Testing of a standardized extract of *Sceletium tortuosum* (Zembrin®) according to OECD Genotoxicity Guidelines 471, 487 and 474

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ABSTRACT

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| **Aims:** The genotoxic safety of the extract Zembrin® of the medicinal plant *S. tortuosum* was evaluated.**Study design:** Genotoxic safety was evaluated performing three standard genotoxicity tests according to OECD guidelines 471, 487 and 474.**Place and Duration of Study:** Assays were performed at Toxi-Coop Zrt., Arácsi út 97 and Ady E. utca 12, 8230 Balatonfüred, Hungary in 2023.**Methodology:** Assays performed under Good Laboratory Practice in compliance with internationally accepted guidelines included the bacterial reverse mutation assay, the *in vitro* mammalian cell micronucleus test and the *in vivo* mouse micronucleus test. **Results:** Zembrin® extract, in concentrations varying from 16 up to 5000 µg/plate, did not change the mutation rates of the various Salmonella strains and the E. coli strain tested with and without metabolic activation in an *in vitro* bacterial reverse mutation assay. Zembrin® extract did also not show an increased frequency of micronuclei with and without metabolic activation in an *in vitro* mammalian cell micronucleus test which was performed using L5178Y tk+/- cells. However, a dose dependent increase was observed at the two higher concentrations (2500 and 5000 μg/mL) in the absence of metabolic activation. Since results were not clearly negative in the initial *in vitro* genotoxicity test, a confirmatory *in vivo* test was conducted. In an *in vivo* mouse micronucleus test in which the test substance was administered intravenously, frequency of micro nucleated polychromatic erythrocytes was not increased.**Conclusion:** Therefore, on the basis of the results of the three genotoxicity tests, we conclude that Zembrin® is not of genotoxic concern.  |

*Keywords: Sceletium tortuosum, Zembrin® , mesembrine-type alkaloids, genotoxicity, OECD guideline 471, OECD guideline 474, OECD guideline 487*

1. INTRODUCTION

*Sceletium tortuosum* (L.) N.E.Br. (*S. tortuosum*) is a succulent plant from South Africa. Traditionally, this plant is mainly masticated, smoked or drank as a tea used for the relief of pains and for euphoric effects (Manganyi, 2021). *S. tortuosum* extracts have a safe history of use in various peoples and products based on these plant extracts are currently widely available through the internet. *S. tortuosum* is currently also investigated for its clinical potential in treating anxiety (Reay, 2020) and depression (Dimpfel, 2016), relieving stress in healthy individuals, and enhancing cognitive functions (Chiu, 2014). These pharmacological actions are attributed to its phytochemical constituents referred to as mesembrine-type alkaloids. The crude extracts and commercially available standardized extracts of *S. tortuosum*, like Zembrin® have a wide spectrum of biological activities in *in vitro* or animal *in vivo* studies (Olantunji, 2022). No specific alkaloid or other component in Zembrin® could be identified as the sole compound causing the antidepressant-like activity (Gericke, 2024).

Zembrin® was reported to be a potent blocker in serotonin transporter binding assays and exhibited high inhibitory effects on phosphodiesterase- 4, while no cytotoxic effect was reported (Harvey, 2011). Also, a pharmaco-fMRI study, showed that amygdala reactivity to fearful faces under low perceptual load conditions was attenuated after a single 25 mg dose of Zembrin® in healthy volunteers. These results showed attenuating effects of *S. tortuosum* on the threat circuitry of the human brain and provide supporting evidence that the dual serotonin reuptake and phosphodiesterase inhibition of Zembrin® (Terburg, 2013). Although these potential benefits may be attractive from a health perspective, a possible health claim still needs to be substantiated (Collins, 2022).

The proprietary standardized and characterized extract Zembrin®, was shown to be safe and well tolerated in pre-clinical studies (Murbach, 2014) including a fourteen-day repeated dose oral toxicity study and a ninety-day repeated dose oral toxicity study in rats. The publication by Murbach et al (Murbach,2014), is a prequel to the present paper; it is authored essentially by the same affiliations as the present paper text and there is a partly overlap in text used. Zembrin® was also well tolerated by healthy human subjects when provided once daily for 3 months at 8 mg and 25 mg dosages (Nell, 2013).

Still the number of toxicological evaluations of Zembrin® is limited. The purpose of this paper is to report on the genotoxic safety of Zembrin® by describing Zembrin®’s effects in a set of three genotoxicity tests according to OECD guidelines and under Good Laboratory Practice: a bacterial reverse mutation assay was conducted following recognized international standards, employing a range of Salmonella typhimurium and Escherichia coli strains to evaluate potential mutagenic effects. (OECD 471). Also an *in vitro* mammalian cell micronucleus test was performed using L5178Y tk+/- cells to assess chromosomal damage (OECD 487), and in an *in vivo* mouse micronucleus test involved administering the test substance intravenously, focusing on the analysis of bone marrow cells for genotoxic markers (OECD 474). These tests follow the EFSA Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011). Also, the European Chemicals Agency (ECHA) provides similar comprehensive guidance on genotoxicity testing strategies, particularly within the framework of the REACH regulation. A key resource is the "Guidance on Information Requirements and Chemical Safety Assessment – Chapter R.7a," which offers detailed information on mutagenicity and genotoxicity testing strategies, including the selection of appropriate test methods and the interpretation of results (https://echa.europa.eu/-/updated-guidance-on-testing-for-mutagenicity ).

Genotoxic safety reporting in the public domain is compulsory for regulatory purposes like Novel Foods in EU (https://food.ec.europa.eu/safety/novel-food\_en) and UK (https://acnfp.food.gov.uk/ACNFPNovelFoodAssessments) and GRAS (Generally Recognized As Safe; https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras) in the USA.

2. material and methods

**2.1 Test materials**

The test material was Zembrin® (manufactured by Nektium Pharma, S.L., Calle Las Mimosas 8, Poligono Industrial de Arinaga, 35118 Agüimes, Las Palmas, Gran Canaria, Spain) for HG&H Pharmaceuticals (Pty) Ltd., The Braes, 193 Bryanston Drive, Bryanston 2191, South Africa), a proprietary standardized 2:1 hydroethanolic extract of the dried leaves and stems of *Sceletium tortuosum* and/or *Sceletium crassicaule*. Zembrin® was provided for use as a test article (lot nr SCE0420-2003 for all three assays), along with specifications, certificates of analysis, and material safety data sheets, by HG&H Pharmaceuticals (Pty) Ltd. A brief description of Zembrin® and its manufacturing process is provided in (Murbach, 2014).

The final product – Zembrin® – is a food-grade, non-GMO, certified allergen-free and BSE free product (no animal derivatives), is soluble in water and is standardized to an alkaloid content of 0.35– 0.45% w/w. The relative amounts of major alkaloids as determined by HPLC-DAD are: mesembrenone + mesembrenol ≥ 60%, mesembrine <20% and mesembranol >5%. The chemical structures of the active alkaloids are provided in (Murbach, 2014). Compositional analyses have identified the major constituents of Zembrin® to be carbohydrates, ash, protein, and fatty acids. Minor constituents include alkaloids and minerals. The compositional analysis of the product used in these three studies are shown in Table 1.

**Table 1. Composition of Zembrin® extract of *Sceletium tortuosum***

|  |  |
| --- | --- |
| **Analysis** | **Result (% dry weight)** |
| Moisture | 3.65 |
| Maltodextrin | <70 |
| Silicon dioxide | <2 |
| Total alkaloids | 0.433 |
|  | **Result (% of total alkaloids)** |
| Mesembrone + mesembrenol | 83.58 |
| Mesembrine | 4.48 |
| Mesembranol | 11.95 |
|  | **Result (ppm)** |
| Arsenic | 0.14 |
| Lead | 0.082 |
| Cadmium | <0.01 |
| Mercury | <0.005 |
| Total heavy metals | 0.237 |
| Residual solvents (ethanol) | <5000 |

**2.2 Assays**

The assays reported below were conducted under Good Laboratory Practice in compliance with the internationally accepted guidelines. Assays were performed at Toxi-Coop Zrt., Arácsi út 97 and Ady E. utca 12, 8230 Balatonfüred, Hungary.

The bacterial reverse mutation assay was conducted following recognized international standards, employing a range of Salmonella typhimurium and Escherichia coli strains to evaluate potential mutagenic effects according to the OECD Guidelines for Testing of Chemicals No 471, adopted 21st July 1997, corrected 26th June 2020 (OECD, 2020); the Council Regulation (EU) (EC) No 440/2008; B13/14 MUTAGENICITY: REVERSE MUTATION TEST USING BACTERIA, dated May 30, 2008; the ICH Guideline S2 (R1): Genotoxicity testing and data interpretation for pharmaceuticals intended for human use, November 2011 and EPA Health Effects Test Guidelines, OPTTS 870.5100, EPA 712-C-98-247, August 1998. The test doses for the bacterial reverse mutation test were determined on the basis of solubility tests and concentration range finding tests. No inhibitory effect of the of the test substance was observed in the concentration range finding test. Triplicate concentrations of 16, 50, 160, 500, 1600 and 5000 µg/plate were freshly prepared in ultrapure water (ASTM Type 1) with Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537, and Escherichia coli tester strain WP2 *uvrA* in the presence and absence of S9 liver microsomal fraction prepared from phenobarbital and 5,6-benzoflavone-induced rats according to Matsushima (Matsushima, 1976). The Mutation Factor was calculated by dividing the mean value of the revertant counts by the exact mean value of the solvent control. Biological relevance of the results was assessed according to the criterion of the OECD guidelines.

The *in vitro* mammalian cell micronucleus test was performed using L5178Y tk+/- cells to assess chromosomal damage according to OECD Guidelines for Testing of Chemicals No 487: *in vitro* The mammalian cell micronucleus test adopted 29th July 2016 and the Council Regulation (EC) No 2017/735, Annex Part B. B.49: *in vitro* mammalian cell micronucleus test dated: 14 February 2017 (OECD, 2023). Concentrations for the mutation assay were based on the results of preliminary dose selection assays. Duplicate concentrations of 1250, 2000, 2500 and 5000 µg/mL were incubated for 4 hours without metabolic activation with cultured L5178Y tk+/- 3.7.2c cells and concentrations of 2000, 3000, 4000 and 5000 µg/mL were incubated for 4 hours with metabolic activation. Cells were incubated and harvested after 24 hours. Similarly, 111.1, 333.3, 1000, 1500, 2000, 2500 and 3000 µg/mL concentrations were incubated for 24 hours up to harvest without metabolic activation. In order to cover the expected cytotoxicity range of 0-55 ±5% the highest concentration of 5000 µg/mL in the 4-hour treatment in the presence of metabolic activation and the concentrations of 2000, 2500 and 3000 µg/mL after 24-hour treatment in the absence of metabolic activation system were excluded from further micronuclei evaluation. The Relative Increase in Cell Counts (RICC) was calculated as an indicator for the cytotoxicity activity. The Cytotoxicity Index was calculated as 100 - RICC. Micronuclei were scored blind by trained technicians in at least 2000 cells per concentration.

The *in vivo* mouse micronucleus test involved administering the test substance intravenously, focusing on the analysis of bone marrow cells for genotoxic markers according to OECD Guidelines for Testing of Chemicals No 474 (2016) and the EPA Health Effects Test Guidelines, OPTTS 870.5395 (OECD, 2014). The potential genotoxic activity of Zembrin® extract was examined in bone marrow of NMRI mice. The doses for the Micronucleus Test were determined according to a preliminary intravenous toxicity test, in which the maximal tolerable dose was based on death and clinical signs of the test substance. Also, sex differences were evaluated in the preliminary test showing that the toxic effects were similar in both sexes. The doses selected were 125, 250 and 500 mg Zembrin® extract per kg body weight. Male mice were used. Negative (vehicle) control and a positive control group were included with a constant treatment volume (10 mL/kg body weight). The test item and negative (vehicle) control item were administered intravenously into tail vein two times at 24-hour intervals. The positive control cyclophosphamide was administered once, intraperitoneally with a treatment volume of 10 mL/kg body weight. In the low (125 mg/kg), mid (250 mg/kg) and high (500 mg/kg) dose groups and solvent control group were sampled at 24 hours after the second treatment. Cyclophosphamide (60 mg/kg bw) treated animals were sampled at 24 hours post-treatment. Five animals per dose groups were used. Four thousand polychromatic erythrocytes (PCEs) were scored per animal by a trained technician to assess micro nucleated cells. Twenty- seven male NMRI mice were intravenously injected with 125, 250 and 500 mg/kg bw twice at a 24 hour interval. Animals were regularly tested for toxic signs and mortality. Twenty-four hours after the second treatment bone marrow was obtained from two exposed femurs. Bone marrow smears were stained with Giemsa (10%) solution for 25 minutes, rinsed dried and mounted and 4000 polychromatic erythrocytes (PCEs) were scored per animal. The frequency of micro nucleated cells was expressed as percent of PCEs. Also, the proportion of immature erythrocytes was determined by counting a total of at least 500 erythrocytes.

**2.3 Statistical analyses**

A statistical evaluation was not considered necessary for the bacterial reverse mutation assay: the test substance did not produce a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the doses. It was considered to be non-mutagenic in this system. For statistical analysis of the *in vitro* mammalian cell micronucleus test using L5178Y tk+/- cells to assess chromosomal damage, the nonparametric χ2 test was performed to verify the results and regression analysis (Microsoft Excel) was performed to examine dose-related increases. Statistical analysis of the *in vivo* mouse micronucleus test which involved administering the test substance intravenously, was performed with SPSS PC+ software (SPSS, Inc., Chicago, IL) to evaluate the frequency of micro nucleated polychromatic erythrocytes and the proportion of immature among total (immature + mature) erythrocytes. Linear trend analysis (Microsoft Excel) was used to examine dose-related increases. For all studies, differences with a p-value smaller than 0.05 were considered significant.

3. results

**3.1 The bacterial reverse mutation assay**

The tester strains demonstrated the specific phenotype characteristics and were in line with the corresponding historical control data ranges, as were the negative (ultrapure water, no increase) and positive (diagnostic mutagens, more than 3-fold increase) control conditions. Each S9 fraction used had the appropriate biological activity. The revertant colony numbers were above the corresponding historical control data range in the confirmatory mutation test, in the case of Escherichia coli WP2 *uvrA* with 2-Aminoanthracene with metabolic activation, namely 514 versus 141-454 for the historical data range. The higher revertant counts were considered to show a greater strain sensitivity of the applied strain (Table 2). The revertant colony numbers of the parallel investigated untreated and DMSO control plates were slightly higher or lower than the ultrapure water (ASTM Type I) control plates and the higher or lower revertant counts of these controls remained in line with the corresponding historical control data ranges (data not shown). In summary, the actual values of untreated, vehicle and positive controls were in line with the criteria for validity of the assay.

**Table 2. Summary results of the genotoxicity tests with Zembrin® according to OECD Guideline 471. Mean values (Mean) and mutation rates (MR) of controls and Zembrin® at various concentrations in the bacterial reverse mutation assay without (-S9) and with (+S9) metabolic activation using various strains of Salmonella typhimurium and E. coli WP2*uvrA*.**

|  |  |  |
| --- | --- | --- |
|  | *Salmonella typhimurium* tester strain | *E coli* WP2*uvrA* |
|  | TA98 | TA100 | TA1535 | TA 1537 |
|  | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
|  | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR | Mean  | MR |
| Initial Mutation Test |
| *Negative controls* |  |
| Untreated | 16.7 | 0.86 | 25.0 | 0.88 | 72.3 | 0.72 | 70.7 | 0.83 | 10.0 | 1.11 | 9.7 | 0.88 | 8.0 | 0.96 | 7.0 | 1.17 | 55.0 | 0.93 | 66.0 | 1.10 |
| Ultrapure water | 19.3 | 1.00 | 28.3 | 1.00 | 100.0 | 1.00 | 85.3 | 1.00 | 9.0 | 1.00 | 11.0 | 1.00 | 8.3 | 1.00 | 6.0 | 1.00 | 59.3 | 1.00 | 60.0 | 1.00 |
| *Positive controls* | *392.0* | *25.57* | *1114.7* | *52.25* | *957.3* | *9.57* | *689.3* | *10.39* | *900.7* | *100.07* | *120.3* | *8.80* | *237.7* | *35.65* | *174.0* | *27.47* | *765.3* | *12.90* | *331.3* | *5.40* |
| *Zembrin® (µg/plate)* |  |
| 16 | 20.0 | 1.03 | 20.0 | 0.71 | 82.3 | 0.82 | 68.0 | 0.80 | 9.3 | 1.04 | 10.3 | 0.94 | 7.0 | 0.84 | 9.7 | 1.61 | 62.3 | 1.05 | 55.0 | 0.92 |
| 50 | 17.3 | 0.90 | 27.7 | 0.98 | 77.7 | 0.78 | 73.3 | 0.86 | 10.0 | 1.11 | 12.0 | 1.09 | 6.7 | 0.80 | 4.7 | 0.78 | 63.0 | 1.06 | 58.3 | 0.97 |
| 160 | 19.7 | 1.02 | 29.0 | 1.02 | 99.7 | 1.00 | 73.3 | 0.86 | 9.3 | 1.04 | 10.7 | 0.97 | 9.0 | 1.08 | 10.3 | 1.72 | 61.7 | 1.04 | 61.3 | 1.02 |
| 500 | 19.7 | 1.02 | 32.0 | 1.13 | 76.7 | 0.77 | 78.7 | 0.92 | 7.7 | 0.85 | 9.7 | 0.88 | 6.7 | 0.80 | 6.7 | 1.11 | 60.7 | 1.02 | 52.7 | 0.88 |
| 1600 | 16.0 | 0.83 | 24.0 | 0.85 | 83.0 | 0.83 | 85.3 | 1.00 | 8.7 | 0.96 | 9.0 | 0.82 | 8.0 | 0.96 | 6.3 | 1.06 | 61.0 | 1.03 | 64.0 | 1.07 |
| 5000 | 21.3 | 1.10 | 25.7 | 0.91 | 75.0 | 0.75 | 79.0 | 0.93 | 8.7 | 0.96 | 8.7 | 0.79 | 8.3 | 1.00 | 8.0 | 1.33 | 59.3 | 1.00 | 52.3 | 0.87 |
| Confirmatory Mutation Test |
| *Negative controls* |  |
| Untreated | 31.0 | 1.01 | 30.0 | 1.13 | 86.3 | 1.00 | 101.0 | 0.99 | 11.7 | 0.85 | 7.0 | 0.95 | 8.0 | 0.92 | 8.3 | 1.09 | 53.0 | 1.04 | 59.7 | 1.08 |
| Ultrapure water | 30.7 | 1.00 | 26.7 | 1.00 | 86.0 | 1.00 | 102.3 | 1.00 | 13.7 | 1.00 | 7.3 | 1.00 | 8.7 | 1.00 | 7.7 | 1.00 | 51.0 | 1.00 | 55.0 | 1.00 |
| *Positive controls* | *326.7* | *16.9* | *1658.7* | *55.91* | *1098.7* | *12.78* | *994.7* | *10.66* | *1112.0* | *81.37* | *115.7* | *19.28* | *224.0* | *26.88* | *142.7* | *17.12* | *914.7* | *17.93* | *513.7* | *11.59* |
| *Zembrin® (µg/plate)* |  |
| 16 | 30.3 | 0.99 | 38.0 | 1.43 | 70.3 | 0.82 | 85.7 | 0.84 | 11.3 | 0.83 | 10.7 | 1.45 | 9.3 | 1.08 | 9.0 | 1.17 | 47.7 | 0.93 | 65.7 | 1.19 |
| 50 | 31.3 | 1.02 | 40.0 | 1.50 | 75.0 | 0.87 | 99.7 | 0.97 | 13.7 | 1.00 | 7.3 | 1.00 | 9.0 | 1.04 | 9.0 | 1.17 | 41.3 | 0.81 | 50.3 | 0.92 |
| 160 | 21.7 | 0.71 | 31.7 | 1.19 | 70.3 | 0.82 | 97.3 | 0.95 | 11.7 | 0.85 | 10.0 | 1.36 | 8.0 | 0.92 | 6.3 | 0.83 | 50.7 | 0.99 | 62.0 | 1.13 |
| 500 | 26.3 | 0.86 | 35.3 | 1.33 | 75.3 | 0.88 | 87.3 | 0.85 | 10.3 | 0.76 | 9.7 | 1.32 | 7.0 | 0.81 | 7.3 | 0.96 | 54.0 | 1.06 | 57.0 | 1.04 |
| 1600 | 32.7 | 1.07 | 33.3 | 1.25 | 81.7 | 0.95 | 84.3 | 0.82 | 11.7 | 0.85 | 10.3 | 1.41 | 9.0 | 1.04 | 7.0 | 0.91 | 61.0 | 1.20 | 54.0 | 0.98 |
| 5000 | 29.3 | 0.96 | 37.7 | 1.41 | 69.0 | 0.80 | 62.3 | 0.61 | 12.7 | 0.93 | 9.3 | 1.27 | 12.0 | 1.38 | 8.0 | 1.04 | 46.7 | 0.92 | 56.7 | 1.03 |

*MR: Mutation Rate; NPD: 4-Nitro-o-phenylenediamine at TA98 (–S9); SAZ: Sodium azide at TA100 and TA1535 (–S9); 9AA: 9-Aminoacridine at TA1537 (–S9); MMS: Methyl methanesulfonate at E. coli WP2uvrA (–S9); 2AA: 2-Aminoanthracene at all strains (+S9). Ultrapure water was applied as solvent of the test item and the positive control substances SAZ and MMS. The DMSO (not presented) was applied as solvent of the positive control substances NPD, 9AA and 2AA.*

Seven concentrations were investigated in the initial informative toxicity test and six in the main mutation experiments. In these main mutation experiments (initial and confirmatory mutation tests), there were at least five analysable concentrations and a minimum of three non-toxic and non-precipitated concentrations at each tester strain. All criteria for the validity of the performed experiments have therefore been met.

Zembrin® extract, in concentrations varying from 16 up to 5000 µg/plate did not change the mean values and mutation rates of the various Salmonella strains and the E. coli strain tested, both with and without metabolic activation (Table 2). The data show that Zembrin® extract did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. In conclusion, the test item Zembrin® extract has no mutagenic activity on the applied bacterium tester strains under the test conditions used in this study.

**3.2 The *in vitro* mammalian cell micronucleus test using L5178Y tk+/- cells**

The cytotoxicity for the negative and positive controls were within acceptable ranges and compatible with the historical control data. Clear cytotoxicity of 59.10 % was observed in the 4-hour treatment with metabolic activation at a concentration of 4000 μg/mL. Clear cytotoxicity of 55.24 % was also obtained at concentration of 1500 μg/mL after the 24-hour treatment. Due to high cytotoxicity in the 4-hour treatment with metabolic activation, the concentration of 5000 μg/mL and in the 24-hour treatment, the concentrations of 2000, 2500 and 3000 μg/mL were excluded from the micronucleus analysis.

The test item Zembrin® extract did not show significant increases in the frequency of micronuclei compared to the solvent both when examined in the absence and in the presence of metabolic activation at the 4-hour treatments as well as in the absence of metabolic activation after 24-hours (Table 3). However, a dose dependent increase was observed at the two higher concentrations (2500 and 5000 μg/mL) at the 4-hour treatment in the absence of metabolic activation. Clear cytotoxicity of 58.88 % was observed at concentration of 5000 μg/mL in the 4-hour treatment in the absence of metabolic activation (Table 3).

Also, these micronuclei formation values were outside of the historical control range. Micronuclei values after 24 hours treatment at 333.3, 1000 and 1500 μg/mL Zembrin® were also higher than the negative control condition; these higher numbers were however, not dose-related. This means that two out of three criteria of the OECD Guidelines for Testing of Chemicals No 487: *in vitro* mammalian cell micronucleus test adopted 29th July 2016, were met for a clearly positive response in the absence of metabolic activation. Therefore, Zembrin® extract is considered to possibly induce some micronuclei resulting from breakage and / or chromosomal loss, in L5178Y cells. Zembrin® extract is therefore considered as equivocal in this micronucleus test. A dose-response regression analysis of the concentration dependency of the 4-hour treatment without metabolic activation (Figure 1) showed that there was a significant (p=0.0008) dose-dependent increase (r=0.976) in the number of mononuclear events occurring with Zembrin® incubation.

In line with the EFSA Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011), a confirmatory *in vivo* test needs to be conducted in cases of no clear negative results in the initial genotoxicity testing according to OECD guideline 471 (OECD, 2024) and OECD guideline 487 (OECD, 2023). All to be able to conclude on absence or presence of genotoxic potential. Such a third test ideally investigates a similar endpoint as the (equivocal) results of the *in vitro* micronucleus test. Therefore, a third test to evaluate Zembrin®’s toxicity was performed namely the *in vivo* mouse micronucleus test according to OECD guideline 474 (OECD, 2014). In this *in vivo* test, the test substance was administered via the intravenous route to have actual confirmation that the bone marrow cells would be exposed to the test product.

**Table 3. Summary results of the genotoxicity test with Zembrin® according to OECD Guideline 487. Relative increase in cell counts (RICC), Cytotoxicity Index (Cytotoxicity), Micronuclei (MN) numbers and frequencies of controls and various Zembrin ® concentrations.**

|  |  |
| --- | --- |
| Treatment |  |
| 4-hour treatment  | Concentration (µg/mL) ((µg/mL) | -S9 or +S9 | Mean cell count (x106) | Increase in cell count1 (x106) | RICC (%) | Cytotoxicity Index (%) | Number of MN (x106) | MN frequency (%) |
| DMSO (2% (v/v)) |  | -S9 and +S9 | 7.15 and 7.652 | 5.07 and 5.732 | 100,00 | 0.00 | 6 and 72 | 0.6 and 0.72 |
| 4-NQO | 0.025 | -S9 | 5.90 | 3.82 | 75.33 | 24.67 | 28 | 2.8 |
| Colchicine | 0.040 | -S9 | 5.90 | 3.82 | 75.33 | 24.67 | 24 | 2.4 |
| Cyclophosphamide | 3.13 | +S9 | 5.07 | 3.14 | 54.88 | 45.12 | 37 | 3.7 |
| Zembrin® | 1250 | -S9 | 6.90 | 4.81 | 95.07 | 4.93 | 7 | 0.7 |
|  | 2000 | -S9 | 6.43 | 4.35 | 85.86 | 14.14 | 8 | 0.8 |
|  | 2500 | -S9 | 5.93 | 3.85 | 75.99 | 24.01 | 10 | 1.0 |
|  | 5000 | -S9 | 4.17 | 2.08 | 41.12 | 58.88 | 12 | 1.2 |
|  | 2000 | +S9 | 7.30 | 5.38 | 93.89 | 6.11 | 8 | 0.8 |
|  | 3000 | +S9 | 6.07 | 4.14 | 72.34 | 27.7 | 8 | 0.8 |
|  | 4000 | +S9 | 4.27 | 2.34 | 40.90 | 59.10 | 7 | 0.7 |
|  | 5000 | +S9 | 4.00 | 2.08 | 36.24 | 63.76 | - | - |
| 24-hour treatment |  |  |  |  |  |  |  |  |
| DMSO (1% (v/v)) |  | -S9 | 8.17 | 5.25 | 100.00 | 0.00 | 7 | 0.7 |
| 4-NQO | 0.025 | -S9 | 6.20 | 3.28 | 62.54 | 37.46 | 32 | 3.2 |
| Colchicine | 0.011 | -S9 | 6.17 | 3.25 | 61.90 | 38.10 | 22 | 2.2 |
| Zembrin® | 111.1 | -S9 | 8.03 | 5.12 | 97.46 | 2.54 | 7 | 0.7 |
|  | 333.3 | -S9 | 7.50 | 4.58 | 87.30 | 12.70 | 14 | 1.4 |
|  | 1000 | -S9 | 6.67 | 3.75 | 71.43 | 28.57 | 13 | 1.3 |
|  | 1500 | -S9 | 5.27 | 2.35 | 44.76 | 55.24 | 14 | 1.4 |
|  | 2000 | -S9 | 0 | -2.92 | 55.56 | 155.56 | - | - |
|  | 2500 | -S9 | 0 | -2.92 | 55.56 | 155.56 | - | - |
|  |  |  |  |  |  |  |  |  |
|  | 3000 | -S9 | 0 | -2.92 | 55.56 | 155.56 | - | - |

*1Increase in cell count was calculated as mean cell count minus relevant control condition 2Mean cell counts, number of MN and MN frequency apply to the -S9 and the +S9 conditions respectively; RPMI1640 was applied as solvent of the test item and the positive control substance colchicine and cyclophosphamide. DMSO, dimethyl sulfoxide, was applied as solvent of the positive control substance 4-Nitroquinoline N-oxide: 4-NQO. Cytotoxicity index was calculated as 100 – RICC.*

**Figure 1. Graphical display of results of the genotoxicity test with Zembrin® according to OECD Guideline 487: dose-response curve of the mononuclear (MN) events observed after 4-hour treatment of Zembrin ® without metabolic activation.**

**3.3 The *in vivo* mouse micronucleus test**

The frequencies of MPCEs in negative and positive control mice were compatible with the historical control data for this laboratory. The positive control cyclophosphamide treated mice (60 mg/kg body weight) showed a large, significant increase in the MPCE number compared to the negative and historical controls. Thus, the study is considered valid.

The two times intravenous administration of 125 mg/kg body weight, 250 mg/kg body weight and 500 mg/kg body weight of Zembrin® extract did not induce increases in the frequency of micro nucleated polychromatic erythrocytes (MPCEs) in male mice compared to the negative and to the historical control groups (Table 4). The proportion of immature among total (immature + mature, viz (PCE/PCE+NCE)) erythrocytes was lower in the dose group of 500 mg/kg body weight as compared to the vehicle and historical control groups at 24 hours after the second treatment. The reduction of immature among total erythrocytes was considered biologically relevant and confirm the exposure of the target tissue cells, which could be expected as a result of the intravenous administration of the test substance.

No significant increases in the frequency of MPCEs were seen in the groups of mice treated with Zembrin® extract compared to the negative and to the historical control groups. So, in conclusion, Zembrin® extract did not show any genotoxic activity in this *in vivo* mouse micronucleus test, upon administering the test substance intravenously.

**4. DISCUSSION**

The studies presented are the first *in vitro* and *in vivo* genotoxic studies reported on Zembrin®, a commercial extract of *S. tortuosum*. The results provide evidence for the genotoxic safety of the extract.

**Table 4. Summary results of the genotoxicity test with Zembrin® according to OECD Guideline 474. Number of micronucleated polychromatic erythrocytes (MPCE) and polychromatic erythrocyte proportion of total mature (NCE) and immature (PCE) erythrocytes induced by controls and various concentrations of Zembrin ®**

|  |  |  |
| --- | --- | --- |
| Treatment | MPCE | PCE/PCE +NCE) |
|  | Mean | SD | Mean | SD |
| *Controls* |  |  |  |  |
| Vehicle Control(Water) | 6.00 | 0.71 | 0.54 | 0.02 |
| Cyclophosphamide60 mg/kg bw | 141.2\* | 5.17 | 0.39\*\* | 0.02 |
| *Zembrin®*  |  |  |  |  |
| 125 mg/kg bw | 5.60 | 1.34 | 0.53 | 0.01 |
| 250 mg/kg bw | 5.80 | 0.84 | 0.52 | 0.01 |
| 500 mg/kg bw | 6.00 | 1.58 | 0.47\*\* | 0.00 |

The bacterial reverse mutation assay was conducted following recognized international standards, employing a range of Salmonella typhimurium and Escherichia coli strains to evaluate potential mutagenic effects. This test was performed according to the state-of-the-art regulatory demands and showed no induction of gene mutations neither in the absence nor in the presence of metabolic activation. The *in vitro* mammalian cell micronucleus test was performed using L5178Y tk+/- cells to assess chromosomal damage, adhering strictly to regulatory guidelines. Results of this test were equivocal in that after 4- hour treatments in the presence of metabolic activation no micronucleus formation could be observed in L5178Y cells, whereas in some conditions in the absence of metabolic activation some non-significant micronucleus formation could be observed. Therefore, the results of this specific test was considered equivocal and a third genotoxic test, the *in vivo* mouse micronucleus test was performed. In this *in vivo* mouse micronucleus test, which involved administering the test substance intravenously, focusing on the analysis of bone marrow cells for genotoxic markers, no increases in micro nucleated polychromatic erythrocytes were observed. It was therefore concluded that Zembrin® did not show any genotoxic activity in this *in vivo* test.

Therefore, on the basis of the results of the three genotoxicity tests and in line with the EFSA Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011), we conclude that Zembrin® is not of genotoxic concern.

Zembrin® is intended to be used as a nutraceutical, as an ingredient in functional foods and specific parts of our diet. The recommended consumption is 25 mg per day. This corresponds to 0.357 mg/kg bw/day in a 70 kg adult. In the assays reported here the concentrations used were much higher, namely, up to 500 mg/kg bw/day, which is about 1400 times higher than the recommended consumption. Recommended consumption is also much lower than a previously proposed NOAEL of 600 mg/kg bw/day (Murbach, 2014). Previous safety assessments have been showing similar non- cytotoxic effects in other non-regulatory assays (Harvey, 2011).

The crude extracts and commercially available standardized extracts of *S. tortuosum* have displayed a wide spectrum of potential biological activities (e.g. antimalarial, anti-oxidant, neuromodulatory, immunomodulatory, anti-HIV, neuroprotection) in *in vitro* or *in vivo* studies (Chiu, 2014), whereas the main interest in *S. tortuosum* has focused on its potential for enhancing cognitive function and managing anxiety and depression.

Neurocognitive effects, as well as safety and tolerability of Zembrin ® were evaluated in a proof-of-concept study, in which healthy volunteers consumed 25 mg Zembrin® daily for 3 weeks. Zembrin® significantly improved cognitive set flexibility and executive function. Also, positive changes in mood and sleep were found and Zembrin® was well tolerated (Chiu, 2014). However, while the plant extract has been studied in clinical populations, this has only been in healthy subjects, so that further study in pathological states remains to be done.

5. Conclusion

The present *in vitro* and *in vivo* genotoxicity studies provide a basis for further studies evaluating health-promoting applications of extract *S. tortuosum* like Zembrin® in functional foods, beverages, and dietary supplements. The data presented here indicate that Zembrin ® has no genotoxic effects based on studies performed conform the officially accepted norms and procedures.

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