



Absence of genotoxicity of purified *Aloe vera* whole leaf dry juice as assessed by an *in vitro* mouse lymphoma *tk* assay and an *in vivo* comet assay in male F344 rats

Jiang Hu^{a,*}, Mel Lloyd^b, Cheryl Hobbs^c, Peter Cox^b, Keith Burke^c, Gareth Pearce^b, Michael A. Streicker^c, Quanyin Gao^a, Vasilios Frankos^a

^a Herbalife Nutrition, Torrance, CA, 90502, United States

^b Covance Laboratories Ltd., Harrogate, United Kingdom

^c Integrated Laboratory Systems, LLC, Morrisville, NC, 27560, United States

ARTICLE INFO

Edited by Dr. A.M Tsatsaka

Keywords:

Aloe vera
Hydroxyanthracene derivatives
Decolorization
Mutant frequency
% tail DNA
Mutagenicity

ABSTRACT

Hydroxyanthracene derivatives (HAD) are naturally present in the latex layer of *Aloe vera* leaf, predominantly as aloins A, B and aloe-emodin. HAD are typically removed from commercial ingestible aloe products through activated charcoal filtration (decolorization). Current research aimed to evaluate genotoxic potential of a purified aloe whole leaf dry juice containing 0.3 ppm of total aloins and non-detectable aloe-emodin (LOD = 0.01 ppm) in the L5178Y mouse lymphoma assay (MLA; OECD 490) and *in vivo* comet assay (OECD 489). No marked increases in mutant frequency at the *tk* locus were observed in the MLA at concentrations up to 5000 µg/mL for 3 h and 24 h (-S9), and up to a precipitating concentration of 3000 µg/mL for 3 h (+S9) compared to concurrent vehicle control. Relative total growth at the highest analyzable concentrations at 3 h (±S9) and 24 h (-S9) ranged from 64 to 133 %. In the comet assay, no statistically significant increases in DNA strand breaks were detected in the colon or kidney following oral gavage of 500, 1000 or 2000 mg/kg/day in male F344 rats for 2 days compared to concurrent vehicle control. Overall, these findings demonstrated the test article containing minimal HAD is not genotoxic under the described experimental conditions.

1. Introduction

Aloe vera (*L.*) *Burm. f.* has a long history of traditional use for its medicinal properties, with documented records dating back to the 4th century B.C. [1,2]. Various preparations from aloe leaves have reportedly been used internally for indications such as colds, sore throat, indigestion, gastrointestinal irritations and constipation [2–5]. More recent studies have shown that *Aloe vera* may have antioxidant, immunostimulatory and anti-inflammatory properties, and its consumption has been linked to potential health benefits, such as increasing absorption of vitamins, alleviating gastroesophageal reflux symptoms, lowering serum LDL-cholesterol and glucose levels, and improving skin elasticity [4,6–10]. *Aloe vera* food and supplement market has grown rapidly in recent years, likely owing to increasing consumer awareness of these purported health benefits.

Hydroxyanthracene derivatives (HAD) are a class of compounds naturally present in plants and with an anthraquinone backbone

structure, including rhein, chrysophanol, aloe-emodin, physcion, dianthrone, and anthrone, along with their glycosides [11]. Because these compounds have potent laxative effects, plant extracts with high levels of these compounds are commonly used in traditional herbal medicine or food supplements to improve bowel function [12–14]. *Aloe vera* leaves contain small amounts (< 1%) of HAD, mainly as aloin (or barbaloin) A, B and aloe-emodin (Fig. 1), which are located in the rind (outer green skin protecting the gel) and latex (yellowish exudate from the rind), but not in the inner leaf (clear fleshy gel) [15,16]. Aloins and aloe-emodin in aloe leaves have been found to be genotoxic in bacterial mutation and mammalian cell assays *in vitro* as well as in several *in vivo* genotoxicity assays [17–19], and are considered to be the main contributing agents to the colonic carcinogenicity observed in a 2-year cancer bioassay of an orally administered *Aloe vera* whole leaf extract (containing approximately 6400 ppm aloin A and 71 ppm aloe-emodin) in F344 N rats [18,20].

Due to concerns related to the potential genotoxicity and

* Corresponding author at: 950 W190th Street, Torrance, CA, 90502, USA.

E-mail address: jianghu@herbalife.com (J. Hu).

<https://doi.org/10.1016/j.toxrep.2021.03.007>

Received 5 October 2020; Received in revised form 5 March 2021; Accepted 6 March 2021

Available online 9 March 2021

2214-7500/© 2021 The Authors.

Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

carcinogenicity risk associated with HAD in *Aloe vera*, the process for aloe production has undergone continuous improvement [21]. It is now a common practice to remove HAD through a multi-step purification process in the commercial production of aloe products [22]. *Aloe vera* whole leaf (rind, inner leaf and latex) and inner leaf gel are typically subjected to a series of filtration steps using activated charcoal, a process known as decolorization, to remove pigmented substances and HAD, resulting in a purified product with only residual amounts of HAD as unavoidable impurities. The International Aloe Science Council (IASC), a self-regulating trade association, has set a quality standard of less than 10 ppm of total aloins for all *Aloe vera* leaf juice products intended for oral consumption [23]. These purified (*a.k.a.* decolorized) aloe products are not known to have any laxative properties linked to HAD due to their insignificant levels. Most recently, the European Commission (EC) has proposed regulatory measures to prohibit the use of preparations from the leaf of *Aloe species* containing HAD in foods and food supplements, and set the level of 1 ppm for aloe-emodin/emodin and the level of 1 ppm for the sum of aloins A and B as the threshold for its harmonized risk management approach [24].

Numerous published studies have shown that *Aloe vera* leaf preparations with < 10 ppm of aloins were not mutagenetic in prokaryotic and eukaryotic test systems [20,25–27]. These studies were considered in the International Agency for Research on Cancer (IARC) evaluation of *Aloe vera* and summarized in Table 1 [18]. However, results from one study by Guo *et al.* [28] that was not included in the IARC evaluation have generated some uncertainty regarding the genotoxic potential of decolorized aloe material. In that study, a decolorized aloe whole leaf extract with 63 ppm of aloins was tested positive for mutagenic effect in a mouse lymphoma *tk* assay after 24 h treatment in the absence of metabolic activation [28]. Notably, positive mutagenicity was found only at dosing concentrations of 6–8 mg/mL, which is higher than the maximum of 5 mg/mL as typically recommended in regulatory testing guidelines for MLA. This decolorized aloe whole leaf extract significantly increased reactive oxygen species (ROS) levels in the cell culture system, and induced a mutation spectrum different from that of the non-decolorized aloe whole leaf extract tested in the same study. These findings have led to the concern that constituents other than HAD might be present in aloe leaves and contribute to mutagenic activities [17,28]. Since *in vivo* evidence of genotoxicity for purified *Aloe vera* preparations is scarce, the question remains as to whether commercial preparations derived from *Aloe vera* whole leaves that adhere to the IASC quality standard and contain only insignificant amounts of HAD are genotoxic.

In view of contradictory genotoxicity evidence on decolorized (*a.k.a.* purified) *Aloe vera* preparations in the literature, further studies conducted in accordance with internationally recognized genotoxicity testing recommendations [30–32], are warranted to assess whether the genotoxic effect observed *in vitro* is of toxicological relevance to human health. The present research aimed to evaluate the mutagenic and clastogenic potential of a purified *Aloe vera* whole leaf preparation containing trace HAD as unavoidable impurities in the MLA. Furthermore, a comet assay was conducted in F344 N rats to examine its

genotoxic potential *in vivo*.

2. Materials & methods

Both the comet and mouse lymphoma assays were conducted following applicable Good Laboratory Practice (GLP) regulations and in accordance with OECD Test Guidelines 489 and 490 [33,34], respectively. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.1. Test article

A commercially available purified *Aloe vera* whole leaf dry juice (Lot# 715HB10YK03) was used in both studies. The test article was supplied by Herbalife Nutrition (Torrance, CA, USA) and manufactured by Pharmachem, LLC (Descoto, TX, USA) from fresh harvested *Aloe vera* leaves. In brief, aloe whole leaves were washed and then macerated mechanically into a slurry, which was subsequently pasteurized and filtered to remove insoluble components and contaminants. The resulting juice was treated with food-grade activated charcoal, a process known as decolorization, to remove chlorophylls and anthraquinones. After removal of activated charcoal, the resulting material was subjected to additional filter presses to produce a clarified juice, which was further concentrated and dried to produce the purified *Aloe vera* whole leaf dry juice powder. The test article was a light tan color dry powder comprised of naturally occurring polysaccharides (aloe acemannan), minerals, glucose, malic acid and other organic acids. The identity of the test article was confirmed by proton nuclear magnetic resonance ($^1\text{H NMR}$) spectrometry. Quantitation of aloin A, aloin B and aloe-emodin in the test article was carried out using the validated AOAC Official Method 2016.09 with reverse-phase high performance liquid chromatography (RP-HPLC) [35]. Chemical composition of the test article is summarized in Table 2.

2.2. Mouse lymphoma *tk* assay

Test article stock solutions were prepared under subdued lighting in purified water at the maximum limit concentration of 50 mg/mL. Purified water diluted 10-fold in the culture medium was used as the negative vehicle control. The positive controls methyl methanesulphonate (MMS) in the absence of S9 and benzo[*a*]pyrene (B[*a*]P) in the presence of S9 were formulated in anhydrous analytical grade dimethyl sulphoxide (DMSO) and protected from light. The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was acquired from Molecular Toxicology Inc. (Boone, NC, USA) and prepared from male Sprague Dawley rats induced with Aroclor 1254. Lyophilized (Mutazyme™) S9 mix was reconstituted with purified water to provide a 10 % S9 mix just prior to use, and the final concentration of S9 fraction was 1% (v/v) for all cultures treated in the presence of S9. The master stock of L5178Y *tk*^{+/-} (3.7.2C) mouse lymphoma cells originated from Dr. Donald Clive (Burroughs Wellcome Co., Research

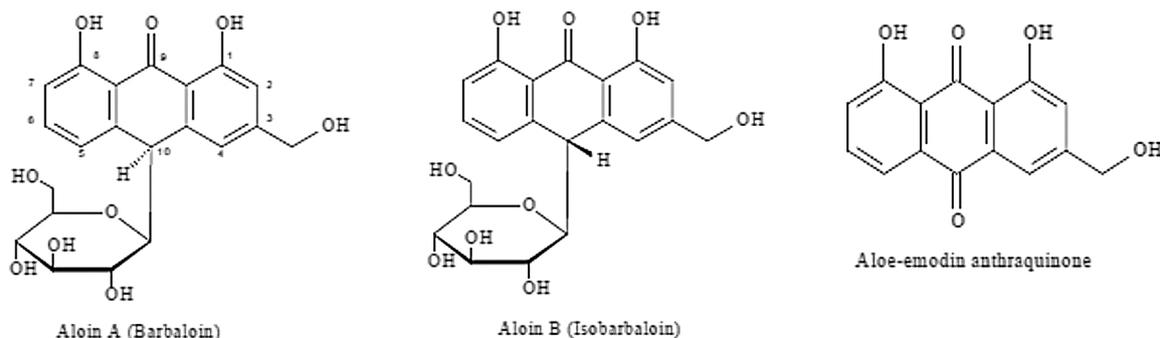


Fig. 1. Chemical Structures of Aloin A, Aloin B and Aloe-emodin.

Table 1
Reported Genetic and Related Effects of *Aloe vera* Inner Leaf, Gel and Decolorized Whole Leaf Materials (Adapted from IARC *Aloe vera* Monograph, Table 4.1, 2016).

Test system	Results		Dose (LED or HID)	<i>Aloe vera</i> preparation	Reference
	Without exogenous metabolic system	With exogenous metabolic system			
<i>Salmonella typhimurium</i> , TA100, reverse mutation	negative	negative	NR	Stabilized gel; aloin A and B ≤ 10 ppm	Sehgal et al. [29]
<i>Salmonella typhimurium</i> , TA98, TA100, TA1535, TA 1537, reverse mutation	negative	negative	10 mg/plate	Qmatrix® inner leaf fillet; aloins < 10 ppm	Williams et al. [27]
<i>Salmonella typhimurium</i> , TA98, TA100, reverse mutation	negative	negative	6 mg/plate	Decolorized whole leaf extract, aloin A at 60–200 ppm	Boudreau et al. [20]
<i>Salmonella typhimurium</i> , TA97, TA98, TA100, TA1535, reverse mutation	negative	negative	10 mg/plate	Gel, aloin A at 1100–1400 ppm	Boudreau et al. [20]
<i>Salmonella typhimurium</i> , TA98, TA100, reverse mutation	negative	negative	21 × initial concentration ^a	Decolorized whole leaf extract; aloin A at 0.868 ppm, aloin B at 1.335 ppm, and aloe-emodin at 0.2 ppm	Sehgal et al. [26]
<i>Escherichia coli</i> , WP2 <i>uvrA</i> /pKM101	negative	negative	6 mg/plate	Decolorized whole leaf extract, aloin A at 60–200 ppm	Boudreau et al. [20]
<i>Escherichia coli</i> , WP2 <i>uvrA</i> /pKM101	negative	negative	3 mg/plate	Gel, aloin A at 1100–1400 ppm	Boudreau et al. [20]
<i>Escherichia coli</i> , SOS DNA damage repair assay	negative	negative	10 × initial concentration ^a	Stabilized gel; aloin A and B ≤ 10 ppm	Sehgal et al. [29]
<i>Escherichia coli</i> , SOS DNA damage repair assay	negative	negative	21 × initial concentration ^a	Decolorized whole leaf extract; aloin A at 0.868 ppm, aloin B at 1.335 ppm, and aloe-emodin at 0.2 ppm	Sehgal et al. [26]
Chromosomal aberrations, Chinese hamster lung cells	negative	negative	10 mg/plate	Qmatrix® inner leaf fillet; aloins < 10 ppm	Williams et al. [27]
Mouse lymphoma L5178Y/TK ^{+/-} cells	positive	negative	6 mg/mL	Decolorized whole leaf extract; aloin A at 63 ppm	Guo et al. [28]
Male ICR mice, micronucleus formation in bone-marrow cells	negative		5000 mg/kg bw, po	Qmatrix® inner leaf fillet; aloins < 10 ppm	Williams et al. [27]

LED, lowest effective dose; HID, highest ineffective dose; NR, not reported; po, per oral.

^a Dosing concentrations as reported in the original publication, and dosing concentrations in mg/mL or mg/plate cannot be deduced based on the information reported.

Table 2
Chemical Composition of Purified *Aloe vera* Whole Leaf Dry Juice.

Test	Result
Appearance	Light tan free flowing powder
pH	4.59
Moisture content (w/w%)	4%
Polysaccharides (w/w%)	5.01 %
Malic acid (w/w%)	13.98 %
Citric acid (w/w%)	9.0 %
Isocitric acid lactone (w/w%)	4.6 %
Lactic acid (w/w%)	5.22 %
Glucose (w/w%)	9.82 %
Fructose (w/w%)	3.37 %
Potassium (mg/g)	66.69
Calcium (mg/g)	61.05
Total of aloin A and B ^a (ppm)	0.3
Aloe-emodin ^a (ppm)	ND
Cadmium (ppm)	< 0.1
Arsenic (ppm)	1.7
Lead (ppm)	0.01
Mercury (ppm)	< 0.5

ND, not detected; ^a LOD =0.01 ppm, LOQ =0.02 ppm.

Triangle, NC, USA) and were stored as frozen working stocks in liquid nitrogen. Each batch of frozen cells was purged of *tk*^{-/-} mutants and checked for spontaneous mutant frequency (MF) and affirmed that they were mycoplasma free. For each experiment, frozen stocks were thawed rapidly, the cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640; Life Technologies, Carlsbad, CA, USA) containing L-glutamine and HEPES supplemented with heat-inactivated horse serum, penicillin and streptomycin, pluronic and sodium pyruvate and incubated at 37 ± 1 °C under 5% (v/v) CO₂ in air for growth.

The test article was assessed in a cytotoxicity range-finder and a mutation experiment, each conducted under three test conditions, 3 h with and without S9 and 24 h without S9. In the range-finder

experiment, the final concentrations of test article ranged from 156.3–5000 µg/mL. Only single cultures were used for test article and vehicle treatment controls and no positive controls were included. In the mutation experiment, the final concentrations of test article ranged from 250 to 5000 µg/mL. Duplicate cultures were used except for positive controls, for which single cultures were used at two independent concentrations. During the exposure period of both experiments, approximately 1 × 10⁷ cells were placed in sterile centrifuge tubes for the 3 h treatments and approximately 4 × 10⁶ cells were placed in sterile tissue culture flasks for the 24 h treatment. For treatment cultures, 2 mL of vehicle or test article formulation or 0.2 mL of positive control solution (plus 1.8 mL of purified water) were added. For 3 h treatments, S9 mix or 150 mM KCl was added. After the exposure period, cells were centrifuged (200 × g), washed with tissue culture medium and resuspended in RPMI 10 with an adjusted density of 2 × 10⁵ cells/mL. Solubility of the test article in culture medium was assessed at the beginning and end of each treatment by the observation of visible precipitation. Osmolality and pH measurements in pre- and post-treatment media were taken in the cytotoxicity range-finder experiment.

During the mutation expression period, cells were transferred to tissue culture flasks (where necessary) and incubated for two days during which the *tk*^{-/-} mutations were expressed. At the end of the expression period, cytotoxicity was assessed in the range-finder experiment by measuring suspension growth (SG) and relative suspension growth (RSG). In the mutation experiment, cytotoxicity was measured by relative total growth (RTG). Cell densities in the cultures were adjusted to 1 × 10⁴ cells/mL, and then plated for viability and trifluorothymidine (TFT) resistance. Viability was evaluated by plating 0.2 mL cell suspension at 8 cells/mL of each culture into each well of two 96-well microtitre plates (an average of 1.6 cells/well), which were incubated at 37 ± 1 °C in a humidified atmosphere with 5% CO₂ until scoreable (12–13 days). Mutation (TFT resistance) was assessed by plating 0.2 mL cell suspension at 1 × 10⁴ cells/mL into each culture of

four 96-well microtitre plates (an average of 2000 cells/well) in medium containing TFT at a final concentration of 3 µg/mL; the plates were incubated until scoreable (14 days).

The total number of wells containing a colony were scored initially for viability and mutation. For the mutation plates, the number of wells containing large colonies and those containing small colonies were scored for the negative and positive control cultures, and for any test article concentrations that induced an MF exceeding the sum of the vehicle control MF plus the Global Evaluation Factor (GEF). For microwell assays, the GEF is defined as 126 mutants per 10⁶ viable cells according to Moore *et al.* [36]. The relevance of increases in MF (total wells with colonies) by comparison with concurrent controls and the GEF was evaluated according to the recommendations of the Mouse Lymphoma Assay Workgroup [36]. Linear regression was performed on the ranked MF against ranked dosing concentrations to test for a linear trend. The assay acceptance and evaluation criteria were applied in adherence to OECD Guideline 490.

2.3. Comet assay

Seven to eight-week-old male Fischer 344/N Hsd rats (Envigo Laboratories, Frederick, MD, USA) were used in the comet assay. The male animals of this model was selected because 1) F344/N rats were tested in the National Toxicology Program (NTP) 2-year carcinogenicity study previously which found clear evidence of carcinogenicity in the colon for the orally administered non-decolorized *Aloe vera* whole leaf extract; and 2) a more pronounced effect was observed in males as compared to females in the study [20]. The colon and kidney were selected as target organs for comet analysis. The kidney was examined because orally administered aloe-emodin was previously shown to cause increases in DNA strand breaks in male Oncins France 1(OFl) mice [37]. Systemic exposure to the test article or HAD was not measured because 1) the levels of aloins and aloe-emodin in the test article were very low and non-detectable, respectively; 2) *in vivo* carcinogenicity associated with ingestion of *Aloe vera* and its HAD has only been detected in the colon (the site of direct contact) in the F344 rat model; and 3) ingested aloins have very low oral bioavailability [38,39].

Animals were housed with two to three per cage, fed Certified Purina Pico Chow No. 5002 (Ralston Purina Co., St. Louis, MO, USA) with access to reverse osmosis treated tap water *ad libitum* in a controlled-temperature room (23 ± 1 °C) with a 12-h dark/light cycle. All husbandry procedures were in compliance with the United States Department of Agriculture's (USDA 2017) Animal Welfare Act Regulations, 9 CFR 1, and animals were handled and treated according to the Guide for the Care and Use of Laboratory Animals [40].

The oral doses of purified *Aloe vera* whole leaf dry juice tested were 500, 1000 and 2000 mg/kg bw/day. Dose selection was based on the results of a 90-day oral toxicity study of the same material (different lot) in F344 rats in which the no-observed-adverse-effect-level (NOAEL) was found to be >1845 mg/kg bw/day, the highest dose tested [41], and designed to meet the limit dose requirement for non-toxic test materials as per OECD Guideline 489. The dose formulations were prepared in deionized water at concentrations of 50, 100, and 200 mg/mL. Deionized water was used as the vehicle control. The positive control, ethyl methanesulphonate (EMS), was prepared in 0.9 % saline daily at a concentration of 15 mg/mL and administered at an oral dose of 150 mg/kg bw/day.

After a 7-day acclimation period, 25 animals were randomly assigned to one of the five groups (n = 5/group) using a stratification procedure to ensure that mean body weight was not statistically different among the groups. Dose formulations were administered *via* oral gavage at a dose volume of 10 mL/kg body weight for two consecutive days. Animals were monitored twice daily for mortality and moribundity. Clinical observations were performed daily prior to dose administration and at termination. Cage side observations were performed at 1 h ± 30 min post dose. Body weights of individual animals

were measured within two days of arrival, for allocation, prior to daily dose administration, and at termination. Approximately 3 h ± 30 min after the final dose administration on Day 2, animals were euthanized for tissue collection.

For the colon tissue collection, the entire colon was removed from each animal. Cold mincing solution (magnesium- and calcium-free Hanks Balanced Salt Solution, 10% v/v DMSO, and 20 mM EDTA, pH 7.4–7.7, Invitrogen, Carlsbad, CA, USA) was injected into the colon to facilitate the removal of fecal material. A 4–5 cm length of colon was removed from the distal end and flushed extensively with mincing solution from both directions. The distal section of colon was cut longitudinally and placed in cold mincing solution, then transferred to a dish, scraped lightly and rinsed into the same dish with 1–2 mL of mincing solution. The tissue was transferred to a new dish and scraped with more pressure to harvest the epithelium. The scraped colon epithelial tissue was rinsed over the dish with ~500 µL of mincing solution and the spatula was rinsed with ~500 µL to remove any adhering cells. The mincing solution in the dish containing the epithelial cells was pipetted up and down several times to break up clumps of cells, then aliquoted into two microfuge tubes and placed on ice. For the kidney tissue collection, a section was collected from the center of the right kidney of each animal. Two small sections of kidney tissue were placed in separate microcentrifuge tubes containing 1 mL of cold mincing solution and rapidly minced to generate duplicate samples. All tissue samples were flash frozen in liquid nitrogen and stored at or below –80 °C in a freezer until processed.

For each animal, one of the duplicate tubes containing frozen tissue samples was transferred from the –80 °C freezer to dry ice, thawed in room temperature water bath, and then placed immediately on wet ice. Samples remained on wet ice for the duration of the slide making process. A portion of the cell suspension of each tissue sample was diluted with 0.5 % NuSieve GTG low melting point agarose (Lonza, Morristown, NJ, USA), dissolved in phosphate buffer at 37 °C, and layered onto duplicate commercially available Flare™ slides (Trevigen, Gaithersburg, MD, USA). The volume of the cell suspension did not decrease the percentage of low melting point agarose by more than 10% (*i.e.*, not below 0.45%). The slides were immersed in chilled lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, with 10 % DMSO and 1% Triton X-100 added fresh) overnight in a refrigerator under a light proof condition. After this incubation period, the slides were rinsed in neutralization solution (0.4 M Trizma base, pH 7.5) to remove residual detergent and salts prior to the alkali unwinding step. Slides were randomly placed onto the platform of a submarine-type electrophoresis unit and submerged in cold alkaline electrophoresis solution (300 mM NaOH, 1 mM Na₂EDTA; pH > 13). The slides were left to unwind under refrigerated conditions for 20 min, then electrophoresed at 4 °C for 20 min at 25 V (0.7 V/cm) with a current of approximately 300 mA. After electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for 5 min and then dehydrated by immersion in absolute ethanol (≥ 99.6 %, Pharmco-AAPER, Shelbyville, KY, USA) for 5 min and allowed to air dry. Slides were stored at room temperature in a desiccator until stained and scored.

Air-dried slides were stained with SYBR™ Gold (Invitrogen, Carlsbad, CA, USA) and 150 cells were scored per sample at 200x total magnification using Comet IV Image Analysis Software (Version 4.3.2, Instem, UK). Slides were coded and scored for the percentage of migrated DNA (% tail DNA) without knowledge of their identity. The % tail DNA endpoint measurement was used to characterize the extent of DNA migration, calculated as the intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage. “Hedgehogs” (*i.e.*, small or no visible head, or the head and tail appear separate) were tabulated but not scored.

The final body weight, body weight gain, and DNA damage results were analyzed using Statistical Analysis System version 9.2 (SAS institute, Cary, NC, USA). Homogeneity of variance was analyzed using the Levene's test, and normality was assessed using the Shapiro-Wilk test.

Homogeneous data were analyzed using one-way analysis of variance (ANOVA) and test article-treated groups were compared to the concurrent vehicle control group using a one-tailed Dunnett's test. Dose-dependent changes were evaluated using a linear regression model. Data that were not homogeneous and normally distributed were transformed using a log transformation and then reanalyzed. Positive control data were analyzed by the Student *t*-test. The body weight and body weight gain data are presented as mean \pm standard deviation (SD) for each group. For % tail DNA, the median value from the 150 cells scored was calculated for each animal, and then group means and standard deviations were calculated. The assay acceptance and evaluation criteria were applied in adherence to OECD Guideline 489.

3. Results

3.1. Mouse lymphoma tk assay

The MLA performed under the described experimental conditions met the acceptance criteria in accordance with OECD 490. For the concurrent vehicle controls, the proportions of small colony mutants in the absence and presence of S9 ranged from 33% to 46%, and mean MF were 76.48, 107.05 and 87.73 per 10^6 viable cells for the 3 h treatments in the absence and presence of S9 and 24 h treatment in the absence of S9, respectively. Substantial increases in the number of both small and large colony mutants were observed following the treatment with positive control chemicals MMS and B[a]P, with the mean total MF rising above 300 per 10^6 viable cells. The values from negative and positive control groups were concordant with the laboratory's historical control data under similar experimental conditions. No marked changes in osmolality or pH were observed in the culture medium treated with the test article at the highest concentration (5000 $\mu\text{g/mL}$) compared to the concurrent vehicle control. The results of cytotoxicity range-finder experiment and mutation experiment are presented in Tables 3 and 4, respectively.

In the range-finder experiment, among six tested concentrations ranging from 156.3–5000 $\mu\text{g/mL}$, no post-treatment precipitate was observed in the absence of S9, but post-treatment precipitate was observed at the two highest concentrations in the presence of S9 (2500 and 5000 $\mu\text{g/mL}$) for the 3 h treatment. No post-treatment precipitate was observed after the 24 h treatment in the absence of S9 for nine tested concentrations ranging from 19.53–5000 $\mu\text{g/mL}$. The test article was not cytotoxic, as indicated by the 3 h treatment RSG values of 88% and 91% for the highest analyzable concentrations of 5000 and 2500 $\mu\text{g/mL}$ in the absence and presence of S9, respectively. The RSG value for the highest concentration tested (5000 $\mu\text{g/mL}$) was 81 % after the 24 h treatment in the absence of S9 (Table 3).

In the mutation experiment, following the 3 h treatment period, post-

Table 3
Relative Suspension Growth in Range-Finder Experiment.

Concentration ($\mu\text{g/mL}$)	3 h Treatment – % RSG		Concentration ($\mu\text{g/mL}$)	24 h Treatment – %RSG –S9
	–S9	+S9		
0	100	100	0	100
156.3	90	95	19.53	91
312.5	92	98	39.06	90
625	89	87	78.13	90
1250	92	90	156.3	89
2500	88	91 pp	312.5	82
5000	88	NE pp	625	87
			1250	85
			2500	87
			5000	81

%RSG, percent relative suspension growth; pp, precipitation noted at end of treatment incubation period.

NE, not evaluated due to observation of precipitate.

treatment precipitate was observed at the two highest concentrations in the presence of S9 (3000 and 5000 $\mu\text{g/mL}$) but no post-treatment precipitate was observed in the absence of S9. Therefore, the highest analyzable concentrations for the 3 h treatment were 5000 $\mu\text{g/mL}$ in the absence of S9 and 3000 $\mu\text{g/mL}$ (the lowest precipitating concentration) in the presence of S9. Following the 24 h treatment in the absence of S9, no post-treatment precipitate was observed at any concentration and 5000 $\mu\text{g/mL}$ was the highest concentration analyzed. The RTG values were 67 % and 133% at the highest analyzable concentrations following the 3 h treatments in the absence and presence of S9, respectively. The RTG value following the 24 h treatment in the absence of S9 was 64 % at 5000 $\mu\text{g/mL}$. Under each treatment condition, the MF at the test article concentrations plated, with or without S9, were all less than the sum of the mean MF of the vehicle control plus the GEF, and the linear trend tests on MF were non-significant (Table 4), indicating a negative result under each treatment condition.

3.2. Comet assay

All animals survived to the scheduled termination without showing signs of moribundity. There were no abnormal observations in the animals that received the test article or 150 mg/kg bw/day EMS except for one animal in the 2000 mg/kg bw/day dose group that had diarrhea at 1 h post-dose on Day 2. No statistically significant changes were observed in final body weight or body weight gain of the groups administered purified aloe dry juice compared to the concurrent vehicle control, while EMS resulted in a statistically lower mean body weight gain compared to the vehicle control group (Table 5).

The % tail DNA results for the colon and kidney are presented in Fig. 2. EMS as the positive control resulted in a statistically significant increase in % tail DNA with respect to the vehicle control ($p < 0.05$) in both tissues. Orally administered purified *Aloe vera* whole leaf dry juice up to 2000 mg/kg bw/day did not cause a statistically significant induction of DNA strand breaks in the colon epithelial cells or kidney cells compared to the concurrent vehicle control ($p > 0.05$). A statistically significant decrease in DNA damage was observed in the kidney cells of the group that received 1000 mg/kg bw/day ($p = 0.01$). This decrease in DNA damage in the mid-dose group coupled with the lack of any dose response is not deemed to be biologically relevant. Since none of the test article treated groups exhibited a statistically significant increase in DNA damage for either kidney or colon, there was no dose-related response, and the values were consistent with laboratory's historical control data, the comet assay was interpreted as negative in these tissues under the experimental condition.

4. Discussion

Mutagenic and genotoxic potential of various *Aloe vera* preparations (whole leaf or inner leaf) has been investigated in prokaryotic and eukaryotic test systems with and without metabolic activation [18]. Non-decolorized aloe whole leaf preparation as well as aloe HAD were found to be mutagenic in both *in vitro* and *in vivo* assays [17–19,42,43,37,44,45]. With regard to aloe preparations containing trace or no anthraquinones; such as purified aloe whole leaf preparations, inner leaf gel and aloe acemannan; bacterial mutation assays have consistently shown negative outcomes [20,26,27,29,46]. However, results of *in vitro* studies in mammalian cell models appear to be limited and inconsistent. Williams *et al.* [27] demonstrated that an *Aloe vera* inner leaf gel material with <10 ppm aloins (Qmatrix®) did not induce chromosomal aberrations in Chinese hamster lung cells, whereas a decolorized aloe whole leaf extract with 63 ppm of aloin A was found to be mutagenic in a MLA at dosing concentrations of 6000–8000 $\mu\text{g/mL}$ in the absence of S9 [28]. Of note, the level of aloin impurities in the material tested by Guo *et al.* [28] was ~6 times higher than the industry standard set by IASC for *Aloe vera* leaf juice preparations intended for oral consumption.

In light of the discrepancies in the outcome among *in vitro*

Table 4
Relative Total Growth and Mutant Frequency in Main Experiment.

3 h Treatment –S9			3 h Treatment +S9			24 h Treatment –S9		
Concentration (µg/mL)	%RTG	MF	Concentration (µg/mL) ^a	%RTG	MF	Concentration (µg/mL)	%RTG	MF
0	100	76.48	0	100	107.05	0	100	87.73
250	81	87.52	250	138	93.42	250	111	69.15
500	84	70.84	500	123	89.19	500	85	107.51
1000	74	91.14	750	118	91.16	1000	97	85.55
2000	83	94.26	1000	114	86.08	2000	92	89.27
3000	80	89.06	1500	135	88.50	3000	86	86.39
4000	73	97.10	2000	163	77.43	4000	75	103.28
5000	67	82.73	2500	134	73.32	5000	64	91.63
MMS 15	44	437.21	3000pp	133	72.99	MMS 5	44	811.51
MMS 20	38	539.00	B[a]P 2	60	615.15	MMS 7.5	33	980.42
			B[a]P 3	43	1110.29			

MF, mutant frequency; RTG, relative total growth; pp, precipitation observed at end of treatment incubation period.

^a A concentration of 5000 µg/mL was tested in the presence of S9 but discarded following the treatment incubation period due to precipitation; Linear trend test on MF for 3 h treatment –S9: p-value = 0.1165, not significant; Linear trend test on MF for 3 h treatment +S9: p-value = not significant (negative trend); Linear trend test on MF for 24 h treatment: p-value = 0.1759, not significant.

Table 5
Body Weight and Changes in Male F344 Rats.

Dose Level (mg/kg bw/day)	Initial Body Weight (g, mean ± SD) ^a	Final Body Weight (g, mean ± SD) ^a	Body Weight Change (g, mean ± SD) ^a
0	160.7 ± 5.4	167.0 ± 5.9	6.3 ± 1.2
500	159.9 ± 6.5	165.1 ± 8.5	5.2 ± 3.0
1000	160.4 ± 5.7	163.8 ± 6.9	3.4 ± 3.6
2000	163.7 ± 5.9	168.7 ± 5.1	5.0 ± 1.3
EMS – 150	162.0 ± 6.5	158.2 ± 7.0	–3.9 ± 2.7 ^b

SD, standard deviation.

^a Calculated from individual animal data.

^b Statistically significant difference compared to the concurrent vehicle control (p < 0.0001).

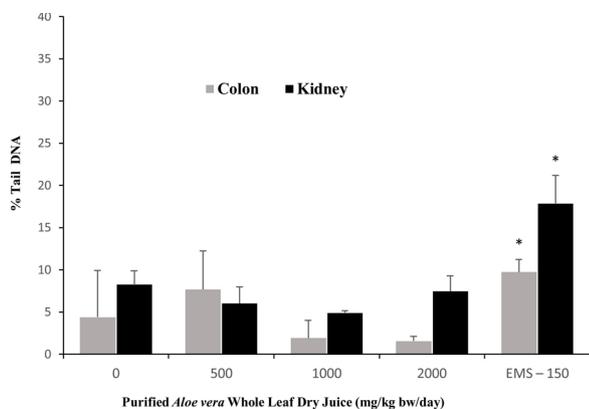


Fig. 2. Comet assay mean % tail DNA (mean ± SD) in male F344N rats (n = 5 animals/group; 750 cells/group/tissue) after oral exposure to purified aloe whole leaf dry juice. * P < 0.05 compared to the concurrent vehicle control.

genotoxicity assays of decolorized *Aloe vera* preparations in the literature, we evaluated a purified *Aloe vera* whole leaf dry juice following the EFSA (2012) and OECD [32] recommended genotoxicity testing strategy, which included the OECD guideline compliant *in vitro* MLA and *in vivo* comet assay with an appropriate animal model and target organs. The MLA was intentionally selected in lieu of other *in vitro* mammalian cell assays because of the positive mutagenicity found by Guo *et al.* [28] in the same assay. The present study demonstrated that the purified *Aloe vera* whole leaf dry juice with 0.3 ppm of total aloins and non-detectable aloe-emodin did not induce mutations in the MLA at concentrations up to 5000 µg/mL (the maximum concentration recommended for testing

mixtures according to OECD 490) in the absence of S9 and up to the precipitating concentration of 3000 µg/mL in the presence of S9 under the experimental conditions. This negative finding is consistent with that of Guo study in which no mutagenicity was detected for its decolorized aloe whole leaf extract up to 5000 µg/mL. Nevertheless, there are notable differences between the two studies. The purified aloe whole leaf dry juice in the present study contained substantially lower levels of aloins than the extract in the Guo study (0.3 vs. 63 ppm). The decolorized aloe extract in the Guo study markedly reduced pH and raised osmolality in the culture medium, whereas the test article in the present study did not cause these effects at similar dosing concentrations. Additionally Guo *et al.* found that its decolorized aloe extract induced substantial ROS in the culture system, which appeared to coincide with increases in MF at very high dosing concentrations. Emodin, a structural related HAD, was found to increase ROS levels and cause DNA damage in the cultured human peripheral blood lymphocytes (HPBLs) *in vitro* [47], suggesting that aloe HAD-induced ROS may contribute to the genotoxic effect in the test system. This raises further questions as to whether mutagenic response observed by Guo *et al.* is, at least in part, an indirect outcome of excessive oxidative stress, a condition known to compromise genome stability and cause damage of DNA and chromosomes in the cell culture system [48].

Considering that cell culture systems have inherent deficiencies (impaired DNA repair capability, lack of antioxidant enzymes and ADME) and are more susceptible to false positive outcomes, *in vivo* tests offer more relevant evidence for extrapolation to humans with respect to cancer risk. An appropriately performed comet assay is regarded as a useful tool to evaluate *in vivo* genetic toxicity as a follow-up to positive or equivocal *in vitro* findings and as a secondary measure of genotoxicity in a tissue other than bone marrow to inform potential carcinogenicity risk in humans [30,49–51]. *In vivo* evidence related to genotoxic effects of *Aloe vera* material is scarce. A search of the literature yielded only one published study, an *in vivo* micronucleus assay evaluating orally administered *Aloe vera* inner leaf gel with < 10 ppm aloins (Qmatrix®) in the Institute for Cancer Research (ICR) mice [27], which found a negative outcome for induction of micronuclei in bone marrow cells. Since ingested aloins and aloe-emodin have very poor bioavailability, and systemic exposure to the test article or aloins was not measured by Williams *et al.*, its negative findings could be due to a lack of exposure in the bone marrow compartment [17,52]. The comet assay is increasingly being used to evaluate DNA damaging or protective effects of natural botanicals added to food products [53–55]. In the present comet assay, purified *Aloe vera* whole leaf dry juice at oral doses of up to 2000 mg/kg bw/day (the limit dose for nontoxic materials according to OECD 489) did not induce DNA strand breaks in the cells isolated from either the kidney or colon epithelium of male F344 rats. It should be noted that

comet assays conducted in F344 rats and reporting data in the colon and kidney tissues are dearth in the literature, so do the historical control data. O'Donoghue *et al.* [55] reported a comet assay in F344 rats, in which the % tail DNA values for the colon and kidney appear to be slightly lower for the vehicle control, and are comparable for the EMS treated positive control when compared to the values observed in the present assay. Since within-assay variance is very small for both studies, different methods and procedures of tissue sampling and handling could conceivably account for the discernable variability between different laboratories. Nevertheless, the results from the present comet assay clearly corroborate the negative findings from the MLA performed using the same batch of test material, and provide further evidence that oral exposure to purified aloe preparation may not cause genetic toxicity *in vivo*.

In the NTP carcinogenicity study exposure to non-decolorized *Aloe vera* whole leaf extract *via* drinking water significantly increased the incidence of adenoma and carcinoma in the large intestine of F344 rats [20]. This carcinogenic effect on the colon was later reproduced in the 13-week study using the same animal model in which preneoplastic lesions in the mucosal and goblet cells of the large intestine were observed following the oral administration of aloins [52]. Notably, no treatment-related cancerous lesions were observed in any internal organs in these studies of non-decolorized aloe whole leaf extract. Coupled with the findings from Guo *et al.* [28] showing that mutagenic activity only occurred in the absence of metabolic activation, the evidence strongly suggests that the genotoxic target organ linked to aloe whole leaf is the site of contact. Typically, the stomach and upper intestinal tract would be considered suitable target organs for evaluation in the comet assay for orally administered substances that may act at the site of direct contact [33]. However, these sites do not appear to be the carcinogenic targets as evidenced by the absence of neoplastic lesions in the forestomach and small intestine of F344 rats in the NTP carcinogenicity study. Moreover, there is no evidence that other structurally related HAD compounds (e.g., emodin, sennosides, danthron) exhibited carcinogenic effect on the stomach or small intestine when administered orally in rodent cancer bioassays [56–60]. These findings support that the colon, rather than the stomach and small intestine, is the most relevant target organ in the present comet assay.

With respect to the kidney, Nesslany *et al.* [37] found that oral administration of aloe-emodin at 2000 mg/kg bw/day induced DNA damage in kidney cells of male OF1 mice. In our comet assay no genotoxicity was detected in the kidney of male F344 rats treated with the purified aloe whole leaf dry juice. This is not surprising considering that the test article did not contain any detectable level of aloe-emodin. The negative outcome from the present study supports that oral exposure to purified aloe preparation may not pose carcinogenic risk for the kidney. This appears to corroborate with the observations in the NTP carcinogenicity study in which aloe whole extract containing aloe-emodin as high as 71 ppm did not induce any neoplastic lesions in the kidney of F344 rats.

One potential limitation in our research is related to the maximum dosing concentration employed in the MLA. The OECD Guideline 490 recommends the limit dose concentration in the MLA to be the lower of 10 mM, 2 mg/mL, or 2 μ L/mL, or determined by a precipitating or limiting cytotoxicity concentration. However, for mixtures of uncertain composition, the top concentration may need to be higher (e.g., 5 mg/mL) in the absence of sufficient cytotoxicity in order to increase the exposure to individual components of the test article. In our work, the purified aloe whole leaf dry juice was tested only up to 5 mg/mL in the absence of S9, at which level it did not induce significant cytotoxicity or cause interfering precipitation in the cell culture. Since Guo *et al.* [28] found that positive responses in the absence of S9 occurred only at higher concentrations (6 mg/mL and above) of the decolorized aloe extract, the data from the present MLA alone might not be sufficient to rule out the effects observed at higher dosing concentrations by Guo *et al.* Instead of testing higher dose concentrations in the MLA, we opted

to perform *in vivo* comet assay to assess genotoxicity potential in a relevant rat model and at target organs germane to carcinogenic effects of aloe whole leaf to assure that an outcome with clinical relevance to human exposure could be achieved.

5. Conclusions

In summary, the results from current research demonstrate the absence of genotoxicity for a purified *Aloe vera* whole leaf dry juice containing insignificant amount of HAD in the mouse lymphoma *tk* assay under testing conditions compliant with the OECD 490 guideline. Furthermore, *in vivo* comet assay conducted per the OECD 489 guideline confirms that this material did not induce DNA damage in the colon and kidney of male F344 rats, reflecting an appropriate *in vivo* model and target organs relevant to human cancer risk associated with consumption of aloe whole leaf and/or its HAD constituents. The evidence generated herein is of important value in informing hazard identification and carcinogenicity risk assessment pertaining to purified aloe leaf preparations containing very low levels of HAD as technically unavoidable impurities. Nonetheless, further research is likely warranted to elucidate the mechanism(s) of the carcinogenic effect of non-decolorized *Aloe vera* whole leaf preparation or aloe HAD observed in the rat model in order to fully comprehend their relevance to dietary exposure to *Aloe vera* products and to devise appropriate risk mitigation strategies.

CRedit authorship contribution statement

Jiang Hu: conceptualization, methodology, original manuscript preparation. **Mel Lloyd:** study director, methodology, data interpretation and reporting for the mouse lymphoma assay, manuscript reviewing and editing. **Cheryl Hobbs:** methodology and execution, data interpretation for the comet assay, manuscript reviewing and editing. **Peter Cox:** formulation analysis for the mouse lymphoma assay. **Keith Burke:** formulation analysis for the comet assay. **Gareth Pearce:** methodology and execution for the mouse lymphoma assay. **Michael A. Streicker:** study director for comet assay. **Quanyin Gao:** test article characterization. **Vasilios Frankos:** conceptualization, methodology.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgement

Funding for this research was provided by Herbalife Nutrition, a manufacturer of nutritional products that contain purified *Aloe vera* whole leaf dry juice. The conduct, data collection and interpretation of the mouse lymphoma *tk* and comet assays were carried out independently by Covance Laboratories and Integrated Laboratory Systems, respectively.

References

- [1] I. Mehta, "History of *Aloe vera*" – (a magical plant), *IOSR J. Humanities and Soc. Sci.* 22 (8) (2017) 4.
- [2] R. Upton, *American Herbal Pharmacopoeia Monograph: Aloe vera Leaf, Aloe vera Leaf Juice, Aloe vera Inner Leaf Juice*, American Herbal Pharmacopoeia, Scotts Valley, CA, 2012.
- [3] K. Eshun, Q. He, *Aloe vera*: a valuable ingredient for the food, pharmaceutical and cosmetic industries - a review, *Crit. Rev. Food Sci. Nutr.* 44 (2) (2004) 91–96, <https://doi.org/10.1080/10408690490424694>.

- [4] U. Nandal, R.L. Bhardwaj, *Aloe vera* for human nutrition, health and cosmetic use - a review, *Int. Res. J. Plant Sci.* 3 (3) (2012) 38–46.
- [5] T. Reynolds, A.C. Dweck, *Aloe vera* leaf gel: a review update, *J. Ethnopharmacol.* 68 (1–3) (1999) 3–37.
- [6] G. Kaithwas, P. Singh, D. Bhatia, Evaluation of *in vitro* and *in vivo* antioxidant potential of polysaccharides from *Aloe vera* (*Aloe barbadensis miller*) gel, *Drug Chem. Toxicol.* 37 (2) (2014) 135–143, <https://doi.org/10.3109/01480545.2013.834350>.
- [7] P. Pressman, R. Clemens, A.W. Hayes, *Aloe vera* at the frontier of glycolipids and integrative medicine: health implications of an ancient plant, *SAGE Open Medicine* 7 (2019), <https://doi.org/10.1177/2050312119875921>.
- [8] S. Sadoyu, C. Rungruang, T. Wattanavijitkul, R. Sawangjit, A. Thakkinstant, N. Chaiyakunapruk, *Aloe vera* and health outcomes: an umbrella review of systematic reviews and meta-analyses, *Phytother. Res.* 35 (2) (2020) 1–22.
- [9] M. Sánchez, E. González-Burgos, I. Iglesias, M.P. Gómez-Serranillos, Pharmacological update properties of *Aloe vera* and its major active constituents, *Molecules* (Basel, Switzerland) 25 (6) (2020) 1324, <https://doi.org/10.3390/molecules25061324>.
- [10] N. Suksomboon, N. Poolsup, S. Punthanitarn, Effect of *Aloe vera* on glycaemic control in prediabetes and type 2 diabetes: a systematic review and meta-analysis, *J. Clin. Pharm. Ther.* 41 (2) (2016) 180–188, <https://doi.org/10.1111/jcpt.12382>.
- [11] M. Fouillaud, Y. Caro, M. Venkatchalam, I. Grondin, L. Dufossé, Anthraquinones, in: L.M.L. Nollet, J.A. Gutierrez-Urbe (Eds.), *Phenolic Compounds in Food - Characterization and Analysis*, CRC Press, 2018 p 131.
- [12] EFSA Panel on Dietetic Products Nutrition and Allergies, Scientific opinion on the substantiation of a health claim related to hydroxyanthracene derivatives and improvement of bowel function pursuant to article 13(5) of regulation (EC) No 1924/2006, *EFSA J.* 11 (10) (2013) 3412, <https://doi.org/10.2903/j.efa.2013.3412>.
- [13] EMA, in: E.M. Agency (Ed.), *European Union Herbal Monograph on Aloe barbadensis Mill. and on Aloe (Various Species, Mainly Aloe ferox Mill. and Its Hybrids), folii Succus Siccatus*, 2017 p 9.
- [14] Health Canada, in: Health Canada (Ed.), *Natural Health Product Monograph - Aloe vera Oral*, 2018.
- [15] M.K. Park, J.H. Park, N.Y. Kim, Y.G. Shin, Y.S. Choi, J.G. Lee, K.H. Kim, S.K. Lee, Analysis of 13 phenolic compounds in *Aloe species* by high performance liquid chromatography, *Phytochem. Anal.* 9 (4) (1998) 186–191.
- [16] D. Sacchi, P. Bogoni, G. Prociada, *Aloe* exudate: characterization by reversed phase HPLC and headspace GC-MS, *J. Agric. Food Chem.* 49 (10) (2001) 4526–4530, <https://doi.org/10.1021/jf010179c>.
- [17] EFSA Panel on Food Additives and Nutrient Sources added to Food, Safety of hydroxyanthracene derivatives for use in food, *EFSA J.* 16 (1) (2018) e05090, <https://doi.org/10.2903/j.efa.2018.5090>.
- [18] IARC, *Aloe Vera - Carcinogenicity of Some Drugs and Herbal Products*, vol 108, International Agency for Research on Cancer, 2016.
- [19] F. Lanouar, I. Boughattas, M. Mkhini, N. Bousserrhine, M. Banni, Evidence of the genotoxic effect of aloin on earth worms *Eisenia andrei*, *Focus on Sci.* 3 (2017) 1–6, <https://doi.org/10.21859/focsci-03041467>.
- [20] M.D. Boudreau, F.A. Beland, J.A. Nichols, M. Pogribna, Toxicology and carcinogenesis studies of a nondecolorized [corrected] whole leaf extract of *Aloe barbadensis miller* (*Aloe vera*) in F344/N rats and B6C3F1 mice (drinking water study), *Nat. Toxicol. Program Tech. Rep. Ser.* 577 (2013) 1–266.
- [21] K.S. Ahlawat, B.S. Khatkar, Processing, food applications and safety of *Aloe vera* products: a review, *J. Food Sci. Technol.* 48 (5) (2011) 525–533, <https://doi.org/10.1007/s13197-011-0229-z>.
- [22] IASC, *Aloe vera* Processing, 2012, in: <https://www.iasc.org/Resources/AloeVeraProcessing.aspx>.
- [23] IASC, Certification Program - Policies & Operational Procedures, 2019, https://www.iasc.org/Portals/19/Documents/Certification/19_0730_IASC%20Certification%20Program_SOP%20Final.pdf?ver=2019-07-30-161006-300.
- [24] European Commission, Draft COMMISSION REGULATION (EU) Amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as Regards Botanical Species Containing Hydroxyanthracene Derivatives, *Comitology Register* <https://ec.europa.eu/transparency/comitology-register/screen/documents/067676/3/consult?lang=en>. Accessed December 15, 2020, 2020.
- [25] X. Guo, N. Mei, *Aloe vera*: a review of toxicity and adverse clinical effects, *J. Environ. Sci. Health Part C Environ. Carcinog. Ecotoxicol. Rev.* 34 (2) (2016) 77–96, <https://doi.org/10.1080/10590501.2016.1166826>.
- [26] I. Sehgal, W.D. Winters, M. Scott, K. Kousoulas, An *in vitro* and *in vivo* toxicologic evaluation of a stabilized *Aloe vera* gel supplement drink in mice, *Food Chem. Toxicol.* 55 (2013) 363–370, <https://doi.org/10.1016/j.fct.2013.01.012>.
- [27] L.D. Williams, G.A. Burdock, E. Shin, S. Kim, T.H. Jo, K.N. Jones, R.A. Matulka, Safety studies conducted on a proprietary high-purity *Aloe vera* inner leaf fillet preparation, qmatrix®, *Regul. Toxicol. Pharm.* 57 (1) (2010) 90–98, <https://doi.org/10.1016/j.yrtph.2010.01.002>.
- [28] X. Guo, S. Zhang, S.L. Dial, M.D. Boudreau, Q. Xia, P.P. Fu, D.D. Levy, M.M. Moore, N. Mei, *In vitro* investigation of the mutagenic potential of *Aloe vera* extracts, *Toxicol. Res.* 3 (6) (2014) 487–496, <https://doi.org/10.1039/C4TX00053F>.
- [29] I. Sehgal, W.D. Winters, M. Scott, A. David, G. Gillis, T. Stoufflet, A. Nair, K. Kousoulas, Toxicologic assessment of a commercial decolorized whole leaf *Aloe vera* juice, lily of the desert filtered whole leaf juice with aloe sorb, *J. Toxicol.* (2013), 802453, <https://doi.org/10.1155/2013/802453>, 12.
- [30] EFSA Scientific Committee, Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, *EFSA J.* 9 (9) (2011) 2379, <https://doi.org/10.2903/j.efa.2011.2379>.
- [31] EFSA Scientific Committee, Genotoxicity assessment of chemical mixtures, *EFSA J.* 17 (1) (2019) e05519, <https://doi.org/10.2903/j.efa.2019.5519>.
- [32] OECD, Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines, 58, 2015.
- [33] OECD, Test No. 489: *In Vivo Mammalian Alkaline Comet Assay*, 2016.
- [34] OECD, Test No. 490: *In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene*, 2016.
- [35] D. Kline, V. Ritruithai, S. Babajanian, Q. Gao, P. Ingle, P. Chang, G. Swanson, Quantitative analysis of aloins and aloin-emodin in *Aloe vera* raw materials and finished products using high-performance liquid chromatography: single-laboratory validation, first action 2016.09, *J. AOAC Int.* 100 (4) (2017) 1154–1157, <https://doi.org/10.5740/jaoacint.2016.09>.
- [36] M.M. Moore, M. Honma, J. Clements, G. Bolcsfoldi, B. Burlinson, M. Cifone, J. Clarke, R. Delongchamp, R. Durward, M. Fellows, B. Gollapudi, S. Hou, P. Jenkinson, M. Lloyd, J. Majeska, B. Myhr, M. O'Donovan, T. Omori, C. Riach, R. San, L.F. Stankowski Jr., A.K. Thakur, F. Van Goethem, Isao Wakuri, I. Yoshimura, Mouse lymphoma thymidine kinase gene mutation assay: follow-up meeting of the International workshop on genotoxicity testing—Aberdeen, Scotland, 2003—assay acceptance criteria, positive controls, and data evaluation, *Environ. Mol. Mutagen.* 47 (1) (2006) 1–5, <https://doi.org/10.1002/em.20159>.
- [37] F. Nesslany, S. Simar-Meintières, H. Ficheux, D. Marzin, Aloe-emodin-induced DNA fragmentation in the mouse *in vivo* comet assay, *Mutation Res. Genetic Toxicol. Environ. Mutagenesis* 678 (1) (2009) 13–19, <https://doi.org/10.1016/j.mrgentox.2009.06.004>.
- [38] M.Y. Park, H.J. Kwon, M.K. Sung, Plasma, tissue and urinary levels of aloin in rats after the administration of pure aloin, *Nutr. Res. Practice* 2 (1) (2008) 17–21, <https://doi.org/10.4162/nrp.2008.2.1.17>.
- [39] C.P. Yu, C.S. Shia, H.J. Lin, Y.W. Hsieh, S.P. Lin, Y.C. Hou, Analysis of the pharmacokinetics and metabolism of aloe-emodin following intravenous and oral administrations in rats, *Biomed. Chromatogr.* 30 (10) (2016) 1641–1647, <https://doi.org/10.1002/bmc.3735>.
- [40] National Research Council. *Guide for the Care and Use of Laboratory Animals*, 8th ed., National Academies Press, 2011.
- [41] A. Shao, A. Broadmeadow, G. Goddard, E. Bejar, V. Frankos, Safety of purified decolorized (low anthraquinone) whole leaf *Aloe vera* (*L. burm. F.*) juice in a 3-month drinking water toxicity study in F344 rats, *Food Chem. Toxicol.* 57 (2013) 21–31, <https://doi.org/10.1016/j.fct.2013.03.002>.
- [42] Y. Li, Y. Luan, X. Qi, M. Li, L. Gong, X. Xue, X. Wu, Y.F. Wu, M. Chen, G.Z. Xing, J. Yao, J. Ren, Emodin triggers DNA double-strand breaks by stabilizing topoisomerase II-DNA cleavage complexes and by inhibiting ATP hydrolysis of topoisomerase II, *Toxicol. Sci.* 118 (2) (2010) 435–443, <https://doi.org/10.1093/toxsci/kfq282>.
- [43] S.O. Mueller, H. Stopper, Characterization of the genotoxicity of anthraquinones in mammalian cells, *Biochimica et Biophysica Acta (BBA) - General Subjects* 1428 (2–3) (1999) 406–414, [https://doi.org/10.1016/S0304-4165\(99\)00064-1](https://doi.org/10.1016/S0304-4165(99)00064-1).
- [44] S.O. Müller, I. Eckert, W.K. Lutz, H. Stopper, Genotoxicity of the laxative drug components emodin, aloe-emodin and danthron in mammalian cells: topoisomerase II mediated? *Mutation Res. Genetic Toxicol.* 371 (3–4) (1996) 165–173, [https://doi.org/10.1016/S0165-1218\(96\)90105-6](https://doi.org/10.1016/S0165-1218(96)90105-6).
- [45] D. Wolffe, C. Schmutte, J. Westendorf, H. Marquardt, Hydroxyanthraquinones as tumor promoters: enhancement of malignant transformation of C3H mouse fibroblasts and growth stimulation of primary rat hepatocytes, *Cancer Res.* 50 (20) (1990) 6540–6544.
- [46] R.W. Fogleman, T.E. Shellenberger, M.F. Balmer, R.H. Carpenter, B.H. McAnalley, Subchronic oral administration of acemannan in the rat and dog, *Vet. Hum. Toxicol.* 34 (2) (1992) 144–147.
- [47] S.R. Brkanac, M. Gerić, G. Gajski, V. Vujčić, V. Garaj-Vrhovac, D. Kremer, A. M. Domijan, Toxicity and antioxidant capacity of *Frangula alnus* Mill. bark and its active component emodin, *Regul. Toxicol. Pharm.* 73 (3) (2015) 923–929.
- [48] B. Halliwell, Cell culture, oxidative stress, and antioxidants: avoiding pitfalls, *Biomedical Journal* 37 (3) (2014) 99–105, <https://doi.org/10.4103/2319-4170.128725>.
- [49] S. Brendler-Schwaab, A. Hartmann, S. Pfühler, G. Speit, The *in vivo* comet assay: use and status in genotoxicity testing, *Mutagenesis* 20 (4) (2005) 245–254, <https://doi.org/10.1093/mutage/gei033>.
- [50] EFSA Panel on Food Contact Materials Enzymes Flavourings and Processing Aids, Guidance on the data required for the risk assessment of flavourings to be used in or on foods, *EFSA J.* 8 (6) (2010) 1623, <https://doi.org/10.2903/j.efa.2010.1623>.
- [51] ICH, International Conference on Harmonisation; Guidance on S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, 2012.
- [52] M.D. Boudreau, G.R. Olson, V.P. Tryndyak, M.S. Bryant, R.P. Felton, F.A. Beland, From the cover: aloin, a component of the *Aloe vera* plant leaf, induces pathological changes and modulates the composition of microbiota in the large intestines of F344/N male rats, *Toxicol. Sci.* 158 (2) (2017) 302–318, <https://doi.org/10.1093/toxsci/kfx105>.
- [53] E.I. Fawzy, A.I. El Makawy, M. Mahmoud El-Bamby, H.O. Elhamalawy, Improved effect of pumpkin seed oil against the bisphenol-a adverse effects in male mice, *Toxicol. Rep.* 5 (2018) 857–863.
- [54] C.A. Hobbs, M. Koyanagi, C. Swartz, J. Davis, R. Maronpot, L. Recio, S. Hayashi, Genotoxicity evaluation of the naturally-derived food colorant, gardenia blue, and its precursor, genipin, *Food and Chem. Toxicol.* 118 (2018) 695–708, <https://doi.org/10.1016/j.fct.2018.06.001>.
- [55] J.L. O'Donoghue, C. Beevers, A. Buard, Hydroquinone: assessment of genotoxic potential in the *in vivo* alkaline comet assay, *Toxicol. Rep.* 8 (2021) 206–214.

- [56] A. Lydén-Sokolowski, A. Nilsson, P. Sjöberg, Two-year carcinogenicity study with sennosides in the rat: emphasis on gastro-intestinal alterations, *Pharmacology* 47 (Suppl 1) (1993) 209–215, <https://doi.org/10.1159/000139860>.
- [57] J.M. Mitchell, U. Mengers, S. McPherson, J. Zijlstra, P. Dettmar, R. Gregson, J. C. Tigner, An oral carcinogenicity and toxicity study of senna (*Tinnevelly senna* fruits) in the rat, *Arch. Toxicol.* 80 (1) (2006) 34–44, <https://doi.org/10.1007/s00204-005-0021-9>.
- [58] H. Mori, S. Sugie, K. Niwa, M. Takahashi, K. Kawai, Induction of intestinal tumours in rats by chrysazin, *Br. J. Cancer* 52 (5) (1985) 781–783, <https://doi.org/10.1038/bjc.1985.257>.
- [59] H. Mori, S. Sugie, K. Niwa, N. Yoshimi, T. Tanaka, I. Hirono, Carcinogenicity of chrysazin in large intestine and liver of mice, *Jpn. J. Cancer Res. Gann* 77 (9) (1986) 871–876.
- [60] National Toxicology Program, Toxicology and carcinogenesis studies of emodin (CAS NO. 518-82-1) feed studies in F344/N rats and B6C3F1 mice, *Nat. Toxicol. Program Tech. Rep. Ser.* 493 (2001) 1–278.