetic controls (without treatment)														
F/58	1978	21	1	0	2000	0.011 (23)	564	2000	209	244	0	3017	66.29	2.04
F/70	1977	20	3	0	2000	0.013 (26)	353	2000	44	63	0	2460	81.3	1.93
M/41	990	10	0	0	1000	0.01 (10)	209	1000	24	14	0	1247	80.19	1.87
M/48	992	7	1	0	1000	0.009 (9)	114	1000	57	42	0	1213	82.44	2.02
M/63	1980	18	2	0	2000	0.011 (22)	431	2000	162	183	4	2780	71.94	2.04
M/74	1985	14	1	0	2000	0.008 (16)	879	2000	72	40	0	2991	66.87	1.76
Diabetic patients treated with an aqueous extract of *Cecropia obtusifolia* (13.5 g daily)														
F/42[c]	2981	15	3	1	3000	0.008 (24)	853	3000	129	157	0	4139	72.48	1.9
F/59[c]	983	16	1	0	1000	0.018 (18)	139	1000	127	222	3	1491	67.07	2.3
F/58[d]	2937	44	16	3	3000	0.028 (85) +	1184	3000	91	131	2	4408	68.06	1.81
F/58[e]	3969	26	5	0	4000	0.009 (36)	1136	4000	297	380	1	5814	68.8	1.99
M/49[f]	2611	11	4	0	2626	0.007 (19)	203	2626	422	540	14	3805	69.01	2.35
M/48[e]	3954	40	6	0	4000	0.013 (52)	974	4000	358	353	5	5690	70.3	2.02

[a] Satistical diagnoses according to a non-parametric 2 × 2 Chi-square tables, Fisher's exact test.
[b] Statistical diagnoses according to a parametric Student's *t*-test.
[c] 32 days.
[d] 38 days.
[e] 85 days.
[f] 71 days.

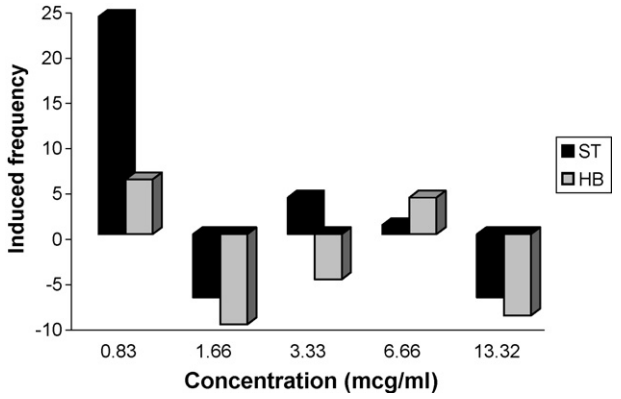

Fig. 3. Induced frequency of total spots obtained after acute treatment of *Drosophila melanogaster* larvae with an extract of *Cecropia obtusifolia*.

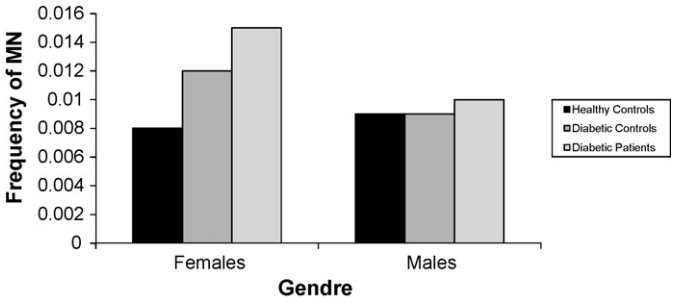

Fig. 4. Frequency of micronuclei obtained in controls and diabetic patients after treatment with an extract of *Cecropia obtusifolia*.

two different times during the treatment. Between 1200 and 5800 cells were counted to obtain the CBPI. A total of at least 2000 binucleated cells were analyzed for each individual (experiment and replicate) with several exceptions in which blood samples of diabetic patients treated or not were to greasy. The numbers of cells with one, two, three, or four nuclei were recorded. The proportion of binucleated cells was between 66% and 82%, while the CPBI varied between 1.8 and 2.5; thus, the phytotherapeutic extract was not cytotoxic. No differences due to gender were observed (Fig. 4). The results for genotoxicity are negative with the exception of the treated diabetic patient (female, 58 years) that yielded a significant positive result for the frequency of MN when compared to the healthy and diabetic (untreated) controls. Lifestyle-based influences on individual variability in sensitivity to the complex mixture could explain these results.

4. Discussion

The wing somatic mutation and recombination test for *Drosophila melanogaster* is a versatile and sensitive eukaryotic short-term assay that has been used for the determination of genotoxic activity of chemical compounds with different structures (Vogel et al., 1999). The present study indicates that aqueous extracts of *Cecropia obtusifolia* did not induce somatic mutation and mitotic recombination in the wing somatic assay of *Drosophila melanogaster* at concentrations ranging from 0.83 to 13.32 mg/ml in both test crosses with basal (ST) or increased (HB) P450 biotransformation capacity.

Cytogenetic biomarkers are the most frequently used endpoints in human population studies. Their sensitivity for measuring exposure to genotoxic agents and their role as early predictors of cancer risk have contributed to this success (Bonassi et al., 2005). The frequency of micronuclei (MN) in peripheral blood lymphocytes in conjunction with the cytokinesis-block assay is among the most popular and effective biomarker of disease used for evaluating the effect of environmental exposure to genotoxic agents (Fenech et al., 1999). The results reported in this paper clearly showed that in diagnosed diabetic type 2 patients the phytotherapeutic extract of "guarumbo" was unable to induce any cytotoxic or genotoxic effect after the administration of aqueous extracts of this plant between 32 and 85 days of treatment.

Very few studies had been performed to detect the genotoxic or antigenotoxic capacities of the main constituents of *Cecropia obtusifolia*. Phytosterols are present in several common vegetable oils such as coconut, corn, olive, peanut, soybean and sunflower (Swern, 1979). Stigmasterol is present in significant proportion in most of these oils, while β-sitosterol is present at a high percentage in all of them (Unilever, 1998). No acute, subchronic, and/or chronic toxicity was observed after oral or dermal treatment of rabbits and dogs with phytosterols at concentrations ranging from 2% to 4% (Andersen, 1993). PEG-5 Soy sterol was not cytotoxic to *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 or TA1538. The compound, with or without metabolic (S9) activation, did not induce reverse mutations in those bacterial strains (Cosm. Ingr. Rev. Exp. Panel, 2004; Henkel Corp., 1995). No significant incidence of chromosome aberrations or change in mitotic index was observed in human lymphocytes exposed in vitro to phytosterols and phytosterol esters (Hungtindon Life Sciences Ltd., 1997). Also, neither evidence of unscheduled DNA synthesis in rat liver hepatocytes exposed in vivo to plant sterol esters nor induction of micronuclei in rat bone marrow cells were found (Covance Laboratories Ltd., 1999a,b). No carcinogenicity was observed when soy sterols were administrated orally to mice, intraperitoneally to rats or injected into the gastric antrum of guinea pigs (Andersen, 1993). Several lines of evidence are available to suggest that plant phytosterols inhibit the induction of tumors in animals; dietary plant phytosterols appear to inhibit colonic cancer development prior to adenoma formation (Ling and Jones, 1995); sitosterol had an inhibitory effect on the tumor-promoting activity of 12-0-tetracanoylphorbol-13-acetate (TPA) in mouse skin following initiation by 7,12-dimethylbenz(a)anthracene (Yasukawa et al., 1991) and coadminstration of *N*-methyl-*N*-nitrosourea and β-sitosterol to rats produced a significant fewer colon tumors (benign or benign and malignant) compared to rats given the carcinogen alone (Raicht et al., 1980). There are no reports in the literature on the possible genotoxic/antigenotoxic effects of chlorogenic acid and isoorientin, the main compounds of the phytoextract employed in this study. Thus the most relevant observation is that in diabetic type 2 patients, after receiving 13.5 g of *Cecropia obtusifolia* extract daily, which contained 2.91 mg of chlorogenic acid and 2.4 mg of isoorientin, no genotoxic effects were produced.

In conclusion the negative results obtained in the in vivo wing somatic assay of *Drosophila melanogaster*, at the concentrations tested, clearly show that the phytotherapeutic extract of *Cecropia obtusifolia* is not genotoxic. Further experiments are needed to verify the possible antigenotoxic properties of the phytoextract in flies. In diagnosed diabetic type 2 patients treated with "guarumbo" daily with 13.5 g of the aqueous extract during between 32 and 85 days no statistically significant increases were observed for cytotoxicity and/or genotoxicity between control and diabetic blood samples. Thus the lack of toxicity, cytotoxicity and/or genotoxicity of this complex mixture validates its use in folk medicine for the management of diabetic type 2 patients and indicates that it can be safely manufactured as a phytomedicine.

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