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Lack of *in vivo* genotoxic effect of dried whole *Aloe ferox* juice

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ABSTRACT

Aloe ferox Mill is widely used as a traditional herbal medicine for the treatment of a broad spectrum of illnesses given its laxative, anti-inflammatory, bitter tonic, anti-oxidant, antimicrobial and anti-cancer properties.

Using the *in vivo* alkaline comet assay in animals (OECD 489), this study investigated the potential *in vivo* genotoxicity of dried *Aloe ferox* juice at dose levels of 500, 1000, and 2000 mg/kg/day in mice. *Aloe ferox* showed no genotoxic activity in preparations of single cells from the colon of the treated Hsd:ICR (CD-1) male mice. No statistically significant increase in DNA migration over the negative control was observed by analysis of variance for both comet parameters, tail moment and tail intensity, apart from the positive control ethyl methanesulphonate that induced clear and statistically significant increases in DNA migration parameters over the concurrent controls. The new reported scientific evidence unequivocally demonstrates that dried *Aloe ferox* juice containing hydroxyanthracene derivatives does not induce DNA damage in preparations of single cells from colon in *in vivo* comet genotoxicity studies. This suggests that the hyperplastic changes and mucosal hyperplasia observed after long-term administration of *Aloe vera* non-decolourised whole leaf extract may be attributed to an epigenetic effect of the material under investigation.

1. Introduction

Aloe ferox Mill. (= A candelabrum A. Berger), commonly known as bitter aloe or Cape aloe is a variable species indigenous to the coastal region of the Cape and other parts of South Africa. The 'ferox' (ferocious) in the botanical name comes from the thorny, sharp, reddish spines of the leaves.

Aloe ferox has been used since ancient times as a medicine. The bitter latex, similarly known as Cape aloe, is widely used as a laxative in Africa and Europe and is also considered to have anti-oxidant, bitter tonic, anticancer, antimicrobial, and anti-inflammatory properties. *Aloe ferox* is used in cosmetics, food supplements, and in the food and flavouring industry [1,2].

Many bitter molecules have been isolated from *Aloe ferox* leaf exudate. The most important of them are the chromones aloeresin A and aloesin and the hydroxyanthracene derivative (anthrone derivative) aloin. The two diastereoisomers of aloin (A and B) are present in the exudate [1,2]. Other hydroxyanthracene derivatives present in *Aloe ferox* are the anthraquinones aloe-emodin, emodin, rhein, and physcion. Other constituents are sugars, amino acids, and organic acids (see also sample characterization below).

The World Health Organization (WHO) recommended that products "*containing anthraquinone glycosides should not be used continuously for longer than 1–2 weeks*" (thus for extended periods) and identified a safe individual dose as "corresponding to 10−30 mg hydroxyanthraquinones per day" [3]. The European Medicines Agency (EMA), in its herbal monographs on Aloe species containing hydroxyanthracene derivatives, concluded that their "*short-term use in case of occasional constipation can be regarded as safe*" and identified a safe daily dosage of 20−30 mg for adults, the elderly and adolescents over 12 years of age [4].

In support of the safety of Aloe-based preparations, a recent study was conducted with the aim of assessing the toxicological profile of Aloe vera soft capsule (ASC), through acute, subacute toxicity and genotoxicity tests. In the acute toxicity study, no mortality or behavioral changes were observed, indicating the LD_{50} was higher than 15,000 mg/kg body weight. In the subacute toxicity test rats were fed on diet blended with different doses of ASC equivalent to 832.5, 1665 and 3330 mg/kg body weight, no significant changes up to a maximum concentration of 3330 mg/kg body weight. In the genotoxicity study, ASC showed no mutagenic activity in the Ames test and no evidence of potential to induce bone marrow micronucleus or testicular chromosome aberrations in ICR mice exposed to 10,000 mg/kg body weight [5].

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The estimated exposure to hydroxyanthracene derivatives from the recommended daily doses of food supplements indicates intakes of 24.83 mg/person per day of sennoside B, 78.8 mg/person per day of rhein, 26 mg/person per day of glucofrangulin A, 24 mg/person per day of barbaloin and 51 mg/person per day of aloin A + B.; the data relating to exposure to emodin and aloe-emodin are not known and are likely to be on the same order of magnitude [6].

The European Food Safety Authority (EFSA) Food Additives and Nutrient Sources added to Food (ANS) Panel concluded that "*hydroxyanthracene derivatives should be regarded as genotoxic and carcinogenic unless there are specific data to the contrary, [...] and that there is a safety concern for extracts containing hydroxyanthracene derivatives although uncertainty persists*" [6]. The only study judged reliable by the ANS Panel was the one in which aloe-emodin was administered by the oral route to male (OF1) mice at doses of 500, 1000 and 2000 mg/kg bw in an *in vivo* rodent comet assay conducted in accordance with unspecified international recommendations [7].

Since Aloe herbal preparations contain hydroxyanthracene derivatives, based on the possible harmful effect on health identified by the EFSA, the Commission decided to place extracts from the leaf of Aloe species containing hydroxyanthracene derivatives, together with aloe-emodin, emodin and danthron extracts, in Part A (ban on the use in food) of Annex III of Regulation (EC) no. 1925/2006 of the European Parliament and of the Council to ensure the high level of health protection in accordance with the precautionary principle provided for in Article 7 of Regulation (EC) 178/2002.

Subsequently, *in vivo* genotoxicity tests (alkaline comet test - OECD 489) carried out in mice following oral gavage of doses of 0, 500, 1000 and 2000 mg/kg/day of a high-titre aloe-emodin showed that the tested material did not induce DNA strand breakage in preparations of single cells from colon and kidney [8]. A similar experiment was also conducted on a preparation of *Aloe ferox* resin to determine whether or not the whole matrix had any genotoxic effect *in vivo*.

2. Materials and methods

2.1. Extract preparation

Dried Aloe ferox juice was kindly supplied by Erbofrut s.n.c., Roccavione (Italy). The characterization of dried *Aloe ferox* juice (provided by Indena, SpA, Milan, Italy) is reported in Table 1. Anthraquinones were identified by ultra-high-performance liquid chromatography, chromones by nuclear magnetic resonance analysis, and mono- and disaccharides (fructose, galactose, glucose, fructose, lactose, maltose) by using a gas chromatographic method after derivatization. A Karl Fisher system was used to detect water, and total amino acids were determined by high-performance liquid chromatography. Metals were assayed using inductively coupled plasma mass spectrometry, while inorganic and organic anions were assayed by ionic chromatography.

Ethyl methanesulfonate (EMS, Sigma) was used as a positive control and prepared in water.

2.2. Animals and treatments

Twenty-three male Hsd:ICR (CD-1) mice were supplied by Envigo RMS s.r.l. (San Pietro al Natisone, Italy), and allowed five days for acclimatisation and quarantine. During this period, the health status of the animals was assessed by daily observations.

The animals were housed up to 5 animals/cage, in polisulphone H-temp solid bottomed cages with nesting material provided in suitable bedding bags. Animal room controls were set to maintain temperature and relative humidity at 22 °C ± 2 °C and 55 % ±15 %, respectively. The animals were kept in a 12 h light/dark cycle.

Food and drinking water were supplied ad libitum. The animals were maintained on a commercially available laboratory rodent diet 4 RF 21 (Mucedola S.r.l., Settimo Milanese, Italy).

At 6–7 weeks old, the animals were treated with the test substance (0.5 % carboxymethylcellulose) or the positive control (EMS). Five animals per group were dosed twice with the vehicle alone or with the extract at the selected dose levels of 500, 1000, and 2000 mg/kg bw/day at 0 h and 24 h. The remaining three animals were treated with EMS, as a positive control, which was given at a dose of 150 mg/kg bw/day. Dried *Aloe ferox* juice in corn oil (Sigma, Germany) was freshly prepared each day. Treatments were administered by oral gavage. Animals were killed 3–6 hours after the treatment by asphyxiation with carbon dioxide.

Table 1
Composition of dried *Aloe ferox* juice.

Antraquinones	%	Chromones	%	Other analytes	%
Aloin (A+B)	8.30	Aloesin	28.70	Glucose	0.30
Aloe-emodin	0.20	Aloeresin	32.20	Water	6.70
Rhein	0.03			Total amino acids	0.55
Emodin	0.01			Metals	0.12
Chrysophanol	0.01			Inorganic anions	0.05
Physcion	n.d.			Organic ions	0.27

The % represents w/w. The sum is 77.44 %.

2.3. Sample preparation

A section of colon was removed from each animal and washed in ice-cold mincing solution consisting of phosphate-buffered saline (PBS) with 20 mM ethylenediaminetetraacetic acid (EDTA) and 10 % dimethyl sulfoxide (DMSO). The section was incubated on ice in mincing solution for about 40 min, then washed and minced using scissors to release cells. Cells were poured into a Falcon tube and filtered through a cell strainer filter. The cells were then centrifuged at 4 °C, resuspended in cold PBS without Ca^{2+} and Mg^{2+} to a final concentration of 1×10^5 cells/mL and kept on ice until slide preparation.

2.4. Alkaline comet assay and slide analysis

Slides were prepared with the Trevigen® Comet Assay Kit (Bio Techne, Italy). A suspension of 50 µL for each sample of cells was added to 500 µL of Low Melting Agarose. An aliquot of 50 µL of this suspension was placed onto a glass microscope slide. At least three slides were prepared for each sample. Once ready, each slide was put in a pre-cooled lysis solution overnight at 4 °C in the dark and then incubated for 20 min in an alkaline electrophoresis buffer (pH > 13) to allow DNA unwinding. After that step, electrophoresis was performed for 25 min at 30 V and 300 mA with a Bio-Rad power supply, on ice. At the end of the electrophoresis, the slides were immersed in 0.3 M sodium acetate in ethanol for 30 min, then dehydrated in absolute ethanol for about 2 h, and dipped in 70 % ethanol for 5 min.

Slides were stained with 12 µg/mL ethidium bromide, examined with the Comet Assay IV system (Perceptive Instruments, UK) connected to a fluorescence microscope (Nikon Eclipse E400). About 150 cells were scored for each animal. DNA damage was evaluated as the extent of DNA migration through the parameters of % of tail intensity and Olive tail moment. Completely damaged cells were not scored in the 150 cells, even if counted.

2.5. Statistical analysis

All analyses were based on the responses of individual animals. The median tail intensity and the median tail moment for each slide were determined and the median values were calculated for each animal. Differences between control and treated groups were assessed using Dunnett's test for variance analysis. The homogeneity of the data was verified by Barlett's test before Dunnett's test. The criteria for statistical significance were $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

Following treatment with the test substance, no signs of adverse effects were observed in animals in any of the treatment groups. Slight body weight loss was randomly observed in the intermediate and low dose groups. This was not considered related to treatment since no body weight loss was seen in the high-dose group and slight body weight loss was also noted in some animals in the vehicle-treated and positive control groups.

The percentages of highly damaged cells (% clouds and hedgehogs) in the comet slides are shown for the colon preparations in Table 2. The control treatment with vehicle only was not cytotoxic (i.e., group mean of less than 30 % clouds or hedgehogs), indicating the correct preparation of the cell suspensions. Treatment with the test substance did not cause DNA damage which could have interfered with the comet analysis.

The positive control, EMS, induced clear and statistically significant increases in DNA migration parameters over the negative control (vehicle).

Analysis of variance for both comet parameters showed no statistically significant differences among groups. Variances of data were found to be homogeneous using Bartlett's test, hence differences between each treated group and the control group were assessed by Dunnett's *t* test which indicated that DNA migration was not statistically significantly increased in the treatment groups compared to the negative control group. Based on the stated criteria, the test substance was considered negative in the comet assay (Table 3).

4. Discussion

The European Commission proposed placing aloe-emodin and all the extracts in which this substance is present and leaf extracts of *Aloe* species containing hydroxyanthracene derivatives in Part A (ban on the use in food) of Annex III of Regulation (EC) no. 1925/2006 of the European Parliament and of the Council, due to alleged genotoxic and carcinogenic effects of hydroxyanthracene derivatives identified by the EFSA [6].

Aloe deprived of hydroxyanthracene derivatives (residual aloins 0.3 ppm) through an activated charcoal filtration process (decolourisation) was demonstrated to be non-genotoxic [7] when administered orally at doses of 500, 1000 and 2000 mg/kg bw/day to male F 344 rats for 2 consecutive days. Furthermore, *in vivo* genotoxicity comet tests carried out in mice following oral gavage of doses of 250, 500, 1000 and 2000 mg/kg bw/day of a high-titre aloe-emodin recently documented that the test material did not induce DNA strand breakage in single-cell preparations from colon and kidney [8].

Since these findings have at least partially mitigated the uncertainty regarding the alleged genotoxicity of hydroxyanthracene derivatives such as aloe-emodin, a new *in vivo* genotoxicity study (*in vivo* alkaline comet assay in mice - OECD 489) was conducted to test the potential genotoxicity of dried *Aloe ferox* juice, at doses of 500, 1000 and 2000 mg/kg bw/day. Dried *Aloe ferox* juice showed no genotoxic activity in preparations of single cells from the colon of the treated Hsd:ICR (CD-1) male mice.

The choice of conducting the genotoxicity experiment using the *in vivo* alkaline comet assay in mice was made as this test makes it possible to concentrate the experimentation on specific tissues that have been the subject of toxicological attention in previous studies while it was considered that the *in vivo* mammalian erythrocyte micronucleus (MN) test (OECD TG 474) was not suitable for a complex mixture such as that of a botanical preparation as it would be difficult to detect systemically or to prove the contact of the components of the mixture with the bone marrow [8].

Treatment with the test substance did not cause statistically significant increases in DNA migration over that of the negative control, as determined by analysis of variance for both comet parameters, tail moment and tail intensity. In contrast, the positive control, EMS, induced clear and statistically significant increases in DNA migration parameters over those of the negative control.

The new reported scientific evidence unequivocally demonstrates that both dried *Aloe ferox* juice containing hydroxyanthracene derivatives and aloe-emodin alone do not induce DNA damage in single-cell preparations from colon or kidney in *in vivo* genotoxicity studies (*in vivo* alkaline comet assay in mice - OECD 489) [8].

The hyperplastic changes and mucosal hyperplasia observed after long-term administration of *Aloe vera* non-decolourised whole leaf extract, whole leaf powder of *Aloe arborescens* Miller *var. natalensis* Berger or emodin in mice and rats may be attributed to an epigenetic effect of the material under investigation [9–14].

Repeated orally administration of *Aloe* botanical preparations showed that toxic effects are clearly linked to the initially damaged intestinal mucosa, caused by the diarrhoea persisting during the early phase of treatment. This continuous phenomenon is followed by lymphoid and goblet cell hyperplasia of the mesenteric lymph nodes associated with inflammation, necrosis, and hyperplastic changes in the intestine, colon and caecum. At high doses (4%), long-term treatments may result in adenomas and carcinomas (1/42), which are confined within the mucosal wall and do not metastasise (EFSA ANS Panel, 2018). The mechanism of action of the potential carcinogenicity of aloe-containing species may be a tumour-promoting effect at diarrhoeagenic doses, most probably due to gel polysaccharides releasing sugar monomers such as mannose (*Aloe vera*) [14] or, in particular, glucose and galactose (*Aloe ferox*) [15].

The intake of botanical preparations with laxative activity for very long periods (months, years) can cause a fluid imbalance that, over time, induces irritation of the intestinal tract that is manifested clinically as diarrhoea, which could play a role in the aetiology of the neoplasms observed in the large bowel.

In order to avoid this risk, the WHO very appropriately recommended that products "*containing anthraquinone glycosides should not be used continuously for longer than 1–2 weeks*" (thus for extended periods) and identified a safe individual dose as "*corresponding to 10–30 mg hydroxyanthraquinones per day*" [3]. Likewise in its herbal monographs on *Aloe* species containing hydroxyanthracene derivatives, the EMA concluded that their "*short-term use in case of occasional constipation can be regarded as safe*" and identified a safe maximum daily dosage of 30 mg

Table 2
Assessment of cytotoxicity of dried *Aloe ferox* juice on colon cells.

Treatment/dose mg/kg/day	% highly damaged colon cells
Vehicle	11.1
Aloe-emodin	
500	7.66
1000	4.18
2000	6.57
EMS	
150	11.1

Table 3
Evaluation of genotoxic damage by the alkaline comet test (pH > 13) in colon cells of mice treated orally with different doses of dried *Aloe ferox* juice.

	Colon cells	
Treatment mg/kg	Tail moment (arbitrary units)	Tail intensity (%)
Vehicle	0.0713 ± 0.0584	0.967 ± 0.757
Aloe extract		
500	0.0645 ± 0.0529	0.940 ± 0.715
1000	0.0492 ± 0.0494	0.816 ± 0.727
2000	0.0685 ± 0.0809	1.130 ± 0.893
EMS		
150	0.608 ± 0.0713***	7.82 ± 0.893***

Group mean ± SD.
*** $p < 0.001$ *vs* vehicle, ANOVA followed by Dunnett's test.

[16].

In issuing their opinion, the EFSA ANS Panel highlighted some gaps and uncertainties that need to be analysed in more depth with regard to reliability, relevance and the consistency of the lines of evidence.

In conclusion, the results of recent studies clearly demonstrate that neither aloe-emodin nor whole *Aloe* extract is genotoxic. Furthermore, they urge reconsideration of the mechanism by which botanical preparations containing hydroxyanthracene derivatives are supposed to cause the formation of neoplasms in treated animals, since the hypothesis that genotoxic effects trigger the formation of neoplasia is no longer plausible.

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CRediT authorship contribution statement

Corrado Galli: study concept, methodology, writing, including preparation of the original draft. **Serena Cinelli:** resources, data management, investigation. **Paola Ciliutti:** resources, data management, investigation, formal analysis. **Gloria Melzi:** project administration. Marina Marinovich: supervision, writing, reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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