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

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety of a tincture derived from *Gentiana lutea* L. (gentian tincture). It is intended to be used as a sensory additive for all animal species. The product is a water/ethanol solution, with a dry matter content of approximately 4.3% and it contains on average 0.0836% polyphenols (of which 0.0463% are flavonoids and 0.0027% xanthones) and 0.0022% gentiopicroside. The additive is intended for use in complete feed or drinking water up to a maximum level of 50 mg tincture/kg for all animal species, except horses, for which the proposed use is 200 mg/kg in complete feed. In a previous assessment, due to the genotoxic potential identified in vitro for xanthones (gentisin and isogentisin) and gentiopicroside the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) could not conclude on the safety of the additive for long-living animals and on risks of genotoxicity and carcinogenicity for dermal exposure of unprotected users. The additive did not raise safety concern for short-living animals, consumers and the environment. The applicant has provided information in the form of literature to address the previously identified genotoxic activity of xanthones and gentiopicroside and the risk for the user. Considering that the literature identified provided no new evidence, the FEEDAP Panel reiterated that it is not in a position to conclude on the safety of the additive for long-living and reproductive animals. No conclusions could be drawn on the potential of the additive to be a dermal/eye irritant or a skin sensitiser. When handling the tincture, exposure of unprotected users to xanthones (gentisin and isogentisin) and gentiopicroside cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

Keywords: sensory additives, flavouring compounds, *Gentiana lutea* L., tincture, gentian tincture, xanthones, gentiopicroside

Requestor: European Commission

Question number: EFSA-Q-2022-00466

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1. Introduction

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

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition and, in particular, Article 9 defined the term of the authorisation by the Commission.

The applicant FEFANA asbl[2,3] is seeking a Community authorisation of the product gentian tincture (*Gentiana lutea* L.) to be used as a flavouring additive in feed for all animal species (Table 1).

Table 1: Description of the substances

Category of additive	**Sensory additives**
Functional group of additive	Flavourings
Description	Gentian tincture
Target animal category	All animal species
Applicant	FEFANA asbl
Type of request	New opinion

On 18 March 2021, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ("Authority"), in its opinion on the safety and efficacy of the product, could not conclude on the safety of the product.

The Commission gave the possibility to the applicant to submit supplementary information and data in order to complete the assessment and to allow a revision of EFSA's opinion. The new data have been received on 18 July 2022.

In view of the above, the Commission asks EFSA to issue a new opinion on the safety of *Gentiana lutea* L. (gentian tincture) from Botanically defined flavourings BDG 12 – Gentianales as a feed additive for all animal species based on the additional data submitted by the applicant, in accordance with Article 29(1) (a) of Regulation (EC) 178/2000.

1.2. Additional information

The FEEDAP Panel issued an opinion on the safety and efficacy of gentian tincture derived from the roots of *G. lutea* L. when used as a sensory additive in feed for all animal species (EFSA FEEDAP Panel, 2021). Considering the potential concern for genotoxicity identified *in vitro* for the constituents xanthones (gentisin and isogentisin) and gentiopicroside, and the lack of an appropriate *in vivo* test, the FEEDAP Panel could not conclude on the safety of the additive for 'long-living animals (companion animals, horses and animals for reproduction)'[4] and on risks of genotoxicity and carcinogenicity for dermal exposure of users. No safety concern was identified for short-living animals (animals for fattening), for consumers and the environment. The applicant has now provided information intended to address the concerns previously identified for long-living animals and the user regarding the genotoxic potential of xanthones and gentiopicroside.

A tincture from *G. 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).

[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] FEFANA asbl, Avenue Louise 130 A, Box 1, 1,050 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 42133–35,221 Chateaubourg Cedex.
[4] Referred in this opinion to as long-living animals and reproductive animals (including those animals reared for laying/breeding/reproduction).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of supplementary information[5] to a previous application on the same product.[6]

In accordance with Article 38 of the Regulation (EC) No 178/2002[7] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39 e of the same Regulation, and of the Decision of EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[8] a non-confidential version of the supplementary information has been published on Open.EFSA.[9]

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.

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[10] 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 target species (EFSA FEEDAP Panel, 2017), Genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011), Clarification of some aspects related to genotoxicity assessment (EFSA Scientific Committee, 2017a) Guidance on the assessment of the biological relevance of data in scientific assessments (EFSA Scientific Committee, 2017b), 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), General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic when used as feed additives (EFSA FEEDAP Panel, 2021).[11]

3. Assessment

The additive under assessment, gentian tincture, is produced from the roots of *G. lutea* L. by extended extraction with a water/ethanol mixture. The additive is intended for use as sensory additive (functional group: flavouring compounds) up to a maximum level of 50 mg tincture/kg complete feed or 50 mg tincture/kg water for drinking for all animal species, except horses, for which the proposed use level is 200 mg/kg complete feed.

3.1. Characterisation of the additive

The additive was previously shown to contain 95.7% water/ethanol solvent and a dry matter content of about 4.3% (EFSA FEEDAP Panel, 2021). The identified constituents of the dry matter fraction were ash (0.10% of the tincture) and 0.0836% polyphenols (of which 0.0463% are flavonoids and 0.0027% xanthones) and 0.0022% gentiopicroside.

[5] Dossier reference: EFSA-Q-2022-00466.
[6] Dossier reference: FAD-2010-0321.
[7] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p.1–48.
[8] Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements
[9] Available at: https://open.efsa.europa.eu/questions/EFSA-Q-2022-00466
[10] 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.
[11] https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf

3.2. Safety

In the previous opinion, the FEEDAP Panel assessed the genotoxicity of the gentian tincture, following the recommendation of the EFSA Scientific Committee (EFSA SC) on the Genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b) and the principles set in the scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011) and in the opinion on the clarification of some aspects related to genotoxicity assessment (EFSA Scientific Committee, 2017a). The Panel concluded that 'the mixture under assessment contains individual components, the xanthones gentisin and isogentisin and gentiopicroside, for which *in vitro* data indicate a potential concern for genotoxicity'. Based on the Statement on Genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b), when a potential concern for genotoxicity is identified *in vitro*, additional *in vivo* genotoxicity data are needed to complete the assessment.

In the risk assessment for target animal species, in a worst-case scenario, xanthones and gentiopicroside were assumed by the FEEDAP Panel to be genotoxic. Then, the Panel followed the general approach to assess the safety for the target species of botanical preparations containing substances that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021) and applied the threshold of toxicological concern (TTC).

For substances that have the potential to be mutagenic, i.e. xanthones and gentiopicroside, the TTC concept was applied in a specified way, considering the different lifespan of the target species and the biological relevance of genotoxicity and carcinogenicity as endpoints (EFSA FEEDAP Panel, 2021). For long-living animals and reproductive animals (including those animals reared for laying/breeding/reproduction), considering their long lifespan and the likelihood to develop cancer, the threshold of the TTC of 0.0025 μg/kg body weight (bw) per day was applied. For short-living animals (animals for fattening), the TTC for non-genotoxic substances was applied when comparing estimated exposures with the relevant thresholds established based on non-neoplastic endpoints. Xanthones and gentiopicroside were allocated to Cramer class III.

For short-living animals, the intake at the proposed use level was below the TTC value for Cramer class III compounds, suggesting that the use of the additive did not raise safety concern.

For long-living animals and reproductive animals, the intake of xanthones and gentiopicroside at the proposed use level of the additive in feed exceeded the TTC value of 0.0025 μg/kg bw per day. Therefore, no conclusion could be drawn, and generation of further data would have been required.

The incomplete data set on the genotoxicity of the additive did not allow conclusions to be drawn on the risks of genotoxicity and carcinogenicity from dermal exposure of users. In addition, in the absence of data the Panel could not conclude on the additive's potential to be a dermal/eye irritant or a skin sensitiser.

The additional information provided in response to the previous opinion was assessed and the outcome is summarised in the next section.

3.2.1. Toxicological studies

3.2.1.1. Genotoxicity studies, including mutagenicity

In the previous assessment, the Panel highlighted the need of *in vivo* genotoxicity data to address the potential concern identified *in vitro* for the individual components, the xanthones and gentiopicroside.

The applicant provided a literature search on the genotoxicity of extracts of *G. lutea* and its individual components gentiopicroside, gentisin and isogentisin.[12] Some publications on the metabolism of gentiopicroside were also provided.

The papers retrieved on the extracts of *G. lutea* reported *in vitro* studies with the Comet assay (Djukanovic et al., 2019; Valenta Šobot et al., 2020; Cvetković et al., 2020a,b, 2022).

Two studies addressed the *in vitro* genotoxicity of gentiopicroside. The first study was aimed at investigating the antigenotoxic potential of *G. lutea* extracts and of the component gentiopicroside (Cvetković et al., 2020b). The results of the alkaline comet assay showed that gentiopicroside induced DNA damage at the highest concentration tested (50 μg/mL). The second study was already considered in the previous assessment (Mustafayeva et al., 2010) and the Panel disagreed with the interpretation of the results given by the study authors, i.e. that oxidative DNA damage would be

[12] Technical Dossier/Annex I_Bibliographic data concerning genotoxicity and carcinogenicity of gentiana lutea extract.

responsible for the mutagenic effect of gentiopicroside. Based on the increase of DNA damage observed with the Comet assay and the statistically significant dose-related increase of micronuclei (in the absence and presence of metabolic activation for both tests) the Panel already concluded that gentiopicroside may act as a genotoxic DNA reactive mutagen (EFSA FEEDAP Panel, 2021).

Concerning gentiopicroside, the *in vivo* studies retrieved were dealing on metabolism (Chang-Liao et al., 2012; Feng et al., 2014; Han et al., 2014; Wang et al., 2014, 2015, 2020; Xiong et al., 2017). The data indicated that gentiopicroside is hydrolysed by β-glucosidase in the gastrointestinal tract to produce the hemiacetalaglycone, which was readily converted by ring opening to a dialdehyde intermediate metabolite (Han et al., 2014). This reactive metabolite can react with proteins and DNA to form covalent mono-adducts and crosslinks. This reaction is typical for the chemical group of iridoids, to which gentiopicroside belongs. In addition, the *in vivo* study confirmed the formation of DNA reactive metabolites with α,β-unsaturated carbonyl functions, observed *in vitro* after treatment of gentiopicroside with β-glucosidase (Zeng et al., 2013). The α,β-unsaturated carbonyl functions represent structural alerts for genotoxic activity, as described in the previous opinion (EFSA FEEDAP Panel, 2021). The formation of covalent adducts of gentiopicroside with DNA could be responsible for the genotoxic activity.

No studies were retrieved on the genotoxicity of xanthones.

Overall, the information retrieved from the literature did not include appropriate *in vivo* genotoxicity tests for the individual components, the xanthones, gentisin and isogentisin, and gentiopicroside, for which *in vitro* data indicate a potential concern for genotoxicity.

3.2.1.2. Other studies

Other studies investigating the clinical effects (Anour et al., 2005) and the anti-inflammatory properties of herbal drugs including gentian root (Jund et al., 2012; Wölfle et al., 2017) were considered not relevant for the current assessment.

3.2.1.3. Conclusions on genotoxicity

No new data were submitted on the *in vivo* genotoxicity of xanthones and gentiopicroside that would allow the FEEDAP Panel to change its previous conclusion on the genotoxicity of the additive under assessment.

3.2.2. Safety for the target species

In the absence of data on the *in vivo* genotoxicity of xanthones and gentiopicroside, the FEEDAP Panel does not change its previous conclusions on the safety for the target species.

Therefore, the FEEDAP Panel reiterates that it is not in a position to conclude on the safety of the additive for long-living and reproductive animals.

3.2.3. Safety for the user

In the previous opinion (EFSA FEEDAP Panel, 2021), no conclusions could be drawn on the potential of the additive to be a dermal/eye irritant or a skin sensitiser. Owing to the concern for potential genotoxicity identified for some constituents, namely xanthones (gentisin and isogentisin) and gentiopicroside, the FEEDAP Panel could not conclude on risks of genotoxicity and carcinogenicity following dermal exposure of unprotected users.

The literature search performed by the applicant did not retrieve publications that addressed the concerns for irritancy, sensitisation and genotoxicity previously identified by the Panel.

Since no data on the irritancy and sensitisation were provided, the previous conclusions apply. The FEEDAP Panel notes that when handling the tincture, exposure of unprotected users to the potential genotoxicants xanthones (gentisin and isogentisin) and gentiopicroside cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

4. Conclusions

In the absence of appropriate *in vivo* genotoxicity tests on xanthones and gentiopicroside, the FEEDAP Panel reiterates that it is not in a position to conclude on the safety of the additive for long-living and reproductive animals (including those animals reared for laying/breeding/reproduction).

No conclusions can be drawn on the potential of the additive to be a dermal/eye irritant or a skin sensitiser. When handling the tincture, exposure of unprotected users to the potential genotoxicants

gentiopicroside and xanthones (gentisin and isogentisin), cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

5. Recommendations

The specification should ensure that the concentration of xanthones and gentiopicroside in gentian tincture should be as low as possible and should not exceed 0.0027% xanthones and 0.0022% gentiopicroside.

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Abbreviations

bw	body weight
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
SC	EFSA Scientific Committee
TTC	threshold of toxicological concern