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Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based quantification of hydroxyanthracene derivatives in *Aloe vera* (L.) Burm. f. gel commercial beverages and preliminary safety evaluation through in vitro genotoxicity studies

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Abstract

Estimating the hydroxyanthracene derivative (HAD) concentrations and assessing the genotoxicity of several marketed *Aloe vera* (L.) Burm. f. gel beverages are the goals of this study. The results showed that five commercial samples contained aloin A at concentrations ranging from 6.05 to 337.98 ng/mL and aloin B at concentrations ranging from 8.84 to 346.89 ng/mL. Four commercial samples contained aloin A and B in concentration higher than 1 ppm, while one sample contained less than 1 ppm. Aloe-emodin was detected in three samples with concentrations ranging from 80.30 to 109.40 ng/mL. Different strains of bacteria were used to perform the Ames test on the samples with the lowest and highest HAD concentrations, and no signs of mutagenicity were found in experiments with samples that increased in concentration (0.0016–5 μL/mL). In accordance with published data and in contrast to findings acquired using products containing *A. vera* latex, which is rich in HADs and whose genotoxicity is extensively proven, even the sample with the highest concentrations of aloin A and B does not show any in vitro genotoxicity, suggesting the importance to standardize the operating procedures to obtain *A. vera* gel to minimize the content of HADs in the commercial formulations.

KEYWORDS

aloe gel beverage, *Aloe vera* leaves, bacterial reverse mutation (Ames) test, hydroxyntracences, UHPLC-MS analysis

Alessandro Di Minno, Maria Vittoria Morone and Hammad Ullah are contributed equally to this work.

1 | INTRODUCTION

Aloe vera (L.) Burm.f. (Aloaceae) is a plant with elongated leaves that can grow a maximum length of 60 cm. Large basal leaves (12–16 on average per plant) are shielded by a thick cuticle and are characterized by mucilaginous gel consisting mainly of water (about 99%), while the remaining portion is formed by over 200 bioactive compounds including carbohydrates, proteins and amino acids, lipids, enzymes, vitamins, minerals, anthraquinones/anthrones, and chromones (Ahlawat & Khatkar, 2011; Elferjane et al., 2023). More than 350 species of *aloe* have been recognized to date, with *A. vera* being the most common (Foster et al., 2011; Hęś et al., 2019). Owing to their numerous health, esthetic, medical, and skin-care benefits, *aloe* plants are well-known and have been utilized as topical and oral beneficial agents for ages (Prisa, 2022). These days, they are among the most important medicinal plants in terms of economic impact. They are frequently employed in primary healthcare, where, in addition to being a rich source of beneficial phytochemicals, they are essential in the treatment of many diseases through the modification of biochemical and molecular pathways (Salehi et al., 2018). Depending on the part of the leaf used, the extraction method, and processing, *A. vera*-based food products could contain different concentrations of hydroxyanthracene derivatives (HADs—such as aloin A and B, emodin, and aloe-emodin), which are known to exert carcinogenic activities (Guo & Mei, 2016). Aloe gel and whole leaf extracts, however, have different concentrations of HADs and, thus, distinct toxicity profiles (Boudreau et al., 2017; Chen et al., 2010; Heidemann et al., 1996; Mori et al., 1985, 1986; Müller et al., 1996; Nesslany et al., 2009; OECD, 2020). Gelum sine cutie, or aloe gel, which is made from the inner leaf of the plant, is the main ingredient in *A. vera* gel beverages. However, because of inadequately precise production methods, some HADs have been shown to be contaminants in these beverages, diffusing from the external leaf tissues during the extraction process. On the other hand, *A. vera* preparations known as succus ex foliis (whole leaf extract) or folium (leaf) may include elevated levels of HADs, deriving from aloe latex found in the cells of the fibrovascular bundles close to the epidermis of aloe leaves. *Gelum sine cute* is used for throat health, whereas *succus ex foliis* is traditionally used for its depurative effects and short-term to treat occasional constipation (Foster et al., 2011).

The presence of HADs in *A. vera* leaves and in their food products has led the European Commission (EC) to release a Regulation (UE) N. 2021/468, which amended Annex III of Regulation (EC) N. 1925/2006 and listed HADs containing *Aloe* leaf extracts in prohibited food ingredients (Annex III, Part A). Given the severity of HAD harmful effects on health, no daily intake of HADs, which does not give rise to concern for human health, could be established. This conclusion is based on the European Food Safety Authority's assessment of the safety of HADs when used in food, which was published in a scientific opinion on 22 November 2017 (Chen et al., 2010). Nevertheless, as regards the analytical determinations of the levels of HADs, the Standing Committee on Plants, Animals, Food, and Feed Section General Food Law of the EC (Fouillaud et al., 2018) reported that "*products ready for use after preparation in accordance with the manufacturer's instructions containing an analyzed level higher than or equal to* 1 ppm *aloe-emodin and/or* 1 ppm *emodin and/or* 1 ppm *aloin A + aloin B provide clear evidence of presence of these substances in the products and are therefore of concern for public health. The sum of the analyzed contents of aloin A and aloin B can be used to quantify the total HAD content in preparations from the leaf of Aloe species, since aloin A and B are the most commonly occurring HADs in Aloe species*". The Commission further stated that the levels of 1 ppm for aloe-emodin/emodin and 1 ppm for the total of aloin A and B are currently the lowest that can be accurately measured in laboratories across the EU and can, therefore, be proposed as the limit of quantification in an EU harmonized risk management strategy. However, neither the Regulation (UE) 2021/468 nor the instructions from the Competent Authorities have offered any guidance on the analytical approach appropriate to detect HADs quantitatively and qualitatively, with a known limit of detection (LOD) and limits of quantification (LOQ) (limit of quantification), respectively. Moreover, in the available literature, none of the studies assessed the genotoxicity of aloe gel extracts and HAD concentrations (Baldi et al., 2021), allowing to establish a relationship between the toxicological property and HAD levels.

This justifies research aimed at evaluating aloin A, aloin B, emodin, and aloe-emodin in various batches of aloe gel-based beverages. This will enable the development of a high-sensitivity, quick, repeatable, and simple UHPLC-MS analytical methodology to determine HADs in commercial products and therefore to monitor their accordance with the current European legislation. In addition, to try to clarify the relationship between the concentration of HADs in *A. vera* gel commercial beverages and the possible mutagenic activity exerted by HADs, the samples with the highest and lowest HAD concentrations were subjected to the evaluation of point mutations, which involve substitution, addition, or deletion of one or a few DNA base pairs using the bacterial reverse mutation test (Ames test).

2 | MATERIALS AND METHODS

2.1 | Materials

Aloe gel samples (Table 1) were provided by seven different producers: samples 1A, 2A, 3A, 4A, and 5A were provided from one producer; samples 1B, 2B, and 3B from the second producer; 1C, 1D, 1E, 1F, and 1G from the other five producer. *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* ECWP2UvrA were purchased from Trinova Biochem (TRINOVA Biochem GmbH, Giessen Germany). Sodium azide (NaAz; for strain TA1535), 2-amino-anthracene (2AA; for strains TA1535, TA1537, and ECWP2UvrA), 2-nitro-fluorene (2NF; for strains TA98 and TA100), benzo-pyrene (BaP; for strains TA98 and TA100), 4-nitroquinoline-N-oxide (NQO; for strain ECWP2UvrA), 9-amino-

TABLE 1 Limits of detection (LOD) and quantification (LOQ), precision (repeatability), and accuracy (recovery %) of the analytical procedure for the analysis of aloin A and aloin B.

Variable	Aloin A	Aloin B	
Limits of detection (LOD) ng/mL	0.025	0.002	
Limits of quantification (LOQ) ng/mL	0.082	0.008	
Repeatability			
CV% concentrations	1.258	0.803	
CV% retention time	0.272	0.287	
Recovery (%, low concentrations)	85.87	80.49	±3.80
Recovery (%, medium concentrations)	86.29	91.66	±3.79
Recovery (%, high concentrations)	91.35	97.66	±4.45

acridine-HCl (9AC; for strain TA1537), and rat liver S9 fraction were also purchased from Trinova Biochem.

2.2 | UHPLC-MS determination of HADs

Prior to analysis, aloe (liquid commercial formulation) was extracted. Each bottle was shaken, and a 6-mL test portion was added to a 16 mL of extraction solution (40:60, ACN:H_2O) in a test tube and vortexed for one min, followed by 10 min sonication at room temperature and 10 min centrifugation at 6000 rpm at 4°C. The supernatant was collected and filtered through a 0.45 μm polytetrafluorethylene (PTFE) filter (Phenomenex). UHPLC-MS/MS analysis was carried out with a Shimadzu Nexera (Shimadzu) UHPLC consisting of two LC 30 AD pumps, a SIL 30AC autosampler, a CTO 20AC column oven, and a CBM 20A controller. The system was coupled online to a triple quadrupole LCMS 8050 (Shimadzu) equipped with an electrospray ionization (ESI) source. The separation was performed on Luna Omega Polar C18 column with geometry (L × I.D) 5 cm × 2.1 mm, 1.6 μm (Phenomenex) employing as mobile phases: (A) H_2O plus 0.1% HCOOH and (B) ACN plus 0.1% HCOOH, with the following gradient: 0 min, 5% B, 0.01–2.50 min; 5%–70% B, 2.50–3 min; 70%–99% B, hold for 1 min; returning to initial conditions in 0.01 min. The flow rate was set to 0.5 mL/min, column oven was set to 45°C, and 2 μL of extract was injected. The ESI was operated in negative mode. MS/MS analysis was carried out in multiple reaction monitoring, employing as transitions for aloin B 417.00 > 297.00 (quantifier ion), Q1 pre bias 16.0 V, collision energy: 20.0 V, Q3 pre bias 19.0 V; aloin A 417.00 > 297.00 (quantifier ion), Q1 pre bias 30.0 V, collision energy: 18.0 V, Q3 pre bias 30.0 V; aloe-emodin 269.30 > 240.00 (quantifier ion), Q1 pre bias 14.0 V, collision energy: 21.0 V, Q3 pre bias 15.0 V; emodin 269.00 > 225.05 (quantifier ion) Q1 pre bias 11.0 V, collision energy: 26.0 V, Q3 pre bias 26.0 V; danthron 239.00 > 211.05 (quantifier ion) Q1 pre bias 19.0 V, collision energy: 29.0 V, Q3 pre bias 25.0 V. Dwell time 10 msec. Interface temperature, desolvation line temperature, and heat block temperature were set, at 300°C, 200°C, and 400°C, respectively. Nebulizing gas, drying (N2), and heating gas (air) were set, to 3, 10, and 10 L/min, respectively. To quantify the content of HADs, each standard stock solution (1 mg/mL) was prepared in methanol and six points calibration curves were built in the range obtained in a concentration range of 1–250 ng/mL (R^2 0.998).

2.2.1 | Analytical validation procedures

Repeatability was established by triplicate injections of sample and solutions at low, medium, and high concentrations of the calibration curve employing the same chromatographic conditions, the same analyst at the same day; results were expressed as CV%. LOD and LOQs for concentration and retention time were also defined. Recovery was assessed by spiking known amounts of each standard at low, medium, and high concentrations.

LOD and LOQs were calculated by the ratio between the standard deviation (SD) (s) of the response and the slope of the calibration curve (S) multiplied by 3 and 10, respectively, according to the following formulas:

$$\mathrm{LOD} = 3\,x\left(\frac{\sigma}{S}\right) - \ LOQ = 10\,x\,(\sigma/S)$$

where σ is the SD of the slope and S is the slope of the calibration curve.

The SD (s) of the response was based on the calibration curve that contains samples in the range of the LOQ and was measured as the SD of y-intercepts of regression lines.

2.3 | Ames test

A mutagenicity test was performed following the principles and procedures defined by OECD guideline 471 (Reynolds, 2004). Briefly, four different strains of *Salmonella typhimurium* (strains TA98, TA100, TA1535, and TA1537) and an *Escherichia coli* strain called ECWP2UvrA were cultured and used in the exponential phase of the growth bacterial curve. A negative control plate was used for DMSO, positive control plates contained different chemicals depending on the bacterial strains, and aloe plates containing aloe solubilized into DMSO at different concentrations (0.0016, 0.005, 0.016, 0.05, 0.16, 0.5, 1.6, and 5 mg/mL). Different tubes with a volume of 0.1 mL of aloe were prepared for each aloe concentrations in a range between 0.0016, and 5 mg/mL, 0.1 mL of fresh bacterial culture (containing approximately 10^8 viable cells) were added with 0.5 sterile buffer, and 2.0 mL of overlay agar. Metabolic activation (S9-mix) was used with or without a post-mitochondrial fraction concentration of 7% v/v. Each tube with different bacterial solutions were plated on minimal agar plates and incubated, after solification for 72 h at 37°C. Then, the number of revertant colonies for each plate was counted and compared with the negative control group. Each experiment was performed in triplicate and the results were expressed as the number of revertant colonies per plate and mean ± SD. Detailed descriptions of the method employed are reported in the Supporting Information S1.

2.3.1 | Acceptance of the test

The test was accepted based on the following standards: (i) All experimental conditions specified by OECD guideline 471 (Reynolds, 2004) were examined; (ii) The criteria used to choose the aloe top test concentration (5 mg/mL), taking into account aloe solubility, were in line with those outlined in OECD guideline 471; (iii) when comparing the number of revertant per plate between the test concentrations and the concurrent negative controls, none of them showed a statistically significant increase; (iv) neither a concentration-related rise nor any other trend could be found; (v) all results were below the historical range of negative control data; (vi) when comparing the number of revertant with the concurrent negative controls, all of the positive controls showed statistically significant increases.

2.4 | Statistical analysis

Numerical variables were expressed as the mean and SD of the replicates, median and interquartile range, frequency, or percentage, where appropriate. All the analyses were performed by SPSS version 27 (IBM SPSS Statistics) following the protocol described in OECD guideline 471 (Kirkland et al., 1989).

3 | RESULTS

3.1 | UHPLC-MS qualitative and quantitative analysis

To identify HADs in *A. vera* gel commercial beverages, the analyses were first carried out on standard compounds (aloin A, aloin B, emodin, and aloe-emodin) with the combined use of reverse-phase UHPLC coupled with a mass spectrometer. The new UHPLC-MS approach produced complete separation of HADs in 5 min, as illustrated in Figures 1 and 2. The analytical procedure precision (repeatability), accuracy (recovery percentage), LOD, and LOQ were determined (Table 1). Then, the determination of the concentrations of aloin A and B, aloe-emodin, and emodin in the 13 samples of *A. vera* gel commercial beverages was performed. Table 2 lists the mean concentrations (±SD) of aloin A and B and aloe-emodin in 13 batches of the commercial *A. vera* gel beverage. The only substances found in samples 1A, 2A, 3A, 4A, and 5A were aloin A (6.05 ± 0.44–337.98 ± 3.98 ng/mL) and aloin B (8.84 ± 0.29–346.89 ± 0.99 ng/mL), but aloe-emodin and emodin were not determined. Thus, all the samples had a concentration of less than 1 ppm, which is within the range permitted by the current regulations. Aloe-emodin was detected in three other samples, 1B, 2B, and 3B, with values ranging from 80.30 ± 7.44–109.40 ± 2.56 ng/mL. In contrast to all other samples examined, the concentrations of aloin A (1.67 ± 0.85–4.12 ± 0.38 ng/mL) and aloin B (2.69 ± 0.45–4.41 ± 0.36 ng/mL) were lower. Conversely, in samples 1C, 1D, 1E, and 1G, aloin A and B levels were consistently greater than 1 ppm; although in the sample 1F, they were within the current regulations' permissible limit (i.e., 175.28 ± 0.48 ng/mL for aloin A and 151.16 ± 0.25 ng/mL for aloin B).

3.2 | In vitro genotoxicity of hydroxyanthracene derivatives

The bacterial reverse mutation test was performed on strains of *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and *E. coli* (WP2urA), each of which represents a distinct type of mutation caused by a substance, to look for potential point mutations (i.e., substitution, addition, or deletion of one or a few DNA base pairs) induced by aloe gel. Concurrent with the test trials, the genetic backgrounds of the used bacterial strains were changed (data not shown). Then, for each bacterial strain, a positive control was made, which demonstrated appreciable increases in the number of revertant colonies in accordance with previous laboratory findings. This served to validate the sensitivity of the test method and the activity of the S9-mix.

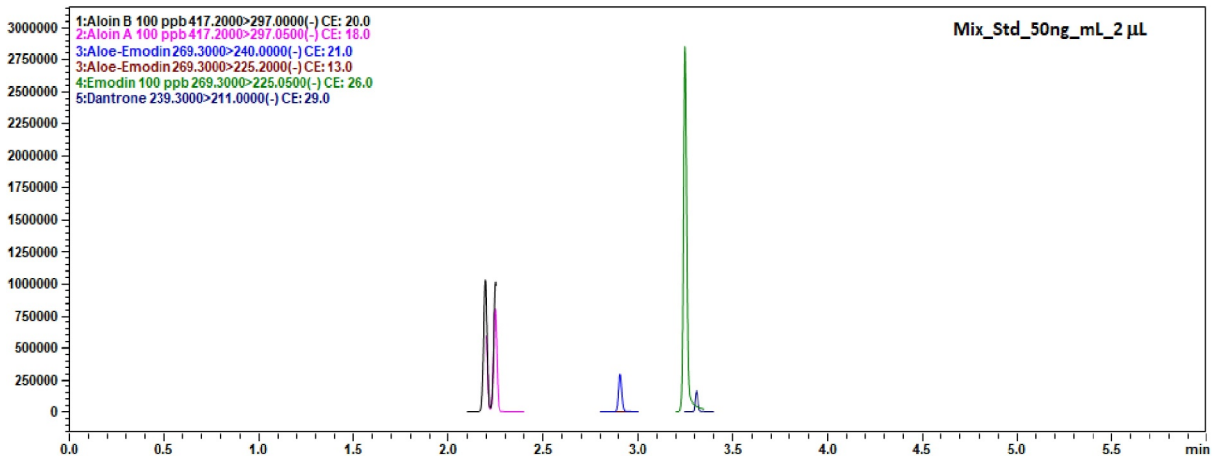

FIGURE 1 UHPLC method to resolve at the baseline aloin B, aloin A, aloe-emodin, emodin, and danthrone*. Chromatogram of standard compounds.

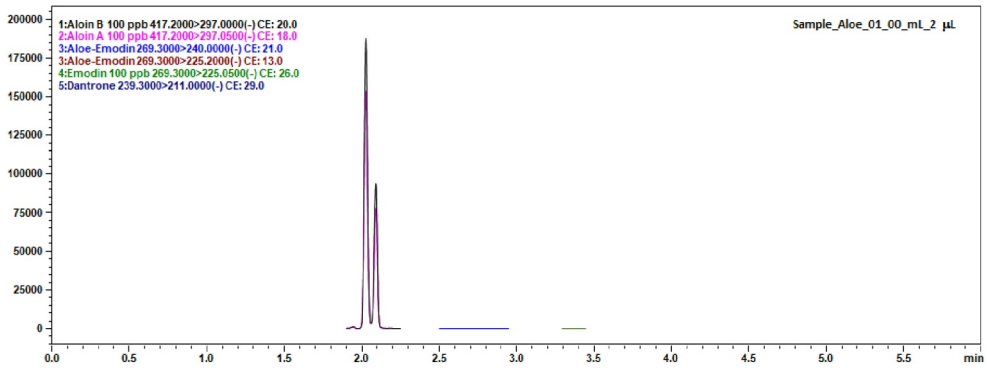

FIGURE 2 UHPLC chromatogram of sample 1.

TABLE 2 Concentrations (±SD) of aloin A and B and aloe-emodin in different batches of *Aloe vera* (provided by 7 different producers).

Sample number	Aloin A (ng/mL)	Aloin B (ng/mL)	Aloe emodin (ng/mL)
1A	129.98 ± 2.44	135.35 ± 0.71	Not detectable
2A	268.01 ± 2.76	286.92 ± 2.42	Not detectable
3A[a]	337.98 ± 3.98	346.89 ± 0.99	Not detectable
4A[a]	6.05 ± 0.44	8.84 ± 0.29	Not detectable
5A	179.16 ± 4.91	193.51 ± 1.96	Not detectable
1B	1.67 ± 0.85	2.69 ± 0.45	86.82 ± 4.52
2B	4.12 ± 0.38	4.41 ± 0.36	80.30 ± 7.44
3B	1.95 ± 0.30	2.73 ± 0.14	109.40 ± 2.56
1C	1597.60 ± 0.16	1851.25 ± 4.08	< LOD[b]
1D	701.67 ± 0.73	762.71 ± 5.22	255.10 ± 3.15
1E	542.71 ± 0.23	549.98 ± 0.21	727.47 ± 1.35
1F	175.28 ± 0,83	151.16 ± 0.25	1062.78 ± 1.56
1G	665.67 ± 0.42	742.45 ± 0.38	158.10 ± 1.32

[a]Analyzed also in Ames tests.
[b]Limits of detection.

In summary, samples 3A and 4A were subjected to the Ames test. Sample 3A had the highest quantities of aloin A (337.98 ± 3.98 ng/mL) and aloin B (346.89 ± 0.99 ng/mL), while sample 4A had the lowest values of aloin A (6.05 ± 0.44 ng/mL) and aloin B (8.84 ± 0.29 ng/mL). When increasing concentrations (from 0.0016 to 5 mg/mL) of these substances were tested with various bacterial strains (*S. typhimurium* and *E. coli*), no indications of toxicity or precipitation were found. Tables 3 and 4 display the mean number (±SD) of revertant colonies per plate for the two tested samples, 3A and 4A, respectively.

4 | DISCUSSION

Aloe gel supplementation has been shown to have protective and physiological effects by a substantial body of scientific evidence. However, the use of aloe gel as an ingredient of food supplements, or foods for medical purposes is limited due to the presence of HADs, which are primarily the result of inaccurate production procedures, and the unfavorable safety profile of HADs, even at low concentrations. Thus, a UHPLC-MS method was developed for this study in order to identify and measure HADs in different commercial batches of aloe gel and to assess their carcinogenic potential in vitro using the bacterial reverse mutation test.

Aloin A and aloin B were found in the samples of aloe gel 1A, 2A, 3A, 4A, and 5A, where sample 4A had the lowest HAD concentration, while 3A had the highest HAD concentration. However, aloe-emodin and emodin were not detected in these samples. In comparison to samples 1A–5A, samples 1B, 2B, and 3B, obtained from another producer, contained lower concentrations of aloin A and B and aloe-emodin, which was detected in these samples in the range of 80–109 ng/mL, suggesting that aloin A and B were oxidized, resulting in the formation of aloe-emodin. In the remaining samples (i.e., 1C, 1D, 1E, 1F, and 1G), the concentration of aloe-emodin ranged from 58.10 to 1062.78 ng/mL. Aloin and aloe-emodin concentrations therefore varied significantly between various commercial samples. It is noteworthy to highlight that in the majority of products on the market analyzed in this study, the concentration of aloe-emodin and/or emodin and/or aloin A + aloin B is lower than 1 ppm in line with the

TABLE 3 Number of revertant per plate in the presence of aloe *sample* 3A in the absence (S9-mix) and in the presence (+S9 mix) of metabolic activation.

	S. typhimurium TA98		*S. typhimurium* TA100		*S. typhimurium* TA1535		*S. typhimurium* TA1537		*E. coli* WP2 urA	
Dose (μL/mL)	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**
Negative control	51 ± 9	40 ± 8	87 ± 13	183 ± 9	64 ± 8	29 ± 2	49 ± 6	31 ± 4	38 ± 4	41 ± 2
Positive control	1202 ± 18	1237 ± 27	1297 ± 29	1464 ± 66	223 ± 15	206 ± 13	172 ± 19	191 ± 32	178 ± 21	215 ± 16
Aloe 0.0016	95 ± 13	42 ± 11	107 ± 13	203 ± 19	57 ± 13	35 ± 8	51 ± 19	41 ± 5	41 ± 13	49 ± 5
Aloe 0.0050	104 ± 17	54 ± 7	130 ± 13	184 ± 16	103 ± 7	20 ± 3	24 ± 12	42 ± 4	33 ± 13	46 ± 14
Aloe 0.0160	178 ± 11	58 ± 9	162 ± 17	187 ± 9	127 ± 15	19 ± 5	44 ± 18	44 ± 15	54 ± 12	72 ± 12
Aloe 0.0500	147 ± 20	55 ± 8	143 ± 21	174 ± 12	121 ± 13	26 ± 5	116 ± 9	63 ± 8	41 ± 17	41 ± 15
Aloe 0.1600	165 ± 24	75 ± 9	140 ± 29	232 ± 19	123 ± 8	27 ± 3	34 ± 9	28 ± 4	42 ± 16	68 ± 10
Aloe 0.5000	137 ± 18	52 ± 6	116 ± 14	223 ± 17	119 ± 12	23 ± 7	121 ± 8	57 ± 6	29 ± 11	71 ± 8
Aloe 1.6000	132 ± 10	54 ± 12	115 ± 14	210 ± 20	127 ± 13	29 ± 3	110 ± 11	73 ± 11	24 ± 7	100 ± 12
Aloe 5.0000	149 ± 15	57 ± 14	205 ± 16	188 ± 10	115 ± 3	43 ± 5	119 ± 6	44 ± 4	51 ± 16	86 ± 6

Note: Negative control: water (100 μL/mL); Positive control: 4-nitroquinoline-N-oxide (1.0 μg/mL) in the absence of S9 and 2 aminoanthracene (20 μg/mL) in the presence of S9. Data is presented as Mean ± SD.

TABLE 4 Number of revertant per plate in the presence of aloe sample 4A in the absence (S9-mix) and in the presence (+S9 mix) of metabolic activation.

	S. typhimurium TA98		*S. typhimurium* TA100		*S. typhimurium* TA1535		*S. typhimurium* TA1537		*E. coli* WP2 urA	
Dose (μL/mL)	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**	**−S9 mix**	**+S9 mix**
Negative control	57 ± 17	41 ± 7	137 ± 24	207 ± 12	47 ± 4	28 ± 4	58 ± 17	32 ± 4	53 ± 9	45 ± 9
Positive control	892 ± 24	1295 ± 16	1234 ± 33	1593 ± 53	247 ± 14	213 ± 18	175 ± 9	217 ± 10	175 ± 12	175 ± 12
Aloe 0.0016	123 ± 10	70 ± 6	62 ± 22	210 ± 13	49 ± 15	28 ± 3	58 ± 5	78 ± 8	56 ± 22	48 ± 9
Aloe 0.0050	102 ± 17	46 ± 6	24 ± 12	208 ± 24	24 ± 2	50 ± 9	118 ± 9	42 ± 7	37 ± 10	53 ± 8
Aloe 0.0160	130 ± 16	63 ± 12	48 ± 20	224 ± 16	52 ± 17	41 ± 9	119 ± 14	63 ± 9	53 ± 22	56 ± 8
Aloe 0.0500	160 ± 14	73 ± 9	34 ± 17	196 ± 19	26 ± 8	31 ± 5	75 ± 5	52 ± 9	39 ± 13	38 ± 6
Aloe 0.1600	129 ± 18	55 ± 11	28 ± 3	192 ± 18	28 ± 3	34 ± 17	87 ± 2	64 ± 7	46 ± 5	77 ± 8
Aloe 0.5000	121 ± 20	74 ± 8	39 ± 18	196 ± 12	39 ± 18	38 ± 17	131 ± 20	44 ± 8	47 ± 11	50 ± 8
Aloe 1.6000	146 ± 12	48 ± 9	28 ± 8	223 ± 21	33 ± 15	25 ± 6	113 ± 10	39 ± 6	44 ± 7	79 ± 9
Aloe 5.0000	153 ± 11	64 ± 10	20 ± 4	224 ± 9	20 ± 4	20 ± 3	61 ± 11	55 ± 7	33 ± 4	66 ± 11

Note: Negative control: water (100 μL/mL); Positive control: 4-nitroquinoline-N-oxide (1.0 μg/mL) in the absence of S9 and 2 aminoanthracene (20 μg/mL) in the presence of S9. Data is presented as Mean ± SD.

highest acceptable level as reported by the Standing Committee on Plants, Animals, Food, and Feed Section General Food Law of the EC (Fouillaud et al., 2018).

Thus, pending the establishment and adoption of harmonized standard operating procedures (SOPs) to produce aloe gel the availability of simple, repeatable analytical techniques for HAD measurement is essential in this field. The UHPLC-MS technique described here offers a high-sensitive, fast, simple, and repeatable way to identify aloin A and B and its derivative aloe-emodin. The current UHPLC method completely separated aloin B, aloin A, aloe-emodin, and emodin in time less than 5 min, as shown in Figure 1. Aloin A and B can be reliably quantified thanks to its detection and quantification limits, precision (repeatability), and accuracy (recovery percentage). The high variability of the concentrations of aloin A and B in the analyzed commercial samples is surely due to the extraction method used to obtain the aloe gel that leads to higher concentrations of aloin if the process of separation of the aloe parenchyma from the mesophyll and rind is not accurate, being these two parts the richest in latex, which, in turn, consists of HADs. Moreover, a potential explanation of the high concentration of aloe-emodin and low concentration of aloin A and B in some commercial samples could be that initially the aloe gel was obtained with an inaccurate

production method that did not allow the gel isolation and caused contamination of the gel with aloin A and B that, in turn, have degraded into aloe-emodin, which is formed by the oxidative cleavage of the glycosidic linkage of aloin, being one of the main degradation products that is formed at pH ranging from 2 to 5 (Ding et al., 2014). Another explanation for the presence of aloe-emodin could be that Aloe products, being rich in water and sugar contents, are susceptible to microbial contamination, particularly from lactic acid bacteria (*Lactobacillus* species), which are able to convert aloin (present at low concentration) into aloe-emodin by the cleavage of the C–C glycosidic bond. In view of this, International aloe scientific council (IASC) declared that lactic acid levels ≥10% in the finished product are a good indicator of bacterial contamination (Bejar, 2019). It is necessary to specify that this second possible explanation is not in contradiction with the known antibacterial properties of aloin since the antibacterial activity of aloin is exerted at a concentration higher than 1 mg/mL. In contrast, in the analyzed aloe gel samples, the aloin concentration is lower and consistent with the contamination by lactic bacteria (Gokulan et al., 2019).

As far as mutagenicity is concerned, the number of revertant colonies in any strain did not increase when the five bacterial strains were exposed to samples 3A (which showed the highest aloin A and B concentration, corresponding to 684,87 mg/mL) and 4A (which showed the lowest aloin A and B contents corresponding to 14,89 ng/mL) at increasing concentrations (0.0016–5 mg/mL), either in the presence or absence of S9-mix, as compared to the negative control (without aloe gel). In fact, the number of revertant colonies at a given aloe gel concentration and under experimental conditions (±S9-mix on various strains) was not increased to the value of the positive control and is close to the value of the negative control's revertant colonies without any discernible mutagenic activity under the used experimental conditions. Overall, both the aloe samples (3A and 4A) did not exhibit mutagenic properties under the testing conditions, according to the obtained results. These results are in agreement with those obtained by Hayes et al. (2022) that recently showed that a stabilized *A. vera* juice product derived from the inner filet and marketed as a beverage currently sold in the European Union containing 8–10 ppm aloin and a mixture of purified aloin A and B were non-mutagenic based on Ames test, an in vitro cytotoxic assay, and an in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes. Moreover, our results are in agreement with those reported by Sehgal et al. (2013) that studied the aloe juice certified by the IASC as regards the quality standards for microbiology testing, production, storage, *A. vera* content, and aloin content (aloin content <10 ppm) and found that it had no effect on the bacterial reverse mutation assay both in the presence or in the absence of S9 extract metabolic activation. Other investigations report similar results following the study of aloe gel with low HAD concentration (Tanaka et al., 2012; Williams et al., 2010).

Finally, our results are in line with those from Hu et al. (2021) that showed the absence of genotoxicity for a *A. vera* whole leaf dry juice purified through activated charcoal filtration (decolorization) containing an insignificant amount of HADs (0.3 ppm of total aloins and non-detectable aloe-emodin) in the L5178Y mouse lymphoma assay (OECD 490) and in vivo comet assay (OECD 489). On the other hand, our results are in contrast with the data obtained using products containing the *A. vera* latex HADs showing clear evidence of in vivo carcinogenic activity, which led the International Agency for Research on Cancer (IARC) to classify HADs as a possible human carcinogen (Group 2B) (Guo & Mei, 2016).

5 | CONCLUSION

In conclusion, the innovative aspect of this work is the examination of genotoxic activity using commercial samples that had known HAD concentrations. The findings of this study indicate that there are notable variations in the concentrations of aloin A, aloin B, and aloe-emodin among different commercial samples of aloe gel. Additionally, the findings demonstrated that neither the sample with highest nor with lowest HAD concentration exhibited any genotoxic activity. Thus, it is imperative to conduct market surveillance by meticulous monitoring of the levels of HAD concentrations in aloe products. SOPs are required to lower the risk of HAD contamination of aloe gel and, as a result, the presence of harmful compounds in foods based on aloe gel that are prohibited and may affect consumer health.

AUTHOR CONTRIBUTIONS

Alessandro Di Minno: Conceptualization, methodology, software, writing—original draft. **Maria Vittoria Morone**: Formal analysis, validation, writing—review & editing. **Hammad Ullah**: Data curation, validation, writing—original draft, writing—review & editing. **Eduardo Sommella**: Data curation, investigation, resources. **Daniele Giuseppe Buccato**: Data curation, resources, visualization. **Lorenza Francesca De Lellis**: Data curation, formal analysis, resources. **Pietro Campiglia**: Formal analysis, software, validation. **Anna De Filippis**: Methodology, software, writing—original draft. **Massimiliano Galdiero**: Methodology, software, writing—original draft. **Maria Daglia**: Conceptualization, methodology, project administration, supervision, visualization, writing—review & editing.

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Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

ETHICS STATEMENT

Ethics approval statement is not applicable to the current study.

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