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An investigation of the genotoxic potential of a well-characterized yerba mate extract

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ABSTRACT

Yerba Mate (*Ilex paraguariensis*) is historically used as a beverage and its extracts are considered traditional medicine in South America. Extract use has been expanding to North American and European markets and the currently available genetic toxicology literature indicate discrepancies in genotoxicity findings for yerba mate. As botanical extract use expands, assumption in safety should be made with caution assuring a good understanding of the test material characterization. Authoritative agencies suggest a two-step paradigm to investigate genotoxicity, and this was implemented to evaluate the safety of yerba mate hydroxycinnamic acid extract. Four OECD compliant assays were employed: bacterial reverse mutation, *in vitro* micronucleus and a parallel *in vivo* micronucleus, and comet assay. No evidence of mutagenicity was observed in the *in vitro* Ames assay, but the results of an *in vitro* micronucleus study were inconclusive. However, oral gavage treatment of rats for the *in vivo* micronucleus and comet assays demonstrated negative findings. The results from this battery of tests, supports that this yerba mate hydroxycinnamic acid extract is not anticipated to pose genotoxicity concerns. A high-level comparison of results to other available genotoxicity literature on yerba mate is presented with emphasis on the importance of identity when drawing conclusions on botanicals.

1. Introduction

Yerba mate, or simply mate (*Ilex paraguariensis* A.St.-Hil., Aquifoliacea), is a woody evergreen tree with bright red fruits and small white flowers. Before European colonization of Latin and South America, yerba mate was routinely enjoyed by local populations in the same way tea or coffee is today. Yerba Mate remains a popular beverage in the subtropical regions of South America, such as Brazil, Uruguay, Argentina, and Paraguay, and is made by steeping the leaves of *I. paraguariensis* in hot water [2,4,17,19,27,28,40]. The steeped beverage is known to have high rates of consumption in its native regions, reaching up to one liter per day [17]. In the case of tea preparations, the leaves from the plant may contain stems and are typically blanched, dried and aged (Heck and Mejia, 2007) prior to use. The daily consumption level is estimated at 200–300 mg/kg-bw/day [34] and ranging from 3 to 5 kg/person/year in Brazil to 6–10 kg/person/year in Uruguay, with 12–23 g of yerba mate generating 1 liter of beverage [4,5,15,34]).

In the United States, the natural extract of yerba mate is generally recognized as safe [33] and is also considered to be GRAS for flavor uses by the Flavor and Extract Manufacturers Association (FEMA; [6]). Flavor uses on average are 40 ppm with average maximum use levels at 1000 ppm in products including beverages, cheeses, condiments, confections, frozen dairy, fruit ices, gelatins and puddings and a number of others food categories [6]. Additionally, in Europe, it is recognized for a safe history of use [12] and in the UK as a novel food [14]. Yerba mate (various preparations) also is used as a traditional medicine in South American regions and for its mood-altering effects and can also be utilized as a laxative and diuretic [17,25].

Perhaps due to the popularity of the tea, interest in yerba mate extracts continues to increase as evidenced by the emergence of a variety of dietary supplements in the marketplace. Grzesiuk et al. [16] point out that although there is interest in mate due to the beneficial substances, questions have been raised as to its effect on DNA. The International

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Agency for Research on Cancer (IARC) reviewed the cancer risk of mate and concluded that evidence for genotoxicity of mate was weak, but that the studies had methodological limitations [20]. It is worth noting that the focus of IARC's review was on consumption of mate as a very hot beverage.

It is a generally accepted tenet of toxicology and a requirement of authoritative agencies that genotoxicity potential is investigated as part of establishing safety for ingredients intended to be added to dietary supplements or food, whether for flavor or technical effect. In particular, the GRAS designation is specific to intended usage. Thus, although the tea version of yerba mate may be considered GRAS, differences in solvent extractions of plant materials or preparation methods of tea leaves can lead to extracts that vary in composition [17,29]. Not surprisingly, in the case of botanicals there has been a movement emphasizing the importance that safety data should be tied to the specific identity of the botanical material as much as possible [32].

A search of the published literature specific to genotoxicity assays for *Ilex paraguariensis* leaf indicates there are nine studies which report various and inconsistent conclusions (Table 1). Yerba mate was reported to be positive for mutagenicity in the Ames assay [13,19] with varying results in the presence and absence of S9. Assays designed to investigate clastogenicity, aneugenicity, and cytotoxicity also resulted in a variety of inconsistent findings, suggesting a positive response by several investigators [2,13,19,36], while others concluded negative findings for genotoxicity [1,16,2,24,35,3]. A possible explanation for the different results between reported findings is likely due to inconsistencies in yerba mate materials tested as a consequence of preparation and harvesting practice differences. A key factor of the published literature search was focused on better understanding the characterization of the test substances used in publications to ascertain those that were similar to the yerba mate extract tested in the standard genotoxicity assays reported herein. Unfortunately (and this remains a common problem for botanical materials), most of the published studies lack detail on the preparation of the yerba mate test material (identity). Identity and efforts to characterize test material is a key component within a GRAS conclusion. This was an issue raised in the work of Bidau et al. [2], who reported there are differences when using commercially available yerba mate versus laboratory-created test material.

Table 1
Summary of literature on yerba mate leaf genotoxicity.

Reference	Test Material*	Study Type	Highest Dose Tested	Results
[1]	Tea infusion (5% yerba mate)	in vivo MN with Wistar Rats	50 mg/kg	Not genotoxic
[2]	Yerba mate aqueous extract	Allium and Microwell Test	20 g/L	Not cytotoxic but genotoxic**
[3]	Commercial yerba mate powder	in vitro MN (exfoliated buccal cells)	4 teas daily	Not genotoxic
[13]	Yerba mate leaf aqueous extract	Ames with and without S9	150 mg/plate	Mutagenic without S9 and mutagenic in TA102 with S9
		in vitro chromosomal aberration human lymphocyte	750 µg/mL	Clastogenic
		in vivo chromosomal aberration with Wistar Rats	2 g/kg/day	Clastogenic
[16]	Yerba mate aqueous extract	Allium Test	not reported	Not genotoxic
[19]	Yerba mate leaf aqueous extract	Ames with and without S9	50 mg/plate	Mutagenic without S9
[24]	Commercial yerba mate powder	in vivo Comet assay	2 g/kg/day	Not genotoxic
[35]	Yerba mate leaf aqueous extract	in vitro human lymphocyte	1400 µg/mL	Not genotoxic
[36]	Commercial yerba mate powder	in vitro human lymphocyte	1000 µg/mL	Genotoxic and cytotoxic

MN, micronucleus; S9, S9 metabolic activation
*No further details provided
** A commercial sample was tested alongside a lab made sample, the commercial sample was genotoxic in Allium assay

A comparison of the available reports demonstrates that some authors indicated tea and tea infusions, while others suggested use of leaves and stems that were prepared in various states. It is important to apply caution when drawing conclusions on safety without understanding the identity of the botanical and characterization of the test material. For this reason, the objective of the studies presented here are to document the genotoxicity potential of a well-characterized yerba mate extract that is refined to >95% hydroxycinnamic acid constituents harvested with consistent practices with non-smoked methods for drying leaves. The goal of this work is to bring attention to the consideration that the characterization and confidence in test material is important since findings may vary across different extracts. The extract associated with the results in the findings reported here was also consistently sourced from a specific geographical region of Argentina from April to October. Since Yerba Mate may be used in a variety of products as a flavor or considered for use in dietary supplements and teas, documentation of safety data on well-defined extracts and the noting of the variance in genotoxicity findings across extracts is an important addition to the peer reviewed literature so that discerning choices can be made in product formulation and in substantiation of safety.

2. Materials & methods

Four separate toxicity assays were conducted at Charles River Laboratories during the timeframe of January-May 2020. All the genotoxicity and mutagenicity evaluations described herein were conducted according to the Organisation for Economic Co-operation and Development (OECD) guidelines and with Good Laboratory Practices (GLP). Two assays (Ames OECD 471 and the *in vitro* micronucleus assay in TK6 cells OECD 487) were performed at the Skokie, IL, USA, location. Additionally, two assays (the *in vivo* micronucleus OECD 474 and an oral gavage comet study OECD 489) were performed at the Mattawan, MI, USA, location. The same lot material was used for all four assays. The OECD guidelines were followed; general assay materials and methods are described below.

2.1. Test material characterization and dose formulation analyses

The mate extract used was prepared from leaves of the yerba mate plant. Although it is possible that some stems from the leaves were present, this was considered to have an insignificant impact on the phenolic compositions as stem phenolic profile is nearly identical to the leaf, and the leaf has a higher percentage of phenolics overall. The raw material was sourced from plants grown in the Corrientes Precinct of Argentina, between April and October, and analysis of leaves indicated no significant variation in phenolic distributions among two harvested batches. The harvested leaves, representing the leaves of a mature bush, were gathered at the same time post-planting. Within the first 24 hours, leaves undergo an expedient drying process (approximately 20–30 seconds) by which they're exposed to the direct heat of flames, causing steam heat which creates a crackling in the epidermis. This process inactivates the protoplasm and enzymes. Leaves are then further dried to reduce remaining moisture content to 3–6%, which is done through a slower drying process where steamed leaves are placed on a

drying belt, between rotating tubes, or stretched across tacuara canes, where heat is applied through the utilized instruments. Approximately 13.5 kg of dried leaves creates 1 kg of final extract.

Refined yerba mate extract powder was produced by Cargill using a toll manufacturer (Lot No. YM20190402). High performance liquid chromatography with ultraviolet absorbance detection (wavelength of 324 nm) was used to determine the overall extract composition (Table 2), which was primarily composed of hydroxycinnamic acids (yerba mate extract) in test formulations. Results met the protocol-specified acceptance criteria for concentration acceptability for formulations (i.e., analyzed concentration was ≥ 90% of target concentrations). For the Ames assay, test material was stored at ambient temperature and prepared as a stock formulation with sterile water at concentrations up to 50 mg/mL. The dosing volumes were 100 µL per plate. For the micronucleus assay in TK6 cells, test material was stored at ambient temperature and prepared with sterile water in a stock solution of 200 mg/mL for the range finder and 50 mg/mL for the repeat micronucleus assay. For the *in vivo* studies, material was stored at ambient temperature and prepared with sterile water at concentrations up to 200 mg/mL. No test article was detected in the analyzed vehicle (sterile water; Center Medical Supply, Lot No. Y293977) administered to the control group.

2.2. Metabolic activation

The Aroclor™ 1254-induced rat liver S9 fraction (Lot Nos. 3954 and 4051) was purchased from Molecular Toxicology, Inc. The metabolic activation mixture was prepared fresh daily and maintained on ice throughout the assay. For the bacterial reverse mutation assay, the S9 mixture was prepared at a concentration of 7.5% (v/v). Additional constituents in the mixture included magnesium chloride (0.4 M) – potassium chloride (1.65 M); glucose-6-phophate (1 M); nicotinamide adenine dinucleotide phosphate (NADP; 0.1 M); phosphate-buffered saline; and sterile distilled deionized water. For the *in vitro* micronucleus assay, S9 (0.3 mL) was diluted with Roswell Park Memorial Institute (RPMI) 1640 plus L-glutamine supplemented with penicillin-streptomycin (1.5 mL). It was then combined with B-nicotinamide adenine dinucleotide phosphate reduced form Type III (NADPH; 36.5 mg).

2.3. Cellular test systems

In the bacterial reverse mutation assay (Ames test), strains were used in accordance with OECD TG 471. Strains employed were as follows: *Salmonella typhimurium* strains TA1537, TA98, TA100, TA1535, and *Escherichia coli* strain WP2*uvrA*. Clones were stored at −60°C to −80°C. Prior to each test, clones were incubated overnight for 8–9 hours in Oxoid nutrient broth at 36°C to 38°C with shaking. For all strains, between 10^8 and 10^9 cells/mL culture was used.

For the *in vitro* micronucleus assay, TK6 human lymphoblasts were obtained from Pfizer Global Research and Development (Garoton, CT) and subcloned at Charles River Skokie. Only cells free of mycoplasma contamination were used for testing. TK6 stock was maintained in RPMI 1640 and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin Complete Culture Medium (CCM). At 24 hours prior to treatment, cultured cells were set up in vented T-75 cm^2 flasks in CCM at a cell density of $1.3–1.8\times10^5$ cells/mL. Cultures were incubated at 36°C to 38°C and 4–6% CO2 in upright flasks for 22–26 hours.

Table 2
Composition of test material.

Compounds	Concentration %
Mono- and di-caffeoylquinic acids	96.33
Feruloyl-, coumaroyl- acids	1.24
Total Hydroxycinnamic Acids	97.57*

* The remaining approximately 2% of the product included plant lignins, tannins, and residual protein.

Male Sprague-Dawley rats were obtained from Charles River Laboratories, Raleigh, NC, USA. They were received at 7 weeks old, weighing between 188–277 g. There were 60 male rats in total, broken into 10 groups (6/group). Of these 10 groups, groups 1–5 were used for the *in vivo* micronucleus and groups 6–10 were used for the *in vivo* comet assay. Animals were housed in solid bottom cages, with *ad libitum* tap water and food (Certified Rodent Diet #5002, PMI Nutrition International, Inc.). Temperature was kept at 68°–79 °F, and humidity was maintained between 30%–70%, with a 12-hour day/night cycle. Animal enrichment was provided. Rodents were observed twice a day for morbidity, mortality, injury, and access to food and water. Animals were also observed for signs of toxicity on Days 1, 2, and 3, prior to dosing, and 1–2 hours after each dose. Clinical observations (e.g., skin, eyes, oral cavity, tremors) were made on Days 1 and 3. Body weights were measured and recorded at reception of subjects and on Day 1 and prior to termination (Day 3). Food consumption was measured and recorded at pretreatment (Day −7), on Day 1, and prior to termination (Day 3). Laboratory animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals from the National Research Council.

2.4. Experimental procedures

For the Ames assay, based on results of the range finding assay (results not shown), concentrations tested using the plate incorporation method were 100, 250, 500, 1000, 2500, and 5000 µg/plate. For each concentration level and for each condition, the mean revertant count and standard deviation (SD) were determined. Vehicle control for the assay was sterile water obtained from MediaTech (Lot No. 21718004). Positive controls for tests without metabolic activation were ICR-191 acridine (0.5 µg/plate; Sigma-Aldrich Inc., Lot No. SLBR0485V) for strain TA1537, 2-nitrofluorene (2.5 µg/plate; Sigma-Aldrich Inc., Lot No. S43858V) for strain TA98, sodium azide (1.0 µg/plate; Sigma-Aldrich Inc., Lot No. MKBX7529V) for strains TA100 and TA1535, and 4-nitroquinoline-N-oxide (2.0 µg/plate; Sigma-Aldrich Inc., Lot No. A0391011) for WP2 *uvrA*. With metabolic activation, 2-aminoanthracene (Sigma-Aldrich Inc., Lot No. STBD3302V) was used across all strains as a positive control: *S. typhimurium* (2.5 µg/plate), and WP2 *uvrA* (10 µg/plate).

For the *in vitro* micronucleus assay, TK6 human lymphoblasts were tested both in the presence and absence of metabolic activation for short incubations (4 hours) and in the absence of activation for long incubation (27 hours). Vehicle control for the assay was sterile water obtained from MediaTech (Lot No. 21718004). Positive control concentrations were mitomycin-C at 0.125 µg/mL and 0.0625 µg/mL in 4-hour cultures without activation, vinblastine sulfate at 0.0030 µg/mL and 0.0025 µg/mL in 27-hour cultures without activation, and cyclophosphamide at 11.9 µg/mL and 4.7 µg/mL in cultures with activation. Harvest times were at 44 hours for the 4-hour exposure with and without metabolic activation (a 40-hour recovery period). Harvest time for the 27-hour exposure was approximately 27 hours with no recovery period. Micronucleus frequencies were analyzed in 1000 or 3000 cells per culture. The highest concentration selected for evaluation of micronucleated cells was the concentration in which 50–60% cytotoxicity was observed, or in the absence of cytotoxicity the highest concentration tested. Cytotoxicity was determined using a coulter counter and relative population doubling method in accordance with OECD practice.

For the *in vivo* bone marrow micronucleus test, rats (6/group) were administered a dose of 500, 1000, and 2000 mg/kg-bw/day of test material, once daily for 3 days (at 0, 24, and 48 hours post first dose), via gavage. Vehicle control for the assay was sterile water obtained from Central Medical Supply (Lot No. Y293777). Positive controls were cyclophosphamide at 10 mg/kg-bw/day and ethyl methanesulfonate at 200 mg/kg-bw/day. In brief, the bone marrow was extracted from

femurs in groups 1–5 on Day 3, approximately 3 hours following the last dose. Marrow was aspirated 2–3 times from the right femur using a syringe containing fetal bovine serum (FBS) into a centrifuge tube. Suspension was centrifuged at 300 g for 5 minutes and an appropriate amount of supernatant was removed. The pellet was resuspended in FBS. Using a 23-gauge needle, one drop of the cell suspension was used to prepare a smear. Slides (4/animal) were prepared and allowed to dry overnight before being placed in 100% methanol for 20 minutes. Slides were blinded to dose group when labeled. To ensure a sufficient number of animals per group were available for terminal procedures and statistical evaluation (n=5), an extra animal was dosed in each group. If not needed as a replacement animal, the sixth animal in each group was euthanized and discarded.

The *in vivo* mammalian alkaline comet assay was carried out similarly to the bone marrow micronucleus assay, using the same dosing structure, doses, route of exposure, and SD rats (6/group). Vehicle control was the same, but positive control was ethyl methanesulfonate at 200 mg/kg-bw/day. This procedure was carried out with SD rat groups 6–10. Lateral segments of the left hepatic lobe and a portion of the duodenum were harvested approximately 3 hours after the last dose. Liver samples were minced, and duodenums were scraped to release epithelial cells. Slides were placed in lysis solution overnight and washed with neutralization buffer prior to electrophoresis. Slides were stained with Sybr Gold™ solution prior to scoring. Up to 150 cells from each tissue were scored using the Comet Assay IV Image Analysis System (Instem, United Kingdom). The clouds produced were tabulated, and frequency of tail intensity calculated for each tissue per animal. Like the micronucleus cohort of the study, an extra animal was dosed in each group to ensure a sufficient number was available for terminal procedures and proper statistical evaluation (n=5). If not needed, the sixth animal in each group was euthanized and discarded.

2.5. Data analysis and statistical analysis

For the reverse bacteria mutation assay at each concentration level, and under each condition, the mean revertant count and SD were determined. A positive mutagenicity response would result from an induced increase of revertants per plate with increasing concentration. The increase should be at least two times the vehicle control for strain with high spontaneous levels (TA100) and three times for those with lower levels (TA1537, TA98, TA1535, and WP2 *uvrA*). To properly account for all revertants, Charles River Computer Systems employed the ProtoCol 3 Colony Counter Software (ver 1.0.2.0).

For the *in vitro* micronucleus assay, a 1-tailed Fisher's Exact test was performed on the total number of micronucleated cells, comparing the treated groups to the results obtained from the concurrent vehicle control group. A positive result for inducing micronuclei is if a statistically significant increase ($p \leq 0.05$) in the mean percentage of micronucleated cells was observed at one or more dose levels and did not fall within the historical control data. The detection of dose-response trends, if needed, was carried out using the Cochran-Armitage test ($p \leq 0.05$).

For the *in vivo* mammalian alkaline comet assay, percent tail intensity (%TI) was measured using Comet IV (Perceptive Instrument, UK). The median %TI was calculated for animals and each tissue. Percent TI was analyzed using a generalized linear model assuming a beta distribution followed by Dunnett's two-sided test to determine if the means of each treated group were significantly different than the vehicle control group ($p \leq 0.05$).

3. Results

3.1. Bacterial reverse mutation assay results

A range-finding study at concentrations up to 5000 μg/plate produced no cytotoxicity and no precipitates were observed (data not shown). At doses ranging from 100 to 5000 μg/plate, no increase in the number of revertant colonies was observed for any of the strains tested with and without metabolic activation (Table 3).

3.2. In vitro micronucleus assay results

Initially, the range-finding assay was conducted to determine the highest concentration which resulted in 50%-60% cytotoxicity. Doses tested ranged from 3.91–2000 ug/mL. Based on the cytotoxicity range finding, the 4-hour treatment without metabolic activation was conducted using the three highest doses (500, 1000, and 2000 μg/mL). All three doses produced micronucleated cells that were significant for trend using the Cochran-Armitage Test. However, the increases were within the 95% confidence interval of the vehicle control (Table 4). In the presence of metabolic activation, the 4-hour treatment doses did not elicit a statistically significant increase in %MN in PCE. Precipitates were not observed for the 4-hour treatment with or without metabolic activation and no changes in pH were observed. The 27-hour treatment without metabolic activation was repeated due to excessive cytotoxicity, and included doses: 62.5, 125, 250, 500, 1000, and 2000 μg/mL. In the repeat, 27-hour treatment without metabolic activation (doses of 250, 300, 350, 400, 450, and 500 μg/mL), there was a statistically significant increase in %MN at the 300 μg/mL dose. However, there were no statistically significant increases in %MN for trend using the Cochran-Armitage Test, and increases were not higher than the 95% confidence interval when compared to vehicle control.

The range finding study for the 4- and 27-hour incubations evaluated concentrations ranging from 62.5 to 2000 μg/mL, and 15.6–500 μg/mL, respectively. Precipitate formation was not observed for either incubation period. No cytotoxicity was observed at any tested concentrations for the 4-hour incubation; therefore, 500, 1000, and 2000 μg/mL were tested. Comparatively, excessive cytotoxicity (>60%) was observed at highest concentrations tested and concentrations ranging from 250 to 500 μg/mL were tested. Based on observed toxicity, doses tested in the main study were 250, 300, and 350 μg/mL.

Overall, the induction of micronuclei was equivocal under the 4-hour treatment without metabolic activation and were negative in the 27-hour treatment without metabolic activation and in the 4-hour treatment with metabolic activation.

3.3. In vivo micronucleus and comet assay results

According to OECD TG 474 and OECD TG 489, *in vivo* micronucleus and mammalian alkaline Comet assay, responses are similar between males and females and therefore, studies could be performed in either sex. In all three doses tested, up to the limit dose of 2000 mg/kg-bw/day, there were no significant changes in results when compared to the control groups. In the micronucleus assay, there were no significant increases in %MN in PCEs when compared to the vehicle control group, and none of the rats exposed to the test material exhibited evidence of bone marrow cytotoxicity (Table 5). In the comet assay, there were no significant increases in %TI of liver or duodenum samples when compared with vehicle control (Table 6). All animals survived to scheduled necropsy, and there were no observed effects on body weight and diet for all three doses tested throughout the length of the study.

4. Discussion

The study results described herein investigated the genotoxic potential of an aqueous 95% hydroxycinnamic acid yerba mate extract made predominantly from leaves of *Ilex paraguariensis*. Traditional testing paradigms investigate genotoxicity using a stepwise approach starting with two *in vitro* tests to investigate gene mutations (Ames) and structural and numerical chromosomal changes followed by *in vivo* assays if positive or equivocal findings are indicated (RedBook 2000; [10, 11]). Standard batteries (Ames, micronucleus *in vitro*, micronucleus *in vivo*, and comet) were conducted following this approach. The results

Table 3
Summary of bacterial reverse mutation assay (Ames test) results.

Concentration (µg/plate)	Bacterial Reverse Mutation Assay									
	TA98		TA100		TA1535		TA 1537		WP2 *uvrA*	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Hydroxycinnamic Acids										
0	10 ± 3	14 ± 3	91 ± 9	83 ± 6	13 ± 4	7 ± 2	4 ± 2	6 ± 4	39 ± 11	49 ± 12
100	14 ± 5	14 ± 3	91 ± 10	93 ± 11	11 ± 4	10 ± 4	4 ± 5	6 ± 2	34 ± 2	49 ± 5
250	13 ± 5	17 ± 7	93 ± 7	100 ± 14	8 ± 3	13 ± 2	7 ± 1	4 ± 4	35 ± 5	45 ± 8
500	11 ± 2	15 ± 5	88 ± 10	97 ± 20	10 ± 3	7 ± 2	5 ± 3	3 ± 3	40 ± 3	42 ± 9
1000	14 ± 1	17 ± 3	90 ± 6	96 ± 10	11 ± 1	11 ± 4	5 ± 2	6 ± 1	28 ± 4	40 ± 4
2500	16 ± 0	12 ± 1	75 ± 4	95 ± 9	7 ± 1	15 ± 2	4 ± 2	5 ± 0	42 ± 5	35 ± 3
5000	14 ± 6$^{NP\ n}$	17 ± 2$^{NP\ n}$	97 ± 7$^{NP\ n}$	79 ± 3$^{NP\ n}$	8 ± 3$^{NP\ n}$	10 ± 3$^{NP\ n}$	2 ± 1$^{NP\ n}$	4 ± 1$^{NP\ n}$	30 ± 3$^{NP\ n}$	39 ± 5$^{NP\ n}$
Positive Controls*	1664 ± 561	2381 ± 26	563 ± 4	2852 ± 28	549 ± 39	304 ± 17	219 ± 16	207 ± 37	1174 ± 46	248 ± 51

Data are presented as mean ± SD.
-S9, without S9 metabolic activation; +S9, with S9 metabolic activation. NP, No precipitate; n, Normal bacterial background lawn
*Positive controls without S9 were as follows: TA98 - 2-Nitrofluorene; TA100 - Sodium azide; TA1535 - Sodium azide; TA1537 - ICR-191 Acridine; WP2 uvrA - 4-nitroquinoline-N-oxide. Positive control with S9 - 2-Aminoanthracene

Table 4
Summary of *in vitro* micronucleus assay with TK6 human lymphoblasts results.

Concentration (µg/mL)	4 Hour Exposure without S9			
	Cytotoxicity (%)	Mean % MN	Mean Fold Increase	Trend***
Hydroxycinnamic Acids				
0	0	0.33	1.00	n/a
500	11	0.92**	2.75	≤0.01
1000	27	0.98**	2.95	≤0.01
2000	54	1.43**	4.30	≤0.01
0.125 MMC-C	28	5.33**	16.00	≤0.01
Concentration (µg/mL)	27 Hour Exposure without S9			
	Cytotoxicity (%)	Mean % MN	Mean Fold Increase	Trend***
Hydroxycinnamic Acids				
0	0	0.70	1.00	n/a
250	17	1.15	1.64	0.2391
300	50	1.30*	1.86	0.2391
350	58	1.05	1.50	0.2391
0.003 VIN	41	8.25**	11.79	n/a
Concentration (µg/mL)	4 Hour Exposure with S9			
	Cytotoxicity (%)	Mean % MN	Mean Fold Increase	Trend***
Hydroxycinnamic Acids				
0	0	0.40	1.00	n/a
500	0	0.35	0.88	0.9337
1000	2	0.30	0.75	0.9337
2000	17	0.40	1.00	0.9337
11.9 CP	34	6.00**	15.00	n/a

*** Cochran-Armitage Test is performed on the number of cells with micronuclei. ≤0.05 = significant.
*** Significantly different from control group (Chi-square test), p≤0.001.
MMC-C, Mytomycin C; VIN, Vinblastine Sulfate; CP, Cyclophosphamide
* $p<0.05$
** $p<0.01$ using Fisher's Exact Test 1-Tailed Test. MMC-C: Mytomycin C

suggest this yerba mate extract does not pose genotoxicity concerns.

Multiple guidance documents address considerations for assessing the safety of botanicals, yet currently, there is no harmonization of guidance for such testing. It is typical to see published genotoxicity data that lacks a clear definition (identification) of test material [27,28,32]. Groups like The Botanical Safety Consortium continue to discuss, support, and guide the need for better publicly available documentation regarding the safety profiles of botanical materials that are of interest for foods and dietary supplements [22]. The European Food Safety Authority (EFSA) in its 2018 genotoxicity assessment of chemical mixtures opinion also emphasizes the importance of characterization. Yet EFSA also recognizes that mixtures (even of natural origin) can pose challenges to characterization. All of these groups indicate that a robust description of identity should include scientific name, synonyms, common names, part use, geographical origin, growth and harvesting conditions, and solvents used for extraction. In this publication, and consistent with newer expectations for botanical material, we have

Table 5
Summary of *in vivo* bone marrow micronucleus assay results.

Concentration (mg/kg/day)	*in vivo* Bone Marrow Micronucleus Assay				
	MN PCEs/ 4000 PCEs	%MN-PCEs	PCEs	NCEs	PCE:TE Ratio
Hydroxycinnamic Acids					
0	3 ± 1	0.09 ± 0.03	237 ± 5	263 ± 5	0.47 ± 0.01
500	3 ± 1	0.07 ± 0.03	237 ± 16	263 ± 16	0.47 ± 0.03
1000	3 ± 0.4	0.08 ± 0.01	234 ± 13	266 ± 13	0.47 ± 0.03
2000	3 ± 1	0.07 ± 0.02	239 ± 11	261 ± 11	0.48 ± 0.02
10 CP and 200 EMS	149 ± 32	3.72 ± 0.81*	215 ± 12	285 ± 12	0.43 ± 0.02*

Data are presented as mean ± SD.
* $p<0.01$. MN, Micronucleated; PCE, Polychromatic Erythrocyte; NCE, Normochromatic Erythrocyte; TE, Total Erythrocyte; CP, Cyclophosphamide; EMS, Ethyl Methanesulfonate.

Table 6
Summary of *in vivo* mammalian alkaline comet assay results.

Concentration (mg/kg/day)	Total Cloud Frequency (% of total)		Mean %TI	
	Liver	Duodenum	Liver	Duodenum
Hydroxycinnamic Acids				
0	16 (2.1%)	215 (28.7%)	2.00 ± 0.46	12.5 ± 5.1
500	17 (2.3%)	72 (9.6%)	1.90 ± 1.2	10.6 ± 2.8
1000	26 (3.5%)	56 (7.5%)	3.97 ± 6.1	12.8 ± 7.7
2000	23 (3.1%)	60 (8.0%)	2.44 ± 0.96	11.7 ± 2.9
200 EMS	18 (2.5%)	44 (5.9%)	29.0 ± 6.3*	31.3 ± 5.0 *

Data for Mean %TI are presented as mean ± SD.
* $p<0.05$; 2-tailed Dunnett's T-test EMS, Ethyl Methanesulfonate; %TI, frequency of tail intensity.

provided additional detail with regards to the source and chemical identity of the extract to help bring clarity for future considerations of safety. This level of clarity could not be found in the previously documented genotoxicity assays (Table 1), making it difficult for any reader to apply the published data broadly. A future research opportunity would be to review the totality of existing data for all published extracts with great detail documenting identity based on geographical practices and descriptions given in a review fashion.

To investigate genotoxicity of this well-characterized extract, we followed the standard two-step paradigm (*in vitro* followed by *in vivo* testing). First, mutagenicity was investigated with the Ames test. In contrast to previous findings (Fonesca et al., 2000 and Leitao and Braga, 1994) indicating mutagenic concern, our data for the Ames assay was negative. Fonesca et al. (2000) reported findings on mate chimarrao extract that was prepared by filtering and freeze drying a boiled preparation of commercially available yerba mate dried leaves. Leitao and Braga (1994) created mate infusions using burned or dry mate leaves in boiling distilled water to a final concentration of 200 mg/mL. Both preparations mimic more of a tea type of preparation. In a review by Heck and Mejia (2007), the typical process for tea preparation is described well, and they note that drying can be done within a chamber that contains a filtered or non-filtered smoke and heat combination. While the extract we tested was clearly not produced from smoked mate leaves, the type of leaves used cannot be discerned for other references discussed herein. Not only can such practices create heat-formed chemicals that could infuse the leaves, but the drying method used has been noted to impact the caffeine content [29]. Although all are similar in that leaf material and water extractions were utilized; the style of preparation of the material and hence different identity profiles could explain the difference in findings suggesting an explanation for the discrepancies between the reports.

The second standard *in vitro* test is conducted to better understand potential for macro effects on chromosomal material. Thus, the extract was tested in the *in vitro* micronucleus assay in the humanTK6 cell line. We found that in the 4-hour/40-hour recovery exposure scenario, there was a trend of increasing MN, but it was within historical control range, and this was only in the absence of metabolic activation. No trend was observed in the presence of S9. In the longer exposure scenario (27 hours with no S9) there was a statistically significant increase in MN at the 300 ug/mL dose but with no evidence of a trend or biological relevance. Although the results were deemed equivocal, the findings still support that in the presence of normal metabolizing enzymes and processes, genotoxicity is not likely to occur. These results are also interesting as we found significant cytotoxicity at doses >500 ug/mL without S9, and at >1000 ug/mL when S9 was present. In contrast, Fonesca et al. (2000) had previously reported (Table 1) evidence of clastogenicity in an *in vitro* chromosomal assay that maintained exposure for 24 hours to the tea-type of test material in human lymphocytes at concentrations up to 750 ug/mL with no effects of S9 and a mitotic index diminished by 42%. Although a different assay, in our micronucleus work we found that the 27-hour exposure with no S9 had excessive cytotoxicity at doses above 300 ug/mL. This may further support that the constituent profiles or the mixtures were different, which supports the conclusion that the extracts are not comparable. The yerba mate used by Vargas Alves et al. [35] was from naturally dried mate leaves and a tea-like infusion was made and used in a micronucleus assay using peripheral blood lymphocytes. The authors intentionally wanted test material to be like the tea that is consumed. No evidence of genotoxicity or cytotoxicity was reported with exposures ranging from at much higher doses of 175–1400 ug/mL. In contrast, Wnuk et al.[36] used a tea-like preparation that was made from a dried commercial product from a different geographical region (Argentina). They reported significant cytotoxicity and evidence of micronucleus formation which were demonstrated to represent an aneugenic mechanism at much lower doses (10 ug/mL; [36]). These authors went on to investigate the possible contribution of caffeine and questioned the presence of organophosphate compounds. More examples of cytotoxicity [16,2] could be discussed, but that is beyond the scope of the work reported here. There have been hypotheses that yerba mate may provide protective anti-cancer effects due to cytotoxicity to cancer cells [17,36], and the discrepancies we highlight in cytotoxic potency may be of interest for further investigation.

Following the two-step paradigm, the equivocal finding we reported in the T6 cell line in the 4-hour exposure when metabolic activation was not present required *in vivo* investigation. Both an *in vivo* micronucleus and a comet assay were conducted with the refined yerba mate extract and found to be negative for genotoxicity. Animals received refined extract by oral gavage. The combined assay approach has been promoted as a more comprehensive *in vivo* assessment of potential genotoxicity [26]. While the *in vivo* micronucleus assay results helped to confirm that there were no concerns from the equivocal *in vitro* finding, the favorable comet assay allows for confidence that there was no evidence of DNA repair or broad-spectrum DNA damage [9]. The strength of the combination is valuable in that it better represents, as Recio et al. [26] points out, "dynamic whole-animal physiological processes such as uptake and systemic distribution by the circulatory system, Phase I and Phase II metabolism and intact elimination and excretory systems that cannot be entirely *recreated in vitro*". In the traditional genetox paradigm, the *in vivo* negatives are required to placate any concerns that may have been raised *in vitro*. Thus, the findings reported here for this refined 95% hydroxycinnamic acid yerba mate extract should give confidence that this particular extract poses no genotoxic concern. Interestingly, all *in vivo* genotoxicity assays that were available on yerba mate extracts (Table 1: [1,3,13,24]) also concluded negative findings. In contrast to *in vitro* work, these findings suggest that despite preparation differences, when studied *in vivo*, yerba mate in various forms does not indicate a genotoxic concern. One research area that could provide valuable insight into this statement and help eliminate confusion over yerba mate terminology (tea/infusion/extract) would be to investigate each of these types of preparation following a good characterization of constituents *in vivo*. Such an approach could benefit validation of read across efforts. This type of comparison could be important to further investigate any assumption that all yerba mate preparations can automatically be considered non-genotoxic.

Understanding genotoxicity not only is expected for regulatory compliance of food ingredients and dietary supplements, but also gives helpful insight into the interpretation of potential increased cancer risk. Regarding yerba mate tea and possible cancer risk, there are case-control reports of links to head and neck cancer as well as bladder cancer [8,7,23,30]. However, people in the geographic regions that have this risk typically consume yerba mate as steaming hot tea. As beverages at or above 65°C are defined by the IARC as a group 2 A probable carcinogen [18]. However, the IARC lists mate beverages as a Group 3 (not classifiable) for cancer so long as it is "not very hot" [18].

In contrast to concern with botanical materials, there have been numerous studies to identify compounds within edible and medicinal plants that may protect humans against DNA damage. A review by Lopez-Romero et al. [21] discusses the various phytochemicals of interest for such anticarcinogenic benefits which includes hydroxycinnamic acids. This group of compounds has been less likely to be associated with risk and more often associated with benefit and can be found in fruits, vegetables, cereals, coffee, tea and wine [31]. The ever-growing interest in lifestyle intervention through diet suggests it will remain important to publicly document risks and benefits of plant-based materials. As interest in yerba mate continues to grow, risk managers and risk assessors should remain aware of the subtle differences in preparation that can lead to discrepancies in safety profiles. Our work suggests that yerba mate, as a source of hydroxycinnamic acids in the form of a refined extract as described herein, poses no genotoxic concerns.

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CRediT authorship contribution statement

T.P.Dubnicka:Resources, Methodology, Investigation, Formal analysis, Data curation. P.Heard:Resources, Methodology, Investigation, Formal analysis, Data curation. **Cody N.Rabert**:Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **C. Doepker**:Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Conceptualization. **A. Eapen**:Resources, Funding acquisition, Conceptualization. **N.Choksi**: Writing – original draft, Validation, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Doepker, Rabert, Heard, Dubnicka, Choksi reports financial support was provided by Cargill. Doepker, Rabert, Choksi reports a relationship with ToxStrategies that includes: employment. Eapen reports a relationship with Cargill that includes: employment. Heard, Dubnicka reports a relationship with Charles River Laboratories that includes: employment. Doepker, Rabert, Heard, Dubnicka, Choksi reports a relationship with Cargill that includes: consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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