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Antioxidant Activity and Skin Sensitization of Eugenol and Isoeugenol: Two Sides of the Same Coin?

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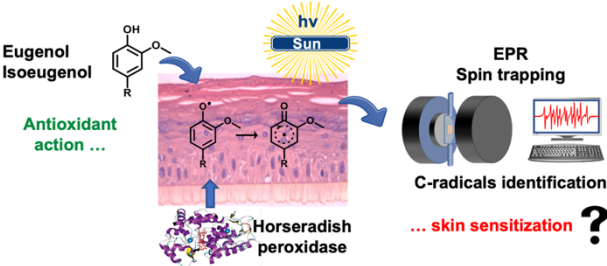
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Keywords

Eugenol, isoeugenol, skin sensitization, radical mechanisms, reconstructed human epidermis, enzymatic activation, solar irradiation, electron paramagnetic resonance, spin trapping

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Abstract

Eugenol and isoeugenol are well acknowledged to possess antioxidant and thus cytoprotective activities. Yet both compounds are also important skin sensitizers, compelling the cosmetics and fragrance industries to notify their presence in manufactured products. While being structurally very similar, they show significant differences in their sensitization properties. Consequently, eugenol and isoeugenol have been the subject of many mechanistic studies where final oxidation forms, electrophilic *ortho*-quinone and quinone methide, are blamed as the reactive species forming the antigenic complex with nucleophilic residues of skin proteins inducing skin sensitization. However, radical mechanisms could compete with such electrophilic-nucleophilic pathway. The antioxidant activity results from neutralising reactive oxygen radicals by release of the phenolic hydrogen atom. The so-formed phenoxyl radicals can then fully delocalize upon the structure becoming potentially reactive towards skin proteins at several positions. To obtain in-depth insights of such reactivity we investigated *in situ* the formation of radicals from eugenol and isoeugenol using electron paramagnetic resonance combined with spin trapping in reconstructed human epidermis (RHE), mimicking human skin and closer to what may happen *in vivo*. Two modes of radical initiation were used, exposing RHE to (i) horseradish peroxidase (HRP), complementing RHE metabolic capacities and mimicking peroxidases present *in vivo* or (ii) solar light using a AM 1.5 solar simulator. In both experimental approaches, where the antioxidant character of both compounds is revealed, oxygen- and carbon-centred radicals were formed in RHE. Our hypothesis is that such carbon radicals are relevant candidates to form antigenic entities prior to conversion into electrophilic quinones. On this basis, these studies suggest that pro- or prehapten fingerprints could be advanced depending of the radical initiation method. The introduction of HRP suggested eugenol and isoeugenol behave as prohaptens, while exposed to light a prehapten nature could be highlighted.

1. Introduction

Allergic contact dermatitis (ACD) is the most prevalent form of immunotoxicity in humans. It is an inflammatory disorder induced by exposure of the skin to chemicals present in our environment and our daily life. It is clinically characterised by an eczematous dermatitis that can be mild to severe, acute and short lived, or chronic. Alinaghi et al. found that at least 20% of the general population are allergic to common environmental skin allergens,¹ and this proportion is growing. The socio-economic expenses in the EU related to fragrance allergy seen in around 5% of the population² is in between 11 and 16 billion € per year (clinical treatments, hospital admissions, sick leave, etc.).³ Furthermore, ACD accounts for 20 to 30% of all occupational skin diseases.⁴ ACD has thus a detrimental effect on the quality of life and represents social, economic and critical psychological burden.⁵ Because contact allergens exist in very common products used in everyday life, industrial and occupational settings, it is difficult to remove potential allergens from ordinary life and prevent the onset of ACD in association with allergic inflammation.⁶ Hence, a better understanding of the structural motifs and mechanisms involved in skin sensitization is essential to improve risk assessment procedures and protection of consumers.

In this manuscript we bring new insights on the mechanism of action of eugenol (Eug; CAS Registry Number 97-53-0) and its positional isomer isoeugenol (IsoEug; CAS Registry Number 97-54-1). These two alkoxyphenols are natural compounds belonging to the group of *ortho*-methoxy phenols containing a propenyl group in the *para* position (Figure 1). Widely used as fragrance materials in perfumes and perfumed products, both are well-known skin sensitizers responsible for fragrance ACD. Eug is a colourless to slightly yellow liquid obtained mainly from clove oil and cinnamon leaf oil but occurring also in small amounts in other essential oils. It is a versatile compound widely used as flavouring agent in cosmetic and food products due to the spicy aroma and clove odour.⁷ It is also commonly used in dentistry as analgesic and sedative agent, in surgical cement materials and dental pastes.⁸ On the other hand, IsoEug occurs in many essential oils, mostly with Eug, but not as the main component. It is a yellowish viscous liquid with a fine clove odour widely used in fragranced products.⁹ In its natural state it is a mixture of *cis/trans* stereoisomers, the proportions of which vary. In contrast, in the synthetic commercially available product the *trans* isomer largely dominates. Both compounds are well acknowledged to possess cytoprotective activities as antioxidants, but are also recognised to be prooxidants with cytotoxic consequences.^{10–13} Such deleterious aspect is well-known from dermatologists specialized in ACD and compel the cosmetics and fragrance industries to notify their presence in manufactured products. As a matter of fact, Eug and IsoEug belong to the list of 26 cosmetic fragrance ingredients qualified as skin sensitizers that are mandatory to label in EU consumer goods when the concentration exceeds 100 ppm in rinse-off products and 10 ppm in leave-on products.¹⁴ The rate of skin allergic reactions to

both is so widespread that they are included in fragrance mix I, the screening allergen mixture used since its introduction in the late 1970s to diagnose fragrance contact allergy, complemented today with fragrance mix II.^{15,16}

The skin sensitization potential of Eug and IsoEug has been the subject of numerous mechanistic studies as they exhibit significant differences in their sensitization properties while being structurally very close. The murine local lymph node assay, widely used during the last decades to assess the sensitization potency of chemicals, classified Eug as weak sensitizer whereas IsoEug was considered as moderate-strong sensitizer.¹⁷ This has been confirmed for example by the study of the frequency of sensitization to the 26 fragrance contact allergens in consecutive patients in a multicentre project from the German Information Network of Departments of Dermatology.¹⁸ Skin sensitizers are chemicals triggering T-cell mediated allergic reactions after repeated skin contact,¹⁹ although they are unable to directly activate an immune response. Following penetration of the compound into the skin, the opening event is chemical in a way that immunogenicity is triggered through a chemical reaction of the sensitizer with endogenous skin proteins.²⁰ The so-formed stable antigenic conjugates will be then revealed and processed for presentation to the immune system.²¹ Two distinct mechanistic pathways have been so far suggested for skin sensitization to Eug and IsoEug. The first one involves a demethylation process (most probably enzymatically) followed by the formation of a catechol which can be then oxidised to an electrophilic *ortho*-quinone able to react with nucleophilic amino acids in the skin. The second suggests the initial formation of a phenoxy radical (PhO[•]) that can evolve to form a reactive also electrophilic quinone methide intermediate (Figure 1). Structure-activity relationship studies involving substituted derivatives proposed that while Eug could react through the demethylation pathway, IsoEug favours the formation of PhO[•].²²

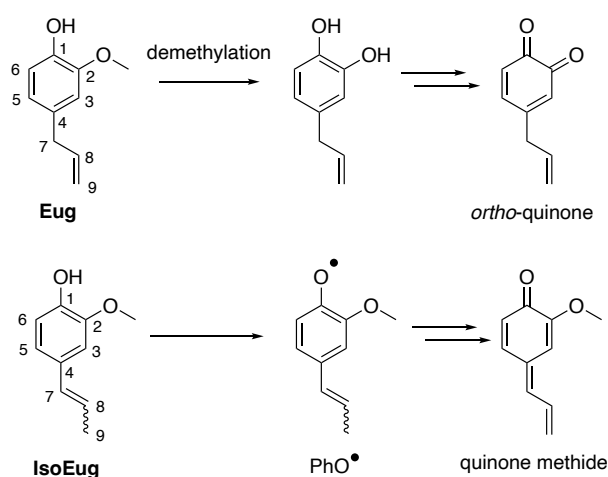


Figure 1. Chemical structures of eugenol (Eug) and isoeugenol (IsoEug) and proposed pathways for skin sensitization

Among Eug and IsoEug antioxidant-cytoprotective activities is the aptitude to trap reactive oxygen radicals by donation of the phenolic hydrogen atom. However, the cytotoxicity of *ortho*-

methoxy phenols has been associated to the so-formed PhO[•] radicals.¹² The formal initial PhO[•] radical possesses several resonant structures as shown in Figure 2, where the unpaired electron can be localized on different carbon atoms. In addition, substituents in *para*-position having conjugated double bonds allow further delocalization on the substituent itself such as for IsoEug (Figure 2). While measuring the antioxidant activity of Eug and IsoEug with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, Bortolomeazzi et al. investigated the byproducts from the reactions between the compounds and the DPPH radical.²³ The formation of a complex mixture of dimeric species was demonstrated, with dehydrodiisoeugenol and dehydrodieugenol being the main reaction products (Figure 2). These dimers are well known because of the great deal of interest over the years on the synthesis of natural lignans with a broad range of biological activities.^{24,25} The biosynthesis and synthetic pathways of dimeric lignans from phenolic phenylpropenoids occur *via* oxidative processes which convert the phenols into the above-described radicals, pertinent candidates for carbon-carbon and carbon-oxygen bond formation.

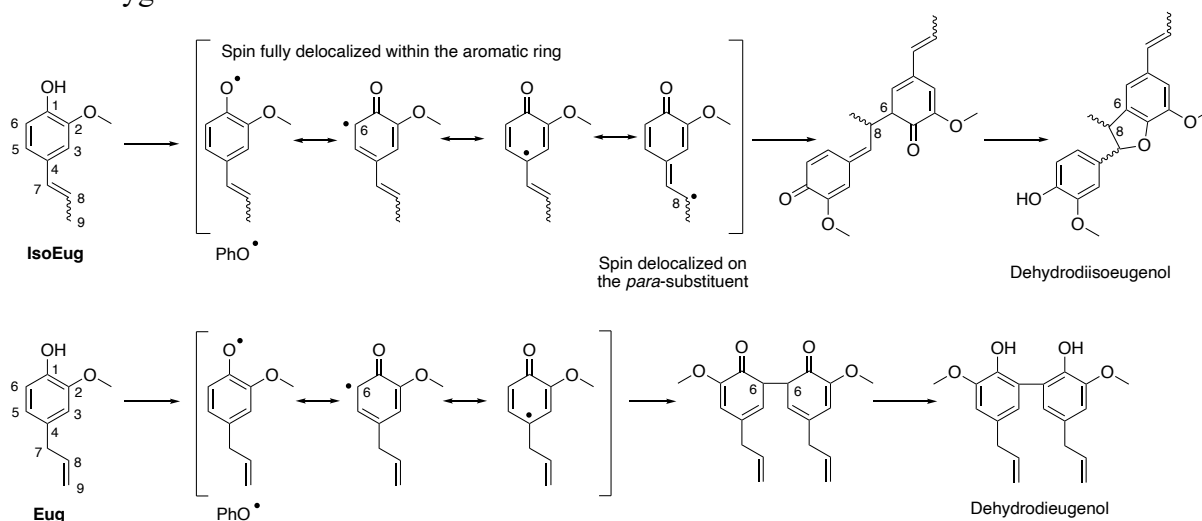


Figure 2. Delocalization of the unpaired electron of formal initial PhO[•] radicals and reactivity via carbon-carbon bond formation to afford dimer lignans dehydrodiisoeugenol (involving one radical at C6 and one at C8) and dehydrodieugenol (involving two C6 radicals)

If the antioxidant and prooxidant potentials of Eug and IsoEug have been widely studied especially in the context of skin sensitization, the fate of such radicals formed in the epidermis and their mechanism of action is far from being unravelled. To this end, the work reported herein investigates radicals in skin exposed to Eug and IsoEug by electron paramagnetic resonance-spin trapping (EPR-ST). Such radicals could react with skin proteins prior to final conversion into electrophilic quinones, being thus suitable candidates for antigenic entities formation. Two distinct modes of radical initiation were highlighted here: (i) an enzymatic approach using horseradish peroxidase (HRP), mimicking *in vivo* peroxidases and (ii) a photo-exposure to solar light. Preliminary results and EPR fingerprints were first obtained in solution and then used as reference for EPR-ST

investigations into a reconstructed human epidermis (RHE) 3D model, mimicking human skin and thus closer to real-life scenarios.

2. Materials and methods

2.1. Chemicals. Eug, IsoEug, guaiacol, veratrole, methyl eugenol (MeEug) and methyl isoeugenol (MeIsoEug), HRP enzyme (activity per mass 164 U.mg⁻¹), Dispase II enzyme (activity per mass 0.5 U.mg⁻¹) and reagents to prepare buffer solutions were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and used as received. Acetone for analysis (99.8%) was acquired from Carlo Erba Reagents (Val de Reuil, France). 4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer (10 mM, pH 6.8) was prepared with 1.19 g HEPES in 400 mL deionized water, 4 g sodium chloride and 0.1 g potassium chloride. To attain pH 6.8, sodium hydroxide pellets were added. If the pH went too high, it was lowered back by carefully adding hydrogen chloride until the pH remained stable to 6.8. Deionized water was added for a final volume of 500 mL. The spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was synthesized as reported in the literature.²⁶

2.2. Equipment. EPR spectra were recorded at room temperature (295 K) on a X-band spectrometer (EMXplus, Bruker Biospin GmbH, Germany) equipped with a high-sensitivity resonator (Bruker Biospin GmbH, Germany). The *g* calibration standard was Bruker “strong pitch” of known *g* factor 2.0028. Principal spectrometer settings were microwave power 5 mW in solution and 10 mW in RHE, modulation amplitude 2 G and sweep time 40 s. Multiple scans were accumulated (up to 10) to obtain decent Signal to Noise ratio (S/N). Experimental EPR spectra were analysed by means of computer simulation using labmade scripts based on Easyspin toolbox under Matlab (MathWorks) environment.²⁷ For photo-exposure studies, the illumination source was an Oriel 3A Solar simulator (AM 1.5G spectrum), commonly used to mimic sun irradiance on Earth.²⁸

2.3. EPR-ST. EPR-ST is an unavoidable tool to detect low yields of short-lived radical species enabling also potential identification and characterization. A diamagnetic spin trap reacts with transient reactive radicals forming a longer-life paramagnetic spin adduct (Figure 3).²⁹ The spin trap used in this study was DEPMPO as (i) it is well tolerated *in vivo* at relatively high concentrations,³⁰ (ii) is able to detect both oxygen centred (O-) and C-radicals in a wide range of experimental conditions (pH, temperature, solvent,...) and (iii) EPR fingerprints of the given spin adducts are strongly influenced by the trapped radicals.^{31–33}

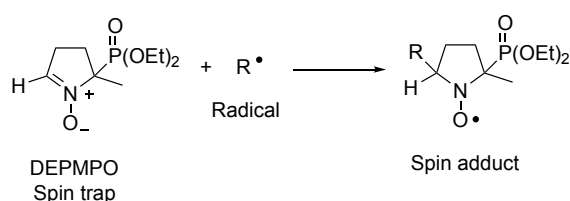


Figure 3. Schematic description of DEPMPO mechanism of ST

2.3.1. *EPR Studies in Solution.* Stock solutions were prepared for the target compounds (133.3 mM in acetone), DEPMPO (333.3 mM in HEPES), and in the case of enzymatic activation for HRP (33.3 U.mL⁻¹ in HEPES) and hydrogen peroxide (H₂O₂, 233.3 mM in HEPES). 15 μL of DEPMPO solution were mixed with 15 μL of target compound solution, and in the case of enzymatic activation 15 μL of HRP and H₂O₂. HEPES buffer was added to the solution for a final volume completed to 100 μL. This way, final concentrations in the reaction mixture were 50 mM DEPMPO, 20 mM target compound, and in the case of enzymatic activation 5 U.mL⁻¹ of HRP and 32 mM H₂O₂. The reaction mixture was subjected to stirring, further introduced into an EPR capillary tube (Hirschmann, 20 μL), sealed on both ends and EPR spectra registered. In the case of photo-exposure, the DEPMPO-target compound mixture was subjected to the light of the solar simulator during 5-10 min before being introduced into the EPR capillary tube and EPR spectra registered. Control experiments in solution with the enzymatic system HRP-H₂O₂ and with photo-exposure are shown in the Supporting Information (S.1 and S.2).

2.3.2. *EPR Studies in RHE.* RHE models are a great alternative to the use of human or animal tissues, as they amend important legal and ethical issues. We used RHE AlternaSkin™ (0.6 cm², Cell Alternativ[®], Trosly Breuil, France), reconstructed from a monolayer culture of keratinocytes and fibroblasts (L929, SIRC) by using a vegetable origin serum allowing the growth of the matrix.³⁴ Normal human epidermal keratinocytes (NHEK, primary foreskin cells) are cultured on a 0.6 cm² inert polycarbonate membrane and at the air-liquid interface allowing cell differentiation and reconstruction of an epidermis.³⁵ The RHE so obtained is histologically similar to human skin regarding the morphology and includes a *stratum corneum* (penetration aspects), being thus adept to mimic what may happen *in vivo*. RHE samples, maintained in agarose gel upon receipt, were placed in culture medium furnished by Cell Alternativ[®] (1.5 mL/well for a 12-well plate). The culture medium was beforehand freshly prepared by warming it at 37 °C and adding a complement also provided by Cell Alternativ[®] (volume of complement 1/200 to the maintenance medium). RHE were then placed in an incubator during 24 h (37 °C, 5% CO₂, saturated humidity atmosphere). After this time, the culture medium was replaced with 1.5 mL/well every 24 h or 2.5 mL/well every 48-72 h.

For the experimentations, the RHE to be treated was first put in a Dispase II solution (1.5 mL/well) during 5 min with the aim of removing the RHE from its polycarbonate support. Dispase II was prepared in HEPES buffer diluted in water (75:25) in order to have an enzymatic activity of the solution of 2.4 U.mL⁻¹. The RHE was then pre-treated topically with DEPMPO in dimethylsulfoxide/HEPES 1:1 (400 mM, 30 μL) and incubated during 15 min (37 °C, 5% CO₂, saturated humidity). After this time, pre-treated RHE was placed in an EPR tissue cell equipped with a silica window (Willmad, #ER162TC-Q) and the target compound (100 mM in acetone, 30 μL) was

topically applied to the epidermis taking care to ensure that the solution was only applied to it. The EPR tissue cell was then closed and EPR spectra registered.

For EPR studies in RHE with integrated HRP, the enzyme was incorporated systemically. For this purpose, 48-72 h prior to the pre-treatment with DEPMPO, HRP (30 U.mL⁻¹) was added to the culture medium (2.5 mL/well) and incubated (37°C, 5% CO₂, saturated humidity) until use. Noteworthy, in contrast to EPR-ST studies in solution no H₂O₂ was used for radical initiation. The RHE was then processed as described above.

For EPR studies in photo-exposed RHE, DEPMPO pre-treated RHE was washed with HEPES solution before topical application of the target compound and the sample was placed in the EPR tissue cell as described above. After the first EPR acquisition (t_0), the RHE was exposed to sunlight for 10 min prior to the next EPR acquisition (t_{10}).

2.4. Computational Density Functional Theory Calculations. Calculation studies were completed by means of Gaussian 09 package (Gaussian, 2009; version D.01) at Density Functional level of Theory (DFT) with ω B97XD functional. Atoms were described by the 6-31+G** basis set,³⁶ and water solvent modelled through a polarized continuum model.³⁷ Structures were optimized and the nature of the encountered stationary point categorized by a frequency calculation. Gibbs free energies (ΔG) were extracted from the frequency calculation done within the harmonic approximation, and are given in kcal.mol⁻¹.

3. Results

3.1. EPR-ST Studies in Solution. In order to trap radical species rising from phenolic compounds in solution, a radical initiation step is mandatory. In our case either (i) an enzymatic HRP/H₂O₂ system or (ii) a photo-induced energy supply was introduced. HRP is a peroxidase of the oxidoreductase enzyme class. It is a large alpha-helical glycoprotein which binds heme as redox cofactor.³⁸ Peroxidases contribute to lignification and participate in the tissue covering of plants, in addition to regulating H₂O₂ level within organisms by reducing it and oxidising another substrate. Antioxidants of phenolic structure such as Eug and IsoEug are known target substrates of HRP,³⁹ that results in the formation of oxidised substrates (PhO[•]) and H₂O (Figure 4).^{39,40} Accordingly, HRP/H₂O₂ was used in our study as radical initiator. This system allows mimicking physiological and biological environments in which the antioxidant features of Eug and IsoEug are highlighted by the generation of PhO[•] radicals. EPR-ST was then used to identify the radical species resulting from such antioxidant conditions.

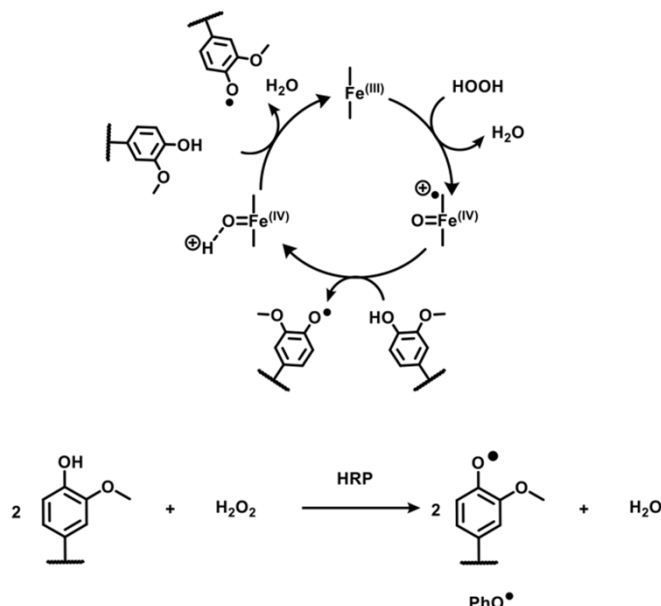


Figure 4. Enzymatic catalytic cycle and outcome of the reaction of HRP in presence of H₂O₂ and phenolic antioxidant substrates

Other substrates were also examined to highlight the importance of the phenolic chemical function in the formation of a PhO[•] radical intermediate: (i) guaiacol, constituting the shared core of Eug and IsoEug and (ii) analogue compounds with methylated phenolic function (veratrole, MeEug and MeIsoEug) (Figure 5).

EPR-ST investigations in solution point towards EPR fingerprints testimony of radical intermediates issued from PhO[•] very similar for Eug, IsoEug and guaiacol (Figure 5; See Supporting Information S.1 for control experiments). Two types of spin adducts were identified on the basis of the analysis and deconvolution of the hyperfine coupling constants (*hfccs*), compared with existing databases and previous ST studies carried out in the laboratory (see Figure 6 for example on Eug).^{41,42}

Taking as example Eug, up to four spin adducts were identified. On the one hand, the *hfccs* of two spin adducts were typical of two C-radicals (Figure 6b; $a_H = 20.7$ G, $a_N = 14.7$ G, $a_P = 47.3$ G; $a_H = 21.2$ G, $a_N = 14.2$ G, $a_P = 46.2$ G) that are consistent with electronic delocalization of PhO[•] along the carbon skeleton of the molecules. Moreover, the obvious similarity between *hfccs* obtained for Eug, IsoEug and guaiacol suggests that the trapped C-radicals belong to the common core of their chemical structure. On the other hand, two spin adducts observed with *hfccs* $a_H = 10.5$ G, $a_N = 13.8$ G and $a_P = 50.5$ G and $a_H = 8.4$ G, $a_N = 13.7$ G and $a_P = 42.2$ G (Figure 6c), could be compatible with DEPMPO-superoxide spin adducts and the addition *cis/trans* of the radical trapped on both faces of the nitron.^{31,32} It is generally accepted that, in the presence of H₂O₂, peroxidases can catalyze the generation of the superoxide radical O₂^{•-} upon oxidation of the substrate. The PhO[•] radicals formed in the case of phenolic-like substrates may react with oxygen and O₂^{•-} is produced.”⁴³⁻⁴⁵ Yet, it cannot be excluded, based solely on *hfccs* values, the presence of spin adducts corresponding to the trapping of PhO[•] radicals in the reaction mixture. No signal in the EPR-ST experiments was observed in the case of veratrole, MeEug and MeIsoEug (Figure 5) even if cannot be excluded that radicals were present in the mixtures. Still, this result pointed out the importance of the phenolic function in the generation of the trapped radical intermediates in our experimental conditions.

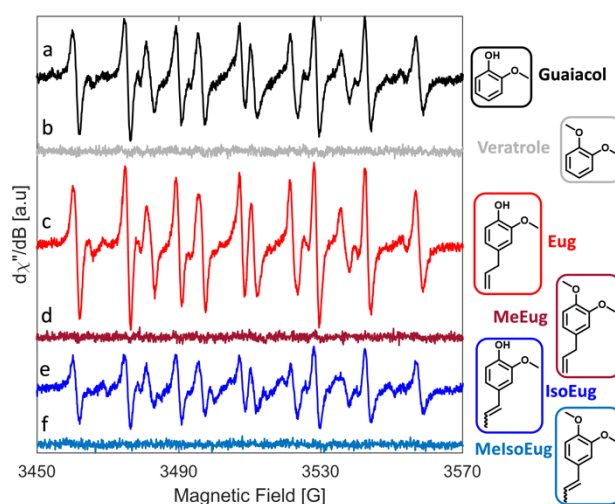


Figure 5. EPR-ST spectra in solution with DEPMPO (50 mM)/HRP-H₂O₂ (5 U.mL⁻¹, 32 mM) and target compound (20 mM) (a) guaiacol, (b) veratrole, (c) Eug, (d) MeEug, (e) IsoEug and (f) MeIsoEug

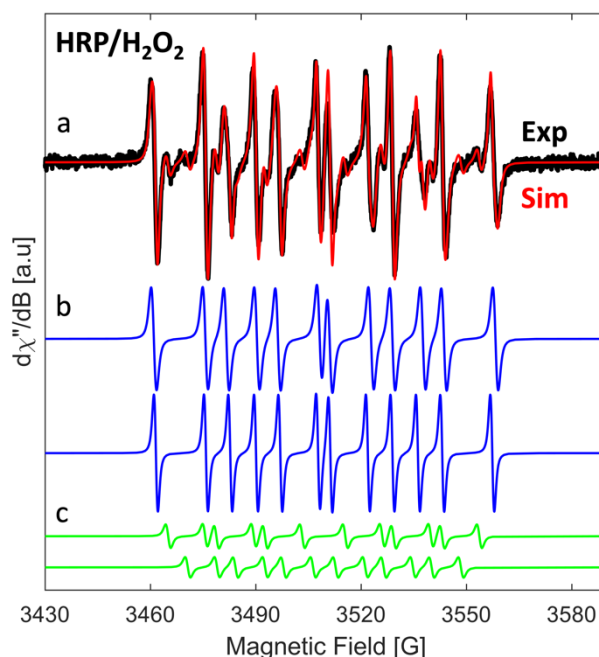


Figure 6. (a) EPR experimental spectra (Exp) of mixture Eug (20 mM)/DEPMPO (50 mM)/HRP-H₂O₂ (5 U.mL⁻¹, 32 mM) in solution with overlaid simulation (Sim). Deconvoluted spin adducts (b) DEPMPO-R (two different C-radicals trapped), and (c) DEPMPO-OR. Values of *hfccs* are indicated in Table S.1 (Supporting Information). Control experiments with only Eug or DEPMPO did not give any signal.

In a second phase, compounds were exposed to solar light using a AM1.5G solar simulator. By essence light energy is able to photo-excite molecules, and can facilitate photo-oxidation of excited compounds. While it has been reported that under UV irradiation (pulse laser, λ 266 nm) phenolic derivatives release a proton and an electron and oxidise to PhO[•] radicals,⁴⁶ it was also proposed that exposure to sunlight could induce such reactions.⁴⁷ Solutions of target molecules in the presence of DEPMPO were irradiated for 5 min prior to the acquisition of EPR spectra, shown in Figure 7. Despite of a lower S/N, both Eug and IsoEug exhibit similar *hfccs* to those obtained enzymatically (See Supporting Information S.2 for control experiments). For the methylated analogues veratrole and MeIsoEug no photo-induced radical intermediates were observed (see Supporting Information S.3). Surprisingly, irradiation seemed to initiate the photo-oxidation of MeEug. This is indeed supported by DFT calculations (see below) that bring to light two oxidation sites of Eug: the phenoxy function and the methylene group of the allyl chain in *para* position, that appears more easily oxidizable. The latter can give rise to distinct C-radicals when compared to PhO[•] radical, yet it was not highlighted here.

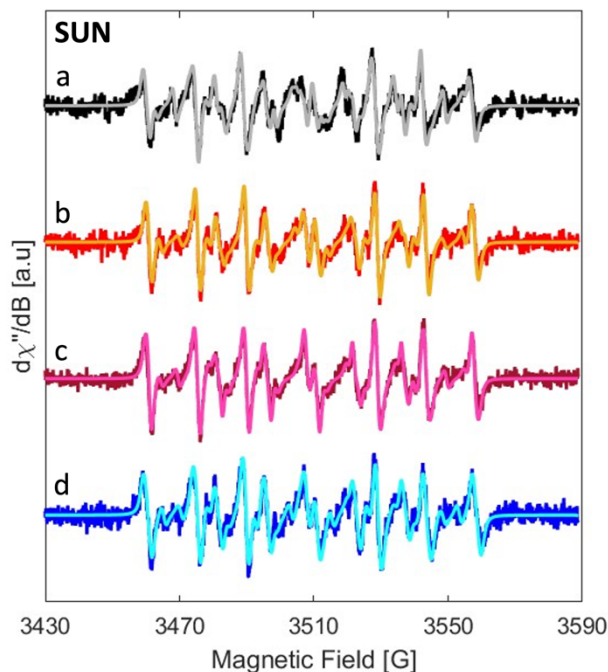


Figure 7. EPR-ST experimental spectra in solution for DEPMPPO (50 mM)/target compound (20 mM)/hv 5 min together with simulations for: (a) guaiacol, (b) Eug, (c) MeEug and (d) IsoEug. Values of $hfccs$ are indicated in Table S.1 in the Supporting Information. See also S.6 for deconvolution. Control experiments with only DEPMPPO (50 mM)/ hv 5 min did not give any signal.

3.2. EPR-ST Studies in HRP Pre-Treated RHE. Following the studies in solution, similar investigations were carried out in RHE to highlight the formation of reactive radical intermediates induced by skin exposure to Eug and IsoEug. The general methodology had been developed elsewhere.⁴¹ Briefly, RHE were placed in 1.5 mL HEPES, pre-treated topically with DEPMPPO in dimethylsulfoxide/HEPES 1:1 (400 mM, 30 μ L, dose/surface area 20 μ mol.cm⁻²) and incubated during 15 min (37 °C, 5% CO₂) to allow a homogeneous diffusion of the spin trap within the skin model. Then, pre-treated RHE was removed from its support and placed on a flat EPR quartz tissue cell equipped with a silica window (Wilmad®, #WG-806-Q). Target compound was then topically applied to the RHE to mimic an individual's exposure. Specifically, 30 μ L of a solution of skin sensitizer in acetone were applied homogeneously in a concentration approaching the one used to diagnose sensitized patients by patch testing. In the case of Eug and IsoEug this concentration was 100 mM in order to obtain a dose/surface area of 5 μ mol.cm⁻² (equivalent to patch test 2% in petrolatum).⁴⁸ The tissue cell was then immediately placed into the spectrometer cavity and the EPR spectrum registered. Unexpectedly, no EPR signal was detected in these experimental conditions, pointing to ineffective radical initiation (not shown). As Eug and IsoEug have been defined as prohaptenes,²² they require metabolic activation *i.e.* chemical transformation to react with skin proteins.⁴⁹ The question therefore was whether metabolism of RHE as received was able to initiate such pre-activation. To say inducing the PhO[•] generation, core element of the antioxidant activity of

these phenolic derivatives. The prohapten-type behaviour of Eug and IsoEug was consequently promoted within the RHE by strengthening the skin model metabolism. To this end, we enzymatically stimulated the RHE to obtain a system closer to what can be found *in vivo*, by systemically incorporating HRP into the RHE, 48 to 72 h upstream to the EPR-ST investigation. HRP (30 U.mL⁻¹) was added to the culture medium (2.5 mL) and incubated (37°C, 5% CO₂, saturated humidity atmosphere). To ensure that such treatment was not impairing *per se* the skin model, cell viability after introduction of HRP was assessed *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The results pointed out that such skin model treatment neither impact the cell viability nor point toward skin irritation (Supporting Information S.4).⁵⁰ In a second step, the difference in transepithelial-transendothelial electrical resistance (TEER) was measured before and after 48/72 h of HRP incubation. TEER is a time-tested technique to monitor living cells that measures electrical resistance of a skin model and provides insight on the permeability and compactness of the tissue through their tight junctions.⁵¹ TEER investigations did not exhibit any significant transepithelial resistance changes *a posteriori* to HRP incorporation (Supporting Information S.5). Worthy of note, no H₂O₂ was added in the EPR investigations of RHE. In such experimental conditions, several spin adducts could be identified and characterised in HRP-treated RHE, pointing to the radical intermediates identified in solution using the enzymatic approach: C-radicals and oxygen-type radicals (Figure 8, upper panel) with *hfccs* summarized in Table S.1 (Supporting Information, see also S.6 for deconvolution and S.7 for control experiments). If the S/N observed in RHE was lower to the one observed in solution, this can ensue from the complexity of the epidermal matrix that offers numerous pathways for the reduction of spin adducts as well as for the direct neutralisation of radical intermediates. As forecasted from investigations in solution, no spin adducts were identified in the case of compounds with methylated phenolic function (veratrole, MeEug and MeIsoEug), that hinder the formation of PhO[•] radical. However, it was more startling while considering the guaiacol study where no radical was detected *a contrario* to parallel investigations in solution *via* the enzymatic approach.

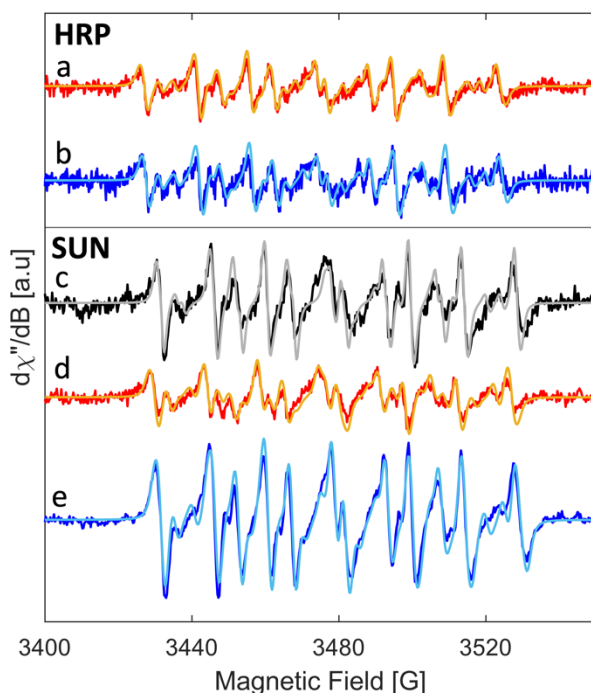


Figure 8. Spin adducts (experimental spectra, Eug in red, IsoEug in blue, guaiacol in black and corresponding simulations in lighter colours) obtained in RHE with DEPMPPO ($20 \mu\text{mol.cm}^{-2}$)/target compound ($5 \mu\text{mol.cm}^{-2}$). Upper panel: HRP pre-treated RHE and (a) Eug and (b) IsoEug. Lower panel: photo-exposed RHE and (c) guaiacol, (d) Eug and (e) IsoEug. Values of *hfccs* are indicated in Table S.1 in the Supporting Information. See also Supporting Information S.6 for deconvolution.

3.3. EPR-ST Studies in Photo-Exposed RHE. The experiments carried out were alike to the above-described protocol, yet integrating a photo-exposure step of the RHE within the flat cell and prior EPR acquisition. In this respect, the “sun” should be able to play the role of radical initiator as demonstrated in the studies in solution. It should be stressed that to ensure that the radicals are formed within the epidermis and avoid artefact on the surface, the RHE were carefully washed with HEPES buffer after incubation of the spin trap before applying the targeted compound. Spectral signatures akin to those observed in solution were thus observed (Figure 8, lower panel; see Supporting Information S.6 for deconvolution, S.8 for control experiments and Table S.1 for *hfccs*). Experiments were also carried out with guaiacol to identify whether this common backbone to the two targeted mono-alkoxyphenols was involved in C-radicals generation. Similar EPR fingerprints with similar *hfccs* to that induced by Eug and IsoEug were found, supporting that C-radicals seemed to be located on the aromatic ring.

4. Discussion

Eug and IsoEug were first investigated for their propensity to induce radical intermediates in solution following two triggering events. On the one hand, an enzymatic HRP/H₂O₂ oxidative system was used with the objective of mimicking peroxidases existing *in vivo*. Such tandem is known to react with phenolic derivatives leading to the PhO[•] radical.^{38,39} On the other hand, as sun exposure is known to be widely implicated in various skin diseases,⁵² the target elements were exposed to solar irradiation using AM 1.5G solar simulator. In presence of HRP/H₂O₂, Eug and IsoEug were placed in oxidative conditions where they could behave as antioxidants leading to PhO[•]. Further delocalization of the so-formed radical upon the structure produced radical forms on carbon atoms of the same structure trapped by DEPMPO with characteristic *hfccs* (Supporting Information Table S.1). The radical species observed showed that the carbon forms are more important than those where the unpaired electron is on the oxygen. In the context of our EPR-ST experiments, comparative studies with guaiacol, veratrole, MeEug and MeIsoEug confirmed the need for the phenol group to originate the radicals trapped. Noteworthy, photo-exposure of sample solutions brought similar outcomes to light. Interestingly, molecules methylated on the phenolic chemical function showed that Eug was likely to generate similar radical signatures by photo-exposure without going through the PhO[•] radical. Indeed, EPR-ST of MeEug irradiated solutions raised the formation of C-radicals which was not the case with HRP/H₂O₂. A possible explanation was given by DFT investigations where the energetically favourable pathways of radical formation were highlighted for both Eug and IsoEug. DFT suggested a difference in the initial radical formation for both compounds (Figure 9). While for IsoEug the expected preferential oxidation site was the phenoxy function, it was the methylene group of the allyl chain in *para* position of Eug which was stressed. This gives rise to a C-radical able to electronically delocalize upon the structure on carbon atoms (Figure 10) which are not the same as those proposed after formation of PhO[•] radical (Figure 2). Such behavior can be explained by the difference in the delocalization scheme of the π system of both compounds. In IsoEug, the radical is formed on the easiest stressed position namely the OH function. In Eug, one must consider that the double bond is not conjugated with the aromatic ring and that formation of the radical on the methylene position leads to structural rearrangement with conjugation of the double bond with the aromatic ring. Such increase of π system delocalization is the source of the greater sensitivity of this position for the formation of the radical for Eug, whereas formation of the PhO[•] radical would not lead to a such increase of the π system disfavoring this position. Same analysis as for Eug is valid for MeEug. Hence, an HRP/H₂O₂ oxidizing medium would favor radical initiation through the most sensitive position PhO[•] radical for IsoEug and C-radical for Eug. Similarly, energy supply (solar irradiation) would favor the formation of a radical on the *para*-substituent of Eug as seen for MeEug.

These EPR-ST preliminary studies in solution gave an outline allowing further characterization of the radicals formed in the RHE model.

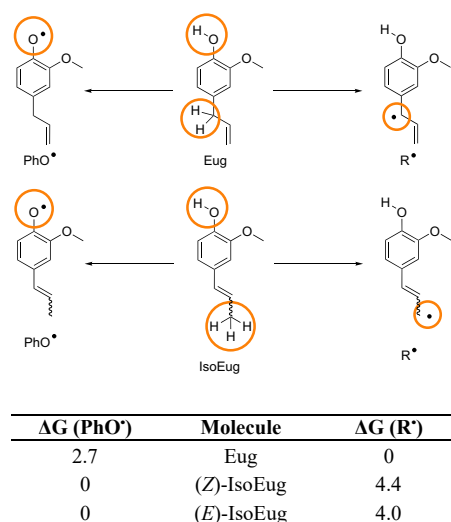


Figure 9. DFT computational studies for radical formation from Eug and IsoEug. The table shows increments in ΔG values ($\text{kcal}\cdot\text{mol}^{-1}$) between the most stable radical upon calculations (then referenced $0 \text{ kcal}\cdot\text{mol}^{-1}$) and the second potential radical that could also be formed. In the case of IsoEug, PhO \cdot is the most stable radical, while for Eug it is a C-radical located on the propenyl substituent.

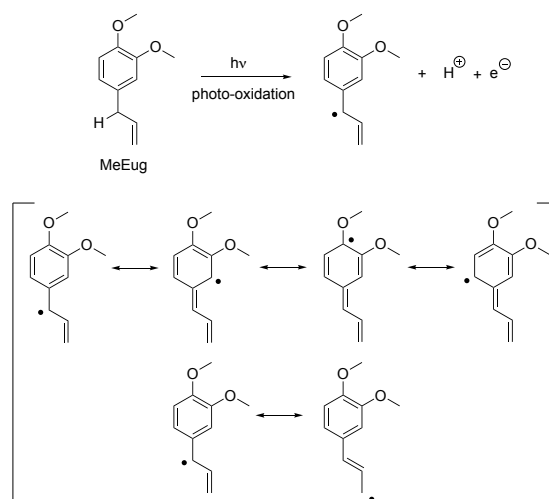


Figure 10. Possible photo-oxidation of MeEug, first radical formed on the allyl substituent and resonance forms

Preliminary investigations using RHE as received did not reveal the presence of radicals as it is typically the case when studying *e.g.* allylic hydroperoxides issued from autoxidation of natural terpenes.⁴¹ Therefore, the introduction of a *stimulus* to induce radical initiation into the epidermal environment was shown to be necessary. As endogenous peroxidases were found to be involved in various skin diseases,⁵³ and based on the studies in solution, HRP was introduced into the RHE to enzymatically strengthen the skin model. Special care was taken not to alter neither the cell viability (MTT assay) nor the permeability and compactness of the tissue through tight junctions (TEER investigations). We demonstrated that a spectral signature alike to those observed in solution was obtained for Eug and IsoEug. C-radicals were trapped within the RHE treated with HRP, meaning by enhanced metabolic reactivity. The fact that H₂O₂ was not added in these experiments approached real conditions of presence of peroxidases in the skin. This could be in line with the proposed prohapten nature of both Eug and IsoEug, needing a metabolic conversion to become reactive and thus skin sensitizers.²² Results underline the importance of the enzymatic action within RHE, notably peroxidases here, necessary for the generation of radical species in the epidermis. Although no H₂O₂ was supplemented in the RHE investigations, such oxidizing compounds could have been produced

in cellulo being sufficient for Eug and IsoEug activation.⁵⁴ Indeed, control experiments in solution had shown that if H₂O₂ was not introduced into the reaction medium containing the phenolic derivatives in the presence of HRP and DEPMPO, no paramagnetic signature was observed (Supporting Information S.1).

As for preliminary studies in solution, RHE with the two targeted mono-alkoxyphenols were exposed to sunlight. It has been shown that such exposure can likewise trigger or at least step up ACD, especially in the case of Eug.⁵⁵ Similar EPR fingerprints as those obtained in solution post irradiation were observed, testimony of C- and O-centered radicals (for control experiments see Supporting Information S.2). Interestingly, guaiacol exhibited a comparable EPR-ST signature to the one induced by Eug and IsoEug, supporting the hypothesis that C-radicals seemed to stem from the common aromatic ring. Under these conditions, spin adducts were equally observed in RHE from guaiacol in contrast to what was observed in HRP pre-treated RHE. Overall, these experiments made it possible to observe, for the first time, radicals from Eug and IsoEug in photo-exposed RHE. This finding was however surprising, as to our knowledge neither has been reported as photoallergens. Photoallergic contact dermatitis is a critical photosensitivity skin pathology caused by combined exposure to photoreactive chemicals in contact with the skin under exposure to UV and/or visible light.⁵⁶ A prehapten behaviour could then also be considered for Eug and IsoEug. Prohaptens and prehaptens are *a priori* inert to skin proteins and necessitate a beforehand transformation to induce antigenic complexes. The difference lies in the origin of the chemical transformation necessary to reach the hapten stage. Physico-chemical transformations such as air oxidations (*e.g.* autoxidation) or photo-mediated transformations allow a prehapten to become a sensitizing compound.^{57,49} The possibility of IsoEug being a prehapten through photo-oxidation processes has been recently proposed, although *via* action of visible light and not through radical mechanisms.⁵⁸ In our case, “real life” photo-exposure scenarios (*i.e.*, sunlight) seems sufficient to induce radical intermediates, pointing to the critical necessity to establish the related mechanism for the target compounds in the photo-exposed RHE. It has been reported that IsoEug dimerization occurring through radical mechanisms involved singlet oxygen (¹O₂). ¹O₂ was produced either *via* a photosensitizer in presence of molecular oxygen or in presence of peroxides, both typical conditions to produce PhO[•].^{24,59} Also, ¹O₂ has been evidenced while exposing keratinocytes and human skin *in vivo* to UVA light yet at higher irradiance.⁶⁰ It could therefore be envisioned that ¹O₂ triggers radical initiation in photo-exposed RHE.

Last but not least, EPR-ST investigations in RHE were carried out with the amounts of Eug and IsoEug used in clinical ACD diagnostic tests. Both compounds are tested at 1% (w/w) in petrolatum (pet.) when they are included as two of the eight ingredients of fragrance mix I, used for the diagnosis of fragrance contact allergy and tested at 8% (w/w) in pet.⁶¹ However, when tested individually a

concentration of 2% (w/w) for each in pet. is recommended.⁴⁸ The critical factor for sensitization and elicitation of ACD is the dose per unit area.⁶² For the commonly used Finn Chambers[®] (8 mm diameter, inner area 0.5 cm²) to apply sensitizers on the back of the patients for patch testing diagnosis of ACD, it is recommended a dose of allergen per unit area of *ca.* 40 mg.cm⁻².⁶¹ This corresponds to a dose per unit area of approximately 0.8 mg.cm⁻² when Eug or IsoEug (2% pet.) are tested in small Finn Chambers. In our studies, 0.8 mg.cm⁻² for a RHE of 0.6 cm² inner area correspond to 0.48 mg of target compound topically applied. We thus applied to the RHE 30 μ L of a 100 mM solution in acetone of Eug or IsoEug, equivalent to 3 μ mol applied to 0.6 cm² surface, *i.e.*, 5 μ mol.cm⁻² and a dose per unit area of 0.82 mg.cm⁻². Accordingly, in our experimental conditions C- and O-radicals were observed in RHE with Eug and IsoEug concentrations in the range of those used for elicitation of ACD and diagnosis of patients. The EPR-ST methodology we are employing is thus sensitive enough and in good agreement with what may happen in human skin *in vivo*.

To summarize, Eug and IsoEug could become skin sensitizers after exerting their antioxidant activity, and this through radical-type mechanisms. Studies reported here give new insights on the chemical transformations of both compounds within the skin involving radical intermediates. Indeed, we have shown that radicals can be formed in a model of RHE after topical application in conditions where the antioxidant behaviour can occur. Moreover, a pro- or prehapten fingerprint could be advanced according to the method used for radical initiation. On the one hand, the introduction of HRP into the RHE, to complement the metabolism capacities, proposed the ability of Eug and IsoEug to perform as prohaptens. On the other hand, studies with RHE exposed to sun light illustrated a possible prehapten nature. C-radicals formed in any case could react with proteins in the skin before they evolve towards the final oxidation form, quinone or quinone methide, that has historically been blamed for Eug and IsoEug skin sensitization potential.

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Abbreviations

ACD, allergic contact dermatitis; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DFT, density functional theory; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; EPR, electron paramagnetic resonance; Eug, eugenol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *hfccs*, hyper fine coupling constants; HRP, horseradish peroxidase; IsoEug, isoeugenol; MeEug, methyl eugenol; MeIsoEug, methyl isoeugenol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Pet, petrolatum; PhO[•], phenoxy radical; RHE, reconstructed human epidermis; ROS, reactive oxygen species; ST, spin trapping; TEER, transepithelial-transendothelial electrical resistance

Supporting Information

EPR control experiments in solution with HRP/H₂O₂, EPR control experiments in solution with photo-exposure, Veratrole and MeIsoEug in solution with photo-exposure, MTT cell viability assay of RHE doped with HRP, TEER assay of RHE doped with HRP, Deconvolution of EPR experimental spectra, EPR control experiments in HRP pre-treated RHE, EPR control experiments in photo-exposed RHE, Table S.1. *hfccs* Values

Authors Contribution

Yannick Port-Lougarre: Performing all experiments and data interpretation

Christophe Gourlaouen: DFT studies

Bertrand Vileno: Responsible EPR studies, conceptualization, establishment mode of action, discussion-data interpretation.

Elena Giménez-Arnau: Conceptualization, establishment mode of action, discussion-data interpretation.

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