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Test Report

Report No.:	**2102096/15169**	**Date:** 2021-11-17
Client:	Rübig Gesellschaft m.b.H. & Co. KG. Griesmühlstraße 4 4600 Wels	
Subject:	Niro Spiegel unbehandelt Niro Spiegel Nitropep-behandelt	
Task:	Extraction according to EN ISO 10993-12:2012 Test for *in-vitro* cytotoxicity according to ISO 10993-5:2009 Test for *in vitro* irritation according to ISO 10993-23:2021	
Order:	2021-07-09	
Date of sampling:	—	
Location of sampling:	No samples taken by OFI staff Samples provided by the client	
Receipt of samples:	2021-10-11	

Nicht akkreditierte Verfahren sind als solche gekennzeichnet.
Non-accredited procedures applied have been named as such.

1 SCOPE OF WORK

According to the order the samples provided were extracted under accreditation according to 10993-12:2012. There are no significant changes between EN ISO 10993-12:2012 and ISO 10993-12:2021 (updated edition), relevant for the analysis.

The tests for cytotoxicity and irritation are performed under accreditation according to ISO 10993-5:2009, and for irritation according to ISO 109953-23:2021.

All tests applied are subject to a quality assurance program according to EN/ISO/IEC 17025:2017.

2 SCOPE OF APPLICATION

The results given in this Test Report have been obtained under the specific conditions of the individual tests. As a rule, they are not the only criteria for assessing the product in question and its suitability for a specific purpose of application.

3 SAMPLE MATERIAL

Our client submitted the following samples for the purpose of testing:

Table 1: Provided samples

Sample	Internal Sample Code
Niro Spiegel unbehandelt	804a
Niro Spiegel Nitropep-behandelt	804b

Other documents submitted by our client:

No (other) documents submitted.

4 TESTS

Testing took place from 2021-11-02 to 2021-11-12

The tests were carried out in the individual technical departments within the scope of competence of the authorized signatories according to the OFI QM manual.

4.1 Preparation of Extracts

4.1.1 Cytotoxicity

Samples were extracted with cell culture media (DMEM, including 10% foetal bovine serum) at 37 °C for 24 hours. Following surface/volume ratio was used: 3 cm²/mL. Three independent extractions were prepared.

4.1.2 Irritation

The samples were extracted with sterile 0.9% sodium chloride solution and with sesame oil at 37 °C for 72 hours using a surface/volume ratio of 3 cm²/mL. For each solvent two independent extractions were prepared.

4.2 Test for *in-vitro* cytotoxicity according to ISO 10993-5

Testing of samples was performed according to the EN ISO 10993-5:2009 using the mammalian fibroblast cell line L-929 (ATCC Number CCL-1, Lot 13) in consideration of SOP 350.006 (grundlegende Zellkulturtechnik) and SOP 350.004 (Zytotoxizitätsprüfung).
For testing a sub-confluent single cell layer was prepared. 10^5 cells/mL were seeded and incubated for 24 hours at 37°C and 5% CO_2. Afterwards, sample extracts were added, whereby different dilution steps of each extract were prepared (undiluted, and 1:2 dilution). Cells were incubated with sample extracts at 37 °C and 5% CO_2 for 24 ± 1 hours. The determination of cytotoxicity was done according to the EN ISO 10993-5:2009 using a microscope (Type Nikon Eclipse TS 100, magnification: 200x). Reactivity was estimated according to Table 1 in EN ISO 10993-5:2009.
A zinc sulphate solution in the range of 5.6 to 90 mg/L was used as positive control. According to internal standards, the first 3 dilutions (90 – 22.5 mg/L) are intended to show a cytotoxic reaction, the last 2 dilutions (11.3 – 5.6 mg/l) are supposed to have no impact on the viability of the cells. An HDPE plastic granulate, extracted in the same way as the samples, and untreated medium served as negative control.

4.3 Test for *in vitro* irritation according to ISO 10993-23

The testing of the samples was performed according to ISO 10993-23:2021. Skin equivalents were obtained from MatTek and incubated for 18-24 hours with the sample extracts. The irritation, defined as loss of viability of the skin keratinocytes, was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Viable cells reduce MTT to formazan, resulting in a color change from yellow to dark blue, which can be detected with a photometer (Victor$^{3\,TM}$ 1420 Multilabel counter).

1% SDS (Sodium Dodecyl Sulphate) was used as a positive control. Sesame oil and 0.9% sodium chloride solution served as negative controls.

5 RESULTS

5.1 Results of cytotoxicity testing

The results of the analysis for cytotoxicity of the sample extracts are shown in Table 2. Furthermore, pictures have been taken of the cells treated with the sample extracts (Figure 1 to Figure 6).

Table 2: Determination of the cytotoxic reaction of the sample extracts, positive and negative control after 24 hours incubation, optical evaluation

Sample	Cell morphology	Reactivity (3 independent extracts)
Niro Spiegel unbehandelt – undiluted	50% round cells and growth inhibition	3/2/3
Niro Spiegel unbehandelt – 1:2 diluted	10% growth inhibition	1/0/0
Niro Spiegel Nitropep-behandelt - undiluted	70% round cells and growth inhibition	3/2/3
Niro Spiegel Nitropep-behandelt – 1:2 dilution	100% typical form of fibroblasts	0/0/0
Positive control $ZnSO_4$		
90 mg/L	100% round cells	4/4/4
45 mg/L	100% round cells	4/4/4
22.5 mg/L	70% round cells	3/3/3
11.3 mg/L	100% typical form of fibroblasts	0/0/0
5.6 mg/L	100% typical form of fibroblasts	0/0/0
Negative control		
Reference material (HDPE)	100% typical form of fibroblasts	0/0/0
Untreated L-929 cells	100% typical form of fibroblasts	0/0/0

Evaluation scheme:
Grades of reactivity
0 = no cytotoxic reactivity
1 = very low cytotoxic reactivity
2 = low cytotoxic reactivity
3 = moderate cytotoxic reactivity
4 = strong cytotoxic reactivity

The achievement of a numerical grade greater than 2, based on Table 1 in the ISO 10993-5, is considered a cytotoxic effect.

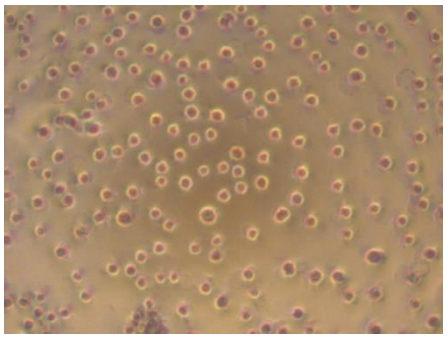

Figure 1: Positive control: 90mg/L zinc sulphate: 100% round cells

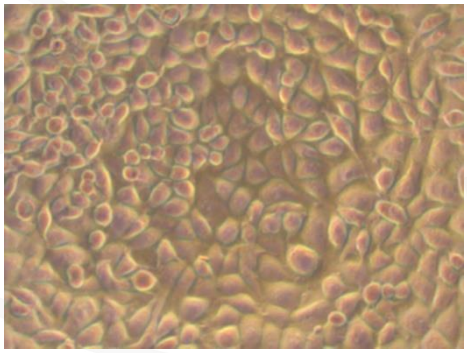

Figure 2: Negative control (untreated L-929 cells): 100% typical form of fibroblasts

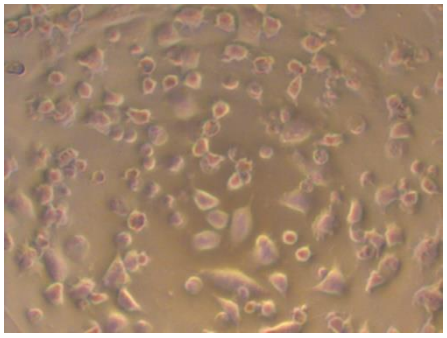

Figure 3: Sample “Niro Spiegel unbehandelt” undiluted: 50% round cells and growth inhibition

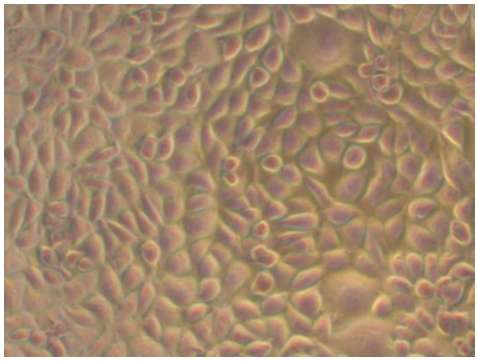

Figure 4: Sample " Niro Spiegel unbehandelt " 1:2 diluted: 10% growth inhibition

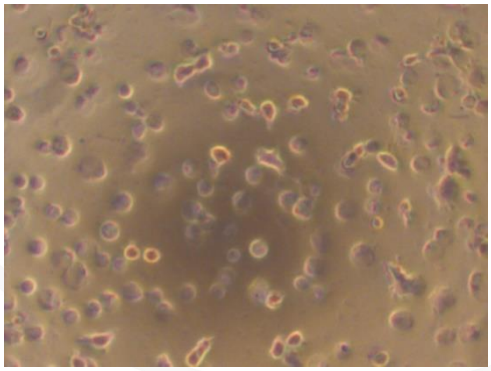

Figure 5: Sample " Niro Spiegel Nitropep-behandelt " undiluted: 70% round cells and growth inhibition

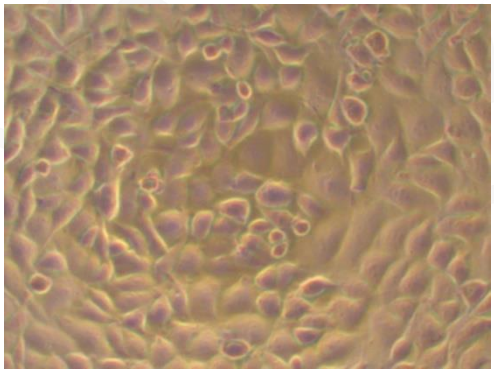

Figure 6: Sample " Niro Spiegel Nitropep-behandelt " 1:2 diluted: 100% typical form of fibroblasts

5.2 Results of testing for Irritation

The results of the skin irritation tests for extracts are shown in Table 3 and further illustrated in Figure 7.

Table 3: Determination of the viability of the skin equivalents after 18 hours incubation with sample extracts (arithmetic mean ± standard deviation)

Sample	sesame oil – Viability [%]	0.9% sodium chloride solution- Viability [%]
Positive control	4±0	4±0
Negative control	100±2	100±1
Niro Spiegel unbehandelt	107±4	113±4
Niro Spiegel Nitropep-behandelt	113±3	107±4

Evaluation scheme: According to ISO 10993-23:2021 a decrease of the viability of the skin models of more than 50% indicates a skin irritating potential. As shown in Table 3 and Figure 7, the extracts of the samples had no significant influence on the viability of the keratinocytes in the 3D skin model.

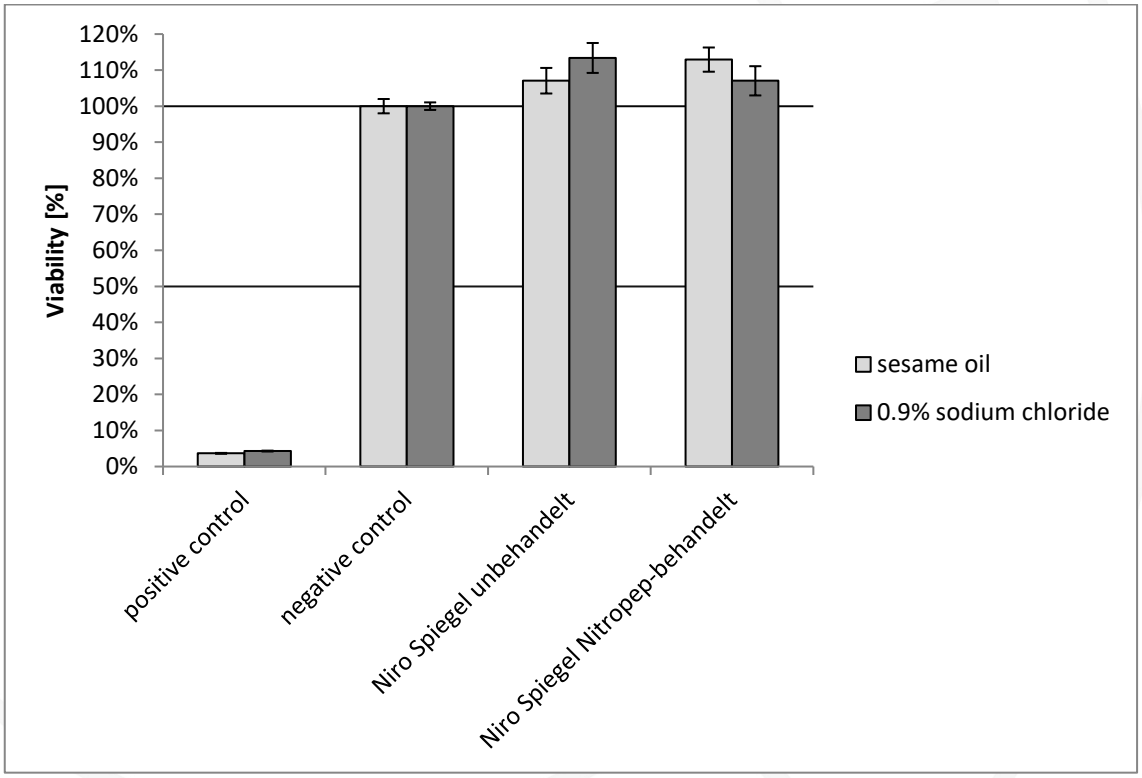

Figure 7: Viability of the skin equivalents after treatment with the sample extracts (error bars show the standard deviation)

6 SUPPLEMENTARY STATEMENT ON THE TEST RESULTS[1]

6.1 Cytotoxicity

At optical evaluation, extracts of the sample “Niro Spiegel unbehandelt” and “Niro Spiegel Nitropep-behandelt” showed low to moderate cytotoxic reactivity (Reactivity 2-3). At a dilution of 1:2 the samples showed no cytotoxic reactivity anymore.

Any cytotoxic effect can be of concern. However, it is primarily an indication of potential for in vivo toxicity and the device cannot necessarily be determined to be unsuitable for a given clinical application based solely on cytotoxicity data.

6.2 Irritation

The provided samples did not show irritating effects according to ISO 10993-23:2021.

[1] Non-accredited procedure

This Test Report No. **2102096/15169** comprises
11 sheets with 3 table(s), 7 figure(s) and 0 appendix(es).

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