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PII: S0273-2300(21)00107-0

DOI: https://doi.org/10.1016/j.yrtph.2021.104967

Reference: YRTPH 104967

To appear in: *Regulatory Toxicology and Pharmacology*

Received Date: 3 February 2021

Revised Date: 28 April 2021

Accepted Date: 26 May 2021

Please cite this article as: Galli, C.L., Cinelli, S., Ciliutti, P., Melzi, G., Marinovich, M., Aloe-emodin, a hydroxyanthracene derivative, is not genotoxic in an *in vivo* comet test, *Regulatory Toxicology and Pharmacology*, https://doi.org/10.1016/j.yrtph.2021.104967.

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,

TITLE: Aloe-emodin, a hydroxyanthracene derivative, is not genotoxic in an *in vivo* comet test

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Keywords:

Aloe-emodin

Hydroxyanthracene

In vivo rodent Comet assay

Kidney

Colon

Highlights:

Aloe-emodin hazard identification

Aloe-emodin is not genotoxic in vivo Comet rodent assay

Aloe-emodin is safe in food supplements and herbal medicinal products

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ABSTRACT

Aloe-emodin, one of the molecules belonging to the group of hydroxyanthracene derivatives, was recently described as genotoxic *in vivo*. Indeed, the EFSA judged that aloe-emodin, together with other similar molecules (emodin and danthron) and extracts from the leaf of *Aloe* species containing hydroxyanthracene derivatives, could represent a risk factor for colorectal cancer mediated by a genotoxic effect.

Given the marked uncertainty regarding the conclusions in the opinion of the EFSA ANS Panel and conflicts in the epidemiological data on which the opinion is based, a new *in vivo* study (*in vivo* alkaline comet assay in mice - OECD 489) was conducted to test the potential genotoxicity of aloe-emodin at doses of 250, 500, 1000 and 2000 mg/kg bw/day on preparations of single cells from the kidney and colon of treated male mice. Following treatment with the test item, no clinical signs were observed in animals in any treatment group. Slight body-weight loss was randomly observed in all groups treated with the test item and was more evident in the groups dosed at 1000 and 2000 mg/kg bw/day.

Under these experimental conditions, aloe-emodin showed no genotoxic activity. Possible oxidative damage to colon tissues could not be excluded based on the results obtained after repair enzyme treatment.

INTRODUCTION

Plants containing hydroxyanthracene derivatives are extensively used in food supplements and herbal medicinal products for their laxative effect. Anthracene derivatives are widely distributed in the plant kingdom, especially in botanicals such as the *Hypericum*, *Rheum*, *Rhamnus* and *Aloe* genera (Thomson, 1986).

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl) anthraquinone) is a dihydroxyanthraquinone belonging to the family of hydroxyanthracene derivatives. It is present in the pericyclic tubules of *Aloe* latex, an exudate from the *Aloe* plant, and also in *Cassia occidentalis*, *Rheum genus*. and *Polygonum multiflorum* Thunb.

It has been shown in animal experiments that at least 20-25% of an oral dose of aloe-emodin will be absorbed. However, the bioavailability of aloe-emodin is much lower than the absorption, because the compound is quickly oxidized to rhein and conjugated. Maximum plasma values of aloe-emodin were reached 1.5-3 hours after oral administration (Dong et al., 2020; Lang, 1993). Anthranoid metabolites are eliminated mainly via faeces, but also renally as glucuronides and sulphates (Teuscher and Lindequist, 2012).

The Food Additives and Nutrient Sources added to Food (ANS) Panel of the European Food Safety Authority (EFSA) recently deliberated that hydroxyanthracene derivatives should be "*considered 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*" (EFSA ANS Panel, 2018).

The present research aims to characterize the relationship between the development of neoplasms in rodents and whether the cause of this phenomenon may be due to a genotoxic or possibly epigenetic mechanism. The genotoxic potential of aloe-emodin was investigated in a number of *in vitro* assays, including mutation and micronucleus assays in mouse L5178Y cells, by kinetochore analysis, and through topoisomerase II and comet assays. Aloe-emodin reduced the amount of monomer DNA generated by topoisomerase II, indicating that it was capable of inhibiting topoisomerase II-mediated decatenation, and increased the fraction of DNA moving into comet tails at concentrations of 50 μM in single-cell gel-electrophoresis assays. The results of these assays indicate that aloe-emodin is genotoxic *in vitro* (Mueller and Stopper, 1999; Müller et al., 1996).

Four *in vivo* studies were conducted to investigate the genotoxicity of aloe-emodin and emodin. In these studies, rats or mice were administered aloe-emodin or emodin orally for periods from 4 hours

to 9 days. Analyses were conducted on bone marrow cells by micronucleus testing or in mouse foetal melanoblasts with the mouse spot test. The results showed no evidence of compound-induced increases of micronuclei, mutation induction or clastogenicity, although blood concentrations of aloe-emodin in the animals reached levels in the range of genetically active concentrations *in vitro* (Brusick and Mengs, 1997). The EFSA ANS Panel, however, considered these studies insufficiently reliable for methodological reasons (EFSA ANS Panel, 2018).

The only study deemed reliable and done with a protocol "*essentially compliant with the current OECD Guideline 489: in vivo mammalian alkaline comet assay*" was performed on both isolated kidney cells and colon cells from male OF1 mice, in which aloe-emodin induced DNA primary damage as observed between 3 and 6 h after two oral doses of 500, 1000 or 2000 mg/kg bw (Nesslany et al., 2009).

In relation to the marked uncertainty reported in the conclusions in the opinion of the EFSA ANS Panel, a new *in vivo* genotoxicity study (*in vivo* alkaline comet assay in male mice - OECD 489) was conducted to test the potential toxicity of aloe-emodin (purity 97.12%), at dose levels of 250, 500, 1000 and 2000 mg/kg bw/day selecting the two target organs, kidney and colon, of the rodent neoplastic developments. The choice of doses was based on the possible dose-effect relationship down to the lowest dose and mice were preferred to rats to avoid creating major discrepancies from the protocol followed in the paper published by Nesslany (Nesslany et al., 2009).

MATERIALS AND METHODS

Source

Aloe-emodin (kindly supplied by Zhengzhou Yuanli Biological Technology Co., Ltd.) suspensions with a purity 97.12 % (as determined by high performance liquid chromatography).

Solubility trial

A solubility trial of the test item was performed using 0.5% carboxymethylcellulose. A homogeneous suspension, feasible for dosing, was obtained at 200 mg/mL after 15 minutes of magnetic stirring. Based on this result, the maximum dose level of 2000 mg/kg bw/day was selected for administration of the test item using a dose volume of 10 mL/kg.

Test material preparation

Aloe-emodin suspensions were freshly prepared for each day's work in 0.5% carboxymethylcellulose (batch SLBT7528, obtained from Sigma) in deionized water (Sigma, Germany). Ethyl methanesulfonate (EMS; batch BCBV9352, Sigma) was used as a positive control and prepared in water of injectable grade (batch 20D0703, obtained from Eurospital).

Animals and treatments

Thirty-three male Hsd:ICR (CD-1) mice were supplied by Envigo RMS srl (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 on a 12 hour 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 9 to 10 weeks old the animals were treated with the test substance (five animals/group), the vehicle (0.5% carboxymethylcellulose) or the positive control (EMS).

Five animals were dosed twice by oral gavage with the vehicle alone or with the extract at the dose levels of 250, 500, 1000, and 2000 mg/kg/day at 0 hours and 24 hours. Three animals were treated with EMS, as a positive control, at a dose of 150 mg/kg/day. The amount of aloe-emodin to

administer was calculated for each animal according to its body weight. Treatments were administered by oral gavage. Animals were killed by asphyxiation with carbon dioxide 3 to 6 hours after the last dose.

Colon preparation

For each animal, a section of colon was removed 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 specimens were incubated in mincing solution for about 40 minutes and then washed and minced using scissors to release cells. The cells were poured into a Falcon tube and filtered through a cell strainer filter. Samples were centrifuged at 4°C, resuspended in cold PBS without Ca^{2+} or Mg^{2+} to a final concentration of 1×10^5 cells/mL and kept on ice until slide preparation.

Kidney preparation

Kidneys were removed from each animal. One of the organs was fixed in 10% neutral buffered formalin with part of the colon section in order to evaluate histopathological changes related to cytotoxicity that could induce increases of DNA migration. The other kidney was placed in an ice-cold Petri dish with mincing solution. The tissue was minced using scissors to release cells. Cells were poured into a Falcon tube and filtered through a cell strainer filter. After centrifugation at 4°C, cells were resuspended in cold PBS without Ca^{2+} or Mg^{2+} to reach a concentration of 1×10^5 cells/mL. Samples were kept on ice until slide preparation.

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. Every slide was put in a pre-cooled lysis solution overnight at 4°C in the dark. DNA unwinding was achieved by incubation for 20 minutes in an alkaline electrophoresis buffer (pH >13). Electrophoresis was performed for 25 minutes at 30V and 300 mA with a Bio-Rad power supply, on ice. The slides were immersed in 0.3 M sodium acetate in ethanol for 30 minutes, then dehydrated in absolute ethanol for 2 hours and immersed in 70% ethanol for 5 minutes.

Slides were stained with 12 µg/mL ethidium bromide. A total of 150 cells for each animal were examined with the Comet Assay IV system (Perceptive Instruments, UK) connected to a fluorescence microscope (Nikon Eclipse E400). DNA damage was evaluated as the extent of DNA migration via the parameter of % tail intensity and tail moment (the product of the proportion of tail intensity and the displacement of tail centre of mass relative to the centre of the head).

Tissue damage and cytotoxicity were evaluated on the basis of the number of necrotic and apoptotic cells ("clouds" and "hedgehogs") scored out of 150 cells examined for each animal.

Modified comet assay

After incubation with lysis solution, additional slides were incubated at 37°C with the enzyme hOGG1 for 35 minutes in order to evaluate DNA oxidative lesions. Results that provide a measure of strand breaks and oxidized bases (SB + OX) were compared with those obtained using the standard procedure which gives an estimate of the background DNA strand breaks (SB) (Smith C.C. et al., 2006).

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 the Dunnett's

test. If the variance of data was not homogeneous, a modified *t* test (Cochran and Cox) was applied. The criteria for statistical significance were $p < 0.05$, $p < 0.01$ and $p < 0.001$.

The statistical significance of differences among groups in the modified comet assay was assessed as for the standard assay, using the absolute values of tail moment and tail intensity. In addition, a two-way analysis of variance was performed in which enzymatic treatment (factor 1) and dose levels (factor 2) were fitted as categorical variables and the statistical significance of their interaction was calculated.

RESULTS

General observations

Following treatment with the test item, no clinical signs were observed in animals in any treatment group. As a result of the excretion of the test item, orange spots were observed in the litter of treated animals. Slight body-weight loss was randomly observed in all groups treated with the test item and was more evident in individual animals dosed at 1000 and 2000 mg/kg bw/day (Table 1).

Table 1: Body weight of animals after treatment with aloe-emodin.

	Body weight (g) – Group mean		
Treatment/dose mg/kg/day	Allocation	Day 1	Day 2
Vehicle	37	37	37
Aloe-emodin			
250	37	37	37
500	38	38	38
1000	38	39	38
2000	39	40	39
EMS			
150	37	38	37

Tissue damage and cytotoxicity assessment

The percentages of highly damaged cells (% clouds and hedgehogs) in kidney and colon in the comet slides are shown in Table 2. There was no tissue damage (i.e., less than 30% clouds or

hedgehogs) in the group administered the vehicle, indicating the correct preparation of the cell suspensions. Treatment with the test item did not cause DNA damage which could have interfered with the comet analysis.

Table 2: Tissue damage and cytotoxicity of aloe-emodin in colon and kidney cells

	% highly damaged cells/group Tissue	
Treatment/dose mg/kg/day	**Colon**	**Kidney**
Vehicle	4.53	1.92
Aloe-emodin		
250	3.60	4.39
500	2.24	1.81
1000	2.45	1.30
2000	2.34	0.70
EMS		
150	4.73	4.04

***In vivo* comet assay in mice**

Table 3: Evaluation of genotoxic damage by the alkaline comet test (pH >13) in colon cells of mice treated orally with different doses of aloe-emodin.

Treatment mg/kg	**Tail moment** (arbitrary units)	**Tail intensity** (%)
Vehicle	0.0476±0.0509	0.810±0.792
Aloe-emodin		
250	0.0514±0.0422	0.868±0.562
500	0.111±0.1041	1.688±1.339
1000	0.0850±0.0634	1.204±0.838
2000	0.0502±0.0518	0.896±0.826
EMS		
150	0.340±0.203**	4.50±2.43**

Mean ± standard deviation of median values from three slides for each animal

For all groups treated with aloe, not significant for analysis of variance; not significant for

Bartlett's test. ** p < 0.01 *vs* vehicle, Student's *t*-test

Table 4: Evaluation of genotoxic damage by the alkaline comet test (pH >13) in kidney cells of mice treated orally with different doses of aloe-emodin.

Treatment mg/kg	**Tail moment** (arbitrary units)	**Tail intensity** (%)
Vehicle	0.0598±0.0078	0.940±0.148
Aloe-emodin		
250	0.0380±0.0283	0.708±0.402
500	0.0528±0.0388	0.778±0.450
1000	0.0272±0.0205	0.470±0.256
2000	0.0406±0.0298	0.696±0.390
EMS		
150	0.393±0.1850**	5.467±2.175**

Mean ± standard deviation of median values from three slides for each animal

For all groups treated with aloe, not significant for analysis of variance; not significant for Bartlett's test. ** p < 0.01 *vs* vehicle, Student's *t*-test.

Analysis of variance of both comet parameters, i.e., tail moment and tail intensity, did not show any statistically significant differences among groups in the preparations of single cells from the colon and kidneys (Tables 3 and 4, respectively). 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 there was no statistically significant increase in DNA migration over that of the negative control in colon and kidneys of any treatment group.

Table 5: Evaluation of genotoxic damage by the alkaline comet test (pH >13) in colon cells of mice treated orally with aloe-emodin at different doses after enzymatic treatment.

Treatment mg/kg	**Tail moment** (arbitrary units)	**Tail intensity** (%)

Vehicle	0.09±0.027	1.53±1.55
Aloe-emodin		
250	0.12±0.067	1.86±0.87
500	0.24±0.068**	3.71±1.15
1000	0.29±0.098***	4.49±1.34
2000	0.24±0.060**	3.54±0.81

Mean ± standard deviation of median values from three slides for each animal

For all groups treated with aloe, not significant for analysis of variance; not significant for Bartlett's test. **p < 0.05, ***p < 0.001 *vs* vehicle, Dunnett's test

For colon tissue after enzyme treatment, no statistically significant dose-related increases in tail moment were observed starting from 500 mg/kg bw/day (Table 5) that might suggest oxidative damage. The two-way analysis of variance showed that the enzymatic treatment had a statistically significant effect and both comet parameters were generally higher following treatment with hOGG1.

Table 6: Evaluation of genotoxic damage by the alkaline comet test (pH >13) in kidney cells of mice treated orally with aloe-emodin at different doses after enzymatic treatment.

Treatment mg/kg	**Tail moment** (arbitrary units)	**Tail intensity** (%)
Vehicle	0.18±0.07	2.55±0.93
Aloe-emodin		
250	0.11±0.06	1.83±0.67
500	0.18±0.03	2.73±0.52
1000	0.29±0.12	4.00±1.55
2000	0.16±0.06	2.34±0.82

Mean ± standard deviation of median values from three slides for each animal

For all groups treated with aloe, not significant for analysis of variance; not significant for Bartlett's test.

For kidney cells, enzymatic treatment was a significant factor ($p < 0.001$) in explaining the observed variation in the data (Table 6). Tail intensity and tail moment were generally higher in the

presence of hOGG1 and the interaction between enzymatic treatment and dose levels was significant for both comet parameters. However, when differences between each treated group and the negative control group were assessed by Dunnett's *t* test, no statistically significant increase in DNA migration was observed at any dose level.

In conclusion, it can be stated that under the experimental conditions reported, aloe-emodin does not induce DNA strand breakage in single cell preparations of kidney and colon from male mice following oral gavage when tested up to the highest required concentration of 2000 mg/kg bw/day.

Based on the overall experimental evidence aloe-emodin can be considered as having no genotoxic potential.

DISCUSSION

Hydroxyanthracene derivatives are a class of chemical substances naturally present in different botanical species and used in food to improve bowel function.

The EFSA ANS Panel recently deliberated that hydroxyanthracene derivatives should be considered as genotoxic and carcinogens "unless there are specific data on the contrary" (EFSA ANS Panel, 2018).

Since hydroxyanthracene derivatives, such as aloe-emodin, are essential components of many herbal preparations, based on the possible harmful effect on health identified by the EFSA, the European Commission decided to place aloe-emodin and all the extracts in which this substance is present 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 a high level of health protection in accordance with the precautionary principle provided for in Article 7 of Regulation (EC) 178/2002 (Commission Regulation (EU) 2021/468 of 18 March 2021).

Most of the experiments conducted with aloe-emodin *in vitro* with bacteria and mammalian cells have shown a genotoxic effect (Chen et al., 2010; Heidemann et al., 1996; Mueller and Stopper,

1999), with a reduction of the amount of monomer DNA generated by topoisomerase II, indicating that the compound is capable of inhibiting topoisomerase II-mediated decatenation.

On the other hand, most of the *in vivo* genotoxicity experiments, in which animals received doses up to 2000 mg/kg bw, showed negative results, even if the data were considered by the ANS Panel to be insufficiently reliable since a validated protocol was not strictly followed (Heidemann et al., 1993, 1996; Mengs et al., 1997).

In the *in vivo* micronucleus test in rats given single oral doses of up to 1500 mg/kg bw, aloe-emodin failed to induce any genotoxic activity at both sampling times of 24 and 48 h after treatment. In the *in vivo* chromosome aberration assay in rat bone marrow, aloe-emodin demonstrated no clastogenic potential when tested at doses up to 2000 mg/kg bw by the oral route with two sampling times, 24 and 48 h after treatment. In a mouse spot-test, results showed that aloe-emodin was not mutagenic when given orally at doses of 20, 200 and 2000 mg/kg bw on Day 9 of pregnancy. Finally, in the *ex vivo* unscheduled DNA synthesis test, no primary DNA damage was observed in hepatocytes from male Wistar rats, treated orally once with 100 and 1000 mg/kg bw aloe-emodin and sampled either 4 or 16 h after treatment (Heidemann et al., 1996). No emodin genotoxicity was documented in a mouse bone marrow assay (Mengs et al., 1997).

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 (Nesslany et al., 2009). The authors claimed that there was a linear dose-related increase in DNA strand breaks in both tissues studied. In the kidney, this increase was statistically significant, but using a two-sample *t*-test, only at the highest dose tested, with average tail moments of the individual animals per group of 3.78, 4.77 and 4.92 at 500, 1000 and 2000 mg/kg/day, respectively *vs.* a value of 3.01 in the respective control group. The authors noted a dose-response relationship in colon cells, since the average tail moments for the three or four animals per group

were 9.36, 8.85 and 10.06 at doses of 500, 1000 and 2000 mg/kg/day aloe-emodin, respectively *vs.* a value of 4.28 in the control group.

For these reasons, the EFSA ANS Panel issued its opinion that there is a safety concern for extracts containing hydroxyanthracene derivatives "although uncertainty persists" (EFSA ANS Panel, 2018).

In order to address the uncertainties identified by the EFSA ANS Panel, we conducted an *in vivo* alkaline comet assay of aloe-emodin in mice, with the same experimental design as that used by Nesslany *et al*. (2009), but following standard guidelines i.e., the OECD Guideline for the Testing of Chemicals No. 489, utilizing Hsd: ICR (CD -1) male mice and adding a further dose of 250 mg/kg bw.

Samples were added to the standard OECD protocol in order to measure DNA strand breaks and oxidative DNA damage by enzymatic treatment. The enzyme-treated slides provide a measure of strand breaks and oxidized bases (SB + OX), while the reference slides, treated with enzyme buffer alone, provide an estimate of the background DNA strand breaks (SB). Assuming a linear dose response for tail % intensity as a function of DNA damage, subtraction of SB from (SB + OX) gives an estimate of DNA strand breaks from oxidized pyrimidines/altered purines (Ding *et al*., 2016).

As can be seen from Tables 2 and 3, aloe-emodin did not induce DNA damage in preparation of single cells from colon and kidneys following oral gavage at doses of 250, 500, 1000, and 2000 mg/kg/day under the standard reported experimental conditions. Furthermore, no statistically significant increases in tail moment and tail intensity were observed over those in the vehicle-treated control group at any dose level.

As expected (Smith *et al*., 2006), tail intensity and tail moment were generally higher in the presence of hOGG1 in both tissues in the vehicle and aloe-emodin-treated groups.

When differences between each treated group and the negative control group were assessed by Dunnett's *t* test, no statistically significant increase in DNA migration was observed at any dose

level in kidney cells, indicating that there was no induction of DNA strand breaks or oxidative DNA damage, invalidating even more strongly the hypothesis that the alleged genotoxicity of aloe-emodin is due to the formation of reactive oxygen species which in turn have a genotoxic effect.

For colon tissue, following the enzymatic treatment, statistically significant increases in break sites were observed above 500 mg/kg bw/day, although no dose-response relationship was identified, suggesting possible oxidative damage for colon tissue based on results obtained after repair enzyme treatment. This outcome is unlikely to be associated with the treatment as it is not dependent on the dose of aloe-emodin administered, hence its interpretation requires further study. It is concluded that high-titre aloe-emodin does not induce DNA strand breakage in single cell preparations of colon and kidney from male mice following oral gavage at doses of 250, 500, 1000 and 2000 mg/kg bw/day although possible oxidative damage can be hypothesised for colon tissue based on results obtained after repair enzyme treatment.

CONCLUSION

Repeated orally administration of some, but not all, hydroxyanthracene derivatives shows that the toxic effects are clearly linked to the initially damaged intestinal mucosa caused by lasting diarrhoea that occurs during the early phase of treatment. The continuous phenomenon is followed by lymphoid and Goblet cell hyperplasia of the mesenteric lymph nodes associated in rodents with inflammation, necrosis, renal toxicity, hyperplastic changes in the intestine, colon and caecum (Matsuda et al., 2008). At high doses (1.5% aloe vera non-decolourised whole leaf extract), long term treatments in rats may result in adenomas and carcinomas confined within the mucosa wall but not metastasizing (NTP, 2013; Boudreau et al., 2013). The Mechanism of Action (MoA) is likely due to a tumour promoting effect at a diarrheagenic doses (Yokohira et al., 2009), rather than a mechanism mediated by a genotoxic effect (Nesslany et al. 2007).

The potential genotoxic-event-mediated carcinogenic risk of long-term use of hydroxyanthracene derivatives with laxative effect is still very weak and more experimental data on characterized

materials are needed together with more reliable, relevant and consistent epidemiological studies (Nusko et al. 2000; Dukas et al. 2000; Watanabe et al. 2004; Kojima et al. 2004; Park et al. 2009; Zhang et al. 2013; Citronberg et al. 2014).

FUNDING BODY INFORMATION

This work was supported by the Italian Society of Toxicology (SITOX).

ACKNOWLEDGEMENTS

We thank Rachel Stenner and Robert Burns of Language Consulting Congressi, Milan, for English proofreading

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 INTERESTS

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

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Highlights:

Hazard assessment of hydroxyanthracene derivatives

In vivo negative genotoxicity of Aloe-emodin

Critical aspects of botanical hazard assessment

FUNDING BODY INFORMATION

This work was supported by the Italian Society of Toxicology (SITOX).

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: