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Safety and efficacy of a feed additive consisting of a tincture derived from roots of *Gentiana lutea* L. (gentian tincture) for use in all animal species (FEFANA asbl)

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of a tincture from the roots of *Gentiana lutea* L. (gentian tincture) when used as a sensory feed additive for all animal species. The product is a [redacted] solution, with a dry matter content of approximately 4.3%. The product contains on average 0.0836% polyphenols (of which 0.0463% are flavonoids and 0.0027% xanthones) and 0.0022% gentiopicroside. The FEEDAP Panel concludes that gentian tincture is safe at the maximum proposed use level of 50 mg/kg complete feed for short-living animals (animals for fattening). The FEEDAP Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed. Considering the genotoxic potential of gentiopicroside and xanthones (gentisin and isogentisin), no conclusions can be drawn for long-living animals (companion animals, horses and animals for reproduction). No safety concern would arise for the consumer from the use of gentian tincture up to the highest safe level in animal nutrition. In the absence of data, no conclusions can be drawn on the potential of the tincture to be a dermal/eye irritant or a skin sensitiser. The data available do not allow to conclude on risks of genotoxicity and carcinogenicity for dermal exposure. Use of the tincture derived from *G. lutea* as a flavour in animal feed is not expected to pose a risk for the environment. Since *G. lutea* and gentian root extract are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary for the tincture under application.

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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 a feed additive shall submit an application in accordance with Article 7 and in addition, Article 10(2) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of seven years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG),[2,3] for authorisation/re-evaluation of two preparations (namely gentian tincture and cats claw extract (sb)) belonging to botanically defined group (BDG) 12 - *Gentianales* when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). During the assessment, the applicant withdrew the application for cats claw extract (sb).[4]

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) and under Article 10(2) (re-evaluation of an authorised 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 27 September 2019.

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 product gentian tincture (*Gentiana lutea* L.), when used under the proposed conditions of use (see Section 3.2.3).

1.2. Additional information

A tincture from *Gentiana lutea* L. (gentian tincture) is currently authorised as a feed additive according to the entry in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 (2b natural products – botanically defined). It has not been assessed as a feed additive in the EU.

There is no specific EU authorisation for any *G. lutea* preparation when used to provide flavour in food. However, according to Regulation (EC) No 1334/2008[5], flavouring preparations produced from food, may be used without an evaluation and approval as long as 'they do not, on the basis of the scientific evidence available, pose a safety risk to the health of the consumer, and their use does not mislead the consumer'.

'Gentian root (*Gentianae radix*)' is described in a monograph of the European Pharmacopoeia 10.0 (PhEur, 2020a). The roots are defined as the dried, fragmented underground organs of *Gentiana lutea* L.

'Gentian tincture (*Gentianae tinctura*)' is described in a monograph of the European Pharmacopoeia 10.0 (PhEur, 2020b). It is defined as the tincture produced from one part of comminuted gentian roots and five parts of ethanol (70% *V/V*) by a suitable procedure.

The European Medicines Agency (EMA) published a herbal monograph and an assessment report on *Gentiana lutea* L., radix and its preparations including tinctures (EMA, 2018a, b).

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] On 13/3/2013, EFSA was informed by the applicant that the applicant company changed to FEFANA asbl, Avenue Louise 130 A, Box 1, 1050 Brussels, Belgium.
[3] On 27 February 2019, EFSA was informed by the applicant about the transfer of contact point for this application to Manghebati SAS, zone de la Basse Haye – BP 42133 – 35221 Chateaubourg Cedex.
[4] On 27 February 2019, EFSA was informed about the withdrawal of the application on cats claw extract (sb).
[5] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Regulation (EC) No 1601/91 of the Council, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34.

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[6] in support of the authorisation request for the use of gentian tincture as a feed additive.

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 phytochemical markers in cinnamon tincture. The Executive Summary of the EURL report can be found in Annex A.[7]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of gentian tincture is in line with the principles laid down in Regulation (EC) No 429/2008[8] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), 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), Guidance on the assessment of the biological relevance of data in scientific assessments (EFSA Scientific Committee, 2017), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a), Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b), Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee, 2019c).

3. Assessment

The additive under assessment, gentian tincture, is derived from the roots of *Gentiana lutea* L. and is intended for use as sensory additive (functional group: flavouring compounds) in feed for all animal species.

3.1. Origin and extraction

Gentiana lutea L. is a perennial herb belonging to the Gentianaceae family, commonly referred to as great yellow gentian. It is native to central and southern Europe.

The gentian tincture is produced from the roots of *G. lutea* (from France) by extended extraction with a [redacted] mixture [redacted] for [redacted] (plant:solvent ratio [redacted]) under ambient conditions. After this period, the tincture is recovered by pressing to separate solid and liquid phases and the extracted solution is then clarified by filtration.

3.2. Characterisation

3.2.1. Characterisation of the tincture

The gentian tincture is a brown liquid, with an average density of 983 kg/m^3 (range: 981–984 kg/m^3) and a pH of 4.27 (4.21–4.38).[9] It is soluble in water.

[6] FEED dossier reference: FAD-2010-0321.
[7] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2010-0321-bdg12.pdf
[8] 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.
[9] Technical dossier/Supplementary information July 2020/Annex_II_3_Results of analysis.

Table 1 summarises the results of proximate analysis of five batches of the additive.[10] The solvent represents about 95.67% of the additive leaving a dry matter (DM) content of about 4.33%. The dry matter consists of ash (2.22% of the DM fraction, on average) and a plant-derived organic fraction (97.78% of the DM fraction), which includes proteins (1.46%), lipids (0.11%) and carbohydrates (96.2%).

Table 1: Proximate analysis of a tincture derived from *Gentiana lutea* L. based on the analysis of five batches (mean and range). The results are expressed as % (w/w)

Constituent	Mean	Range
	% (w/w)	% (w/w)
Dry matter	4.33	3.85–4.59
Ash	0.10	0.08–0.11
Organic fraction	4.23	3.77–4.49
Proteins	0.06	0.04–0.09
Lipids	0.005	0.001–0.009
'Carbohydrates'	4.16	3.71–4.40
Solvent	95.67	95.41–96.15

The constituent described as 'carbohydrate' in Table 1 represents the fraction of organic matter remaining after subtraction of the values for protein and lipids. It will contain a variety of plant-derived compounds including phenolic compounds, in addition to any carbohydrate present.

The fraction of secondary metabolites was characterised in the same batches of the tincture and the results are summarised in Table 2. The tincture was shown to contain polyphenols (0.0836%) determined by spectrophotometry (at 760 nm) and expressed as gallic acid equivalents, and 10 unidentified flavonoids (0.0463%), separately determined by high-performance thin layer chromatography (HPTLC) and expressed as rutin equivalents.[11]

The applicant performed a literature search to identify substances of concern in *G. lutea* and its aqueous and ethanol-water extracts.[12] Among the compounds identified, xanthones (mainly gentisin 0.03–0.07% and isogentisin 0.06–0.11%) and secoridoids (mainly gentiopicroside, 2–3.5% up to 9.5%) have been reported to occur in *G. lutea* roots. Gentiopicroside has been reported in ethanolic and methanolic preparations.

The applicant estimated the content of gentiopicroside and xanthones (gentisin and isogentisin) by HPTLC. The content of gentiopicroside determined as hyperoside equivalent was on average 0.0022%[13] and the content of xanthones quantified as isogentisin equivalent was on average 0.0027%.[14] The FEEDAP Panel notes that there is uncertainty in the quantification of gentiopicroside by HPTLC with ultraviolet (UV) detection, as it was determined using a standard with a 30% difference in the molecular weight. Further uncertainty results from differences in the molecular absorption at the detection wavelength (254 nm) of hyperoside and gentiopicroside. The maximum wavelengths of hyperoside and gentiopicroside are 257 and 235 nm, respectively (Outuki et al., 2015; Olennikov et al., 2019). Therefore, it is likely that the concentration of gentiopicroside is underestimated.

The identified secondary metabolites account on average for 2.0% of the dry matter content of the tincture (range: 1.2–2.8%) and other plant constituents for about 98%.

[10] Technical dossier/Supplementary information November 2020.
[11] Technical dossier/Supplementary information July 2020/ Section_II_Identity and Annex II_4.
[12] Technical dossier/Supplementary information July 2020/Annex II_4_Bibliographic data concerning composition of gentian and gentian extracts.
[13] Technical dossier/Supplementary information July 2020/Annex II_7_Detailed report of gentiopicroside quantification.
[14] Technical dossier/Supplementary information July 2020/Annex II_8_Detailed report of xanthones quantification.

Table 2: Characterisation of the fraction of secondary metabolites of a tincture derived from *Gentiana lutea* L. based on the analysis of five batches (mean and range). The results are expressed as % (w/w)

Constituent	Mean	Range
	% (w/w)	% (w/w)
Total polyphenols	0.084	0.052–0.107
Flavonoids	0.046	0.031–0.056
Xanthones	0.003	0.002–0.004
Gentiopicroside	0.002	0.0001–0.006

No information on the concentrations of impurities in the tincture is given. The applicant controls contamination at the level of the raw material, including knowledge of the cultivation conditions and pesticides applied. Specifications are set with suppliers covering heavy metals (cadmium < 1 mg/kg, mercury < 0.1 mg/kg and lead < 10 mg/kg) and arsenic (< 2 mg/kg), and microbial contamination.[15] [redacted][16] Analysis of impurities in the tincture apparently is made on irregular basis and does not form part of the Hazard Analysis and Critical Control Points Plan.

3.2.2. Stability

The shelf-life of the tincture is declared by the applicant to be at least 36 months when stored in tightly closed containers under standard conditions. No evidence was provided to support this claim.

3.2.3. Conditions of use

The additive is intended for use in feed for all animal species. The applicant proposes a maximum concentration of 50 mg gentian tincture/kg complete feed or 50 mg/kg water for drinking for all animal species, except horses, for which the proposed use is 200 mg/kg complete feed.

3.3. Safety

The safety assessment is based on the highest proposed use levels, which is 200 mg/kg complete feed for horses and 50 mg/kg complete feed for all other species.

No studies were performed with the additive under assessment.

The additive under assessment, gentian tincture, is a mixture consisting of 95.67% of a water/ethanol mixture. The concentration of plant-derived compounds is about 4.33% of the tincture. The dry matter included ash, protein, lipids and carbohydrates, which are not of concern and are not further considered.

Among the secondary plant metabolites, phenolic compounds including flavonoids were quantified but not identified. They will be assessed based on considerations at the level of the assessment group (see Section 3.3.3). These compounds will be readily metabolised and excreted and are not expected to accumulate in animal tissues and products.

The following sections focus on the compounds of concern identified by the literature search performed by the applicant and analytically determined in the tincture, the xanthones gentisin and isogentisin and gentiopicroside.

3.3.1. Absorption, distribution, metabolism and excretion

The applicant provided a literature search on the pharmacokinetics of gentiopicroside. A study in mice showed that the oral administration of gentiopicroside at 150 mg/kg body weight (bw) is followed by a rapid absorption (Wang et al., 2004). The serum peak level of 55 μg/mL was reached at 1 h and rapidly decreased to 8 μg/mL at 2 h. The half-life of gentiopicroside was 2.8 h and its bioavailability was about 40%. The compound was widely distributed in tissues, with a maximum residence time of 2 h in muscle.

[15] Technical dossier/Supplementary information July 2020/Annex II_5_technical data gentian (raw material).
[16] Technical dossier/Supplementary information July 2020/[redacted]

Wang et al. (2007a,b) evaluated the bioavailability of gentiopicroside in the rat orally administered as pure compound or in decoction preparations of Gentianae radix at 150 mg gentiopicroside/kg bw. In both studies, the data showed a faster absorption and a lower bioavailability of gentiopicroside when given as pure compound as compared to preparations (Cmax of 5.8 μg/mL at 0.75 h and T1/2 of 3.4 h vs. Cmax of 10.5 μg/mL at 1.6 h and T1/2 of 6.2 h). The area under the curve (AUC) values of gentiopicroside were about 2.5-fold higher after decoctions administration and the half-life two times higher. The FEEDAP Panel notes that the decoction preparations are expected to have different composition as compared to the tincture under assessment, and thus, the data cannot be directly compared.

No information was made available on the pharmacokinetics of xanthones (gentisin and isogentisin).

3.3.2. Toxicology

Genotoxicity

The literature search provided by the applicant on the toxicity of xanthones[17] and gentiopicroside[18] indicated a mutagenic potential of xanthones. In addition to the publications provided by the applicant, the FEEDAP Panel found several references, which identified a genotoxicity concern also for gentiopicroside. The findings reported in the literature for xanthones and gentiopicroside are described and discussed in this section.

The mutagenicity of a methanol extract of Gentianae radix was investigated in *Salmonella* Typhimurium strain TA 100 by Morimoto et al. (1983). The extract was mutagenic in the presence of S9-mix. After fractionation, the mutagenicity was assigned to gentisin (1,7-dihydroxy-3-methoxyxanthon) and isogentisin (1,3-dihydroxy-7-methoxyxanthon) showing dose-dependent mutagenic activity. Because of limited solubility, a plateau was observed with gentisin at concentrations above 10 μg/plate. Additional evidence of mutagenic activity for gentisin and isogentisin derived from the study by Matsushima et al. (1985) investigating the potential of a variety of structurally related xanthones to induce gene mutations in *S.* Typhimurium strains TA 100 (detecting point mutations), TA 97, TA98 and TA 2637 (detecting frameshift mutations). Xanthone itself was not mutagenic, whereas tri- and tetrahydroxy-derivatives were mutagenic. In particular, a significant increase in the number of revertant colonies was observed in the presence of metabolic activation after treatment with isogentisin (positive in all the tester strains) and gentisin (positive in TA 97 and 2637). Noticeably, a stronger mutagenic response was induced in the three *S.* Typhimurium strains detecting frameshift mutations than in TA100, specifically sensitive to point mutations. It was also observed that methylation of 1,3,7-trihydroxyxanthone (gentisin) in different positions could modulate the potency of the mutagenic effect, since isogentisin (methylated in position 7) showed higher mutagenicity than gentisin, methylated in position 3. Supporting evidence for the mutagenic activity of the two xanthones gentisin and isogentisin was also provided by a study performed on a variety of structurally related hydroxy-anthraquinones (Westendorf et al., 1990), since anthraquinones and xanthones have similar chemical structures, with a slight difference in the middle of the three membered ring system, which should allow read across. The study showed that mutagenicity of hydroxyanthraquinones was more pronounced in frameshift than point mutation strains, in agreement with the results obtained with hydroxyxanthones by Matsushima et al. (1985). These data point to a mutagenic mechanism via DNA intercalation. This is also supported by the results of a quantitative structure–activity relationship (QSAR) analysis carried out for gentisin and isogentisin, identifying xanthones as structural alerts for which a non-covalent DNA binding mechanism via intercalation was proposed and supported by the literature. The investigation by Matsushima et al. (1985) additionally identified the 1,3-dihydroxy structure as an alert with high impact on the mutagenic activity since isogentisin and norswertianolin, the most potent among the xanthones investigated, presented two hydroxy groups in position 1 and 3. However, as a glycoside, norswertianolin requires the presence of β-glucosidase added to the S9-mix prior to be mutagenic. The 1,3-dihydroxy structure was also identified as an alert to mutagenicity in the investigation with hydroxyanthraquinones by Westendorf et al. (1990). The results of this study are described in Appendix A, where the structure similarity between hydroxyxanthones and hydroxyanthraquinones is highlighted and a possible mechanism for the special reactivity of the common 1,3-dihydroxystructure is proposed.

[17] Technical dossier/Supplementary information July 2020/Annex III_4_Bibliographic data about xanthones.
[18] Technical dossier/Supplementary information July 2020/Annex III_5_Bibliographic data about gentiopicroside.

Gentiopicroside belongs to the chemical class of secoiridoids. These compounds are glycosides produced by plants for defence purposes (Konno et al., 1999). The aglycones released after hydrolysis of the glycosidic bond form highly reactive electrophilic structures, which bind covalently to macromolecules. A reaction scheme has been proposed by Zeng et al. (2013) showing that after treatment of gentiopicroside with β-glucosidase, a series of isomerisations led to the formation of four metabolites bearing α,β-unsaturated carbonyl functions, which represent structural alerts for genotoxic activity. An *in silico* analysis performed using the VEGA-QSAR platform as prediction tool for mutagenicity and carcinogenicity (Sobot et al., 2020) identified low reliability prediction for mutagenicity and predicted non-carcinogenic effect using the carcinogenicity models Computer-Assisted Estimation for Synthetic Accessibility (CAESAR 2.1.9) and Istituto Superiore di Sanità (ISS 1.0.2) while opposite predictions were identified by the carcinogenicity models developed by the Istituto di Ricerche Farmacologiche Mario Negri (IRFMN/Antares and IRFMN/ISSCAN-CGX models[19]). The genotoxicity prediction obtained by the *in silico* approach was further tested *in vitro* in human peripheral blood mononuclear cells applying the alkaline Comet assay and the micronucleus test. The authors reported significant increase of DNA damage induced by gentiopicroside treatment. However, the Panel noted that anomalous values of micronucleus frequency were reported for the negative control cultures (100 MN/1,000 binucleated cells). These values are significantly above the average frequencies reported in literature by the Organization for Economic Co-operation and Development (OECD, 2014). Also the results of the Comet assay appeared unusual, because in negative controls, the percentage of tail DNA is generally 10 times lower than the value reported in this study. On this basis, the study was not considered reliable for the assessment of *in vitro* genotoxic effects. The potential capacity of gentiopicroside to induce gene mutations, primary DNA damage and chromosome damage was also evaluated by Mustafayeva et al. (2010) applying the Ames test in S. Typhimurium strains TA97a, TA98, TA100, TA102 as well as the alkaline Comet assay and the micronucleus test in Chinese hamster ovary (CHO) cells. The authors reported a mutagenic effect of gentiopicroside in *S.* Typhimurium strain TA102 and interpreted the effect as positive and suggestive of a mechanism involving oxidative DNA damage. The FEEDAP Panel does not agree with this interpretation because the increased mutation rates were not dose dependent and the increases compared to the negative control were not biologically relevant. A statistically significant increase of DNA damage was observed with the Comet assay both in the absence and presence of metabolic activation; similarly, gentiopicroside induced a statistically significant dose-related increase of micronuclei in the absence and presence of S9 mix. Overall, the results suggest that the compound may act as a genotoxic DNA-reactive mutagen. This is confirmed by the chemical nature of the metabolites formed after hydrolysis of gentiopicroside.

Overall, the available data raise concern that the xanthones gentisin and isogentisin as well as gentiopicroside have a genotoxic potential. Therefore, following the recommendation of the EFSA Scientific Committee (EFSA SC) on the Genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b), it is concluded that the additive as a whole raises concern for its potential of genotoxicity.

Other toxicological studies

The applicant did not provide toxicological studies in laboratory animals to support the safety of the additive.

3.3.3. Safety for the target species

The applicant did not provide tolerance studies or toxicological studies in laboratory animals to support the safety of the additive for the target species. In the absence of tolerance studies and/or data from repeated dose toxicity studies in laboratory animals performed with the additive under assessment or its individual components, the threshold of toxicological concern (TTC) is applied to derive maximum safe feed concentrations for the individual known components of the tincture.

For the components, for which no concern for genotoxicity has been identified the TTC values of Cramer structural class I–III were assigned (EFSA FEEDAP Panel, 2017b).

For the components that have the potential to be genotoxic mutagens, i.e. xanthones and gentiopicroside, the TTC concept is applied in a specified way depending on the lifespan of the target

[19] ISSCAN: carcinogenicity database from Istituto Superiore della Sanità, CGX: Carcinogenicity Genotoxicity eXperience data set.

species and the biological relevance of genotoxicity and carcinogenicity as endpoints (EFSA Scientific Committee, 2017):

- For long-living animals (companion animals, horses and reproduction animals), considering their long lifespan and the likelihood to develop cancer, the threshold of the TTC of 0.0025 μg/kg bw per day is applied. This value has been established for potential DNA-reactive mutagens and/or carcinogens in human risk assessment (EFSA Scientific Committee, 2019c) and is considered applicable in this context
- Due to their short lifespan, cancer risk is not a relevant concern for short-living animals under farming conditions (animals for fattening). For those animals, the TTC for non-genotoxic substances is applied when comparing estimated exposures with the relevant thresholds established based on non-neoplastic endpoints

Phenolic compounds including flavonoids

Among the identified secondary metabolites, 0.084% is phenolic in nature including 0.046% flavonoids.

At the maximum proposed use level of 50 mg gentian tincture/kg complete feed, the concentration of the phenolic fraction after subtraction of values for flavonoids and xanthones (on average 0.035% and up to 0.061%, measured by the Folin–Ciocalteu method) would be on average 0.017 mg/kg feed and at maximum 0.031 mg/kg feed. Although the individual compounds were not identified, phenolic acids are assigned to Cramer Class I. The available data indicate that phenolic compounds would be well below the maximum acceptable concentration in feed for Cramer Class I (ranging from 0.3 mg/kg feed for poultry to 1.5 mg/kg feed for salmonids and dogs). For horses, at the maximum proposed use level of 200 mg/kg feed, the average concentration of polyphenols would be 0.069 mg/kg feed (maximum 0.123 mg/kg feed), which is below the maximum acceptable concentration of 1.3 mg/kg for Cramer Class I compounds in feed for horses.

At least 10 unidentified flavonoids were detected and quantified (as rutin equivalents) accounting together for 0.046% on average (maximum 0.056%). At the proposed use level of 50 mg gentian tincture/kg complete feed, this would correspond to 0.023 mg/kg feed (maximum 0.028 mg/kg feed). The available data indicate that flavonoids would be in the range of maximum acceptable concentrations in feed for Cramer Class III (ranging from 0.02 mg/kg feed for poultry to 0.08 mg/kg feed for salmonids and dogs). For horses, at the maximum proposed use level of 200 mg/kg feed, the average concentrations of flavonoids would be 0.093 mg/kg feed (maximum 0.113 mg/kg), which is 1.3–1.6 fold higher than the maximum acceptable concentration of 0.07 mg/kg for Cramer Class III compounds in feed for horses.

When used in water for drinking, the intake of the additive via water would be two to three times higher than the intake via feed for poultry, pigs and rabbits (EFSA FEEDAP Panel, 2010). At the maximum proposed use level of 50 mg/kg for the use in water for drinking, the concentration of flavonoids would be above the maximum acceptable concentration for these species.

Substances of concern: xanthones and gentiopicroside

Low concentrations of xanthones (on average: 0.003%, range: 0.002–0.004%) were detected in the additive under assessment. The use of gentian tincture at the proposed use levels in feed of 50 mg/kg complete feed would result in an average concentration of 1.4 μg xanthones/kg feed (maximum 1.8 μg/kg feed). The corresponding value for horses, at proposed use level of 200 mg/kg is 5.4 μg xanthones/kg feed (maximum 7.2 μg/kg).

Similarly, when considering gentiopicroside (on average: 0.002%, range: 0.0001–0.006%), the use of gentian tincture at the proposed use levels in feed of 50 mg/kg complete feed would result in an average concentration of 1.1 μg gentiopicroside/kg feed (maximum 3.1 μg/kg feed). The corresponding value for horses, at proposed use level of 200 mg/kg is 4.4 μg gentiopicroside/kg feed (maximum 12.2 μg/kg).

The average and maximum intake of xanthones and gentipicroside for the different target species is reported in Table 3.

Table 3: Target animal intake of xanthones and gentiopicroside (as μg/kg bw per day) at the maximum proposed use level of the additive in feed for each species. The values of xanthones and gentiopicroside in feed are calculated considering the average and the maximum analysed values in the additive

Target species	Daily feed intake	Body weight	Use level	Xanthones		Gentiopicroside	
				Average	Max	Average	Max
	kg DM/day	kg	mg/kg	μg/kg bw per day		μg/kg bw per day	
Chickens for fattening	0.158	2	50	0.121	0.162	0.099	0.274
Laying hens	0.106	2	50	0.081	0.108	0.066	0.184
Turkey for fattening	0.176	3	50	0.091	0.121	0.074	0.204
Piglet	0.88	20	50	0.068	0.090	0.055	0.152
Pig for fattening	2.2	60	50	0.057	0.076	0.046	0.128
Sow lactating	5.28	175	50	0.046	0.061	0.038	0.104
Veal calf (milk replacer)	1.89	100	50	0.027	0.036	0.022	0.061
Cattle for fattening	8	400	50	0.031	0.041	0.025	0.069
Dairy cows	20	650	50	0.048	0.063	0.039	0.107
Sheep/goat	1.2	60	50	0.031	0.041	0.025	0.069
Horse	8	400	200	0.123	0.164	0.100	0.277
Rabbit	0.1	2	50	0.077	0.102	0.063	0.173
Salmon	0.0021	0.12	50	0.028	0.037	0.023	0.062
Dog	0.25	15	50	0.026	0.035	0.021	0.059
Cat	0.06	3	50	0.031	0.041	0.025	0.069
Ornamental fish	0.00054	0.012	50	0.007	0.009	0.006	0.016

Long-living animals (companion animals, horses and species for reproduction)

For long-living animals, the intake of xanthones ranges from 0.026 μg/kg bw per day in dog to 0.123 μg/kg bw per day in horses (maximum intake in the range 0.035–0.164 μg xanthones/kg bw per day). These intake levels are on average 10- to 49-fold (14- to 65-fold when considering the maximum intake values) higher than the TTC value of 0.0025 μg/kg bw per day established for potential DNA reactive mutagens and/or carcinogens in human risk assessment (EFSA Scientific Committee, 2019c).

For gentiopicroside, the intake ranges from 0.021 μg/kg bw per day in dog to 0.100 μg/kg bw per day in horses (maximum intake in the range 0.059–0.277 μg gentiopicroside/kg bw per day). These intake levels are on average 9- to 40-fold (24 to 111-fold when considering the maximum intake values) higher than the TTC value of 0.0025 μg/kg bw per day established for potential DNA reactive mutagens and/or carcinogens in human risk assessment (EFSA Scientific Committee, 2019c).

For long-living animals, the TTC value is exceeded, and generation of further data would be required. In the absence of carcinogenicity studies with xanthones and gentiopicroside, no conclusion can be drawn on the use of gentian tincture for long-living animals.

Short-living animals (fattening animals)

For short-living animals, the TTC based on non-cancer endpoints has been applied. For these species, the average intake of xanthones ranges from 0.028 μg/kg bw per day in salmonids to 0.121 μg/kg bw per day in chickens for fattening (maximum intake in the range 0.037–0.162 μg xanthones/kg bw per day). These average intake levels are 12- to 54-fold (9- to 41-fold when considering the maximum intake values) lower than the TTC value for Cramer class III compounds (1.5 μg/kg bw per day), indicating that there is a low probability of adverse effects.

Similarly, for gentiopicroside, the average intake ranges from 0.023 μg/kg bw per day in salmonids to 0.099 μg/kg bw per day in chickens for fattening (maximum intake in the range 0.062–0.274 μg xanthones/kg bw per day). These average intake levels are 15- to 24-fold (5- to 24-fold when considering the maximum intake values) lower than the TTC value for Cramer class III compounds (1.5 μg/kg bw per day), indicating that there is a low probability of adverse effects.

When used in water for drinking at the maximum proposed use level of 50 mg/kg, the intake of xanthones and gentiopicroside of non-ruminants would be below the TTC value for Cramer class III compounds (EFSA FEEDAP Panel, 2010).

3.3.3.1. Conclusions on safety for the target species

The FEEDAP Panel concludes that gentian tincture is safe at the maximum proposed use level of 50 mg/kg complete feed for short-living animals (animal species for fattening). The maximum proposed use level of 50 mg/kg water for drinking is considered not safe. The Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed. Considering the genotoxic potential of gentiopicroside and xanthones (gentisin and isogentisin), no conclusions can be drawn for long-living animals (companion animals, horses and animals for reproduction).

3.3.4. Safety for the consumer

The roots of *G. lutea* and their preparations including extracts and tinctures are added to a wide range of food categories as spice or for flavouring purposes. Although individual consumption figures for the EU are not available, the Fenaroli's handbook of flavour ingredients (Burdock, 2010) cites values of 0.0026 mg/kg bw per day for gentian root extract.

No data on residues in products of animal origin following the use of the tincture under assessment were made available. The phenolic compounds, including flavonoids and xanthones, will be readily metabolised and excreted and are not expected to accumulate in animal tissues and products. A relevant increase of the uptake of these compounds by humans consuming products of animal origin is not expected. For gentiopicroside, the available data indicate that it is absorbed, metabolised and rapidly excreted and is not expected to accumulate in animal tissues and products (see Section 3.3.1).

Considering the reported human exposure due to direct use of gentian root and its preparations in food (Burdock, 2010) and traditional medicinal use, it is unlikely that consumption of products from animals given gentian tincture at the proposed maximum use level would significantly increase human background exposure.

Consequently, no safety concern would be expected for the consumer from the use of gentian tincture up to the highest safe use level in animal nutrition.

3.3.5. Safety for user

No specific data were provided by the applicant regarding the safety of the additive for users.

The applicant provided information according to Classification, Labelling and Packaging (CLP) Regulation (EC) 1272/2008[20] concerning the presence of ethanol in the tincture.[21]

No conclusions can be drawn on the additive's potential to be a dermal/eye irritant or a skin sensitiser. The data available do not allow to conclude on risks of genotoxicity and carcinogenicity for dermal exposure.

3.3.6. Safety for the environment

G. lutea is a native species to Europe where it grows spontaneously and is cultivated for decorative purposes. Use of the tincture under the proposed conditions of use in animal feed is not expected to pose a risk for the environment.

3.4. Efficacy

Gentian (*G. lutea* L.) and gentian root extract are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009), by the Flavour and Extract Manufactures Association (FEMA) with the reference numbers 2506.

Since gentian (*G. lutea* L.) and gentian root extract are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary.

[20] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ L 353, 31.12.2008, p. 1–1355.

[21] H319: causes serious eye irritation (relevant for dermal exposure).

4. Conclusions

The FEEDAP Panel concludes that gentian tincture is safe at the maximum proposed use level of 50 mg/kg complete feed for short-living animals (animal species for fattening). The maximum proposed use level of 50 mg/kg water for drinking is considered not safe. The Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed. Considering the genotoxic potential of gentiopicroside and xanthones (gentisin and isogentisin), no conclusions can be drawn for long-living animals (companion animals, horses and animals for reproduction).

No safety concern would arise for the consumer from the use of gentian tincture up to the highest safe level in animal nutrition.

In the absence of data, no conclusions can be drawn on the potential of the tincture to be a dermal/eye irritant or a skin sensitiser. The data available do not allow to conclude on risks of genotoxicity and carcinogenicity for dermal exposure.

Use of the tincture derived from *G. lutea* as a flavour in animal feed is not expected to pose a risk for the environment.

Since *G. lutea* L. and gentian root extract are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary for the tincture under application.

5. Documentation as provided to EFSA/Chronology

Date	Event
05/11/2010	Dossier received by EFSA. Botanically defined flavourings from Botanical Group 12 - Gentianales for all animal species and categories. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)
24/02/2011	Reception mandate from the European Commission
26/02/2013	EFSA informed the applicant (EFSA ref. 7150727) that, in view of the workload, the evaluation of applications on feed flavourings would be re-organised by giving priority to the assessment of the chemically defined feed flavourings, as agreed with the European Commission
24/06/2015	Technical hearing during risk assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products": data requirement for the risk assessment of botanicals
27/09/2019	Application validated by EFSA – Start of the scientific assessment
04/11/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety for target species, safety for the consumer, safety for the user and environment*
17/07/2020	Comments received from Member States
06/11/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
24/11/2020	Reception of supplementary information from the applicant – scientific assessment restarts
18/03/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
AUC	area under the curve
$BMDL_{10}$	Benchmark Dose Lower Confidence Limit for a 10% response
CAESAR	Computer Assisted Estimation for Synthetic Accessibility
CGX	Carcinogenicity Genotoxicity eXperience dataset
CHO	Chinese hamster ovary
DM	dry matter
EMA	European Medicines Agency
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FEMA	Flavour and Extract Manufactures Association
FFAC	Feed Flavourings authorisation Consortium of FEFANA (EU Association of Specialty Feed Ingredients and their Mixtures)
FLAVIS	The EU Flavour Information System
FL-no	FLAVIS number
HPTLC	high performance thin layer chromatography
IRFMN	Istituto di Ricerche Farmacologiche Mario Negri
ISS	Istituto Superiore di Sanità
ISSCAN	carcinogenicity database from Istituto Superiore della Sanità

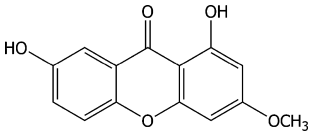
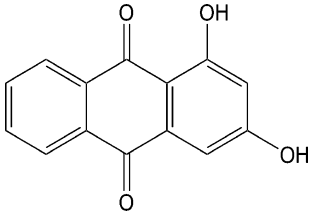
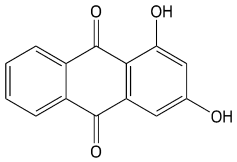
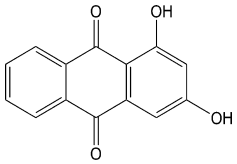
MOE(T)	Combined (total) Margin of exposure
OECD	Organization for Economic Co-operation and Development
QSAR	Quantitative Structure - Activity Relationship
SC	EFSA Scientific Committee
TG	technical guidance
TTC	threshold of toxicological concern
UV	ultraviolet

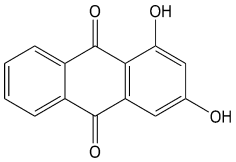
Appendix A – Genotoxicity of hydroxyxanthones and hydroxyanthraquinones

Table A.1 summarises the results of the investigation by Matsushima et al. (1985) on the mutagenicity of the hydroxyxanthones gentisin and isogentisin in *Salmonella* Typhimurium (Section 3.2.2) and the results of the study with hydroxyanthraquinones by Westendorf et al. (1990), highlighting the structural similarities between the two classes of compounds.

In the study by Westendorf et al. (1990), the compounds were tested for their genotoxic potential also in in vitro systems using mammalian cells, such as the HPRT gene mutation assay in V79 cells, the induction of unscheduled DNA synthesis in primary rat hepatocytes (UDS test) and the induction of malignant transformations in C3H mouse fibroblasts. The three 1,3-dihydroxyanthraquinones (1,3-DHA, purpurin and emodin) tested were positive in all these test systems, whereas hydroxyanthraquinones without this structure were only mutagenic in *Salmonella* TA 1537. This makes it likely that the same could be due for the analogue hydroxyxanthone derivatives, which would strengthen the genotoxic alert.

Table A.1: Genotoxicity of hydroxyxanthones (Matsushima et al., 1985) and hydroxyanthraquinones (Westendorf et al., 1990)

Structure	Mutagenic in *Salmonella*		Mutagenic in V79 HPRT assay	UDS in primary rat hepatocytes	Transformation of C3H mouse fibroblasts	Reference
	TA1537 or TA97 (*)	Other strains				
Gentisin 	+	TA2637	N.D.	N.D.	N.D.	Matsushima et al. (1985)
Iso-Gentisin 	++	TA2637 TA98 TA100	N.D.	N.D.	N.D.	Matsushima et al. (1985)
Purpuroxanthin 	++	TA1535 TA1538	+	+	+	Westendorf et al. (1990)
Emodin 	++		+	+	+	Westendorf et al. (1990)

Structure	Mutagenic in *Salmonella*		Mutagenic in V79 HPRT assay	UDS in primary rat hepatocytes	Transformation of C3H mouse fibroblasts	Reference
	TA1537 or TA97 (*)	Other strains				
Physcion 	(+)		–	–	N.D.	Westendorf et al. (1990)

(*): Mutagenicity in TA1537 or TA97 with S9-mix: (+) = borderline activity; + = 2- to 5-fold background; ++ = > 5-fold background.

A possible chemical mechanism for the special reactivity of 1,3-dihydroxyxanthones and 1,3–dihydroxyanthraquinones is shown in Figure A.1. The compounds can easily accept a free electron from an oxidising system provided by CYP450 present in the S9-mix and stabilise the radical by a widespread resonance system. Intercalation brings the radical close to the DNA which may lead to DNA damage. Methylation of the hydroxgroup in position 3 reduces the possible resonance intermediates and thus the stability of the radical. This may explain the lower mutagenic potential of gentisin compared with iso-gentisin. The same is due for the anthraquinone pair of emodin and physcion. Experimental evidence with hydroxyxanthones from non-bacterial test systems to confirm this hypothesis is not available.

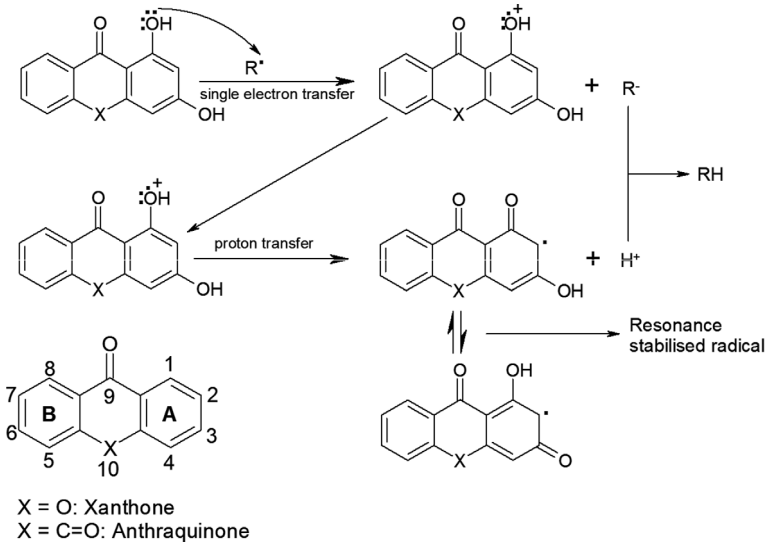


Figure A.1: Possible chemical mechanism showing the reactivity of 1,3-dihydroxyxanthones and 1,3–anthraquinones, based on a hypothesis by Zafar et al. (2019)

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Gentian tincture from botanically defined flavourings Group BDG 12 – Gentianales

In the current application an authorisation is sought under Article 4(1) and Article 10(2) for *gentian tincture* from *botanically defined flavourings group BDG 12 - Gentianales* under the category/functional group (2 b) 'sensory additives'/'flavouring compounds', according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all animal species and categories.

In the updated dossier, the Applicant described *gentian tincture* as brown aqueous/alcoholic preparation from *Gentiana lutea L.* roots, containing a mixture of chemical components naturally present in the plant such as polyphenols, flavonoids, xanthones (gentisin and isogentisin) and gentiopicroside as major constituents.

The *feed additive* is intended to be incorporated directly into *feedingstuffs* or in combination with other flavouring substances (flavouring premixtures) with proposed maximum levels ranging from 50 to 600 mg *feed additive*/kg *feedingstuffs* (or *water* for drinking) depending on the target animal species.

The Applicant proposed to characterise the *feed additive* (*gentian tincture*) by the determination of the content of dry matter, ash, total polyphenols, total flavonoids, xanthones (gentisin and isogentisin) and gentiopicroside. According to the Applicant, the use of high-performance thin-layer chromatography (HPTLC) profiles as a fingerprint for the identification of the *feed additive* is considered a reliable way to identify the *feed additive*.

For the identification and characterisation of the *feed additive,* the EURL recommends the above-mentioned methods based on gravimetry, spectrophotometry and HPTLC to determine the contents of dry matter, ash, total polyphenols, total flavonoids, xanthones (gentisin and isogentisin) and gentiopicroside.

The Applicant did not provide experimental data or analytical method for the determination of *gentian tincture* in *premixtures* and *feedingstuffs*, as the unambiguous determination of the *feed additive* added to the matrices is not achievable experimentally.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.