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# Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay

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**Abstract**

When substances are developed in the aim to be a constituent of personal care products, and to be applied on the skin, it is necessary to carry out an assessment of potential phototoxic hazard. Phototoxicity is skin reaction caused by concurrent topical or systemic exposure to specific molecule and ultraviolet radiation. Most phototoxic compounds absorb energy particularly from UVA light leading to the generation of activated derivatives which can induce cellular damage. This type of adverse cutaneous response can be reproduced in vitro using different models of phototoxicity such as the validated 3T3 Neutral Red Uptake (NRU) phototoxicity assay. In the present study we utilised two different cell lines (the murine fibroblastic cell line 3T3 and the rabbit cornea derived cell line SIRC) to compare the photo-irritation potential of a strong phototoxic compound, chlorpromazine, to a weaker composite, such as 8-methoxypsoralen and Bergamot oil. After comparison of the different systems, five other essential oils were tested with both cell lines. Cellular damage was evaluated by the NRU cytotoxicity test or by MTT conversion test.


*Keywords:* Phototoxicity; In vitro; MTT; Neutral red; Essential oils

## 1. Introduction

Phototoxicity (photo-irritation) can be defined as a skin inflammatory reaction elicited by topical application or systemic administration of chemicals and subsequent exposure to light, particularly UVA radiation (Epstein, 1983). This type of adverse response can occur following exposure to exogenous compounds like drugs or cosmetic products. Therefore when chemicals/ingredients are intended for dermatological products, the evaluation of phototoxic potential of the ingredients represents a crucial step in risk assessment during product development.

Over the past few years, several in vitro test methods have been suggested as valid substitutes for in vivo animal testing of photo-irritant. Numerous in vitro methods have been developed to assess the phototoxic potentials of chemicals by both academic and industrial laboratories. These can be assigned to two general groups: (a) those using cells and tissues for screening purposes and (b) tests focusing on a specific mechanism of phototoxicity. Thus, a broad spectrum of cell and tissue culture systems has been developed for assessing the phototoxic potentials of chemicals. Among them, primary human keratinocyte cultures had not shown any obvious advantage compared to the

*Abbreviations*: 3T3 NRU phototoxicity test, in vitro 3T3 cell neutral red uptake phototoxicity test; CPZ, Chlorpromazine; ECACC, European collection of animal cell cultures; DMEM, Dulbecco's medium containing 10% newborn bovine serum; HBSS, Earle's buffered salt solution; NBS, newborn bovine serum; FBS, foetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NR, neutral red; NRU, neutral red uptake; PBS, phosphate buffered saline; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulphate; PIF, photo-irritation factor; SD, standard deviation; UV, ultraviolet; $EC_{50}$, effective concentration of test material causing 50% reduction of cell viability.

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validated 3T3 NRU phototoxicity assay (Api, 1997; Spielmann et al., 1998a,b). These studies support the observation of Maier et al. (1991) that keratinocytes (either primary cultures or permanent cell lines) are less sensitive than fibroblasts to irradiation. However, in organotypic tests and for certain specific purposes it is preferable to use keratinocytes, since in vivo they are the first target cell type exposed to sunlight. Nevertheless, in several in vitro phototoxicity tests, keratinocytes have been shown to be less sensitive than fibroblasts to the effect of UV light (Clothier et al., 1999). Further standardisation of in vitro toxicity using human keratinocytes is essential. Result should be compared with those obtained using other cell types (e.g. A431 human epidermal cell line, 3T3 murine fibroblast cell line or V79 hamster fibroblast cell line). Human keratinocytes appear however less useful than fibroblasts for screening purposes. Among cell types exposed constantly to sunlight, ocular cells, and especially corneal epithelial and/or endothelial appear to be interesting too (Roberts, 2001). Moreover, these cells contain very efficient defense systems that serve to protect against oxidative and photo-induced damage. Thus, corneal cells could be used in a simple screening procedure.

The 3T3 NRU phototoxicity test has been scientifically validated in the EC as one alternative method for skin phototoxicity (Api, 1997; Spielmann et al., 1998a,b). Yves Rocher currently uses the 3T3 NRU phototoxicity test within the context of EU test guidelines (2000/33/EC, 2000; Directive 67/548/EEC, 2004) and OECD guideline 432 adopted on 13th April 2004 (OECD, 2004). Complementary in vitro methods can also be set up using human skin models (Edwards et al., 1994; Augustin et al., 1997; Jones et al., 2001). Such models have been shown to be able to identify phototoxic potential of both soluble and insoluble substances (Api, 1997; Bernard et al., 2000). This type of model allows application of test materials to the air-exposed surface and modification of usage concentrations and formulations, thus mimicking the in vivo situation.

At the present time essential oils are found in many products, including antiseptic, liniments, soaps, deodorants, flavours and cosmetic products. The current widespread use of essential oils in pharmacy and industry necessitates research on their toxicity. This report presents the assessment of the phototoxic hazard of some of these compounds. An initial assay, i.e. a pre-screen, was carried out by identifying ingredients at relevant wavelengths of the UV/visible absorption spectrum, and hence discerning their potential photoreactivity to sunlight (Lovell, 1993). Subsequently, the in vitro 3T3 cell neutral red uptake phototoxicity test (3T3 NRU phototoxicity test) was performed. This test was realized on two different cell lines, the murine fibroblast cell line 3T3 and the rabbit cornea derived cell line SIRC.

This study aims to compare the ability of two different cell lines to assess the toxic effect of weak phototoxic compounds, 8-methoxypsoralen and Bergamot oil, in relations to a strong phototoxic composite, chlorpromazine; all of them were used as positive controls. An irritant, but non-phototoxic compound, sodium dodecyl sulphate (SDS), was used as a negative control. Cell viability was the end point chosen for this study and cellular damage was evaluated by NRU or by MTT conversion test. Additionally, the study intends to demonstrate the fact that the two cell lines can both be used to evaluate phototoxicity, as illustrated by the investigation we performed on several essential oils.

## 2. Materials and methods

### 2.1. Tests substances and preparation

Essential oils used for this study were supplied from internal company sources. Main components of each sample were indicated in Table 1. Four reference molecules were chosen to assess the validity of the method: (1) chlorpromazine (CPZ), which is highly phototoxic, was purchased from Sigma-Aldrich Chemical (Sigma-aldrich C0982—Saint Quentin Fallavier—France); (2) 8-methoxypsoralen (Sigma-aldrich M3501) and (3) Bergamot essential oil (internal source), both of which are mildly phototoxic, were used as positive phototoxic controls in the cell-based assay; and (4) sodium dodecyl sulphate (Sigma-aldrich L4509), an irritative but non-phototoxic, was used as a negative control. All these molecules have been used previously in the 3T3 NRU phototoxicity test inter-laboratory validation (Spielmann et al., 1998b).

### 2.2. Absorption spectra

The absorption spectrum of the different ingredients was measured in absolute ethanol (VWR) using a Hewlett

Table 1
Composition of the essential oils studied (only main compounds were reported)

| INCI name | CAS number | Main components (%) |
|---|---|---|
| *Citrus aurantium dulcis* (Orange) oil | 8008-57-9/8028-48-6 | Limonene (92.5), myrcene (3.9), linalol (1.7) |
| *Lemongrass cymbopogon citratus* oil | 8007-02-1 | Citral (70–90), geraniol (1.5–7.5), geranyl acetate ( 1.5–5.5), limonene (0.5–4) |
| *Fusanus spicatus* wood oil (Santal oil) | 8024-35-9 | α and β Santalol (40–50), α and β bisabolol (2–12.5) |
| *Zingiber officinalis* oil (Ginger oil) | 8007-08-7 | Zingiberene (30), limonene (2–3.1), cis-γ-bisabolene (7), citronnellol (2) |
| *Daucus carota sativa* (Carrot) seed oil | 8015-88-1 | Carotol (50), α and β pinene (5–10), β caryophyllene (4), geranyl acetate (3), linalol (2) |
| Bergamot oil | 8007-75-8 | Limonene (25–32), linalool (16–41), linalyl acetate (11–41), bergamottin (1–3) |

Estimated values are obtained from RIFM database and/or internal assays.