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SCIENTIFIC OPINION

Scientific Opinion on additional scientific data related to the safety of preparations of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, *Rhamnus purshiana* DC., *Rhamnus frangula* L. and *Cassia senna* L., submitted pursuant to Article 8(4) of Regulation (EC) No 1925/2006

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) | Dominique Turck | Torsten Bohn | Jacqueline Castenmiller | Stefaan De Henauw | Karen Ildico Hirsch-Ernst | Helle Katrine Knutsen | Inge Mangelsdorf | Harry J. McArdle | Androniki Naska | Kristina Pentieva | Alfonso Siani | Frank Thies | Sophia Tsabouri | Marco Vinceti | AnnaMaria Rossi | Ariane Titz | Thibault Fiolet | Alexandre Maciuk

Correspondence: nif@efsa.europa.eu

Abstract

The Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver a scientific opinion on the safety of plant preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC. and from the leaf or fruit of *Cassia senna* L., which have been placed under Union scrutiny in Part C of Annex III in accordance with Article 8(4) of Regulation (EC) No 1925/2006. The NDA Panel reviewed the additional scientific data submitted during the period of scrutiny and the public consultation by interested parties. The pertinent scientific data were in vitro and in vivo genotoxicity studies on the plant preparations under consideration. All the results of the genotoxicity studies on plant preparations were negative. However, the plant preparations that were tested in the submitted studies were not sufficiently characterised with respect to the content of total and individual hydroxyanthracene derivatives (HADs) and components other than HADs. The studies confirmed the presence of [redacted], known to be genotoxic in vivo, and [redacted], shown to be genotoxic in vitro. In line with the EFSA Scientific Committee statement on genotoxicity assessment of chemical mixtures, considering the presence of an in vivo genotoxic compound, the plant preparations used in these studies have to be considered of concern for genotoxicity. Thus, the safety of preparations containing HADs from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC. cannot be established based on the submitted studies.

KEYWORDS

[redacted], cascara, [redacted], frangula, hydroxyanthracene derivatives, rhubarb, senna

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1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

1.1.1 | Background

Regulation (EC) No 1925/2006 harmonises the rules on the addition of vitamins and minerals and of certain other substances to foods. Article 8 of Regulation (EC) No 1925/2006[1] provides for a procedure for the regulatory management of a substance other than vitamins or minerals, or an ingredient containing a substance other than vitamins or minerals added to foods or used in the manufacture of foods that may present a potential risk to consumers.

1.1.2 | Terms of Reference

On 18 March 2021, pursuant to Article 1(2) of Commission Regulation (EU) 2021/468,[2] amending Annex III, Part C, of Regulation (EC) No 1925/2006, the Commission has placed the following entries under scrutiny:

- 'preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids containing hydroxyanthracene derivatives';
- 'preparations from the leaf or fruit of *Cassia senna* L. containing hydroxyanthracene derivatives';
- 'preparations from the bark of *Rhamnus frangula* L., *Rhamnus purshiana* DC. containing hydroxyanthracene derivatives'.

The European Commission requests the European Food Safety Authority's (EFSA) opinion on whether the new scientific data submitted by food business operators or any other interested parties demonstrate the safety of substances placed under Union scrutiny in accordance with Article 8(4) of Regulation (EC) No 1925/2006.

1.2 | Interpretation of the Terms of Reference

Recital 11 of Commission Regulation (EU) 2021/468[3] lays out the reasons for placing under Union scrutiny preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC. The reason for that decision is that '*scientific uncertainty persists about whether such preparations contain the substances listed in Annex III, Part A of Regulation (EC) No 1925/2006*'. These prohibited substances are aloe-emodin, emodin, danthron and preparations from the leaf of *Aloe* species containing hydroxyanthracene derivatives (HADs). As danthron is a synthetic compound not naturally present in plant preparations and was considered by the ANS Panel[4] only for read-across purposes (EFSA ANS Panel, 2018), the Panel interprets that danthron is not to be further considered in the present opinion. In the context of the terms of reference and the reasons given in the recitals of Commission Regulation (EU) 2021/468 for placing the substances referred to in the terms of reference under scrutiny, the Panel understands that EFSA is requested to assess, based on the data provided by food business operators (FBOs) or any other interested parties:

1. whether the plant preparations under evaluation contain aloe-emodin and/or emodin, which have been put in part A of the Regulation (EC) No 1925/2006 and/or contain other HADs, which, according to the Scientific Opinion of the ANS Panel, should be considered as genotoxic and carcinogenic unless there are specific data to the contrary (EFSA ANS Panel, 2018)
2. in case of the absence of genotoxic carcinogenic HADs (as demonstrated by appropriate analytical methods), the genotoxic and carcinogenic potential of the plant preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L., *Rhamnus purshiana* DC., following the principles given in the Statement of EFSA Scientific Committee on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019).

The Panel notes that HADs are inherent constituents of preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC.

[1]Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. OJ L 404, 30.12.2006, p. 26–38.

[2]Commission Regulation (EU) 2021/468 of 18 March 2021 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards botanical species containing hydroxyanthracene derivatives. OJ L 96, 19.3.2021, p. 6–8.

[3]Commission Regulation (EU) 2021/468 of 18 March 2021 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards botanical species containing hydroxyanthracene derivatives. OJ L 96, 19.3.2021, p. 6–8.

[4]The EFSA Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS Panel).

The term 'preparation', as defined by the EFSA Guidance on the safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), covers all preparations obtained from botanical materials (e.g. whole, fragmented or cut plants, plant parts, algae, fungi and lichens) by various processes (e.g. pressing, squeezing, extraction, fractionation, distillation, concentration, drying up and fermentation).

The mandate does not cover a re-assessment of the safety of individual HADs mentioned in Annex III, Part A of Regulation (EC) No 1925/2006 (i.e. aloe-emodin, emodin and danthron) or of the plant preparations included in Annex III, Part A of the same Regulation (i.e. preparations from the leaf of Aloe species containing HADs).

The mandate of the European Commission focuses on 'substances placed under Union scrutiny', but it has been clarified that it refers to the plant preparations placed under scrutiny. The scientific evaluation is limited to the data provided to EFSA by FBOs and any other interested parties.

1.3 | Previous assessment on hydroxyanthracene derivatives

In a favourable opinion of the EFSA NDA Panel on a health claim on HADs and the improvement of bowel function (EFSA NDA Panel, 2013), in relation to the restrictions of use, the NDA Panel had noted that the use of stimulant laxatives for more than 2 weeks should be avoided owing to *'the danger of electrolyte imbalance, impaired function of the intestine, and dependence on laxatives*' (EFSA NDA Panel, 2013). In that context, Member States raised concerns about the safety of HADs.

Therefore, in 2016, the European Commission initiated the procedure in the framework of Article 8(2) of Regulation (EC) No 1925/2006. In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002,[5] the European Commission asked EFSA to:

- review the existing scientific data on the possible link between the intake of HADs and a harmful effect on health;
- provide advice on a daily intake of HADs that does not give rise to concerns about harmful effects to health, for the general population, and as appropriate, for vulnerable subgroups of the population.

In 2018, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) issued an opinion on the safety of HADs (EFSA ANS Panel, 2018) in the framework of Article 8(2) of Regulation (EC) No 1925/2006.

In its opinion (EFSA ANS Panel, 2018), the EFSA ANS Panel limited its assessment to HADs from plant species with an established effect on bowel function owing to their content of HADs, as evaluated in the opinion of the NDA Panel (EFSA NDA Panel, 2013). The EFSA ANS Panel (2018) identified the possible carcinogenic risk of long-term use of anthranoid-containing laxatives as the specific concern that may have triggered the mandate. Therefore, the assessment performed by the EFSA ANS Panel (2018) was focused on the evaluation of the genotoxic and carcinogenic potential of HADs in preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC., which are used as stimulant laxatives.

In its opinion (EFSA ANS Panel, 2018), the EFSA ANS Panel concluded that '*the hydroxyanthracenes, emodin, aloe-emodin and the structurally related substance danthron, have been shown to be genotoxic in vitro*'. '*Furthermore, aloe-emodin was shown to be genotoxic in mice, the whole leaf aloe extract was carcinogenic to rats and there was evidence of carcinogenicity of the structural analogue danthron in both rodent species. Given that aloe-emodin and emodin may be present in the extracts, the Panel concluded that hydroxyanthracene derivatives should be regarded as genotoxic and carcinogenic unless there are specific data to the contrary, such as for rhein, and that there is a safety concern for extracts containing hydroxyanthracene derivatives, although uncertainty persists. The Panel was unable to provide advice on a daily intake of hydroxyanthracene derivatives that does not give rise to concerns about harmful effects to health, for the general population, and as appropriate, for vulnerable subgroups of the population*.'

Based on this assessment, aloe-emodin, emodin and danthron and preparations in which these substances are present as well as preparations from the leaf of Aloe species containing HADs were placed in Part A of the Annex III to Regulation (EC) No 1925/2006 (substances that cannot be added to foods or used in the manufacture of foods).

Preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC. containing HADs were placed under Union scrutiny in Part C of Annex III to Regulation (EC) No 1925/2006 since there was a possibility of harmful effects on health associated with the use of these plants and there was uncertainty about whether these preparations contain the substances listed in Annex III, Part A of Regulation (EC) No 1925/2006. Data allowing sufficient characterisation of these plant preparations in Part C were lacking at the time of the scientific assessment of the ANS Panel due to the absence of pertinent data (only partial information was available on senna extracts and senna fruits) (EFSA ANS Panel, 2018). Without a complete characterisation of the botanical extracts included in Part C, uncertainty remained as to whether they contain the prohibited substances listed in Part A.

In 2022, the European Commission asked EFSA to provide technical assistance in relation to the assessment of two scientific publications on aloe-emodin and dried aloe ferox juice (Galli et al., 2021a, 2021b), submitted by the Società Italiana Tossicologia (SITOX) on 24 January 2022. EFSA was asked to assess whether these publications were sufficient to revise

[5]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

the conclusions of the Scientific opinion of the EFSA ANS Panel on the safety of HADs (EFSA ANS Panel, 2018). After having been evaluated by the EFSA cross-cutting Working Group (ccWG) on Genotoxicity, EFSA concluded that the two studies by Galli et al. (2021a, 2021b) (two in vivo comet assays in mice) did not provide evidence that would warrant a revision of the conclusions of the EFSA ANS Panel on the safety of HADs for use in food (EFSA, 2022).

2 | DATA AND METHODOLOGIES

2.1 | Data

During the period of Union scrutiny, foreseen by Article 8(4) of Regulation (EC) No 1925/2006, two interested parties (Laboratoires ORTIS and Feder Botanicals Italia (FEI)) submitted data.

FEI submitted:

- an in vitro cytotoxicity assay in Caco-2 cells on single HADs (aloin A and B, aloe-emodin, emodin, cascaroside A, sennoside A and B, frangulin A and B, glucofrangulin A and B, rhein, physcion, chrysophanol and rhein-8-glucoside) and on plant preparations two bark dried extracts *of Rhamnus frangula* L., three bark samples of *Rhamnus frangula* L., one bark powder of *Rhamnus frangula* L., one bark dried extract of *Rhamnus purshiana* DC., two dried extracts of *Rhamnus purshiana* DC. (plant part not reported), one bark of *Rhamnus purshiana* DC., one bark powder of *Rhamnus purshiana* DC., one root powder of *Rheum palmatum* L., one rhizome powder of *Rheum palmatum* L., one rhizome powder of *Rheum rhaponticum* L., four leaf extracts of *Cassia angustifolia* Vahl, two leaf powders of *Cassia angustifolia* Vahl, one fruit powder of *Cassia angustifolia* Vahl, five extracts and one sample of *Cassia angustifolia* Vahl (plant part not reported) and proteomic profile data.
- in vitro and in silico investigations on intestinal bioaccessibility, cell viability of Caco-2 cells, pro-inflammatory parameters (interleukin (IL)-6 and IL-1β) and reactive oxygen species levels in Caco-2 cells after the exposure to single HADs (sennoside B, cascaroside A, aloin A, glucofrangulin A, rhein, emodin, aloe-emodin, danthrone) and plant preparations (three dry root and rhizome samples of *Rheum palmatum* L. or *Rheum officinale* Baill., leaf samples of *Cassia alexandrina* L., bark samples of *Rhamnus frangula* L., bark samples of *Rhamnus purshiana* DC. and two different *Aloe ferox* Mill. dried juices.
- a narrative review including studies on the pharmacokinetics of HADs and on toxicological studies on emodin, rhein, aloin, aloe-emodin, sennosides, aloe preparations, cascara preparations, frangula preparations, senna preparations and rhubarb preparations. There was only one study on genotoxicity (Wu et al., 2021): one bacterial reverse mutation assay on *Aloe vera* soft capsules. It should be noted that preparations from the leaf of Aloe species containing HADs are placed in Part A of Annex III of Regulation (EC) No 1925/2006 (prohibited substances) and are therefore not relevant for the current mandate. The other studies in the narrative review did not provide information on genotoxicity.

The Panel notes that none of the studies submitted by FEI were related to the genotoxic or carcinogenic potential of plant preparations placed under scrutiny. Therefore, the data submitted by FEI were not considered further.

ORTIS submitted three reports on the root of *Rheum palmatum* L. and/or *Rheum officinale* Baill.:

- a bacterial reverse mutation test in TA1535, TA1537, TA98, TA100 and TA102 Salmonella Typhimurium strains.
- an in vitro micronucleus assay in human lymphocytes.
- a combined in vivo comet assay and micronucleus assay (in femoral erythrocytes) in Sprague–Dawley (SD) rats.

In accordance with Article 38 of Regulation (EC) No 178/2002[6] and taking into account the protection of confidential information and of personal data in accordance with Articles 39, 39a to 39e of the same Regulation and of the Decision of the EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[7] the non-confidential versions of the data submitted by FEI and ORTIS are published in OpenEFSA.[8]

According to Art. 32c (2) of Regulation (EC) No 178/2002 and according to the Decision of EFSA's Executive Director laying down the practical arrangements on the pre-submission phase and public consultations, EFSA carried out a public consultation (PC-0706) from 31 October to 21 November 2023 on the non-confidential version of the submissions by FEI and ORTIS. The outcome of the public consultation is described in Appendix A to this Scientific Opinion. EFSA received comments from the Società Italiana Tossicologia (SITOX), Hermes Arzneimittel GmbH and Finzelberg GmbH & Co. KG. Among those comments, 11 additional genotoxicity studies from SITOX and Finzelberg GmbH & Co. KG. were provided to EFSA and are also addressed in the scientific evaluation.

[6]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, pp. 1–48.

[7]Decision available online: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements

[8]The non-confidential version of the dossier, following EFSA's assessment of the applicant's confidentiality requests, is published on Open.EFSA and is available at the following link: https://open.efsa.europa.eu/questions/EFSA-Q-2022-00790

These genotoxicity studies included:

- five bacterial reverse mutation assays in TA98, TA100, TA1535, TA1537 and TA102 Salmonella Typhimurium strains, on preparations of *Rheum palmatum* L. (plant part not reported), *Rhamnus purshiana* DC. (plant part not reported), *Rhamnus frangula* L. (bark) and *Cassia senna* L. (leaves and fruits),
- five in vitro micronucleus tests in human lymphocytes on preparations of *Rheum palmatum* L. (plant part not reported), *Rhamnus purshiana* DC. (plant part not reported), *Rhamnus frangula* L. (bark) and *Cassia senna* L. (leaves and fruits),
- one publication (Melzi et al., 2022) on a bacterial reverse mutation test and an in vitro micronucleus test in human lymphocytes on the rhizome of *Rheum palmatum* L. This publication was mentioned in the comments provided by Hermes Arzneimittel GmbH and SITOX during the public consultation.

2.2 | Methodologies

The scientific evaluation was solely based on studies submitted during the period of scrutiny and the public consultation. Only studies on genotoxicity were considered. No studies on carcinogenicity have been submitted.

The present evaluation was performed in line with EFSA's Scientific guidance documents on risk assessment of substances that are both genotoxic and carcinogenic (EFSA Scientific Committee, 2005), on genotoxicity testing strategies (EFSA Scientific Committee, 2011, 2017) and on uncertainty analysis in scientific assessments (EFSA Scientific Committee, 2018), as well as on the Statement of the EFSA Scientific Committee on the assessment of chemical mixtures (EFSA Scientific Committee, 2019) and the Technical Report on harmonised approach for reporting reliability and relevance of genotoxicity studies (EFSA Scientific Committee, 2023). Accordingly, the reliability was scored using numerical values, where 1 corresponded to 'Reliable without restriction', 2 corresponded to 'Reliable with restrictions', 3 corresponded to 'Not reliable' and 4 corresponded to 'Not assignable' and the relevance of the test system and study results were categorised into 'high', 'limited' or 'low' relevance. After being evaluated by the ccWG on Genotoxicity, the study results were presented as positive, negative, equivocal or inconclusive.

The outcome of the assessment of the genotoxicity studies carried out by the ccWG on Genotoxicity is presented in Appendix B and was submitted to the NDA Panel for its consideration and decision.

3 | ASSESSMENT

Fourteen studies received during the period of scrutiny and the public consultation investigated the genotoxic potential of plant preparations from *Rheum palmatum* L., *Rhamnus purshiana* DC., *Rhamnus frangula* L. and *Cassia senna* L.

3.1 | Characterisation of the plant preparations under assessment

The source material of the plant preparations placed under Union scrutiny according to Regulation (EC) No 1925/2006 is listed in Table 1.

The Plants of the World Online database was used to identify synonyms of the scientific names and the common names.

TABLE 1 Plant and plant parts that are the basis of preparations placed under Union scrutiny according to Regulation (EC) No 1925/2006.

Botanical name	Common name	Plant part
Rheum palmatum L. (synonyms: *Rhabarbarum palmatum* (L.) Moench, *Rheum potaninii* Losinsk and *Rheum qinlingense* Y. K.Yang, D. K. Zhang & J. K. Wu)	Chinese rhubarb	Root, rhizome
Rheum officinale Baill. (synonyms: *Rheum baillonii* F. Heim and *Rheum pichonii* Pierre ex A. Chev.)		
Senna alexandrina Mill. (synonym: *Cassia senna* L.)	Senna, Alexandrian senna	Leaf, fruit
Frangula alnus Mill. (synonym: *Rhamnus frangula* L.)	Alder buckthorn, frangula	Bark
Frangula purshiana (DC.) A.Gray ex J.G.Cooper (synonym: *Rhamnus purshiana* DC.)	Californian buckthorn, Cascara	

Test items and their relative composition in HADs are described for each study included in the scientific assessment in Table 2. There was no information on the absolute concentrations of HADs.

TABLE 2 Description and composition in hydroxyanthracene derivatives of the test items used in the studies.

Study	Plant preparations			
	Botanical name	**Part**	**Type of extract**	**HADs composition**
ORTIS				
Two certificates of analysis of the same batch of rhubarb extract were provided, reporting different results with respect to the content of individual HADs in the extracts and total HADs				
Bacterial reverse mutation test In vitro micronucleus assay	*Rheum palmatum* L. and/or *Rheum officinale* Baill. or their hybrids (as described in PhEur monograph 0291) Batch CPXAD16191003-3	Root	[redacted]	[redacted]
In vivo comet assay in rats and in vitro micronucleus assay in human lymphocytes	*Rheum palmatum* L. and/or *Rheum officinale* Baill. or their hybrids (as described in PhEur monograph 0291) Batch 21191498	Root	[redacted]	[redacted]
Finzelberg GmbH & Co. KG				
No information on aloe-emodin and emodin contents in the certificates of analysis. Comments from the public consultation indicated the presence of aloe-emodin and emodin and provided information on the type of extract used				
5 bacterial reverse mutation tests	'Senna' (botanical name not reported)	Leaves	Aqueous	HADs expressed as sennoside B: 6.3%
	Rhamnus purshiana DC. *'Cascara'* (as described in PhEur 0105)	Bark	Hydroalcoholic	Hydroxyanthracene glycosides expressed as cascaroside A: 19.1% Cascaroside expressed as cascaroside A: 68.8%
	'Frangulae cortices extractum siccum' *Frangula alnus* Mill.[b]	Bark	Hydroalcoholic	Glucofrangulins expressed as glucofrangulin A: 21.3%
	'Extr. Rhei radix spir. sicc'. *Rheum palmatum* L. and/or *Rheum officinale* Baill.[b]	Root	Hydroalcoholic	HADs expressed as rhein: 4.51%
	'Senna' (botanical name not reported)	Fruit	Hydroalcoholic	Hydroxyanthracene glycoside expressed as sennoside B: 20.30%
SITOX				
No information on aloe-emodin or emodin contents were reported in the study report (certificate of analysis). In a separate letter, SITOX provided an average content across all extracts of aloe-emodin (0.06%–0.23%), emodin (0.07%), rhein (0.16%) for the extracts tested in the five in vitro micronucleus tests and botanical names of the source materials				
5 in vitro micronucleus tests in human lymphocytes	*Cassia senna* L.	Fruits	Solvent: cultured medium with 10% deionised water	Hydroxyanthracene glycosides expressed as sennoside B: 21.6%
	Cassia senna L.	Leaves	Aqueous	Hydroxyanthracene glycosides expressed as sennoside B: 6.4%
	Rhamnus frangula L.	Bark	Hydroalcoholic extract > 15%	Glucofrangulins expressed as glucofrangulin A: 16.1%
	Rheum palmatum L.	Not reported	Hydroalcoholic extract 5%	Not reported
	Rhamnus purshiana DC., (as described in PhEur 0105)	Not reported	Solvent: cultured medium with 1.0% dimethyl sulfoxide	Glycosides expressed as cascaroside A: 19.1%. Cascarosides expressed as cascaroside A: 77.5%

(Continues)

TABLE 2 (Continued)

Study	Plant preparations			HADs composition
	Botanical name	**Part**	**Type of extract**	
Melzi et al. (2022)[a]				
Bacterial reverse mutation test, in vitro micronucleus test in human lymphocytes	*Rheum palmatum* L.	Rhizome	Hydroalcoholic	Aloin (A + B) n.d., aloe-emodin (0.37%), rhein (0.54%), emodin (0.31%), chrysophanol (0.10%), physcion (0.07%)

[a]Melzi et al. (2022) was mentioned in the comments from SITOX and Hermes Arzneimittel GmbH during the public consultation.

[b]Not formally specified by the applicant.

In the studies provided by ORTIS, two analyses of the same batch CPXAD16191003-3 of rhubarb extract using two different high-performance liquid chromatography methods provided different results with respect to the content of individual HADs in the extracts and total HADs ([redacted]). Part of the differences can be explained by the sampling differences and the different limits of detection for single HADs.

In the studies submitted by Finzelberg GmbH & Co. KG, no information on the content in aloe-emodin and emodin was available in the certificates of analysis. However, in the comments provided by Finzelberg GmbH & Co. KG during the public consultation, it was written *'all [the extracts] containing relevant amounts of hydroxyanthracenes including aloe-emodin and emodin'*.

The Panel notes that the plant preparations used in the submitted studies are not sufficiently characterised with respect to the exact contents of total and individual HADs. The Panel also notes that the other compounds of the plant preparations are not sufficiently characterised.

Overall, the studies submitted during the scrutiny period and from the public consultation confirm the presence of genotoxic HADs ([redacted] both listed in Part A of Regulation (EC) No 1925/2006) in the plant preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC.

3.2 | Genotoxicity assessment

Genotoxicity studies were submitted to EFSA by ORTIS, SITOX and Finzelberg GmbH & Co. KG. A detailed description of the studies and the outcome of the evaluation by the ccWG on Genotoxicity are provided in Appendix B.

3.2.1 | Bacterial reverse mutation assays

As described by the ccWG on Genotoxicity (Appendix B), induction of reverse mutations by extracts from *Rheum palmatum* L., *Rhamnus frangula* L., *Cassia senna* L. and *Rhamnus purshiana* DC. were investigated in Salmonella Typhimurium TA1535, TA1537, TA98, TA100 and TA102 strains by plate incorporation and pre-incubation methods with and without metabolic activation. The tested extract concentrations ranged from 16.8 up to 5000 µg/plate (ORTIS, Study no. FSR-IPL 191207; Finzelberg, 2011, 2016, 2018, 2019a, 2019b). No information on aloe-emodin and emodin concentrations was given in the reports provided by Finzelberg GmbH & Co. KG. The ORTIS study reported [redacted] and [redacted] concentrations in the extracts to be [redacted] and [redacted], respectively. No increase in the number of mutants was observed in any of these studies.

Negative results were also reported for *Rheum palmatum* L. dried rhizomes investigated in a study by Melzi et al. (2022) in Salmonella Typhimurium TA1535, TA1537, TA98 and TA100 and in *Escherichia coli* WP2 uvrA with and without metabolic activation. The concentrations of aloe-emodin and emodin in these extracts were 0.37% and 0.31%, respectively.

3.2.2 | In vitro micronucleus tests

As described by the ccWG on Genotoxicity (Appendix B), seven in vitro micronucleus tests, performed in human lymphocytes with and without metabolic activation and according to Organisation for Economic Co-operation and Development Test Guidelines 487, have been conducted with rhubarb extracts.

The genotoxic potential of *Rheum palmatum* L. extract ([redacted]) was investigated in cultured human peripheral blood lymphocytes, with and without metabolic activation (ORTIS, Study no. FSR-IPL 201103). Concentrations of 1315, 3024 and 4000 µg/mL were used in a 4-h treatment with and without S9-mix and concentrations of 614.4, 768 and 960 µg/mL were used in a 24-h treatment. No genotoxic activity was revealed in the absence or presence of metabolic activation.

Melzi et al. (2022) tested *Rheum palmatum* L. extract (containing aloe-emodin 0.37% and emodin 0.31%). Concentrations of 195–5000 µg/mL were used in a 3-h treatment, with and without S9-mix, and in a continuous (31 h) treatment without S9-mix. The authors did not find a significant induction of micronuclei nor a concentration response. *Rheum palmatum* L. hydroalcoholic extract 5% was tested in concentrations ranging from 16.2 to 2500 µg/mL in a 3-h treatment with and without S9-mix and in a 28-h treatment without S9-mix. Results were negative (SITOX, 2023b).

Similarly, a study on *Rhamnus purshiana* DC. dry extract in concentrations from 13 to 2000 µg/mL was negative. No information on aloe-emodin and emodin concentrations was provided (SITOX, 2023a). *Rhamnus frangula* L. dry hydroalcoholic extract > 15% (no information on aloe-emodin and emodin concentrations) was tested in an in vitro micronucleus test in human lymphocytes in a 3-h treatment with and without S9 metabolic activation and in a longer 24-h treatment without S9 mix. Due to precipitation, the top concentrations were 816 and 1143 µg/mL, respectively. No statistically significant increases in the frequency of micronuclei were observed in any of the tested conditions (SITOX, 2023c). A study with *Cassia senna* L. fruit and leaf dry extracts with concentrations up to 5000 µg/mL with or without metabolic activation also did not show any induction of micronuclei in human lymphocytes (SITOX, 2023d, 2023e).

3.2.3 | Combined in vivo comet assay and micronucleus test of rhubarb extract

As described by the ccWG on Genotoxicity (Appendix B), a combined comet assay and micronucleus test in vivo were conducted to investigate the genotoxic potential of the root rhubarb extract containing [redacted] and other HADs as described in Table 2 (ORTIS, ERBC Study no. A4454). Male SD rats were treated by oral gavage for 24 and 45 h with 500, 1000 and 2000 mg/kg per day of rhubarb extract or carboxymethyl cellulose (negative controls). No significant increase in DNA tail intensity (%) and tail moment were detected in cells isolated from the colon and liver in any treatment group. No induction of micronuclei in polychromatic erythrocytes (PCE) was found in any treatment group. In addition, no reduction of the PCE/(PCE + NCE) ratio was observed at any dose indicating that there was no bone marrow toxicity, and that target tissue exposure could not be demonstrated.

Summary and overall assessment

Seven in vitro bacterial reverse mutation tests, one combined in vivo comet assay and micronucleus test in SD rats and six in vitro micronucleus tests in human lymphocytes reported negative results for extracts from *Rheum palmatum* L. (root or rhizome), *Rhamnus purshiana* DC. (bark), *Rhamnus frangula* L. (bark) and *Cassia senna* L. (leaves and fruits). All these plant preparations contained aloe-emodin and emodin.

The previous EFSA Opinion on HADs (EFSA ANS Panel, 2018) concluded that aloe-emodin and emodin are genotoxic in vitro. Additionally, DNA damage caused by aloe-emodin in comet assays has been observed in mice colon (EFSA ANS Panel, 2018).

The Panel notes that all the submitted genotoxicity tests were conducted with plant preparations containing low concentrations of [redacted] and other HADs. In this situation, the absence of genotoxic results cannot be used to rule out the genotoxicity concern, originating from the presence of a genotoxic component in the plant preparation (EFSA Scientific Committee, 2019). This is in line with the EFSA Scientific Committee statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019) that recommends that chemically defined substances have to be assessed individually for their potential genotoxicity and that '*If a mixture contains one or more chemical substances that are individually assessed to be genotoxic in vivo via a relevant route of administration, the mixture raises concern for genotoxicity*'.

Based on the genetic endpoint, bacterial reverse mutation test, micronucleus tests in vitro and in vivo, comet assay in vivo used in the submitted studies are considered as test systems with high relevance for hazard identification according to the Technical report on Harmonised approach for reporting reliability and relevance of genotoxicity studies (EFSA Scientific Committee, 2023).

Therefore, the NDA Panel considers, in line with the conclusion of the ccWG on Genotoxicity, that despite the high relevance of the test assays used in the submitted studies, the relevance of the result is low, owing to the presence of genotoxic compounds in the tested plant preparations and the partially and/or insufficient characterisation of the plant preparations with respect to the content of total and individual HADs. According to the 2017 EFSA Scientific Committee opinion on the clarification of some aspects related to genotoxicity assessment, '*If, based on the overall assessment, concern for genotoxicity remains, establishing an HBGV is not considered appropriate*'. Thus, no safety threshold can be established for a mixture containing genotoxic compounds. In this case, conducting an intake assessment of these plant preparations containing genotoxic HADs would be inappropriate.

The Panel considers that the safety of preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC cannot be established based on the submitted studies.

4 | CONCLUSIONS

The Panel concludes that:

- the plant preparations that were tested in the submitted studies were not sufficiently characterised with respect to the content of total and individual HADs and components other than HADs;
- the additional data submitted confirm the presence of [redacted], known to be genotoxic in vivo, and [redacted], known to be genotoxic in vitro in the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, in the leaf or fruit of *Cassia senna* L. and in the bark of *Rhamnus frangula* L., and *Rhamnus purshiana* DC.;
- in line with the EFSA Scientific Committee statement on genotoxicity assessment of chemical mixtures, considering the presence of a compound that is genotoxic in vivo, the plant preparations containing HADs used in these studies must be considered of concern for genotoxicity; and
- the safety of plant preparations from the root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L. and *Rhamnus purshiana* DC. containing HADs cannot be established based on the submitted studies.

5 | DOCUMENTATION AS PROVIDED TO EFSA

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ORTIS, ERBC Study no. A4454 (EFSA-2022-00013810).

SITOX. (2023a). Cascara dry extract Ph. Eur: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182503).

SITOX. (2023b). Chinese rhubarb dry hydroalcoholic extract 5%: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182502).

SITOX. (2023c). Frangula dry hydroalcoholic extract > 15%: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182501).

SITOX. (2023d). Sennae fructus dry extract: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182504).

SITOX. (2023e). Senna Leaf Dry Extract Eur. Ph.: Micronucleus Test in Human Lymphocytes In vitro (ICCR Study Number: 397611).

6 | STEPS TAKEN BY EFSA

1. The files submitted by FEI and ORTIS pursuant to Article 8(4) of Regulation (EC) No 1925/2006 were received by EFSA within 24 months from the date on which the substances have been listed in Part C of Annex III to Reg (EC) No 1925/2006.
2. The scientific evaluation started on 08 April 2023.
3. In line with EFSA's policy on openness and transparency, a public consultation on non-confidential data submitted by FEI and Ortis was launched from 31 October 2023 to 21 November 2023. During the public consultation, additional studies were received and were included in the scientific evaluation (see Appendix A).
4. The Scientific Committee cross-cutting Working Group (ccWG) on Genotoxicity agreed on a list of questions for the food business operators to provide additional information about data submitted during the period of scrutiny. EFSA sent an additional data request letter to the FBOs (ORTIS and FEI) on 06 December 2023. The clock was stopped on 06 December 2023.
5. FEI and Ortis provided additional information on 20 December 2023. The clock restarted on 20 December 2023. An extension of the assessment deadline of 3 months applied.
6. The studies submitted during scrutiny period together with additional information provided by FEI and Ortis, as well as the additional studies submitted during public consultation have been evaluated by the ccWG on Genotoxicity. On 04 March 2024, the (ccWG) on Genotoxicity issued a technical report (in Appendix B) evaluating the genotoxicity aspects of the data received.
7. During its meeting on 20 March 2024, the NDA Panel, having considered the technical report of the (ccWG) on Genotoxicity and reviewed the data, adopted an opinion on additional scientific data related to the safety of preparations from root or rhizome of *Rheum palmatum* L., *Rheum officinale* Baillon and their hybrids, from the leaf or fruit of *Cassia senna* L. and from the bark of *Rhamnus frangula* L., and *Rhamnus purshiana* DC; submitted pursuant to Article 8(4) of Regulation (EC) No 1925/2006.

ABBREVIATIONS

ANS	EFSA Panel of Food Additives and Nutrients Sources added to Food
ccWG	cross-cutting Working Group
FBO	food business operator
FEI	Feder Botanicals Italia
HADs	hydroxyanthracene derivatives

HBGV	Health-based guidance value
IL-6	Interleukin 6
IL-1β	Interleukin-1 beta
NCE	Normochromatic erythrocytes
n.d.	Non detected
NDA	Panel on Dietetic Products, Nutrition and Food Allergies
NIF	Nutrition and Food Innovation
OECD TG	Organisation for Economic Co-operation and Development Test Guidelines
PCE	polychromatic erythrocytes
SC	Scientific Committee
SD	Sprague–Dawley
SITOX	Società Italiana Tossicologia (Italian Society of Toxicology)
WG	Working Group

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CONFLICT OF INTEREST
If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR
European Commission

QUESTION NUMBER
EFSA-Q-2022-00790

PANEL MEMBERS
Dominique Turck, Torsten Bohn, Jacqueline Castenmiller, Stefaan De Henauw, Karen Ildico Hirsch-Ernst, Helle Katrine Knutsen, Alexandre Maciuk, Inge Mangelsdorf, Harry J McArdle, Androniki Naska, Kristina Pentieva, Alfonso Siani, Frank Thies, Sophia Tsabouri and Marco Vinceti.

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APPENDIX A

Outcome of the public consultation 'Assessment of new scientific data related to Hydroxyanthracenes derivatives for use in food, pursuant to Article 8(4) of Regulation (EC) No 1925/2006 for safety evaluation of substances placed under Union scrutiny in Part C of Annex III' (PC-0706)

Four comments were submitted by four contributors. The comments are published on the EFSA web page as received (https://open.efsa.europa.eu/consultations/a0c0900000KyF5kAAF).

General comments

Contributor/Organisation	Comment and reply
Società Italiana di Tossicologia, Italy	**Comment 1**: Studies conducted by the Italian Society of Toxicology (SITOX) on the extracts of HAD-containing plants subjected to the monitoring period under Article 8 of Reg. 1925/06: Frangula dry hydroalcoholic extract > 15%: Micronucleus Test in Human Lymphocytes in vitro **Comment 2**: The files uploaded are: - A comment signed by Prof. Corrado L. Galli, SITOX Past President and reports on studies conducted by the Italian Society of Toxicology (SITOX) on the extracts of HAD-containing plants subjected to the monitoring period under Article 8 of Reg. 1925/06: (1) L 100999, Senna Leaf Dry Extract Eur. Ph.: Micronucleus Test in Human Lymphocytes In vitro (2) 0192305MBM Sennae fructus dry extract: Micronucleus Test in Human Lymphocytes In vitro (3) Chinese rhubarb dry hydroalcoholic extract 5%: Micronucleus Test in Human Lymphocytes In vitro (4) 0125330 Cascara dry extract Ph. Eur: Micronucleus Test in Human Lymphocytes In vitro (5) Frangula dry hydroalcoholic extract > 15%: Micronucleus Test in Human Lymphocytes In vitro And another publication with the title "Lack of genotoxicity of rhubarb (rhizome) in the Ames and micronucleus in vitro tests", Gloria Melzi, Corrado Galli, Paola Ciliutti, Cristina Marabottini and Marina Marinovich, Toxicology Reports 9 (2022) 1574?1579 **Comment 3**: Extracts of Rheum palmatum L., Rhamnus purshiana DC., Rhamnus frangula L., and Cassia senna L. are used as supplements or in traditional medicine, mainly for their laxative properties. These species contain hydroxyanthracene derivatives, considered as genotoxic and possibly related to colon-rectal cancer development. The aim of this research was to evaluate, using a micronucleus assay in vitro, the genotoxic potential of Rheum palmatum L., Rhamnus purshiana DC., Rhamnus frangula L. (bark) and Cassia senna L. (leaves and fruits) extracts. Concentration ranges evaluated were from 0 to 2000 µg/mL for Rhamnus purshiana DC, from 0 to 2500 µg/mL for Rheum palmatum L. and Rhamnus frangula L., and from 0 to 5000 µg/mL for Cassia senna L. The cytokinesis-block proliferation index was calculated to analyze the possible cytotoxicity induced by the treatments. Hydroxyanthracenes content varied between 0.06% and 0.23% for aloe-emodin, and 0.07% and 0.16% for emodin and rhein. No cytotoxicity was detected at the tested concentrations. Micronucleus results showed lack of genotoxicity for all the extracts tested. These results demonstrate that the Rheum palmatum L., Rhamnus purshiana DC, Rhamnus frangula L., and Cassia senna L. extracts do not induce micronuclei formation in human lymphocytes in vitro. As an indication, an attempted safety evaluation was made by estimating the dose of aloe-emodin to which a consumer of food supplements would be exposed by consuming products containing the tested Cassia senna and Rhamnus frangula. The dose of ingested aloe-emodin would be 0.00228 and 0.00657 mg/kg bw, in the worst case, for Cassia senna (800 mg) and Rhamnus frangula (200 mg) in a 70 kg individual. Such doses are tens of thousands of times lower than the doses that did not produce any genotoxic effects in in vivo tests. The risk characterization exercise carried out shows that the toxicological data on the hazard identification of different botanical extracts, which are very well characterized for their anthracene derivatives content, combined with worst-case data for a realistic exposure, indicate that the risk deriving from the use of the test extracts can be considered negligible **Reply**: additional studies provided by SITOX have been considered in the scientific assessment
HERMES ARZNEIMITTEL GMBH, Germany	**Comment 4**: Points to consider for the assessment of the mutagenicity or genotoxicity of anthraquinones from Rheum palmatum L. and Rheum officinale Baillon, radix as an infusion (herbal tea preparation, food grade): The aim of our statement enclosed is not to assess and evaluate the entire scientific publications, but to share our thoughts from a subordinate level referring to the design of the different approaches to assess mutagenicity and genotoxicity and the validity of the conclusions drawn with special regard to the publication by Melzi et al. (2022) (available open access via https://doi.org/10.1016/j.toxrep.2022.07.017). Melzi and coworkers show a lack of genotoxicity of rhubarb (rhizome) in the Ames and micronucleus in vitro tests using an solid soft extract from a 60 % ethanolic (V/V) fluid extract of ground rhubarb rhizome **Reply**: Melzi et al., 2022 publication has been considered in the scientific assessment
Finzelberg GmbH & Co. KG, Germany	**Comment 5**: Likewise, to the comments and information submitted by ORTIS no mutagenic effects in the Reverse Mutation Assay was identified for commercially available extracts from of Senna leaves and fruit, Rhamnus prushiana bark extract, Rheum palmatum and Rhamus frangula extract all containing relevant amounts of hydroxyanthracenes including aloeemodin and emodin. A commercial dry extract of Senna leaves, extracted with water DER 4-6:1, was examined in the 5 Salmonella typhimurium strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 in two independent experiments, each carried out without and with metabolic activation. No mutagenic effect was identified (Report Extr. Senna leaves sicc.). Mutagenicity study of the Extr. sennae e fruct. spir. sicc. 20% Hydroxyanthracene glycoside (DER 7-12:1, extracted with ethanol 60%) in the Salmonella typhimurium reverse mutation assay showed also negative results and did not reveal any mutagenic effects (Report AMES Extr. Seanna leaves fructus). A commercial extract from Rheum palmatum extracted with ethanol 70% (DER 2-5:1) showed only negative results in the reverse mutation assay using bacteria Salmonella typhimurium, with and without metabolic activation (Report AMES Extr. Rhei rad. spir. sicc.). No toxic effects in the reverse mutation assay using bacteria Salmonella typhimurium, with and without metabolic activation were noted using a commercial extract from Rhamnus prushiana bark (Cascara), produced with ethanol 75% and standardised to 18%–22% hydroxyanthracen glycosides (Report AMES Extr. Cascara sicc.). No toxic effects in the reverse mutation assay using bacteria Salmonella typhimurium, with and without metabolic activation were noted using a commercial extract from Rhamnus frangula produced with ethanol 60% (DER 5-7:1) and standardised to 18%–22% glucofrangulins The presented results demonstrate that commercially produced extracts form.Senna leaves and fruits, Rhamnus prushiana, Rhamnus frangula, Rheum palmatum did not show toxic effects in the reverse mutation assay, although significant concentrations of aloeemodin and emodin are typical for those extracts **Reply**: additional studies provided by Finzelberg GmbH & Co. KG have been considered in the scientific assessment

APPENDIX B

Request for assistance from the Nutrition and Food Innovation (NIF) Unit on the evaluation of new scientific data on rhubarb (*Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids), frangula (*Rhamnus frangula* L.), cascara (*Rhamnus purshiana* DC.) and senna (*Cassia Senna* L.)

B.1 | INTRODUCTION

The cross-cutting Working Group (ccWG) on Genotoxicity was asked by the Nutrition and Food Innovation (NIF) Unit to review the new scientific data submitted by interested parties pursuant to Article 8(4) of Regulation (EC) No 1925/2006 for the safety evaluation of substances placed under Union scrutiny in Part C of Annex III. In particular, the WG was asked to provide scientific advice on whether the submitted data are sufficient to demonstrate the safety of the several plant preparations containing hydroxyanthracene derivatives (HADs), namely rhubarb (*Rheum palmatum* L., Rheum officinale Baill. and their hybrids), frangula (*Rhamnus frangula* L.), cascara (*Rhamnus purshiana* DC.) and senna (*Cassia Senna* L.) preparations.

B.2 | METHODOLOGIES

The assessment of the data has been conducted independently by the ccWG Genotoxicity and submitted to the NIF Unit for its consideration and decision. The assessment was performed in line with EFSA guidance documents (EFSA, 2023; EFSA Scientific Committee, 2005, 2011, 2018, 2019). Accordingly, the reliability was scored using numerical values, where 1 corresponded to 'Reliable without Restriction', 2 corresponded to 'Reliable with Restrictions', 3 corresponded to 'Not reliable' and 4 corresponded to 'Not assignable' and the relevance of the test system and study results were categorised into high, limited or low relevance. Only study results of high and limited relevance were to be considered further. After being evaluated by the ccWG Genotoxicity, the study results were presented as positive, negative, equivocal or inconclusive. A tabular format was used to transparently structure the outcome of the evaluations.

B.3 | ASSESSMENT

B.3.1 | Genotoxicity studies on rhubarb, frangula, cascara and senna preparations

Several studies investigating the genotoxic potential of extracts from different plants, namely rhubarb (***Rheum palmatum*** L., ***Rheum officinale*** Baill. and their hybrids), frangula (***Rhamnus frangula*** L.), cascara (***Rhamnus purshiana*** DC.) and senna (***Cassia Senna*** L.), are described below and summarised in Table B.1.

The ccWG Genotoxicity noted that all the extracts covered by the present mandate are complex mixtures containing a substantial fraction of unidentified components. For such mixtures, the EFSA Scientific Committee (EFSA Scientific Committee, 2019) recommends that first the chemically defined substances be assessed individually for their potential genotoxicity. If the mixture contains one or more chemical substances that are evaluated to be genotoxic in vivo via a relevant route of administration, the whole mixture raises concern about genotoxicity. On this basis, considering that aloe-emodin was shown to be genotoxic in vivo, the mixture has to be considered of concern for genotoxicity if in a botanical extract the absence of this component cannot be demonstrated by appropriate analytical methods. This is independent of the outcome of experiments conducted on the whole extract.

For these reasons, all the studies on plant extracts were evaluated to be of low relevance.

B.3.1.1 | Bacterial reverse mutation assays

Induction of reverse mutations by extracts from rhubarb, *Rhamnus frangula* L., *Cassia Senna* fruit and leaves, Rheum radix and cascara sicc. was investigated in Salmonella Typhimurium TA1535, TA1537, TA98, TA100 and TA102 strains by plate incorporation and pre-incubation methods with and without metabolic activation (S9 mix from Aroclor 1254-induced rat liver). The tested extract concentrations ranged from 16.8 up to 5000 μg/plate (ORTIS, Study no. FSR-IPL 191207; Finzelberg, 2011, 2016, 2018, 2019a, 2019b). No information on aloe-emodin and emodin concentrations was reported in Finzelberg reports. The ORTIS study reported [redacted] and [redacted] concentrations in the extracts to be [redacted] and [redacted] respectively. No increase in the number of mutants was observed in any of these reports.

Negative results were also reported for Rhubarb dried rhizomes investigated in a study by Melzi et al. (2022) in S. typhimurium TA1535, TA1537, TA98 and TA100 and in *E. coli* WP2 uvrA with and without metabolic activation. The concentrations of aloe-emodin and emodin in these extracts were 0.37% and 0.31%, respectively.

B.3.1.2 | In vitro micronucleus tests

Several in vitro micronucleus tests, performed in human lymphocytes with and without metabolic activation and according to Organisation for Economic Co-operation and Development Test Guidelines 487, have been conducted with rhubarb extracts. The genotoxic potential of rhubarb extract (containing [redacted]) was investigated in cultured human peripheral blood lymphocytes, with and without

metabolic activation (ORTIS, Study no. FSR-IPL 201103). Concentrations of 1315, 3024 and 4000 μg/mL were used in a 4-h treatment with and without S9-mix, and concentrations of 614.4, 768 and 960 μg/mL were used with a 24-h treatment. No genotoxic activity was revealed in the absence or presence of metabolic activation.

Melzi et al. (2022) tested rhubarb extract (containing aloe-emodin 0.37% and emodin 0.31%) in concentrations 195–5000 μg/mL and did not find significant induction of micronuclei nor concentration response. Similarly, a study on cascara dry extract in concentrations from 13 to 2000 μg/mL was negative. No information on aloe-emodin and emodin concentrations was provided (SITOX, 2023a). Chinese rhubarb dry hydroalcoholic extract 5% (suspended or dissolved in dimethyl sulfoxide) was tested in concentrations ranging from 16.2 to 2500 μg/mL with negative results (SITOX, 2023b).

Frangula dry hydroalcoholic extract > 15% (no information on aloe-emodin and emodin concentrations) was tested in an in vitro micronucleus test in human lymphocytes in a short 3-h treatment with and without S9 metabolic activation and in a longer 24-h treatment without S9 mix. Due to precipitation, the top concentrations were 816 and 1143 μg/mL, respectively. No statistically significant increases in the frequency of micronuclei were observed in any of the tested conditions (SITOX, 2023c).

A study with Senna fructus and leaf dry extracts with concentrations up to 5000 μg/mL with or without metabolic activation also did not show any induction of micronuclei in human lymphocytes (SITOX, 2023d, 2023e).

B.3.1.3 | Combined in vivo comet assay and micronucleus test of rhubarb extract

A combined comet assay and micronucleus test in vivo was conducted to investigate the genotoxic potential of rhubarb extract (ORTIS, ERBC Study no. A4454). Male Sprague–Dawley (SD) rats were treated by oral gavage for 24 and 45 h with 500, 1000 and 2000 mg/kg per day of rhubarb extract or carboxymethycellulose (negative controls). No significant increase in DNA tail intensity (%) and tail moment were detected in cells isolated from colon and liver in any treatment group. The presence of micronuclei in polychromatic erythrocytes (PCE) and the numbers of normal and micronucleated normochromatic erythrocytes were used to assess aneugenicity. No induction of micronuclei in any treatment group was found.

B.3.2 | Other studies on HADs and HAD-containing extracts

Studies investigating endpoints other than genotoxicity and studies conducted on preparations not covered by the present mandate (i.e. Aloe vera preparations) were submitted to EFSA. Although these studies are considered not relevant for the present assessment, they are described below and reported in Table B.2 for transparency.

B.3.2.1 | Studies investigating endpoints other than genotoxicity

Two studies addressing aspects other than genotoxicity were submitted to EFSA by Feder Botanicals Italia (FEI). In the first one (Feder Botanicals Italy, Study 1) the cytotoxicity of several HADs was analysed when applied to Caco-2 cells as single compounds (aloin A and B, aloe-emodin, emodin, cascaroside A, sennoside A and B, frangulin A and B, glucofrangulin A and B, rhein, physcion, chrysophanol and rhein-8-glucoside) or as several whole cell extracts of plant-containing HADs. In addition, a comparison of proteomic profiles in the same cell line exposed to single compounds or whole cell extracts was also performed.

In the second study (Feder Botanicals Italy, Study 2), cytotoxicity, cytokine release and reactive oxygen species (ROS) production were compared in Caco-2 exposed to single HADs (sennoside B, cascaroside A, aloin A, glucofrangulin A, rhein, emodin, aloe-emodin and danthrone) or to several dry extracts of plant-containing HADs.

The results of these studies do not provide relevant information on the genotoxic potential of these compounds.

B.3.2.2 | Genotoxicity studies on Aloe vera Soft Capsules

A narrative review was submitted to EFSA by FEI (Feder Botanicals Italy, Narrative review) describing pharmacokinetics and toxicological studies on HADs and HAD-containing preparations. Among the studies cited in the narrative review, there was only one study on genotoxicity and was therefore assessed by the WG: one bacterial reverse mutation assay on Aloe vera Soft Capsules (ASC). The other studies in the narrative review did not provide information on genotoxicity.

The genotoxic potential of the content of ASC was investigated in a limited number of *S. typhimurium* strains (TA97, TA98, TA100 and TA102) with negative results. The reported aloin content in the capsule was in the range 0.32%–0.38%.

ASC were also tested in a chromosome aberration test in primary spermatocytes, conducted by oral gavage on male ICR mice. The study included five groups: a control group (olive oil, oral gavage), a positive control group and aloe vera + olive oil groups (2500, 5000 and 10,000 mg/kg bw). The treatment was provided once daily for five successive days. ASC did not induce chromosome aberrations at any of the tested doses.

Finally, Wu et al. (2021) also studied potential increases in micronuclei in the bone marrow of female and male mice. Animals were treated twice in a 24-h interval by oral gavage with either olive oil (negative control) or the ASC at a dose of 2500, 5000, 10,000 mg/kg bw. No evidence of the induction of bone marrow micronuclei was found.

It should be noted that preparations containing aloe vera are not permitted for use in foods (Regulation (EC) No 1925/2006) and therefore not relevant to the current mandate.

B.4 | CONCLUSION

The extracts from rhubarb (*Rheum palmatum* L., *Rheum officinale* Baill. and their hybrids), frangula (*Rhamnus frangula* L.), cascara (*Rhamnus purshiana* DC.) and senna (*Cassia Senna* L.) were tested for genotoxicity in several in vitro and in vivo studies.

All these studies showed negative results; however, the ccWG Genotoxicity noted that the tested extracts were complex mixtures and that aloe-emodin, known to be genotoxic in vivo (EFSA ANS Panel, 2018), is naturally present in these botanical extracts. As outlined in the EFSA Scientific Committee statement on genotoxicity assessment on chemical mixtures, '*if the mixture contains one or more chemical substances that are evaluated to be genotoxic in vivo via a relevant route of administration, the whole mixture raises concern about genotoxicity*' (EFSA Scientific Committee, 2019).

On this basis, considering that aloe-emodin was shown to be genotoxic in vivo, the mixture has to be considered of concern for genotoxicity if in a botanical extract the absence of this component cannot be demonstrated by appropriate analytical methods. This is independent of the outcome of experiments conducted on the whole extract.

Overall, the ccWG on Genotoxicity concluded that there is a safety concern for the genotoxicity of the plant preparations included in this mandate.

Documentation as provided to EFSA

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Finzelberg. (2019b). Reverse Mutation Assay using Bacteria (Salmonella typhimurium) with Extr. Rhei radix spir. sicc. (Eurofins Munich Study No.: 188181).

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SITOX. (2023b). Chinese rhubarb dry hydroalcoholic extract 5%: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182502).

SITOX. (2023c). Frangula dry hydroalcoholic extract > 15%: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182501).

SITOX. (2023d). Sennae fructus dry extract: Micronucleus Test in Human Lymphocytes in vitro (ICCR Study Number: 2182504).

SITOX. (2023e). Senna Leaf Dry Extract Eur. Ph.: Micronucleus Test in Human Lymphocytes In vitro (ICCR Study Number: 397611).

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TABLE B.1 Genotoxicity studies on rhubarb, frangula, cascara and senna preparations.

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vitro bacterial mutagenicity						
Bacterial reverse mutation test TA1535, TA1537, TA98, TA100, TA102 strains GLP, OECD Guideline no 471	0, 50, 150, 500, 1500 and 5000 µg/plate A dose of 3000 mg/plate was added for strain TA100 (+S9) Plate incorporation and pre-incubation method in presence and in absence of S9 metabolic activation	Rhubarb extract (CPXAD16191003-3) [redacted] Solvent: sterile water	**Negative** No increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration Decreases in the number of mutants is reported in some strains with no apparent toxicity e no consistency between the type of assay (plate or pre-incubation) and the presence or absence of S9 mix	2 No explanation is provided on the decrease in the number of mutants in the apparent absence of toxicity and no consistency for strain, concentration and type of assay Minor deviations from the study plan are reported Inconsistent information provided on the composition in HADs of the extract	High/Low (See Section B.3.1)	ORTIS, Study no. FSR-IPL 191207 (EFSA-2022-00014085)
Bacterial reverse mutation test TA98, TA100, TA1535, TA1537, TA102 strains GLP study OECD Guideline No. 471	0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Aroclor 1254-induced rat liver Positive controls: yes	Dry extract of senna leaves, DER 4–6:1 Hydroxyanthracene derivatives calculated as sennosid B: 6.3%	**Negative** No increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration No toxicity	1	High/Low (See Section B.3.1)	Finzelberg (2011)
Bacterial reverse mutation test TA98, TA100, TA1535, TA1537, TA102 strains GLP study OECD Guideline No. 471	0, 16.8, 53, 168, 530, 1680 or 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Aroclor 1254 -induced rat liver Positive controls: yes	Extr. sennae e fruct. spir. sicc. 20% Hydroxyanthracene glycoside (DER 7–12:1; 60% EtOH)	**Negative** No increase in the number of mutant colonies in any tested strains at any concentration neither in the presence or the absence of S9 Cytotoxicity (> 50%) at 5000 µg/plate and precipitation	1	High/Low (See Section B.3.1)	Finzelberg (2016)
Bacterial reverse mutation test TA98, TA100, TA1535, TA1537, TA102 strains GLP study OECD Guideline No. 471 Europhins Munich Study No. 3188180	0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Phenobarbital and b naphthoflavone induced rat liver Positive controls: yes	Cascara dry extract Ph. Eur. 85% native extract 15% excipient (Lactose Monohydrat Ph. Eur.) No information on aloe-emodin and emodin concentrations	**Negative** No increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration No toxicity	1	High/Low (See Section B.3.1)	Finzelberg (2018)

(Continues)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
Bacterial reverse mutation test TA98, TA100, TA1535, TA1537, TA102 strains GLP study OECD Guideline No. 471	0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Phenobarbital and b naphthoflavone induced rat liver Positive controls: yes	Frangulae corticis extractum siccum Ph. Eur. 80%–99% native extract 1%–20% excipient (Maltodextrin Ph. Eur.)	**Negative** No increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration No toxicity	1	High/Low (See Section B.3.1)	Finzelberg (2019a)
Bacterial reverse mutation test TA98, TA100, TA1535, TA1537, TA102 strains GLP study OECD Guideline No. 471	0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Phenobarbital and b naphthoflavone induced rat liver Positive controls: yes	Extr. Rhei radix spir. Sicc. (rhubarb) 70%–96% native extract 4%–30% excipient (1–27% Maltodextrin Ph. Eur., 3% Silica, colloidal anhydrous Ph. Eur.) No information on aloe-emodin and emodin concentrations	**Negative** No increase in the number of mutant colonies in any tested strains at any concentration neither in the presence or the absence of S9 Some toxicity in TA100 strain at 2500 and 5000 µg/plate. No cytotoxicity in any of the other strains	1	High/Low (See Section B.3.1)	Finzelberg (2019b)
Bacterial reverse mutation test TA1535, TA1537, TA98, TA100, *Escherichia coli* WP2 uvrA	0, 313, 625, 1250, 2500 and 5000 µg/plate Plate incorporation and pre-incubation assays +/– S9 from Moltox, (Molecular Toxicology, Inc) Positive controls: yes	Rhubarb (dried rhizomes, from China) extract (solid soft extract obtained from a 60% ABV ethanolic fluid extract of ground rhubarb rhizome) Aloe-emodin 0.37% Emodin 0.31% Rhubarb (dried rhizomes, from China) extract (solid soft extract obtained from a 60% ABV ethanolic fluid extract of ground rhubarb rhizome) Aloe-emodin 0.37% Emodin 0.31%	**Negative** No significant increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration No toxicity	1	High/Low (See Section B.3.1)	Melzi et al. (2022)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vitro chromosomal damage						
In vitro micronucleus test in Human peripheral blood lymphocytes from 18 to 35 years healthy non-smoker subjects 2000 binucleated cells/ concentration GLP, OECD guideline 487, ICH S2 (R1)	(1) Without S9-mix, 4-h treatment +24 h recovery period 4000–3024.6 – 1315 µg/mL Positive control = mitomycin C 0.075 µg/mL (2) Without S9-mix, 24-h treatment without recovery period (continuous treatment) 4000–3024.6 – 1315 µg/mL Positive control: cyclophosphamide 10 µg/mL (3) With 5% S9-mix, 4-h treatment + 24 h recovery period 960–768 – 614.4 µg/mL Positive control = mitomycin C 0.075 µg/mL griseofulvin 10 µg/mL	Rhubarb extract (CPXAD16191003-3) [redacted] Test item prepared in sterile water	**Negative** No genotoxic activity was revealed in absence of metabolic activation, with a short or a continuous treatment or in presence of metabolic activation with a short-term treatment Reduction in number of micronuclei in cells treated with the rhubarb extract	2 In short-term treatments (with and without S9-mix) recommended cytotoxicity was not achieved Number of micronuclei in negative control (4 h without treatment) exceeded historical control Inconsistent information provided on the composition in HADs of the extract	High/Low (See Section B.3.1)	ORTIS, Study no. FSR-IPL 201103 (EFSA-2022-00014086)
In vitro micronucleus test in human lymphocytes from four healthy donors under 35 years old OECD TG 487 GLP study	Treatment 3 h with and without S9 MIX, continuous (31 h) treatment without S9 mix. Concentrations: 195–5000 µg/mL. Cytochalasin B (6 µg/mL) Positive controls: 3 h -S9: Mitomycin C 3 h + S9: Cyclophosphamide (CP) Continuous treatment: Colchicine (Col) Cytotoxicity: CBPI	Rhubarb (dried rhizomes, from China) extract (solid soft extract obtained from a 60% ABV ethanolic fluid extract of ground rhubarb rhizome) Aloe-emodin 0.37% Emodin 0.31%	**Negative** No induction of micronuclei, no concentration response Cytotoxicity Short treatment + and –S9 highest conc. Appr 20% cytotoxicity Continuous treatment: max conc. 69% cytotoxicity	2 Only 1000 binucleated cells per concentration per cell culture were scored	High/Low (See Section B.3.1)	Melzi et al. (2022)

(Continues)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vitro micronucleus test in human lymphocytes Exp 1- female donor 19 year old Exp 2 – male donor 24 year old OECD TG 487 GLP study	Two independent experiments Solvent: Dimethyl sulfoxide (1%) EXP 1 Short treatment: 3 h + and –S9 EXP 2 Continuous treatment: –S9 Concentrations from 13.0 to 2000 µg/mL Cytotoxicity – preliminary exp – CBPI PC -S9: Mitomycin C (MMC) 3-h treatment Vinblastine – continuous treatment PC + S9: Cyclophosphamide CPA	Cascara dry extract Ph. Eur, batch 20011908, brownish powder Glycosides expressed as cascaroside A: 19.1%. ascarosides expressed as cascaroside A: 77.5% No information on aloe-emodin and emodin concentrations in the study report	**Negative** No induction of micronuclei when tested up to precipitating concentrations Recommended cytotoxicity was not achieved in any of treatment conditions due to precipitation	1	High/Low (See Section B.3.1)	SITOX (2023a)
In vitro micronucleus test in human lymphocytes EXP I – female donor 19 years old EXP II – male donor 20 years old EXP III – male donor 23 years old OECD TG 487 GLP study	Three independent experiments EXP I Short: 3 h + and –S9 EXP II and III 28-h treatment -S9 Concentrations from 16.2 to 2500 µg/mL. Due to precipitation, top concentrations were: EXP I 466 µg/mL EXP II 550 µg/mL - not analysed for micronuclei due to solubility problem EXP III 579 µg/mL Cytotoxicity – preliminary exp – CBPI Solvent: Dimethyl sulfoxide (1%) PC -S9: Mitomycin C (MMC) 3 h treatment Vinblastine – continuous treatment PC + S9: Cyclophosphamide CPA	Chinese rhubarb dry hydroalcoholic extract 5% Batch 20B0029100 Brown powder suspended (Experiment I) or dissolved (Experiment II and III) in dimethyl sulfoxide No information on aloe-emodin and emodin concentrations in the study report	**Negative** No induction of micronuclei when tested up to precipitating concentrations in EXP1 In EXP III after 28-h treatment with 579 µg/mL, significant increase (1.10%) of micronucleated cells but within the historical control data (0.00%–1.19%) In EXP I both + and –S9 recommended cytotoxicity was not achieved up to precipitation concentration In EXP I without S9 the concurrent solvent control value of 0.95% MN cells exceeded the historical control data (0.01%–0.92%) but were within min and max of historical control	1	High/Low (See Section B.3.1)	SITOX (2023b)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vitro micronucleus test in human lymphocytes EXP I – male donor 31 years old EXP II – male donor 24 years old OECD TG 487 GLP study	Two independent experiments EXP I Short: 3 h + and –S9 EXP II 28-h treatment - S9 Concentrations from 16.2 to 2500 µg/mL. Due to precipitation, top concentrations were: EXP I 816 µg/m EXP II 1143 µg/mL Cytotoxicity – preliminary exp – CBPI Solvent: Dimethyl sulfoxide (1%) PC -S9: Mitomycin C (MMC) 3-h treatment Vinblastine – continuous treatment PC + S9: Cyclophosphamide CPA	Frangula dry hydroalcoholic extract > 15% Batch 20B0078300 Brown powder No information on aloe-emodin and emodin concentrations in the study report	**Negative** Non-mutagenic when tested up to precipitating concentrations Cytotoxicity in EXP I: –S9 no toxicity +S9 highest conc. 44.2% EXP II: 28-h treatment 13.2%	1	High/Low (See Section B.3.1)	SITOX (2023c)
In vitro micronucleus test in human lymphocytes EXP I – male donor 23 years old EXP II – male donor 20 years old OECD TG 487 GLP study	2 independent experiments EXP I Short: 3 h + and –S9 EXP II 28 h treatment -S9 Concentrations from 32.5 to 5000 µg/mL Top conc in main EXP: 5000 µg/mL Cytotoxicity – preliminary exp. – CBPI Solvent: Deionised water PC -S9: Mitomycin C (MMC) 3 h treatment Vinblastine – continuous treatment PC + S9: Cyclophosphamide CPA	Sennae fructus dry extract Batch 21001501 Sennoside B: 21.6% No information on aloe-emodin and emodin concentrations in the study report	**Negative** Non-mutagenic when tested up to the highest required concentration No cytotoxicity in any of conditions	1	High/Low (See Section B.3.1)	SITOX (2023d)

(Continues)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vitro micronucleus test in human lymphocytes EXP I- male donor 26 years old EXP II – female donor 22 years old OECD TG 487 GLP study	2 independent experiments EXP I Short: 3 h + and –S9 EXP II 28 h treatment -S9 Concentrations from 18.9 to 5000 µg/mL Due to precipitation top conc. in EXPI –S9 was 544 µg/mL: +S9 952 µg/mL EXP II 533 µg/mL Cytotoxicity – preliminary exp. – CBPI Solvent: culture medium, sonicated 5 min PC -S9: Mitomycin C (MMC) 3 h treatment Vinblastine – continuous treatment PC + S9: Cyclophosphamide CPA	Senna Leaf Dry Extract Eur. Ph. Batch SEN211125/3/80 Brown powder Sennoside B: 6.4% No information on aloe-emodin and emodin concentrations in the study report	**Negative** Non-mutagenic when tested up to precipitating concentrations No cytotoxicity in any of conditions	1	High/Low (See Section B.3.1)	SITOX (2023e)
In vivo DNA damage						
In vivo single cell gel Comet Assay in Sprague–Dawley SD rats (two assays) 7–8 weeks old for main assays ICH S2 (R1), OECD Guideline no 474, no 489 Five male/group Colon and liver cells	Oral gavage 500, 1000 and 2000 mg/kg per day, at 0, 24 h (Day 2) and 45 h (Day 3) Solvent: 0.5% carboxylmethylcellulose Positive control: ethyl methanesulfonate by oral gavage (150 mg/kg bw per day)	Rhubarb extract (CPXAD16191003-3) [redacted]	**Negative** No statistically significant increase in the % of DNA tail intensity was observed in any treatment group. Experiment 1 – High DNA damage in control cells – exp terminated Experiment 2 – only comet assay performed (with some modification) Significant decreases in tail intensity observed at the intermediate and high-dose levels in colon and liver cells. Only lowest concentration – non-significant increase (liver) Body weight loss observed in all treated groups Piloerection was observed in the high-dose group [redacted] was determined in rat plasma	2 Unclear high level of DNA damage in control	High/Low (See Section B.3.1)	ORTIS, ERBC Study no. A4454 (EFSA-2022-00013810)

TABLE B.1 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
In vivo chromosomal damage						
In vivo micronucleus test in Sprague–Dawley SD rats 7–8 weeks old for main assays Erythrocytes from femur of rats ICH S2 (R1), OECD Guideline no 474, no 489	Oral gavage 500, 1000 and 2000 mg/kg per day, at 0, 24 h (Day 2) and 45 h (Day 3) Solvent: 0.5% carboxylmethylcellulose Positive control: Mitomycin C by intraperitoneal route (10 mL/kg bw per day)	Rhubarb extract (CPXAD16191003-3) [redacted]	**Negative** No induction of micronuclei in polychromatic erythrocytes in the bone marrow of treated rats Bone marrow cell toxicity: no inhibitory effect on erythropoietic cell division at any dose level Body weight loss observed for all the test groups. Soft faeces were found in the high-dose group [redacted] were determined in rat plasma	1	High/Low (See Section B.3.1)	ORTIS, ERBC Study no. A4454 (EFSA-2022-00013810)

TABLE B.2 Other studies on HADs and HAD-containing extracts.

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/Relevance of the result	Reference
In vitro cytotoxicity Proteomic profile Caco-2 human colorectal carcinoma cells	Quantification of the HAD analytes in the extracts by Ultimate 3000 UPLC system (ThermoFisher Scientific) coupled with a high-resolution Q-Exactive Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer Viability: AlamarBlue reagent Treatment time: 48 h Solvent: Dimethyl sulfoxide Proteomics: LC–MS/MS	1–20 ppm single compounds: aloin A and B, aloe-emodin, emodin, cascaroside A, sennoside A and B, frangulin A and B, glucofrangulin A and B, rhein, physcion, chrysophanol and rhein-8-glucoside Whole cell extracts: six samples *of Rhamnus Frangula*, five samples of *Rhamnus purshiana*, two samples of *Rheum palmatum,* one sample of *Rheum raponticum* and 15 samples of *Cassia angustifolia*	**Cytotoxicity** Various HAs are toxic to Caco-2 cells when delivered as single compounds but not as whole cell extract **Proteomics:** Different protein profiles were identified in Caco-2 cells exposed to single molecules (proteins involved in apoptotic and DNA damage process) and whole cell extracts (proteins involved in cell proliferation and negative regulation of the apoptotic process)	3 These results do not provide information on the genotoxic potential of these compounds	Low (See Section B.3.2)	Feder Botanical Italy (FEI), Study 1 (EFSA-2022-00013219)
In vitro and in silico investigation on intestinal bioaccessibility, viability, inflammatory and oxidative impact Caco-2 cells	Simulated digestion: treatment time 4 and 24 h ROS measurements by H2-DCF-DA IL-6 and IL-1b by ELISA kits Cytotoxicity: CCK-8 assay (colorimetric method)	0.10–0.01 mg/mL for single HADs 0.15–0.01 mg/mL for extracts Single compounds: sennoside B, cascaroside A, aloin A, glucofrangulin A, rhein, emodin, aloe-emodin, danthrone Three dry extracts of *Rheum palmatum* L. or *Rheum officinale* Baillon, roots and rhizomes (rhubarb), *Cassia alexandrina L.*, leaves (senna), *Rhamnus frangula L.* cortex (frangula), *Rhamnus purshiana* DC. cortex (cascara), two different *Aloe ferox* Mill and dried juices (*aloe*)	**Cytotoxicity:** No cytotoxicity of whole cell extracts while some toxicity of the single compounds **Cytokine release:** No significant release by whole cell extracts **ROS production:** No significant changes induced by whole cell extracts	3 These results do not provide information on the genotoxic potential of these compounds	Low (See Section B.3.2)	Feder Botanical Italy (FEI), Study 2 (EFSA-2022-00013038)

TABLE B.2 (Continued)

Test system/Test object	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/Relevance of the result	Reference
Bacterial reverse mutation test TA97, TA98, TA100, TA102 strains	0, 8, 40, 200, 1000, 5000 µg/plate of Aloe vera soft capsules Plate assay +/– S9 from MOLTOX, Molecular Toxicology Inc., USA Positive controls: yes	Aloe vera soft capsules prepared by the HEALTHMAY biotechnology Co. Ltd in HuBei Every 100 g Aloe vera Soft Capsules contained 0.32–0.38 g aloin, 45–55 g xylo-oligosaccharide, 44.5–54.5 g sunflower seed oil and 0.15 g beeswax.	**Negative** No increase in the number of mutant colonies neither in the presence or the absence of S9 in any tested strains at any concentration	2 Limited number of strains used The tested concentration tested is related to the capsule content	High/Low The test material is out of scope for the present mandate (see Section B.3.2)	Wu et al. (2021) – cited in Feder Botanicals Italy (FEI), Narrative review
In vivo mouse chromosome aberration assay in primary spermatocytes	ICR mice, male 5 per group 832.5, 1665 and 3330 mg/kg body weight of ASC ASC dissolved in 15 mL olive oil PC mitomycin colchicine by intraperitoneal on the ninth day after the last treatment 100 metaphase division phase of primary spermatocyte in each mouse	Aloe vera soft capsules prepared by the HEALTHMAY biotechnology Co. Ltd in HuBei Every 100 g Aloe vera Soft Capsules contained 0.32–0.38 g aloin, 45–55 g xylo-oligosaccharide, 44.5–54.5 g sun flower seed oil and 0.15 g beeswax	**Negative** No induction of chromosomal aberrations in mouse primary spermatocytes No evidence of toxicity	2	High/Low The test material is out of scope for the present mandate (see Section B.3.2)	Wu et al. (2021) – cited in Feder Botanicals Italy (FEI), Narrative review
In vivo micronucleus test in bone marrow of mice	Fifty ICR mice, five male, five female 2500, 5000, 10,000 mg/kg bodyweight Oral gavage Two exposures – 0 and second 24 h after PC 40 mg cyclophosphamide	Aloe vera soft capsules prepared by the HEALTHMAY biotechnology Co. Ltd in HuBei Every 100 g Aloe vera Soft Capsules contained 0.32–0.38 g aloin, 45–55 g xylo-oligosaccharide, 44.5–54.5 g sun flower seed oil and 0.15 g beeswax	**Negative** No increased micronucleus frequency in bone marrow No evidence of toxicity	2	High/Low The test material is out of scope for the present mandate (see Section B.3.2)	Wu et al. (2021) – cited in Feder Botanicals Italy (FEI), Narrative review