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Safety and efficacy of a feed additive consisting of *Macleaya cordata* (Willd.) R. Br. extract and leaves (Sangrovit® extra) for all poultry species (excluding laying and breeding birds) (Phytobiotics Futterzusatzstoffe GmbH)

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of *Macleaya cordata* (Willd.) R. Br. extract and leaves (Sangrovit® Extra) when used as a zootechnical feed additive (functional group: other zootechnical additives) for all poultry species (excluding laying and breeding birds). The additive is standardised to contain a concentration of the sum of the four alkaloids sanguinarine, chelerythrine, protopine and allocryptopine of 1.25%, with 0.5% sanguinarine. Owing to the presence of the DNA intercalators sanguinarine and chelerythrine, a concern for genotoxicity was identified. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) had no safety concerns when the additive is used at the recommended level of 150 mg/kg complete feed (corresponding to 0.750 mg sanguinarine/kg complete feed) for chickens for fattening and other poultry species for fattening. No conclusion can be drawn for poultry reared for laying/breeding. The use of Sangrovit® Extra in poultry species for fattening at the maximum recommended level was considered of low concern for consumers. The additive was shown to be irritant to the eyes but not irritant to skin or a skin sensitiser. The FEEDAP Panel could not exclude the potential of the additive to be a respiratory sensitiser. When handling the additive, exposure of unprotected users to sanguinarine and chelerythrine may occur. Therefore, to reduce the risk, the exposure of users should be reduced. The use of Sangrovit® Extra as a feed additive under the proposed conditions of use was considered safe for the environment. The additive Sangrovit® Extra had the potential to be efficacious in improving performance of chickens for fattening at 45 mg/kg complete feed. This conclusion was extended to chickens reared for laying/breeding and extrapolated to all poultry species for fattening or reared for laying/breeding.

Keywords: zootechnical additives, performance enhancers, *Macleaya cordata* extract, SANGROVIT® extra, sanguinarine, safety, efficacy

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Phytobiotics Futterzusatzstoffe GmbH[2] for the authorisation of the additive consisting of *Macleaya cordata* extract and leaves (Sangrovit® Extra), when used as a feed additive for all poultry species (excluding laying and breeding birds) (category: zootechnical additive; functional group: other zootechnical additives).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 18 November 2021.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the feed additive consisting of *M. cordata* extract and leaves (Sangrovit® Extra), when used under the proposed conditions of use (see **Section 3.2.6**).

1.2. Additional information

The additive under assessment, Sangrovit® Extra, consists of *Macleaya cordata* extract and leaves. It has not been previously authorised as a feed additive in the European Union.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of *Macleaya cordata* extract and leaves (Sangrovit® Extra) as a feed additive. The dossier was received on 27/5/2021 and the general information and supporting documentation is available at https://open.efsa.europa.eu/questions/EFSA-Q-2021-00454.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance sanguinarine in Sangrovit® Extra and in premixtues and feedingstuffs.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Macleaya cordata* extract and leaves (Sangrovit® Extra) is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity,

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Phytobiotics Futterzusatzstoffe GmbH, Wallufer Str. 10a, D-65343, Eltville, Germany.
[3] FEED dossier reference: FAD-2021-0054.
[4] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2021-0054_en
[5] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019a) Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee, 2019b) and General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021).[6]

3. Assessment

The additive under assessment (Sangrovit® Extra) consists of a *Macleaya cordata* (Willd.) R. Br. extract and *M. cordata* processed leaves. It is intended for use as a zootechnical additive (functional group: other zootechnical additives) in feed for all poultry species (excluding laying and breeding birds).

3.1. Origin

The genus *Macleaya* is a member of the poppy family (Papaveraceae) and is native to China and Japan.

The additive Sangrovit® Extra contains two botanical ingredients derived from *M. cordata*:

- *Macleaya cordata* extract (MCE) [redacted]
- *M. cordata* processed leaves.

and three defined excipients.

[redacted]

3.2. Characterisation

3.2.1. *Macleaya cordata* extract (MCE)

[redacted]

The main active substance in the additive is the alkaloid sanguinarine (SG), which is the most abundant of the quaternary benzophenanthridine and protopine alkaloids present in the fruit and leaves of *M. cordata*. Other alkaloids present in the additive are the isoquinoline derivatives chelerythrine (CH), protopine (PRO) and allocryptopine (ALL), and as described in literature dihydrosanguinarine (DHSG), dihydrochelerythrine (DHCH) and a variety of other alkaloids (Lin et al., 2018).

The structural formula and the chemical abstract (CAS) numbers of SG and other structurally related alkaloids is presented in Figure 1.

The applicant provided data on the full characterisation of MCE,[8] including the quantification of the four main alkaloids SG, CH, PRO and ALL determined by high performance liquid chromatography

[6] https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf
[7] Technical dossier/Section II/Annex_II_3_1.Conf.
[8] Technical dossier/Supplementary information March 2023/Annex_II_1_3_3, Annex_II_1_3_5_Ph_Eur_Conf, Annex_II_1_3_9_Conf.

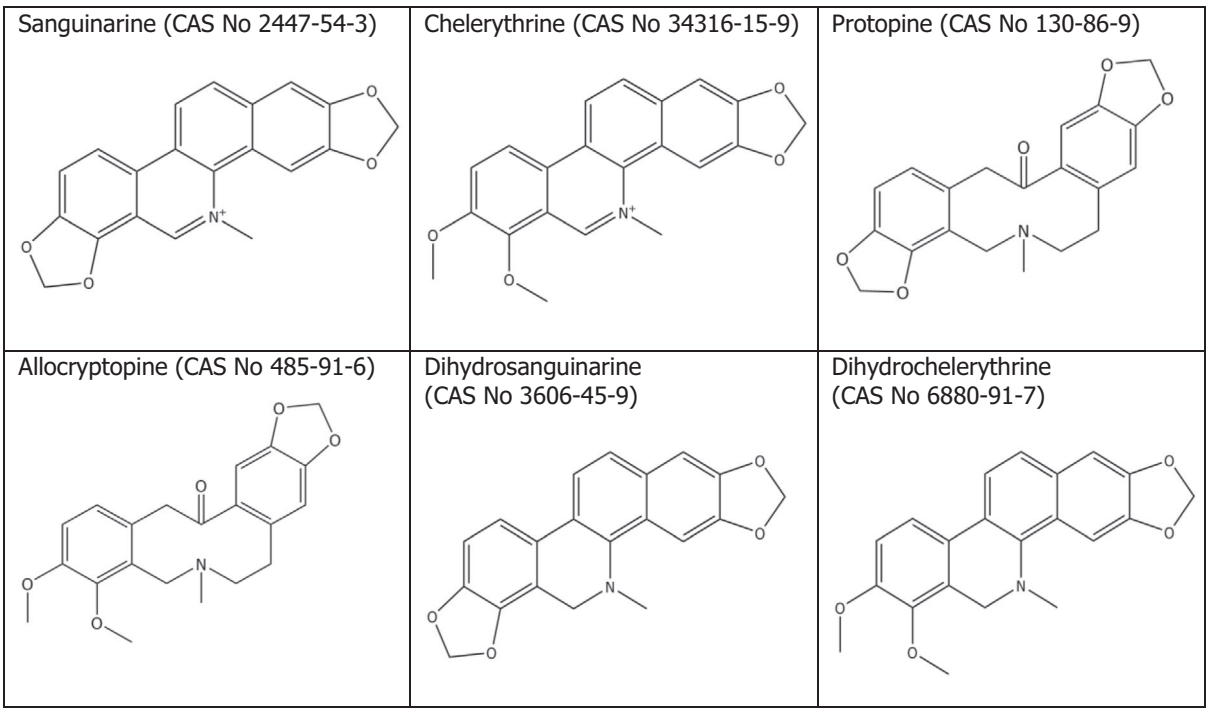


Figure 1: Molecular structures and CAS numbers of sanguinarine (SG, iminium form), chelerythrine (CH, iminium form), protopine (PRO), allocryptopine (ALL), dihydrosanguinarine (DHSG) and dihydrochelerythrine (DHCH)

(HPLC) with fluorescence detection (FLD),[9,10] the content of total alkaloids determined according to the method described in the European Pharmacopoeia for the monograph 'Chelidonii herba' and expressed as chelidonine (PhEur, 2022a),[11] and the content of other secondary plant metabolites, i.e. total flavonoids determined by colorimetric assay at 490 nm and expressed as quercetin dihydrate equivalent (QEq). The results are summarised in Table 1.

[redacted][12] In

Table 1: Characterisation of *Macleaya cordata* extract: secondary plant metabolites

[redacted]

In MCE, ethanol was in the range 0.11-0.23%, sodium <0.01-0.016% and sulfur 5.95-6.66%.[13]

[9] Technical dossier/Supplementary information March 2023/Annex_II_1_3_3_Conf.
[10] Technical dossier/Supplementary information July 2022/Annex_II_6_13.
[11] Technical dossier/Supplementary information March 2023/Annex_II_1_3_5_Ph_Eur_Conf.
[12] Technical dossier/Supplementary information March 2023/Annex_II_1_3_10_Conf.
[13] Technical dossier/Section II/Annex_II_1_4_1_5.

3.2.2. *Macleaya cordata* processed leaves

[redacted][14]

The applicant provided data on the full characterisation of *M. cordata* processed leaves.[15] The results are summarised in Table 2.

Table 2: Characterisation of *Macleaya cordata* processed leaves: secondary plant metabolites

[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]		[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

[redacted][12]

3.2.3. Characterisation of the additive

[redacted]

The additive Sangrovit® Extra consists of *M. cordata* extract [redacted] and *M. cordata* processed leaves [redacted] The additive is formulated with [redacted] The additive is specified to contain a minimum of 4,000 mg SG/kg additive and at maximum 7,000 mg SG/kg additive.[16] Analysis of 15 batches of the additive (manufactured in 2019–2023) showed compliance with these specifications[17] (see Table 3).

Table 3: Composition of Sangrovit® Extra, as specified and batch to batch variation based on the analysis of 15 batches (mean and range expressed as %)

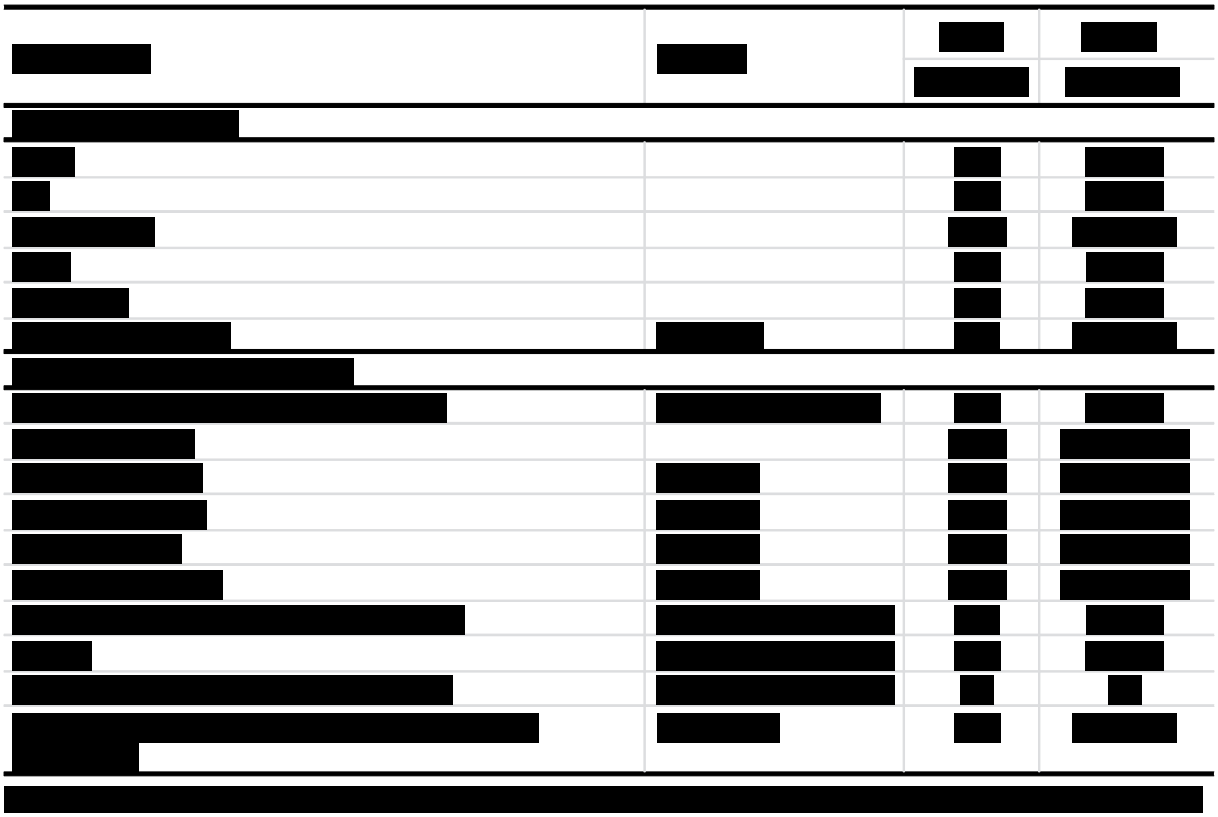
[redacted]	[redacted]	[redacted]
[redacted]		
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[14] Technical dossier/Section II/Annex_II_3_1_Conf.
[15] Technical dossier/Supplementary information March 2023/Annex_II_1_3_4, Annex_II_1_3_5_Ph_Eur_Conf, Annex_II_1_3_7, and Annex_II_1_3_9_Conf.
[16] Technical dossier/Supplementary information July 2022/SIn_reply_page 5.
[17] Technical dossier/Supplementary information July 2022/Annex_II_1_3_1, SIn_spontaneous_080722/Annex_CoAs_Sangrovit and Supplementary information March 2023/Annex_II_1_3_2_Conf.

The same 15 batches were analysed for CH [redacted] PRO [redacted] and ALL [redacted][18]

The applicant provided the full characterisation of the additive based on five batches. This includes a proximate analysis,[19] the content of total alkaloids[20] expressed as chelidonine (PhEur, 2022a), the quantification of the four main alkaloids determined by HPLC-FLD,[21] the content of total polyphenols including tannins[22] expressed as pyrogallol (PhEur, 2022b) and of total flavonoids.[23] DHSG and DHCH were not individually analysed, but they are covered by the analysis of total alkaloids. [redacted]

Table 4: Characterisation of Sangrovit® Extra: proximate analysis and secondary plant metabolites



[18] Technical dossier/Supplementary information_July 2022/Annex_II_1_3_1.
[19] Technical dossier/Supplementary information March 2023/ Annex_II_1_3_2_Conf.
[20] Technical dossier/Supplementary information March 2023/ Annex_II_1_3_4_Ph_Eur_Conf.
[21] Technical dossier/Supplementary information March 2023/Annex_II_1_3_2_Conf.
[22] Technical dossier/Supplementary information March 2023/Annex_II_1_3_6_Conf.
[23] Technical dossier/Supplementary information March 2023/Annex_II_1_3_9_Conf.

The presence of 112 alkaloids has been reported in the different parts (aerial parts, leaves, fruits, roots or stems) of *M. cordata* (Lin et al., 2018). Some were identified by different analytical techniques including high performance liquid chromatography *tandem* mass spectrometry (HPLC/MS–MS) and nuclear magnetic resonance (NMR), others were reported as intermediate products in SG and CH biosynthetic pathways.[24] Some of these alkaloids (chelidonine, coptisine, berberine and stylopine) were below the limit of detection (LOD, < 0.01%).[25]

[redacted][12]

3.2.3.1. Impurities

Three batches of the additive were analysed for impurities. The concentrations of toxic elements were: cadmium and arsenic below the corresponding limit of quantification (LOQ), lead 0.92–3.35 mg/kg, and mercury < LOQ-0.03 mg/kg.[26]

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar dioxin-like polychlorinated biphenyls (co-planar PCBs) were analysed in three batches.[8] The calculated (upper bound) levels of dioxins and the sum of dioxins and dioxin-like-PCBs were 0.06–0.34 ng WHO-PCDD/F-TEQ/kg and 0.09–0.48 ng WHO-PCDD/F-PCB-TEQ/kg, respectively (results expressed in 88% dry matter). Non dioxin-like PCBs ranged from 0.07 to 1.7 μg/kg additive.

Pesticide residues were not detected in a multiresidue analysis.[27]

The analysis of aflatoxin B1, deoxynivalenol, zearalenone and ochratoxin A showed values below the corresponding LOD.[28]

Microbiological contamination was analysed. Total plate counts were 1.7–2.2 × 10^5 colony-forming units (CFU), yeast < 10^4 CFU and moulds 2.4–6.2 × 10^3 CFU. *Salmonella* spp. was absent in 25 g and *Escherichia coli* < 100 CFU/g.[29]

The FEEDAP Panel considers that the microbial contamination and the amounts of the detected impurities do not raise safety concerns.

3.2.4. Physical properties of the additive

The additive appears as a reddish-orange, dry granular powder, with a density of 350–450 kg/m^3. The additive is insoluble in water.

The dusting potential of three batches of the additive was determined using the Stauber-Heubach method and showed values on average of 0.19 g/m^3 (range 0.18–0.20 g/m^3) (g airborne dust per m^3 of air).[30]

Particle size distribution, determined by laser diffraction in three batches of the additive, resulted in 0.06%, 0.69%, 3.16% and 7.82% of particles below 1, 10, 50 and 100 μm, respectively.[31] The particle size of the dust was not provided.

3.2.5. Stability and homogeneity

The shelf life of the additive (four batches) was studied when stored at 21°C in light-tight packaging for 24 months by monitoring SG as the phytochemical marker. Losses at the end of the storage period ranged from 0% to 15%.[32]

The stability of the additive (one batch) in a premixture for poultry was studied when supplemented to achieve 25 mg SG/kg and stored at 21°C for 3 months. There were no losses of SG at the end of the storage period.[33]

[24] Technical dossier/Supplementary information March 2023.
[25] Technical dossier/Supplementary information July 2022.
[26] Technical dossier/Section II/Annex_II_1_4_1_2. Limit of detection (LOQ): cadmium and mercury 0.20 mg/kg, arsenic 0.50 mg/kg.
[27] Technical dossier/Section II/Annex_II_1_4_1_4. Limit of quantification (LOQ): 0.02–0.05 mg/kg.
[28] Technical dossier/Section II/Annex_II_1_4_1_3. Limit of detection (LOD): aflatoxin B1 0.3 μg/kg, deoxynivalenol 0.5 mg/kg, zearalenone 10 μg/kg and ochratoxin A 0.5 μg/kg.
[29] Technical dossier/Section II/Annex_II_1_4_1_1.
[30] Technical dossier/Section II/Annex_II_1_5_1.
[31] Technical dossier/Section II/Annex_II_1_5_2.
[32] Technical dossier/Section II/Annex_II_1_3.
[33] Technical dossier/Section II/Annex_II_4_1_3.

The stability of the additive (one batch) in mash broiler feed was studied when supplemented at 0.60 mg SG/kg feed and stored at 21°C for 3 months. Losses of SG at the end of the storage period were 8%.[34] The stability during pelleting at 60°C was investigated and ranged between 92% and 96%.[35]

The capacity for homogeneous distribution of the additive in feed was studied in 11 subsamples of a broiler mash feed when supplemented at 0.75 mg SG/kg feed. The coefficient of variation (CV) for the SG concentration was 6.8%.[36]

3.2.6. Conditions of use

The additive is intended to be used in feed for all poultry species (to slaughter or point of lay) to provide a minimum SG content in complete feed of 0.225 mg/kg feed and a maximum content of 0.750 mg/kg feed. As the levels of SG in the final additive range between 4,000 and 7,000 mg SG/kg Sangrovit® Extra, these levels are achieved with an amount of the additive ranging between 45 and 150 mg Sangrovit® Extra/kg complete feed.

3.3. Safety

To support the safety of the additive for the target species the applicant provided a tolerance study in chickens for fattening made with the additive under assessment (Sangrovit® Extra). The applicant also provided a structured literature search covering the safety for the target species[37] (details are given in section 3.3.5.2). Toxicological studies, including genotoxicity, sub-chronic toxicity studies and studies aimed at demonstrating the safety for the user (skin and eye irritancy and skin sensitisation) were submitted. *M. cordata* extract (MCE) was also tested for genotoxicity.

The FEEDAP Panel notes that several studies[38] described in the next sections were not performed with the additive under assessment, Sangrovit® Extra (containing 0.4–0.7% SG [redacted]), but Sangrovit® a more concentrated test item containing ~ 1.5–1.9% SG [redacted] The differences in the composition are shown in Table 5.[39]

Table 5: Composition of Sangrovit® Extra and of Sangrovit®, the test item used in absorption, distribution, metabolism and excretion (ADME) and toxicological studies, including genotoxicity studies and in the studies on the safety for the user

[redacted]

[34] Technical dossier/Section II/Annex_II_4_1_1.
[35] Technical dossier/Section II/Annex_II_4_1_2.
[36] Technical dossier/Section II/Annex_II_4_2.
[37] Technical dossier/Section III/Annex_II_1_1 and Bibliography_Sect_III.
[38] Several toxicological studies (Zdarilova et al., 2008; Stiborova et al., 2008) were made with Sangrovit® (a test item containing 1.35% SG and 5% isoquinoline alkaloids). The alkaloid composition was (g/kg): sanguinarine (13.51 ± 0.25); chelerythrine (6.90 ± 0.09); α-allocryptopine (20.26 ± 1.96); protopine (4.30 ± 0.54); homochelidonine (1.63 ± 0.13); dihydrosanguinarine (0.25 ± 0.01) and traces of oxysanguinarine, oxychelerythrine and dihydrochelerythrine.
[39] Technical dossier/Supplementary information_spontaneous_ 080722/Sangrovit_vs_Sangrovit_Extra.

Considering the higher concentrations of the active components of the additive and of the plant material in the test item Sangrovit® (at expenses of wheat), compared to the additive under assessment, the Panel considers the test item Sangrovit® as a worst-case scenario, and therefore relevant for the present assessment.

The studies and publications which were considered relevant are described in the corresponding sections.

3.3.1. Absorption, distribution, metabolism and excretion

The applicant carried out a structured literature search using PubMed on the absorption, distribution, metabolism and excretion (ADME) of the alkaloids present in *M. cordata*, SG, CH, PRO, ALL, DHSG and DHCH.[40] The literature search (no time limits) was conducted in February 2022 and identified 32 publications. *In vitro* (cellular fractions or hepatocytes) and *in vivo* studies were retrieved for SG, CH, or SG plus CH, in laboratory animal models and in some food producing animals. For PRO and ALL a rat study was retrieved. Fourteen publications were considered relevant by the FEEDAP Panel and are described in the next sections, where the biological activities of the dihydrometabolites of SG and CH, DHSG and DHCH are also addressed.

3.3.1.1. Sanguinarine and chelerythrine

Experimental models

In a single dose study, male Wistar rats were given 10 mg SG/kg bw by gavage (Večeřa et al., 2007). Blood samples were collected immediately after administration and at several times up to 88 h and plasma was analysed by high performance liquid chromatography-electrospray mass spectrometry (HPLC-ESI-MS, LOD: 598 fg and 858 fg for SG and DHSG, respectively). Absorption of SG was rapid, the maximum plasma peak level being attained at 2 h after administration, as well as of its principal metabolite DHSG, with levels of 192.3 and 545.9 ng/mL, for the parent compound and metabolite, respectively. The area under the curve (AUCs $_{0-\infty}$) were 380 and 1,269 ng/mLh for SG and DHSG, respectively. The 3-fold higher level of DHSG as compared with that of the parent compound, demonstrates the rapid metabolism of SG. The half-life was 3 h for both SG and DHSG. Another group of three animals were given, also by gavage, 10 mg/kg bw together with ^{3}H-labelled SG (177 kBq/100 g bw). After 3.5 h, blood and faeces were collected, the animals exsanguinated, and liver, heart, kidney, spleen, brain, muscle, fat and intestine removed for radioactivity measurement. The % of radioactivity in plasma at 3.5 h after administration was 0.058%, indicating a very low absorption of SG. The highest level of radioactivity, about 32% of the administered dose, was present in the content of the small intestine, followed by colon content (7.4%), rectum content (1.56%), small intestine tissue and liver (about 1.5% each). The levels in the other analysed samples were all in the range of 0.007% in muscle to 0.09% in erythrocytes. The radioactivity amount excreted in urine was about 0.4% of the administered dose.

Two feeding studies in rats were available. In the first study, male Wistar rats, eight per group, were fed for 109 days a standard diet or the same diet added with 120 mg/kg of a test item containing 640.3 mg/g SG and 219.9 mg/g CH (Psotova et al., 2006). The calculated mean quantity of ingested alkaloids was 10.5 mg SG/kg bw per day and 3.7 mg CH/kg bw per day. Faeces were collected at days 50 and 109, urine at day 109; at necropsy, blood, liver, muscle, heart, kidney and intestine were collected for determination of alkaloid contents by HPLC-UV/fluorescence detectors (LOD/LOQ: 0.003/0.006 μg/g). The highest levels of SG and CH were found in faeces (138.5 and 86.0 μg/g, respectively). In plasma, CH was not detected, the SG level was 0.008 μg/mL and in the analysed tissues values ranged from 0.004 μg/g in muscle to 0.083 μg/g in liver. CH was only detected in liver and kidney (0.024 and 0.009 μg/g, respectively). In urine, SA and CH were not detected. The absorbed percentage of alkaloids was calculated as being 2% of the administered dose.

In another feeding study (Zdarilova et al., 2008), male Wistar rats were fed during 90 days with a standard diet (group 1: control) or with the diet added with a standardised extract Sangrovit® (a test item containing 1.35% SG and 5% isoquinoline alkaloids) at 100 mg/kg (group 2: 1.62 and 1.0 mg/kg feed of SG and CH, respectively), 7,000 mg/kg (group 3: 64.4 and 41 mg/kg feed of SG and CH, respectively) or 14,000 mg/kg (group 4: 147.2 and 93 mg/kg feed of SG and CH, respectively) or 600 mg/kg of another *M. cordata* extract (group 5: 316.5 and 69.7 mg/kg feed, of SG and CH, respectively). At the end of the study, rats were fasted for 12 h and blood collected as well as liver,

[40] Technical dossier/ Supplementary information July 2022/References.

kidney, muscle, myocardium, tongue, ileum, faeces and urine for analysis by HPLC-MS of SG, CH, and their dihydrometabolites DHSG and DHCH. In groups 2, 3 and 4, SG and CH were not found in plasma, urine, and any analysed tissue (LOD not given), except the ileum where CH was detected. The levels of both compounds in faeces were high, and diet concentration dependent. DHSG and DHCH were detected in ileum of the three groups and DHSG in the liver of group 3 (0.04 ng/g) as well as in all analysed tissues of group 4 (liver 0.51, kidney 0.22, tongue 0.10, myocardium 0.07 and muscle 0.08 ng/g). In plasma of rats of group 5, exposed to *M. cordata* extract in the diet, only DHSG (0.33 ng/g) and CH (0.12 ng/g) were present, the low levels demonstrating a limited absorption of the alkaloids and rapid metabolism of SG. Both SG and CH were present in liver, although at very low levels (2.3 and 3.6 ng/g). In none of the other tissues/organs analysed were the alkaloids detected. Referring to the metabolites, DHCH was present in liver and ileum (0.44 and 9.1 ng/g) and DHSG in all tissues (59.3; 3.81; 1.14; 0.14 ng/g in tongue, myocardium, kidney and muscle, respectively). Faeces contained comparatively high levels of both alkaloids and metabolites (273 μg SG/g, 73 μg DHSG/g, 53 μg CH/g and 6.1 μg DHCH/g), pointing to extensive reductive intestinal metabolism and subsequent excretion in faeces.

Huang et al. (2021) carried out a metabolism and tissue distribution study of CH after intragastric administration of 10 mg/kg bw to rats, as well as a metabolism study in rat liver S9 fraction. In samples collected from both the *in vitro* and the *in vivo* models, 12 metabolites of CH were identified by high-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (HPLC/QqTOF-MS; LOQ for CH: 0.5 ng/g). In the rat liver S9 fraction, the reduction of the iminium bond of CH and subsequent O-demethylation was the main metabolic pathway. *In vivo*, the reduction of the iminium bond of CH was the predominant metabolic pathway. Three hours after intragastric administration of CH, no parent compound or metabolites were detected in the plasma of the male and female rats. The Cmax, Tmax and $T_{1/2}$ of CH were 5.06 ng/mL, 1.67 h, and 2.82 h, respectively. Only two metabolites were found in the urine of male rats between 0 and 12 h. Eight and seven metabolites were detected in the faeces of female and male rats during 0–12 h after intragastric administration of CH, respectively, showing that CH is extensively metabolised in the gut and excreted in faeces.

Additionally, a tissue distribution residues study in liver, heart, spleen, lung and kidney was carried out in rats after intragastric administration of a daily dose of 5 mg/kg bw of a *M. cordata* extract containing 40% SG and 20% CH (corresponding to about 2 mg/kg SG and 1 mg/kg CH), for 3 weeks. Rats were killed at 24 or 48 h after the last administration of the extract. Residue contents of SG in organs of rats killed at 24 h/48 h were (ng/g): liver 10.4/9.8; heart 2.9/2.6; spleen 2.0/1.9; lung 2.3/2.0; kidney 3.8/5.7. The corresponding residue values for CH were (ng/g): liver 9.4/20.6; heart 2.8/3.9; spleen 24.0/1.1; lung 1.2/1.7; kidney 1.6/0.9. Data show that both compounds are broadly distributed in tissues.

The distribution of DHSG, the main metabolite of SG formed in the intestine by reduction, was evaluated in male Wistar rats fed with a diet containing DHSG at 97.5 or 478 mg/kg feed, during 90 days, corresponding to an average daily dose of 14 or 58 mg/kg bw per day (Vrublova et al., 2008).[41] After 12 h fasting, rats were killed and plasma, urine, faeces and several tissues/organs were collected for analysis of DHSG. In the low concentration group, no DHSG and SG were detected in plasma (LOD: 178 fg/g for DHSG and 358 fg/g for SG). Ileum contained 16.7 and 84.8 ng/g DHSG, for the lower and higher group levels, respectively (SG not detected), followed by tongue (0.84 and 5.16 ng/g), kidney (0.32 and 1.77 ng/g) and liver (0.21 and 0.56 ng/g). Only in liver SG was detected, at 0.19 and 1.34 ng/g, suggesting the capability of formation of SG in the organ. No DHSG was found in urine. Faeces were, by far, the samples with the highest concentration of compounds, with 109/19.7 and 704/127 μg/g for DHSG/SG, demonstrating that DHSG is mainly excreted in faeces.

The authors also performed a pharmacokinetic study in rats administering by gavage a single dose of DHSG at 9.1 or 91 mg/kg bw. Animals were killed at different time points (three per time point) after compound administration (at 0.5 h up to 36 h) and blood, urine and liver collected for DHSG quantification. Plasma DHSG Cmax was 1.69 and 28.1 ng/mL, attained at 2.0 and 1.0 h, respectively for the low and high doses; the respective $AUC_{0-\infty}$ were 9.88 and 51.86 mg/mL h. The biliary duct of some rats given 91 mg/kg bw was ligated and blood collected from 0.5 till 9 h and plasma prepared for DHSG analysis. This experiment confirmed the enterohepatic circulation of DHSG as suggested by the several plasma peaks noted in the plasma and liver concentration-time curve observed in the

[41] Technical dossier/Supplementary information_July 2022/Vrublova et al., 2008.

kinetic study of the compound. The data also suggest the possibility of the conjugates of DHSG being excreted through bile into the intestine, contributing to the high levels of this SG metabolite in faeces.

An *in vitro* metabolism study of SG and of CH was performed in rat liver microsomes (RLM) of male Sprague–Dawley rats and of SG in the Ad293 human cells transfected with the genes for eight cytochrome P450 (CYP450) isozymes. For comparison of metabolism *in vivo*, three male Sprague–Dawley rats were given a single oral dose of SG, 10 mg/kg bw, and urine was analysed for SG metabolites (Deroussent et al., 2010).[42] *In vitro*, two major metabolites were identified by LC-ESI-MS: one resulting from a ring-cleavage of SG and subsequent *O*-demethylation and a hydroxy-SG, resulting from hydroxylation of an aromatic ring. Other minor metabolites, resulting from oxidative reactions, were identified (epoxy-SG and diol-SG). A ring-cleavage of CH and two *O*-demethylated metabolites were also identified in RLM. In the *in vivo* study, the principal compounds in urine 12 h after oral administration of SG were SG and DHSG. After hydrolysis of urine with β-glucuronidase, two structures were identified, corresponding to a diol-SG and a hydroxy-SG. These two metabolites had been characterised *in vitro*, in the rat liver microsomes and in the human transfected cells, being CYP1A1 and CYP1A2 identified as the only enzymes involved in phase I oxidative reactions of both SG and CH. The data show that *in vivo*, the predominant metabolic pathway of SG is the reductive one, originating DHSG. Some minor metabolites, resulting from an oxidative pathway, are glucuronide-conjugated and excreted in the urine.

In another study, the incubation of human hepatocytes with SG and CH showed that the respective dihydrometabolites were formed as analysed by HPLC/ESI ion-trap MS and HPLC/ESI-QqTOF MS (Kosina et al., 2011). *O*-demethylenation or *O*-demethylation of SG, CH and their dihydrometabolites also occurred, although to a lower extent.

Food producing animals

The pharmacokinetics of SG and CH and their respective dihydrometabolites DHSG and DHCH were evaluated in female chickens after administration of a single dose of Sangrovit® by gavage at 20 mg/kg bw corresponding to 0.384 mg SG/kg bw and 0.286 mg CH/kg bw (Hu et al., 2019). Blood was collected before administration and at several times up to 12 h after administration for analysis by HPLC-MS/MS. Both SG and its metabolite, DHSG, were present in plasma at low levels that declined rapidly. CH and its metabolite, DHCH, were not detected 0.75 h after administration (LOQ for SG and CH was 0.8 ng/mL and for DHSA and DHCH was 0.2 ng/mL). SG was rapidly absorbed and metabolised being the elimination half-life values 1.05 h and 0.83 h for SG and DHSG, respectively. The peak plasma concentration of SG was 1.89 μg/L at 0.9 h, and for DHSG it was 2.49 μg/L at 0.59 h. These data show that the Cmax plasma concentration of the assumed less active metabolite, DHSG, was higher than that of the active parent compound, SG. The AUC $_{0-\infty}$ values for SG and DHSG were 9.9 and 6.1 ng/ml h, respectively.

Recently, Wu et al. (2000), carried out an *in vitro* metabolic study and an *in vivo* pharmacokinetics study of SG in pigs. Incubation of SG with microsomes of intestinal mucosa (added with NADPH), cytosol (added with NADH) and gut flora of pigs showed its reductive biotransformation to DHSG. When pigs were administered a single oral dose of SG at 0.1 mg/kg bw, plasma SG and DHSG reached Cmax (3.41, and 2.41 ng/mL, respectively) at 2.75 h. For SG and DHSG, AUC was 15.6 and 9.1 ng h/ml, and half-life was 2.33 h and 2.20 h, respectively.

A repeated dose administration was also carried out by orally giving to six pigs SG at a dose of 0.1 mg/kg bw three times a day, each dose interval of 8 h, for three consecutive days (Wu et al., 2000). Blood samples were collected at several time points up to 24 h post last dose. AUC for SG and DHSG were 31 and 13 ng h/mL, respectively, higher than after single dose. Also plasma Cmax was higher after repeated dose, 5.9 and 2.9 for SG and DHSG, respectively, although attained at a similar Tmax as for single dose (2.6 h). T1/2 of SG and DHSG was 3.2 h and 2.4 h, respectively. The higher AUC values and plasma Cmax after repeated dose administration of SG are indicative of possible accumulation.

3.3.1.2. Protopine and Allocryptopine

The metabolism, pharmacokinetics and pharmacological activities of protopine have been recently reviewed (Huang et al., 2022).

A biotransformation study of PRO and ALL was carried out in rats after intragastric administration of PRO or ALL at 10 mg/kg bw (Huang et al., 2018a). Blood samples were collected 3 h after dosing and

[42] Technical dossier/Supplementary information_July 2022/Deroussent et al., 2010.

urine and faeces were collected for 0–24 h post-dose and analysed by HPLC-QqTOF MS. No PRO or PRO metabolites were detected in plasma at 3 h after intragastric administration of PRO to female and male rats. In 0–24 h urine of male and female rats PRO and six PRO metabolites were detected and two PRO metabolites in faeces. No ALL or ALL metabolites were detected in plasma at 3 h after intragastric administration of ALL to female and male rats. In the 0–24 h urine of female rats, ALL and five ALL metabolites were detected and ALL and four ALL metabolites in the urine of males. In faeces of both male and female rats ALL and seven ALL metabolites were detected. The results show that ALL was incompletely absorbed from the gastrointestinal tract and can be metabolised in the intestine. PRO and ALL were extensively metabolised in rats, being characterised a total of 10 PRO metabolites and 10 ALL metabolites. The metabolic pathways are common to both compounds, including ring cleavage, demethylation following ring cleavage and glucuronidation.

In parallel, a group of rats was intragastrically given 5 mg/kg of 'Plume Poppy Total Alkaloids' (containing 0.68 mg/kg of ALL and 1.83 mg/kg of PRO), daily, for 3 weeks for tissue distribution evaluation. Organs and cecal contents were collected from animals killed at 24 or 48 h after the last dose and analysed by HPLC-QqTOF MS and HPLC–MS/MS (LOQ for PRO and ALL in tissues was 0.5 ng/g). Some PRO metabolites were identified in cecal contents both at 24 and 48 h and no ALL metabolites were detected. ALL was present in all tissues both at 24 and 48 h, except for kidney at 48 h, as well as in cecal contents (levels ranging from 0.99 to 2.49 ng/g). The highest concentrations of PRO was about 6 ng/g found in cecal contents and in liver at 48 h post dose (6.1 ng/g), being not present in heart. These data indicate that PRO and ALL are distributed in various tissues and appreciably excreted in faeces.

3.3.1.3. Biological activity of the dihydrometabolites of SG and CH

There is a consensus in the literature about the lower biological activity of the dihydroderivatives of SG and CH when compared with the parent compounds, as reviewed by Lin et al., 2018. In cultured human hepatocytes, Vacek et al. (2013), demonstrated the extensive metabolic transformation of CH by phase I and phase II reactions and showed in this *in vitro* model that the main metabolite DHCH was less cytotoxic than the parent compound. Dihydroderivatives of SA and CHE were shown to be markedly less toxic *in vitro* compared to parent alkaloids in human leukocytes and lymphocytes, rat peritoneal mastocytes and primary cultured hepatocytes (Vavrecková et al., 1994 as referenced in Psotova et al., 2006). Similarly, DHSG showed much less cytotoxicity than SG when tested in human leukaemia HL-60 cells (Vrba et al., 2009 as referenced in Kosina et al., 2011). In rat hepatocytes, Gao et al. (2019) evaluated the cytotoxic potential of some alkaloids and the rank order of toxicity was: coptisine > CH > SG > chelidonine > PRO > DHSG. One of the cellular targets of SG is the Na+/K + -ATPase. Janovská et al. (2010), observed *in vitro* that DHSG did not interact with this protein while SG showed a clear inhibitory effect.

An *in vivo* pilot feeding study was made in male Wistar rats by giving DHSG in diet, for 90 days, at concentrations corresponding to the daily intake of 14 or 58 mg/kg bw of DHSG (Vrublova et al., 2008). No adverse effects were observed on feed intake, body weight (BW) and macroscopic examination of the organs. Urine, faeces, blood and several organs were analysed for DHSG and SG (see ADME section), lymphocytes for genotoxicity (Comet assay), liver for analysis of DNA adducts and tongue, gingiva, liver, ileum and heart for histological examination. In plasma obtained at day 90, several biochemical parameters were determined and haematology in total blood. Oxidative stress parameters were evaluated in erythrocytes, liver and plasma. No alterations were observed in any of the endpoints monitored, showing that DHSG is well tolerated up to daily intake of 58 mg/kg bw.

Overall, there is evidence that in hepatocytes the dihydroderivatives of SG and CH are less cytotoxic than the parent compounds. *In vivo*, a repeated dose toxicity study with DHSG did not reveal adverse effects under the conditions of the study at doses up to 58 mg/kg bw per day.

In several laboratory and food producing animals it has been demonstrated that the formation of the dihydroderivatives rapidly occurs in the gut and liver (see ADME section), decreasing the biological activity of the parent compounds.

3.3.2. Residue studies in food-producing animals

The applicant submitted an efficacy study in chickens for fattening, from which some information on residues of SG and CH can be extracted. A tolerance study in chickens and a 90-day study in pigs retrieved from literature are briefly described as they include some data on residues.

Tissue samples were taken from chickens for fattening used in the efficacy study (described in Section 3.4.1).[43] The tissues collected at the end of the study (after 38 days) were muscle and skin+fat in natural proportion and organs (liver and kidneys) from the control group and from the group fed with the maximum recommended level (0.750 mg SG/kg feed). SG, CH, PRO and ALL were quantified by ultraperformance liquid chromatography *tandem* mass spectrometry (UPLC-MS/MS)[44] but not in the other groups. SG and CH were not detected in samples from the control group. In samples from the group fed with the maximum recommended level, SG and CH were not detected in muscle (except one sample with SG at the LOD). SG residues were detected in all skin+fat samples, in all (except two) kidney samples and in all liver samples. CH was below the LOQ (< 0.5 μg/kg) in all kidney, liver and skin+fat samples (except in two skin+fat samples). SG and CH were detected in all excreta samples (> 15 μg/kg).[45] In the samples from the group fed with the maximum recommended level, PRO and ALL residues were below the LOQ in all muscle samples (except one sample with PRO at the LOQ) and in all skin+fat samples. PRO residues were quantifiable in seven kidney samples and in all liver samples. ALL residues were quantifiable in five kidney samples and in all liver samples. PRO and ALL were detected in excreta samples.[46] The results are summarised in Table 6.

Table 6: Residue data of sanguinarine, chelerytrine, protopine and allocryptopine in tissues (skin+fat, kidney, liver and muscle) and excreta samples collected at the end of the efficacy study (after 38 days) from the animals administered with the maximum recommended level (0.750 mg SG/kg feed, n = 14 otherwise indicated)

Sample	Sanguinarine	Chelerythrine	Protopine	Allocryptopine
	μg/kg	μg/kg	μg/kg	μg/kg
Skin+fat	0.83 (< 0.5–2.8)	≤ 0.5–0.8 (n = 2)	< 0.5	< 0.5
Kidney	0.83 (< 0.5–2.9) (n = 12)	< 0.5	0.52 (< 0.5–1.12) (n = 7)	0.73 (< 0.5–1.22) (n = 57)
Liver	0.85 (< 0.5–1.7)	< 0.5	11.87 (4.34–18.3)	2.15 (0.73–3.73)
Muscle	≤ 0.1 (n = 1)	< 0.5	≤ 0.5 (n = 1)	< 0.5
Excreta	> 15[(a)]	> 15[(a)]	1.34–5.00	< 0.5–2.10

Sanguinarine (SG), chelerythrine (CH), protopine (PRO) and allocryptopine (ALL) quantified by ultraperformance liquid chromatography *tandem* mass spectrometry (UPLC-MS–MS, LOD: 0.1 for SG and CH, 0.1–0.24 μg/kg for PRO and ALL, LOQ: 0.5 μg/kg in all tissues).
(a): The concentration of SG and CH in excreta (> 15 μg/kg) was outside the calibration range.

Matulka et al. (2014) carried out a tolerance study in chickens for fattening of a *M. cordata* extract and made in parallel a residue analysis. The test item was the marketed *M. cordata* extract (Sangrovit® containing at least 1.5% SG), given to the animals for 35 days at levels of 100, 500 or 1,000 mg/kg feed (corresponding to 2.0, 8.0 and 15.5 mg SG/kg feed and 1.0, 3.9 and 7.1 mg CH/kg feed, confirmed by analysis). On day 35, animals were killed and tissue and organ samples (liver, kidneys and breast muscular tissue, skin+fat tissue around the breast) were taken for SG and CH analysis. Biological samples as well as feed were analysed for SG and CH contents by a validated HPLC–MS/MS. The LOD was 10 μg/kg in muscle, liver and fat and 15 μg/kg in kidney tissue, for both SG and CH. The LOQ was 45 μg/kg in muscle, liver and fat and 60 μg/kg in kidney tissue for both SG and CH. In feed LOD was 0.05 mg/kg and LOQ was 0.1 mg/kg. At the lower feeding level, 100 mg/kg feed, compounds were not detected in any analysed sample (< 10 or 15 μg/kg). In the 500 mg/kg group, residues of SG were present in two skin+fat samples (89.8 and 56.7 μg/kg). In the 1,000 mg/kg fed group, three skin+fat samples contained SG (55, 117, and 63 μg/kg) as well as one kidney sample (96.6 μg/kg). No CH was detected in any sample. No residues of the compounds were detected in muscle tissues of animals of all groups.

From the two studies carried out in chickens it can be concluded that residues of the main alkaloids (detected in skin+fat, liver and kidney) are very low.

In another study with pigs, animals were fed with *M. cordata* extract (a test item containing 64% SG and 22% CH) for 90 days (corresponding to a daily intake of 2 or 100 mg extract/kg feed, SG:CH

[43] Technical dossier/SectionIV/Annex_IV_3_1.
[44] UPLC-MS–MS, LOD: 0.1 μg/kg for SG and CH, LOD: 0.1–0.24 μg/kg for PRO and ALL; LOQ: 0.5 μg/kg in all tissues for all.
[45] Technical dossier/Section III/Annex_III_2_1_2.
[46] Technical dossier/Supplementary information July 2022/Annex_III_2.1.3, Annex_III_2.1.4.

of 3:1) (Kosina et al., 2004). On day 91, SG was present at the highest level in gingiva followed by liver, intestine and tongue for both extract concentrations. In the low concentration diet group CH was only detected in gingiva and liver, and in the high concentration group gingiva, intestine and liver. No compounds were detected in muscle (LOD: 0.001 μg/mL). The very high contents of both compounds in faeces indicate that this is the principal route of excretion. The data of this study are consistent with those obtained for chickens, the target species of this application.

3.3.2.1. Conclusions on the ADME and residue studies

The data from the studies in rat and in food-producing animals (chickens for fattening and pigs) indicate that after oral administration (by gavage or in feed)

- SG and CH are poorly absorbed as such, being extensively metabolised in the gut, principally by reduction with the production of DHSG and DHCH, which are identified in almost all the analysed samples (liver, kidney, tongue, myocardium and muscle).
- After a single dose administration of SG and in subchronic feeding studies with SG and CH, tissues showed very low levels of the parent compounds and their metabolites, which were mainly present in the ileum, the liver and the tongue.
- Both SG and CH are found in low concentrations in plasma and urine and at highest levels in faeces, as well as their dihydrometabolites. The fraction absorbed and metabolised in liver to conjugated derivatives is excreted via bile to the intestine and subsequently in faeces. This route of excretion is confirmed by the very low levels of the compounds present in urine.
- The biotransformation of SG and CH was similar in rat liver fractions and in human hepatocytes.
- Both in chickens and in pigs SG and CH are absorbed mainly after reduction in gut and rapidly excreted. Quantification of residues in tissues after repeated oral administration of the compounds to pigs gives indication of accumulation.
- PRO and ALL are absorbed in the rat to a greater extent than SG and CH, distributed in various tissues, extensively metabolised after absorption, and excreted in urine and faeces.

3.3.3. Genotoxicity

3.3.3.1. Genotoxicity studies with *Macleaya cordata* extract

The applicant submitted two *in vitro* studies, a reverse mutation test and a micronucleus test in human peripheral blood lymphocytes, performed with *M. cordata* extract (MCE) with a content of total alkaloids of 61.1%, including SG at 43.0% and CH at 18.2% (determined by HPLC–FLD). The studies are described below.

M. cordata extract was tested for the induction of reverse mutations in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102. The experimental protocol was in line with Organisation for Economic Co-operation and Development (OECD) technical guideline (TG) 471.[47] The test item was dissolved in water and tested both in the presence and absence of the metabolic activation system at five concentration levels up to 100 μg extract/plate. Positive and negative controls were included. In the presence and in the absence of S9-mix signs of toxicity were observed at the highest concentrations of 100 μg extract/plate in all tester strains. No indication of mutagenic activity was observed in any experimental condition, while a significantly increased number of revertant colonies was observed in the positive controls.

The *in vitro* micronucleus test in human peripheral lymphocytes (OECD guideline 487) was performed in the presence and absence of metabolic activation at concentrations of 1.25, 2.5, 5, 10 and 20 μg *M. cordata* extract/mL water, selected on the basis of a preliminary dose finding test.[48] A steep increase in cytotoxicity was detected at the highest concentrations analysed for the frequency of micronuclei, in all the experimental conditions (i.e. 10 μg/mL without S9 and 20 μg/mL with S9). No induction of chromosomal damage was observed.

Although the *in vitro* tests performed with *M. cordata* extract gave negative results, the studies show some limitations related to the prevalent toxic activity of the test item, possibly not allowing the expression of a potential genotoxicity. Indeed, SG is a compound with well-known antimicrobial activity

[47] Technical dossier/Section III/Annex_III_2.2.1.1.
[48] Technical dossier/Annex_III_2.2.2.3.

(Schmeller et al., 1997) and the analysis of gene mutation in bacteria could be not appropriate to evaluate the mutagenic activity of the test item.

3.3.3.2. Genotoxicity studies with the formulated additive

In another dataset, the applicant submitted *in vitro* and *in vivo* genotoxicity studies performed with the formulated additive (Sangrovit®, a batch containing 1.67% SG was tested *in vitro* and another batch containing 1.35% SG and 5% isoquinoline alkaloids was tested *in vivo*).[49] All the studies gave negative results. The Panel noted that the limited absorption of the major constituents of the additive (SG ad CH, which are tertiary amines, see Section 3.3.1) could be responsible for the negative results observed *in vivo* and that possible genotoxic effects at first sites of contact (stomach and duodenum) were not evaluated.

3.3.3.3. Studies on the genotoxic potential of sanguinarine

The FEEDAP Panel notes that for mixtures for which not all components have been chemically fully identified, the EFSA Scientific Committee recommends assessing first the chemically defined substances for their potential genotoxicity individually, using all available information (EFSA Scientific Committee, 2019a). Therefore, information on the genotoxic potential of all the identified individual components following the Scientific Committee guidance (EFSA Scientific Committee, 2011, 2017) would be needed to draw conclusion on the genotoxicity of the extract under assessment.

Based on the recommendations of the EFSA Scientific Committee for the genotoxicity assessment of chemical mixtures, the FEEDAP Panel carried out an analysis of the available literature on the genotoxic potential of the main component of *M. cordata* extract, SG and the other alkaloids, in addition to the information provided by the applicant.

Chemical characteristics

SG and chelerythrine show a pH-dependent structural equilibrium between the charged form (iminium) and the uncharged form (alkanolamine). The charged form (iminium), which is prevalent at pH 2–6, has DNA binding affinity and was proven to be a strong intercalator (Sen and Maiti, 1994; Bai et al., 2006), whereas the uncharged form (alkanolamine), which is prevalent at pH 6.5–9.0, does not bind to DNA, but has greater cellular bioavailability due to its greater lipophilicity (Maiti et al., 2002; Maiti and Kumar, 2009). At physiological pH (7.4) both forms are present.

Genotoxicity studies

Literature data show both positive and negative outcomes of *in vitro* and *in vivo* genotoxicity tests with SG from different origins and with a different purity (as reviewed by Croacker et al., 2017).

***In vitro* studies**

- Gene mutation tests

Positive results were observed in two Ames tests with SG chloride (Frankos et al., 1990, as reported by Croacker et al., 2017), while negative results were observed in the *E. coli* (SOS) chromotest (Kevekordes et al., 1999) and *Saccharomyces cerevisiae* mutation test (Frankos et al., 1990).

Two unpublished studies on gene mutation in mammalian cells were reported by Munro et al. (1999); the Panel considered them not informative because the original data are not available for a peer-review scrutiny.

- Micronucleus test

In an *in vitro* micronucleus test performed with SG chloride in human lymphocytes and metabolically competent Hep-G2 human hepatoma cell line, no induction of micronuclei was observed (Kevekordes et al., 2001, as reported in Croacker et al., 2017).

- Comet assay

Positive results were observed with the Comet assay after treatment of murine and human cell lines (reviewed in Croacker et al., 2017) with SG. In particular, Matkar et al. (2008a) demonstrated that single strand breaks and formation of 8-oxodeoxyguanosine (8-oxodG) were induced by SG hydrochloride treatment (purity ≥ 98%) in human colon cancer cells, in addition to the increase of

[49] Technical dossier/ Annex_III_2.2.2.2, Annex_III_2.2.2.4, Annex_III_2.2.3.

gamma-H2AX, a marker of double strand breaks (DSB). Co-treatment with antioxidants, i.e. dithiothreitol, glutathione (GSH), N-acetyl cysteine (NAC) partially prevented these effects (Matkar et al., 2008b), results consistent with the induction of oxidative damage. Another study performed in human cancer cell lines with SG extracted and purified from *Chelidonium majus* L. (purity not reported) showed a significant increase of DNA strand breaks reverted by pretreatment with NAC (Kaminskyy et al., 2006), supporting the key role of oxidative damage. The induction of DNA strand breaks by the same test item was also demonstrated in primary mouse spleen cells and mouse leukaemic cells (Kaminskyy et al., 2008).

- DNA adduct formation

SG (purity 98.1%) isolated from an alkaloid extract of *M. cordata* induced the formation of DNA adducts in calf thymus DNA after metabolic activation with rat hepatic microsomes; the level of DNA adducts, detectable by ^{32}P post-labelling, was concentration dependent. At the lowest concentration of SG tested (1 μM), adducts were undetectable (Stiborova et al., 2002). The Panel notes that it was not clarified whether the adduct was formed from a direct interaction of SG metabolite(s) with DNA or indirectly by reactive oxygen species (ROS) possibly enhanced by the depletion of antioxidant enzymes (Walterova et al., 1981; Ulrichova et al., 2001; Debiton et al., 2003).

***In vivo* studies**

- Chromosomal aberrations and sister chromatid exchanges

Positive results for the induction of chromosomal aberrations and sister chromatid exchanges (SCE) were observed in bone marrow of mice after i.p. administration of SG chloride (purity 98%) at 5, 10 and 15 mg/kg bw (Das et al., 2004). Statistically significant dose-related increases in chromosome damage and SCE frequency were reported. No cytotoxicity was observed in the target tissue. The induction of chromosome aberrations and SCE in mouse bone marrow was confirmed by Ghosh and Mukherjee (2016), after i.p. administration of a single dose of 50 mg/kg bw of SG chloride (purity 98%).

- Comet assay

A Comet assay performed in mice treated i.p. with five doses of SG isolated from argemone oil (purity 86–88%) (1.35, 2.70, 5.40, 10.80 and 21.60 mg SG/kg bw) showed a statistically significant increase of DNA damage in bone marrow and blood cells at the highest doses tested, with a dose-related effect (Ansari et al., 2005).

The increase of DNA strand breaks in bone marrow and blood cells was confirmed in an additional study performed in mice treated i.p. with SG isolated from argemone oil (purity 86–88%) (21.6 mg SG/kg bw). Combined treatment with antioxidants (i.e. alpha-tocopherol, riboflavin) induced a statistically significant reduction of SG-induced DNA damage (Ansari et al., 2006).

SG isolated from argemone oil (purity 86–88%) was also tested for the induction of DNA strand breaks in the frame of a study evaluating its carcinogenic potential in a mouse model for skin cancer (Das et al., 2005). A single dose of the compound was tested, and the Comet assay showed a significant increase of DNA damage in skin cells after twice a week topical application for 25 weeks. No tumours (squamous cell carcinoma) were induced by isolated SG under the same conditions of exposure.

Overall, genotoxic effects were reported both *in vitro* and *in vivo* after treatment with isolated SG. However, the Panel noted that the test item contains 12–14% residues of argemone oil or impurities and that confounding effects cannot be discounted.

- DNA adduct formation

In 90-day feeding studies in pigs (Kosina et al., 2004) and in rat (Vrublova et al., 2008), no induction of DNA adducts in liver were detected by ^{32}P-post labelling after oral administration of SG or DHSG. The Panel noted that plasma levels of SG and DHSG were in the range of 28–130 ng/mL, substantially below the concentration resulting in detectable DNA adduct formation *in vitro* (10 μM corresponding to 3.7 μg/mL of SG chloride). Hence, these negative results obtained *in vivo*, not consistent with the formation of DNA adducts observed *in vitro* (described above) might be related to the limited bioavailability of the tested compounds after oral administration. A different situation may occur in target cells with more intimate contact, such as intestinal mucosa cells.

3.3.3.4. Overall discussion on genotoxicity

The Panel notes that the genotoxic effects of *M. cordata extract* as well as of SG at first sites of contact (stomach and duodenum) after oral exposure have not been investigated. However, the results of the *in vivo* studies with SG administered i.p. show the induction of DNA damage when cells are exposed. These effects do not show tissue specificity being observed in bone marrow, blood cells and skin. The negative results reported for the induction of DNA adducts and DNA damage after oral administration of SG may be due to its limited absorption. These results do not discriminate between the genotoxic effects due to the direct binding of SG/SG metabolite(s) to DNA and the induction of oxidative DNA damage through the increased formation of ROS. Results obtained in toxicity studies measuring markers of oxidative stress (Stiborova et al., 2008; Zdarilova et al., 2008) point to the induction of radical induced damage, a thresholded mechanism. DNA-intercalating activity was also demonstrated for SG in a cellular systems and murine lymphoma cells (Giri and Kumar, 2007; Bai et al., 2006; Kaminskyy et al., 2006). No data are available on the potential mutagenic effect of SG by intercalation mechanism in mammalian cells. However, it has also been proposed that the impairment of the binding or activity of DNA-interacting enzymes, i.e. DNA repair enzymes, may trigger the formation of chromosomal damage. This could be an indirect mechanism underneath the SG-induced increase in chromosomal aberrations.

Overall, SG genotoxicity is related to evidence showing that it has the potential to (i) intercalate between DNA base pairs; (ii) induce DNA strand breaks; (iii) and also induce DNA strand breaks associated to oxidative damage and the formation of ROS. Therefore, SG has the potential to produce ROS in proximity of the double helix of DNA and, therefore, to induce oxidative DNA damage.

Based on the structural similarity with SG, the same conclusions apply to CH.

3.3.3.5. Other alkaloids

PRO and ALL, in contrast to SG and CH, have a macrocyclic non-planar structure[50] which does not allow the compounds to intercalate between DNA base pairs *(*Takahashi et al., 1985; Marek et al., 1998). However, there is evidence showing that PRO and ALL form reversible complexes with DNA by binding non-covalently to the DNA-groove with a preference to G-rich quadruplex DNA (Mandal et al. 2018; Fu et al., 2018). G-quadruplex structures play critical roles in the regulation of key biological processes, such as DNA replication, transcription as well as DNA repair, the latter through interactions with DNA repair proteins (reviewed in Varshney et al., 2020). The non-covalent interaction of ligands with the DNA minor-groove is not associated with direct DNA damage. This is consistent with the results obtained in an *in vitro* Comet assay performed in mammalian cells showing that PRO did not induce DNA strand breaks (Spiess et al., 2022).

In addition, the negative results obtained in mice with different mutagenicity tests (sperm abnormality test, micronucleus test and chromosomal aberration test) confirmed the absence of genotoxic activity of an extract rich in protopine alkaloids (MPTA, containing 35% PRO and 15% ALL) obtained from the acidic waste stream used for the extraction of the benzophenanthridine alkaloids in *M. cordata* (Dong et al., 2022).

Overall, a direct interaction of PRO with DNA is not supported by the experimental data available. Based on the structural similarity between PRO and ALL, this conclusion can be extended to ALL.

3.3.3.6. Conclusions on genotoxicity

The FEEDAP Panel concludes that, based on the available information on the main individual component(s) of the extract and considering the uncertainty in the interpretation of the negative outcome of the *in vitro* genotoxicity tests performed with the extract and in the mode of action, a concern for genotoxicity remains owing to the presence of the DNA intercalators sanguinarine and chelerythrine.

3.3.4. Sub-chronic toxicity studies

A 90-day study was done with 24 Wistar rats with 100, 7,000 or 14,000 mg Sangrovit®/kg feed (corresponding to 1.35, 94.5 and 189 mg SG/kg feed and to 5, 330 and 660 mg isoquinoline alkaloids/kg feed) (following OECD guideline 408, 1998 update). Mean absolute kidney weights of the highest two doses groups were significantly lower than those of controls. These differences were not present when weights were expressed relative to BW and there was no evidence of any histopathological changes. Haematological

[50] Technical dossier/Supplementary information March 2023/Annex_III_2_1_8_Conf.

and chemical parameters did not show effects. Oxidative stress parameters (thiobarbituric acid reactive substances, total antioxidant capacity in plasma or liver homogenates, GSH and superoxide dismutase (SOD) in erythrocytes and liver homogenate, glutathione peroxidase) and content of cytochrome P450 were measured. No differences among the three groups were observed, except for a significant increase in the hepatic content of SOD and GSH in the mid and top doses (Stiborova et al., 2008).

In another 90-day study, 30 Wistar rats were fed with 100, 7,000 or 14,000 mg Sangrovit®/kg feed (corresponding to 1.35, 94.5 and 189 mg SG/kg feed and to 5, 330 and 660 mg isoquinoline alkaloids/kg feed) or 600 mg/kg feed of quaternary benzo[c]phenanthridine alkaloids from *M. cordata* (FQBA). Body and organ weights, clinical chemistry and haematological markers, oxidative stress parameters, morphological structure of tongue, liver, ileum, kidney and heart samples and total cytochrome P450 in liver were monitored. The results showed no statistically significant differences in any parameter between control and treated animals, except for the group treated with 14,000 ppm Sangrovit® where there were higher values of reduced glutathione level and superoxide dismutase (Zdarilova et al., 2008). From the results of this study a NOAEL of 95 mg/kg feed (corresponding to 7.7 mg/kg bw per day[51]) was identified for SG.

Chronic oral toxicity studies, carcinogenicity studies and reproduction toxicity studies including prenatal developmental toxicity with *M. cordata* extract were not submitted.

3.3.5. Safety for the target species

According to the General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP, 2021), genotoxicity and carcinogenicity endpoints are not considered relevant for short-living animals. Short-living animals are defined as those animals raised for fattening whose lifespan under farming conditions makes it very unlikely to develop cancer as a result of the exposure to genotoxic and/or carcinogenic substances in the diet. Therefore, for these species, the safety evaluation of additives containing substances which are genotoxic and carcinogenic can be based on the outcome of the tolerance trials in the target species (EFSA FEEDAP Panel, 2021).

In the context of this assessment, the definition of short-living animals includes poultry species for fattening but does not include poultry species reared for laying/reproduction purposes, which are considered to be long-living/reproductive animals and for which genotoxicity and carcinogenicity endpoints are considered relevant.

3.3.5.1. Tolerance trial in chickens for fattening

The applicant provided one combined tolerance/efficacy study in chickens for fattening[52] to support the safety for all poultry for fattening or reared for laying/breeding.

A total of 2,688 one-day-old male chicks (Ross 308) were distributed in 112 pens in groups of 24 chickens and randomly allocated to seven dietary groups (16 replicates per group). Two basal diets (starter from day 1 to 21; grower from day 22 to 42), based on maize, wheat and soyabean meal were either not supplemented (control) or supplemented with Sangrovit® Extra to provide 45 (minimum recommended level), 60, 90, 120, 150 (1× maximum recommended level) and 3,000 mg/kg feed (20×). The level of the additive in the feed was confirmed based on the analysis of SG and CH as markers.[53] The experimental diets were offered *ad libitum* in mash form for 42 days.

At day 21, four birds per replicate (pen) were randomly removed in order to be used for a digestibility trial (not undertaken). Consequently, from day 22 to 42, the number of animals was 2,240 with 112 pens in groups of 20 birds. The removal of the animals did not affect the overall performance of the treatments.

Mortality and health status were checked daily, and the most likely cause of death or reason for culling recorded. The birds were weighted at the start of the trial (day 1). Thereafter, pen BW and feed intake were recorded on days 21 and 42. The average daily feed intake (ADFI), average daily gain (ADG) and feed to gain ratio (F:G) were calculated and corrected for mortality for each feeding period (days 1 to 21, and 22 to

[51] NOAEL in mg/kg feed converted in mg/kg bw per day using the default value of 0.081 for male rat and for subchronic studies according to the EFSA Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data (EFSA Scientific Committee, 2012).

[52] Technical dossier/Section III/Annex_III.1.

[53] These doses were intended to deliver respectively 0.244, 0.325, 0.487, 0.680, 0.812 and 16.242 mg sanguinarine per kg feed (confirmed by analysis). The additive also delivered chelerythrine respectively at the dose of 0.119, 0.159, 0.239, 0.318, 0.398 and 7.96 mg/kg feed (confirmed by analysis).

42) and the overall experiment. On day 42, blood was collected from two birds per pen (32 samples per treatment) and analysed for biochemical[54] and haematological[55] parameters.

The data were analysed with a generalised linear model with the treatment as fixed effect. Group means were compared with Tukey's test. A non-inferiority test was performed comparing the performance parameters between the maximum level applied (20×) and the control (non-inferiority margins considered were BW 42 days = −76.3 g; mortality-corrected ADG 1–42 days = −1.88 g/days; ADFI 1–42 days = −2.26 g/days; F:G 1–42 days = −0.031). The statistical significance for each analysis was set at 0.05.

Mortality was on average 1.9% with no differences between groups. According to the non-inferiority test, the data on ADFI and F:G data showed not to be inferior at the highest level in comparison with the control diet. The non-inferiority comparison was not possible for the final BW and average daily gain data, as the average values of the chickens supplemented with 3,000 mg/kg were higher than the control group. At day 42, the dietary supplementation of chickens with 45 and 90 mg Sangrovit® Extra/kg feed showed higher final BW (2,841; 2,982; 2,967 g for the C, 45 and 90 mg/kg groups, respectively) and average daily gain (37.1; 38.8; and 39.1 g/day) compared to the control. The average daily feed intake was lower in the 150 and 3,000 mg/kg groups in comparison with the control (105.1; 100.1; and 100.5 g/day for the control, 150 and 3,000 mg/kg groups, respectively). The feed to gain ratio was improved from 45 mg additive/kg feed compared to the control (1.65; 1.53; 1.56; 1.56; 1.56; 1.55; 1.56; and 1.56 for control, 45, 60, 90, 120, 150 and 3,000 mg/kg groups, respectively). The blood haematology and biochemistry data showed no differences between the control and any of the supplemented groups in any of the parameters analysed, except from the content of creatine kinase in the 90 mg/kg group (43.8 U/L), which was higher than the control (28.1 U/L).

According to the results of the tolerance trial, the FEEDAP Panel has no safety concerns when the additive is used at the recommended level of 150 mg/kg complete feed (corresponding to 0.750 mg SG/kg complete feed) for chickens for fattening.

3.3.5.2. Literature search

The applicant provided a literature search[56] which identified 17 publications, including studies assessing the effect of the supplementation of the feed or water for drinking of chickens for fattening with Sangrovit®, Sangrovit® water soluble (WS)[57] or other *Macleaya cordata* extracts, all of them containing SG as major active substance. Five of them include studies in which a challenge with avian pathogens (clostridia, *E. coli* and/or *Clostridium perfringens*) was performed[58]; while another[59] only evaluated the effect of Sangrovit® on the birds' caecal microflora activity and fatty acid profile. Therefore, these references could not be considered for the assessment of the safety.

Matulka et al. (2014) assessed the effect on chickens for fattening of increasing levels of the dietary supplementation of Sangrovit® up to 1,000 mg/kg (corresponding to levels of SG > 15 mg/kg feed) for 35 days. The experimental design was compliant with the requirements for tolerance trials included in the Guidance for the safety of the target species (2017). The study included the monitorisation of the general health status, zootechnical performance (BW, feed intake, BW gain and feed to gain ratio), blood haematology[60] and biochemistry[61], and gross pathology[62] of the birds. The results of the study showed no negative effect of the supplementation of the extract up to 1,000 mg/kg in any of the parameters

[54] Urea, uric acid, creatinine, cholesterol, bilirubin, glucose, magnesium, phosphorus, calcium, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), amylase, creatine kinase (CK), aspartate transaminase (AST), alanine transaminase (ALT), total protein, albumin, globulin, lactate dehydrogenase (LDH), potassium, sodium, chlorine, acute phase proteins (C-reactive protein, ovotransferin).

[55] Red blood cells (RBC), haemoglobin, haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), heterophils, lymphocytes, fibrinogen, prothrombin time.

[56] Technical dossier/Section III/Annex_III_1_1 and Bibliography_Sect_III.

[57] Water soluble *Macleaya cordata* extract (1% sanguinarine/kg).

[58] Hasan et al. (2020), Aljumaah et al. (2020), Xue et al. (2017), Hussein et al. (2020), Mathis et al. (2016).

[59] Juskiewicz et al. (2013).

[60] Haematocrit (HCT), haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), erythrocytes count.

[61] Glucose, calcium, inorganic phosphorus, cholesterol, triglycerides, phospholipids, uric acid, urea, creatinine, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and total bilirubin.

[62] External skin, eyes and any injuries, feet, ears, head and tail, mouth and anus, gut (oral cavity, oesophagus, stomach, upper, mid and lower small intestine, caecum and colon), pancreas, spleen, liver/gall bladder, kidneys, genitals, abdominal fat, omentum, heart and lungs, skeletal muscle and fat.

evaluated in chickens compared to the control group. The results of this study showed that high levels of Sangrovit®, a product with similar composition but more concentrated than the additive under assessment, is well tolerated above the maximum recommended levels for Sangrovit® Extra.

The rest of the submitted publications included long-term trials (longer than 35 days) assessing the effect of the supplementation of the feed or water of chickens for fattening with *M. cordata* extracts (levels of SG from 0.19 to 15 mg/kg feed) on the health status, zootechnical performance, blood haematology/biochemistry and/or other traits (carcass quality, organs weight, intestinal health).[63] Most of the studies showed no negative impacts of the dietary supplementation with the test items used on any of the parameters monitored in the birds. In one of them (Khadem et al., 2014), the increasing supplementation of water for drinking with Sangrovit® WS from 25 to 100 mg/L (equivalent to 0.28, 0.55 and 1.11 mg SG/L) showed a tendency to increase the mortality of the chickens (with 2, 3, 7 and 8% mortality for the control, 25, 50 and 100 mg/L groups, respectively). The authors indicated that the observed mortality was mostly associated with the typical symptoms of coccidiosis. No other adverse effect of the supplementation of the additive on the zootechnical performance parameters or organs' relative weight was observed.

3.3.5.3. Conclusions on safety for the target species

Based on the results of the tolerance trial in chickens for fattening, the Panel has no safety concerns when the additive is used up to the maximum recommended level of 150 mg/kg complete feed (corresponding to 0.750 mg SG/kg complete feed) for chickens for fattening. This conclusion is extrapolated to other poultry for fattening.

When considering long living/reproductive animals, genotoxicity and carcinogenicity endpoints are considered relevant according to the General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021). Therefore, no conclusions can be drawn for poultry reared for laying and breeding purposes, which are considered long living/reproductive animals.

3.3.6. Safety for the consumer

3.3.6.1. Assessment of consumer exposure

The FEEDAP Panel performed an exposure assessment following the methodology described in the Guidance on consumer safety (EFSA FEEDAP Panel, 2017b) (Appendix A).

The residue values for SG and CH from the residue study in chickens for fattening (see section 3.3.2, converted from μg/g to mg/kg) were used as input data for the exposure calculation and are reported in Table 7.[44] For SG, quantified in all fat and liver samples (n = 14) and in the majority of kidney samples (n = 12), the arithmetic mean plus two standard deviations was calculated. For SG in muscle (n = 1), the highest value corresponding to the LOD was considered. CH was not detected in muscle and was quantified only in two skin+fat samples (the highest analysed value was used). In the other samples, where CH was detected (> LOD) but not quantified (< LOQ), a value corresponding to half the LOQ was used.

Table 7: Input values on sanguinarine and chelerythrine content in food of animal origin following the use of the additive at the maximum recommended level (0.750 mg SG/kg feed) and used for the consumer exposure assessment

Animal product	Sanguinarine	Chelerythrine	Sanguinarine + chelerythrine
	(mg/kg wet tissue or product)		
Fat	0.0021	0.00080	0.00290
Kidney	0.0022	0.00025	0.00245
Liver	0.00162	0.00025	0.00187
Muscle[(a)]	0.0001	n.d	0.0001

(a): The residue concentration in muscle and skin/fat will be applied to the intake of meat at the following proportions: 90% muscle and 10% skin/fat (EFSA FEEDAP Panel, 2017b).

[63] Hassan et al. (2018), Huang et al. (2018b), Liu et al. (2020), Kyung-Woo et al. (2015), Vieira et al. (2008a), Vieira et al. (2008b), Yeşilbağ et al. (2020), Juskiewicz et al. (2010), El-Sheikh et al. (2019), Khadem et al. (2014).

The results of the dietary exposure to SG and SG + CH for the different population categories are reported in Table 8.

Table 8: Chronic human exposure to sanguinarine and chelerythrine. Maximum highest reliable percentile in ng/kg bw per day

Population class	No. of surveys	Maximum HRP* ng/kg bw/day	% TTC value	Maximum HRP* ng/kg bw per day	% TTC value†
		Sanguinarine		Sanguinarine + chelerythrine	
Infants	6	0.702	28	0.702	28
Toddlers	10	1.172	47	1.255	50
Other children	18	1.239	50	1.448	58
Adolescents	17	0.463	19	0.463	19
Adults	17	0.585	23	0.677	27
Elderly	14	0.362	14	0.434	17
Very elderly	12	0.327	13	0.372	15

*: Highest reliable percentile.
†: TTC value for substances that have the potential to be DNA reactive mutagens and/or carcinogens: 0.0025 μg/kg bw per day (equal to 2.5 ng/kg bw per day).

The estimated mean daily exposure for SG + CH ranged from 0.026 up to 0.367 ng/kg bw per day, the 95th percentile between 0 and 1.401 ng/kg bw per day. The highest exposure at the 95th percentile occurs in the category 'other children'.

In the absence of a reference point for SG and CH, the estimated exposure can be related to the value that, according to threshold of toxicological concern (TTC) approach, is considered of low probability of adverse health effects (EFSA Scientific Committee, 2019b). For substances that have the potential to be DNA reactive mutagens and/or carcinogens, the TTC value corresponds to 0.0025 μg/kg bw per day (equal to 2.5 ng/kg bw per day). For all consumer categories, exposure in the individual countries was constantly below 1.4 ng/kg bw per day, corresponding to 13–56% of the value of TTC. Below this threshold, the probability of adverse effects is low.

The FEEDAP Panel considers that the exposure in all the population groups is of low concern.

The residue values for PRO and ALL from the residue study in chickens for fattening (see Section 3.3.2, converted from mg/g to mg/kg)[64] were used as input data for the exposure calculation and are reported in Table 9.

Table 9: Input values on protopine and allocryptopine content in food of animal origin following the use of the additive at the maximum recommended level (0.750 mg SG/kg feed) and used for the consumer exposure assessment

Animal product	Protopine	Allocryptopine	Protopine + allocryptopine
	(mg/kg wet tissue or product)		
Fat	0.00024	0.00013	0.00037
Kidney	0.00121	0.00163	0.00284
Liver	0.02079	0.00394	0.02473
Muscle(a)	0.00049	0.00022	0.00071

(a): The residue concentration in muscle and skin/fat will be applied to the intake of meat at the following proportions: 90% muscle and 10% skin/fat (EFSA FEEDAP Panel, 2017b). This correspond would to 0.00047 mg PRO/kg, 0.00021 mg ALL/kg and 0.00068 mg PRO + ALL/kg.

The results of the dietary exposure to PRO, ALL and PRO+ALL for the different population categories are reported in Table 10.

[64] Technical dossier/Supplementary information July 2022/Annex_III_2_1_4.

Table 10: Chronic human exposure to protopine (PRO), allocryptopine (ALL) and their sum (PRO + ALL). Maximum highest reliable percentile in ng/kg bw per day

Population class	No. of surveys	Maximum HRP* ng/kg bw per day			% TTC value†		
		PRO	ALL	PRO + ALL	PRO	ALL	PRO + ALL
Infants	6	3.30	1.44	4.78	0.22	0.10	0.32
Toddlers	10	5.77	1.96	7.81	0.38	0.13	0.52
Other children	18	7.33	1.91	9.11	0.49	0.13	0.61
Adolescents	17	2.18	0.97	3.17	0.15	0.06	0.21
Adults	17	6.58	1.33	7.92	0.44	0.09	0.53
Elderly	14	1.32	0.59	1.91	0.09	0.04	0.13
Very elderly	12	2.64	0.54	3.18	0.18	0.04	0.21

*: Highest reliable percentile.
†: TTC value for CC III: 1.5 μg/kg bw per day (equal to 1,500 ng/kg bw per day).

In the absence of a reference point for PRO and ALL, the estimated exposure can be related to the value that, according to threshold of toxicological concern (TTC) approach, is considered of low probability of adverse health effects (EFSA Scientific Committee, 2019b). For substances belonging to Cramer class III, the TTC value corresponds to 1.5 μg/kg bw per day (equal to 1,500 ng/kg bw per day). For all consumer categories, exposure in the individual countries was constantly below 10 ng/kg bw per day, corresponding to < 1% of the value of TTC. Below this threshold, the probability of adverse effects is low.

3.3.6.2. Conclusions on safety for the consumer

The FEEDAP Panel considers that the use of Sangrovit® Extra in poultry species for fattening at the maximum dose proposed is of low concern for the consumer.

3.3.7. Safety for the user

The applicant also provided studies on skin and eye irritancy and skin sensitisation performed with a similar, more concentrated formulation (Sangrovit®, a test item containing 1.67% SG). Considering the similarity in composition between the test item (Sangrovit®) and the additive under assessment (Sangrovit® Extra, see Table 5), the FEEDAP Panel considered the studies submitted relevant to the present assessment.

3.3.7.1. Effect on respiratory system

The dusting potential the additive (up to 0.20 g/m^3)[65] and the particle size distribution (3% of particles are smaller than 50 μm)[66] indicated the exposure of users via respiratory route while handling the additive is considered low.

However, the FEEDAP Panel notes that the additive contains SG and CH, which are suspected to have a genotoxic potential. When handling the additive, exposure of unprotected users to SG and CH may occur. Therefore, to reduce the risk, the exposure of users should be minimised.

3.3.7.2. Effect on eyes and skin

An acute skin irritation GLP study was performed with Sangrovit® according to OECD TG 404.[67] Under the conditions of the test the product was classified as non-irritating to skin (UN GHS 'No Category').

An *in vitro* eye irritation test was performed with Sangrovit® according to OECD TG 492 using reconstructed Human Cornea-Like Epithelium.[68] Based on the results obtained the test item was predicted to be an eye irritant. As the test is not recommended for distinction between irritancy (UN GHS Category 2) and serious eye damage (UN GHS Category 1), no conclusion regarding the classification of the test item could be made.

[65] Technical dossier/Section II/Annex_II_1_5_1.
[66] Technical dossier/Section II/Annex_II_1_5_2.
[67] Technical dossier/Section III/ Annex_III_3_1_2_1.
[68] Technical dossier/Section III/ Annex_III_3_1_2_3.

A skin sensitisation test with Sangrovit® in guinea pigs (Magnusson and Kligman maximisation test) was conducted according to EC method B.6. (96/54/EC) and OECD TG 406.[69] Under the conditions of the test the product was considered not to be a dermal sensitiser.

The results of the studies submitted showed that the test item Sangrovit® is irritant to the eyes but not irritant to skin or a skin sensitiser. The same conclusion is extended to the additive under assessment, Sangrovit® Extra.

3.3.7.3. Conclusions on safety for the user

On the basis of the studies submitted, the additive was shown to be irritant to the eyes but not irritant to skin or a skin sensitiser. The FEEDAP Panel cannot exclude the potential of the additive to be a respiratory sensitiser. When handling the additive, exposure of unprotected users to sanguinarine and chelerythrine may occur. Therefore, to reduce the risk, the exposure of users should be minimised.

3.3.8. Safety for the environment

M. cordata is a natural occurring plant primarily distributed in temperate areas in North America (east of the Mississippi River at elevations below 1,000 m) and eastern Asia (China and Japan).[70] It was introduced in Europe in the late part of the 18th century, where it is widely distributed as an ornamental garden plant.

SG, the main component of *M. cordata* extract, is widely distributed in nature and can be found in plants of the families Papaveraceae, Fumariaceae and Rutaceae.[71]

The use of Sangrovit® Extra in animal nutrition at the proposed conditions of use is not expected to increase the concentration of SG and other alkaloids in the environment.

3.4. Efficacy

3.4.1. Efficacy for chickens for fattening

A total of six trials (including the tolerance-efficacy one described above in Section 3.3.5, which is referred in this section as Trial 6) in chickens for fattening were submitted sharing a similar design. The details on the study design are provided in Table 11 and the main results in Table 12.

Table 11: Trial design and use level of the efficacy trials performed in chickens for fattening

Trial	Total N (birds/rep) Reps/treat	Breed Sex	Duration (starter/ grower/ finisher)	Composition feed (form)	Groups		
					mg additive/ kg feed	mg SG/kg feed	
						Intended	Analysed*
1[72]	1,950 (22) 14	Ross 308 Male	38 days (1–21/22–42)	Maize, wheat and soyabean meal (mash)	0 45 60 90 120 150	0 0.24 0.33 0.49 0.65 0.81	< 0.05 0.17 0.26 0.49 0.57 0.79
2[73]	270 (15) 9	Cobb 500 Male	42 days (1–21/22–42)	Maize, wheat and soyabean meal (mash)	0 60	0 0.28	< 0.05 0.26
3[74]	600 (15) 8	Cobb 500 Male	42 days (1–14/15–28/ 29–42)	Maize, wheat and soyabean meal (pellets)	0 45 60 120 150	0 0.244 0.325 0.650 0.812	0 0.18 0.26 0.50 0.65

[69] Technical dossier/Section III/ Annex_III_3_1_2_2.
[70] Technical dossier/Section III/Annex III_31.
[71] Technical dossier/Section III/Annex III_ 15 and Annex III_ 39 and supplementary information.
[72] Technical dossier/Section IV/Annex_IV_3_1.
[73] Technical dossier/Section IV/Annex_IV_3_2.
[74] Technical dossier/Section IV/Annex_IV_3_3.

Trial	Total N (birds/rep) Reps/treat	Breed Sex	Duration (starter/ grower/ finisher)	Composition feed (form)	Groups		
					mg additive/ kg feed	mg SG/kg feed	
						Intended	Analysed*
4[75]	480 (16) 10	Ross 308 Male	42 days (1–21/22–42)	Wheat and soyabean meal (mash)	0 45 150	0 0.244 0.812	< 0.05 0.26 0.75
5[76]	480 (16) 10	Ross 308 Male	42 days (1–21/22–42)	Maize and soyabean meal (mash)	0 45 150	0 0.244 0.812	< 0.05 0.21 0.72
6[77]	2,688 (24) 16	Ross 308 Male	42 days (1–21/22–42)	Maize, wheat and soyabean meal (mash)	0 45 60 90 120 150 3,000	0 0.26 0.30 0.45 0.65 0.75 15.0	< 0.05 0.19 0.24 0.54 0.61 0.75 15.6

Total N: total number of animals; birds/rep: number of animals per replicate; Reps/treat: number of replicates per treatment.
*: Average values of the content of sanguinarine (SG) in the different feeds (starter, grower, finisher).

In all trials, the basal diets were either not supplemented (control) or supplemented with Sangrovit® Extra at the corresponding level(s) considered in the studies (see Table 8). The one-day-old male chicks were distributed in pens and randomly allocated to one of the dietary groups. The levels of the additive in the feeds were confirmed by the analysis of SG as marker. In all trials, the feed and water were offered *ad libitum* during the whole experimental period (38–42 days).

In Trial 1, at day 21, two birds per replicate (pen) were randomly removed for the analysis of blood stress biomarkers (data not reported). Consequently, from d22 to d38, the number of animals was 1,680 with 84 pens in groups of 20 birds. The removal of the animals did not affect the overall performance of the treatments.

In all trials, mortality and health status were checked daily, and the most likely cause of death or reason for culling recorded. The birds were weighted at the start of the trial (day 1). Thereafter, pen body weight and feed intake were recorded weekly (for trials 2, 3, 4 and 5) or after each diet change (for trials 1 and 6). The average daily feed intake, average daily gain and feed to gain ratio were calculated and corrected for mortality for each feeding period and the overall experiment. In all cases, the experimental data were analysed with ANOVA with the pen as experimental unit and the treatment as fixed effect. Group means were compared with Tukey's test. Significance level was set at 0.05.

Mortality values were within commercial standards in all trials, with no difference between treatments. In Trials 1, 4, 5 and 6, the supplementation of the diets of chickens with Sangrovit® Extra at the minimum recommended level showed an improvement of the feed to gain ratio in comparison with the control. At the same level of supplementation, it was observed higher final BW and average daily gain in trials 4, 5 and 6, and lower average daily feed intake in Trial 1. In Trial 2, the birds supplemented with 60 mg Sangrovit® Extra/kg feed (1.33× minimum recommended level) showed better feed to gain ratio. In trial 3, the improvement of the feed to gain ratio was only observed at 2.33× the minimum recommended level compared to the control. The Panel notes that the effect of the additive was not consistent within the whole recommended range, showing lower average daily gain and final BW in Trial 1 and no effect in Trials 3, 4 and 5 at 150 mg Sangrovit® Extra/kg feed compared to the control diet.

[75] Technical dossier/Section IV/Annex_IV_3_4.
[76] Technical dossier/Section IV/Annex_IV_3_5.
[77] Technical dossier/Section III/Annex_III_1.

Table 12: Effects of Sangrovit® Extra on the performance of chickens for fattening

Trial	Groups (mg Sangrovit® Extra/kg feed)	Total/average(1) feed intake (g)	Final body weight (g)	Average daily weight gain (g)	Feed to gain ratio	Mortality and culling (%)
1	0	4,134^{a}	2,743^{a}	68.8^{a}	1.58^{c}	2.6
	45	3,948^{b}	2,731^{a}	68.6^{a}	1.52^{ab}	2.9
	60	3,948^{b}	2,726^{ab}	68.3^{a}	1.51^{ab}	3.2
	90	3,960^{b}	2,748^{a}	69.1^{a}	1.51^{a}	1.9
	120	4,055^{ab}	2,723^{ab}	68.5^{a}	1.56^{bc}	1.9
	150	3,929^{b}	2,594^{b}	64.7^{b}	1.56^{c}	5.2
2	0	4,834	3,230	75.8	1.52^{a}	3.7
	60	4,733	3,259	76.5	1.47^{b}	1.5
3	0	4,402	3,201^{a}	75.3	1.39^{a}	5.0
	45	4,410	3,222^{ab}	75.8	1.39^{ab}	5.0
	60	4,423	3,262^{abc}	76.7	1.37^{ab}	4.2
	120	4,414	3,291^{c}	77.4	1.36^{b}	3.3
	150	4,465	3,287^{bc}	77.3	1.38^{ab}	4.2
4	0	4,709	2,967^{b}	70.9^{b}	1.61^{a}	6.9
	45	4,604	3,092^{a}	72.6^{a}	1.51^{b}	4.4
	150	4,579	2,998^{ab}	70.5^{ab}	1.55^{ab}	1.3
5	0	4,500	2,935^{b}	69.9^{b}	1.56^{a}	3.8
	45	4,514	3,025^{a}	71.0^{a}	1.51^{b}	3.8
	150	4,483	2,997^{ab}	70.4^{ab}	1.52^{ab}	1.9
6	0	105.1^{a}	2,841^{c}	70.5^{c}	1.65^{b}	1.8
	45	102.6^{ab}	2,982^{a}	74.1^{a}	1.53^{a}	1.6
	60	102.6^{ab}	2,933^{abc}	72.8^{abc}	1.56^{a}	2.1
	90	103.6^{ab}	2,967^{ab}	73.7^{ab}	1.56^{a}	1.3
	120	102.1^{ab}	2,938^{abc}	73.0^{abc}	1.55^{a}	2.1
	150	100.1^{b}	2,852^{bc}	70.9^{bc}	1.56^{a}	1.6
	3,000	100.5^{b}	2,882^{abc}	71.4^{abc}	1.56^{a}	1.0

^{a,b}: Mean values within a trial and within a column with a different superscript are significantly different $p < 0.05$.
(1): Total feed intake for Trials 1–5; Average daily feed intake for Trial 6.

3.4.1.1. Conclusions on efficacy

The FEEDAP Panel concludes that the additive Sangrovit® Extra has the potential to be efficacious in improving performance of chickens for fattening at 45 mg/kg complete feed. This conclusion can be extended to chickens reared for laying/breeding and can be extrapolated to all poultry for fattening and reared for laying/breeding. The FEEDAP Panel notes discrepancies in the effect of the additive on the performance data of chickens fed at 150 mg/kg complete feed.

3.5. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[78] and Good Manufacturing Practice.

4. Conclusions

The following conclusions apply to Sangrovit® Extra, which consists of a *M. cordata* extract [redacted] and of *M. cordata* processed leaves [redacted] and is formulated to contain a concentration of the sum of the four alkaloids (sanguinarine, chelerythrine, protopine and allocryptopine) of 1.25%, with 0.5% sanguinarine (0.4–0.7%).

Owing to the presence of the DNA intercalators sanguinarine and chelerytrine, a concern for genotoxicity was identified.

[78] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Based on the results of the tolerance trial in chickens for fattening, the Panel has no safety concerns when the additive Sangrovit® Extra is used up to the maximum recommended level of 150 mg/kg complete feed (corresponding to 0.750 mg SG/kg complete feed) for chickens for fattening. This conclusion is extrapolated to other poultry for fattening. No conclusion can be drawn for poultry reared for laying/breeding, which are considered long-living/reproductive animals.

The use of Sangrovit® Extra in poultry species for fattening at the maximum dose proposed is of low concern for the consumer.

The additive was shown to be irritant to the eyes but not irritant to skin or a skin sensitiser. The FEEDAP Panel cannot exclude the potential of the additive to be a respiratory sensitiser. When handling the additive, exposure of unprotected users to sanguinarine and chelerythrine may occur. Therefore, to reduce the risk, the exposure of users should be reduced.

The use of Sangrovit® Extra as a feed additive under the proposed conditions of use is considered safe for the environment.

The additive Sangrovit® Extra has the potential to be efficacious in improving the zootechnical performance of all poultry for fattening or reared for laying/breeding at 45 mg/kg feed.

5. Recommendation

The specification for sanguinarine in Sangrovit® Extra should not exceed 0.7%. The specifications for the sum of the four alkaloids should not exceed the highest analysed concentration (1.4%).

References

Ansari KM, Dhawan A, Khanna SK and Das M, 2005. *In vivo* DNA damaging potential of sangluinarine alkaloid, isolated from argemone oil, using alkaline Comet assay in mice. Food and Chemical Toxicology, 43, 147–153. https://doi.org/10.1016/j.fct.2004.09.005

Ansari R, Mahdavinia M, Sadjadi A, Nouraie M, Kamangar F, Bishehsari F, Fakheri H, Shahriar Semnani d, Arshi S, Zahedi MJ, Darvish-Moghadam S, Mansour-Ghanaei F, Mosavi A and Malekzadeh R, 2006. Incidence and age distribution of colorectal cancer in Iran: Results of a population-based cancer registry. Cancer Letters, 240, 143–147. https://doi.org/10.1016/j.canlet.2005.09.004

Bai LP, Zhao ZZ, Cai Z and Jiang ZH, 2006. DNA-binding affinities and sequence selectivity of quaternary benzophenanthridine alkaloids sanguinarine, chelerythrine, and nitidine. Bioorganic and Medicinal Chemistry, 14, 5439–5445. https://doi.org/10.1016/j.bmc.2006.05.012

Croacker A, King GJ, Pyne JH, Anoopkumar-Dukie S, Simanek V and Liu L, 2017. Carcinogenic potential of sanguinarine, a phytochemical used in 'therapeutic' black salve and mouthwash. Mutation Research-Reviews in Mutation Research, 774, 46–56. https://doi.org/10.1016/j.mrrev.2017.09.001

Das A, Mukherjee A and Chakrabarti J, 2004. Sanguinarine: an evaluation of in vivo cytogenetic activity. Mutation Research, 563, 81–87. https://doi.org/10.1016/j.mrgentox.2004.06.004

Das M, Ansari M, Dhawan A, Shukla Y and Khanna SK, 2005. Correlation of DNA damage in epidemic dropsy patients to carcinogenic potential of argemone oil and isolated sanguinarine alkaloid in mice. International Journal of Cancer, 117, 709–717. https://doi.org/10.1002/ijc.21234 PMID: 15981203

Debiton E, Madelmont JC, Legault J and Barthomeuf C, 2003. Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion. Cancer Chemotherapy and Pharmacology, 51, 474–482. https://doi.org/10.1007/s00280-003-0609-9

Deroussent A, Ré M, Hoellinger H and Cresteil T, 2010. Metabolism of sanguinarine in human and in rat: characterization of oxidative metabolites produced by human CYP1A1 and CYP1A2 and rat liver microsomes using liquid chromatography-tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 52, 391–397. https://doi.org/10.1016/j.jpba.2009.09.014

Dong Z, Tang SS, Ma XL, Tan B, Tang ZS, Li CH, Yang ZH and Zeng JG, 2022. Acute, chronic, and genotoxic studies on the protopine total alkaloids of the *Macleaya cordata* (willd.) R.Br. In rodents. Frontiers in Pharmacology, 13, 987800. https://doi.org/10.3389/fphar.2022.987800

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017a. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2017b. Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017;15(10):5021, 19 pp. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Dujardin B, Galobart J and Innocenti ML, 2017c. Guidance on the assessment of the safety of feed additives for the consumer. EFSA Journal 2017;15(10):5022, 17 pp. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2018. Guidance on the assessment of the efficacy of feed additives. EFSA Journal 2018;16(5):5274, 25 pp. https://doi.org/10.2903/j.efsa.2018.5274

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis V, Bastos ML, Christensen H, Dusemund B, Kouba M, Kos Durjava M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Brock T, Knecht J, Kolar B, Beelen P, Padovani L, Tarrés-Call J, Vettori MV and Azimonti G, 2019. Guidance on the assessment of the safety of feed additives for the environment. EFSA Journal 2019;17(4):5648, 78 pp. https://doi.org/10.2903/j.efsa.2019.5648

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2021. General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic. Available online: https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf

EFSA Scientific Committee, 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379, 69 pp. https://doi.org/10.2903/j.efsa.2011.2379

EFSA Scientific Committee, 2012. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012;10(3):2579, 32 pp. https://doi.org/10.2903/j.efsa.2012.2579

EFSA Scientific Committee, Hardy A, Benford D, Halldorsson T, Jeger M, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Younes M, Aquilina G, Crebelli R, Gürtler R, Hirsch-Ernst KI, Mosesso P, Nielsen E, van Benthem J, Carfi M, Georgiadis N, Maurici D, Parra Morte J and Schlatter J, 2017. Scientific Opinion on the clarification of some aspects related to genotoxicity assessment. EFSA Journal 2017;15(12):5113, 25 pp. https://doi.org/10.2903/j.efsa.2017.5113

EFSA Scientific Committee, More S, Bampidis V, Benford D, Boesten J, Bragard C, Halldorsson T, Hernandez-Jerez A, Hougaard-Bennekou S, Koutsoumanis K, Naegeli H, Nielsen SS, Schrenk D, Silano V, Turck D, Younes M, Aquilina G, Crebelli R, Gürtler R, Hirsch-Ernst KI, Mosesso P, Nielsen E, Solecki R, Carfi M, Martino C, Maurici D, Parra Morte J and Schlatter J, 2019a. Statement on the genotoxicity assessment of chemical mixtures. EFSA Journal 2019;17(1):5519, 11 pp. https://doi.org/10.2903/j.efsa.2019.5519

EFSA Scientific Committee, More SJ, Bampidis V, Benford D, Bragard C, Halldorsson TI, Hernández-Jerez AF, Hougaard BS, Koutsoumanis KP, Machera K, Naegeli H, Nielsen SS, Schlatter JR, Schrenk D, Silano V, Turck D, Younes M, Gundert-Remy U, Kass GEN, Kleiner J, Rossi AM, Serafimova R, Reilly L and Wallace HM, 2019b. Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment. EFSA Journal 2019;17(6):5708, 17 pp. https://doi.org/10.2903/j.efsa.2019.5708

El-Sheikh SM, Khairy MH, Eleiwa NZ, Abdalla OE and El-Monsef AGA, 2019. Effect of antibiotic growth promoter alternatives on growth performance, hemato-biochemical and immunological profile of healthy broiler chickens Zagazig. Veterinary Journal, 47, 21–31. https://doi.org/10.21608/zvjz.2019.6003.1008

Frankos VH, Brusick DJ, Johnson EM, Maibach HI, Munro I, Squire RA and Weil CS, 1990. Safety of Sanguinaria extract as used in commercial toothpaste and oral rinse products. Journal of the Canadian Dental Association, 56(7 Suppl), 41–47. PMID: 2207854

Fu H, Yang P, Hai J and Li H, 2018. Utilization of circular dichroism and electrospray ionization mass spectrometry to understand the formation and conversion of G-quadruplex DNA at the human c-myb proto-oncogene. Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy, 203, 70–76. https://doi.org/10.1016/j.saa.2018.05.079

Ghosh I and Mukherjee A, 2016. Argemone oil induces genotoxicity in mice. Drug and Chemical Toxicology, 39, 407–411. https://doi.org/10.3109/01480545.2015.1135339

Giri P and Kumar GS, 2007. Specific binding and self-structure induction to poly(A) by the cytotoxic plant alkaloid sanguinarine. Biochimica Biophysica Acta, 1770, 1419–1426. https://doi.org/10.1016/j.bbagen.2007.05.005

Gao L, Schmitz HJ, Merz KH and Schrenk D, 2019. Characterization of the cytotoxicity of selected Chelidonium alkaloids in rat hepatocytes. Toxicology Letters, 311, 91–97. https://doi.org/10.1016/j.toxlet.2019.04.031
Hassan HMA, Samy A, Youssef Amani W and Mohamed MA, 2018. Using different feed additives as alternative to antibiotic growth promoter to improve growth performance and carcass traits of broilers using different feed additives as alternative to antibiotic growth promoter to improve growth performance and carcass traits of broilers. Internationa Journal of Poultry Sciences, 17, 255–261. https://doi.org/10.3923/ijps.2018.255.261
Hu NX, Chen M, Liu YS, Shi Q, Yang B, Zhang HC, Cheng P, Tang Q, Liu ZY and Zeng JG, 2019. Pharmacokinetics of sanguinarine, chelerythrine, and their metabolites in broiler chickens following oral and intravenous administration. Journal of Veterinary Pharmacology and Therapeutics, 42, 197–206. https://doi.org/10.1111/jvp.12729
Huang YJ, Cheng P, Zhang ZY, Tian SJ, Sun ZL, Zeng JG and Liu ZY, 2018a. Biotransformation and tissue distribution of protopine and allocryptopine and effects of Plume Poppy Total Alkaloid on liver drug-metabolizing enzymes. Scientific Reports, 11, 537. https://doi.org/10.1038/s41598-017-18816-7
Huang P, Zhang Y, Xiao K, Jiang F, Wang H, Tang D, Liu D, Liu B, Liu Y, He X, Liu H, Liu X, Qing Z, Liu C, Huang J, Ren Y, Yun L, Yin L, Lin Q, Zeng C, Su X, Yuan J, Lin L, Hu N, Cao H, Huang S, Guo Y, Fan W and Zeng J, 2018b. The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. Microbiome, 6, 211. https://doi.org/10.1186/s40168-018-0590-5
Huang CY, Huang YJ, Zhang ZY, Liu YS and Liu ZY, 2021. Metabolism and tissue distribution of chelerythrine and effects of *Macleaya Cordata* extracts on liver NAD(P)H Quinone Oxidoreductase. Frontiers in Veterinary Sciences, 8, 659771. https://doi.org/10.3389/fvets.2021.659771
Huang CY, Kong L, Cao Y and Yan L, 2022. Identification and quantification, metabolism and pharmacokinetics, pharmacological activities, and botanical preparations of protopine: a review. Molecules, 27, 215. https://doi.org/10.3390/molecules27010215
Janovská M, Kubala M, Šimánek V and Ulrichová J, 2010. Fluorescence of sanguinarine: spectral changes on interaction with amino acids. Physical Chemistry Chemical Physics, 12, 11335–11341. https://doi.org/10.1039/b925828k
Juskiewicz J, Gruzauskas R, Zdunczyk Z, Semaskaite A, Jankowski J, Totilas Z, Jarule V, Sasyte V, Zdunczyk P, Raceviciute-Stupeliene A and Svirmickas G, 2010. Effects of dietary addition of *Macleaya cordata* alkaloid extract on growth performance, caecal indices and breast meat fatty acids profile in male broilers. Journal of Animal Physiology and Animal Nutrition, 95, 171–178. https://doi.org/10.1111/j.1439-0396.2010.01037.x
Kaminskyy V, Lootsik MD and Stoika RS, 2006. Correlation of the cytotoxic activity of four different alkaloids, from *Chelidonium majus* (greater celandine), with their DNA intercalating properties and ability to induce breaks in the DNA of NK/Ly murine lymphoma cells. Central European Journal of Biology, 1, 2–15. https://doi.org/10.2478/s11535-006-0001-y
Kaminskyy V, Lin KW, Filyak Y and Stoilka R, 2008. Differential effect of sanguinarine, chelerythrine and chelidonine on DNA damage and cell viability in primary mouse spleen cells and mouse leukemic cells. Cell Biology Intentional, 32, 271–277. https://doi.org/10.1016/j.cellbi.2007.09.004
Kevekordes M-SV, Burghaus CM, Spielberger J, Schmeiser HH, Arlt VM and Dunkelberg H, 1999. SOS induction of selected naturally occurring substances in *Escherichia coli* (SOS chromotest). Mutation Research, 445, 81–91. https://doi.org/10.1016/s1383-5718(99)00141-2
Kevekordes S, Spielberger J, Burghaus CM, Birkenkamp P, Zietz B, Paufler P, Diez M, Bolten C and Dunkelberg H, 2001. Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances. Anticancer Research, 21, 461–469.
Khadem A, Soler L, Everaert N and Niewold T, 2014. Growth promotion in broilers by both oxytetracycline and *Macleaya cordata* extract is based on their anti-inflammatory properties. British Journal of Nutrition, 112, 1110–1118. https://doi.org/10.1017/S0007114514001871
Kosina P, Walterová D, Ulrichová J, Lichnovský V, Stiborová M, Rýdlová H, Vičar J, Krečman V, Brabec MJ and Šimánek V, 2004. Sanguinarin and chelerythrine: assessment of safety on pigs in ninety days feeding experiment. Food and Chemical Toxicology, 42, 85–91. https://doi.org/10.1016/j.fct.2003.08.007
Kosina P, Vacek J, Papoušková B, Stiborová M, Styskala J, Cankař P, Vrublová E, Vostálová J, Šimánek V and Ulrichová J, 2011. Identification of benzo[c]phenanthridine metabolites in human hepatocytes by liquid chromatography with electrospray ion-trap and quadrupole time-of-flight mass spectrometry. Journal of Chromatography B, 879, 1077–1085. https://doi.org/10.1016/j.jchromb.2011.03.023
Kyung-Woo L, Kim J-S, Oh S-T, Kang C-W and An B-K, 2015. Effects of dietary sanguinarine on growth performance, relative organ weight, cecal microflora, serum cholesterol level and meat quality in broiler chickens. The Journal of Poultry Science, 52, 15–22. https://doi.org/10.2141/jpsa.0140073
Lin L, Liu YC, Huang JL, Liu XB, Qing ZX, Zeng JG and Liu ZY, 2017. Medicinal plants of the genus Macleaya (*Macleaya cordata*, *Macleaya microrcarpa*): a review of their phytochemistry, pharmacology, and toxicology. Phytotherapy Research, 32, 19–48. https://doi.org/10.1002/ptr.5952
Liu Z-Y, Ang X-L, Ou S-Q, Hou D-X and He J-H, 2020. Sanguinarine modulate gut microbiome and intestinal morphology to enhance growth performance in broilers. PLoS ONE, 15, e0234920. https://doi.org/10.1371/journal.pone.0234920

Maiti M, Das S, Sen A, Das A, Kumar GS and Nandi R, 2002. Influence of DNA structures on the conversion of sanguinarine alkanolamine form to iminium form. Journal of Biomolecular Structure and Dynamics, 20, 455–464. https://doi.org/10.1080/07391102.2002

Maiti M and Kumar GS, 2009. Biophysical aspects and biological implications of the interaction of benzophenanthridine alkaloids with DNA. Biophysical Reviews, 1, 119–129. https://doi.org/10.1007/s12551-009-0014-7

Mandal P, Sahoo D, Saha S and Chowdhury J, 2018. Sensing of different human telomeric G-quadruplex DNA topologies by natural alkaloid allocryptopine using spectroscopic techniques. The Journal of Physical Chemistry B, 122, 10279–10290. https://doi.org/10.1021/acs.jpcb.8b07856

Marek J, Dostal J and Slavik J, 1998. Crystal structures of α-and β-allocryptopine. Collection of Czechoslovak chemical communications, 63, 416–442.

Matkar SS, Wrischnik LA and Hellmann-Blumberg U, 2008a. Sanguinarine causes DNA damage and p53-independent cell death in human colon cancer cell lines. Chemico-Biological Interactions, 172, 63–71. https://doi.org/10.1016/j.cbi.2007.12.006

Matkar SS, Wrischnik LA and Hellmann-Blumberg U, 2008b. Production of hydrogen peroxide and redox cycling can explain how sanguinarine and chelerythrine induce rapid apoptosis. Archieves of Biochemistry and Biophysics, 477, 43–52. https://doi.org/10.1016/j.abb.2008.05.019

Matulka RA, von Alvensleben S and Morlacchini M, 2014. Tolerance and residue study for standardized *Macleaya cordata* extract added to chicken feed. International Journal of Poultry Science, 13, 368–373. https://doi.org/10.3923/ijps.2014.368.373

Munro IC, Delzell ES, Nestmann ER and Lynch BS, 1999. Viadent usage and oral leukoplakia: a spurious association. Regulatory Toxicology and Pharmacology, 30, 182–196.

PhEur (European Pharmacopoeia), 2022a. Greater Celandine (Chelidonii herba). 11th Edition. Monograph 01/2012:1861. European Directorate for the Quality of Medicines and Health.

PhEur (European Pharmacopoeia), 2022b. Tannins in herbal drugs. 11th Edition. Monograph 01/2008:20814. European Directorate for the Quality of Medicines and Health.

Psotova J, Vecera R, Zdarilova A, Anzenbacherova E, Kosina P, Svobodova HJ, Jirovsky D, Stiborova M, Lichnovsky VJ, Simanek V and Ulrichova J, 2006. Safety assessment of sanguirytrin, alkaloid fraction of *Macleaya cordata*, in rats. Veterinarni Medicina, 51, 145–155. https://doi.org/10.17221/5534-VETMED

Schmeller T, Latz-Brüning B and Wink M, 1997. Biochemical activities of berberine, palmatine and sanguinarine mediating chemical defence against microorganisms and herbivores. Phytochemistry, 44, 257–266. https://doi.org/10.1016/s0031-9422(96)00545-6

Sen A and Maiti M, 1994. Interaction of sanguinarine iminium and alkanolamine form with calf thymus DNA. Biochemical Pharmacology, 48, 2097–2102. https://doi.org/10.1016/0006-2952(94)90510-x1994

Spiess D, Winker M, Dolder Behna A, Gründemann C and Simões-Wüst AP, 2022. Advanced in vitro safety assessment of herbal medicines for the treatment of non-psychotic mental disorders in pregnancy. Frontiers of Pharmacology, 13, 882997. https://doi.org/10.3389/fphar.2022.882997

Stiborova M, Simanek V, Frei E, Hobza P and Ulrichova J, 2002. DNA adduct formation from quaternary benzo[c] phenanthridine alkaloids sanguinarine and chelerythrine as revealed by the 32P-postlabeling technique. Chemico-Biological Interactions, 140, 231–242. https://doi.org/10.1016/s0009-2797(02)00038-8

Stiborova M, Vostalova J, Zdarilova A, Ulrichova J, Hudecek J, Tschirner K and Simanek V, 2008. *Macleaya cordata* extract and Sangrovit genotoxicity assessment *in vivo*. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 152, 35–39. https://doi.org/10.5507/bp.2008.005

Takahashi H, Iguchi M and Onda M, 1985. Utilization of protopine and related alkaloids. XVII. Spectroscopic studies on the ten-membered ring conformations of protopine and α-allocryptopine. Chemical and Pharmaceutical Bulletin, 33, 4775–4782.

Ulrichova J, Dvorak Z, Vicar J, Lata J, Smrzova J, Sedo A and Simanek V, 2001. Cytotoxicity of natural compounds in hepatocyte cell culture models: the case of quaternary benzo[c]phenanthridine alkaloids. Toxicology Letters, 125, 125–132. https://doi.org/10.1016/S0378-4274(01)00430-1

Vacek J, Papoušková B, Kosina P, Galandáková A and Ulrichová J, 2013. Mass spectrometric investigation of chelerythrine and dihydrochelerythrine biotransformation patterns in human hepatocytes. Journal of Chromatography B: Analytical Technology in the Biomedical and Life Sciences, 941, 17–24. https://doi.org/10.1016/j.jchromb.2013.09.037

Varshney D, Spiegel J, Zyner K, Tannahill D and Balasubramanian S, 2020. The regulation and functions of DNA and RNA G-quadruplexes. Natural Reviews. Molecular Cell Biology, 21, 459–474. https://doi.org/10.1038/s41580-020-0236-x

Vavrecková C, Ulrichová J, Hajdúch M, Grambal F, Weigl E and Simánek V, 1994. Effect of quaternary benzo[c] phenanthridine alkaloids sanguinarine, chelerythrine and fagaronine on some mammalian cells. Acta Universitatis Palackianae Olomucensis Facultatis Medicae, 138, 7–10. PMID: 8714083

Večeřa R, Klejdus B, Kosina P, Orolin J, Stiborová M, Smrček S, Vičar J, Dvořák Z, Ulrichová J, Kubáň V, Anzenbacher P and Šimánek V, 2007. Disposition of sanguinarine in the rat. Xenobiotica, 37, 549–558. https://doi.org/10.1080/00498250701230542

Vieira SL, Oyarzabal OA, Freitas DM, Berres J, Peña JEM, Torres CA and Coneglian JLB, 2008a. Performance of broilers fed diets supplemented with sanguinarine-like alkaloids and organic acids. Journal of Applied Poultry Research, 17, 128–133. https://doi.org/10.3382/japr.2007-00054

Vieira SL, Berres J, Reis RN, Oyarzabal OA, Coneglian JLB, Freitas DM, Peña JEM and Torres CA, 2008b. Studies with sanguinarine like alkaloids as feed additive in broiler diets. Brazilian Journal of Poultry Science, 10, 67–71. https://doi.org/10.1590/S1516-635X2008000100010

Vrba J, Dolezel P, Vicar J and Ulrichová J, 2009. Cytotoxic activity of sanguinarine and dihydrosanguinarine in human promyelocytic leukemia HL-60 cells. Toxicology In Vitro, 23, 580–588. https://doi.org/10.1016/j.tiv.2009.01.016

Vrublova E, Vostalova J, Vecera R, Klejdus B, Stejskal D, Kosina P, Zdarilova A, Svobodova A, Lichnovsky V, Anzenbacher P, Dvorak Z, Vicar J, Simanek V and Ulrichova J, 2008. The toxicity and pharmacokinetics of dihydrosanguinarine in rat: a pilot study. Food and Chemical Toxicology, 46, 2546–2553. https://doi.org/10.1016/j.fct.2008.04.013

Yeşilbağ D, Çetin İ, Balcı F, Ardıçlı S, Özyiğit MÖ and Cengiz ŞŞ, 2020. Effects of dietary supplementation of two commercial plant extracts on the growth performance and ileal inflammation score in broiler chickens. Acta Veterinaria Eurasia, 46, 68–74. https://doi.org/10.5152/actavet.2020.19034

Walterova D, Ulrichová PV and Simánek V, 1981. Inhibition of liver alanine aminotransferase activity by some benzophenanthridine alkaloids. Journal of Medicinal Chemistry, 1981, 110–1103. https://doi.org/10.1021/jm00141a019

Wu Y, Zhao NJ, Cao Y, Sun Z, Wang Q, Liu ZY and Sun ZL, 2000. Sanguinarine metabolism and pharmacokinetics study in vitro and in vivo. Journal of Veterinary Pharmacology and Therapeutics, 43, 208–214. https://doi.org/10.1111/jvp.12835

Zdarilova A, Vrublova E, Vostalova J, Klejdus B, Stejskal D, Proskova J, Kosina P, Svobodova A, Vecera R, Hrbac J, Cernochova D, Vicar J, Ulrichova J and Simanek V, 2008. Natural feed additive of *Macleaya cordata*: Safety assessment in rats a 90-day feeding experiment. Food and Chemical Toxicology, 46, 3721–3726. https://doi.org/10.1016/j.fct.2008.09.054

Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ADME	Absorption, distribution, metabolism and excretion
ALL	allocryptopine
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	Analysis of Variance
AST	aspartate transaminase
AUC	area under the curve
BW	body weight
CAS	Chemical Abstracts Service
CH	chelerythrine
CK	creatine kinase
CFU	colony forming unit
CV	coefficient of variation
CYP450	cytochrome P450 monooxygenase families
DHCH	dihydrochelerythrine
DHSG	dihydrosanguinarine
DSB	double strand breaks
EURL	European Union Reference Laboratory
FCR	feed conversion ratio
F:G	feed to gain ratio
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FQBA	quaternary benzo[c]phenanthridine alkaloids from *M. cordata*
GC–MS	gas chromatography–mass spectrometry
GGT	gamma-glutamyl transferase
GSH	glutathione
HPLC	high performance liquid chromatography
HPLC–ESI-MS	high performance liquid chromatography-electrospray mass spectrometry
HPLC–MS/MS	high performance liquid chromatography *tandem* mass spectrometry

HPLC/QqTOF-MS	high-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry
HCT	haematocrit
IUPAC	International Union of Pure and Applied Chemistry
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCE	*Macleaya cordata* extract
MCV	mean corpuscular volume
MG	monograph
NAC	N-acetyl cysteine
NMR	Nuclear magnetic resonance
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
8-oxodG	8-oxodeoxyguanosine
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PhEur	European Pharmacopoeia
PRO	protopine
QEq	quercetin dihydrate equivalent
RLM	rat liver microsomes
ROS	reactive oxygen species
SC	EFSA Scientific Committee
SCE	sister chromatid exchanges
SG	sanguinarine
SOD	superoxide dismutase
TEQ	toxic equivalent
TG	technical guideline
TTC	threshold of toxicological concern
UPLC-MS–MS	ultraperformance liquid chromatography tandem mass spectrometry
UN GHS	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
WHO	World Health Organization

Appendix A – Detailed results of chronic exposure calculation

Table A.1: Chronic dietary exposure of consumers to residues of the sum of sanguinarine and chelerythrine per population class, country and survey (ng/kg body weight per day) based on residue data

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Infants	Bulgaria	523	0.18272	0.70223	95th
Infants	Germany	142	0.02622	0.12784	95th
Infants	Denmark	799	0.03555	0.14827	95th
Infants	Finland	427	0.05814	0.22167	95th
Infants	Italy	9	0.03295	0.00000	50th
Infants	United Kingdom	1,251	0.07833	0.30968	95th
Toddlers	Belgium	36	0.16164	0.32697	90th
Toddlers	Bulgaria	428	0.36714	1.25492	95th
Toddlers	Germany	348	0.06138	0.21556	95th
Toddlers	Denmark	917	0.04619	0.16451	95th
Toddlers	Spain	17	0.24582	0.32051	75th
Toddlers	Finland	500	0.09995	0.35410	95th
Toddlers	Italy	36	0.10181	0.29205	90th
Toddlers	Netherlands	322	0.06903	0.35875	95th
Toddlers	United Kingdom	1,314	0.10901	0.34148	95th
Toddlers	United Kingdom	185	0.12058	0.36192	95th
Other children	Austria	128	0.06526	0.28323	95th
Other children	Belgium	625	0.13724	0.40745	95th
Other children	Bulgaria	433	0.37116	1.44831	95th
Other children	Germany	293	0.06828	0.23389	95th
Other children	Germany	835	0.05237	0.22290	95th
Other children	Denmark	298	0.06276	0.18390	95th
Other children	Spain	399	0.12354	0.41642	95th
Other children	Spain	156	0.17099	0.58281	95th
Other children	Finland	750	0.11417	0.40126	95th
Other children	France	482	0.15022	0.50024	95th
Other children	Greece	838	0.08225	0.29791	95th
Other children	Italy	193	0.09407	0.31390	95th
Other children	Latvia	187	0.07140	0.33826	95th
Other children	Netherlands	957	0.06186	0.26171	95th
Other children	Netherlands	447	0.09344	0.32955	95th
Other children	Sweden	1,473	0.08386	0.24691	95th
Other children	Czechia	389	0.15229	0.61473	95th
Other children	United Kingdom	651	0.11450	0.30908	95th
Adolescents	Austria	237	0.05013	0.19571	95th
Adolescents	Belgium	576	0.04952	0.17901	95th
Adolescents	Cyprus	303	0.05880	0.18925	95th
Adolescents	Germany	393	0.04010	0.17345	95th
Adolescents	Germany	1,011	0.02805	0.14315	95th
Adolescents	Denmark	377	0.04509	0.14382	95th

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Adolescents	Spain	651	0.06980	0.24185	95th
Adolescents	Spain	209	0.09754	0.32456	95th
Adolescents	Spain	86	0.07515	0.28699	95th
Adolescents	Finland	306	0.05370	0.18142	95th
Adolescents	France	973	0.09284	0.32531	95th
Adolescents	Italy	247	0.04688	0.14430	95th
Adolescents	Latvia	453	0.04836	0.20614	95th
Adolescents	Netherlands	1,142	0.06417	0.24705	95th
Adolescents	Sweden	1,018	0.05640	0.18591	95th
Adolescents	Czechia	298	0.10113	0.46345	95th
Adolescents	United Kingdom	666	0.08225	0.22434	95th
Adults	Austria	308	0.05816	0.22412	95th
Adults	Belgium	1,292	0.05027	0.18805	95th
Adults	Germany	10,419	0.03436	0.14610	95th
Adults	Denmark	1,739	0.03221	0.09375	95th
Adults	Spain	981	0.06346	0.22575	95th
Adults	Spain	410	0.05381	0.22531	95th
Adults	Finland	1,295	0.04383	0.18187	95th
Adults	France	2,276	0.07595	0.25434	95th
Adults	Hungary	1,074	0.13007	0.67665	95th
Adults	Ireland	1,274	0.08460	0.22226	95th
Adults	Italy	2,313	0.03391	0.12368	95th
Adults	Latvia	1,271	0.04735	0.18550	95th
Adults	Netherlands	2,055	0.05805	0.20840	95th
Adults	Romania	1,254	0.16427	0.65218	95th
Adults	Sweden	1,430	0.06278	0.19121	95th
Adults	Czechia	1,666	0.07065	0.23198	95th
Adults	United Kingdom	1,265	0.05979	0.16574	95th
Elderly	Austria	67	0.11711	0.19745	95th
Elderly	Belgium	511	0.04233	0.16400	95th
Elderly	Germany	2,006	0.02827	0.11812	95th
Elderly	Denmark	274	0.02554	0.07582	95th
Elderly	Finland	413	0.03199	0.14374	95th
Elderly	France	264	0.07110	0.24434	95th
Elderly	Hungary	206	0.09831	0.43371	95th
Elderly	Ireland	149	0.06618	0.18416	95th
Elderly	Italy	289	0.04697	0.14399	95th
Elderly	Netherlands	173	0.03996	0.16229	95th
Elderly	Netherlands	289	0.03400	0.13130	95th
Elderly	Romania	83	0.13146	0.27836	95th
Elderly	Sweden	295	0.05697	0.18185	95th
Elderly	United Kingdom	166	0.05312	0.14870	95th
Very elderly	Austria	25	0.32043	0.04711	75th
Very elderly	Belgium	704	0.04791	0.16815	95th
Very elderly	Germany	490	0.02675	0.12314	95th
Very elderly	Denmark	12	0.03146	0.03959	75th

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Very elderly	France	84	0.04243	0.15199	95th
Very elderly	Hungary	80	0.08703	0.22234	95th
Very elderly	Ireland	77	0.06204	0.18388	95th
Very elderly	Italy	228	0.03822	0.12186	95th
Very elderly	Netherlands	450	0.03422	0.13016	95th
Very elderly	Romania	45	0.19366	0.37225	90th
Very elderly	Sweden	72	0.03898	0.13492	95th
Very elderly	United Kingdom	139	0.03584	0.10172	95th

Table A.2: Chronic dietary exposure of consumers to residues of the sum of protopine and allocryptopine per population class, country and survey (ng/kg body weight per day) based on residue data

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Infants	Bulgaria	523	1.32257	4.77520	95th
Infants	Germany	142	0.16905	0.86933	95th
Infants	Denmark	799	0.24172	1.00823	95th
Infants	Finland	427	0.39535	1.50738	95th
Infants	Italy	9	0.22408	0.00000	50th
Infants	United Kingdom	1,251	0.56598	2.12883	95th
Toddlers	Belgium	36	1.09913	2.22339	90th
Toddlers	Bulgaria	428	2.52901	7.80844	95th
Toddlers	Germany	348	0.41896	1.46579	95th
Toddlers	Denmark	917	0.31407	1.11869	95th
Toddlers	Spain	17	1.67160	2.17949	75th
Toddlers	Finland	500	0.67964	2.40790	95th
Toddlers	Italy	36	0.69227	1.98591	90th
Toddlers	Netherlands	322	0.46939	2.43951	95th
Toddlers	United Kingdom	1,314	0.74401	2.32209	95th
Toddlers	United Kingdom	185	0.83281	2.43605	95th
Other children	Austria	128	0.44375	1.92600	95th
Other children	Belgium	625	1.03661	2.77066	95th
Other children	Bulgaria	433	2.60709	9.10766	95th
Other children	Germany	293	0.47024	1.71768	95th
Other children	Germany	835	0.35571	1.53047	95th
Other children	Denmark	298	0.42674	1.25051	95th
Other children	Spain	399	0.84011	2.83168	95th
Other children	Spain	156	1.16273	3.96309	95th
Other children	Finland	750	0.90354	3.57840	95th
Other children	France	482	1.00987	3.21088	95th
Other children	Greece	838	0.55933	2.02577	95th
Other children	Italy	193	0.63970	2.13455	95th
Other children	Latvia	187	0.48549	2.30016	95th
Other children	Netherlands	957	0.42066	1.77961	95th
Other children	Netherlands	447	0.63542	2.24091	95th

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Other children	Sweden	1,473	0.57025	1.67898	95th
Other children	Czechia	389	1.04367	4.18015	95th
Other children	United Kingdom	651	0.80343	2.06993	95th
Adolescents	Austria	237	0.34086	1.33082	95th
Adolescents	Belgium	576	0.33711	1.21729	95th
Adolescents	Cyprus	303	0.39985	1.28693	95th
Adolescents	Germany	393	0.27426	1.17946	95th
Adolescents	Germany	1,011	0.18886	0.95975	95th
Adolescents	Denmark	377	0.30660	0.97797	95th
Adolescents	Spain	651	0.47465	1.64459	95th
Adolescents	Spain	209	0.66325	2.20704	95th
Adolescents	Spain	86	0.53822	2.26492	95th
Adolescents	Finland	306	0.36515	1.23367	95th
Adolescents	France	973	0.62863	1.99584	95th
Adolescents	Italy	247	0.34419	0.98123	95th
Adolescents	Latvia	453	0.32886	1.40176	95th
Adolescents	Netherlands	1,142	0.43638	1.67991	95th
Adolescents	Sweden	1,018	0.38352	1.26417	95th
Adolescents	Czechia	298	0.64689	3.17313	95th
Adolescents	United Kingdom	666	0.55780	1.51708	95th
Adults	Austria	308	0.39551	1.52402	95th
Adults	Belgium	1,292	0.35101	1.29016	95th
Adults	Germany	10,419	0.23679	0.97812	95th
Adults	Denmark	1,739	0.21906	0.63753	95th
Adults	Spain	981	0.44331	1.53507	95th
Adults	Spain	410	0.36590	1.53211	95th
Adults	Finland	1,295	0.30621	1.24865	95th
Adults	France	2,276	0.52787	1.68719	95th
Adults	Hungary	1,074	1.03807	2.52848	95th
Adults	Ireland	1,274	0.57400	1.51138	95th
Adults	Italy	2,313	0.25404	0.84105	95th
Adults	Latvia	1,271	0.32978	1.26139	95th
Adults	Netherlands	2,055	0.42440	1.41712	95th
Adults	Romania	1,254	1.53106	7.91600	95th
Adults	Sweden	1,430	0.42643	1.29548	95th
Adults	Czechia	1,666	0.41261	1.62372	95th
Adults	United Kingdom	1,265	0.41020	1.12187	95th
Elderly	Austria	67	1.25370	1.34267	95th
Elderly	Belgium	511	0.30016	1.10948	95th
Elderly	Germany	2,006	0.19088	0.77801	95th
Elderly	Denmark	274	0.17370	0.51560	95th
Elderly	Finland	413	0.22474	1.05713	95th
Elderly	France	264	0.55453	1.49664	95th
Elderly	Hungary	206	0.72116	1.65658	95th
Elderly	Ireland	149	0.49171	1.25228	95th
Elderly	Italy	289	0.41925	0.97912	95th

Population class	Survey's country	Number of subjects	Mean	Highest reliable percentile value	Highest reliable percentile description
Elderly	Netherlands	173	0.26127	1.05066	95th
Elderly	Netherlands	289	0.23030	0.89282	95th
Elderly	Romania	83	1.11860	1.90985	95th
Elderly	Sweden	295	0.41105	1.20149	95th
Elderly	United Kingdom	166	0.41466	0.95907	95th
Very elderly	Austria	25	4.00274	0.32032	75th
Very elderly	Belgium	704	0.34322	1.20034	95th
Very elderly	Germany	490	0.18534	0.80709	95th
Very elderly	Denmark	12	0.21394	0.26923	75th
Very elderly	France	84	0.28658	0.99817	95th
Very elderly	Hungary	80	0.52027	1.24508	95th
Very elderly	Ireland	77	0.42188	1.25040	95th
Very elderly	Italy	228	0.29402	0.82861	95th
Very elderly	Netherlands	450	0.23893	0.88509	95th
Very elderly	Romania	45	1.74879	3.17882	90th
Very elderly	Sweden	72	0.26507	0.91747	95th
Very elderly	United Kingdom	139	0.24114	0.69169	95th