Evaluation of Biologically Inspired Ammonium Xanthommatin as a Multifunctional Cosmetic Ingredient

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We describe the investigation of an organic natural product, ammonium xanthommatin (Xanthochrome), in a series of studies designed to not only assess its impact on endocrine receptor function in vitro but also interrogate its mutagenic potential using bacterial reverse mutation assays. As a multifunctional raw material, ammonium xanthommatin functions as an antioxidant with a broad absorption profile spanning the UV through the visible spectrum, making it an interesting target for cosmetic applications. In solution, ammonium xanthommatin contributes to <30% inhibition of hormonal activities, indicating that it is not an endocrine disruptor. Furthermore, the compound does not cause gene mutations in the bacterial strains used, indicating that it is nonmutagenic. Applications are also described, highlighting xanthommatin's ability to boost the UVA and UVB absorptive properties of traditional chemical UV filters by >50% across all filters tested. In addition to these features, xanthommatin exhibited no phototoxic hazards in vitro when irradiated with UVA and visible light, demonstrating its utility as a multifunctional cosmetic ingredient. Although these findings encourage the use of xanthommatin in cosmetics, they represent only the beginning of the complete in vitro and in vivo data package needed to support safety and efficacy claims for future applications in skin health.

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INTRODUCTION

Growing trends support the application and utility of biologically derived or biologically inspired raw materials in cosmetics, as the industry continues to push toward natural and ecofriendly product offerings designed to replace many nonsustainable and sometimes toxic counterparts (Bom et al., 2020, 2019). Despite the origin of their inspiration or sourcing, these biobased or bioinspired raw materials are still subject to rigorous safety screening and standards before they can be incorporated in over-the-counter products (Bom et al., 2020, 2019). In this study, we describe the application and testing of a recently registered cosmetic ingredient ammonium xanthommatin (Xanthochrome) in vitro. Ammonium xanthommatin (referred to as xanthommatin in this paper) is a naturally occurring chromophore present in arthropods and cephalopods and is formed during the metabolism of tryptophan in these species (Deravi et al., 2014; Futahashi, 2012; Osanai-Futahashi et al., 2016; Riou and Christidès, 2010; Williams et al., 2019, 2016). It has a natural red/yellow color depending on its oxidation state (Futahashi, 2012) that can also be enhanced or muted depending on its formulation specifications. It has also recently been shown to behave as an antioxidant and broad-spectrum absorber that spans the UV through the visible range in vitro (Martin et al., 2019)—two features that highlight its potential as multifunctional cosmetic raw material. Given this exciting potential, our goal is to understand the reactivity of xanthommatin in vitro. Specifically, in this report, we evaluate the effect of xanthommatin on cell viability, endocrine receptor binding and/or inhibition, phototoxicity, and the induction of genetic alterations according to the Organization for Economic Co-operation and Development guidelines.

We chose to focus on the endocrine system as our primary target because it is a major homeostatic system that regulates normal functions in the body, including but not limited to the female reproductive cycle, bone development and growth, cellular proliferation, and behavioral properties (Marty et al., 2011). Disruption or damage to the endocrine system through exogenous factors, such as chemical ingredients, would directly impact one or all of these physiological processes; thus, it is imperative to first test how raw materials designed to interface with the body impact these receptor functions before going to market. One way to test these interactions in vitro is through the steroidogenesis assay that evaluates the production of testosterone and estradiol in cell culture. We chose this assay together with androgen receptor (AR)- and estrogen receptor (ER)-mediated transcriptional activation assays to evaluate how xanthommatin induces (i.e., acts as agonists) or suppresses (i.e., acts as antagonists) AR- or ER-dependent transcription. Beyond the endocrine system, we also investigated the mutagenic capacities of xanthommatin using bacterial reverse mutation assays, where the goal is to evaluate whether the presence of this compound might introduce genetic mutations in vitro

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Abbreviations: AR, androgen receptor; ER, estrogen receptor

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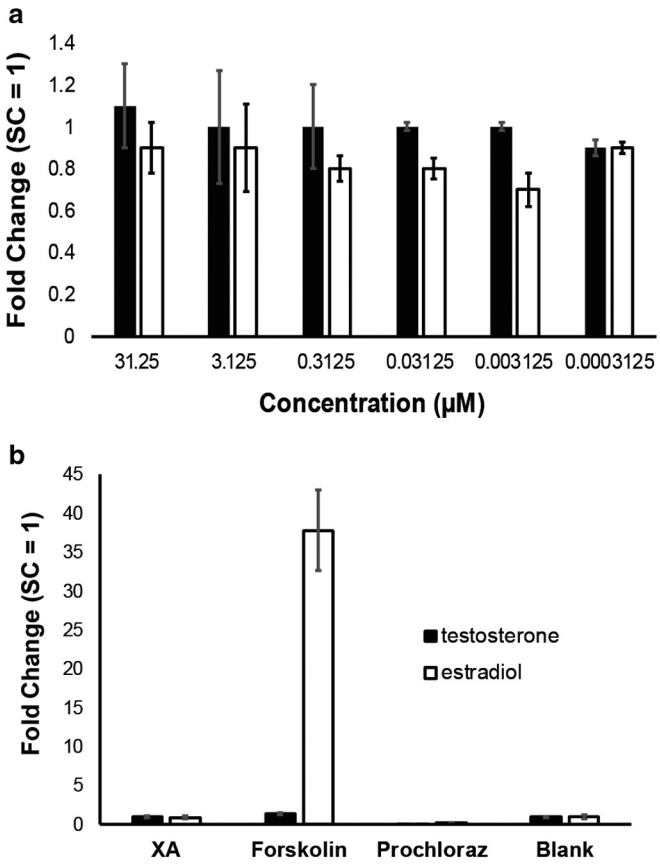

Figure 1. Results from the endocrine disruption test using the steroidogenesis ELISA assay compared with those from the SC. (a) Graphical evaluation of estradiol and testosterone fold change with XA in one trial (n = 3, error is SD). (**b**) Summary of endocrine disruption studies comparing XA (3.125 μM) with forskolin (induces estradiol and testosterone, 1 μM concentration shown in this figure), prochloraz (inhibits estradiol and testosterone, 1 μM concentration shown in this figure), and the solvent (blank). Experimental results are normalized to the solvent control, which had a fold change of 1. We found no cytotoxicity >20% in the tested concentration ranges compared with that in the solvent control. In Figure 1a and b, the black bars represent testosterone, and the white bars represent estradiol as denoted in the legend in Figure 1b. SC, solvent control; XA, xanthommatin.

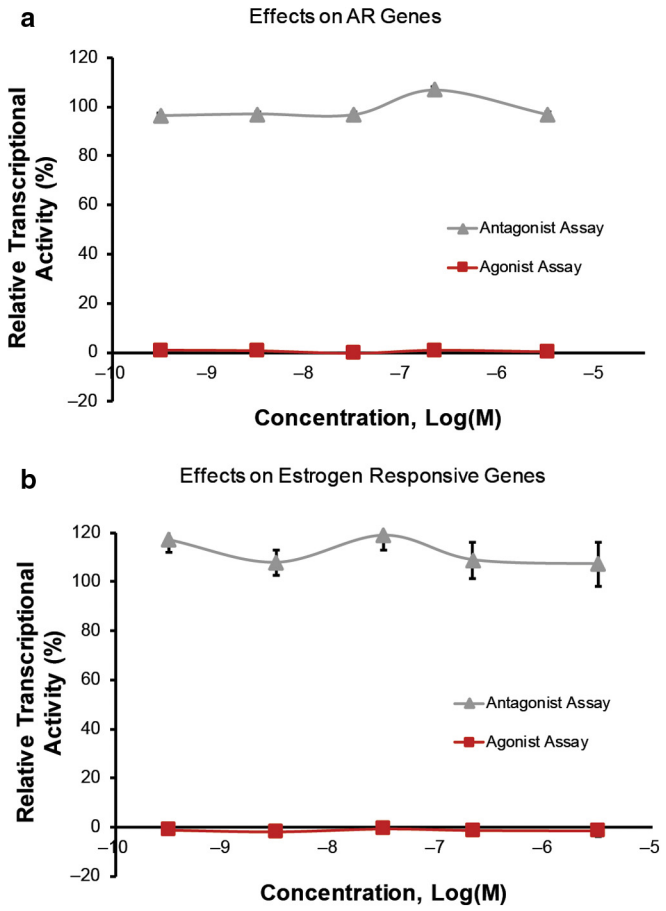

Figure 2. Analysis of agonist and antagonist properties of xanthommatin. Results from the (**a**) AR and (**b**) ER transcriptional activation assays across a broad range of xanthommatin concentrations. In both cases, xanthommatin can be classified as negative in both agonist and antagonist assays when assayed across these two gene types. n = 3; error is SD. AR, androgen receptor; ER, estrogen receptor.

(Ames et al., 1973; Claxton et al., 1987; Maron and Ames, 1983). Finally, we end with a proof-of-concept demonstration that highlights the utility of xanthommatin as a UV filter booster when combined with traditional chemical UV filters and demonstrate that xanthommatin does not elicit any phototoxic responses in the presence of UVA irradiation. Results from this exploratory in vitro data package validate the utility of this bioinspired material as a cytocompatible, multifunctional ingredient, which is an important first step in evaluating its performance for cosmetic applications.

RESULTS

To investigate whether xanthommatin induces or inhibits endocrine receptor function, we first investigated how it affected the production of testosterone and 17β-estradiol in vitro. Before all experiments, cell viability was measured at all concentrations using the MTT assay (Supplementary Table S1). When assayed across a broad concentration range (0.00003125–31.25 μM); xanthommatin exhibited no statistically significant increase or decrease in fold induction of testosterone or estradiol formation compared with the solvent control (Figure 1a and Supplementary Tables S2 and S3). These results indicated that xanthommatin does not behave as a hormonal disrupter within this specified range. When compared directly with forskolin (1 μM) and prochloraz (1 μM), which are known to induce and inhibit testosterone and estradiol activity, respectively, xanthommatin (3.16 μM) exhibited fold changes of 1 ± 0.1 (testosterone) and 0.9 ± 0.21 (estradiol), whereas the fold changes associated with forskolin (1.4 ± 0.15 for testosterone and 37.8 ± 5.2 for estradiol) and prochloraz (0.1 ± 0 for testosterone and 0.2 ± 0.04 for estradiol) indicated notable differences (Figure 1b and Supplementary Tables S2 and 3). When taken together, the findings from the steroidogenesis assays indicate that xanthommatin did not induce estradiol and testosterone expression within human adrenocortical carcinoma cell lines within the specified range.

Next, xanthommatin was assayed against stably transfected AR-EcoScreen (Figure 2a) and human ERα HeLa-9903 (Figure 2b and Supplementary Figure S1) cell lines at multiple concentrations to determine whether xanthommatin has the potential to activate (i.e, act as an agonist) and/or

Table 1. Summary of the Ames Test with XA

Test Item	Concentration (μg/plate)	Number of Revertant Colonies (mean ± SD)									
		TA98		TA100		TA1535		TA1537		WP2 uvrA (pKM101)	
		–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
XA	31.6	33 ± 3	34 ± 9	116 ± 11	125 ± 4	9 ± 3	11 ± 4	13 ± 2	14 ± 8	131 ± 7	175 ± 35
	100	33 ± 6	30 ± 5	126 ± 4	109 ± 17	5 ± 2	8 ± 5	18 ± 4	14 ± 2	172 ± 14	196 ± 11
	316	16 ± 3	33 ± 2	132 ± 13	117 ± 11	9 ± 5	8 ± 3	18 ± 4	15 ± 1	119 ± 9	175 ± 11
	1000	6 ± 2	34 ± 4	116 ± 27	111 ± 15	13 ± 4	10 ± 1	13 ± 2	25 ± 6	167 ± 16	179 ± 15
	2500	16 ± 1	36 ± 5	114 ± 13	127 ± 6	7 ± 4	10 ± 3	8 ± 5	28 ± 8	114 ± 32	150 ± 10
	5000	20 ± 1	35 ± 4	113 ± 4	110 ± 7	10 ± 4	13 ± 4	28 ± 8	29 ± 6	100 ± 12	133 ± 47
Vehicle		24 ± 5	39 ± 13	113 ± 15	120 ± 17	7 ± 3	10 ± 4	13 ± 5	16 ± 5	154 ± 3	252 ± 25
Mutagen I	10	912 ± 137	—	649 ± 26	—	1,176 ± 133	—	95 ± 10	—	1,508 ± 106	—
Mutagen II	2.5	—	2,222±220	—	1,134 ± 141	—	172 ± 9	—	131 ± 23	—	992 ± 45

Abbreviations: w/, with; w/o, without; XA, xanthommatin.
In this table, –S9 = w/o metabolic activation, and +S9 = w/ metabolic activation. n = 3. The vehicle is DMSO. Strain-specific positive controls were included in the assays to show test performance.

suppress (i.e, act as an antagonist) receptor-dependent transcription. These findings are important and have the potential to elucidate whether this compound may activate or block receptor function in a cell type–dependent manner. In the agonist assay, the ability to induce AR-mediated transactivation of luciferase gene expression was determined. In the antagonist assay, the ability to reduce the relative transcriptional activity when coexposed with 5α-dihydrotestosterone (a potent androgen agonist) compared with the ability when exposed to the potent 5α-dihydrotestosterone alone was evaluated. In both cases, xanthommatin neither bound to nor activated the transcription of AR and ER responsive genes, where the average relative transcriptional activity across all concentrations for the antagonist assays was 98.6 ± 4.6% and 108 ± 10.9% for the AR and ER genes, respectively. No 30% inhibitory concentration values could be calculated because the inhibitory effect of xanthommatin was <30% across this concentration range, further supporting that xanthommatin has no antagonistic activity. Conversely, the positive control, 5α-dihydrotestosterone (500 pM), generated a mean luciferase activity of 9.2-fold and 6.5-fold over two independent runs (Supplementary Figure S2). The average relative transcriptional activities in the agonist assays were 0.4 ± 0.5 and –1.5 ± 0.8 for AR and ER genes, respectively. Again, throughout the agonist assays, no PC_{10} value could be calculated because the maximum level of response induced by xanthommatin (i.e., its RPC_{Max}) was below the 10% threshold of the positive control (PC). Specifically, the RPC_{Max} was –0.6% of the response of the positive control (1 nM 17β-estradiol, a potent estrogen agonist) in the first run and was 3.6% in a second independent run. Furthermore, no $logPC_{10}$ values could be calculated, confirming that xanthommatin has no agonistic activity. In all cases, cells maintained >95% cell viability within the specified test concentrations, as validated using the MTT assay.

Given its cytocompatibility and its ability to not disrupt endocrine receptor function in vitro, we expanded this data set in separate bacterial cell lines to next evaluate whether the presence of xanthommatin leads to any adverse genetic mutations within *Salmonella typhimurium* and *E. coli* tester trains (Table 1). Because these bacterial reverse mutation assays directly measure heritable DNA mutations, which have been linked to adverse physiological effects (McCann et al., 1975; Zeiger et al., 1992, 1988), they provide useful information on whether specific mutations occur on application of exogenous raw materials. According to the specific assay employed (e.g., either direct plate incorporation or the preincubation method), the bacteria are first exposed to solutions of the raw material (e.g., xanthommatin in DMSO) with and without metabolic activation. After an incubation time of 48–72 hours, revertant colonies are then counted (Maron and Ames, 1983). At least five different concentrations of xanthommatin were tested with approximately half-log intervals between test points (Table 1 and Supplementary Table S4). Positive, negative, and solvent controls (Gatehouse et al., 1994) were also tested in parallel. Throughout the two independent experiments run, no significant increases in revertant colony numbers were observed across the five tester strains when treated with xanthommatin up to the highest recommended concentration of 5 mg/ml, neither in the presence nor

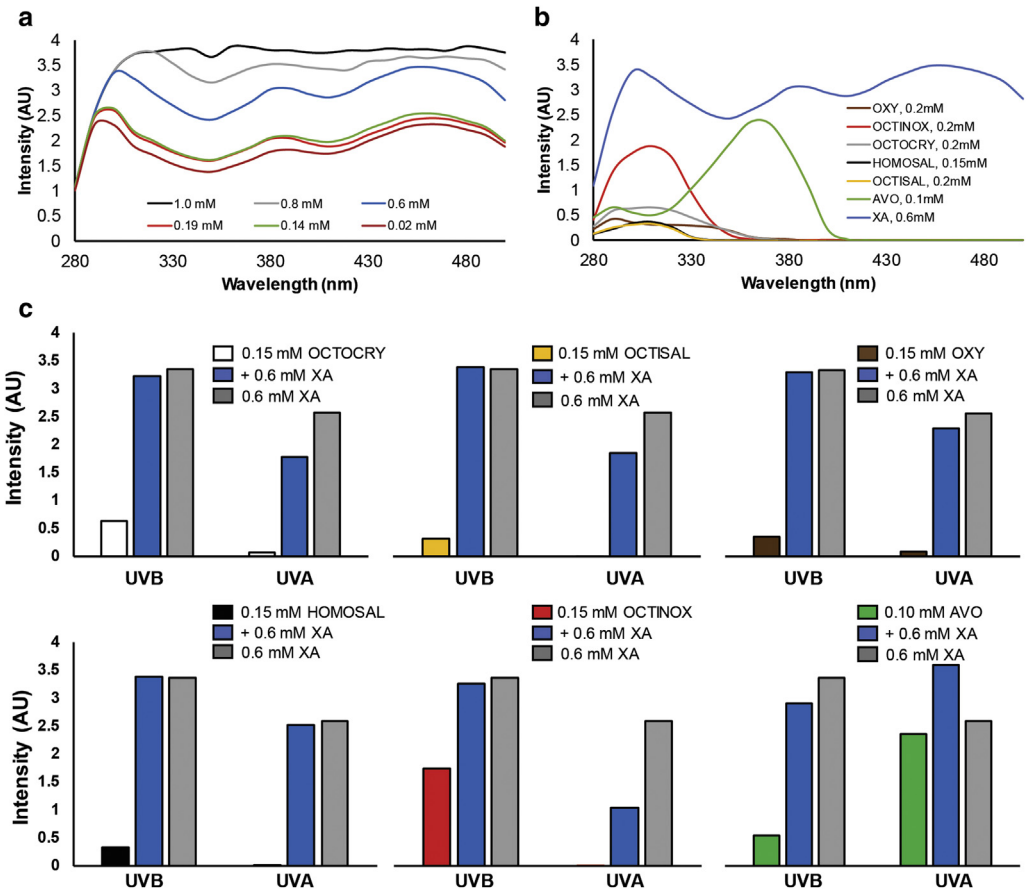

Figure 3. UV filter–boosting properties of XA. Results depicting (**a**) the absorptive properties of varying concentrations of XA in DMSO and (**a**) absorptive properties of XA compared directly with those of traditional UV filters at a fixed concentration (0.1–0.2 mM) where XA is at 0.6 mM in DMSO. (**c**) A comparison of the UVA (recorded at 360 nm) with UVB (recorded at 300 nm) absorbance of traditional UV filters alone, filters blended with XA (+0.6 mM), and XA only (0.6 mM), showing that the addition of XA contributes to the enhanced performance of traditional UV filters, eliciting broad-spectrum protection against solar UV. AU, arbitrary unit; AVO, avobenzone; HOMOSAL, homosalte: OCTINOX, octinoxate; OCTISAL, octisalate; OCTOCRY, octocrylene; OXY, oxybenzone; XA, xanthommatin.

in the absence of metabolic activation, indicating that xanthommatin can be classified as nonmutagenic.

In the first test, xanthommatin precipitation was observed at the highest concentration (5,000 μg/plate) in all tester strains used with metabolic activation. Toxic effects were noted in test concentrations ≥316 μg/plate without metabolic activation in the TA98 tester strain and at 2,500 μg/plate without metabolic activation in the TA1537 tester strain (Table 1). In the second test, precipitation was observed at concentrations ≥2,500 μg/plate in all strains used with metabolic activation. Toxic effects were noted in test concentrations ≥1,000 μg/plate without metabolic activation for TA98 and TA1535; at 316 μg/plate without metabolic activation for TA100, TA1537, and *E. coli* WP2 uvrA (pKM101); at 5,000 μg/plate with metabolic activation for TA98 and TA100; and at 2,500 μg/plate with metabolic activation for *E. coli* WP2 uvrA (pKM101) (Supplementary Table S4). Across all concentrations, xanthommatin did not cause gene mutations by either base-pair changes or frameshifts in the genome when tested against the five strains, further supporting claims that it is a nonmutagenic material.

We next sought to test the performance features of the raw material as a broad-spectrum absorber. Given the growing controversies surrounding the safety of approved organic UV filters (Krause et al., 2012; Lindqvist et al., 2014; Planta, 2011), we asked whether the natural optical features of xanthommatin could be applied as a UV filter booster for low (<0.2 mM) concentrations of organic UV filters. To test this hypothesis, we evaluated xanthommatin's absorbance capabilities alone in solution (Figure 3a) and in combination with Food and Drug Administration–approved organic UV filters (Figure 3b and c) over a spectral range of 280–500 nm. Similar to previous reports (Martin et al., 2019), we observed a clear relationship between increasing concentrations of xanthommatin and the absorption of UV through visible light (Figure 3a). Specifically, the absorptive behavior of xanthommatin (0.6 mM in DMSO) exhibited a broader profile that spanned the UVB through visible light regions than the absorptive behaviors of the pure organic UV filters (0.1–0.2 mM in DMSO). When xanthommatin was combined with the chemical UV filters, a significant increase in both the UVB (~300 nm) and UVA (~360 nm) range was achieved across all filters tested, where we observed at least a 50% increase in UVA and UVB performance on addition with xanthommatin (Figure 3c). These results effectively show that xanthommatin boosts the absorption profiles of organic filters in solution.

Given the UV filter–boosting features of xanthommatin, we next tested its cytotoxicity with and without exposure to a

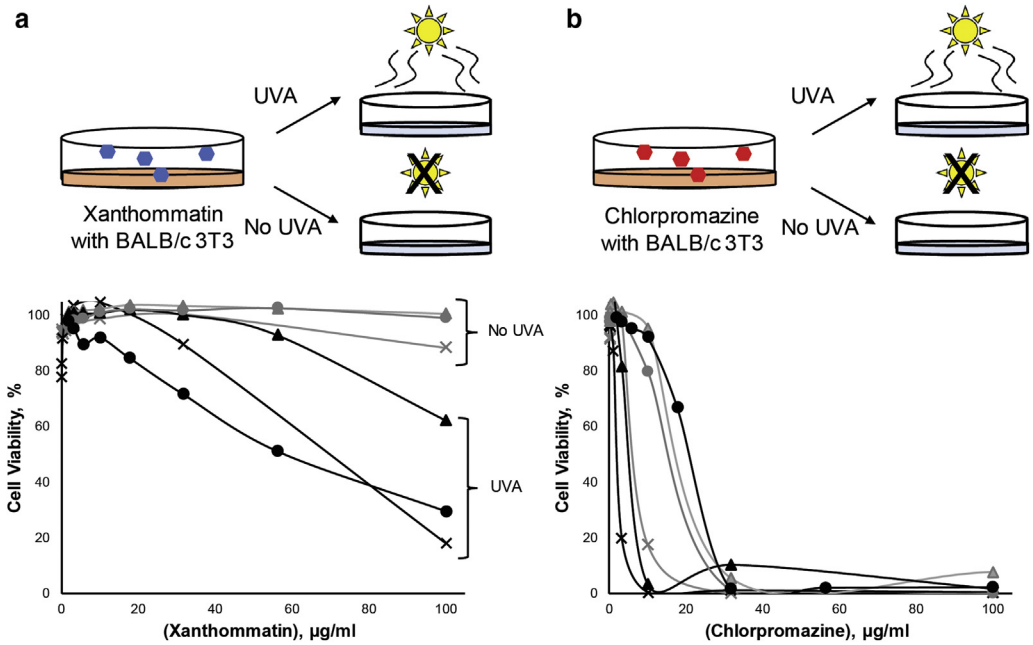

Figure 4. In vitro phototoxicity measurements of xanthommatin with and without exposure to UVA light (from 315 to 400 nm and a measuring range between 0 and 199.9 mW/cm^2). The viability (%) of BALB/c 3T3 fibroblasts reported for each concentration of (**a**) xanthommatin compared with that of (**b**) positive control, chlorpromazine with and without (±) UVA irradiation. In this figure, the gray lines represent data collected in the absence of UVA (–UVA), and the black lines represent data collected with exposure to UVA (+UVA). Data were collected for three independent experiments.

noncytotoxic dose of UVA light (from 315 to 400 nm and a measuring range between 0 and 199.9 mW/cm^2). In these experiments, cytotoxicity was expressed as a concentration-dependent reduction of a neutral red dye uptake within BALB/c 3T3 mouse fibroblast cell lines after treatment both with and without the presence of UVA exposure according to previous protocols (Borenfreund and Puerner, 1985). In the presence of UVA, the cells treated with xanthommatin showed cytotoxic effects, where the relative cell viability at the highest test item concentration across three independent + UVA experiments was 18.1 %, 29.7%, and 62.3% compared with the relative cell viability in the experiments UVA controls (88.5%, 99.3 %, and 100.6% for the three experiments) (Figure 4a). Because no half-maximal effective concentration (i.e., EC_{50})-value could be calculated for the experiments without UVA, a photoirritation factor (PIF = EC_{50} [–UVA]/EC_{50}[+UVA]) could not be calculated. Instead, the mean photoeffect $\left(MPE = \frac{\sum_{i=1}^{n} w_i PE_{Ci}}{\sum_{i=1}^{n} w_i}\right)$ was calculated; where, the photoeffect (i.e., PE) at any concentration (i.e., C) is defined as the product of the response effect and the dose effect concentrations. Mean photoeffect values of 0.019, 0.199, and 0.042 were measured for the three independent experiments with xanthommatin. An mean photoeffect value >0.15 indicates phototoxicity. In our experiments, two of the three datasets were below this threshold; thus, xanthommatin was classified as not phototoxic.

The controls confirmed the validity of the study, in which negative controls of the experiments with UVA exhibited cell viabilities of 86.80% (experiment 1), 92.03% (experiment 2), and 102.39% (experiment 3) relative to the untreated, control experiments without UVA. In contrast, the calculated half-maximal effective concentration values of the positive controls containing chlorpromazine for the experiments without UVA (8.327 µg/ml for experiment 1, 15.530 µg/ml for experiment 2, and 18.809 µg/ml for experiment 3) and for the experiment with UVA (0.221 µg/ml for experiment 1, 0.413 µg/ml for experiment 2, and 0469 µg/ml for experiment 3) were within the validity ranges. The photoirritation factor values for the positive controls were 37.81, 37.74, and 40.33 for the first, second, and third experiments, respectively, further supporting the validity of our claims (Figure 4b).

Because the toxicological endpoint of this in vitro 3T3 Neutral Red Uptake phototoxicity assay was developed and validated in a joint European Cosmetic, Toiletry and Perfumery Association project (Spielmann et al., 1998, 1994), this assay is a well-recognized in vitro alternative to the various in vivo tests in use. According to these guidelines, our data infer that xanthommatin is neither phototoxic nor a photoirritant, where phototoxicity is defined as a toxic response that either occurs after the first exposure to the test chemicals followed by subsequent exposure to light or is induced after systemic administration of a chemical after irradiation.

DISCUSSION

Xanthommatin was assayed across three human cell types and five bacterial strains in vitro, with the aim of determining its propensity to disrupt endocrine receptor function or cause genetic mutations. Specifically, the induction/inhibition potential of xanthommatin was evaluated, and no statistically significant increase or decrease in fold induction of testosterone or estradiol formation was observed when compared with that of the solvent control. This finding was complemented with two additional assays designed to test whether xanthommatin bound to and/or activated AR or ER responsive genes. In both cases, xanthommatin did not significantly alter normal luciferase gene expression. These

data collectively show that xanthommatin does not behave as a hormonal disrupter. When studied using bacterial reverse mutation assays, xanthommatin did not exhibit any mutagenic potential at concentrations <5,000 μg/plate over the five tester strains assayed. These observations were important, considering the potential application for xanthommatin in cosmetics. Specifically, in this report, we showed that xanthommatin contributed to UV filter–boosting activity of low concentrations (<0.2 mM) of Food and Drug Administration–approved chemical UV filters in solution and exhibited no toxicities or irritation in vitro when exposed to UVA light, supporting its classification as a nonphototoxic material. These combined findings are timely considering the growing controversies surrounding the safety profiles of some of the Food and Drug Administration–approved organic UV filters.

Although the lack of hormonal action, negative Ames mutagenicity, and negative phototoxicity results were encouraging results, future studies will be required to fully evaluate the safety profile of xanthommatin in vitro and in vivo in solution and as part of a finished formulation. These include but are not limited to a micronucleus test for genotoxicity, Organization for Economic Co-operation and Development–validated skin irradiation and sensitization assays, and systemic absorption tests using ex vivo human or porcine skin and/or in vitro reconstructed human epidermis skin models. In addition to this next suite of more rigorous safety studies, future tests will also include investigations of allergic reactions on application to the skin. For now, the results from the assays presented in this report represent important and promising first steps that highlight the future of xanthommatin as a powerful cosmetic ingredient.

MATERIALS AND METHODS

Participating organizations and structure of the study

No human or animal studies were conducted in this work. Studies were conducted in accordance with the Organization for Economic Co-operation and Development guidelines number 456 (H295R Steroidogenesis Assay [OECD, 2011] using human cell line—H295R), number 455 (Performance-based Test Guideline for Stably Transfected Transactivation in vitro Assays to Detect Estrogen Receptor Agonist and Antagonists using the human ERα HeLa-9903 cell line [OECD, 2016]), number 458 (Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for detection of Androgen Agonist and Antagonist Activity of Chemicals using the AR-EcoScreen cell line [OECD, 2020a]), number 432 (In vitro 3T3 HRU Phototoxicity Test [OECD, 2019]), number 101 (UV-Vis Absorption Spectra [OECD, 1981]), and number 471 (bacteria reverse mutation test, [OECD, 2020b]) at Eurofins BioPharma Product Testing Munich GmbH (Eurofins Scientific, Planegg, Germany). Eurofins also contributed to technical readout and analysis. All experiments were conducted in compliance with Good Laboratory Practice Regulations. There were no circumstances that may have affected the quality or integrity of the study.

Test and reference chemicals

Ammonium xanthommatin (CAS number 521-58-4) was acquired by Seaspire Skincare as a brown powder (purity > 95%) (Supplementary Figure S3). All remaining reference chemicals, solvents, and buffers were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All tests were conducted with freshly prepared material before use. Xanthommatin-containing solutions were made with DMSO (CAS number 67-68-5, Sigma-Aldrich) and diluted at 1:1,000 with a cell culture medium. In each condition, the solvent was present at a constant volume ratio of 0.1% (v/v). Two independent experiments were done for each condition, with three technical replicates per concentration. Concentrations in the endocrine studies included 3.125, 0.3125, 0.03125, 0.003125, 0.0003125, and 0.00003125 μM. Concentrations >3.125 μM presented solubility issues that interfered with the assay and were not included in these studies. Concentrations for the phototoxicity tests ranged from 0.0316 to 100 μg/ml. Concentrations in the bacteria assays ranged from 31.6 to 5,000 μg/plate.

Overview of steroidogenesis assay

H295R cells were thawed and cultured through passage 4 to achieve 80–90% confluency, where approximately 3.0×10^5 cells were assayed in each of the test concentrations and reference samples (including control and blank) for 48 hours. Testosterone and estradiol concentrations were measured in each sample using an ELISA system (estradiol EIA and testosterone EIA, Cayman Chemicals, Ann Arbor, MI) and were compared with the concentrations of the solvent control and reference materials.

Overview of ERα transactivation assay

Stably transfected human ERα HeLa-9903 cell line was used according to the suppliers' specifications. On reaching up to 90% confluency, cells were washed, and a single-cell suspension at 1×10^5 cells per ml was used to determine luciferase activity after 24 hours.

Overview of AR transactivation assay

An AR-EcoScreen cell line was derived from Chinese hamster ovary cells (CHO-K1). On reaching up to 75–90% confluency, cells were trypsinized, after which time the medium was supplemented with 5% Dextran-coated charcoal–treated fetal bovine serum (5% Dextran-coated charcoal–treated fetal bovine serum DMEM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and resuspended and assayed at a resulting concentration of 9×10^3 cells/well and incubated at 37 °C and 5% carbon dioxide for 24 hours before chemical exposure

Overview of bacterial reverse mutation test

Tester strains TA98, TA1535, and *E. coli* were obtained from Molecular Toxicology (Boone, NC). Tester strains TA100 and TA1537 were obtained from Xenometrix AG (Allschwil, Switzerland). Tester strains of *S. typhimurium* (TA100, TA1535, TA98, and TA1537) and *E. coli* WP2 uvrA (pKM101) were tested without metabolic activation, and tester strains of *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and *E. coli* WP2 uvrA (pKM101) were tested with metabolic activation. The four strains of *S. typhimurium* and one strain of *E. coli* WP2 uvrA (pKM101) with the following characteristics were used: for TA98, his D 3052, rfa–, uvrB–, and frameshift mutations for R-factor; for TA100, his G 46, rfa–, uvrB–, and base-pair substitutions for R-factor; for TA1535, his G 46, rfa–, uvrB–, and base-pair substitutions; for TA1537, his C 3076, rfa–, uvrB–, and frameshift mutations; and for *E. coli* WP2 uvrA (pKM101), trp–, uvrA–, and base-pair substitutions. For the experiments, *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and tester strain *E. coli* WP2 uvrA (pKM101) were used to evaluate the effects of xanthommatin at concentrations ranging from 31.6 to 5,000 μg/plate. All concentrations, including those of the controls, were tested in triplicate across two independent experiments.

Overview of UV filter–boosting demonstrations

Stock solutions of xanthommatin and organic UV filters—oxybenzone, homosalate, octisalate, octinoxate, octocrylene, and avobenzone—were prepared. A 96-well plate was utilized to measure the absorbance of xanthommatin alone and in combination with the organic UV filters. The concentration of all organic UV filters was kept constant while increasing concentrations of Xanthommatin were mixed into each sample well. The absorbance of each well was measured with a Molecular Devices spectrophotometer over the 280–500 nm range with 10 nm steps. All concentrations, including those of the controls, were tested in triplicate across two independent experiments.

Overview of phototoxicity test

The in vitro 3T3 Neutral Red Uptake phototoxicity assay used in this study was developed and validated in a joint EU/COLIPA project from 1992–1997 (Spielmann et al., 1998, 1994) to establish a valid in vitro alternative to the various in vivo tests to detect the phototoxic potential of a test item. In this study, a BALB/c 3T3 mouse fibroblast cell line (CCL-163, clone A31, ATCC, Manassas, VA) was used in standard culture medium containing DMEM with 10% calf serum at 37 ± 1 °C and 5% carbon dioxide at passage numbers 85 in the first experiment, 80 in the second experiment, and 92 in the third experiment. For each test item, two plates were prepared: one for determination of cytotoxicity (without UVA) and the other for determination of photocytotoxicity (with UVA). The cells were incubated for 24 ± 2 hours (5% carbon dioxide, 37 ± 1 °C) until they formed a half-confluent monolayer, during which 100 μl of the test item or just solvent was added to the cells. The cells were then incubated in the dark for 60 minutes. To perform the +UVA part of the assay, the cells were irradiated for 50 minutes through the lid of the 96-well plate using solar simulator SOL-500 equipped with an H1-filter for 1.5–1.7 mW/cm2 (UVA = 4.5–5.1 J/cm^2) in the first experiment, 1.4–1.7 mW/cm2 (UVA = 4.2–5.1 J/cm^2) in the second experiment, and 1.2–1.8 mW/cm^2 (UVA = 3.6–5.4 J/cm^2) in the third experiment. The positions of the plates with low and high irradiance were exchanged after half time of the irradiation (25 minutes). Duplicate plates were kept at room temperature in a dark box for 50 minutes (–UVA exposure time). After the exposition, cells were washed and incubated overnight (18–22 hours), were then analyzed on incubation with neutral red medium for 3 hours, and were then washed with neutral red desorb solution (freshly prepared ethanol/acetic acid) measuring the optical density at 540 nm in a microplate autoreader, using blanks as a reference.

Statistical analysis

Statistical analysis using the Dunnett's test was performed only for the steroidogenesis assay. Differences were considered significant at $P \leq 0.05$ from the solvent control over the two or three independent runs.

Data availability statement

Datasets related to this article can be found at https://data.mendeley.com/datasets/4m36x8vmrk/1, hosted at Mendeley Data.

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CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIALS

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.xjidi.2021.100081.

REFERENCES

Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci U S A 1973;70:2281–5.

Bom S, Fitas M, Martins AM, Pinto P, Ribeiro HM, Marto J. Replacing synthetic ingredients by sustainable natural alternatives: a case study using topical O/W emulsions. Molecules 2020a;25:4887.

Bom S, Jorge J, Ribeiro HM, Marto J. A step forward on sustainability in the cosmetics industry: a review. J Clean Prod 2019;225:270–90.

Bom S, Ribeiro HM, Marto J. Sustainability calculator: a tool to assess sustainability in cosmetic products. Sustain 2020b;12:1437.

Borenfreund E, Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 1985;24:119–24.

Claxton LD, Allen J, Auletta A, Mortelmans K, Nestmann E, Zeiger E. Guide for the Salmonella typhimurium/mammalian microsome tests for bacterial mutagenicity. Mutat Res 1987;189:83–91.

Deravi LF, Magyar AP, Sheehy SP, Bell GR, Mäthger LM, Senft SL, et al. The structure-function relationships of a natural nanoscale photonic device in cuttlefish chromatophores. J R Soc Interface 2014;11:20130942.

Gatehouse D, Haworth S, Cebula T, Gocke E, Kier L, Matsushima T, et al. Recommendations for the performance of bacterial mutation assays. Mutat Res 1994;312:217–33.

Futahashi R, Kurita R, Mano H, Fukatsu T. Redox alters yellow dragonflies into red. Proc Natl Acad Sci USA 2012;109:12626–31.

Krause M, Klit A, Blomerg Jensen M, Søeborg T, Frederiksen H, Schlumpf M, et al. Sunscreens: are they beneficial for health? An overview of endocrine disrupting properties of UV-filters. Int J Androl 2012;35:424–36.

Lindqvist PG, Epstein E, Landin-Olsson M, Ingvar C, Nielsen K, Stenbeck M, et al. Avoidance of sun exposure is a risk factor for all-cause mortality: results from the Melanoma in Southern Sweden cohort. J Intern Med 2014;276:77–86.

Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res 1983;113:173–215.

Martin CA, Rezaeeyazdi M, Colombani T, Dinneen SR, Kumar A, Bencherif SA, et al. A bioinspired, photostable UV-filter that protects mammalian cells against UV-induced cellular damage. Chem Commun (Camb) 2019;55:12036–9.

Marty MS, Carney EW, Rowlands JC. Endocrine disruption: historical perspectives and its impact on the future of toxicology testing. Toxicol Sci 2011;120(Suppl. 1):S93–108.

McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc Natl Acad Sci U S A 1975;72:5135–9.

OECD. OECD guideline for the testing of chemicals, section 1: Test No. 101: UV-VIS absorption spectra. Paris, France: OECD Publishing; 1981.

OECD. OECD Guidelines for the Testing of Chemicals, Section 4: test no. 456: H295R steroidogenesis assay. Paris, France: OECD Publishing; 2011.

OECD. OECD guidelines for testing of chemicals, section 4: test no. 455: performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonist and antagonists. Paris, France: OECD Publishing; 2016.

OECD. OECD guidelines for the testing of chemicals, section 4: test no. 432: in vitro 3T3 NRU phototoxicity test. Paris, France: OECD Publishing; 2019.

OECD. OECD guidelines for testing of chemicals: test no. 458: stably transfected human androgen receptor transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals. Paris, France: OECD Publishing; 2020a.

OECD. OECD guidelines for testing of chemicals, section 4: test no. 471: bacterial reverse mutation test. Paris, France: OECD Publishing; 2020b.

Osanai-Futahashi M, Tatematsu KI, Futahashi R, Narukawa J, Takasu Y, Kayukawa T, et al. Positional cloning of a Bombyx pink-eyed white egg locus reveals the major role of cardinal in ommochrome synthesis. Heredity 2016;116:135–45.

Planta MB. Sunscreen and melanoma: is our prevention message correct? J Am Board Fam Med 2011;24:735–9.

Riou M, Christidès JP. Cryptic color change in a crab spider (Misumena vatia): identification and quantification of precursors and ommochrome pigments by HPLC. J Chem Ecol 2010;36:412–23.

Spielmann H, Balls M, Brand M, Döring B, Holzhütter HG, Kalweit S, et al. EEC/COLIPA project on in vitro phototoxicity testing: first results obtained with a Balb/c 3T3 cell phototoxicity assay. Toxicol In Vitro 1994;8:793–6.

Spielmann H, Balls M, Dupuis J, Pape WJ, Pechovitch G, de Silva O, et al. The international EU/COLIPA in vitro phototoxicity validation study: results of phase II (blind trial). part 1: the 3T3 NRU phototoxicity test. Toxicol In Vitro 1998;12:305–27.

Williams TL, DiBona CW, Dinneen SR, Labadie SF, Chu F, Deravi LF. Contributions of phenoxazone-based pigments to the structure and function of nanostructured granules in squid chromatophores. Langmuir 2016;32:3754–9.

Williams TL, Senft SL, Yeo J, Martín-Martínez FJ, Kuzirian AM, Martin CA, et al. Dynamic pigmentary and structural coloration within cephalopod chromatophore organs. Nat Commun 2019;10:1004.

Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K. Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ Mol Mutagen 1988;11(Suppl. 12):1–157.

Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K. Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. Environ Mol Mutagen 1992;19(Suppl. 21):2–141.