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Assessment of phototoxicity potential of botanicals as cosmetic ingredients using the *in vitro* 3T3 neutral red uptake phototoxicity test

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ABSTRACT

Cosmetics and personal care products are frequently formulated with botanical ingredients due to their beneficial properties, the nature of their composition, and consumers' interest for products with more natural or organic profiles. Compounds that absorb light significantly and are in contact with the skin have potential to become phototoxic upon exposure to sunlight. Here we demonstrate that an *in vitro* test methodology, the 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT), is an effective screening tool in evaluation of botanical ingredients that absorb light in the Ultraviolet/Visible (UV/Vis) range. Thirty-eight prospective botanical ingredients were evaluated in the 3T3 NRU PT assay. Five botanicals were identified to have phototoxicity potential, and were eliminated from consideration for use. Thirty three botanicals were identified to have no phototoxicity potential in the 3T3 NRU PT; and a subset of six were further evaluated in a clinical confirmatory test that corroborated the data obtained using the *in vitro* test. Our results support this *in vitro* test method as a reliable, high throughput model in evaluating a large subset of compounds to efficiently identify those that pose a potential risk and to ensure that marketed cosmetic products do not contain ingredients with phototoxicity potential.

1. Introduction

There have been growing trends in the use of botanicals across several industries including those manufacturing cosmetics and personal care products (Antignac et al., 2011; Ferreira et al., 2021; Api et al., 2022). Botanical ingredients or extracts are naturally-occurring complex chemical mixtures from plants (Rojas et al., 2016; Roe et al., 2018). They can be sourced from the whole plant or from specific parts such as the leaves, roots, flowers, fruits, seeds, or berries. Botanicals are typically used as extracts, oils or isolated by more complex processes such as fermentation (Troyano et al., 2011). Beauty products often include botanicals because of their innate ability to moisturize, cleanse, and perfume, or due to claimed anti-oxidant, anti-inflammatory, and anti-microbial qualities (Corazza et al., 2014; Rojas et al., 2016). The use of naturally derived ingredients is a growing trend in the beauty industry: based on a survey conducted in 2011, approximately one-third of ingredients listed by the International Nomenclature of Cosmetic Ingredients (INCI) system at the Personal Care Products Council were classified as "botanical extracts" (Ferreira et al., 2021).

While these botanical ingredients are often perceived as safer than those that are chemically derived, their safety and efficacy should be evaluated before being formulated in products for human use. Given the typical exposure route of most cosmetics (*e.g.,* applied directly to skin, on face or near eyes, etc.), cosmetic companies often evaluate ocular and dermal safety of prospective compounds (or in final formulation) as part of their due diligence process. These tests can inform on offending compounds that are likely to produce adverse effects, which may include skin or eye irritation/corrosion, skin sensitization, and phototoxicity (also referred to as photoirritation).

Photoirritation is an acute irritation response that occurs after application of a phototoxic compound and subsequent exposure to light (OECD TG 432, 2019; OECD TG 498, 2023). The adverse effects resemble a sunburn-like response, and may include erythema, edema, blistering, and burning (Maibach and Honari, 2014; Hinton and Goldminz, 2020; Guan et al., 2022), all of which may be mitigated in time upon removal of the offending compound. Not all compounds are capable of eliciting phototoxic effects, and they can be identified *via* UV/Vis absorption spectra determinations as presented in the Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 101 (OECD, 1981). This test method is able to identify compounds which have the ability to significantly absorb light in the wavelengths of 290–700 nm, which covers UVB (280–315 nm), UVA

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(315–400 nm), and visible light (>400 nm) (Commission Internationale de l'Eclairage), and as a result may generate reactive species.

In addition to the UV/Vis *in chemico* test method, other *in vitro* test methods, now referred to as New Approach Methodologies (NAMs), have been used in the evaluation of photoirritation potential of ingredients, and ultimately final formulations for decades (Kandarova and Liebsch, 2017). These methods are recommended first steps to rule out phototoxicity hazard potential as presented in the OECD Test Guidelines 432 (OECD, 2019), 495 (OECD, 2019) and 498 (OECD, 2023), as well as the ICH S10 Photosafety Evaluation of Pharmaceuticals Guidance for Industry (ICH, 2015). Both OECD Test Guideline 432 and 498 are test methods used to identify phototoxicity hazard potential based on increased cytotoxicity upon application of test compound to a monolayer of cells (TG 432) or reconstructed human epidermis (RhE) tissue model (TG 498) in the presence of light exposure as compared to the absence of light exposure. These test methods may be used as part of a tiered pre-clinical evaluation to ensure safety prior to human no clinical effect patch testing (ICH, 2015; Ritacco et al., 2022).

The 3T3 NRU PT was used to evaluate 38 cosmetic ingredients, identified as botanicals and that were determined to absorb light significantly. Six ingredients from the group found to have no phototoxicity potential based on the 3T3 NRU PT were formulated in one or more prototype cosmetic formulation (at final desired use concentration) and subject to confirmatory clinical testing. The strategy used in our experiments is presented in detail in Fig. 1.

2. Materials and methods

2.1. Botanical ingredients

Thirty-eight botanicals identified as prospective cosmetic ingredients were evaluated for photosafety as outlined in Fig. 1. The botanicals were provided by Avon Global Research and Development.

2.2. Reagents

Reagents used in the experiments conducted were sourced from commercial vendors (*e.g.*, Sigma, Quality Biologicals, Fisher Scientific, etc.) as indicated in the respective subsequent sections.

2.3. Cell cultures

For the 3T3 NRU PT, the test system was a monolayer of Balb/c 3T3 (clone A31) mouse fibroblast cells obtained from American Type Culture Collection (ATCC) (Manassas, Virginia USA). A stock of cells was reconstituted upon receipt and propagated at Institute for In Vitro Sciences (IIVS) to create working banks of cells designated for experimental use.

2.4. UV/Vis test method

The absorption spectra of the 38 botanical ingredients was determined using methods adapted from OECD TG 101 (OECD, 1981). In this test method, a Molar Extinction Coefficient (MEC) value is calculated using the tested concentration, molecular weight, and absorbance value, and significant absorption is indicated when MEC values exceed a threshold of $\geq$1000 mol L^{-1} cm^{-1} (ICH S10, 2015; OECD TG 432, 2019; OECD TG 498, 2023). However, the botanical ingredients did not have defined molecular weights, and additional considerations were needed under the circumstances. As such, they were evaluated at a concentration of 1 % weight/volume (w/v) which was 10-times the maximum concentration prescribed for the OECD TG 432 3T3 NRU PT. Each ingredient was prepared as a 1 % dilution in ethanol in neutral, acidic, and basic pH buffered conditions, and then the absorption across UV/Vis spectra was determined using the cuvette method. Absorbance values > 0.1 at wavelengths between 290 nm and 700 nm were considered significant.

2.5. 3T3 NRU PT

Thirty-eight botanical ingredients identified to have absorbed light significantly based on the method described in section 2.4 were evaluated in the 3T3 NRU PT (Fig. 1). The procedures were adapted from OECD TG 432 (OECD, 2019) and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) INVITTOX Protocol 78 (ECVAM DB-ALM, 2008) with modifications, as presented in IIVS and EPAA (Vimeo training video). The study design included a solubility assessment to select or confirm the most suitable solvent for the botanical ingredients and at least two experimental trials. The positive control, chlorpromazine, was tested concurrently with the experimental trials. The acceptance of each experimental trial was determined in accordance with the protocol used by IIVS and adapted from the

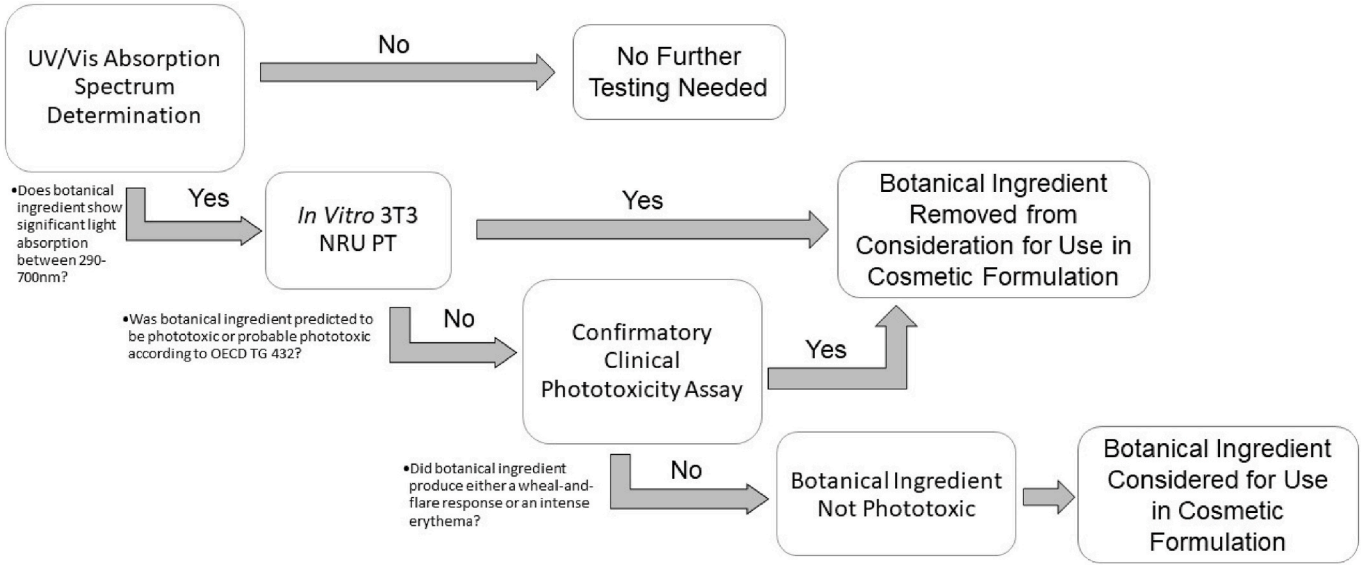

Fig. 1. Testing and safety evaluation strategy used for identification of phototoxicity (*i.e.*, photoirritation) potential of botanical ingredients.

criteria presented in OECD TG 432 (OECD, 2019).

The solubility of each botanical ingredient was evaluated in Hanks' Balanced Salt Solution (HBSS) without phenol red, or in intermediate solvents, dimethyl sulfoxide (DMSO), ethanol, acetone, or a 50:50 mixture of ethanol:water at the highest possible concentrations presented in OECD TG 432.

The results obtained in two independent experiments (identified as either dose range and/or experimental trial) were used to evaluate the phototoxicity potential of the botanical ingredients. A dose range finding experiment using a wide range of concentrations from 0.291 μg/mL to 1000 μg/mL may have been performed to inform concentrations for experimental trials using a narrower range of concentrations. In the dose range trials, the concentrations were prepared *via* serial dilution at a dilution factor of 3.2, or ~ ½ log steps, and in the narrow range trials, the concentrations were prepared *via* serial dilution at a dilution factor of 1.8, or ~ ¼ log steps. The positive control, chlorpromazine, was evaluated at twelve concentrations ranging from 0.156 μg/mL to 100 μg/mL using a dilution factor of 1.8. The solvent (vehicle) used to prepare each botanical ingredient or positive control was included on each test plate.

Two plates were seeded with the Balb/c 3T3 cells (*i.e.,* 10,000 cells/well) for each botanical ingredient or positive control [*i.e.,* one for testing in the presence of UVA/Vis light irradiation (+Irr) and one in the absence thereof (-Irr)]. At least eight concentrations were prepared for each botanical ingredient. Each concentration was applied in six replicate wells per plate, and 12 wells designated as the solvent controls received the respective solvent (vehicle) control containing the solvent at the same concentration as for the treatment groups. After dosing, the plates were incubated at standard culture conditions (*i.e.,* 5 % ± 1 % CO_2, 37° ± 1 °C, in humidified air) for 60 min.

After the initial treatment period, the plates were moved to the appropriate exposure condition for 50 min: the plates designated for irradiation (+Irr) were placed under the solar simulator and received 5 J/cm^2 of total UVA exposure, while the non-irradiated (-Irr) plates were retained in the dark in the same room. For the cultures designated for irradiation (+Irr), a Dermalight SOL3 (UVATECH, California, USA) solar simulator equipped with H1 filter (320–400 nm) was used. The UVA intensity of the solar simulator was monitored throughout the duration of the assays using a Honle Type II (Honle, Germany) radiometer equipped with UVA sensor. An intensity of 1.7 ± 0.1 mW/cm^2 of UVA for 50 min was equivalent to 5 J/cm^2 of UVA.

The treatments were then removed, and the plates rinsed once with 125 μL of HBSS/well. Culture medium containing antibiotics was added to the plates, and the plates incubated for a 24 ± 1 h post-exposure incubation period, after which the viability was assessed using a 33 μg/mL neutral red dye prepared in medium containing 10 % serum. An extraction solvent was added to lyse the cells, and the amount of neutral red retention was quantified on an absorbance plate reader at 550 nm (OD_{550}).

The raw data collected from each trial were input into the Phototox 2.0 software (ZEBET) developed to determine the phototoxicity potential (Holzhütter et al., 1995; Holzhütter, 1997). Relative viability was determined comparing the OD_{550} value of each test well to the mean solvent (vehicle) control OD_{550} value on the respective irradiated or non-irradiated test plate. The IC_{50} values (+Irr and –Irr), Photo Irritancy Factor (PIF) value, and Mean Photo Effect (MPE) value were calculated with the Phototox 2.0 software. An IC_{50} value (*i.e.,* concentration causing 50 % inhibition of neutral red uptake) was determined for each test plate. The IC_{50} values were used to calculate a PIF value by dividing the IC_{50} value (-Irr) by the IC_{50} value (+Irr). If the IC_{50} value was only determined in the presence of irradiation (+Irr), an estimated PIF value (*e.g.*, > PIF) was determined by dividing the highest tested concentration (-Irr) by the IC_{50} value (+Irr). If an IC_{50} value was not obtained in the presence (+Irr) or absence (-Irr) of irradiation (+Irr), the PIF value was not determined. The software also calculated an MPE value. Briefly, the MPE value is a comparison of a set of dose responses across the range of concentrations in the presence and absence of irradiation, and is especially useful when IC_{50} values cannot be calculated (Holzhütter et al., 1997). The PIF and MPE values were used to evaluate phototoxicity according to OECD TG 432 (Table 1): a botanical ingredient was predicted to have no phototoxicity potential, probable phototoxicity potential, or phototoxicity potential.

2.6. Confirmatory clinical testing

Six botanical ingredients that were predicted to have no phototoxicity potential according to the 3T3 NRU PT results were formulated in a prototype cosmetic formulation containing the final desired use concentration, which was ≤0.1 % of the botanical ingredient (Fig. 1). Although 28 ingredients were found to be non-phototoxic in the 3T3 NRU PT, the 6 selected botanical ingredients were prioritized by product development due to existing literature indicating their potential antioxidant and skin-conditioning properties, making them particularly attractive for use in finished cosmetic applications. They underwent confirmatory clinical testing (n = 10) based on the method of Kaidbey and Kligman, 1978 but with exposure to each cosmetic formulation containing the botanical ingredient for 24 h rather than 6 h and exposure to 0.5 Minimal Erythemal Dose (MED) full spectrum solar-simulated radiation in addition to 10 J/cm^2 of UVA. Measurement of the shortest exposure producing a minimally visible faint erythema 20–24 h later was used to determine the MED for each subject on Day 1. On Day 2, an amount of approximately 40 mg of each cosmetic formulation was applied to duplicate (2 × 2 cm) squares of nonwoven cotton cloth and fastened to skin with occlusive tape. After 24 h, one set of patches was removed and the test sites immediately exposed to 10 J/cm^2 UVA plus 0.5 MED full spectrum solar-simulated radiation. The other set of patches served as non-irradiated controls. An adjacent site was similarly treated with a vehicle (petrolatum) and exposed to the same dose of UVA plus 0.5 MED full spectrum solar-simulated radiation and served as an irradiated control. Reactions were graded immediately and again at 24 and 48 h after irradiation. A phototoxic material will produce either a wheal-and-flare response immediately after exposure or an intense erythema at either 24 or 48 h (Kaidbey and Kligman, 1978). The human clinical phototoxicity protocol was approved by an Independent Ethical Review Board (IRB) prior to the initiation of the studies.

3. Results

3.1. UV/Vis absorbance evaluation of the botanical ingredients

Thirty-eight botanical ingredients produced significant absorbance [*i.e.,* > 0.1 absorbance units (AU)] in the wavelengths of interest between 290 nm and 700 nm (data not shown). They were further investigated to determine their phototoxicity potential.

3.2. 3T3 NRU PT used as screening tool of phototoxicity potential of botanical ingredients

Of the thirty-eight botanical ingredients, a total of twenty-four did not induce sufficient cytotoxicity to produce IC_{50} values, and therefore PIF values were not determined (Table 2). A > PIF value using the IC_{50}

Table 1
3T3 NRU PT prediction for phototoxicity (*i.e.*, photoirritation) potential based on OECD TG 432 (2019).

MPE Value	PIF Value	Prediction of Phototoxicity Potential
<0.1	<2.0	No phototoxicity potential
≥0.1 and < 0.15	≥2.0 and < 5.0	Probable (equivocal) phototoxicity potential
≥0.15	≥5.0	Phototoxicity potential

MPE, Mean Photo Effect value; NRU, Neutral Red Uptake; PIF, Photo Irritancy Factor value; PT, Phototoxicity Test.

Table 2
Summary results of phototoxicity (*i.e.*, photoirritation) potential for the 38 botanical ingredients evaluated using the 3T3 NRU PT. A subset of 6 botanicals was subject to subsequent clinical confirmatory testing.

Botanical Name	PIF[a]	MPE[b]	3T3 NRU PT Prediction of Phototoxicity Potential
Erythrina flabelliformis extract	>2.52^	0.160	Phototoxic
Feronia elephantum extract	>3.10^	0.170	Phototoxic
Hymenosporum flavum extract	45.4	0.550	Phototoxic
Lonchocarpus capassus extract	>2.37^	0.224	Phototoxic
Thunbergia laurifolia extract	>2.26^	0.095	Probable Phototoxic
Backhousia citriodora leaf oil	1.11	0.014	Not Phototoxic
Botanical Blend 1: *Citrus nobilis* (Mandarin Orange) peel extract; *Citrus grandis* (Grapefruit) fruit extract; Isopropyl Myristate; *Citrus aurantium dulcis* (Orange) peel extract; *Mangifera indica* (Mango) fruit extract; *Aniba rosaeodora* (Rosewood) wood extract; *Citrus aurantifolia* (Lime) peel extract; *Vanilla planifolia* fruit extract	ND	0.006	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Botanical Blend 2: *Citrus Aurantium dulcis* (Orange) peel extract; *Mangifera indica* (Mango) fruit extract; *Aniba rosaeodora* (Rosewood) wood extract; *Citrus grandis* (Grapefruit) fruit extract; *Citrus nobilis* (Mandarin Orange) peel extract; *Vanilla planifolia* fruit extract; *Citrus aurantifolia* (Lime) peel extract; *Prunus armeniaca* (Apricot) fruit extract	1.76	0.018	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Botanical Blend 3: *Vanilla planifolia* fruit extract; *Prunus armeniaca* (Apricot) fruit extract; *Vitis vinifera* (Grape) fruit extract; Butter extract; *Aniba rosaeodora* (Rosewood) wood extract; *Cinnamomum zeylanicum* bark extract; *Citrus medica limonum* (Lemon) peel extract; *Trigonella foenum-graecum* seed extract; *Theobroma cacao* (Cocoa) extract; *Eugenia caryophyllus* (Clove) fower extract; *Lavandula angustifolia* (Lavender) extract	ND	0.047	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Botanical Blend 4: *Camellia sinensis* leaf extract; *Aniba rosaeodora* (Rosewood) wood extract; *Lavandula angustifolia* (Lavender) extract; *Rosmarinus officinalis* (Rosemary) leaf extract; *Fucus vesiculosus* extract, *Prunus persica* (Peach) fruit; *Vanilla planifolia* fruit extract; Rose extract; *Citrus Aurantium bergamia* (Bergamot) fruit extract; *Coriandrum sativum* (Coriander) seed extract; *Cupressus sempervirens* seed extract; *Jasminum officinale* (Jasmine) flower extract	ND	0.012	Not Phototoxic
Botanical Blend 5: *Cucumis sativus* (Cucumber) fruit extract; *Anthemis nobilis* flower extract; Rose extract; *Citrus medica limonum* (Lemon) peel extract; *Cucumis melo* (Melon) fruit extract	ND	0.012	Not Phototoxic
Cinnamomum zeylanicum leaf oil; *Murraya koenigii* stem extract	1.06	0.001	Not Phototoxic
Citrus grandis (Grapefruit) seed extract	0.917	−0.024	Not Phototoxic
Coleus forskohlii root extract	ND	−0.003	Not Phototoxic
Cupressus sempervirens cone extract	ND	0.027	Not Phototoxic
Gynandropsis gynandra extract	ND	0.015	Not Phototoxic
Harungana madagascariensis extract; *Chamomilla recutita* extract	1.15	0.004	Not Phototoxic
Hedyotis auricularia extract	0.910	−0.036	Not Phototoxic
Helianthus annuus (Sunflower) seed oil; (Matricaria) flower extract	ND	−0.003	Not Phototoxic
Helianthus annuus (Sunflower) seed oil; *Vanilla planifolia* fruit extract	ND	−0.059	Not Phototoxic
Hibiscus sabdariffa flower extract	ND	−0.057	Not Phototoxic
Hippophae rhamnoides fruit extract	ND	−0.046	Not Phototoxic
Lycium chinense fruit extract	ND	−0.078	Not Phototoxic
Mentha piperita (Peppermint) extract	ND	−0.032	Not Phototoxic
Mentha piperita (Peppermint) leaf extract; *Vanilla planifolia* fruit extract	ND	−0.032	Not Phototoxic
Mentha viridis (Spearmint) extract	ND	−0.033	Not Phototoxic
Nymphaea coerulea flower extract	ND	0.026	Not Phototoxic
Petasites hybridus leaf extract	ND	−0.009	Not Phototoxic
Plankton Extract; Arginine ferulate	ND	0.061	Not Phototoxic
***Pouzolzia pentandra* extract**	ND	−0.006	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Radish Root ferment filtrate	ND	−0.006	Not Phototoxic
***Raphia farinifera* extract**	0.930	0.036	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Rhinacanthus nasutus extract	ND	0.034	Not Phototoxic
Royal Jelly; Radish Root ferment filtrate	ND	−0.008	Not Phototoxic
Salix alba (Willow) bark extract	ND	−0.014	Not Phototoxic
***Sapindus rarak* fruit extract**	1.06	−0.019	Not Phototoxic (**Confirmed by clinical confirmatory testing**)
Simmondsia chinensis (Jojoba) seed oil	ND	−0.046	Not Phototoxic
Skeletonema costatum extract	1.52	0.027	Not Phototoxic

IC_{50}, concentration of 50 % inhibition of NRU; MPE, Mean Photo Effect value; ND, Not Determined; NRU, Neutral Red Uptake; PIF, Photo Irritancy Factor value; PT, Phototoxicity Test; UV, Ultraviolet light.

Notes.

- Botanical ingredients are listed first based on the predicted phototoxic potential and then alphabetically within each respective group (phototoxic; probable phototoxic, and non-phototoxic, respectively). The botanicals tested in a subsequent clinical study are presented in bold.
- The MPE and PIF values presented are the average results of at least 2 experiments.
- An MPE value < 0.100 or PIF value < 2.0 indicated no phototoxicity potential; an MPE ≥0.100 and < 0.150 and/or PIF ≥2.0 and < 5.0 indicated probable phototoxicity potential; and an MPE value ≥ 0.150 and/or PIF value ≥ 5.0 indicated phototoxicity potential according to OECD TG 432 (2019).
- ND: a PIF value could not be determined by the Phototox 2.0 software because an IC_{50} value was not calculated + Irr and -Irr.
- [a]PIF = IC_{50} value (− Irr)/IC_{50} value (+Irr).
- [b]MPE = a comparison of the difference between the dose-response curves were carried out by the Phototox 2.0 software.
- ^ represents > PIF since IC_{50} value determined only in presence of irradiation (+Irr); calculated from highest tested concentration (-Irr)/IC_{50} value (+Irr).

(+Irr) and the highest tested concentration (-Irr) was determined for four botanical ingredients, *Erythrina flabelliformis* extract, *Feronia elephantum* extract, *Lonchocarpus capassus* extract, *and Thunbergia laurifolia* extract, since an IC_{50} was produced only + Irr (Table 2). When a PIF value was not determined, the phototoxicity potential was evaluated using the MPE value. Four botanical ingredients resulted in a prediction of phototoxicity potential, and one botanical ingredient resulted in a prediction of probable phototoxicity potential. A total of thirty-three of the botanical ingredients resulted in a prediction of no phototoxicity potential (Table 2).

The results of the 3T3 NRU PT were also analyzed as graphs presenting dose responses as % relative viability over botanical ingredient concentration in the presence (+Irr) and absence (-Irr) of irradiation. Fig. 2 provides examples of representative cellular responses to the treatment with botanical ingredients that showed no cytotoxicity or phototoxicity (panel 2a), cytotoxicity without phototoxicity (panel 2b), no cytotoxicity but phototoxicity potential (panel 2c) or both cytotoxicity and phototoxicity potential (panel 2d).

3.3. Clinical testing

Six of the 33 botanical ingredients that were determined to have no phototoxicity potential in the 3T3 NRU PT and formulated in a prototype cosmetic formulation containing the final desired use concentration (*i.e.*, ≤0.1 % of botanical ingredient), underwent confirmatory clinical testing. None of the botanical ingredients evaluated in the clinical confirmatory testing resulted in adverse reactions (Table 2).

4. Discussion

Botanical and natural substances have been historically in use for medicinal, personal care and industrial purposes. They are generally sourced from the roots, flowers, fruits, leaves or seeds of the plants and are available in a wide variety of preparations, ranging from solvent extracts to distilled essential oils, expressed juices, tinctures, waxes, etc. This diversity of available species and sources facilitates the design of a wide range of finished products for a multitude of intended application purposes.

In recent years, consumers' interest shifted focus to cosmetic, personal or household care products containing such ingredients, leading to an annual growth of the global natural cosmetics market of ~11 % (between 2015 and 2019) (Ferreira et al., 2021). For example, in the United States >70 % of the population has been reported to use an herbal product (Mortimer and Reeder, 2016). Furthermore, within the niche anti-aging cosmetics line, over 70 % of the products on the market contained at least one botanical source ingredient in 2018 (Ferreira et al., 2021).

This surge in the consumers' preference for products containing botanical ingredients is rooted in the perception that they are capable to provide additional benefits (anti-inflammatory, immunomodulatory, anti-oxidant, photoprotective, etc.) over chemical ingredients and that in general "natural" is indicative of a healthy, organic, ecological and safe profile (Antignac et al., 2011). However, contrary to popular beliefs, natural products may induce a variety of toxic effects. For example, furocoumarins are prevalent in plants from the Apiaceae family that include celery (*Apium graveolens*), wild parsnip (*Pastinaca sativa*), and carrot (*Daucus carota*), and in Rutaceae family that includes citrus fruits. The furocoumarins have been determined to be significant contributors

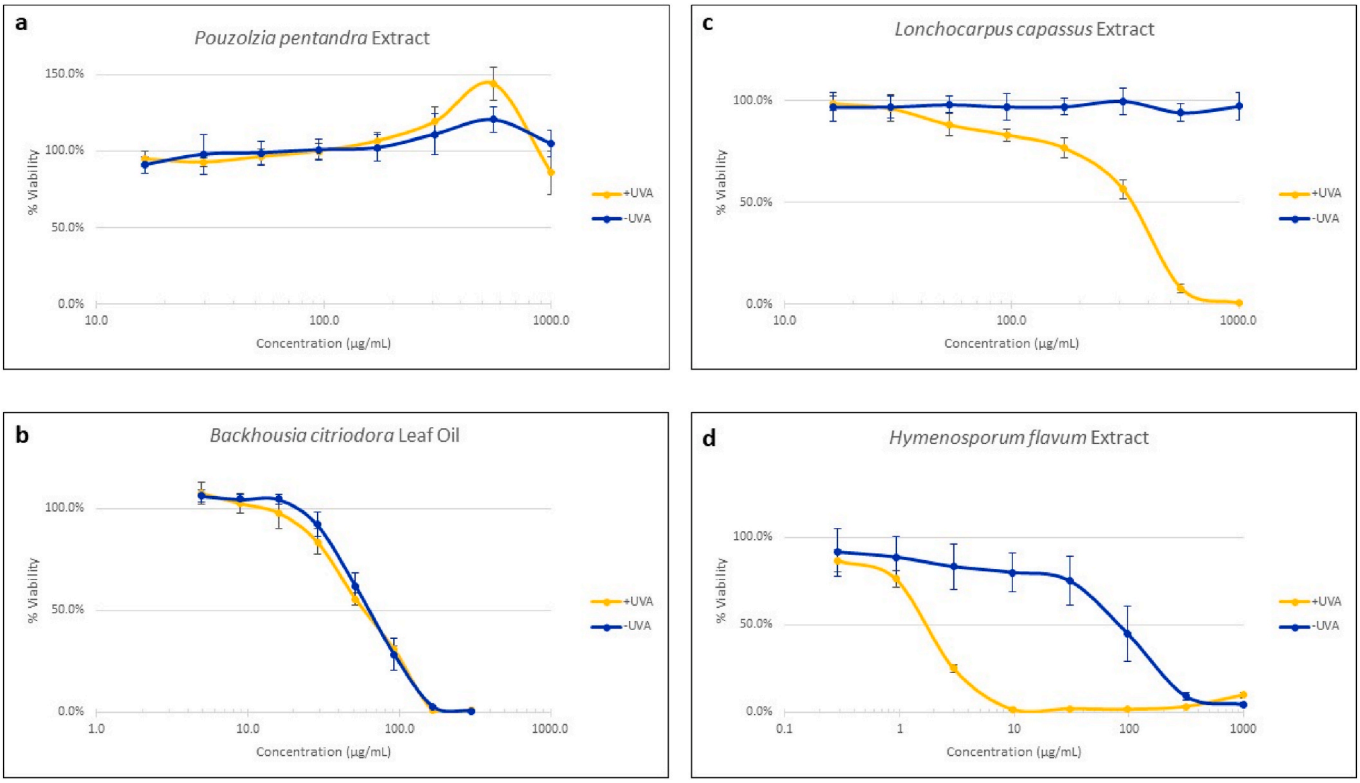

Fig. 2. Representative examples of 3T3 NRU PT results. Percent relative viability (y-axis) over botanical ingredient concentration (x-axis) of responses in the presence of light (yellow line) and absence of light (blue line). At least eight concentrations were evaluated in the presence (+Irr) and absence of irradiation (-Irr) in 6 replicate wells per exposure (+Irr or –Irr), presented as the average relative viability ± 1 standard deviation. Results from the 3T3 NRU PT assay included a botanical ingredient showing no-cytotoxicity or phototoxicity (a); cytotoxicity without phototoxicity (b); no cytotoxicity but phototoxicity potential (c); and cytotoxicity and phototoxicity potential (d). Graphics created using Microsoft Excel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to induction of potent phototoxic reactions (Phachasupap et al., 2012; Paulsen et al., 2014; Kenari et al., 2021; Guan et al., 2022; Petit et al., 2024).

Even though in recent years proposals have been made for the safety assessment of botanicals, particularly for systemic endpoints (Galli et al., 2019), topically applied botanical-based consumer products are not always evaluated for their efficacy or safety within a regulatory setting since they do not fall under Food and Drug Administration (FDA)'s purview (Mortimer and Reeder, 2016; Torres et al., 2020). Under the circumstances, consumer products formulated with botanical ingredients undergo a thorough safety assessment as part of the manufacturers' due diligence strategy.

This can be accomplished by using a number of assays gathered under the umbrella of NAMs. Several of these methods have formal regulatory acceptance under OECD as Test Guidelines, and have undergone rigorous testing, evaluation, and review to ensure their transferability, reproducibility and prediction accuracy of human responses to toxicants and are now validated for regulatory decisions. Other testing strategies that may not have yet reached this status are also available and have been considered sufficient to support product development and safety evaluation. Collectively, these methodologies serve the 3 R's - to reduce, replace, or refine the use of animals in testing.

Photosafety evaluations to address phototoxicity, or photoirritation, using NAMs are well-defined in several regulatory and peer-reviewed documents (ICH S10, 2015; OECD, 2019a,b; Ritacco et al., 2022; OECD, 2023, OECD, 2024). These approaches provide guidance for evaluation of phototoxicity, but not specifically for photoallergy. At Avon Global Research and Development, every cosmetic ingredient was also evaluated to determine its potential for eliciting skin sensitization prior to human clinical testing based on the default established internal procedures. Photoallergy is a delayed, immune-mediated response upon exposure to a photoallergic compound and subsequent exposure to light (ICH S10, 2015) with clinical manifestations that may persist for extended periods of time and may include eczematous eruptions and itching (Maibach and Honari, 2014). While there has been increasing interest and approaches have been proposed to address photoallergy using a battery of regulatory and non-regulatory test methods, our evaluation focused on photoirritation.

In some cases, the test methods may require adaptations to accommodate specific testing needs and thus overcome technical challenges as we show herein for botanical ingredients and the phototoxicity (*i.e.,* photoirritation) endpoint. For example, for the first assay in the tiered testing strategy, the UV/Vis evaluation, additional considerations were included since the cosmetic ingredients were complex mixtures and did not have defined molecular weights. As such, the cosmetic ingredients were prepared at 1 % dilutions, which was 10-times the highest concentration of 1000 μg/mL outlined in OECD TG 432 (OECD, 2019). Further, an absorption threshold of >0.1 AU was used to establish significant absorbance. The 2004 version of OECD TG 432 (OECD, 2019) served as guidance during the collection of our data. In the 2004 version, an MEC value > 10 L mol^{-1} cm^{-1} was considered significant, but this threshold was later updated in 2019 to >1000 L mol^{-1} cm^{-1} as supported by the work of Bauer et al. (2014) and Henry et al. (2009). Nishida et al. (2015) presented a similar approach to evaluating complex cosmetic ingredients using an absorption threshold value, with absorbance of >1.0 considered significant. Bouchard et al. (2023) further investigated the threshold approach presented in Nishida et al. (2015) in evaluation of complex mixtures at three starting concentrations of 32, 10, and 3.2 mg/mL using novel solvents. These updated methodologies and approaches may have minimized the number of ingredients that continued the testing strategy using the 3T3 NRU PT.

Another significant adaptation became necessary in order to overcome a typical challenge often encountered in dilution-based assays when used to test complex compounds, like botanicals: their limited solubility in certain solvents. OECD Test Guidelines prescribe solvents that have shown acceptable use specific for each assay, but these solvents may not fully solubilize more complex mixtures. Additional solvents can be considered, but should be carefully evaluated prior to use for compatibility with the test system to ensure they do not enhance or quench phototoxicity potential or cause excess cytotoxicity (Sheehan et al., 2016; Sadowski et al., 2016; OECD, 2019).

OECD TG 432 (2019) specifies that only soluble concentrations should be evaluated. However, Spielmann et al. (1998) evaluated insoluble concentrations during test method development to understand impacts on assay predictivity, and showed that appropriate predictions of phototoxicity hazard could be determined regardless of full solubility. Further, work of Sadowski et al. (2016) revealed that insoluble concentrations should be considered for appropriate prediction of phototoxicity, using amiodarone, a proficiency chemical with phototoxicity potential (OECD, 2019) as an example.

OECD TG 432 (OECD, 2019) provides a maximum testing concentration of 1000 μg/mL, but there is no guidance on a lowest acceptable concentration for evaluation. Testing only soluble concentrations, especially with limited solubility, includes a caveat induced by the regulatory understanding that negative results in the 3T3 NRU PT generally do not require further photosafety testing (ICH, 2015). Since the 3T3 NRU PT incorporates evaluation of a test compound in the presence (+Irr) and absence (-Irr) of irradiation, useful comparisons between dose responses are possible without full solubility across all concentrations. For example, in our experiments we purposefully evaluated concentrations that were not fully soluble in intermediate solvent, and/or in HBSS. In an attempt to facilitate achievement of solubility, additional measures like sonication and heating at approximately 37 °C were employed for the prepared dilutions. Of the five botanicals determined to have phototoxicity or probable phototoxicity potential, only *Thunbergia laurifolia* extract was fully soluble at all tested concentrations. However, phototoxicity potential was still predicted through comparison of dose response curves, as supported by the PIF and/or MPE values obtained in the experiments. Similarly, four of the six botanicals that underwent clinical testing were not fully soluble in the 3T3 NRU PT: Botanical blend 2, Botanical blend 3, and *Raphia farinifera* extract, prepared in HBSS, and *Pouzolzia pentandra* extract, initially prepared in a 50:50 mixture of ethanol:water. Of note, only Botanical Blend 1, prepared in HBSS, and *Sapindus rarak* fruit extract, prepared in 50:50 ethanol:water, were fully soluble at all concentrations. While not fully soluble at the highest concentrations, the predictions by the 3T3 NRU PT were confirmed by the lack of phototoxicity in clinical testing.

Putting in practice all these adaptations of the assays, we conducted a prospective evaluation of botanical ingredients according to the tiered-testing paradigm outlined in Fig. 1. This photosafety strategy was adapted by Avon Global Research and Development as a very conservative approach given the type of materials evaluated and limited resources available during the collection of this data over several years, before NAMs reached regulatory status. In brief, if a botanical ingredient was determined to have significant absorption in the UV/VIS absorbance assay, and also resulted in a probable phototoxicity or phototoxicity potential (*i.e.,* MPE ≥0.1 and/or PIF ≥2.0) in the 3T3 NRU PT, a risk averse evaluation was upheld by the company dictating the botanical ingredient be removed from consideration for use in further formulation development. A subset of 6 ingredients predicted by the 3T3 NRU PT to have no phototoxicity were further confirmed in the clinical testing and were considered for use in cosmetic products. This thoroughly informed selection for further testing using a confirmatory clinical evaluation upheld a standard of ethics and minimized the risk for adverse phototoxicity effects not only in human subjects included in the study, but also to the general population now able to enjoy products formulated with the promising botanical ingredient.

More recently published work reported on options for further photosafety assessment before removing promising candidates from consideration; these approaches were not available at the time when we tested the botanical ingredients evaluated in our manuscript. The OECD Test Guideline 498 based on Reconstructed human Epidermis (RhE)

tissue models was adopted in 2021 and updated in 2023 for use as a stand-alone test method for evaluating phototoxicity hazard. Additional RhE-based NAMs have proven utility in evaluation of phototoxicity in combination with the 3T3 NRU PT assay (Ceridono et al., 2012; Gaspar et al., 2013; Ritacco et al., 2022). For example, Ritacco et al. (2022) reported the use of a tiered testing paradigm similar to the one used in our experiments: after evaluation of fragrance materials in the UV/VIS assay, the 3T3 NRU PT was used as a first-tier assay. Further, unlike the strategy we used in our experiments, the authors continued the profiling of the fragrance materials using the RhE PT assay only for those that were predicted to have phototoxicity potential in the cell-based assay. At least three concentrations of each fragrance material were evaluated in the RhE PT to establish No Effect Levels (NOELs) for phototoxicity, and the maximum concentration with no phototoxicity potential (based on the prediction model using percent relative viability comparisons between tissues exposed + Irr and –Irr) was used to inform concentrations for the clinical confirmatory test (Ritacco et al., 2022).

Our data predicted four of the extracts to have phototoxicity potential and one as probable phototoxicity as further detailed. We included in our experiments an extract of *Erythrina flabelliformis* from the Fabaceae family. Phytochemical studies of *Erythrina* species showed that alkaloids and flavonoids are their main secondary metabolites which have been shown to be phototoxic (Li et al., 2018; Siewert and Stuppner, 2019; Grosu Dumitrescu et al., 2024). Triterpenes, sterols, stilbenes, coumarins and phenolic acids are also reported in the genus (Kumar et al., 2010; Fankam and Kuete, 2024), of which coumarins have been reported to be also phototoxic (Fu et al., 2013; Siewert and Stuppner, 2019; Petit et al., 2024; Grosu Dumitrescu et al., 2024). Also part of the Fabaceae family is *Lonchocarpus capassus* with a chemical composition for the genum based on terpenoids, flavonoids, saponin, tannins, etc. (Moronkola and Oladosu, 2013; Santos et al., 2024), all demonstrated to have phototoxic activity (Li et al., 2018; Kumar et al., 2010; Siewert and Stuppner, 2019; Petit et al., 2024; Grosu Dumitrescu et al., 2024). *Feronia elephantum* belongs to Rutaceae family which has been identified to contain coumarins as phototoxic components (Fu et al., 2013; Siewert and Stuppner, 2019; Grosu Dumitrescu et al., 2024); other studies on the genus identified alkaloids, furocoumarins as other chemical classes with phototoxic potential (Siewert and Stuppner, 2019; Petit et al., 2024). Finally, *Hymenosporum flavum* belongs to the Pittosporaceae family which has been identified to contain furocoumarins as the potential phototoxic compounds (Petit et al., 2024). The extract of *Thunbergia laurifolia* was predicted to have a probable phototoxic potential; it belongs to the family Acanthaceae and studies indicated flavonoids, alkaloids and triperpenoids as potential ingredients responsible for phototoxic effects (Petit et al., 2024).

Our results indicate that the assays we used were sensitive enough to identify phototoxic botanical ingredients whose activity is supported by the numerous previous studies that investigated their potential. The design of the assays, in particular, the 3T3 NRU PT, also afforded a high-throughput screening tool to select the most promising out of a large number candidates, in a cost-effective, timely, and ethical manner. In sum, we demonstrated that the strategy employed justified its usefulness not only by identifying phototoxic botanical ingredients, but also by confirming in clinical evaluation the non-phototoxic profiles predicted *in vitro*.

5. Conclusion

The results of our experiments generated using the tiered testing paradigm we described herein support the use of *in chemico* and cell-based *in vitro* methodologies for the assessment of phototoxicity potential of botanical ingredients prior to clinical testing. The 3T3 NRU PT is a well-established, validated high throughput assay that can be reliably used as a first-tier method for botanical ingredients profiled for phototoxic potential. As the 3T3 NRU PT assay is known for being a highly sensitive assay, compounds resulting in phototoxicity potential are considered of concern and qualify for follow-up assessment with other considerations given for human risk, while those without phototoxicity potential are considered as such and further testing is generally not warranted (Ceridono et al., 2012; ICH, 2015; Ritacco et al., 2022). Its integration with other assays of higher complexity levels (RhE PT, clinical investigations) provides a solid testing strategy even for challenging materials like botanical ingredients (solubility concerns, no molecular weight, etc.) that need to be evaluated for a multitude of safety endpoints in order to confirm their safety in cosmetic products for human use.

CRediT authorship contribution statement

Allison Hilberer: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lisa Hoffman:** Writing – review & editing, Writing – original draft, Visualization, Resources, Data curation, Conceptualization. **Megan Madrid:** Writing – review & editing, Formal analysis, Data curation. **Ramez Labib:** Writing – review & editing, Visualization, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Gertrude-Emilia Costin:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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