5.4 PHOTOTOXICITY FEASIBILITY STUDY

A described in the Introduction (section 3.4), the aim of the phototoxicity feasibility study was to demonstrate, that the pre-validated human skin model phototoxicity test (H3D PT) (Liebsch *et al*., 1997) can be used as an adjunct test to the validated 3T3 neutral red uptake phototoxicity test (3T3 NRU PT).

It is known that several substances predicted as phototoxic by the 3T3 NRU PT are not phototoxic, when topically applied on skin in low concentrations. This effect is linked to their limited bio-availability in the skin (Liebsch *et al*., 1997, Jones *et al*., 2004, Jírova *et al*., 2005, Liebsch *et al*., 2005). It is assumed, that the bio-availability of the substances can be correctly determined in the *in vitro* skin model test, since the reconstructed human skin models closely resemble the native human epidermis (Liebsch *et al*., 1997, Jones *et al*., 2004; Liebsch *et al*., 2005). Confirmation of this hypothesis (preferably in the validation trial) would lead into the official implementation of the skin model phototoxicity test into sequential testing strategy for substances absorbing UV light (EMEA, 2002).

The obstacle for performing a straightforward study is a lack of suitable chemicals which could be used for evaluation of the hypothesis. The substances should be compatible with both *in vitro* assays and in addition, should not have a dangerous toxicological profile, because the results obtained in H3D PT should be later evaluated on human volunteers. Therefore the selection of appropriate test substances presented the most complicated step in the whole study. Finally, two groups of photo-active compounds were selected; UV filters and essential plant oils containing low amounts of photo-active ingredients.

All substances were first tested in the 3T3 NRU PT and if the result was positive, the test chemicals were further evaluated in the human skin models phototoxicity test (H3D PT). A photo-patch study with human volunteers is currently performed at the National Institute of Public Health, Prague, Czech Republic with aim to evaluate the correct prediction of the skin model test.

5.4.1 RESULTS OBTAINED WITH UV FILTERS

In the phototoxicity feasibility study five cosmetic UV filters and non-coated titanium dioxide were tested. These chemicals have no potency to cause acute photo-irritation in man, however it is known that they may induce in small group of sensitised persons photo-allergy. Due to this effect, it was assumed that some of these chemicals may show over-prediction in the validated 3T3 NRU PT assay and thus could be used for phototoxicity feasibility study.

3T3 NRU PT

Of the six chemicals tested in the validated 3T3 NRU PT, two showed significant phototoxicity. These were: butyl methoxy-dibenzoylmethane (BM-DBM) and titanium dioxide (TiO_2) (Table 37).

Table 37. Results obtained in the 3T3 NRU PT assay.

No	Trade Name	INCI name	Run	Solvent	PIF	MPE	IC 50 mg/l		Classification (OECD 432)
							UV -	UV +	
1	Eusolex 9020	Butyl Methoxy-dibenzoylmethane	1	DMSO (1%)	3.504	0.114	n.d.	19.495	Probably phototoxic
			2	DMSO (1%)	5.158	0.108	n.d.	29.459	Phototoxic
			3	EtOH (1%)	5.451	0.215	n.d.	18.417	Phototoxic
			4	EtOH (1%)	5.363	0.213	n.d.	18.67	Phototoxic
2	Eusolex 232	Phenylbenzimidazole Sulfonic Acid	1	PBS	n.d.	0.084	n.d.	n.d.	Non-phototoxic
3	Eusolex 4300	Benzophenone-3	1	EtOH (1%)	0.965	-0.002	39.515	40.986	Non-phototoxic
			2	EtOH (1%)	1.019	0.094	36.554	36.663	Non-phototoxic
4	Eusolex 6300	4-Methylbenzylidene Camphor	1	EtOH (1%)	2.123	0.0212	6.593	3.119	Probably phototoxic
			2	EtOH (1%)	0.798	-0.012	6.648	8.342	Non-phototoxic
5	Benzophenone-4	2-hydroxy-4-methoxynemzophenone -5 sulphonic acid	1	DMSO (1%)	n.d.	0.137	n.d.	n.d.	Non-phototoxic
			2	DMSO (1%)	n.d.	0.029	n.d.	n.d.	Non-phototoxic
6	Titanium Dioxide	Titanium Dioxide (TiO_2)	1	PBS-disp.	7.296	0.391	n.d.	140.311	Phototoxic
			2	PBS-disp.	4.053	1.093	n.d.	314.908	Phototoxic
			3	PBS-disp.	1.488	0.654	n.d.	n.d.	Phototoxic
7	Chlorpromazine	Positive control	1	PBS	22.669	0.318	30.256	1.336	Phototoxic
			2	PBS	22.806	0.285	23.507	0.988	Phototoxic
			3	PBS	45.883	0.537	29.501	0.644	Phototoxic

„PBS-disp." – dispersion in PBS, n.d. – not decetable. A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

Butyl methoxy-dibenzoylmethane was poorly soluble in PBS therefore it was necessary to pre-dissolve the chemical in an appropriate solvent. Since the BM-DBM was well soluble in both solvents recommended by the OECD TG 428 (ethanol and DMSO) both types of solutions were evaluated in the 3T3 NRU PT assay.

First two runs (solvent PBS, 1% DMSO) revealed the Photo Irritation Factor (PIF) 3.54 and 5.158, which classify the chemical as phototoxic. Similar results were obtained in the other two experiments (solvent PBS, 1% EtOH), where a PIF of 5.451 and 5.363 classified the chemical as a clearly phototoxic. Moreover, in the second solvent the Mean Phototoxic Effect (MPE) was more than 0.15 which would also classify the chemical as

phototoxic. Examples of the dose response curves obtained in the 3T3 RU PT assay of the BM-DBM are given in Figure 39.

In both cases the IC 50 concentration for irradiated part of the experiment can be found in range of 15-30 mg/l, which indicate relatively high phototoxicity of the BM-DBM. No significant differences in results were obtained when the two different solvents were used.

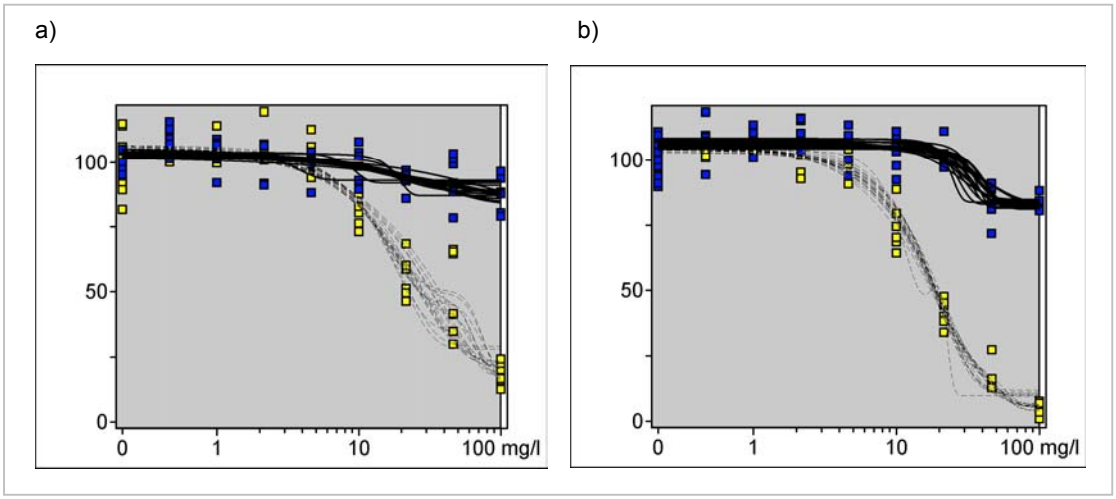

Figure 39. Example of a dose-response curves of butyl methoxy-dibenzoylmethane (graphics generated by Phototox software, version 2.0 (Holzhütter, 1989; Peters and Holzhütter, 2003).
The blue dots represent non-irradiated part of the experiment, the yellow belongs to the irradiated part.
a) BM-DBM in PBS/ DMSO (1%) solution
b) BM-DBM in PBS/ EtOH (1%) solution

Due to the insolubility of titanium dioxide in water, PBS or other solvents, testing on cell monolayers was limited. The dispersions of TiO_2 in a concentration range 10 – 1000 mg/l revealed only low cytotoxicity to 3T3 cells. Surprisingly, although TiO_2 was absorbed into cell organelles (see Figure 40), the basic cell functions seemed to be not disturbed during the two days observation period and in the 3T3 neutral red uptake viability test performed thereafter.

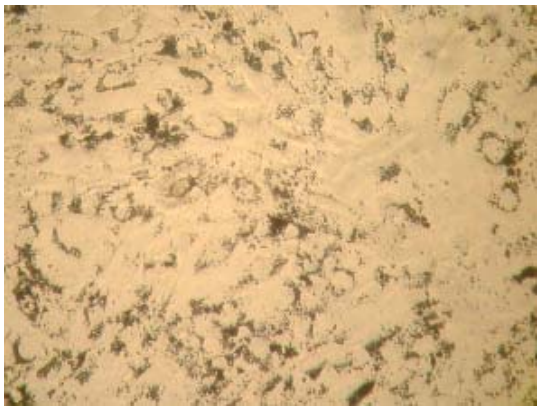

Figure 40. Phase contrast image of TiO_2 particles absorbed in the mouse fibroblast cell line 3T3.

In the 3T3 NRU PT, titanium dioxide revealed clear phototoxicity in all experiments. Although the PIF and MPE factors given in Table 37 indicate phototoxicity, these values are not relevant, because the dose response curves obtained in the NR assay (Figure 41) were affected by an increasing level of particles reflecting light. Except for negative controls and blanks, the TiO_2 particles were present in all wells of the 96 well plates and therefore the absorbance measurements by multiwell reader was not adequate to observed situation. However, the intensity of the phototoxic effect was very clear when the irradiated and non-irradiated plates were compared visually (see Figure 42).

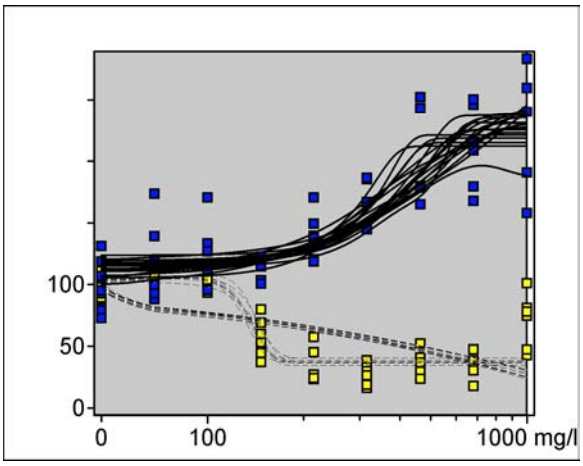

Figure 41. Example of dose-response curves of TiO_2 (graphic generated by Phototox software, version 2.0 (Holzhütter, 1989; Peters and Holzhütter, 2003).
The blue dots represent non-irradiated part of the experiment, the yellow belongs to the irradiated part.

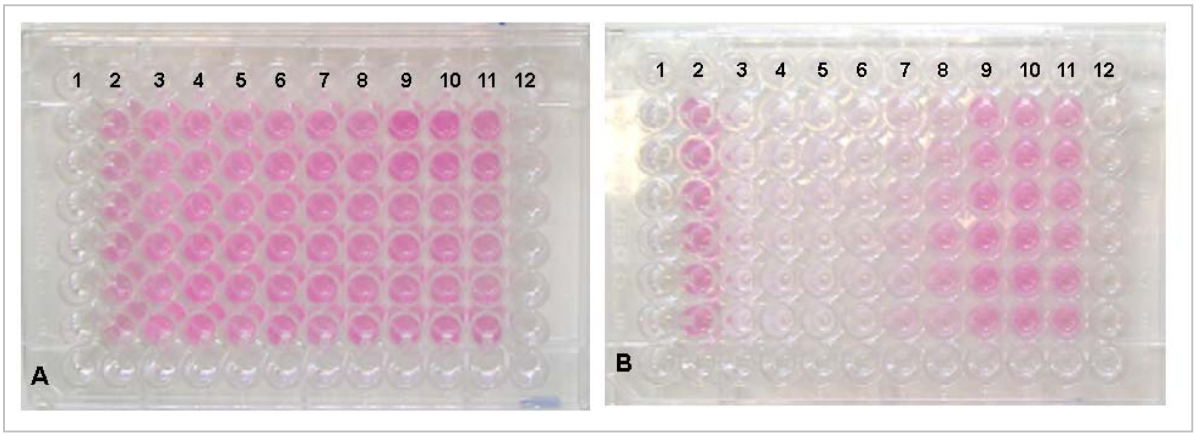

Figure 42. Non irradiated and irradiated part of the experiment with TiO_2.
Eight concentration of TiO_2 in range of 50 -1000 mg/ml were tested (column 3-10). Columns 2 and 11 were negative controls (non treated cells). Non irradiated plate (a) shows the same level of cell viability up to highest concentration of TiO_2, while irradiated plate (b) revealed strong phototoxic effect in range 150 mg/ml -1000 mg/ml of TiO_2 (column 3-8).

H3D PT using EpiDerm model

According the proposed strategy for testing of phototoxic chemicals, butyl methoxy-dibenzoylmethane and TiO_2 were further evaluated in the reconstructed human skin model phototoxicity test (H3D PT) using the pre-validated EpiDerm phototoxicity assay described by Liebsch *et al*. (1997).

Butyl methoxy-dibenzoylmethane was dissolved in sesame oil and tested in five concentrations on reconstructed human epidermal model EpiDerm (EPI-200). In the two independent experiments, no phototoxicity and cytotoxicity was observed up to concentration of 10% (w/v) (Figure 43 a).

Titanium dioxide was tested in five concentrations as suspension in deionised water. Similarly as with butyl methoxy-dibenzoylmethane, no phototoxicity and cytotoxicity was observed up to concentration 10% (w/v) (Figure 43 b).

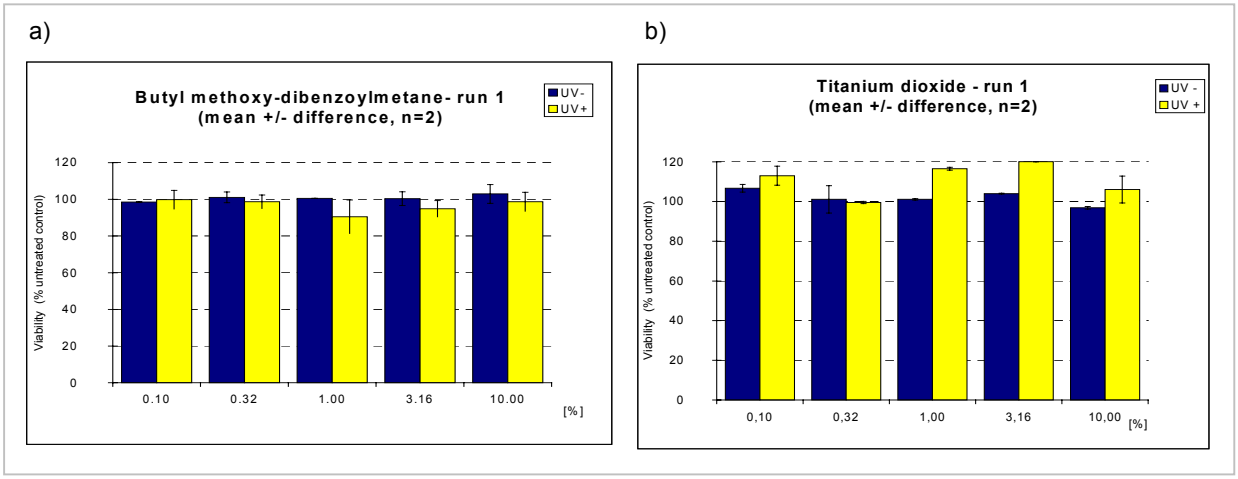

Figure 43. Phototoxicity of (a) butyl methoxy-dibenzoylmethane and (b) titanium dioxide evaluated in the EpiDerm phototoxicity test (representative figures).

Titanium dioxide and Butyl methoxy-dibenzoylmethane are commonly used in cosmetic products and it is known that they do not cause acute phototoxicity in man even if used in relatively high concentrations. However, for completeness of the study, both chemicals will be evaluated in a photo-patch study in human volunteers at the National Institute of Public Health, Prague, Czech Republic.

5.4.2 RESULTS OBTAINED WITH ESSENTIAL PLANT OILS

The second group of substances evaluated in the phototoxicity feasibility were essential plant oils. Impure plant extracts and essential oils frequently show various dermatological “photo–effects”, e.g. photosensitisation, pigmentation and photo-allergy, mainly when used in high concentrations. Therefore, these types of substances were suitable candidates for the estimation of the first non-phototoxic concentration, which could be safely used in humans.

Two essential oils, *Litsea Cubeba* extract and Bergamot oil were evaluated in the both *in vitro* tests. Moreover, Bergamot oil purchased from four different suppliers was analysed with aim to investigate the variability of results within different samples. The first non-phototoxic concentration determined by the H3D PT test was subsequently evaluated in a human patch test at the National Institute of Public Health, Prague.

LITSEA CUBEBA

3T3 NRU PT

Litsea Cubeba was well soluble in ethanol as well as in DMSO and revealed satisfactory solubility also in PBS. The extract was therefore tested in all three solvents in the 3T3 NRU PT to evaluate, if the different solvent has an impact on a final phototoxicity.

In all three solvents, *Litsea Cubeba* proved to be phototoxic (see Table 38 and Figure 44). An extremely high PIF factor (and thus also phototoxicity) was obtained with sample diluted in PBS. This experiment showed also the highest variability, which is most probably linked to the limited solubility of the substance in PBS. Interestingly, samples diluted in DMSO, showed the lowest phototoxicity and according to the OECD TG 432 would *Litsea Cubeba* gain classification only as “probably phototoxic. The differences between the phototoxicity of the three solutions could be explained by different "bio-availability" of photo-active compounds in the three solvents.

Table 38. Litsea Cubeba - phototoxic effect in three different solvents

solvent	Run	PIF	MPE	ET 50 mg/l		Classification
				UV -	UV +	(based on OECD TG432)
EtOH	1	**9.143**	**0.417**	51.319	5.684	Phototoxic
	2	**6.484**	**0.195**	57.159	8.832	Phototoxic
DMSO	1	**3.799**	0.094	36.356	9.576	Probably phototoxic
	2	**3.976**	0.048	38.81	9.766	Probably phototoxic
PBS	1	**59.344**	**0.620**	62.654	1.103	Phototoxic
	2	**44.231**	**0.404**	77.644	1.809	Phototoxic

A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

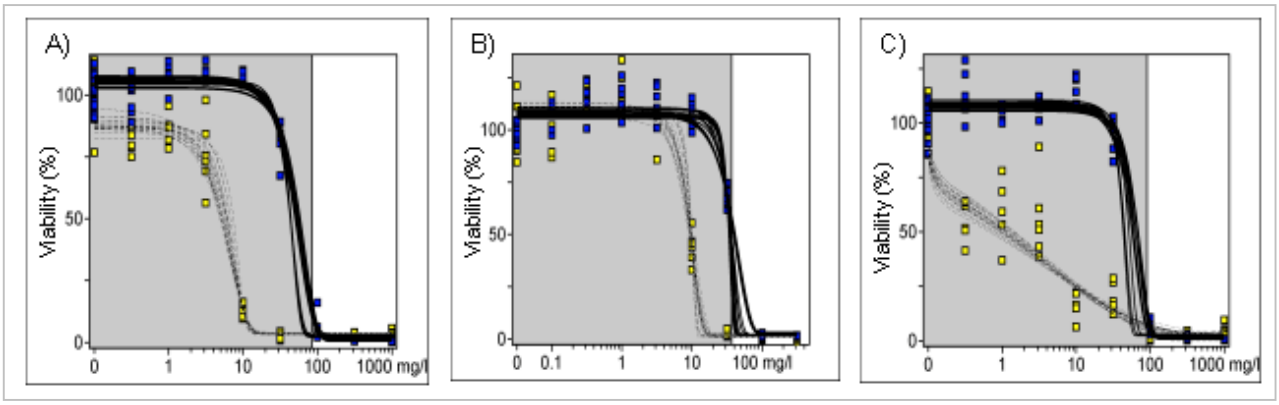

Figure 44. Phototoxicity of Litsea Cubeba in three different solvents:
a) in PBS/DMSO (1%), b) in PBS/EtOH (1%), c) in PBS
(graphic generated by Phototox software, version 2.0 (Holzhütter, 1989; Peters and Holzhütter, 2003).

H3D PT using EpiDerm model

To follow the interesting observation of the phototoxic effect in different solvents and 3T3 NRU PT, two solvents (water and sesame oil) were used in the H3D PT to dilute *Litsea Cubeba* to appropriate test concentrations.

In the two independent experiments (in both solvents) the highest non-phototoxic and at the same time non-cytotoxic concentration was 1 %. Exception was experiment performed in the first run, with *Litsea Cubeba* dissolved in oil, where slight decrease of viability after irradiation in 1% concentration was observed (see Figure 45 b). In the second run, no phototoxic or cytotoxic reaction was observed; the tissue viability compared to negative controls was 95% (UVA + part of the experiment) and 96 % (UVA - part of the experiment). Based on results obtained in both independent experiments, it can be concluded, that *Litsea Cubeba* oil can be regarded as non-phototoxic up to concentration 1%.

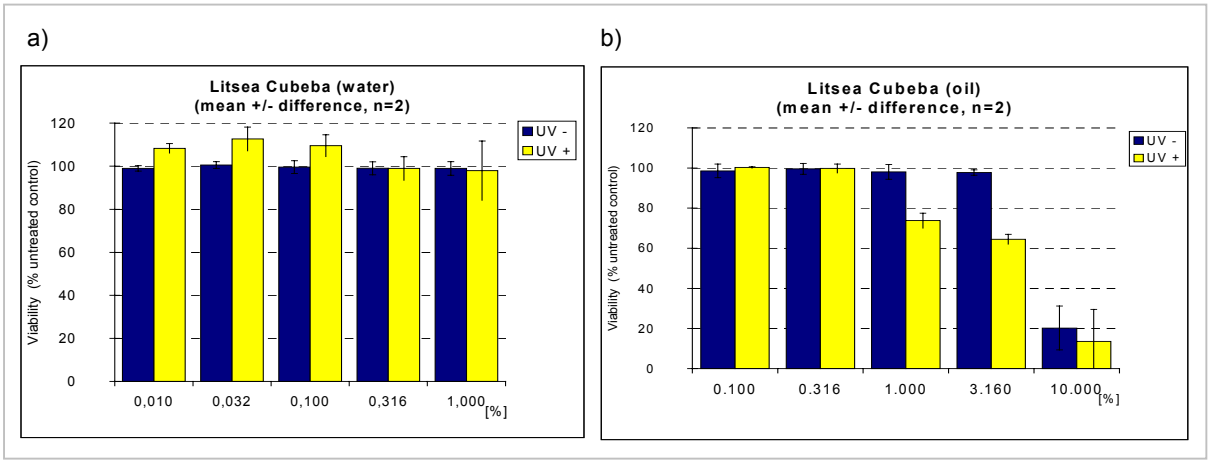

Figure 45. Phototoxicity of *Litsea Cubeba* evaluated in the EpiDerm Phototoxicity test.
a) water (50 µl); highest concentration tested: 1 % b) oil (20 µl); highest concentration tested: 10 %

Human patch testing

In the photo-patch test performed at National Institute of Public Health, Prague, Czech Republic, the test persons were exposed to highest non-phototoxic concentration determined in the H3D PT test (1%) for 30 min and 4 hours. Immediately after irradiation, and also during the following observation time which lasted up to 7 days, Litsea Cubeba caused no phototoxic reaction (Kejlová, personal communication 2005).

BERGAMOT OIL

3T3 NRU-PT assay

Four different samples of Bergamot oil (obtained from four different suppliers – for details see Materials and Methods) were tested in the 3T3 NRU PT. Two phototoxic and two non-phototoxic oils were identified by the 3T3 NRU PT. However, a couple of borderline classifications were also obtained.

Table 39. Bergamot oil "AROMA" - phototoxic effect in three different solvents

Solvent	Run	PIF	MPE	ET 50		Classification
				UV -	UV +	(based on OECD TG432)
EtOH	1	1.218	0.027	36.31	30.22	Not phototoxic
	2	1.067	-0.004	36.45	34.23	Not phototoxic
DMSO	1	1.006	0.005	40.82	40.71	Not phototoxic
	2	1.185	-0.038	30.76	26.34	Not phototoxic
PBS	1	1.075	0.004	170.0	158.5	Not phototoxic
	2	0.905	0.064	210.5	233.5	Not phototoxic

A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

Table 40. Bergamot oil "BIOMEDICA"- phototoxic effect in three different solvents

Solvent	Run	PIF	MPE	ET 50		Classification
				UV -	UV +	(based on OECD TG432)
EtOH	1	1.377	-0.004	58.86	42.78	Not phototoxic
	2	1.178	0.016	54.93	46.68	Not phototoxic
DMSO	1	1.051	0.003	43.26	41.23	Not phototoxic
	2	0.904	-0.017	32.47	36.0	Not phototoxic
PBS	1	1.310	0.113	370.6	283.0	Not phototoxic
	2	1.113	0.031	186.6	167.9	Not phototoxic

A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

Table 41. Bergamot oil "SIGMA" - phototoxic effect in three different solvents

Solvent	Run	PIF	MPE	ET 50		Classification
				UV -	UV +	(based on OECD TG432)
EtOH	1	1.159	0.000	28.07	24.27	Not phototoxic
	2	1.383	**0.147**	38.8	28.07	**Probably phototoxic**
DMSO	1	1.266	0.048	30.09	23.79	Not phototoxic
	2	1.666	0.144	56.74	34.09	Not phototoxic
PBS	1	1.648	0.017	287.2	174.6	Not phototoxic
	2	**2.380**	**0.232**	245.8	103.4	Phototoxic

A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

Table 42. Bergamot oil "SCHUPP"- phototoxic effect in three different solvents

Solvent	Run	PIF	MPE	ET 50		Classification
				UV -	UV +	(based on OECD TG432)
EtOH	1	1.126	0.126	27.04	24.03	Not phototoxic
	2	1.358	**0.156**	24.87	18.38	**Probably phototoxic**
DMSO	1	1.419	0.081	32.19	22.74	Not phototoxic
	2	1.410	0.088	34.74	24.66	Not phototoxic
PBS	1	**2.581**	**0.289**	113.3	44.0	**Phototoxic**
	2	**3.415**	**0.348**	121.6	35.66	**Phototoxic**

A PIF more than 5 and /or MPE more than 0.15 classify the substance as phototoxic (for details about the prediction model see section 4.2.4.1).

As demonstrated in Table 41 and 42, different solvents significantly influence the final prediction. While samples diluted in DMSO and ethanol show almost no phototoxicity, PBS dilutions caused clear phototoxicity in the 3T3 NRU PT. A Similar effect was observed also with *Litsea Cubeba* extract.

EpiDerm Phototoxicity Test

All four samples of Bergamot oil were subjected to EpiDerm phototoxicity test. Because the influence of the solvent was obvious in previously performed experiments, all four samples were diluted both in water and sesame oil to assess the solvent effect. The representative results are shown below.

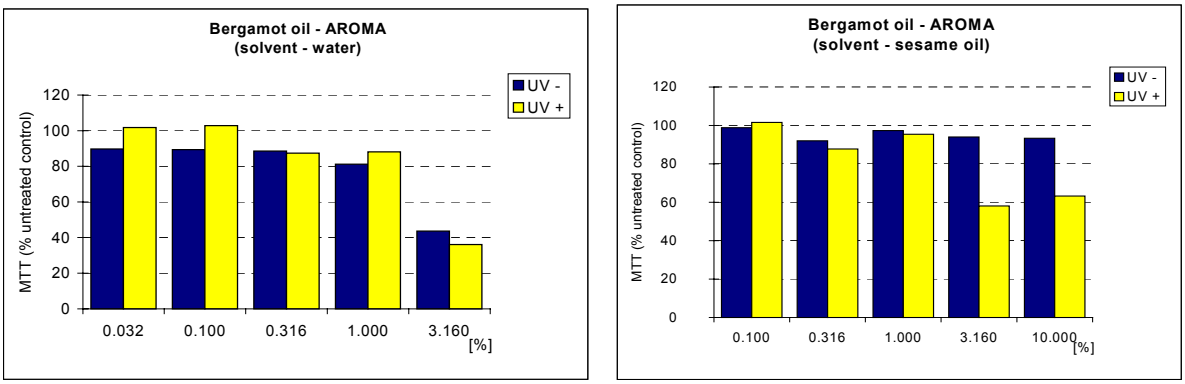

Figure 46. Bergamot oil (supplier AROMA) - Run I. a) water dilutions (volume 50 µl); b) sesame oil dilutions (volume 20 µl)

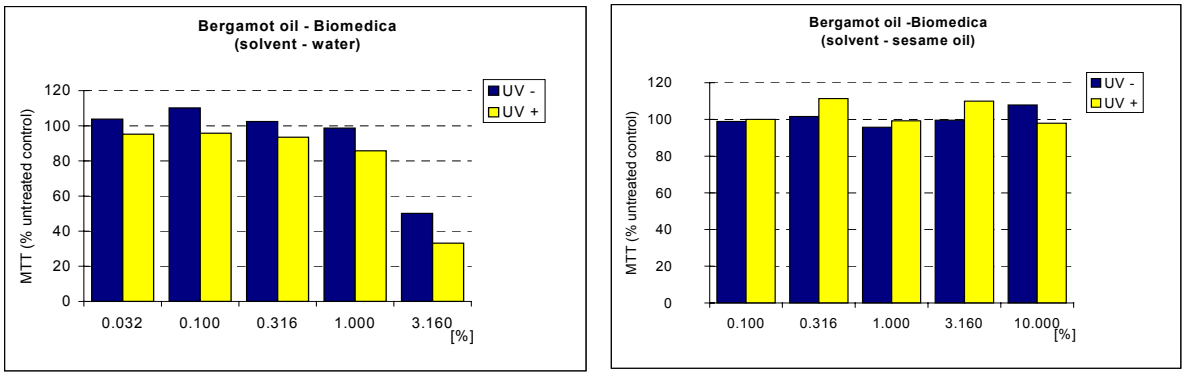

Figure 47. Bergamot oil (supplier Biomedica) - Run I. a) water dilutions (volume 50 µl); b) sesame oil dilutions (volume 20 µl).

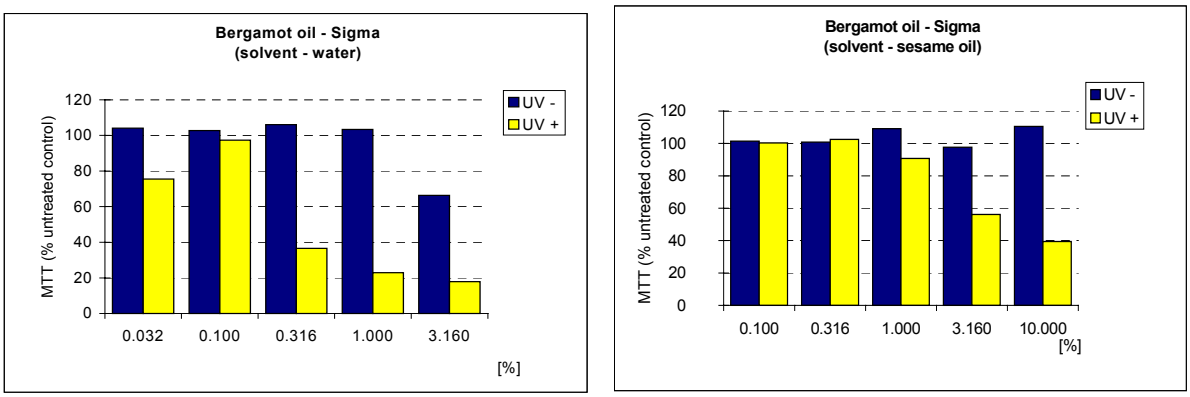

Figure 48. Bergamot oil (supplier Sigma) - Run I. a) water dilutions (volume 50 µl); b) sesame oil dilutions (volume 20 µl).

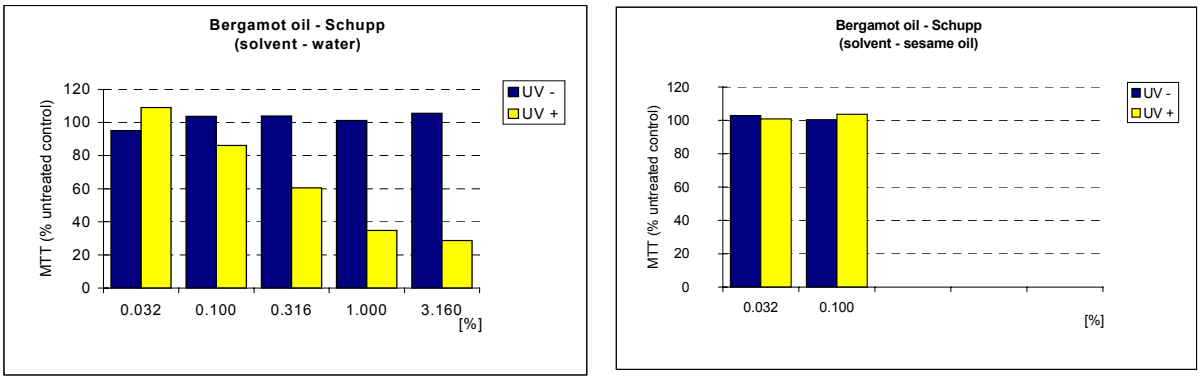

Figure 49. Bergamot oil (supplier Schupp) - Run I. a) water dilutions (volume 50 µl); b) sesame oil dilutions (volume 20 µl).

As can be seen from in Figure 46 and 47, Bergamot oils from Aroma and Biomedica were non-phototoxic in both solvents up to a concentration of 1 %. The first phototoxic reaction and at the same time first cytotoxic reaction was induced in concentration 3.16 %. Slightly different results were obtained with dilutions in oil. Bergamot oil “Biomedica” was non-phototoxic and non-cytotoxic up to highest concentration tested (10%). Bergamot oil “Aroma” revealed phototoxic reactions only in high concentrations: 3.16 % and 10 %. Bergamot oil “Sigma” diluted in water, however, induced clear phototoxic effect in the low concentration of 0.316 %. Interestingly, the phototoxicity of oil solutions was not so high (see Figure 48b). Bergamot oil obtained from supplier “Schupp” was non-phototoxic up to concentration 0.1 % in both solvents (Figure 49).

Summarising the outcome given above, in the H3D PT, Bergamot oil “Biomedica” and “Aroma” (both non phototoxic in the 3T3 NRU PT) proved to non-phototoxic up to concentration 1 %, while Bergamot oils from Sigma and Schupp approved to be safe only up to concentration 0.1 %.

Human patch testing

In the small-scale photo-patch test performed at National Institute of Public Health, Prague, Czech Republic, five test persons were exposed to all four samples of Bergamot oil. Only these test concentrations were used in the human patch test, where no phototoxic and no cytotoxic effect was observed in the previously performed H3D PT. Water/ethanol dilutions of Bergamot oils were tested in human volunteers to mimic their use in cosmetics (e.g. perfumes). The final concentration of Bergamot oil Aroma and Biomedica was 1%, Bergamot oil “Sigma” was tested as and 0.1 % solution and Bergamot Oil Schupp as 0.0316 % solution.

The results of the study are summarised in Table 43. Based on the outcome of the skin model test, it was estimated that Bergamot oils “Aroma” and “Biomedica” were safe for use up to concentration 1 %. Indeed, when tested in man no skin reactions were observed. When testing Bergamot oil “Schupp”, no immediate acute phototoxic reaction (erythem/oedema) was observed, however, slight pigmentation occurred after 72h after irradiation. In case of Bergamot oil “Sigma” slight erythem was observed immediately after irradiation, which changed after 24 h into the pigmentation which persisted until the end of the observation period (7 days).

Table 43. Small-scale human patch test with four Bergamot oils (performed at the National Institute for Public Health, Prague Czech Republic).

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	0.0316 % in water	1% in water	1% in water
Effect – immediate	Erythem (+/ -)	no reaction	no reaction	no reaction
24h after irradiation	Slight pigmentation no erythem or oedema	no reaction	no reaction	no reaction
48 h after irradiation	well developed pigmentation no erythem or oedema	no reaction	no reaction	no reaction
72 h after irradiation	well developed pigmentation no erythem or oedema	slight pigmentation no erythem or oedema	no reaction	no reaction
1 week after irradiation	well developed pigmentation no erythem or oedema	slight pigmentation no erythem or oedema	no reaction	no reaction
Classification	**slightly phototoxic**	**Not acutely phototoxic**	**Not acutely phototoxic**	**Not acutely phototoxic**

exposure - forearm; irradiation dose 5J UVA; 5 test persons. Test performed in the full occlusion

5.4.3 DISCUSSION

Due presence of the barrier function similar to barrier function of human epidermis, the reconstructed human skin models are proposed as an additional tool for verification of positive results of the 3T3 NRU PT and/or for testing of substances incompatible with the 3T3 NRU PT. In contrast to cell cultures, such as mouse fibroblasts used in the 3T3 NRU PT, human skin models permit the topical application of various types of chemicals and preparations, and have less limitations concerning solubility problems. The test substances can be applied to reconstructed human skin models undiluted, at extreme pH values or even as insoluble materials (Liebsch *et al*., 2005).

The experiments performed in current study with UV filters and essential oils covered the challenging cases, where the 3T3 NRU PT provide unsatisfactory or non-relevant results. The human skin model phototoxicity test correctly predicted known non-phototoxic effect of butyl methoxy-dibenzoylmethane and titanium dioxide. These two substances have no potency to cause acute phototoxicity, because they do not penetrate the stratum corneum and thus do not reach the viable cell layers of the epidermis (Lademann *et al*., 1999, Gamer *et al*., 2005 and Trauer *et al*., 2005). However, for a final prove of the predictability of the H3D PT, human patch tests will be performed with the two substances at the National Institute of Public Health, Prague.

Studying essential plant oils, the H3D PT provided relatively reliable prediction of the safe concentration at which no acute phototoxic effect would occur in man. The experiments with *Litsea Cubeba* clearly demonstrated the capability of the skin model test. Some borderline results were observed in the study with bergamot oils and H3D PT, however also the 3T3 NRU PT provided unclear or borderline classifications. It is important to note, that Bergamot oils is not a single, chemically defined substance, yet it consists of more than 20 compounds and many isomers (Jírova *et al*., 2005). Therefore, safety assessment of such a mixtures is quite complicated.

While Bergamot oils “Aroma” and “Biomedica” were correctly predicted by the H3D PT as non phototoxic, the effects of the Bergamot oil Sigma and Schupp were slightly underestimated by the skin model. However, this might be due to the differnt test conditions of the human patch test and H3D PT with regard to the vehicles used. The use of 15 % ethanol/water solution as vehicle might enhance the penetration of the Bergamot oils into the deeper layers of the epidermis. In addition, ethanol might increase the activity of the phototoxic compounds in similar manner as observed in the 3T3 NRU PT. Therefore, the human patch test should be repeated avoiding ethanol. Yet, the tanning effect of the Bergamot oil “Sigma” and Schupp will not be predicable by the simple H3D PT, as the standard reconstructed human skin model EpiDerm does not contain melanocytes. The tanning effect could be studied using e.g. MelanoDerm (MatTek Corporation) or SkinEthic reconstructed human epidermis (SkinEthic laboratories) containing melanocytes. It is also of note, that in the past Bergamot oil was used due to the knowen tanning effect in a cosmetic lotions accelerating tanning, e.g. in solarium.

In summary, the very promising results show the usefulness of the H3D PT in the estimation of the phototoxcity, photopotency and bio-availability of the compounds and mixtures. Still, for reliable evaluation of the ability of the reconstructed human skin model to correctly predict “No Observed Adverse Effect Level Dose” (NOAEL), more experiments, preferably with larger set of well defined chemicals should be performed.

Limitations of the test

The H3D PT is qualified as an adjunct test for further investigating chemicals with possibly false-positive outcomes in the 3T3 NRU PT. In contrast to the 3T3 NRU PT assay, the H3D PT may fail to detect phototoxins that cannot enter the skin via a topical route, but may reach the skin via systemic pathways (e.g. by after oral exposure). The H3D PT may also fail to detect weakly photoreactive chemicals that induce photoallergic reactions only after repeated exposure (Liebsch *et al*., 2005). However, if the H3D PT is used as an adjunct to the 3T3 NRU PT only for chemicals (formulations) intended for topical use, it has hardly

any limitations, because it mimics the *in vivo* situation and can handle solutions, as well as suspensions.

The only limitation known is that a test substance may directly reduce MTT and mimic the dehydrogenase activity of the cellular mitochondria. This is only a problem if during the MTT test (24 hours after exposure to the test substance), sufficient amounts of the test substance are still present on (or in) the tissues. In this case, the (true) metabolic MTT reduction and the (false) direct MTT reduction can be differentiated using the procedure with killed control tissues as described in skin corrosion or skin irritation assays.