# INTERIM REPORT

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## A FEASIBILITY STUDY ON WHETHER THE PREVALIDATED HUMAN 3-D EPIDERMIS MODEL *IN VITRO* PHOTOTOXICITY TEST (EpiDerm-PT) , COULD SUCCESSFULLY BE USED FOR PHOTOTOXIC POTENCY TESTING

31 pages, 11 Figures, 19 Tables, 1 Annex

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# CONTENT

# 1. BACKGROUND OF THE STUDY

Phototoxicity is an acute toxic response that is elicited after initial exposure of the skin to certain chemicals and subsequent exposure to light, or that is induced by skin irradiation after the systemic administration of a chemical substance. The assessment of whether a chemical compound is likely to cause adverse phototoxic effects is necessary for all chemicals that sufficiently absorb certain parts of UV and visible light (solar light) and that are intended for human use (including some pharmaceuticals, cosmetic ingredients, and food additives).

None of more than ten different animal tests used for predicting acute phototoxicity in humans have been scientifically validated. However, it was recently proven in an international EU/ECVAM/COLIPA validation exercise that the phototoxic potential of chemicals in humans can correctly be predicted by the 3T3-NRU *in vitro* phototoxicity test (3T3-NRU-PT). This *in vitro* test, which involves the use of the permanent mouse fibroblast cell line, Balb/c 3T3, gained regulatory acceptance in all EU Member States in June 2000. The test is now widely used in the chemical and cosmetic industries.

Determination of the phototoxic potential of a chemical in the 3T3-NRU-PT is often the first step in a sequential phototoxicity testing strategy. If a chemical provides a *negative* result in the 3T3 NRU-PT, no further testing is required in most instances. However, if the result is *positive*, the chemical may be still applied topically to the skin at safe concentrations, depending on absorption and accumulation of the chemical by the skin. Thus, in addition to the information on phototoxic *potential*, which is assessed in the 3T3-NRU-PT, additional testing may be required to obtain combined information on the *phototoxicity* and *bioavailability* of the chemical in the skin, and on the *relative phototoxic potential* of the chemical, i.e. its *phototoxic potency*.

Ideally, a photopotency test should be performed *in vivo* in human volunteers, but this is often not acceptable for ethical reasons, especially if the chemical may be a photoallergen. Reconstituted, 3-D human skin models could offer an effective means of avoiding the need for confirmatory testing *in vivo* in animals, especially since such models are characterised by having both viable primary skin cells and skin barrier functions. Indeed, based on the promising outcome of an ECVAM-funded prevalidation study on the EpiDerm™ model, the European Medicines Evaluation Agency (EMEA) has suggested, in a recent Draft Guidance Document on Photosafety Testing, that confirmatory testing can be performed on such a skin model

High-quality, reconstituted human skin models are now available from a number of commercial producers. They have been used successfully in the routine safety testing of skin products in various laboratories, since they are directly relevant to the organ of major concern. In contrast to conventional, submerged cell cultures, human 3-D skin models permit the topical application of a wide spectrum of chemicals and finished products. They also offer advantages when the aqueous solubility of the test material is a limiting factor, and test materials can be applied undiluted. In addition, their use in corrosivity testing has shown that these culture models can handle materials with extreme pH values.

Experience suggests that human 3-D models could offer the following advantages when compared to the 3T3-NRU-PT:

1. Neat chemicals or complex mixtures can be applied, simulating more-closely the situation when preparations as applied topically to the skin.
2. Test concentrations can be closer to real exposure conditions, including dermatological patch techniques.
3. Histology can be performed on exposed and control samples.
4. Exposure to light can better be adapted to real-life situations, e.g. exposure time and spectrum of simulated sunlight (a higher dose of short-wave light in the UVB range).
5. Depending on the barrier function of the stratum corneum, absorption and penetration of the original chemicals or molecules created during exposure could provide more-relevant results than tests performed on simpler systems (revealing less false-positive results).

## 1.1 GOAL

The goal of the study is to assess whether the potencies of phototoxic chemicals can correctly be predicted when applying them to the pre-validated *in vitro* EpiDerm Phototoxicity Test. In other words, to test whether the lowest topical phototoxic dose (concentration) determined *in vitro* in the human skin model test, correlates well with the lowest phototoxic dose (concentration) measured *in vivo* in human volunteers.

## 1.2 PARTICIPATING LABORATORIES

It was proposed that ZEBET (BfR, Berlin) should be the main contractor and should perform the EpiDerm Test, since this laboratory has unique experience in the development and prevalidation of *in vitro* tests involving 3-D skin models, and also directed the validation of the 3T3-NRU-PT.

It was further proposed that 3T3-NRU-PT and the human volunteer studies will be undertaken by the National Institute of Public Health, Prague (SZU), Czech Republic, since this laboratory has experience of both the *in vitro* method and of human skin patch testing.

In some cases it was necessary to perform all tests in both laboratories in parallel, therefore a joint training on all methods was performed on 21– 24 October 2003 at ZEBET.

# 2 MATERIALS AND METHODS

## 2.1 3T3 NRT-PT TEST

The 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) is designed to detect the phototoxicity induced by the combined action of a chemical and light by using an *in vitro* cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line. The test identifies compounds that act *in vivo* phototoxic after systemic application, as well as compounds, that act as photo-irritants after topical application and distribution to the skin.

### *2.1.1 Brief description of the test*

**Endpoint and Endpoint Detection**: Cell viability, determined as inhibition of the capacity of the cell cultures to take up vital dye, neutral red;

**Test Parameter**: Determination of PIF factor and MPE

**Test System**: Balb/c 3T3 mouse fibroblast cell line Balb/c 3T3 cells, clone 31, e.g. ECACC # 86110401 (European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK)

**Brief experimental procedure:**

Balb/c 3T3 cells are maintained in culture for 24 hrs for formation of monolayers. Two 96-well plates per test chemical are then pre-incubated with eight different concentrations of the chemical for 1 hr. One plate is then exposed to a dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). The treatment medium is then replaced with culture medium and after 24 hrs cell viability is determined by Neutral Red Uptake for 3 hrs.

Cell viability obtained with each of the eight concentrations of the test chemical is compared with that of untreated controls and the percent inhibition is calculated. For prediction of phototoxic potential the concentration responses obtained in the presence and in the absence of UV irradiation are compared, usually at the EC50 level, i.e. the concentration inhibiting cell viability by 50% of untreated controls. Results were calculated using the "Phototox software - version 2.0"

## 2.2 SKIN MODEL TEST

In contrast to normal cell cultures, such as mouse fibroblasts that are used in the 3T3-NRU-PT, human skin models permit the topical application of various types of chemicals and preparations, and fewer limitations concerning solubility problems. The test materials can be applied to 3-D skin models undiluted, at extreme pH values or even as 'insoluble' materials.

Due to the functional stratum corneum barrier in the human skin or epidermis models, the H3D-PT is not a stand-alone test for assessing the inherent phototoxic potentials of chemicals. In contrast to the 3T3-NRU-PT, the H3D-PT may fail to detect phototoxins that cannot enter the skin via a topical route, but may be sufficiently bioavailable in the skin via systemic pathways, for example, after oral or parenteral exposure. The H3D-PT may also fail to detect weakly photoreactive chemicals that induce photoallergic reactions only after repeated exposure. H3D-PT it is qualified as an adjunct test for further investigating chemicals with possibly false-positive outcomes in the 3T3-NRU-PT, as specified in paragraph 54 of draft OECD TG 432.

### *2.2.1 Brief description of the test*

**Endpoint and Endpoint Detection**: Cell viability, determined as inhibition of the capacity of the cell cultures to take up vital dye - MTT

**Test System**: EpiDerm™ EPI-200 system (MatTek, Ashland, USA).

**Brief procedure:**

The test consists of topical exposure of the EpiDerm tissue to the test material (5 concentrations) followed by an irradiation experiment (UVA dose of 6 J/cm$^2$, +UVA) ) and a dark control experiment (-UVA) after 21 hrs. incubation. After irradiation and rinsing, the tissues are incubated for 21 hrs followed by the determination of the cytotoxic effect (MTT-assay). Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity, measured by production of formazan from MTT at the end of the treatment.

Tissue viability obtained with each of the five concentrations of the test chemical is compared with that of untreated controls and the percent inhibition is calculated. For prediction of phototoxic potential the concentration responses obtained in the presence and in the absence of UV irradiation are compared.

## 2.3 HUMAN PATCH TESTING

The estimation of phototoxicity is an integral part of the required hazard information on cosmetic ingredients (SCCNFP, 2003). A sequential approach was applied, using first in vitro validated methods (validated 3T3 NRU PT and 3D human skin model) and in vivo (photo-patch test in human volunteers). The selection of volunteers and the test method were carried out in compliance with the ethical principles of the Declaration of Helsinki and subsequent revisions (CIOMS, 1993). The study was approved by the Ethical Review Committee of the National Institute of Public Health, Prague.

### *2.3.1 Brief description of the test*

**Endpoint and Endpoint Detection**: erythema and / or oedema development after irradiation of exposed skin areas.

**Brief procedure:**

The photo-patch test in human volunteers was performed on healthy females, age 26-61. Aqueous solutions of the test samples were applied in occlusion, using saturated filter paper discs (Finn Chamber, USA; diameter of 10 mm), on both forearms. The exposure time was 1 h or 4 h, depending on the toxicological profile of the tested chemical. Immediately after removal of the test patch, the irradiation of one forearm followed. The forearm was exposed to a dose of 5 J/cm$^2$ (as measured in the UVA range). The other non-irradiated forearm served as a control. Test reactions were recorded after 4h, 24 h, 48 h, 72 h and 7 days after irradiation. Standard grading scale using scores 0-4 (0 no erythema, 1 very slight, barely perceptible erythema, 2 well-defined erythema, 3 moderate erythema, 4 severe erythema).was used for the determination of the phototoxic effect of the test chemical.

## 2.4 TEST CHEMICALS

### *2.4.1 Phtalocyanines:*

Phthalocyanines belong to a new generation of substances for photodynamic therapy . They can be chelated with a variety of metals, chiefly aluminium and zinc, and these diamagnetic metals enhance their phototoxicity. A ring substitution in phthalocyanines with sulfonated groups will render them water soluble and affect their cellular uptake of the compound. Less sulfonated compounds, which are more lipophilic, exhibit the best membrane-penetrating properties and are the most active. The phthalocyanines are chemically pure compounds that absorb light (UVA maximum of the electromagnetic spectra at 350 nm and in visible region of the spectra between 650 and 700 nm with maximum in 670 nm).

These substances were considered as promising candidates for the estimation of the ability of the H3D-PT test to correctly predict effects (photopotency) of topical phototoxines. Pure monosulfonated, disulfonated, trisulfonated and tetrasulfonated hydroxyaluminium phthalocyanines (and their mixtures) were prepared by sulfonation of hydroxyaluminium phthalocyanine and subsequent chromatographic separation (see **Table 1**). Quality of the products were checked by capillary electrophoresis and HPLC.

**Tab 1. Characterisation of the test substances**

| Number | Composition | $LD_{50}$(mg/kg) |
|---|---|---|
| 951/105 | $ClAlPCS_x$- mixture | 150 |
| 938/292 | $ClAlPCS_x$- mixture | 300-320 |
| 951/166-2 | $ClAlPCS_x$- mixture | 375 |
| 951/198 | $OHAlPCS_2$ -2% mono-, 98% di-sulfonated | 180 |
| 951/188 | $OHAlPCS_3$ -1.4% di, 98.6% tri-sulfonated | 250 |
| 951/193 | $OHAlPCS_4$- 0.2% di, 1.6% tri, 98.2% tetra-sulfonated | >300 |
| 1009/35 | $OHAlPCS_2$ - (new) | >300 |

### *2.4.2 Sulfonated shale oils (class - Bituminous tars)*

Sulfonated shale oils are widely used in pharmaceutical, veterinary and cosmetic industries for their anti-microbial, anti-inflammatory and anti-pruritic effects (Fadrhoncova´, 1990). Bituminous tars are suggested as alternative substances with a similar spectrum of pharmacological action as coal tars (Warnecke, 1999). In contrast to a coal tar (banned from use in cosmetic products due to their mutagenic, carcinogenic and teratogenic effects (Commission Directive 97/45/EC, 1997)), no phototoxicity of bituminous tars has been reported in man. In our study, 2 pharmaceutical preparations (Ichthammol, Ichthyol pale) were tested (Ichthammol and Ichthyol pale) in parallel in 3T3 NRU test, EpiDerm PT test and in humans. Both Ichthammol and Ichthyol Pale are products of destructive distillation of bitumen separated from shale deposits containing fossilised fish (European Pharmacopeia, 1997). They consist of sulphur (about 10%), ammonium sulphate (5–7%), hydrocarbons, nitrogenous bases, acids, and thiophene derivatives (Rietschel and Fowler, 1995)

**Tab 2. Characterisation of the test substances**

| | CAS no. | supplier | relative density | sulphate ash | total sulphur | dry matter |
|---|---|---|---|---|---|---|
| **Ichthyol Pale** | [1340-06-3] | Herbacos -Bofarma, CZ | 1,15-1,25 | max.15%, | 11-13,5%, | 50-56% |
| **Ichthammol** | [8029-68-3] | Herbacos -Bofarma, CZ | 1,04-1,085 | max. 0,3% | max. 20% | 50-56% |

### *2.4.3 Plant extracts and oils*

**Litsea Cubeba**

Litsea Cubeba oil is an aromatic essential oil extracted from the fruits of Litsea Cubeba. It is used as a flavour enhancer in foods, cosmetics, and cigarettes; as a raw material in the manufacture of citral, vitamins A,E, and K, ionone, methyl ionone, and perfumes; and as an anti-microbial and insecticide (Man, L., et al., 2005; Luo, M. 2004). The chemical is volatile and has limited solubility in the water. It seems to have a remarkable anitoxidant effects (Hwang., J.K et al., 2005). Dominating components found in Litsea Cubeba oil are 1,8-cineole (0.2-51.7%), linalool (0.2-91.1%), sabinene (0-41,8%) and also alpha terpinelol.

The Litsea Cubeba oil used in our study was supplied from AROMA Praha, a.s., Czech Republic and BIOMEDICA, s.r.o., Czech republic. The content of active compounds was assessed using HPLC chromatography. The results are summarised in **Table 3.**

**Tab 3. Characterisation of the test substances (GC chromatography)**

| Name of componenets | Litsea Cubeba Biomedica % content | Litsea Cubeba Aroma % content |
|---|---|---|
| a-thujen | 0.12 | 0.13 |
| a-pinen | 2.29 | 2.10 |
| kamfen | 0.83 | 0.60 |
| sabinen | 4.20 | 4.88 |
| ß-pinen | 1.77 | 1.76 |
| myrcen | 1.14 | 1.78 |
| a-phelandren | 0.89 | 0.78 |
| a-terpinen | 0.17 | 0.26 |
| p-cymen | 1.65 | 1.60 |
| limonen | 11.81 | 12.60 |
| cis-ocimen | 0.06 | 0.06 |
| trans-ocimen | 0.13 | 0.10 |
| ?-terpinen | 0.29 | 0.45 |
| p,a-dimethylstyren | 0.84 | 0.78 |
| terpinolen | 0.35 | 0.38 |
| linalool | 2.42 | 2.52 |
| a-citronellal | 2.19 | 1.32 |
| iso-borneol | 0.38 | 0.37 |
| ?-terpinen-4-ol | 2.74 | 3.12 |
| a-terpineol | 1.15 | 1.13 |
| neral (z-citral) | 22.97 | 23.83 |
| linalylacetát | 1.33 | 1.12 |
| geranial (a-citral) | 28.87 | 29.81 |
| $C_{10}H_{16}O_2$ | 0.48 | n.d. |
| nerylacetát | 0.43 | 0.14 |
| a-copaen | 0.19 | 0.24 |
| $C_{15}H_{24}$ | 0.58 | 0.42 |
| ß-karyofylen | 2.40 | 2.13 |
| farnesen | 0.06 | 0.15 |
| a-karyofylen | 0.39 | 0.28 |
| germacren | 0.17 | 0.38 |
| karyofylen oxid | 0.48 | 0.17 |
| Not identified | 6.23 | 4.61 |
| Total | 100.00 | 100.00 |

**Bergamot oils**

Oil of bergamot is an extract from the rind of bergamot orange (Citrus aurantium ssp bergamia). Until a few years ago it had been widely used as an ingredient in cosmetics but was restricted or banned in most countries because of certain adverse effects. More recently, oil of bergamot preparations have been renewed popularity in aromatherapy. Native Oil of Bergamot possess photosensitive and melanogenic properties because of the presence of furocumarines, primarily bergapten (5-Methoxypsoralen [5-MOP]) (Kaddu, et al. 2001, whereas the modified bergamot oils do not suffer from this effect.

In our study Bergamot oils from four different supplieres (Sigma GmBH, Germany; Aroma Praha a.s., Czech Republic; Biomedica s.r.o., Czech Republic; Schupp GmbH, Germany). The content of Photo-active components was determined by GC chromatography.

**Tab 4. Characterisation of bergamot oils used in the study (GC chromatography)**

| | **Content of main photoactive components in Bergamot oil (%)** | | | | |
|---|---|---|---|---|---|
| | Bergapten | Citropten | Bergamotin | Geranial | Neral |
| Bergamot oil - Sigma | 0,13 | 0,13 | 0,86 | 0,04 | 0,48 |
| Bergamot oil - Schupp | 0,18 | 0,19 | 0,83 | n.d. | 0,43 |
| Bergamot oil - Biomedica | n.d. | n.d. | n.d. | n.d. | n.d. |
| Bergamot oil - Aroma | n.d. | n.d. | n.d. | n.d. | n.d. |

n.d.- not detected

### *2.4.4 Cosmetic UV filters:*

Several cosmetic UV filters were evaluated in current study. The selection was focused mainly on chemicals for which adverse photo-toxic effects (as photo-sensitisation and photo-allergy) were reported. Initially the 3T3 NRU-PT test was performed and if the result was positive or borderline, H3D-PT test with EpiDerm EPI-200 model was subsequently performed.

**Table 5: Test chemicals specification**

| **Name** | **INCI name** | **CAS-No.** | **Supplier** |
|---|---|---|---|
| Eusolex 9020 | Butyl methoxy-dibenzoylmethane | 70356-09-1 | Merck |
| Eusolex 2292 | Ethylhexyl Methoxycinnamate, BHT | 5466-77-3 | Merck |
| Eusolex 232 | Phenylbenzimidazole Sulfonic Acid | 27503-81-7 | Merck |
| Eusolex 4300 | Benzophenone-3 | 131-57-2 | Merck |
| Eusolex 6300 | 4-Methylbenzylidene Camphor | 36861-47-9 | Merck |
| TiO2 | TiO2 | | Sigma |
| Benzophenone-4 | 2-hydroxy-4-methoxynemzophenone -5 sulphonic acid | 4065-45-6 | Across Organics |

# 3 Results

## 3.1 PHTHALOCYANINES

Results obtained with the EpiDerm-PT assay indicate that phototoxic potency (LOAEL) decreases with increased degree of sulfonisation of the phthalocyanines. This is not obvious from the results obtained with the 3T3NRU-PT. 3T3NRU-PT which predicted similar EC50 values for all seven phthalocyanines (see **Table 6**). In the human patch test protocol even the strongest phthalocyanine ($OHAIPCS_2$) did not show phototoxicity when topically applied and activated with the same UV-vis light dose as used in the EpiDerm PT.

**Tab 6. Summary of results obtained with testing seven different phthalocyanines in the EpiDerm -PT at ZEBET and the 3T3 NRU-PT test performed at SZU Prague**.

| **Phthalocyanine** | **Sulfonisation** | **EpiDerm data (ZEBET)** | | **3T3 NRU data (SZU)** | | |
|---|---|---|---|---|---|---|
| | | NOAEL % | LOAEL % | EC50 (+UV) | EC50 (-UV) | PIF |
| 951/105 | $ClAIPCS_x$ | 0.003 | 0.01-0.03 | 3.4 | >100 | 29,4 |
| 938/292 | $ClAIPCS_x$ | 0.003 | 0.01 | 3.4 | >100 | 29,4 |
| 951/166-2 | $ClAIPCS_x$ | 0.003 | >0.003-0.01 | 1.7 | >100 | 58,8 |
| 951/198 | $OHAIPCS_2$ | 0.003 | >0.003-0.01 | 0.8 | >100 | 125,0 |
| 951/188 | $OHAIPCS_3$ | 0.003 | 0.03-0.1 | 2.2 | >100 | 12,2 |
| 951/193 | $OHAIPCS_4$ | 0.01-0.03 | 0.1-0.3 | no phototoxicity and cytotoxicity observed | | |
| 1009/35 | $OHAIPCS_2$ new | 0.001 | 0.01 | 1,1 | 100 | 96,9 |

(n = 2 independent dose-response or concentration-response experiments per chemical)

**Conclusion:**

The set of tested phtalocyanines have shown phototoxic potential in the two in vitro phototoxicity tests, the Epiderm human skin model and the 3T3 NRU PT. However, since the same test chemicals did not show any phototoxic potential in human patch testing in healthy human volunteers, phtalocyanines can not be used as reference chemicals for developing an *in vitro* photo-potency test for predicting photo-potency in humans. Therefore it was decided to select different class of phototoxic chemicals to develop photo-potency test.

## 3.2 SULFONATED SHALE OILS

**3T3 NRU PT test:**

Results obtained with the 3T3NRU-PT assay indicate that both pharmaceutical preparation shows phototoxic potency (see **Table 7**). Based on PIF and MPE factor, the 3T3 NRU test classifies "Ichthammol" as strong phototoxic and "Ichthyol pale" as phototoxic formulation

**Tab 7.** Performance in 3T3 NRU test

| **Test** | **3T3 NRU** | | **ET 50 (mg/l)** | | **Classification** |
|---|---|---|---|---|---|
| **substance** | PIF | MPE | + UVA | - UVA | |
| Ichthammol | 97.289 | 0,604 | 6.373 | 619.5 | strong phototoxic |
| Ichthyol pale | 5,132 | 0,292 | 114.7 | 591.1 | phototoxic |

**EpiDerm test :**

In EpiDerm PT-test (H3D-PT test), "Ichthammol" was predicted in concordance with 3T3-NRU PT test as phototoxic. However, the phototoxic potential of "Ichthyol pale" was not observed in the up to highest recommended concentration for use (see **Figure 1**).

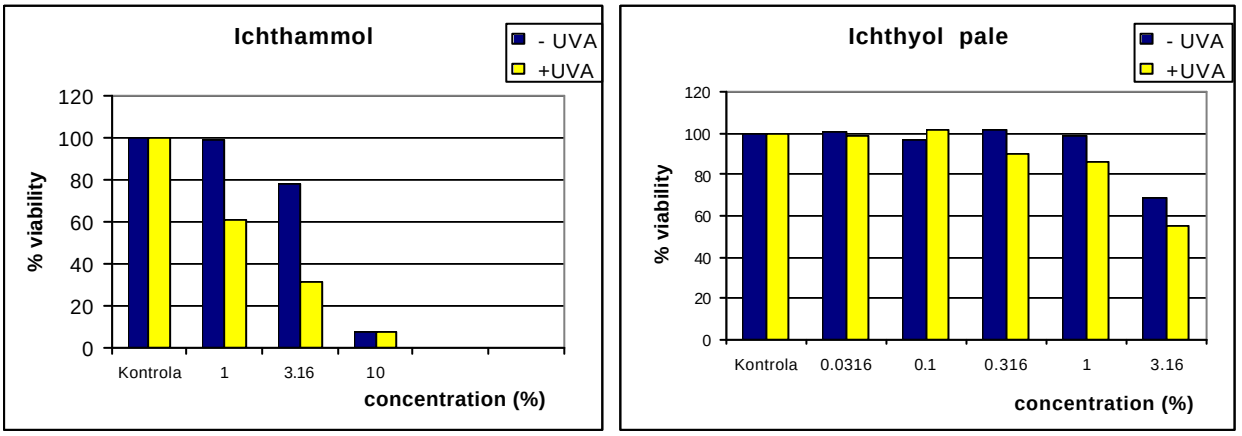


**Figure 1: Performance of the Ichthammol and Ichthyol pale in the H3D-PT test with EpiDerm EPI-200**

**Human patch test:**

In the human patch test protocol slight phototoxic effect of Ichthammol was observed in the concentration of 5 % (topically applied on forearm of test person). Well developed erythema was observed only when 10 % of Ichthammol was tested. Ichthyol pale demonstrated no phototoxic effect at concentration 5 and 10 % .

**Tab 8. Human patch test protocol with Ichthammol**

| **Test substance** | **Ichthammol 5 %** | | **Ichthammol 10 %** | |
|---|---|---|---|---|
| | 30 min | 4 h | 30 min | 4 h |
| immediate effect | no reaction | no reaction | slight erythema (+/-) 4 h after irradiation | erythema (+) 4-6 h after irradiation |
| 24 h after irradiation | no reaction | slight erythema (+/-) | slight erythema (+/-) | slight erythema (+/-) |
| 48 h after irradiation | no reaction | no reaction | no reaction | no reaction |
| 72 h after irradiation | no reaction | no reaction | no reaction | no reaction |
| 7 days after irradiation | no reaction | no reaction | no reaction | no reaction |
| **classification** | **slightly phototoxic** | | **phototoxic** | |

note : exposure : forearm; irradiation dose 5J UVA; solvent - water

**Tab 9: Human patch test protocol with Ichthyol pale**

| **Test substance** | **Ichthyol pale 5 %** | | **Ichthyol pale 10 %** | |
|---|---|---|---|---|
| | 30 min | 4 h | 30 min | 4 h |
| immediate effect | no reaction | no reaction | no reaction | no reaction |
| 24 h after irradiation | no reaction | no reaction | no reaction | no reaction |
| 48 h after irradiation | no reaction | no reaction | no reaction | no reaction |
| 72 h after irradiation | no reaction | no reaction | no reaction | no reaction |
| 7 days after irradiation | no reaction | no reaction | no reaction | no reaction |
| **classification** | **non-phototoxic** | | **non-phototoxic** | |

note : exposure : forearm; irradiation dose 5J UVA; solvent - water

**Conclusion:**

The two sulphonated shale oils (IIchthamol and Ichtyol pale) provided a clearly positive result in the 3T3 NRU PT. In the EpiDerm H3D PT Ichthammol provided positive result in all tested concentrations, while Ichtmammol pale did not show any phototoxic reaction up to highest tested dose. The results obtained in the H3D-test correlate quite well with the response observed in humans.

## 3.3 PLANT EXTRACTS AND OILS

### *3.3.1 LITSEA CUBEBA*

**3T3 NRU PT test:**

Results obtained with the 3T3NRU-PT assay indicate that in all three solvents Litsea Cubeba shows quite significant phototoxic effect (see **Table 10**). Interestingly, extremely high PIF factor was observed, by the sample diluted in PBS. This differences in photopotency may be explained by different "bio-availability" of photo-active compounds in the different solvents used.

**Tab. 10** Litsea Cubeba (AROMA) - phototoxic effect in three different solvents

| solvent | Run | PIF | MPE | ET 50 mg/l | |
|---|---|---|---|---|---|
| | | | | UV - | UV + |
| EtOH | 1 | 9,143 | 0,417 | 51,319 | 5,684 |
| | 2 | 6,484 | 0,195 | 57,159 | 8,832 |
| DMSO | 1 | 3,799 | 0,094 | 36,356 | 9,576 |
| | 2 | 3,976 | 0,048 | 38,81 | 9,766 |
| PBS | 1 | 59,344 | 0,620 | 62,654 | 1,103 |
| | 2 | 44,231 | 0,404 | 77,644 | 1,809 |

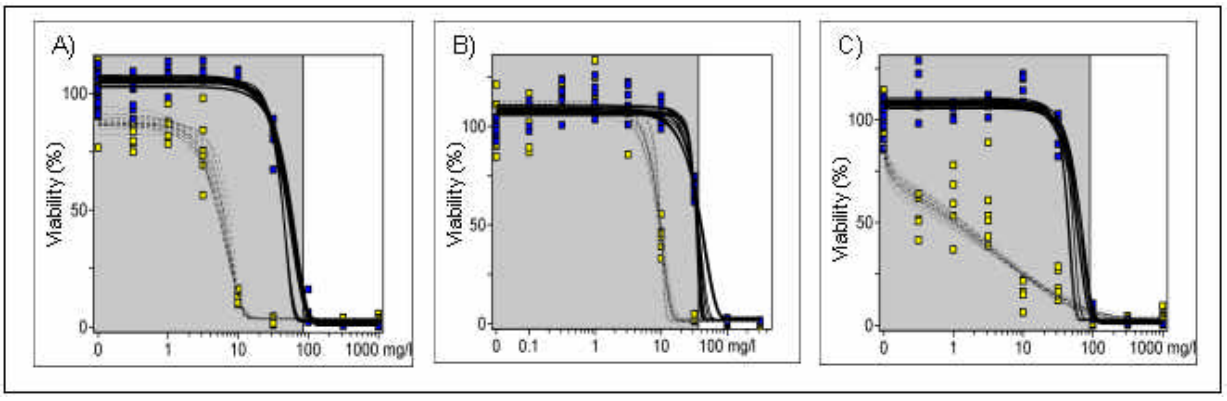


**Figure 2:** Phototoxicity of Litsea Cubeba in three different solvents:
a) in DMSO, b) in EtOH, c) in PBS

**EpiDerm PT- test:**

In the EpiDerm PT-test, the phototoxic effect of Litsea Cubeba was evaluated in two different solvents (water and sesami oil). In both solvents the highest non-phototoxic and at the same time non-cytotoxic concentration was 1 %. Based on results obtained in 2 independent experiment (Figure 3 and Figure 4), we conclude that Litsea Cubeba oil (AROMA Praha, a.s.) can be regarded as non-phototoxic.

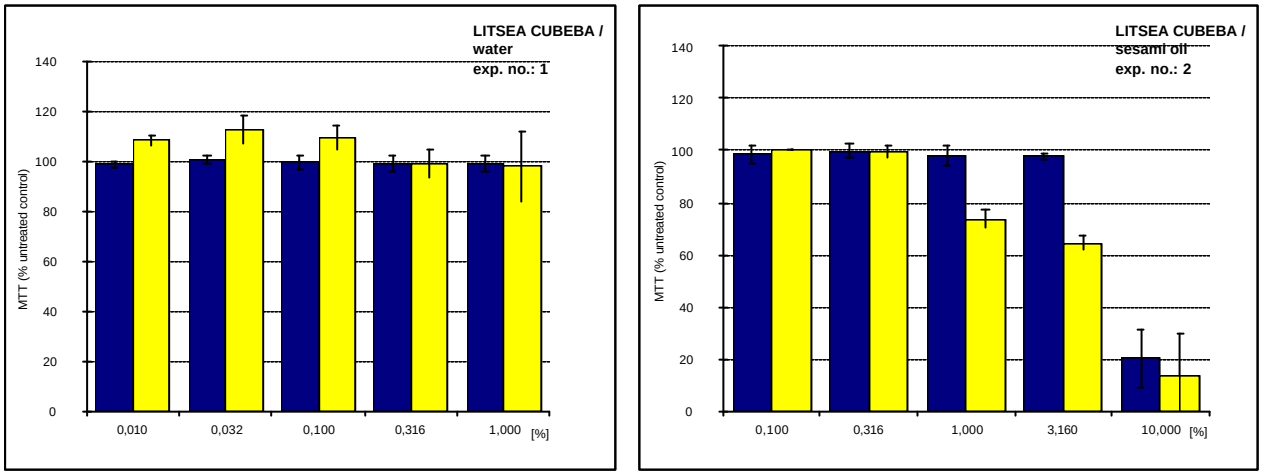


**Figure 3**: Phototoxic effect of Litsea Cubeba (in 2 different solvents) was evaluated on 2 different batches of EpiDerm (Run I).

a ) water (50 µl) - highest concentration 1 %

b) oil (25 µl) - highest concentration 10 %

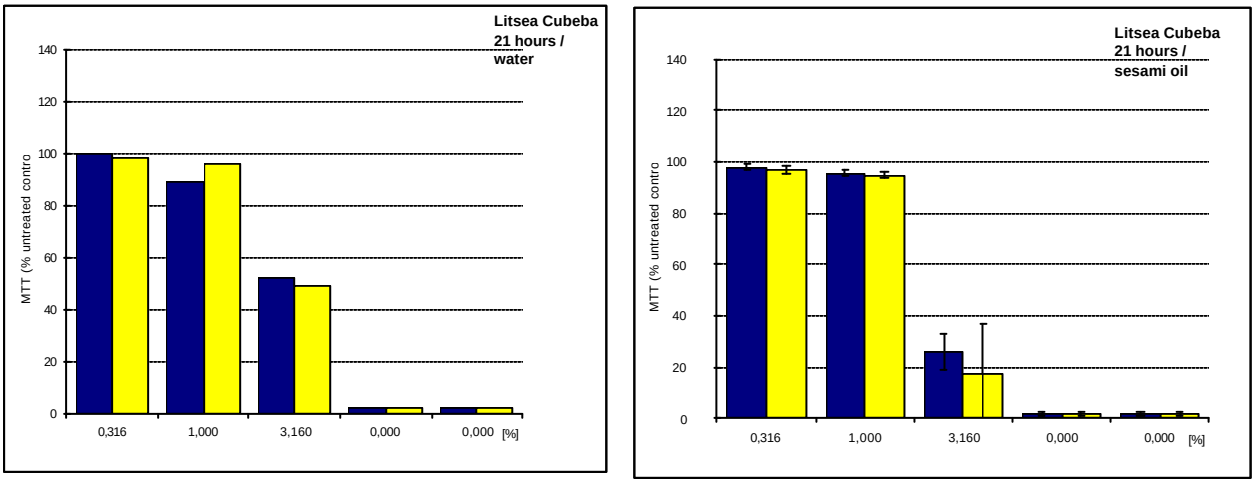


**Figure 4:** Phototoxic effect of Litsea Cubeba evaluated on the same batch of EpiDerm in two different solvents (Run II).

a ) water (50 µl) - highest concentration 3.16 %

b) oil (25 µl) - highest concentration 3.16%

**Human patch testing:**

In the human patch test, the test person was exposed to the test chemical 30 min and 4 hours. No phototoxic effect of Litsea Cubeba was observed in the concentration of 1% (topically applied on forearm of test person).

**Tab. 11 Litsea Cubeba, Human patch test**

| **Test substance** | **Litsea cubeba 1 %** | |
|---|---|---|
| | 30 min | 4 h |
| immediate effect | no reaction | no reaction |
| 24 h after irradiation | no reaction | no reaction |
| 48 h after irradiation | no reaction | no reaction |
| 72 h after irradiation | no reaction | no reaction |
| 7 days after irradiation | no reaction | no reaction |
| **classification** | **non-phototoxic** | |

*water -ethanol solution, 1 test person*

**Conclusion:**

Litsea Cubeba when tested in three different solvents provided a clearly positive phototoxic effect in the 3T3 NRU PT. In the H3D test, the substance did not provided any phototoxicity when tested in water and oil up to 1 %.

The photopatch test conducted in the healthy human volunteers provided negative result without any phototoxic reaction in the concentration of 1%. This result correlates well with outcome of the H3D test. Additional confirmatory testing on healthy volunteers have to be still conducted.

### *3.3.2 BERGAMOT OIL*

**3T3 NRU PT test:**

Amongst four different samples, two phototoxic and two non-phototoxic oils were identified by the 3T3 NRU PT. However, a couple of borderline classifications were also obtained.

**Tab 12. Bergamot oil "AROMA" - phototoxic effect in three different solvents**

| Solvent | Run | PIF | MPE | ET 50 | |
|---|---|---|---|---|---|
| | | | | UV - | UV + |
| EtOH | 1 | 1.218 | 0.027 | 36.31 | 30.22 |
| | 2 | 1.067 | -0.004 | 36.45 | 34.23 |
| DMSO | 1 | 1.006 | 0.005 | 40.82 | 40.71 |
| | 2 | 1.185 | -0.038 | 30.76 | 26.34 |
| PBS | 1 | 1.075 | 0.004 | 170.0 | 158.5 |
| | 2 | 0.905 | 0.064 | 210.5 | 233.5 |

**Tab 13. Bergamot oil "BIOMEDICA"- phototoxic effect in three different solvents**

| Solvent | Run | PIF | MPE | ET 50 | |
|---|---|---|---|---|---|
| | | | | UV - | UV + |
| EtOH | 1 | 1.377 | -0.004 | 58.86 | 42.78 |
| | 2 | 1.178 | 0.016 | 54.93 | 46.68 |
| DMSO | 1 | 1.051 | 0.003 | 43.26 | 41.23 |
| | 2 | 0.904 | -0.017 | 32.47 | 36.0 |
| PBS | 1 | 1.310 | 0.113 | 370.6 | 283.0 |
| | 2 | 1.113 | 0.031 | 186.6 | 167.9 |

**Tab 14. Bergamot oil "SIGMA" - phototoxic effect in three different solvents**

| Solvent | Run | PIF | MPE | ET 50 | |
|---|---|---|---|---|---|
| | | | | UV - | UV + |
| EtOH | 1 | 1.159 | 0.000 | 28.07 | 24.27 |
| | 2 | 1.383 | 0.147 | 38.8 | 28.07 |
| DMSO | 1 | 1.266 | 0.048 | 30.09 | 23.79 |
| | 2 | 1.666 | 0.144 | 56.74 | 34.09 |
| PBS | 1 | 1.648 | 0.017 | 287.2 | 174.6 |
| | 2 | 2.380 | 0.232 | 245.8 | 103.4 |

**Tab 15. Bergamot oil "SCHUPP"- phototoxic effect in three different solvents**

| Solvent | Run | PIF | MPE | ET 50 | |
|---|---|---|---|---|---|
| | | | | UV - | UV + |
| EtOH | 1 | 1.126 | 0.126 | 27.04 | 24.03 |
| | 2 | 1.358 | 0.156 | 24.87 | 18.38 |
| DMSO | 1 | 1.419 | 0.081 | 32.19 | 22.74 |
| | 2 | 1.410 | 0.088 | 34.74 | 24.66 |
| PBS | 1 | 2.581 | 0.289 | 113.3 | 44.0 |
| | 2 | 3.415 | 0.348 | 121.6 | 35.66 |

## EpiDerm PT- test:

In the EpiDerm H3D-PT test, the phototoxicity of Bergamot oils obtained by Sigma and Schupp was clearly demonstrated. The first non-phototoxic concentration of B.O. Schupp and Sigma was 0,1 %. Bergamot oils obtained from Biomedica and Aroma provided non-phototoxic result.

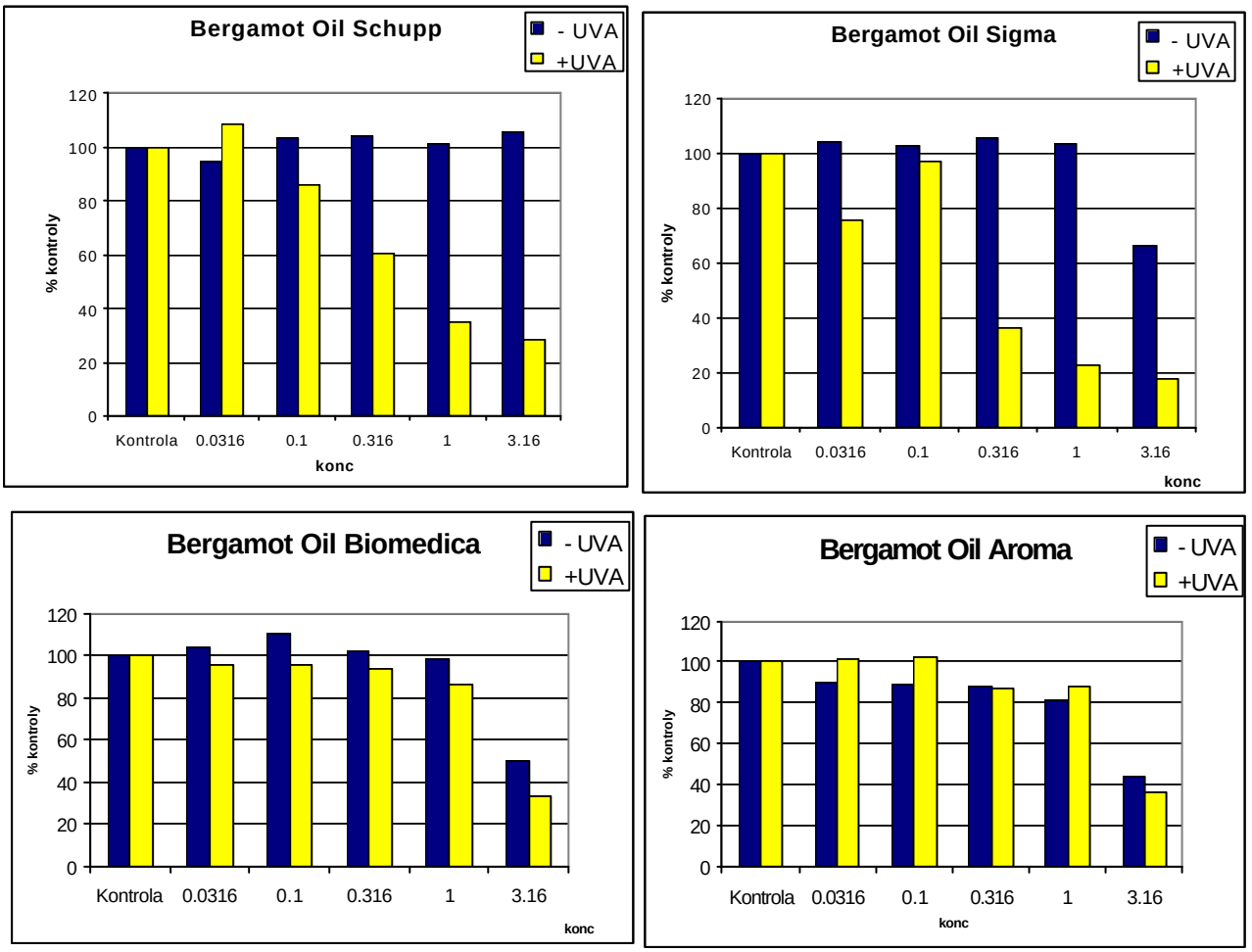


**Figure 5. Bergamot oils diluted in water (dose :50 µl)**

**Human patch testing**

In the human patch tests, the four substances were tested in concentrations determined to be safe with the EpiDerm H3D PT-test. The exposure time was 30 min and observation time lasted up to 7 days. For all samples, the concentrations selected provided to be safe. Only in case of B.O. Sigma, slight skin darkening was observed, which is however known side effect of the bergamot oil.

**Tab 16. Bergamot oil – Human patch test**

| **Test substance** | **Bergamot oil SIGMA** | **Bergamot oil SCHUPP** | **Bergamot oil BIOMEDICA** | **Bergamot oil AROMA** |
|---|---|---|---|---|
| exposure time | 30 min | 30 min | 30 min | 30 min |
| concentration tested | 0,1 % in water | 0,1 % in water | 1% in water | 1% in water |
| effect - immediate | no reaction | no reaction | no reaction | no reaction |
| 24h after irradiation | slight pigmentation<br>no erytheme or oedeme | no reaction | no reaction | no reaction |
| 48 h after irradiation | well developed pigmentation<br>no erytheme or oedeme | no reaction | no reaction | no reaction |
| 72 h after irradiation | well developed pigmentation<br>no erytheme or oedeme | no reaction | no reaction | no reaction |
| 1 week after irradiation | well developed pigmentation<br>no erytheme or oedeme | no reaction | no reaction | no reaction |
| **classification** | **questionable** | **non phototoxic** | **non phototoxic** | **non phototoxic** |

*exposure - forearm; irradiation dose 5J UVA; 3 test persons (diluted in water with addition of EtOH and applied on pads. Test performed in the occlusion)*

**Conclusion:**

Samples from 4 different suppliers were evaluated for phototoxicity. In addition to this study, analytical analysis (applying capillary GC/MS) was performed for identification and quantification of photoactive compounds present in the test samples. Besides bergapten, differences in citropten, bergamottin, geranial and neral content were identified. In can be concluded, that the different phototoxic effect depends on the amount of these components. Amongst 4 different samples, two phototoxic and two non-phototoxic oils were classified by 3T3 NRU PT, however, only on the basis of borderline phototoxicity results.

Surprisingly, even samples classified borderline proved to be clearly phototoxic in the EpiDerm test. In general, the skin model test and human patch test provided concordant results. In both cases, it was estimated that bergamot oils (classified as non-phototoxic by 3T3

NRU PT) were safe for use up to 1 %. The skin model test therefore seems to be a useful tool in the risk assessment, since it enables to set a margin of safety before any testing in humans.

Since different level of phototoxicity was observed in the 3T3 NRU PT test when three different solvents were used, it seems to be important to evaluate this effect also in the H3D PT test. Additional human patch tests in a larger group of human volunteers will still be necessary.

## 3.4 COSMETIC UV FILTERS

**Initial 3T3 NRU PT test:**

Seven UV-filter chemicals (used in cosmetic) were evaluated fot their phototoxicity in the 3T3 NRU PT test. Majority of these substances are known to be photo-allergens, a phototoxic effect can be expected.

Eusolex 9020 (Butyl methoxy-dibenzoylmethane) and Titanium Dioxide were classified to be phototoxic in the 3T3 NRU test. These two substances were further evaluated in the H3D and Human photo-patch test.

**Tab 17. Cosmetic UV filters - Initial 3T3 NRU PT test**

| Name | INCI name | Classification based on the 3T3 NRU-PT test | Remark |
|---|---|---|---|
| Eusolex 9020 | Butyl methoxy-dibenzoylmethane | Phototoxic | Futher test in the H3D-PT test |
| Eusolex 2292 | Ethylhexyl Methoxycinnamate, BHT | Non-phototoxic | |
| Eusolex 232 | Phenylbenzimidazole Sulfonic Acid | Non-phototoxic | |
| Eusolex 4300 | Benzophenone-3 | Non-phototoxic | |
| Eusolex 6300 | 4-Methylbenzylidene Camphor | Non-phototoxic | |
| TiO2 | TiO2 | Phototoxic | Futher test in the H3D-PT test |
| Benzophenone -4 | 2-hydroxy-4-methoxynemzophenone -5 sulphonic acid | Non-phototoxic | |

### *3.4.1 EUSOLEX 9020*

**3T3 NRU PT test:**

Two samples of Eusolex 9020 (Butyl methoxy-dibenzoylmethane), were tested. One sample was kindly provided by Dr. J. EOM (NITR, Korea) and another one was obtained from MERCK. Both samples proved to be strongly phototoxic.

**Tab 18. Eusolex 9020 - 3T3 NRU PT test**

| | **Eusolex 9020** (provided by Dr. EOM, NITR Korea) | | | | | **Eusolex 9020** (provided by Merck, Germany) | | | |
|---|---|---|---|---|---|---|---|---|---|
| **solvent** | **Run** | **PIF** | **MPE** | **ET 50 mg/l** | | **PIF** | **MPE** | **ET 50 mg/l** | |
| | | | | **UV -** | **UV +** | | | **UV -** | **UV +** |
| EtOH | 1 | 3,047 | 0,446 | n.d. | 39,64 | 5,451 | 0,215 | n.d. | 18,417 |
| | 2 | 10,26 | 0,558 | n.d. | 9,759 | not tested | not tested | not tested | not tested |
| DMSO | 1 | 16,254 | 0,521 | n.d. | 6,178 | 5,185 | 0,108 | n.d. | 19,495 |
| | 2 | 17,835 | 0,450 | n.d. | 5,650 | 3,504 | 0,114 | n.d. | 29,459 |
| **classification** | **phototoxic** | | | | | **phototoxic** | | | |

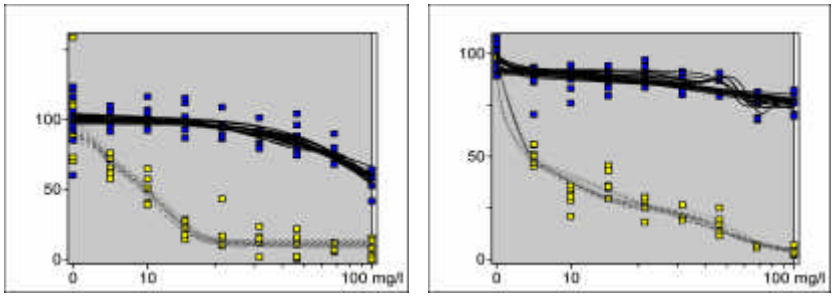


**Figure 6**: Eusolex 9020 (sample provided by Dr. J.H. Eom)

a) solvent Ethanol
b) solvent DMSO

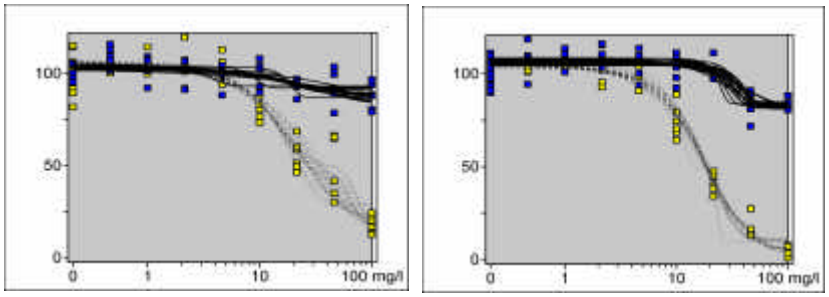


**Figure 7**: Eusolex 9020 (sample provided by Dr. J.H. Eom)

a) solvent Ethanol
b) solvent DMSO

**EpiDerm PT- test:**

In the EpiDerm H3D PT test, Eusolex 9020 proved to be non-phototoxic up to concentration of 10 % when tested as suspension in Sesami oil and Mygliol. Additional penetration studies were performed, to explain differences between the strong phototoxic effect in the 3T3 NRU PT test and H3D-test.

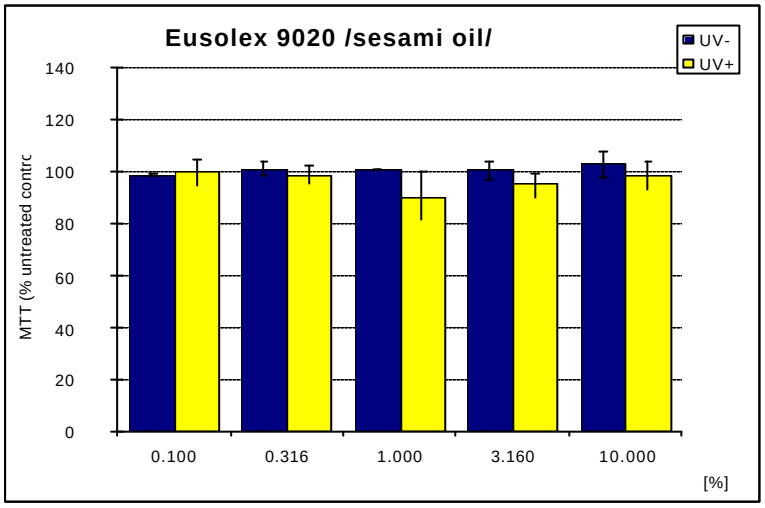


**Figure 8:** Eusolex 9020 – EpiDerm PT test

**Human patch testing**

Based on confidential reports provided by manufacturers of this substance and published data, the chemical is non phototoxic in humans. It is therefore listed in Annex VII of the European Cosmetics Directive 76/768 EEC. Human patch test performed on one human volunteer provided a negative result.

**Conclusion:**

Although Eusolex 9020 provided strong phototoxic effect in the 3T3 NRU-PT test, in the H3D PT test and test on one human volunteer, no phototoxicity was observed. Additional skin penetration studies are performed to confirm the hypothesis, that the substance does not penetrate the epidermis. Additional human volunteers test should be performed.

### *3.4.2 TITANIUM DIOXIDE*

**3T3 NRU PT test:**

Non-coated Titanium Dioxide (Sigma) provided strong phototoxic effect in the 3T3 NRU PT test. However, significant difficulties by testing of this substance revealed. $TiO_2$ is practically insoluble in all three recommended solvents. Therefore, suspensions of this substance were tested. Interestingly, although the cells absorb the substance in significant amount (see Figure 10), no cytotoxicity in the 3T3 NRU test was observed in the non-irradiated cells up to concentration 1000 mg/ml.

**Tab 19. TiO2 - 3T3 NRU PT test**

| | TiO2 (Sigma) | | | | |
|---|---|---|---|---|---|
| **solvent** | **Run** | **PIF** | **MPE** | **ET 50 mg/l** | |
| | | | | **UV -** | **UV +** |
| PBS | 1 | 7,3 | 0,39 | n.d. | 140.31 |
| | 2 | 4,1 | 1,1 | n.d. | 314,9 |
| | 3 | 1,5 | 0,654 | n.d. | n.d. |
| **classification** | | | **phototoxic** | | |

n.d. - not detected

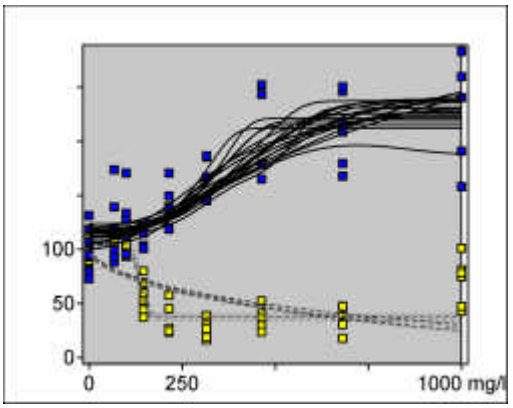


**Figure 9:** $TiO_2$ – 3T3 NRU test

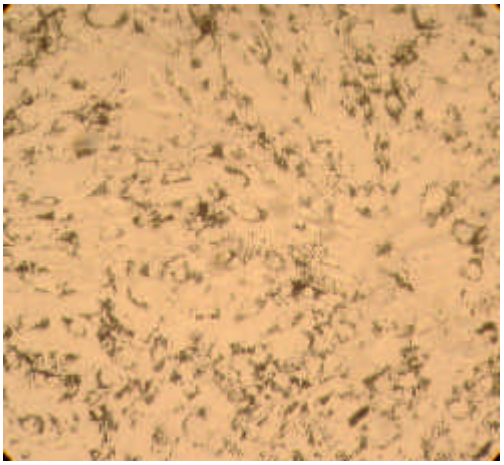

**Figure 11 :** Accumulation of $TiO_2$ in 3T3 cells - no cytotoxicity observed up to highest concentration tested

**EpiDerm PT- test:**

In the EpiDerm H3D PT tests, Titanium dioxide proved to be non-phototoxic up to concentration of 10 %. The chemical was tested as suspension in water and PBS.

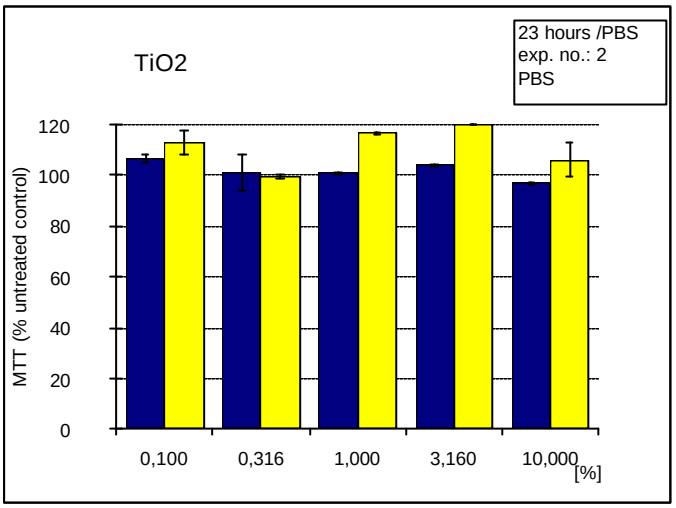


**Figure 10:** $TiO_2$ – EpiDerm PT test

**Human patch testing**

Titanium dioxide showed no phototoxicity when tested on forearm of one volunteer. Human photo-patch tests in a larger group of human volunteers still needs to be performed.

**Conclusion:**

$TiO_2$ provided strong phototoxic effect in the 3T3 NRU-PT test. Due to the insolubility of the substance in the recommended solutions, $TiO_2$ would be claimed in incompatible with the 3T3 NRU PT assay. The chemical would have to be further evaluated in another suitable model.

The advantage of H3D PT test is that it enables to test also substances with limited solubility. As demonstrated above, no phototoxicity was observed in the highest recommended concentration (10%). One human patch test was performed to prove the result of the H3D PT test. No phototoxic effect was observed at the highest tested dose (10%).

# 4 SUMMARY

A set of test chemicals from different chemicals classes were evaluated in two in vitro PT-tests, 3T3 NRU PT and EpiDerm H3D assay, and in addition in the human photo-patch test in healthy human volunteers.

First results indicate, that the EpiDerm H3D-test has ability to correctly predict the phototoxic potential of chemicals with regard to human response. The 3T3 NRU PT is over-predictive, since there is no barrier in this model comparable to barrier of the human or reconstructed skin.

In contract, the EpiDerm H3D-model and PT test provides data that are comparable to situation in humans. However, since the human patch tests were performed only in a limited group of human volunteers, further confirmatory testing is necessary.

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# ANNEX 1

| STUDY MANAGEMENT | |
|---|---|
| **Sponsor**<br><br>ECVAM<br>Institute for Health & Consumer Protection<br>Joint Research Centre, TP 580<br>21020 Ispra (VA),<br>Italy | **Sponsor representative**<br><br>Dr. Valerie Zuang<br>Tel.: +39-0332-78 58 43<br>Fax: +39-0332-78 53 36<br>e-mail: valerie.zuang@jrc.it |
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| | Laboratory contact:<br>Dr. Kristína Kejlová (3T3 NRU PT, H3D-test)<br>Dr. Hana Bendová (human patch tests)<br><br>Tel.: +420-267-082-327<br>e-mail: kejlova@szu.cz |
| **Time period of experiments:** 2003 - 2006 | |