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Plant alkaloids in liquorice roots: genetic damage by matrine and oxymatrine unlikely

The German Federal Institute for Risk Assessment (BfR) examines the health risk of residues

Matrine and oxymatrine are chemical substances belonging to the group of plant alkaloids. They are found in and produced by pagoda trees of the genus *Sophora to* protect themselves against predators such as insects. Residues of the substances were detected in samples of liquorice roots and liquorice root extracts, which are used in the production of liquorice. The residues are probably the result of a mix-up as liquorice roots have a similar appearance to roots of pagoda trees and are harvested together in the wild. The German Federal Institute for Risk Assessment (BfR) has now examined whether matrine and oxymatrine residues pose a health risk to consumers and, in particular, to children.

The evaluation focused on the question of the extent to which the substances may have a genotoxic potential. During the initial assessment, suspected genotoxicity could not be completely ruled out due to uncertainty in the available data. Following the submission of new data, the genotoxic potential could be assessed. The result showed that genotoxicity from matrine and oxymatrine is unlikely.

For substances that have not been comprehensively tested for their toxicities but are not genotoxic - such as matrine and oxymatrine in this case – the threshold of toxicological concern (TTC) of 1.5 micrograms (millionths of a gram) per kilogram of body weight per day can be used as a basis. Based on residue data in liquorice roots and their extracts, this TTC value is not exceeded. Therefore, the probability of negative effects on human health is very low based on current scientific knowledge.

1 Subject of the evaluation

Matrine and oxymatrine are chemical substances belonging to the group of plant alkaloids. They are found in and produced by pagoda trees of the genus *Sophora* to protect

themselves against predators such as insects. Residues of the substances were detected in samples of liquorice roots and liquorice root extracts, which are used in the production of liquorice. The residues are probably the result of a mix-up as liquorice roots have a similar appearance to roots of pagoda trees and are harvested together in the wild. The available data initially revealed a gap in the toxicological assessment. The BfR therefore reviewed the assessments and publications on matrine and oxymatrine. A data gap on genotoxicity was closed on the basis of the studies conducted by BfR and data, which were jointly compiled by the Association of the German Confectionery Industry (BDSI), the European Association Tea & Herbal Infusions Europe (THIE) and the Association of Companies Involved in the Drugs and Chemicals Wholesale and Foreign Trade (Drogen- und Chemikalienverein e.V.).

2 Results

The result of a final weight-of-evidence assessment including also new studies (see below) shows that a genotoxic potential of matrine and oxymatrine is unlikely based on the current scientific and technical knowledge. However, the BfR will continue to investigate both substances as part of its scientific expertise development.

For an initial assessment of the reported analytical findings in liquorice roots and extracts, the resulting consumer exposure can be estimated accordingly and compared with the Threshold of Toxicological Concern (TTC) for toxic substances (Cramer class III) of 1.5 µg/kg body weight (bw) per day (corresponds to 0.0015 mg/kg bw/d). The previous use of the much more critical TTC for potentially DNA-reactive genotoxic substances of 0.0025 µg/kg bw per day is no longer applicable.

Taking into account the residue findings in liquorice roots and extracts reported by Schultz et al., the TTC value of 1.5 µg/kg bw for adults and children was not exceeded.

The BfR points out that on the basis of the available toxicological data on matrine and oxymatrine it is still not possible to derive health-based guidedance values such as the acceptable daily intake (ADI) or the acute reference dose (ARfD) (data gaps).

3 Rationale

3. 1 Background to matrine and oxymatrine

Matrine and oxymatrine are plant alkaloids. Chemically speaking, oxymatrine is the N-oxide of matrine. Both substances are primarily found in roots of the genus *Sophora* (pagoda trees), where high contents of 1 - 2 % can occur especially in dried root material, and the quantity of oxymatrine appears to predominate. However, it is assumed that oxymatrine is converted into matrine during the processing of raw materials. Therefore, based on current knowledge, a joint assessment of both substances appears justified. The genus *Sophora* belongs to the *Fabaceae* (legume) family and comprises around 45 known species of small trees and shrubs. They are particularly native to southern Asia, Australia and the Pacific region, but also to some regions in North and South America.

Dried roots of various *Sophora* species are used in some traditional Chinese medicines. Areas of application include gastrointestinal discomfort and skin eczema but also supposedly inflammation of the liver, heart disease and even cancer. However, some publications report

side effects including hepatotoxicity. This information as well as any antibacterial or antineoplastic efficacy attributed to these alkaloids cannot be verified within the scope of this evaluation.

Matrine is used as an insecticide and fungicide in some Asian countries (such as Bangladesh, China and Vietnam) and is considered a "biopesticide". This seems to be the reason for the designation of matrine and oxymatrine as active substances in plant protection products in the EU although the active substances have not been approved and no joint evaluation has been carried out at EU level. No toxicological limits or specific maximum residue levels (MRLs) have been derived (a default value of 0.01 mg/kg applies in accordance with Regulation (EC) No. 396/2005). Consequently, there is no authorisation for plant protection products containing the active substances in any Member State or, to the knowledge of the BfR, has one ever been granted.

An article by Schultz et al. (2021) shows that matrine findings in liquorice roots or their extracts (obtained from *Glycyrrhiza* spp., which also belong to the *Fabaceae* family) are most likely not due to use as a pesticide. They are rather the result of contamination with the morphologically similar roots of *Sophora* species, which are virtually indistinguishable from liquorice roots after harvesting and further processing. As liquorice roots are not cultivated but collected from the wild and often grow in association with *Sophora* species, a certain degree of mixing is likely. This has been well documented at least from Iran, an important supplier country of liquorice roots and their extracts. Although matrine is also authorised as a pesticide in this country, it is probably used in other regions and especially in other crops. The treatment of wild plants with a pesticide is unlikely.

Therefore, the conclusion that the matrine levels determined by the BDSI in various analysed samples are not due to plant protection product applications but are instead a contamination seems justified. However, matrine and oxymatrine residues are subject to the provisions of Regulation (EC) No. 396/2005 regardless of the route of entry.

3. 2. Evaluations of the assessments prepared by the Technical University of Denmark (DTU) and RDA Scientific Consultants GmbH

The considerations taken in the DTU (2021) statement on matrine and oxymatrine are deemed reasonable to the BfR in the context of the data available for this statement. A genotoxic potential of matrine and oxymatrine could not be ruled out at that time. A recent publication by Heo et al. (2021) is not yet included in the DTU report as it was presumably not yet available at the time of publication. However, due to the limitations of this publication, which are described in more detail below, the conclusions of the DTU were not fundamentally challenged despite this article.

RDA Scientific Consultants GmbH prepared an additional and much more comprehensive report (Orth, 2022), which was also submitted to the BfR and will be discussed below. This report states, among others, that an acceptable daily intake (ADI) for matrine of 0.1 mg/kg bw per day has been established in China. This value was apparently based on a mouse acute toxicity study, in which the active substance was administered subcutaneously. If this is indeed the case, this limit value would be considered unsuitable for risk assessment not only from the perspective of RDA Scientific Consultants GmbH but also from the perspective of the BfR. However, the BfR does not have access to this study or more detailed information

on it. The report further refers to several studies with regard to the genotoxic potential of matrine. However, the majority of the available studies investigated the genotoxicity of *Sophora* extracts and not that of matrine or oxymatrine. There are indications of genotoxic activity based on positive findings in the Ames test (not available at the BfR, reported by Orth, 2022) and in the *in vitro* chromosome aberration test, whereas a micronucleus test on mice is reported to be negative but without evidence of bone marrow exposure (Che, 2015). In the opinion of the BfR and in accordance with the EFSA guidelines, these studies were not suitable for conclusively assessing the genotoxic potential of the substance matrine. While it remains unclear which ingredients can be attributed to positive findings, it cannot be ruled out from the negative findings that the test systems were not sensitive enough to detect possible genotoxic activity due to the dilution of the individual substances in the extract.

In another study (Heo et al., 2021), the genotoxic potential was investigated with the pure substance matrine in an *in vivo* comet assay, which according to the publication was carried out in accordance with OECD Test Guideline (TG) 489. No genotoxic activity was observed under the selected test conditions. However, this study has considerable limitations. For example, the purity of the test substance is not reported. It is also not clear how the maximum dose tested was determined as the previous dose-finding study is not described in detail. As no clinical signs of toxicity were observed in the animals, it is unclear whether the test substance reached the target tissues (liver and blood cells) at all. The latter is particularly relevant as the bioavailability of matrine is reported to be low. Against this backdrop, investigation of local genotoxicity in the gastrointestinal tract (as site of first contact) would have been desirable and should have been carried out in parallel in accordance with OECD Test Guideline 489. In addition, a dose-dependent numerical increase in strand breaks compared to the negative control was observed in the treatment groups[1] . In the opinion of the BfR, the statistical method chosen by the authors (Duncan's multiple comparison method) does not fulfil the requirements of OECD Test Guideline 489 for a group-by-group comparison with the included control. The trend test required by the guideline is also missing. Individual data for a follow-up evaluation are not available.

The report also points out that repeated administration of matrine (among others) appears to have hepatotoxic activity. As RDA Scientific Consultants GmbH considered a genotoxic potential to be refuted, the derivation of a provisional ADI was proposed (Orth, 2022). This was based on the findings from a rat subchronic toxicity study with a *Sophora* extract with a known content of matrine and oxymatrine. A NOAEL of 10 mg extract/kg bw was identified. Based on this NOAEL, a provisional ADI of 0.91 µg/kg bw/day for matrine and 2.135 µg/kg bw/day for oxymatrine was derived, taking into account the known levels of matrine (18.2 mg/g) and oxymatrine (42.7 mg/g) in the extract and applying an extrapolation factor of 200.

This derivation is in principle reasonable for the BfR. However, due to the use of an extract, the reliability of these ADI values is limited. In particular, it should be noted that a complex plant extract was tested and it is not clear whether the observed toxicological effects, including the liver, can actually be attributed to matrine/oxymatrine. In addition, the value for matrine would be lower than and for oxymatrine comparable to the generic TTC[2] for Cramer

[1] The proportion of DNA fragments increased from 1.3 ± 0.2 % in the control animals to 2.7 ± 0.8 % / 4.5 ± 0.4 % / 4.2 ± 0.3 % in the liver cells of the animals treated with 50 / 100 / 200 mg/kg bw, respectively.

[2] TTC value(s) = Threshold(s) of toxicological concern. They are generic, health-based guidance values determined on the basis of a collection of data on toxicological properties using statistical methods. The appropriate TTC value is selected according to the known biological and physicochemical properties of the substance in question.

class III substances of 1.5 µg/kg bw/day (EFSA Scientific Committee, 2019). Thus, as the available data from animal experiments provide support for the sufficient protection of the TTC, the use of this generic value is favoured.

3. 3 Assessment of the newly available studies on genotoxicity

As a first step, the BfR carried out a supplementary assessment of the genotoxic potential of matrine/oxymatrine with *in silico*, i. e., computer-based, predictions based on validated commercial and non-commercial models to improve the database. The results are described in Fischer et al. (2023). However, when applying the criteria established at the BfR for assessing data confidence, it was not possible to attest sufficient reliability of the predictions for these specific substances. The need for experimental testing was thus confirmed.

In the letters dated 7 December 2022 and 2 May 2023, new studies for matrine and oxymatrine (Bacterial Reverse Mutation Assay according to OECD TG 471 and *In vitro* Mammalian Cell Micronucleus Assay according to OECD TG 487) were made available to the BfR (Burns, K., Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Matrine (ASB2022-20926); Burns, K., Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Oxymatrine (ASB2022-20927); Donath, C., *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Matrine (ASB2023-9643); Donath, C., *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Oxymatrine (ASB2023-9644)). The evaluation of all four studies performed under GLP conditions did not show any relevant deviations from the underlying OECD TGs. The results of all four studies were negative.

An Ames test carried out at the BfR also found no evidence of mutagenicity neither for matrine nor for oxymatrine, which independently confirms the results of Burns (2022a, 2022b) (Fischer, B. et al., 2023). A non-OECD-compliant exploratory micronucleus test carried out by the BfR in V79 cells yielded negative results for oxymatrine and equivocal results for use in the regulatory context for matrine with a slight increase in micronuclei in cells already severely injured from high, cytotoxic concentrations.

Based on a final weight-of-evidence assessment, the existence of a biologically relevant genotoxic potential for both substances is considered unlikely in light of the current scientific and technical knowledge. As part of its scientific expertise development, the BfR will continue to work on this topic.

3. 4 Toxicological assessment of the levels of matrine and oxymatrine in liquorice root and preparations as determined by Schultz et al. (2021)

Since genotoxic potential for matrine and oxymatrine can be considered unlikely from the BfR's point of view based on the now available information, the use of the TTC concept taking the TTC for toxic substances (Cramer class III: 1.5 µg/kg bw per day) as the basis is possible as a decision-making guide.

The Acceptable Daily Intake (ADI) for matrine of 0.1 mg/kg bw per day derived from a mouse acute toxicity study with subcutaneous administration from China is not considered reliable (see above). An acute reference dose (ARfD) for matrine and oxymatrine is not known. The

rat subchronic toxicity study with a *Sophora* extract with a known content of matrine and oxymatrine as cited in the expert opinion by Orth (2022) confirms the sufficient protection of the TTC of 1.5 µg/kg bw per day for Cramer class III substances (also see above).

For an initial assessment of the reported analytical findings in liquorice roots and extracts (Schultz et al. (2021)), the resulting consumer exposure can be estimated accordingly and compared with the TTC for toxic substances (Cramer class III) of 1.5 µg/kg bw per day (EFSA Scientific Committee, 2019). This estimate can be used as a basis for risk management decisions. The TTC concept is used to help decide whether exposure to a substance is so low that the likelihood of adverse health effects is low and no further data are required. It is only in the case of the TTC being exceeded that toxicological data are required for a health risk assessment (EFSA Scientific Committee, 2019).

3. 5. Estimation of the short-term intake quantity (IESTI)

The German NVS II model[3] and the EFSA PRIMo model (version 3.1)[4], which are usually applied to estimate the intake levels of pesticide residues, are not sufficiently detailed to depict the consumption of liquorice. Therefore, data from the National Consumption Study II (DISHES module, adults) and the KiESEL study (children 3-4 years), which contain the amounts of liquorice in long-term consumption as provided below, were used directly:

Adults (DISHES, 1012 consumers)

Median	0.09 g/kg bw per day
P95	0.76 g/kg bw per day

Children (KiESEL, 8 consumers)

Median	0.35 g/kg bw per day
Maximum	0.49 g/kg bw per day

The intake estimation was based on the assumption that ready-to-eat liquorice contains at least 3 % liquorice extract[5]. In addition, the median (4.333 mg/kg), the mean residue concentration of 7.195 mg/kg and the P95 of 21.93 mg/kg for "Extract, block" (= raw liquorice) are assumed as content data (Schultz et al., 2021). Overall, the following relationship results for the calculation of the intake quantity:

*Intake (µg/kg bw per day) = Amount consumed (g/kg bw per day) * Concentration (µg/g) * 3 %*

[3] http://www.bfr.bund.de/cm/343/bfr-berechnungsmodell-zur-aufnahme-von-pflanzenschutzmittel-rueckstaenden-nvs2.zip
[4] https://www.efsa.europa.eu/sites/default/files/applications/EFSA_PRIMo_rev3.1.xlsm
[5] https://www.bdsi.de/warenkunde/bonbons-und-zuckerwaren/lakritzwaren/

Exposure estimation for children based on KiESEL study (8 consumers)

Food	Percentile consumption data	Amount consumed (g/kg bw and day)	Percentile content data	Content (µg/g)[1]	Proportion of raw liquorice in food	Intake (µg/kg bw)
Liquorice	Median	0.35	Median	4.333	3 %	0.0455
			Mean value	7.195		0.0756
			P95	21.93		0.230
Liquorice	Maximum	0.49	Median	4.333	3 %	0.064
			Mean value	7.195		0.106
			P95	21.93		0.322

[1] Based on the determined concentration in "Extract, block" (= raw liquorice)

Exposure estimation for adults based on the NVS II study (DISHES, 1012 consumers)

Food	Percentile consumption data	Amount consumed (g/kg bw and day)	Percentile content data	Content (µg/g)[1]	Proportion of raw liquorice in food	Intake (µg/kg bw)
Liquorice	Median	0.09	Median	4.333	3 %	0.0117
			Mean value	7.195		0.0194
			P95	21.93		0.0592
Liquorice	Maximum	0.76	Median	4.333	3 %	0.099
			Mean value	7.195		0.164
			P95	21.93		0.500

[1] Based on the determined concentration in "Extract, block" (= raw liquorice)

Based on the mean content (median, mean value) and the 95th percentile of the measured matrine content in raw liquorice, the long-term intake for all population groups falls below the TTC for toxic substances (Cramer class III) of 1.5 µg/kg bw per day.

It should be noted that for children only 8 consumers were recorded in the KiESEL study and therefore the long-term intake for this consumer group is subject to a high degree of uncertainty. For adults, the amount consumed is based on 1012 consumers and can be considered robust.

3. 6 Health assessment

Following the submission of new valid OECD TG-compliant studies to assess the genotoxic potential of matrine and oxymatrine, it can be stated that both substances have not shown to be genotoxic up to this point. Due to data gaps in the broader toxicological database on matrine and oxymatrine, it is currently not possible to derive substance-specific health-based guidance values (ADI, ARfD). The TTC for toxic substances (Cramer class III) of 0.0015 mg/kg bw per day was therefore used for an initial assessment of the reported analytical findings in liquorice roots and their extracts. Even when high levels of matrine are taken into account (95th percentile of the levels measured in raw liquorice), the long-term intake for all population groups is below this TTC. Therefore, the probability of negative effects on human health, if the levels fall below this value, can be regarded as very low[6]. However, the BfR will continue to investigate matrine and oxymatrine as part of its scientific expertise development.

[6] For further information see also: https://www.efsa.europa.eu/de/topics/topic/threshold-toxicological-concern

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About the BfR

The German Federal Institute for Risk Assessment (BfR) is a scientifically independent institute within the portfolio of the Federal Ministry for Food and Agriculture (BMEL) in Germany. It advises the Federal Government and the States ('Laender') on questions of food, chemical and product safety. The BfR conducts independent research on topics that are closely related to its assessment tasks.

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