

INVESTIGATIVE REPORT

Evaluation of Phototoxic Properties of Fragrances

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Fragrances are widely used in topical formulations and can cause photoallergic or phototoxic reactions. To identify phototoxic effects, 43 fragrances were evaluated *in vitro* with a photohaemolysis test using suspensions of human erythrocytes exposed to radiation sources rich in ultraviolet (UV) A or B in the presence of the test compounds. Haemolysis was measured by reading the absorbance values, and photohaemolysis was calculated as a percentage of total haemolysis. Oakmoss caused photohaemolysis of up to 100% with radiation rich in UVA and up to 26% with radiation rich in UVB. Moderate UVA-induced haemolysis (5–11%) was found with benzyl alcohol, bergamot oil, costus root oil, lime oil, orange oil, alpha-amyl cinnamic aldehyde and laurel leaf oil. Moderate UVB-induced haemolysis was induced by hydroxy citronellal, cinnamic alcohol, cinnamic aldehyde, alpha-amyl cinnamic aldehyde and laurel leaf oil. The phototoxic effects depended on the concentration of the compounds and the UV doses administered. We conclude that some, but not all, fragrances exert phototoxic effects *in vitro*. Assessment of the correlation of the clinical effects of these findings could lead to improved protection of the skin from noxious compounds. **Key words:** photosensitization; phototoxicity; fragrances; essential oils; photohaemolysis.

(Accepted December 21, 2006.)

Acta Derm Venereol 2007; 87: 312–316.

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Fragrances are used in perfumes, cosmetics, shampoos, soaps and other toiletries, as well as in non-cosmetic products such as household cleaners or detergents. Their worldwide use is enormous, e.g. the consumption of cinnamyl alcohol is about 100–1000 metric tonnes per year. The relevant determinant factors of fragrance exposure are quantity of cosmetics used, frequency of use, and concentrations of fragrances in these products (1).

Contact allergy to fragrances is frequent and quite well characterized (2). In addition, some fragrances, including essential oils, such as bergamot oil, 6-methyl coumarin or musk ambrette, are known to induce photosensitivity reactions *in vivo* (3, 4).

Acute skin reactions to photosensitizing compounds may be phototoxic or photoallergic. Photoallergic reactions are T-cell-mediated immunological reactions, while phototoxic reactions are non-immunological events inducing toxic cell damage. Most substances eliciting photoallergic reactions also have a phototoxic potential.

We systematically assessed the *in vitro* phototoxic potential of a series of fragrances to which the skin might be exposed, using a photohaemolysis test. This assay is a standard *in vitro* method used in isolated erythrocytes for measurement of possible phototoxic effects, and is especially suitable for screening investigations.

MATERIALS AND METHODS

Test substances

Tests were performed with the following compounds: alpha-amyl cinnamic aldehyde, 6-methyl coumarin, hydroxycitronellal, alpha-hexyl cinnamic aldehyde (Aldrich Chemie, Steinheim, Germany), coumarin; l-menthol, methyl jasmonate, methyl salicylate, nerol, terpineol, vanillin, bergamot oil, benzyl benzoate, costus root oil, eucalyptus oil, lavender oil, lemongrass oil, lime oil, laurel leaf oil, clove bud oil, orange oil, peppermint oil, rose oil, sandalwood oil, thyme oil, ylang-ylang oil, cedarwood oil, cinnamon bark oil, lemon oil (Caelo-Caesar & Lorenz, Hilden, Germany), oakmoss (Drom, Baierbrunn, Germany), benzaldehyde, benzyl acetate, benzyl alcohol, benzyl salicylate, citronellal, citronellol, methyl benzoate, cinnamic alcohol, methyl cinnamate (Merck, Darmstadt, Germany), isoeugenol (Schuchardt, Hohenbrunn, Germany), eugenol, geraniol, and cinnamic aldehyde (Serva, Heidelberg, Germany). The test substances were dissolved in appropriate solvents (methanol or ethanol) and further diluted in TCM buffer (NaCl 7.0 g; Tris 3.0 g; KCl 0.3 g; MgCl₂ × 6 H₂O, 0.2 g; CaCl₂ × H₂O, 0.147 g; aqua dest. ad 1000 ml; pH 7.4; 280 mOsm/kg).

UV sources

Irradiation was performed with the following ultraviolet (UV) A-rich (i) or UVB-rich (ii) lamps: (i) UVASUN 5000 (Mutzhas, Munich, Germany), emitting in the range 320–460 nm (maximum approximately 375 nm) (UVA irradiance at a distance of 40 cm was 42 mW/cm²) (2); (ii) TL 20 W/12 light bulbs (Philips, Hamburg, Germany) with a main emission in the range 275–365 nm (maximum approximately 315 nm) (irradiance was 1.0 mW/cm² for UVB and 0.4 mW/cm² for UVA at a distance of 40 cm).

Dosimetry

UVA or UVB intensities or doses were measured by an integrating instrument (Centra-UV, Osram, Munich, Germany).

Photohaemolysis test

The test was performed as described previously (5). Briefly, washed human erythrocytes were suspended at a dilution of 1:200 in TCM buffer containing 0.03% human albumin. A 0.4 ml volume of this suspension and a correspondingly prepared erythrocyte-free sample were incubated with 0.1 ml of the test substance preparations for 1 h at 37°C. Concentrations were 10^{-5} mol/l, 10^{-4} mol/l or 10^{-3} mol/l or, with oils, dilutions of 1:100000, 1:10000 and 1:1000. Both samples containing the test substances and accordingly prepared test substance-free erythrocyte samples (blank) were exposed to 0, 5, 25, 50 or 100 J/cm² UVA (UVASUN 5000) or to 0 (0), 500 (0.2), 1000 (0.4) or 2000 (0.8) mJ/cm² UVB (J/cm² UVA) from the TL 20 W/12 light bulbs. During irradiation, samples were kept in a shaking bath at 37°C. 100% haemolysis was obtained by exposure of the erythrocytes to distilled water. After an incubation period of 30 min in the dark, supernatants were recovered by centrifugation. The released haemoglobin in the supernatants was determined as cyanmethaemoglobin after incubating the samples for 15 min with Drabkin's solution (Sigma, St Louis, USA). Haemolysis was determined by reading the absorbance at 550 nm with a MR 700 microplate reader (Dynatech, Denkendorf, Germany). Haemolysis was calculated on the basis of the absorbance data according to the formula:

Haemolysis (%) = $100 \times [\text{test sample} - \text{blank} - \text{erythrocyte-free sample}] / [\text{total haemolysis} - \text{blank}]$

Photohaemolysis was calculated by subtracting the % value obtained with the non-irradiated sample from that found with the irradiated samples. The results are given as mean of independent experiments performed each in triplicate with erythrocytes from 3 different donors. In order to exclude equivocal results, only photohaemolysis >5% was regarded as a meaningful positive finding.

RESULTS

Bergamot oil, cedarwood oil, cinnamon bark oil, costus root oil, laurel leaf oil, lemon oil, lavender oil, oakmoss, sandalwood oil and ylang-ylang oil induced significant haemolysis (>15%) already without UV irradiation at a dilution of 1:1000. These substances were further tested for phototoxic effects at the lower dilutions.

Of the 43 compounds investigated, 11 exhibited phototoxic effects. Oakmoss (1:10000) induced a UV-dose-dependent haemolysis up to 100% (UVA) or 26% (UVB) (Fig. 1). On the basis of data modelled by P. Koepke, the following intensities for a horizontal surface can be calculated: UVA 38 J/cm² and UVB 986 mJ/cm² (assuming 2 h radiation during a cloudless day in summer around noon in Central Europe, latitude of Munich) (6).

The other 10 phototoxic substances caused moderate photohaemolysis up to 11% only with UVB (hydroxy citronellal 10^{-3} mol/l, cinnamic alcohol 10^{-3} mol/l, cinnamic aldehyde 10^{-3} mol/l), only with UVA (benzyl alcohol 10^{-4} mol/l, bergamot oil 1:10000, costus root oil 1:10000, lime oil 1:1000, orange oil 1:1000) or by both UVB and UVA (alpha-amyl cinnamic acid 10^{-3} mol/l, laurel leaf oil 1:10000). The phototoxic effects depended on the concentrations of the compounds and the UV doses administered. Details are shown in Figs. 2 and 3. All other tests did not yield haemolysis >5%.

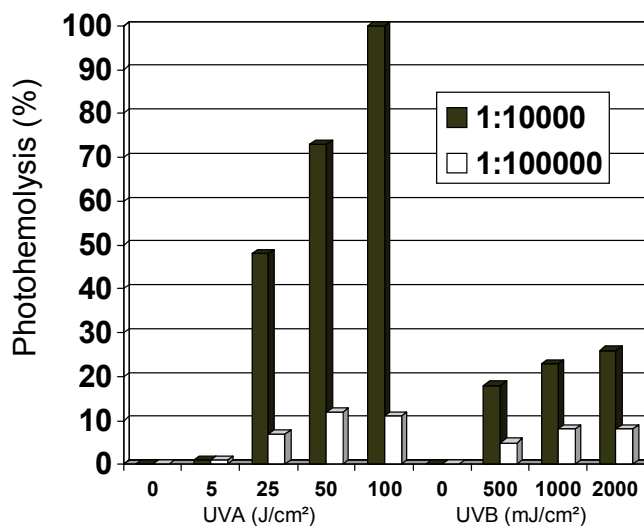


Fig. 1. Photohaemolysis caused by oakmoss at concentrations of 1:10,000 and 1:100,000 and radiation rich in UVA or UVB.

DISCUSSION

Phototoxic substances

Oakmoss exerted prominent phototoxic effects in this *in vitro* assay, photohaemolysis occurring with exposure to both UVB- or UVA-rich sources. Moderate phototoxic effects were induced by UVA in the presence of seven fragrances (benzyl alcohol, bergamot oil, costus root oil, alpha-amyl cinnamic aldehyde, laurel leaf oil, lime oil, orange oil) and by UVB due to incubation with five fragrances (alpha-amyl cinnamic aldehyde, hydroxy citronellal, cinnamic alcohol, cinnamic aldehyde, laurel leaf oil).

Oakmoss is extracted from the lichen *Evernia prunastri*, which grows on the trunk and branches of oak trees. One study showed that the ingredients of oakmoss were detected in 87% of popular perfumes. Similar to our results Addo et al. (7) demonstrated oakmoss-dependent photohaemolysis induced by UVB or UVA. Contact allergy to oakmoss is not infrequent (8), and photosensitivity reactions have also been reported (9). Photo-patch testing revealed reactions to oakmoss in one study (7), but not in another (10). This is in accordance with the notion that photoallergic substances have a phototoxic potential.

Alpha-amyl cinnamic aldehyde also caused UVA- and UVB-induced photohaemolysis in our study. However, no reports of photosensitizing properties of this compound, shown by *in vitro* or *in vivo* tests, could be found.

Bergamot oil, lime oil, orange oil and lemon oil caused moderate UVA-induced photohaemolysis in our study. They are all known photosensitizers. Residual hyperpigmentation from phototoxic reactions to bergamot oil was described in 1916 (11), and 5-methoxypsoralen as the main phototoxic ingredient of bergamot oil was isolated in 1938 (12). Furthermore, the peel of

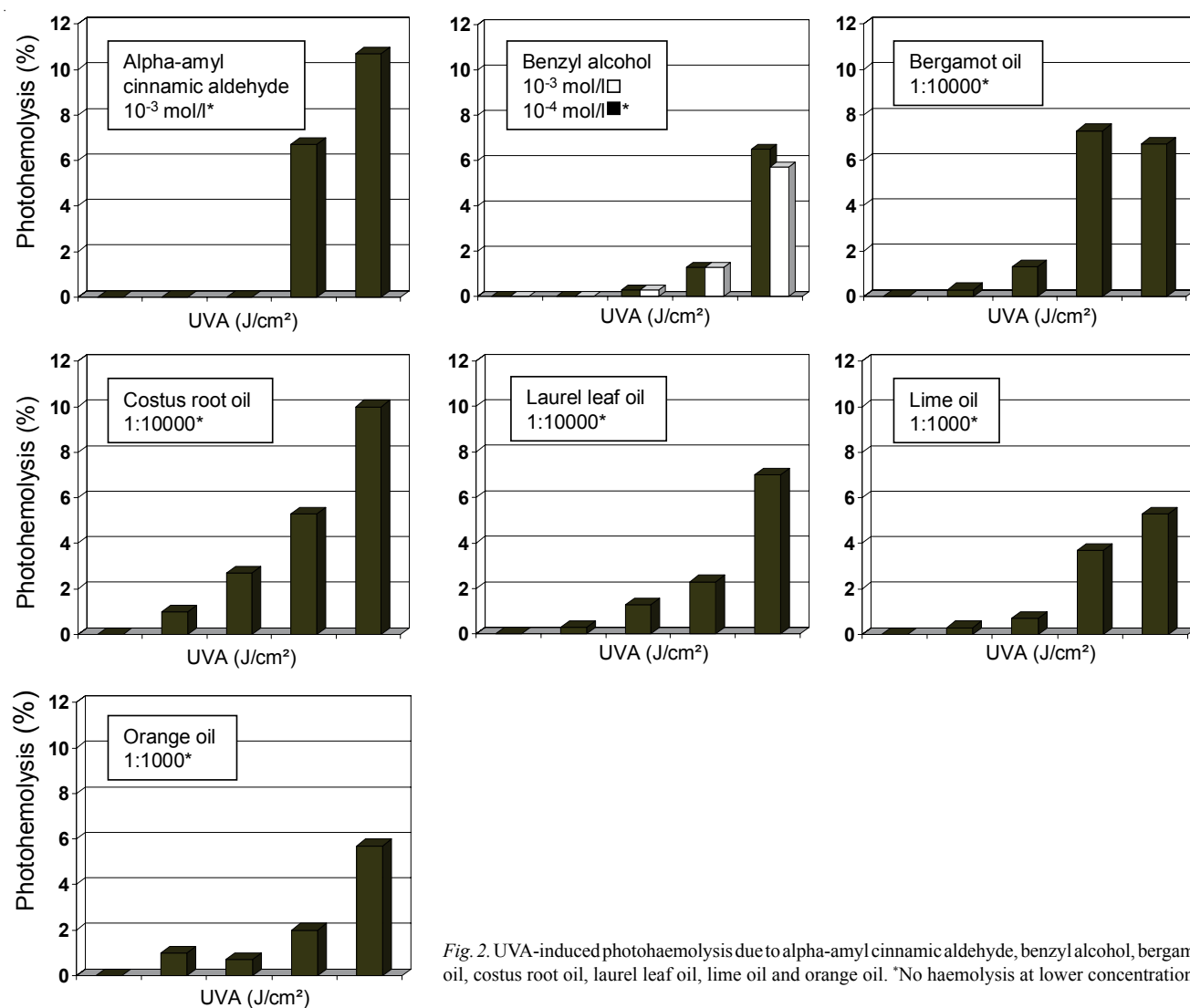


Fig. 2. UVA-induced photohaemolysis due to alpha-amyl cinnamic aldehyde, benzyl alcohol, bergamot oil, costus root oil, laurel leaf oil, lime oil and orange oil. *No haemolysis at lower concentrations.

the fruits bergamot (*Citrus bergamia*), lime (*C. aurantifolia*), orange (*C. aurantium*) and lemon (*C. medica*) are known elicitors of phytophotodermatitis (13). Also, oils of lemon, lavender, lime, sandalwood and cedar are known to elicit cutaneous phototoxic reactions, but lavender, sandalwood and cedar oil did not induce photohaemolysis in our assay. The phototoxic activity of lemon oil has been assessed previously in albino guinea pigs and found to be attributable to bergapten and oxypeucedanin (14).

Costus root oil and laurel leaf oil were found to be phototoxic with UVA exposure in our study. This is in concordance with the results of other *in vitro* studies (7). Photo-patch reactions to costus root oil were found in two patients (7).

Cinnamic alcohol, which was found to be phototoxic with UVB radiation in our study, elicited phototoxic effects with both UVA and UVB exposure in another photohaemolysis test and in a phototoxicity test using plates seeded with *Candida utilis* (7). In another assay,

measuring the photosensitized oxidation of histidine, phototoxicity of cinnamic alcohol could not be demonstrated (15). Photo-patch tests showed no reactions to cinnamic alcohol (10).

We found cinnamic aldehyde-dependent UVB-induced photohaemolysis. However, phototoxic effects of this compound were not observed when cinnamic aldehyde was evaluated for phototoxicity in other assays using human lymphocytes, human keratinocytes, human erythrocytes or murine fibroblasts (15). Positive as well as negative results were obtained using phototoxicity assays with yeast (7, 15, 16) or measurement of histidine oxidation (7, 15). Phototoxicity of cinnamic aldehyde was also shown in a haemoglobin oxidation assay and an *in vitro* study using human leukaemic T-cell lymphoblasts (17). Four out of 76 patients with photodermatoses, evaluated in two studies, exhibited photo-patch reactions to cinnamic aldehyde (7, 10).

Benzyl alcohol was found to be weakly phototoxic in this study, whereas in a previously published study

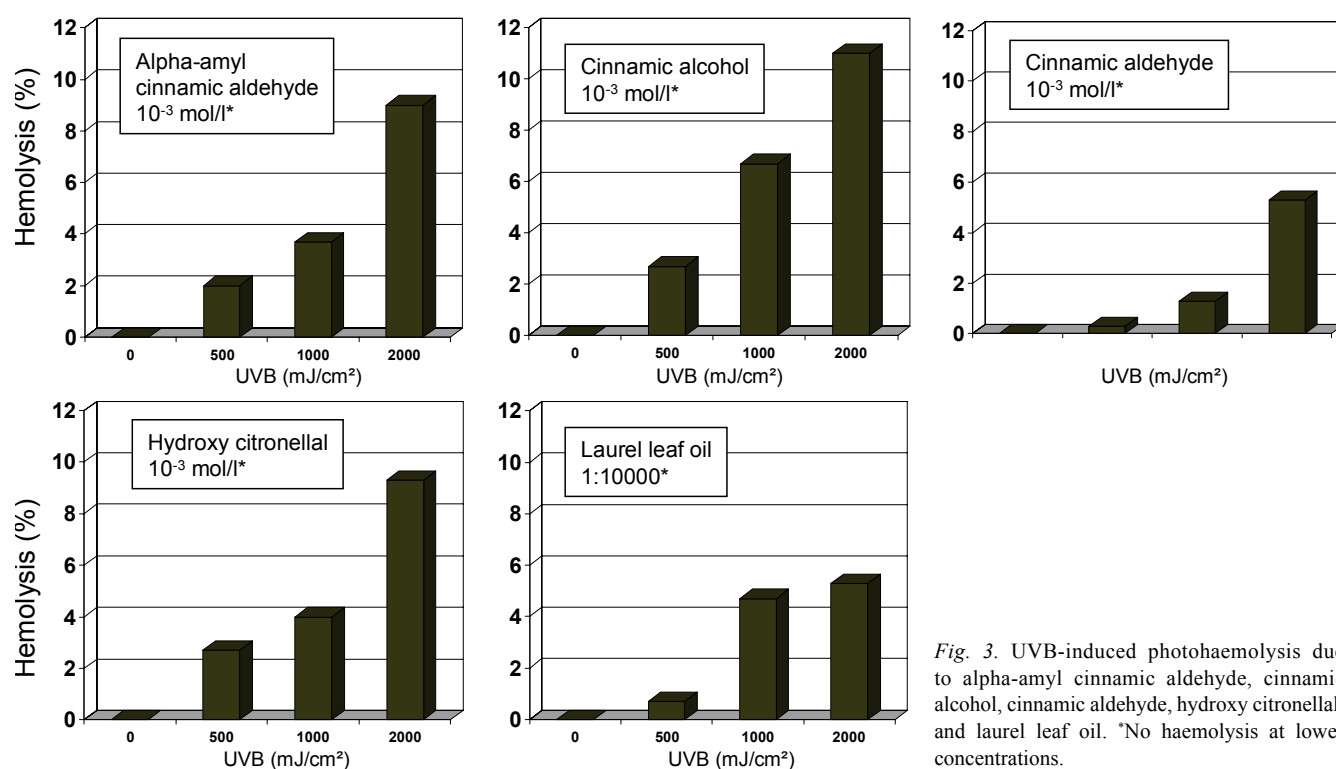


Fig. 3. UVB-induced photohaemolysis due to alpha-amyl cinnamic aldehyde, cinnamic alcohol, cinnamic aldehyde, hydroxy citronellal, and laurel leaf oil. *No haemolysis at lower concentrations.

no phototoxicity was demonstrable (6). Others found that benzyl alcohol-dependent photohaemolysis was induced by UVA and UVB (7).

Similar to our results, UVB-induced photohaemolysis due to hydroxycitronellal has been reported (7). Also a photo-patch test reaction to this compound was found in one patient (7, 10).

Substances not inducing photohaemolysis

6-Methyl coumarin and coumarin did not elicit photohaemolysis in our test system. In addition, other investigators found that coumarin was not, and 6-methyl coumarin was not clearly, phototoxic in a photohaemolysis test, but in a test using *Candida*, UVA-induced 6-methyl coumarin-dependent phototoxicity was shown (7). Phototoxicity of these compounds was also demonstrated in a three-dimensional skin model (18). 6-Methyl coumarin is found in lists of photoallergic drugs and chemicals, and coumarin belongs to the topical agents known to induce direct cutaneous phototoxicity (3). Photo-patch testing revealed phototoxic as well as photoallergic reactions to 6-methyl coumarin (19).

Lavender oil and sandalwood oil did not induce photohaemolysis in our test system. However, a few reports on photosensitivity reactions due to these substances have been published, e.g. one patient with persistent light reaction and a positive photo-patch test to sandalwood oil (20).

Similar to our results isoeugenol, eugenol and geraniol showed no phototoxic reaction in a photohaemoly-

sis test, and results in a test-system using *Candida* were inconclusive (7). Photo-patch testing yielded reactions to eugenol in two patients (7, 10).

Our study yielded negative results, as did other *in vitro* phototoxicity studies assessing benzyl benzoate and benzyl salicylate (7).

All other tested substances, i.e. alpha-hexyl cinnamic aldehyde, benzaldehyde, benzyl acetate, cedarwood oil, cinnamon bark oil, citronellal, citronellol, clove bud oil, eucalyptus oil, lemongrass oil, l-menthol, methyl benzoate, methyl cinnamate, methyl jasmonate, methyl salicylate, nerol, terpineol, vanillin, peppermint oil, rose oil, thyme oil and ylang-ylang oil did not elicit photohaemolysis in our test system. Also, no reports regarding photosensitization by these compounds could be found in the literature.

In conclusion, these data show that the photosensitizing effects of fragrances are detectable by a photohaemolysis test. There is large concordance between our results and those obtained by others in various test systems using yeasts cells, lymphocytes, keratinocytes, erythrocytes, etc. Compounds with phototoxicity *in vitro* are quite abundant (21–23). However, acute phototoxic reactions in patients are not very frequent. Nonetheless photosensitization below the threshold of overt skin disease may add to chronic photo-damage, which is characterized primarily by “photo-ageing” and photocarcinogenesis (3). The long-term use of drugs with phototoxic properties (e.g. non-steroidal anti-inflammatory drugs, anti-diuretics, antibiotics, anti-diabetics, psychotherapeutic drugs) has been found to be associa-

ted with an increased number of solar keratoses, e.g. intra-epithelial carcinomata and precursors of invasive squamous cell carcinoma (24). It is well known that photochemotherapy, using photosensitizing psoralens and UVA radiation to treat skin disease, is associated with an increased number of malignant skin tumours (25). Quinolones, which have been found to be phototoxic in the photohaemolysis test (e.g. ciprofloxacin, enoxacin, ofloxacin) (5), were found to be associated with photocarcinogenesis *in vitro* (26) and *in vivo* (27, 28). Thus, although the photohaemolysis test does not allow disclosure of effects related to DNA damage, phototoxicity demonstrated in this system supports photocarcinogenic risks. This is not surprising, as besides DNA damage, other mechanisms also contribute to UV-induced skin cancer (5, 28, 29, 30).

As UV-enhanced skin tumours have become an increasing health problem, the probable association between exposure to "common" phototoxic compounds and photocarcinogenesis requires further study.

ACKNOWLEDGEMENT

This work was supported by Bayerischer Forschungsverbund (BayForUV).

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