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Synthesis, antioxidant and photoprotection activities of hybrid derivatives useful to prevent skin cancer

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ABSTRACT

Chronic ultraviolet (UV) radiation exposure is a major cause of skin cancer. A novel series of hybrid derivatives (**I**–**VIII**) for use in sunscreen formulations were synthesized by molecular hybridization of *t*-resveratrol, avobenzone, and octyl methoxycinnamate, and were characterized. The antioxidant activity values for **VIII** were comparable than to those of *t*-resveratrol. Compounds **I**–**III** and **VI** demonstrated Sun Protector Factor superior to that of *t*-resveratrol. Compounds **I** and **IV**–**VIII** were identified as new, broad-spectrum UVA filters while **II**–**III** were UVB filters. In conclusion, novel hybrid derivatives with antioxidant effects have emerged as novel photoprotective agents for the prevention of skin cancer.

1. Introduction

The incidence and mortality of skin cancer are increasing worldwide, and skin cancer now accounts for one in three of all diagnosed cancers.[1] The most common skin cancers are melanoma and non-melanoma (basal cell carcinoma and squamous cell carcinoma), with malignant melanoma being the main cause of death.[2] It was estimated that 1 in 49 people will be diagnosed with malignant melanoma during their lifetime, particularly in people exposed to certain risk factors, including constant exposure to ultraviolet (UV) radiation.[3,4]

Although there is widespread concern over the importance of using sunscreen formulations to prevent skin cancer, epidemiologic data are still showing progressive increases in its morbidity and mortality rates. In the United States of America, for example, over 3.5 million cases of skin cancer are diagnosed annually.[5] The preventive behavior is complex and, in most cases, the incorrect use of sunscreen formulations results in inadequate photoprotection. It is well established that chronic exposure to UV radiation is one of the main causes of skin cancer, and clinical studies have demonstrated that increased frequency of sunburns increase the risk of melanoma.[6] UV radiation can directly damage DNA through the formation of DNA adducts and/or ROS generation, which promote tumor formation through different pathways.[7]

UV radiation is divided into ultraviolet A (UVA, 320–400 nm), ultraviolet B (UVB, 280–320 nm), and ultraviolet C (UVC, 100–280 nm). UVA and UVB have direct effects on genetic material.[8] The direct genotoxic effects of UV radiation are due to the formation of dimeric photoproducts between adjacent bases in DNA.[9] UV radiation also has indirect effects mediated by increased Reactive Oxygen Species (ROS) levels, which (a) regulate cell processes associated with malignant transformation; (b) cause DNA damage-induced mutations; (c) alter the activity of the pro-survival pathway; and (d) promote skin aging.[10,11]

It was reported that antioxidants could prevent ROS-induced DNA damage.[12] In particular, *t*-resveratrol has potent antioxidant activity exceeding those of vitamins E and C,[13] and might also protect against UV radiation. Because antioxidant compounds, such as *t*-resveratrol, have chemoprotective properties,[14] the development of novel photoprotectors with antioxidant properties is a

Abbreviations: DPPH·, 2,2-diphenyl-1-picrylhydrazyl radical; *t*-resveratrol, *trans*-resveratrol; FDA, Food and Drug Administration; ROS, Reactive Oxygen Species; UVR, Ultraviolet Radiation; SPF, Sun Protector Factor.

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promising strategy for the development of sunscreen formulations with improved photoprotective properties.

The mechanisms involved in the protective effects of resveratrol and other polyphenols against UV radiation are complex, but seem to involve the modulation of cellular signaling pathways, anti-inflammatory activities, induction of cytokines such as interleukin-12 (IL-12), prevention of UVB-induced immunosuppression, and upregulation of genes encoding nucleotide excision repair (NER) enzymes.[15–18] In an in vivo study using SKH-1 hairless mice, *t*-resveratrol inhibited UVB-induced skin edema and the production of hydrogen peroxide.[19] In another study, *t*-resveratrol protected mouse skin against UVB-induced skin damage by modulating survival pathways.[20] Furthermore, it has been suggested that *t*-resveratrol blocks UVB radiation, in particular.[21]

Avobenzone and octyl methoxycinnamate are widely used in sunscreen formulations to protect against UVA and UVB, respectively. However, these compounds have some limitations, including photo-instability, low efficacy if used alone, absence of antioxidant effects, potential allergenic effects, and incompatibility with other components used in commercial sunscreen formulations.[22–24] Molecular modification strategies, such as hybridization, represent a powerful tool that might facilitate the discovery of new photoprotective compounds that are effective, stable and safe.

In a continuing effort to develop new candidate sunscreens to prevent skin cancer and radiation-induced oxidative stress, this report describes the synthesis, antioxidant activity, and photoprotective effects of novel hybrid derivatives (**I**–**VIII**), which were obtained by molecular hybridization of the prototypes *t*-resveratrol (**1**), octyl methoxycinnamate (**2**), and avobenzone (**3**) (Fig. 1). The selections of each subunit are due contributions to (a) antioxidant effect (phenolic hydroxyl in A, B and D subunits); (b) ability to promote electronic conjugation and shift the absorption maximum to higher wavelengths (aryl-α,β-insaturation in subunit C); and (c) free radical stabilization (subunit E). Therefore, this approach aims to combine UVA and UVB photoprotection and antioxidant activity in the same molecule to improve the efficacy of sunscreen formulations.

2. Chemistry

The synthetic routes for preparing the hybrid derivatives (**I**–**VIII**) are summarized in Scheme 1. The coupling reactions between the previously selected aldehydes and hydrazides were catalyzed with 37% hydrochloric acid, and generated the corresponding hydrazone derivatives (**I**–**VIII**) with yields of 70–99%. The structures of all of the compounds were established by elemental analysis, infrared (IR) spectroscopy, and ^{1}H- and ^{13}C-nuclear magnetic resonance. All compounds were analyzed by high-performance liquid chromatography, and the purities were >98.5%. The ^{1}H-nuclear magnetic resonance spectra of all hybrid derivatives exhibited one peak, corresponding to the ylidenic hydrogen of the *E*-diastereomer.[25]

3. Results and discussion

The new hybrid compounds were generated from hydrazone derivatives (**I**–**VIII**), as *E* diastereoisomers, with excellent yields (70–99%). All hybrid compounds were obtained with a purity >98.5% and were characterized using standard analytical methods.

The antioxidant activities of the hybrid compounds (**I**–**VIII**) and positive controls were evaluated by measuring their free radical scavenging capacities against test compounds using the adapted DPPH· microplate assay (Table 1). This assay measures the hydrogen-donating ability of antioxidants to convert the stable DPPH free radical to 2,2-diphenyl-1-picrylhydrazyl, which is accompanied by a change in color from deep-violet to light-yellow. All compounds were incubated in the microplate for 60 min at concentrations of 1000, 300, 100, and 35 μM. Although the test was also conducted for 30 min, the results were similar for both times (results not shown). All compounds, except **VI**, had antioxidant properties. The most potent compounds were **I** (inhibitory concentration 50% [IC_{50}] = 275 μM), **VII** (IC_{50} = 88.2 μM), and **VIII** (IC_{50} = 109.6 μM). The compounds **VII** and **VIII** were more potent than *t*-resveratrol (IC_{50} = 110 μM) but were less potent than ascorbic acid (IC_{50} = 64.2 μM), which was used as a control. The presence of α,β-unsaturated carbonyl moieties for **V**–**VIII** seems to increase the compound's antioxidant activity if at least one of the aryl rings is substituted with a hydroxyl group at the *para* position. As previously reported by some authors, *N*-acyl hydrazone spacer could extend delocalized π-electron in the structure. This effect allows conjugated electrons to flow between the aromatic rings and for this subunit to react with high-energy oxygen species.[26–29] This effect is particularly relevant because UV radiation induces ROS, which promote skin cancer development and photoaging.

In addition, in vitro photoprotection analyses were determined using an Optometric 290S analyzer (SPF-290S; Optometrics, Ayer, MA, USA), and the results were manipulated using WinSPF software version 4.1 (Optometrics). High correlations between SPF-290S in vitro measurements and in vivo tests were reported for a variety of formulations, including creams, lotions, gels, and sprays.[30] Sun Protector Factor (SPF), UVA Protection Factor, UVA/UVB ratio, and the critical wavelength (λ_C) were determined using a stable neutral cream containing 7% of compounds **I**–**VIII**, avobenzone, benzophenone-3, *t*-resveratrol and octyl methoxycinnamate (Table 2). Avobenzone and benzophenone-3 as UVA filters and resveratrol and octyl methoxycinnamate as UVB filters were used as reference. Table 2 shows that the SPF values for compounds **I**–**III** and **VI** were superior to that of resveratrol (SPF = 2). All compounds demonstrated superior activity to that of benzophenone-3 (SPF = 1). Interestingly, the most active compound (**II**) (SPF = 5) is structurally related to resveratrol (1), having an *N*-acyl-hydrazone subunit that extends electron conjugation (Scheme 1). Generally, organic UV filters contain a chromophore that is conjugated by the π-electron system. Therefore, increasing the conjugation, until certain limits, shifts the absorption maximum to higher wavelengths, increasing the molecule's ability to absorb UV radiation. Very large conjugation systems could have maxima of absorption bathochromic shifted out of the UV range.

All synthesized compounds were less active than octyl methoxycinnamate (SPF = 13), however this filter has a lot of inconveniences such as photo-instability and absence of antioxidant effects. Compounds **IV**, **V**, **VII** and **VIII** had similar activities to that of *t*-resveratrol. Polonini et al. developed several *t*-resveratrol analogs as potential photoprotectors, but the SPF value of the most active compound was only 1.42 times greater than that of *t*-resveratrol.[31] Despite the apparent reductions in SPF values of *t*-resveratrol analogs, it is important to acknowledge that sunscreen formulations contain multiple filters to achieve high SPF values.

The UVA protection factor values of compounds **II**, **III**, and **VI** were 1.5–2 times greater than that of *t*-resveratrol, but were less than that of avobenzone. Meanwhile, the UVA protection factor values of compounds **I**, **IV**, **V**, **VII**, and **VIII** were similar to that of resveratrol. Sunscreen formulations that can block UVA are desirable because this type of radiation has immunosuppressive and mutagenic effects in humans and animals.[32,33] The critical wavelength (λ_C), another UVA parameter, is classified by the United States Food and Drug Administration into five categories, as follows: 0 (λ_C <325 nm), 1 (325–335), 2 (335–350), 3 (350–370), and 4 ($\geqslant$370).[34] According these categories, compounds **I** and **IV**–**VIII** were scored as '4', like avobenzone and benzophenone-3,

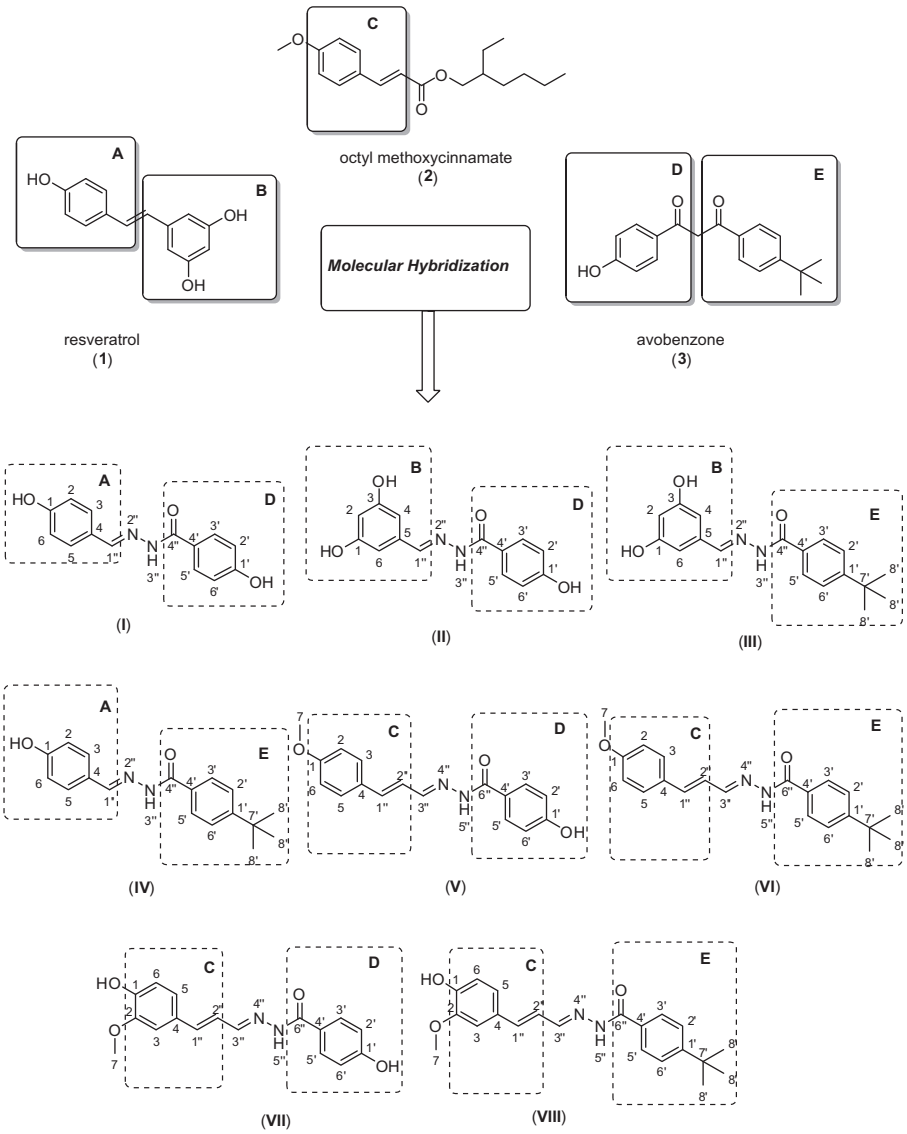
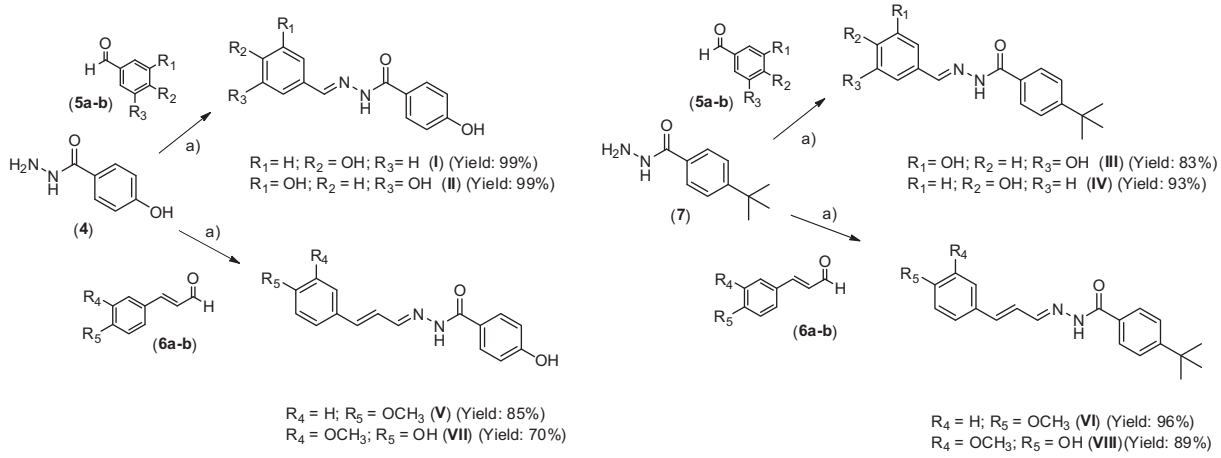


Figure 1. Molecular hybridization among *t*-resveratrol, octyl methoxycinnamate and avobenzone subunits to design new photoprotector compounds.



Scheme 1. Reagents and conditions: (a) EtOH, HCl., rt, 24 h (70–99%).

Table 1
Radical scavenging activity of compounds **I–VIII** expressed as the percent reduction of the absorbance of DPPH at 519 nm (λ) after incubation for 60 min. Ascorbic acid and *t*-resveratrol were used as standards. The values represent means ± the standard deviation of the data ($n = 3$)

Compounds	1000 μM	300 μM	100 μM	35 μM	IC_{50} (μM)
I	65.0 ± 3.4	54.4 ± 9.2	38.0 ± 9.2 [a]	18.4 ± 4.7 [a,b]	275
II	34.2 ± 5.9	26.5 ± 6.7	11.2 ± 9.0	5.6 ± 6.3	–
III	31.3 ± 1.2	20.1 ± 1.4	10.9 ± 1.2	7.0 ± 2.9	–
IV	38.7 ± 1.4	28.9 ± 0.7	15.0 ± 1.4	10.2 ± 2.5	–
V	2.8 ± 1.6	−2.6 ± 1.4	−1.5 ± 0.6	−3.4 ± 3.2	–
VI	−7.1 ± 2.2	−5.6 ± 2.3	−8.3 ± 1.7	−6.6 ± 2.2	–
VII	75.5 ± 3.1 [a,b]	77.2 ± 6.0 [a,b]	56.7 ± 1.4	34.8 ± 6.0	88.2
VIII	76.1 ± 3.8 [a,b]	75.1 ± 3.3 [a,b]	45.6 ± 0.1 [a]	20.0 ± 0.4 [a,b]	109.6
Ascorbic acid	81.2 ± 2.3	80.7 ± 1.4	77.9 ± 1.7	18.8 ± 8.6	64.2
t-Resveratrol	73.8 ± 0.3	71.3 ± 1.1	45.5 ± 0.3	20.5 ± 3.0	110

[a] $p < 0.05$ compared with *t*-resveratrol.
[b] $p < 0.05$ compared with ascorbic acid.

Table 2
Photoprotection activity of compounds **I–VIII**, *t*-resveratrol, octyl methoxycinnamate, avobenzone and benzophenone-3

Compounds	SPF[a]	UVAPF[b]	UVA/UVB ratio	λ_C (nm)
I	3 ± 0.1	2 ± 0.1	0.8	376
II	5 ± 0.2	4 ± 0.1	0.6	368
III	4 ± 0.3	3 ± 0.1	0.6	366
IV	2 ± 0.1	2 ± 0.1	0.8	379
V	2 ± 0.1	2 ± 0.1	1.1	388
VI	4 ± 0.2	4 ± 0.1	1.0	386
VII	2 ± 0.1	2 ± 0.1	1.2	389
VIII	2 ± 0.1	2 ± 0.1	1.1	387
t-Resveratrol	2 ± 0.1	2 ± 0.1	0.6	365
Octyl-methoxycinnamate	13 ± 0.1	–	–	334
Avobenzone	–	39 ± 1.0	1.8	382
Benzophenone-3	1 ± 0.1	–	0.6	371

[a] SPF: Sun Protection Factor.
[b] UVAPF: UVA Protection Factor.

whereas *t*-resveratrol and compounds **II–III** were scored as '3'. Octyl methoxycinnamate was scored as '1'. It was reported that sunscreen formulations with a λ_C near 400 nm, including those scored as '4' could block a broad spectrum of UV radiation.[31] The UVA/UVB ratio represents a ratio of the areas under the curves for UVA and UVB radiation in the range of 290–400 nm. It is related to UVA photoprotection and is the basis for the Boots Star Rating classification.[35] Using this classification, benzophenone-3 was scored as '2 stars' displaying 'good' protection against UVA. Compounds **II** and **III** were scored as '3 stars', like *t*-resveratrol. This category displays 'superior' protection against UVA. Meanwhile, compounds **I** and **IV** was scored as '4 stars', which represents 'maximum' protection against UVA. Compounds **V–VIII** had the highest scores, of '5 stars', which represents 'ultra' protection against UVA. Taken together, these results indicate that compounds **I** and **IV–VIII** had superior UVA blocking to *t*-resveratrol, but were inferior to avobenzone. Therefore, compounds **I** and **IV–VIII** are effective UVA filters while compounds **II** and **III** are effective UVB filters.

4. Conclusions

A novel series of hybrid derivatives (compounds **I–VIII**) were synthesized by molecular hybridization of *t*-resveratrol, avobenzone, and octyl methoxycinnamate, and were characterized by elemental analysis, IR spectroscopy, and ^1H and ^{13}C NMR. All of the compounds, except **VI**, demonstrated antioxidant activity using the DPPH$^{\cdot}$ method. The most potent molecules were **VII** (IC_{50} = 88.2 μM), and **VIII** (IC_{50} = 109.6 μM), with activities superior to that of *t*-resveratrol (IC_{50} = 110 μM). The SPF values for **I–IV** and **VI** were also greater than that of *t*-resveratrol (SPF = 2), with compound **II** having the highest SPF (5). Compounds **I** and **IV–VIII** were broad-spectrum UVA filters while **II** and **III** were UVB filters, with greater SPF values than *t*-resveratrol. In conclusion, the hybrid derivatives with antioxidant effect are novel prototypic compounds that could be used in sunscreen formulations used for the prevention of skin cancer.

5. Experimental

5.1. General

Melting points were measured with an electrothermal melting-point apparatus (SMP3, Bibby Stuart Scientific) in open capillary tubes and are uncorrected. Infrared spectra (KBr disc) were produced on an FTIR-8300 Shimadzu and the frequencies expressed in cm^{-1}. ^1H NMR and ^{13}C NMR spectra were scanned on a Bruker DRX-400 (300 MHz) NMR spectrometer using DMSO-d_6 as the solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane. The coupling constants are reported in hertz (Hz) and signal multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), multiplet (m). Elemental analyses (C, H, and N) were performed on a Perkin Elmer model 240C analyzer and the data were within ±0.4% of the theoretical values. HPLC analysis was performed on a Shimadzu LC-10AD chromatograph equipped with a model SPD-10A UV–vis detector (Shimadzu). All compounds were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. The compounds were separated on a reversed phase C18 column (5 μm particle, 250 mm × 4.6 mm I.D.) Shimadzu Shim-pack CLC-ODS (M). HPLC-grade solvents (acetonitrile, methanol, acetic acid, and toluene) were used in the analyses and were bought from a local supplier. The progress of all reactions was monitored by TLC, which was performed on 2.0 × 6.0 cm^2 aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under UV light (254 nm and 365 nm) and treated with iodine vapor. Merck silica gel (70–230 mesh) was used for preparative column chromatography. Reagents and solvents were purchased from commercial suppliers and used as received.

The compounds 4-hydroxybenzhydrazide (**4**), 4-hydroxybenzaldehyde (**5a**), 3,5-dihydroxybenzaldehyde (**5b**), *trans*-*p*-methoxycinnamaldehyde (**6a**), 4-hydroxy-3-methoxycinnamaldehyde (**6b**) and 4-(*tert*-butylbenzoic)hydrazide (**7**) were purchased commercially from Sigma–Aldrich (St. Louis, MO, USA).

5.2. Chemistry

5.2.1. General procedures for the preparation of hybrid derivatives (I–VIII)

The corresponding aromatic aldehyde (**6a–b**; or **7a–b**) (1.64 mmol) was added to a solution of previously selected

hydrazide derivatives (**5** or **8**) (1.64 mmol) in 15 mL of ethanol, in the presence of 0.1 mL of 37% hydrochloric acid. The reaction was stirred for 24 h at room temperature and monitored by TLC. Afterwards, the solvent was partially concentrated at reduced pressure and the resulting mixture was poured into cold water. The precipitate was collected by filtration, washed with cold ethanol and dried under vacuum to give the hybrid derivatives containing the *N*-acyl hydrazone subunit (**I**–**VIII**). All compounds were recrystallized in ethanol in excellent yields (70–99%). If necessary, the compounds can be purified by column chromatography (flash silica, eluent: 40% ethyl acetate; 60% hexane).

5.2.1.1. (*E*)-4-Hydroxy-N′-(4-hydroxybenzylidene)benzohydrazide (I)[36]. White solid. Yield: 99%, mp: 270–273 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3470 (O—H), 3265 (N—H), 1645 (C=O amide), 1610 (C=N imine), 1600 and 1550 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.44 (1H; s, N—$\underline{H}_{amide}$, $H_{3''}$); 9.95 (2H; s; O—$\underline{H}$, H_1 and $H_{1'}$); 8.32 (1H; s, C—$\underline{H}_{imine}$, $H_{1''}$); 7.78–7.81 (2H; d, $H_{3'}$ and $H_{5'}$); 7.53–7.56 (2H; dd, J_{orto} = 8,3 Hz; H_3 and H_5); 6.82–6.87 (4H; m; H_2, H_6, $H_{2'}$ and $H_{6'}$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.61 ($C_{4''}$); 160.47 (C_1); 159.19 ($C_{1'}$); 147.26 ($C_{1''}$); 129.51 ($C_{4'}$); 128.68 (C_4); 125.47 ($C_{3'}$ and $C_{5'}$); 124.06 (C_3 and C_5); 115.66 ($C_{2'}$ and $C_{6'}$); 114.94 ($C_{2'}$ and $C_{6'}$) ppm. Anal. Calcd for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.95; H, 5.03; N, 11.27.

5.2.1.2. (*E*)-N′-(3,5-Dihydroxybenzylidene)-4-hydroxybenzohydrazide (II). White solid. Yield: 98%, decomposition: 269 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3401 (O—H), 3208 (N—H), 1647 (C=O amide), 1607 (C=N imine), 1601 and 1551 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.50 (1H; s, N—$\underline{H}$; $H_{3''}$); 10.10 (1H; s; O—$\underline{H}$; $H_{1'}$); 9.42 (2H; s; O—$\underline{H}$; H_1 and H_3); 8.21 (1H; s, C—$\underline{H}_{imine}$; $H_{1''}$); 7.77–7.79 (2H; dd, J_{orto} = 8.7 Hz and J_{meta} = 2.0 Hz, $H_{3'}$ and $H_{5'}$); 6.83–6.85 (2H; dd, J_{orto} = 8.8 Hz and J_{meta} = 2.0 Hz, $H_{2'}$ and $H_{6'}$); 6.57 (2H; s; H_4 and H_6); 6.24 (1H; dd; J_{meta} = 2.2 Hz, H_2) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.69 ($C_{4'}$); 160.62 ($C_{1'}$); 158.67 (C_1 and C_3); 147.24 ($C_{1''}$); 136.19 (C_5); 129.62 ($C_{4'}$); 123.94 ($C_{3'}$ and $C_{5'}$); 115.00 ($C_{2'}$ and $C_{6'}$); 105.10 (C_6 and C_7); 104.31 (C_2) ppm. Anal. Calcd for $C_{14}H_{12}N_2O_4$: C, 61.76; H, 4.44; N, 10.29. Found: C, 61.98; H, 4.71; N, 10.44.

5.2.1.3. (*E*)-4-(*tert*-Butyl)-N′-(3,5-dihydroxybenzylidene)benzohydrazide (III). White solid. Yield: 83%, decomposition: 233 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3420 (O—H), 3265 (N—H), 2963 (C—$\underline{H}$ methyl); 1648 (C=O amide), 1630 (C=N imine), 1601 and 1545 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.64 (1H; s, N—$\underline{H}$; $H_{3''}$); 9.43 (2H; s; O—$\underline{H}$; H_1 and H_3); 8.23 (1H; s, C—$\underline{H}_{imine}$; $H_{1''}$); 7.81–7.84 (2H; dd, J_{orto} = 8.4 Hz, $H_{3'}$ and $H_{5'}$); 7.51–7.54 (2H; dd, J_{orto} = 8.5 Hz, $H_{2'}$ and $H_{6'}$); 6.58–6.59 (2H; dd, J_{meta} = 1.8 - Hz, H_4 and H_6); 6,24–6.26 (1H; dd; H_2); 1.31 (9H; s, $H_{8'}$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.92 ($C_{4''}$); 158.55 (C_1 and C_3); 154.57 ($C_{4'}$); 147.85 ($C_{1''}$); 136.02 (C_5); 130.67 ($C_{1'}$); 127.42 ($C_{3'}$ and $C_{5'}$); 125.20 ($C_{2'}$ and $C_{6'}$); 105.14 (C_4 and C_6); 104.43 (C_2); 34.66 ($C_{7'}$); 30.89 ($C_{8'}$) ppm. Anal. Calcd for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.87. Found: C, 69.53; H, 6.77; N, 8.96.

5.2.1.4. (*E*)-4-(*tert*-Butyl)-N′-(4-hydroxybenzylidene)benzohydrazide (IV). White solid. Yield: 93%, mp: 263–265 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3446 (O—H), 3286 (N—H), 2966 (C—$\underline{H}$ methyl); 1622 (C=O amide), 1607 (C=N imine), 1599 and 1547 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.56 (1H; s, N—$\underline{H}$; $H_{3''}$); 9.91 (1H; s; O—$\underline{H}$; H_1); 8.35 (1H; s, C—$\underline{H}_{imine}$; $H_{1''}$); 7.83–7.85 (2H; dd, J_{orto} = 8.4 Hz, $H_{3'}$ and $H_{5'}$); 7.51–7.57 (4H; m, H_3, H_5, $H_{2'}$ and $H_{6'}$); 6.83–6.86 (2H; dd, J_{orto} = 8.8 Hz, H_2 and H_6); 1.31 (9H; s, $H_{8'}$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.77 ($C_{4''}$); 159.34 (C_1); 154.39 ($C_{4'}$); 147.85 ($C_{1''}$); 130.88 ($C_{1'}$); 128.77 (C_4); 127.37 ($C_{3'}$ and $C_{5'}$); 125.15 (C_3, C_5, $C_{2'}$ and $C_{6'}$); 115.68 (C_2 and C_6); 34.65 ($C_{7'}$); 30.91 ($C_{8'}$) ppm. Anal. Calcd for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 73.12; H, 7.13; N, 9.78.

5.2.1.5. (*E*)-4-Hydroxy-N′-((*E*)-3-(4-methoxyphenyl)allylidene)benzohydrazide (V). Yellow solid. Yield: 85%, decomposition: 271 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3290 (O—H), 3286 (N—H), 2966 (C—$\underline{H}$ methyl); 1618 (C=O amide), 1604 (C=N imine), 1582, 1551 and 1450 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.46 (1H; s, N—$\underline{H}$; $H_{5''}$); 10.10 (1H; s; O—$\underline{H}$; H_1); 8.17–8.19 (1H; d, C—$\underline{H}_{imine}$; J = 6.9 Hz, $H_{3''}$); 7.77–7.80 (2H; d, J_{orto} = 8.8 Hz, $H_{3'}$ and $H_{5'}$); 7.55–7.58 (2H; dd, J = 8.8 Hz, $H_{1''}$ and $H_{2''}$); 6.94–6.97 (4H; m, H_2, H_3, H_5 and H_6); 6.84–6.87 (2H; d, J_{orto} = 8.7 Hz, $H_{2'}$ and $H_{6'}$), 3.79 (3H; s, H_7) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.49 ($C_{6''}$); 160.53 ($C_{1'}$); 159.78 (C_1); 149.19 ($C_{3''}$); 138.21 ($C_{2''}$); 129.57 ($C_{4'}$); 128.67 ($C_{1''}$); 128.50 (C_3 and C_5); 123.95 ($C_{3'}$ and $C_{5'}$); 123.49 (C_4); 114.93 ($C_{2'}$ and $C_{6'}$); 114.27 (C_2 and C_6); 55.19 (C_7) ppm. Anal. Calcd for $C_{17}H_{16}N_2O_3$: C, 68.91; H, 5.44; N, 9.45. Found: C, 69.13; H, 5.79; N, 9.51.

5.2.1.6. (*E*)-4-(*tert*-Butyl)-N′-((*E*)-3-(4-methoxyphenyl)allylidene)benzohydrazide (VI). Yellow solid. Yield: 96%, mp: 188–190 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3472 (O—H), 3221 (N—H), 2956 (C—$\underline{H}$ methyl); 1657 (C=O amide), 1606 (C=N imine), 1581, 1550 and 1452 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.58 (1H; s, N—$\underline{H}$; $H_{5''}$); 8.18–8.21 (1H; d, C—$\underline{H}_{imine}$; J_{orto} = 8.0 Hz, $H_{3''}$); 7.81–7.84 (2H; d, J_{orto} = 8.4 Hz, $H_{3'}$ and $H_{5'}$); 7.51–7.59 (4H; m, J_{orto} = 8.8 Hz, J_{meta} = 4.6 Hz, $H_{1''}$, $H_{2''}$, $H_{2'}$ and $H_{6'}$); 6.95–6.97 (4H; m, H_2, H_3, H_5 and H_6); 3.79 (3H; s, H_7); 1.31 (9H; s, $H_{8'}$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 162.78 ($C_{6''}$); 159.85 (C_1 and $C_{4'}$); 149.86 ($C_{3''}$); 138.67 ($C_{2''}$); 130.74 ($C_{1'}$); 128.62 ($C_{1''}$); 128.58 (C_3 and C_5); 127.41 ($C_{3'}$ and $C_{5'}$); 125.16 ($C_{2'}$ and $C_{6'}$); 123.37 (C_4); 114.28 (C_2 and C_6); 55.21 (C_7); 34.65 ($C_{7'}$); 30.90 ($C_{8'}$) ppm. Anal. Calcd for $C_{21}H_{24}N_2O_2$: C, 74.97; H, 7.19; N, 8.33. Found: C, 74.86; H, 6.98; N, 8.02.

5.2.1.7. (*E*)-4-Hydroxy-N′-((*E*)-3-(4-hydroxy-3-methoxyphenyl) allylidene)benzohydrazide (VII). Yellow solid. Yield: 70%, mp: 241–243 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3364 (O—H), 3252 (N—H), 2953 (C—$\underline{H}$ methyl); 1622 (C=O amide), 1609 (C=N imine), 1587, 1553 and 1454 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.41 (1H; s, N—$\underline{H}$, $H_{5''}$); 10.07 (1H; s, O—$\underline{H}$, $H_{1'}$); 9.32 (1H; s, O—$\underline{H}$, H_1); 8.14–8,16 (1H; d, C—$\underline{H}_{imine}$, J_{orto} = 7.7 Hz; $H_{3''}$); 7.77 (2H; d, J_{orto} = 8.8 Hz, $H_{3'}$ and $H_{5'}$); 7.21 (1H; d, J_{meta} = 1,7 Hz, H_6); 6.98–7.00 (1H; dd, J_{orto} = 8.3 Hz and J_{meta} = 1.5 Hz, H_3); 6.88–6.90 (2H; d, J_{orto} = 10.8 Hz, $H_{1''}$ and $H_{2''}$); 6.83 (2H; dd, J_{orto} = 8.7 Hz, $H_{2'}$ and $H_{6'}$); 6.75–6.77 (1H; d, J_{orto} = 8.1 Hz, H_5); 3.81 (3H; s, H_7) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.37 ($C_{6''}$); 161.02 ($C_{1'}$); 155.65 (C_1); 149.80 (C_2); 148.33 ($C_{3''}$); 148.16 ($C_{2''}$); 139.50 ($C_{1''}$); 130.05 (C_4); 128.13 ($C_{4'}$); 123.33 ($C_{3'}$ and $C_{5'}$); 121.58 (C_5); 115.99 ($C_{2'}$); 115.42 (C_3); 110.57 (C_6); 56.06 (C_7) ppm. Anal. Calcd for $C_{17}H_{16}N_2O_4$: C, 65.38; H, 5.16; N, 8.97. Found: C, 65.71; H, 5.33; N, 8.85.

5.2.1.8. (*E*)-4-(*tert*-Butyl)-N′-((*E*)-3-(4-hydroxy-3-methoxyphenyl)allylidene)benzohydrazide (VIII). Yellow solid. Yield: 89%, mp: 244–246 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3368 (O—H), 3213 (N—H), 2962 (C—$\underline{H}$ methyl); 1630 (C=O amide), 1601 (C=N imine), 1585, 1551 and 1450 (C=C). NMR ^1H (300 MHz, DMSO-d_6) δ: 11.59 (1H; s, N—$\underline{H}$, $H_{5''}$); 9.98 (1H; s, O—$\underline{H}$, H_1); 8.15–8.18 (1H; d, J_{orto} = 8.5 Hz, C—$\underline{H}_{imine}$, $H_{3''}$); 7.80–7.83 (2H; d, J_{orto} = 8.4 Hz, $H_{3'}$ and $H_{5'}$); 7.51–7.53 (2H; d, J_{orto} = 8.4 - Hz, $H_{2'}$ and $H_{6'}$); 7.22 (1H; d, J_{orto} = 1.8 Hz, H_6); 6.99–7.02 (1H; dd, J_{orto} = 8.4 Hz and J_{meta} = 1.7 Hz, H_3); 6.91–6.93 (2H; d, J = 5.6 Hz, $H_{1''}$ and $H_{2''}$); 6,76–6,78 (1H; d, J_{orto} = 8.2 Hz, H_5); 3.81 (3H; s, H_7); 1.30 (9H; s, $H_{8'}$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.27 ($C_{6''}$); 154.99 (C_1 and $C_{4'}$); 150.53 (C_2); 148.35 ($C_{2''}$ and $C_{3''}$); 139.98

($C_{1''}$); 131.19 (C_4); 128.08 ($C_{1'}$); 127.89 ($C_{3'}$ and $C_{5'}$); 125.70 ($C_{2'}$ and $C_{6'}$); 121.70 (C_5); 116.00 (C_3); 110.61 (C_6); 56.07 (C_7); 35.14 ($C_{7'}$); 31.37 ($C_{8'}$) ppm. Anal. Calcd for $C_{21}H_{24}N_2O_3$: C, 71.57; H, 6.86; N, 7.95. Found: C, 71.82; H, 6.91; N, 8.14.

5.3. Antioxidant activity

The antioxidant activity of hybrid compounds (**I–VIII**), ascorbic acid (Sigma–Aldrich®) and *t*-resveratrol (Sigma–Aldrich®) were performed using adapted DPPH· methodology in microplate.[37–39] A 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) stock solution was prepared in methanol at a concentration 105.3 μM. In order to enhance the solubility, the compounds (**I–VIII**) were prepared by co-solvent method using as solvent DMSO which final concentration was maintained below 0.1% in the test. The blank sample was prepared using 150 μL of DMSO and 1350 μL in methanol. The compounds (**I–VIII**) and positive controls were diluted in DMSO at concentration of 10 mM and then diluted with methanol for 1 mM. Samples and standards were prepared in the following concentrations: 1000, 300, 100, 35 μM. After, 50 μL of each test compound stock solution and 100 μL of DPPH· solution was dispensed into each well of a 96-well microplate, in triplicate. The plate was wrapped in aluminum foil and kept at 37 °C and it was read in an Biotech microplate reader (model Synergy H1/G5 2.00) using 519 nm filter after 30 and 60 min. The percent scavenging was calculated using the expression $[(Abs_{control} - Abs_{evaluated\ compounds})/Abs_{control}] \times 100$, where $Abs_{control}$ is absorption of the control sample that not contain any inhibitor and $Abs_{evaluated\ compounds}$ is the absorption measured in the presence of the compounds (**I–VIII**) and positive controls (ascorbic acid and *t*-resveratrol). The results are expressed as means ± the standard deviation of the data ($N = 3$ experiments). The data were analyzed statistically with Tukey test at a significance level of $p < 0.05$.

5.4. Photoprotection assay

5.4.1. Samples

The compounds (**I–VIII**) were incorporated in a neutral cream at 7%. This cosmetic neutral cream containing dibutyl adipate (4%), C12–C15 alkyl benzoate (8%), emulfeel® (4%), triglyceride of caprylic–capric acid (6%) and deionized water (quantity sufficient to 100%) have demonstrated stability, possibility of manipulating at room temperature and lack of interference in photoprotective effect. As a reference standard, avobenzone, octyl methoxycinnamate, benzophenone-3 and *t*-resveratrol in a neutral cream at 7% were used. All reference standards were purchased commercially from Sigma–Aldrich (St. Louis, MO, USA).

5.4.2. In vitro photoprotection studies

In vitro photoprotection analyses were determined using an Optometric 290S analyser (SPF-290S) (Littleton, Massachusetts, United States) and the latest version of WinSPF software. A 1-cc syringe is used to spread out 0.11 g of each sample over a Transpore® tape substrate area (70.7 × 70.7 mm) at the rate of 2 μL/cm^2, as recommend by U.S. FDA. The results were expressed as an average of data after 25 scans of the samples performed in different points on the Transpore® tape. Data were collected in the range of 290 nm to 400 nm, with accumulated data at intervals of 2 nm. All sample data is compared to a reference scan in order to compute the transmittance. Afterwards, the WinSPF software automatically converts measurements to SPF values and/or Boots Star ratings using according calculations methods.[29,30] In addition, UVAPF estimation, UVA/UVB ratio and critical wavelength (λ_c) were calculated. The results are expressed as means ± the standard deviation of the data ($N = 3$ experiments). The data were analyzed statistically with Tukey test at a significance level of $p < 0.05$ using Graph Pad Prism® statistical software, version 5.01.

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