Universitätsklinikum Jena

Klinik für Hautkrankheiten

Qualitäts-zertifiziert nach DIN EN ISO 9001:2008

Universitätsklinikum Jena · Klinik für Hautkrankheiten · Erfurter Str. 35 · 07743 Jena

smartfiber AG

Breitscheidstr. 154

07407 Rudolstadt

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07743 Jena

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10.02.2012

study report:

In vitro-Cytotoxicity

Textile Sample 1201003

(24h Extract)

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1. Quality certificate

Zertifikat

QUALITÄTSMANAGEMENTSYSTEM – DIN EN ISO 9001: 2008

Hiermit wird bestätigt, dass das

Universitätsklinikum Jena
Klinik für Dermatologie und
dermatologische Allergologie
Erfurter Straße 35
07740 Jena
Deutschland

Inhaber des Zertifikates Nr. **FS 519135/5409D**

ein Qualitätsmanagementsystem gemäß **DIN EN ISO 9001:2008** *für den folgenden Geltungsbereich anwendet:*

Dermatologie, Allergologie, Berufsdermatologie, Andrologie, Dermato-Histologie, Dermato-Onkologie, Hautphysiologie/Skin Study Center, Forschungslabor, Diagnostisches Labor, Operative Dermatologie, Laser, Photodermatologie, Proktologie, Phlebologie, Wundheilung

Für und im Namen von BSI:

Geschäftsführung, BSI Management Systems (Deutschland)

Ursprünglich zertifiziert: **12.12.2003** Letzte Ausgabe: **06.10.2009** Ablaufdatum: **16.10.2012**

QMS/EMS-TGA-ZM 08-92

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2. General information

2.1 Test items

Textile (Sample 1201003)

2.2 Reference items

-

2.3 Sponsor

smartfiber AG
Breitscheidstr. 154
07407 Rudolstadt

2.4 Test facility

Klinik für Hautkrankheiten
Universitätsklinikum Jena
Erfurter Straße 35
D-07740 Jena
Germany
Study director: PD Dr. Uta-Christina Hipler

2.5 Operating schedule

Start of experiment: 27.01.2012
End of experiment: 09.02.2012
Date of final report: 10.02.2012

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3. GLP and quality assurance statement

I assure that the Test facility complies with the Principles of Good Laboratory Practice. Appropriate and technically valid Standard Operating Procedures are established for the described tests. The Test facility is certified according to DIN EN ISO 9001:2008.

10. 2. 2012 — Date

Study director: PD Dr. Uta-Christina Hipler

4. Summary

In this study, the effect of the extract from the textile sample 1201003 on human HaCaT keratinocytes was determined (extraction ratio: 1g:50mL, extraction time: 24h, extraction temperature: 37°C) according to DIN ISO 10993-12 *in vitro.*
The luminometric ATP assay, the Pierce BCA Protein Assay and the LDH Cytotoxity Detection Kit were used.

Neither the extract (1g:50mL) nor its dilutions had a cytotoxic effect on human HaCaT keratinocytes *in vitro.* Medium was used as control.

5. Background

The skin is the major interface between body and environment. It is the most versatile human organ and plays a key role in protecting the body against environmental influences, preserving homeostasis, and regulating body temperature. The skin is also involved in absorption, sensory perception, metabolic processes and immunological functions. Textiles are the tissues with the longest and most intense contact to the human skin. They are widely used for clinical and cosmetic purposes. Hence, such textiles have to be evaluated according to their potential risks. Biocompatibility is the central request for materials and devices that come into direct contact with human tissue [DIN EN ISO 10993-1]. The determination of toxic effects is part of the initial evaluation process stipulated by ISO standards. In vitro-cytotoxicity is tested according to DIN EN ISO 10993-5. Samples can be analysed in direct and indirect contact with cells, respectively, as well as in form of extracts [DIN EN ISO 10993-5, DIN EN ISO 10993-12]. A wide variety of cell lines are suitable for biocompatibility tests and are commonly used for *in vitro* cytotoxicity testing such as fibroblasts from human skin, buccal mucosa, periodontal membrane or embryonic lung, cultures of human keratinocytes and HaCaT cells, different murine cell lines (C3H-L, Balb/c 3T3, L929, liver and spleen cell lines, others), and T-lymphocytes from lymph nodes. Nevertheless, the general opinion is that toxicity tests *in vitro* will be more convincing when performed with cells that are homologous with the human tissue concerned. In accordance, appropriate cell lines for testing of local skin compatibility are human dermal fibroblasts and epidermal keratinocytes [Wiegand & Hipler 2009, Wiegand & Hipler 2008]. Determination of cell proliferation and cell viability has become the key technology to assess biocompatibility of materials like medical textiles *in vitro*. Tests that measure cellular growth are capable to express toxic effects via loss of proliferation capacity or reduction of living cells. These negative effects can be quantified by methods that determine cell death. A characteristic sign for necrotic cell death is the degradation of the cell membrane and activation of the inflammatory response. Cell membrane damage leads to release of LDH (lactate dehydrogenase) from the cytosol into the supernatant [Wiegand & Hipler 2009, Wiegand & Hipler 2008]. The reaction of cells to cytotoxic substances is often accompanied by the release of inflammatory cytokines. Analysis of cellular interleukin 6 and 8 release can be used to detect proinflammatory effects, which would not be detected with cytotoxicity assays alone [Hipler & Wiegand 2011, Wiegand et al. 2010, Wiegand & Hipler 2009, Wiegand & Hipler 2008].

6. Description of materials and test methods

6.1 Preparation of sample material

Extract of sample material was produced according to DIN EN ISO 10993-12 by incubating 1g sample material with 50 mL Dulbecco's modified Eagle medium (DMEM, Lot 359P072, 12/2012) without fetal calf serum (FCS) for 24h at 37°C.
The extract was sterile filtrated and supplemented with 10% FCS (Lot 05372935, 12/2014, Promocell). Extract dilutions of the original extract (100% - extraction ratio 1g:50mL) in DMEM were used for the *in vitro* assays: 75% - 0.75g:50mL, 50% - 0.5g:50mL, 25% - 0.25g:50mL, 10% - 0.1g:50mL, 1% - 0.01g:50mL and 0.1% - 0.001g:50mL.

6.2 Cell culture of HaCaT keratinocytes

The human HaCaT keratinocytes were a gift from Prof. Dr. N.E. Fusenig, DKFZ, Germany.
Human HaCaT keratinocytes were cultured in DMEM supplemented with 1% antibiotic-antimycotic solution (10000 U/mL penicillin, 10000 µg/mL streptomycin, 25 µg/mL amphotericin) and 10% fetal calf serum. The cells were cultured for 7 days in 75cm^2 cell culture flasks (Greiner bio-one, Germany) at 37°C and in humidified atmosphere containing 5% CO_2 atmosphere. For experiments, the cells were harvested through trypsin-EDTA treatment and seeded into 96-well plates (Greiner bio-one, Germany) at a density of 30,000cells/cm^2. After 48 hours, the culture medium was replaced by either fresh DMEM (control) or extract dilutions as indicated. As cytotoxicity control Triton X-100 was used.

6.3 ATP bioluminescence assay

Determination of cell proliferation was carried out on the basis of a luminometric ATPLite™-M assay (Lot 69-11442, 02/2013, PerkinElmer) according to the manufacturer's recommendations. In brief, 50µL lysis solution was added to the wells containing treated or control cells. Subsequently, 50µL substrate solution (luciferase/*D*-luciferin) was added to each well. After incubation, the luminescence was measured using a microplate luminometer (LUMIstar Galaxy, BMG Labtech). ATP concentrations were calculated on the basis of a standard curve.

6.4 Determination of cellular protein content

Determination of cell proliferation was carried out by measurement of total protein content. In brief, cell culture medium was removed completely, and cells were washed two times with PBS. Human HaCaT keratinocytes were lysed with 0.1% Triton-X 100 in PBS at 95°C for 10 min. After cooling, the plates were stored at -20°C until testing. Total protein content was determined by the Pierce BCA Protein Assay (Lot MK166712, Thermo Scientific) based on the biuret reaction. The method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline solution with the colorimetric detection of Cu^{+} using bichinoninic acid (BCA). The assay was performed as recommended by the manufacturer using the microplate procedure protocol. Absorbance was measured by means of the microplate photometer FLUOstar Galaxy (BMG Lab Technologies GmbH). Protein concentrations were calculated on the basis of a standard curve.

6.5 Detection of cytotoxicity (LDH)

Determination of *in vitro* cytotoxicity was carried out by measurement of lactate dehydrogenase (LDH) activity in cell culture supernatants. LDH is released by loss of membrane integrity from cytosol to the cell culture medium. LDH activity was determined by the Cytotoxicity Detection Kit (Lot 12173400, 07/2012, Roche Diagnostics). In this assay, LDH activity is measured with an enzymatic test. LDH catalyze the reaction from lactate to pyruvate together with the conversion of NAD^{+} to NADH + H^{+}. A catalyst (diaphorase) mediates the conversion of a tetrazolium salt to formazan. The absorption of the formazan dye can be measured at 490nm.

The assay was performed as recommended by the manufacturer using the microplate procedure protocol. In brief, 80µL of cell culture supernatant were collected and transferred to a new 96-well plate. 80µL tetrazolium dye solution was added and after incubation absorbance was measured by means of the microplate photometer FLUOstar Galaxy (BMG Lab Technologies GmbH). Cytotoxicity was calculated in comparison with positive control (Triton X-100):

$$\text{Cytotoxicity [\%]} = \frac{OD_{sample} - OD_{control}}{OD_{positive\ control} - OD_{control}} \times 100\ \%$$

6.6 Statistics

Experiments and measurements were performed in duplicate each. All values are expressed as means ± SD (standard deviation). One-way analysis of variance was carried out to determine statistical significances (Microsoft® Excel 2000). Differences are considered statistically significant at a level of $p < 0.05$. Asterisks indicate significant deviations from the control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

7. Archiving

The following records will be stored in the archives of the Klinik für Hautkrankheiten, Universitätsklinikum Jena according to the GLP regulations:

A copy of the final report, the study plan and a documentation of all raw data generated during the conduct of the study will be stored for at least 4 years after completion of the study.

Unused test items and reference items are stored for at least 12 month after completion of the study.

Materials and samples that are unstable may be disposed of before that time and without sponsor's prior consent.

Records and reports of the maintenance and calibration of apparatus, validation documentation for computerized systems and the historical file of all Standard Operating Procedures (SOPs) is stored in accordance with the appropriate authorities.

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8. Results and discussion

8.1 Determination of cell proliferation by ATP bioluminescence assay

The original extract (100% - extraction ratio 1g:50mL) and dilutions of the original extract in DMEM were used for the *in vitro* assay: 75% - 0.75g:50mL, 50% - 0.5g:50mL, 25% - 0.25g:50mL, 10% - 0.1g:50mL, 1% - 0.01g:50mL and 0.1% - 0.001g:50mL.
Human HaCaT keratinocytes were incubated with the different extract dilutions for 1h, 24h and 48h. Medium was used as control.
Neither the extract (1g:50mL) nor its dilutions had a cytotoxic effect on human HaCaT keratinocytes *in vitro* (Fig. 1).

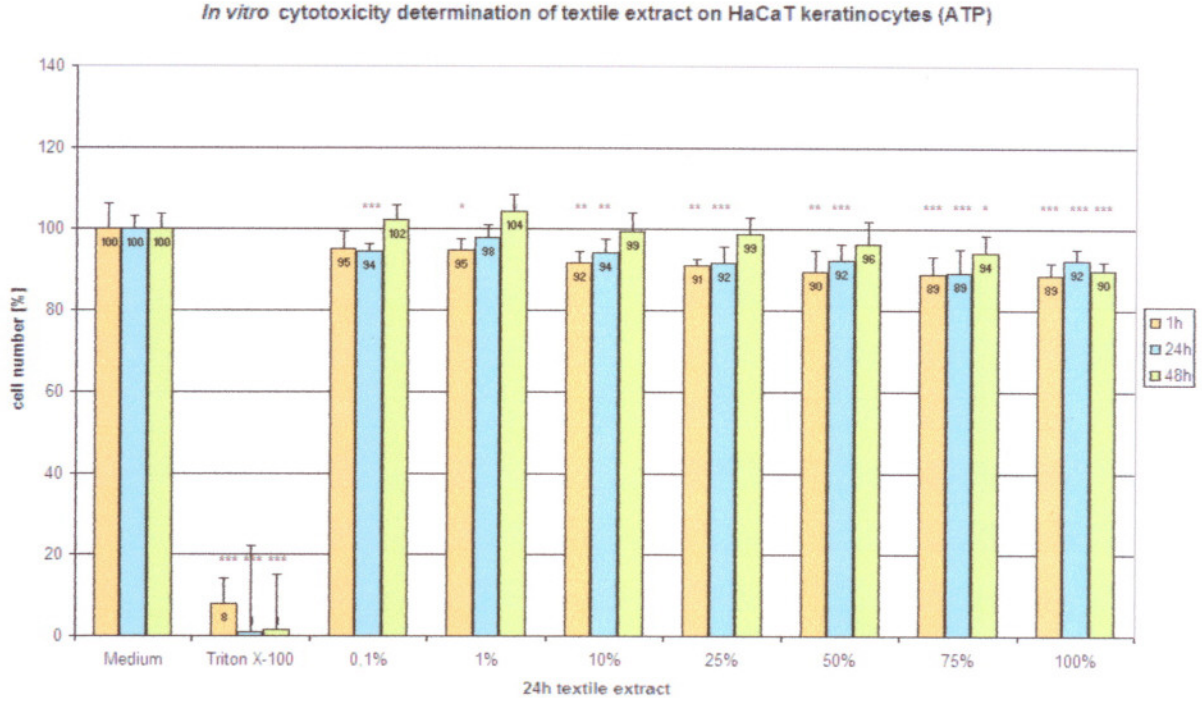

Figure 1: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. Cell proliferation was measured with the ATP bioluminescence assay.

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8.2 Determination of cell proliferation by BCA Protein Assay

The original extract (100% - extraction ratio 1g:50mL) and dilutions of the original extract in DMEM were used for the *in vitro* assay: 75% - 0.75g:50mL, 50% - 0.5g:50mL, 25% - 0.25g:50mL, 10% - 0.1g:50mL, 1% - 0.01g:50mL and 0.1% - 0.001g:50mL.

Human HaCaT keratinocytes were incubated with the different extract dilutions for 1h, 24h and 48h. Medium was used as control.

Neither the extract (1g:50mL) nor its dilutions had a cytotoxic effect on human HaCaT keratinocytes *in vitro* (Fig. 2).

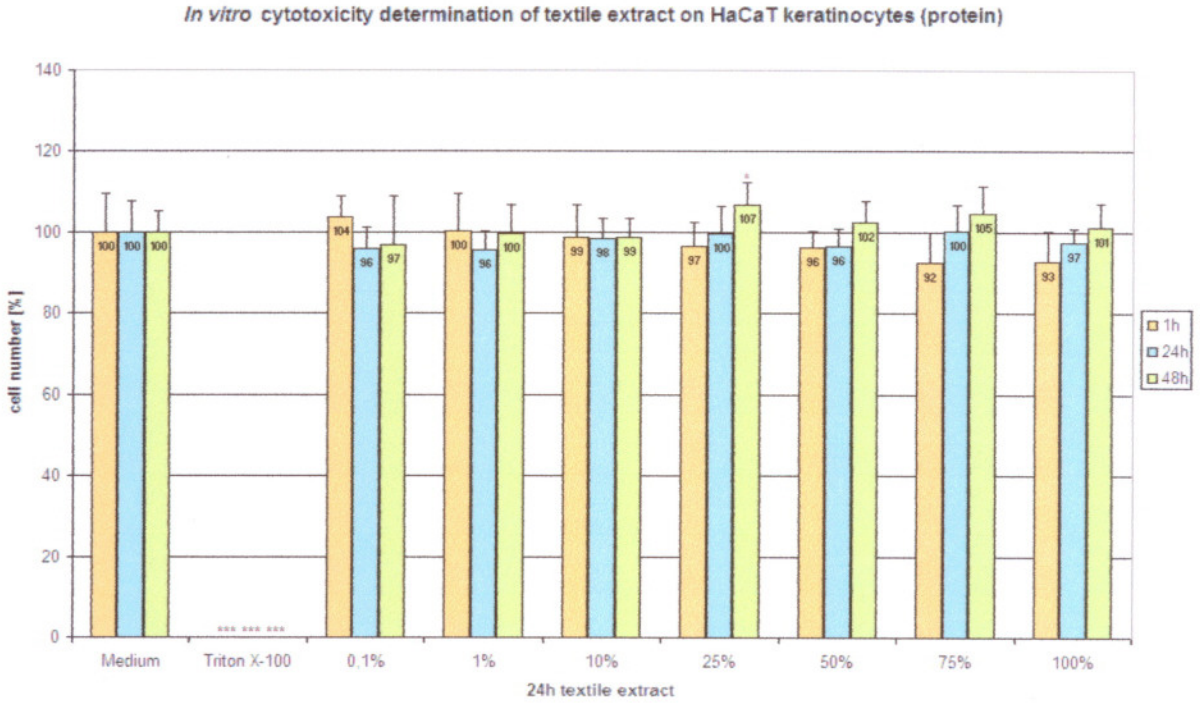

Figure 2: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. Cell proliferation was measured with the BCA Protein Assay.

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8.3 Detection of cytotoxicity by LDH measurement

The original extract (100% - extraction ratio 1g:50mL) and dilutions of the original extract in DMEM were used for the *in vitro* assay: 75% - 0.75g:50mL, 50% - 0.5g:50mL, 25% - 0.25g:50mL, 10% - 0.1g:50mL, 1% - 0.01g:50mL and 0.1% - 0.001g:50mL.
Human HaCaT keratinocytes were incubated with the different extract dilutions for 1h, 24h and 48h. Medium was used as control.
Neither the extract (1g:50mL) nor its dilutions had a cytotoxic effect on human HaCaT keratinocytes *in vitro* (Fig. 3).

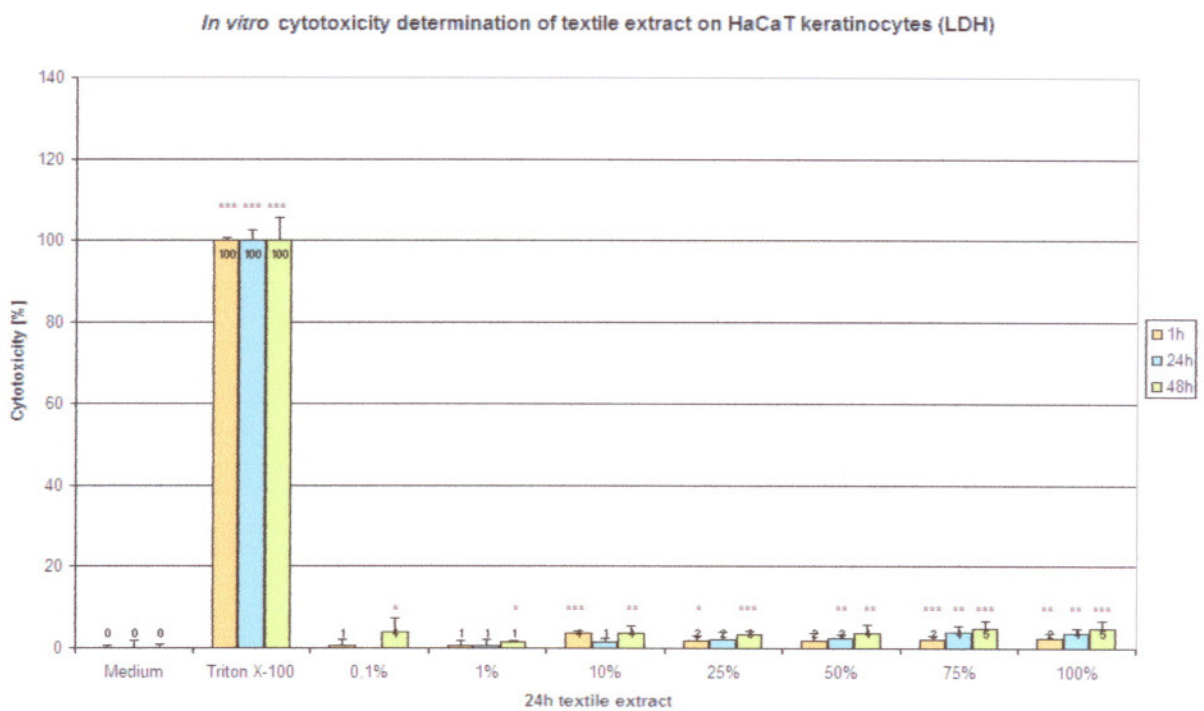

Figure 3: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. LDH release was determined with the Cytotoxicity Detection Assay.

9. Appendix

9.1 Abbreviations

cm^2	square centimeters
°C	grad Celsius
h	hour
IL-6	interleukin 6
IL-8	interleukin 8
M	molar
µg	microgram
mg	milligrams
mL	milliliters
µL	microliters
mm	millimeters
mM	millimolar
n.d.	not determined
PBS	phosphate buffered saline
U	units

9.2 Tables and Figures

9.3 References

DIN EN ISO 10993-1 April 2010 Biologische Beurteilung von Medizinprodukten Teil 1: Beurteilung und Prüfung im Rahmen eines Risikomanagementsystems

DIN EN ISO 10993-5 Juni 2007 Biologische Beurteilung von Medizinprodukten Teil 5: Prüfung auf in-vitro-Zytotoxizität

DIN EN ISO 10993-12 Februar 2008 Biologische Beurteilung von Medizinprodukten Teil 12: Probenvorbereitung und Referenzmaterialien

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Wiegand C, Fluhr JW, Elsner P, Hipler UC. in Cellulose: Structure and properties, derivatives and industrial uses, eds. A. Lejeune & T. Deprez, Nova Science Publishers, 2010

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9.4 Measurement data

Table 1: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. Cell proliferation was measured with the ATP bioluminescence assay. (n=4, Fig.1)

time [h]		control	Extract concentration [%]						
		medium	0.1%	1%	10%	25%	50%	75%	100%
1	ATP-content [nM]	15302	14580	14498	14055	13939	13706	13603	13564
	SD [nM]	966	626	425	391	220	712	609	421
	Cell number [%]	100	95	95	92	91	90	89	89
	SD [%]	6	4	3	3	2	5	4	3
24	ATP-content [nM]	31640	29885	31036	29779	29038	29221	28240	29218
	SD [nM]	976	616	906	1050	1195	1167	1685	820
	Cell number [%]	100	94	98	94	92	92	89	92
	SD [%]	3	2	3	4	4	4	6	3
48	ATP-content [nM]	68107	69597	71140	67700	67364	65699	64213	61266
	SD [nM]	2720	2755	2905	3183	2730	3715	2838	1353
	Cell number [%]	100	102	104	99	99	96	94	90
	SD [%]	4	4	4	5	4	6	4	2

Table 2: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. Cell proliferation was measured with the BCA Protein Assay. (n=4, Fig. 2)

time [h]		control	Extract concentration [%]						
		medium	0.1%	1%	10%	25%	50%	75%	100%
1	Protein content [µg/mL]	38	39	38	37	36	36	35	35
	SD [µg/mL]	4	2	4	3	2	1	3	3
	Cell number [%]	100	104	100	99	97	96	92	93
	SD [%]	10	5	9	8	6	4	7	8
24	Protein content [µg/mL]	86	83	83	85	86	83	86	84
	SD [µg/mL]	7	4	4	4	6	4	6	3
	Cell number [%]	100	96	96	98	100	96	100	97
	SD [%]	8	5	5	5	7	4	7	3
48	Protein content [µg/mL]	171	166	171	169	183	175	179	173
	SD [µg/mL]	9	20	12	8	11	9	12	10
	Cell number [%]	100	97	100	99	107	102	105	101
	SD [%]	5	12	7	5	6	5	7	6

Table 3: Effect of 24h textile extract on human HaCaT keratinocytes after incubation of 1, 24 or 48h. LDH release was determined with the Cytotoxicity Detection Assay. (n=4, Fig. 3)

time [h]		control	Extract concentration [%]						
		medium	0.1%	1%	10%	25%	50%	75%	100%
1	Cytotoxicity [%]	0	0.7	0.5	3.6	1.9	1.9	2.2	2.5
	SD [%]	0.7	1.4	1.3	0.8	1.3	2.0	0.9	1.2
24	Cytotoxicity [%]	0	0	0.7	1.4	2.2	2.6	3.9	3.6
	SD [%]	2.0	0.8	1.4	1.1	1.9	0.9	1.5	1.2
48	Cytotoxicity [%]	0	4.0	1.4	3.8	3.2	3.8	4.9	5.0
	SD [%]	0.9	3.3	0.6	1.9	1.1	2.0	2.0	1.8