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The SOS Chromotest applied for screening plant antigenotoxic agents against ultraviolet radiation

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In this work, we investigated the usefulness of the SOS Chromotest for screening plant antigenotoxic agents against ultraviolet radiation (UV). Fifty Colombian plant extracts obtained by supercritical fluid (CO_2) extraction, twelve plant extract constituents (apigenin, carvacrol, β-caryophyllene, 1,8-cineole, citral, *p*-cymene, geraniol, naringenin, pinocembrin, quercetin, squalene, and thymol) and five standard antioxidant and/or photoprotective agents (curcumin, epigallocatechin gallate, resveratrol, α-tocopherol, and Trolox®) were evaluated for their genotoxicity and antigenotoxicity against UV using the SOS Chromotest. None of the plant extracts, constituents or agents were genotoxic in the SOS Chromotest at tested concentrations. Based on the minimal extract concentration that significantly inhibited UV-genotoxicity (CIG), five plant extracts were antigenotoxic against UV as follows: *Baccharis nítida* (16 μg mL^{-1}) = *Solanum crotonifolium* (16 μg mL^{-1}) > *Hyptis suaveolens* (31 μg mL^{-1}) = *Persea caerulea* (31 μg mL^{-1}) > *Lippia origanoides* (62 μg mL^{-1}). Based on CIG values, the flavonoid compounds showed the highest antigenotoxic potential as follows: apigenin (7 μM) > pinocembrin (15 μM) > quercetin (26 μM) > naringenin (38 μM) > epigallocatechin gallate (108 μM) > resveratrol (642 μM). UV-genotoxicity inhibition with epigallocatechin gallate, naringenin and resveratrol was related to its capability for inhibiting protein synthesis. A correlation analysis between compound antigenotoxicity estimates and antioxidant activity evaluated by the oxygen radical absorbance capacity (ORAC) assay showed that these activities were not related. The usefulness of the SOS Chromotest for bioprospecting of plant antigenotoxic agents against UV was discussed.

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Introduction

Skin exposure to solar radiation has been a major public concern because of its carcinogenicity.[1–3] UV induces DNA damage,[4] principally by producing the cyclobutane pyrimidine dimer (CPD) and (6-4) pyrimidine pyrimidone photoproducts (6-4PP), which cause mutations[5] that can lead to inflammatory responses, immunosuppression and skin cancer.[6–8] Therefore, preventive protection strategies using photoprotective agents are successful to reduce the harmful effect of solar radiation on healthy human populations.[9]

Phytochemicals are emerging as a class of genoprotective agents less toxic than synthetic photoprotective compounds.[10] Plants have metabolic pathways that naturally produce medicinal compounds;[11] therefore, they can be sources of antigenotoxic/antimutagenic compounds that prevent cellular mutations involved in skin cancer and aging by regulating UV-induced mutability.[10,12,13] These plant compounds operate by different mechanisms such as: absorbing photons directly, strengthening the endogenous antioxidant system, stimulating DNA repair mechanisms or melanogenesis, among others.[14]

The risk of UV exposure to human skin has been mainly assessed using empirical indices such as the minimum erythema dose (MED) and other UV indices based on erythema.[15] The index based on erythema has also been used to measure the ability of sunscreens to protect against UV. For example, the solar protection factor (SPF) indicates how much a sunscreen increases the capacity of natural skin defense against erythema or redness before burn. However, erythema is a risk indicator with the threshold UV dose of MED. The effect of UV doses lower than MED cannot be evaluated using erythema; whereas UV genotoxicity would be expected for the skin even at those low dose ranges.[16] In addition, compounds used as the active principle of sunscreens are selected mostly based on their absorbance UV spectra.[17] This criterion is not applicable to those phytochemicals that protect against UV by

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stimulating cellular capability to repair DNA damage.[12] To overcome these limitations, the use of the DNA dosimeter has been proposed as a complement to improve the actual system that measures sunscreen genoprotective efficacy.[18]

In vitro bacterial assays are useful tools to evaluate the antigenotoxic properties of natural compounds due to their sensitivity, simplicity and flexibility to design experimental procedures to study protective mechanisms.[19] The SOS Chromotest,[20] a colorimetric assay developed for carcinogen detection at Pasteur Institute (France), has been used to study antigenotoxic compounds against UV.[21] The assay exploits the inducible DNA replication–cell division coupling system (SOS response) present in *E. coli*[22] (Fig. 1). When *E. coli* cells are exposed to UV, CPD and 6-4PP are produced, which introduce structural distortions in DNA.[23] Since these photoproducts are an obstacle for chromosome replication,[24] they induce the SOS response.[22] Thus, the SOS Chromotest is highly sensitive to UV.[25]

In spite of the genetic differences between bacteria and human cells, there are several reasons that support the use of the SOS Chromotest to identify compounds with potential DNA protective activity in humans: (i) a DNA damage inducible system like bacterial SOS response that involves CPD repair, cell cycle arrest and increased antioxidant defenses and melanogenesis has been described in human skin cells.[26] Therefore, the predictive capability for DNA protection in human skin cells of the studied samples could be expected when the SOS Chromotest is used, because this assay is highly sensitive to UV-induced DNA damage.[25] (ii) It has been hypothesized that the suppression of SOS repair in *E. coli* comprises a possible antimutagenicity mechanism and protective effects of common vegetables.[27] (iii) Using UV-treated isogenic *E. coli* strains, we have previously identified critical genes for cell survival and SOS induction.[28] The simultaneous use of these strains could provide preliminary information about the protection mechanisms by which natural compounds act against UV-induced genotoxicity. (iv) Plant extract cytotoxicity can produce false positive antigenotoxicity results with an *in vitro* assay.[29] The SOS Chromotest overcomes this limitation by measuring alkaline phosphatase activity in PQ37 cells, which reflects constitutive protein synthesis and bacteria cell density.[30] Therefore, samples that showed a UV-genotoxicity inhibitory effect related to their cytotoxic activity can be discarded during screening. (v) The SOS Chromotest is a validated genotoxicity assay[31] that compared to other methods allows greater throughput screening.[32]

We are developing a project aimed at identifying plants from Colombian flora as potential sources of compounds with genoprotective activity against UV. In this work, the usefulness of a modified protocol of the SOS Chromotest to detect the antigenotoxic activity of phytochemical extracts against UV has been investigated. This is proposed as a reliable procedure for large-scale and rapid evaluation of plants that can be a source of genoprotective compounds against UV-induced genotoxicity.

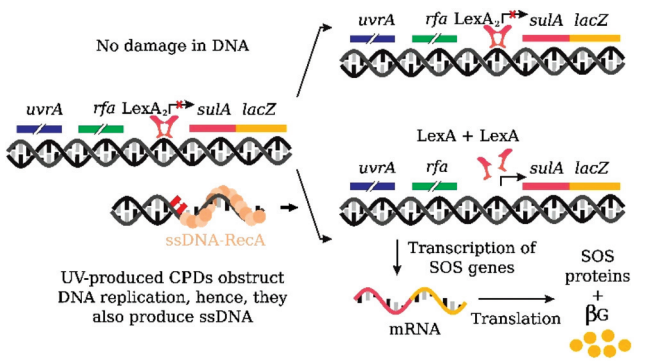

Fig. 1 SOS Chromotest basis. The assay exploits the SOS response system in *E. coli* by fusion of a lacZ reporter gene to *sfiA* (*sulA*), a SOS gene responsible for temporarily inhibiting the cell division after DNA damage. Following DNA damage, the lacZ reporter gene is expressed when RecA binds to ssDNA at stalled replication forks, cleaving LexA enabling the transcription of the lacZ gene. β-Galactosidase, the gene product of lacZ, cleaves an indicator compound to produce a colored or fluorescent product that can be monitored using a plate reader. The quantity of colored/fluorescent product is directly proportional to the amount of damage induced.

Material and methods

Chemicals

Reference mutagen 4-nitroquinoline-1-oxide (4-NQO) and the standard compounds apigenin (95%), carvacrol (98%), β-caryophyllene (98%), citral (95%), 1,8-cineole (99%), curcumin (94%), *p*-cymene (99%), epigallocatechin gallate, EGCG (95%), fluorescein disodium (98%), geraniol (98%), naringenin (95%), pinocembrin (95%), quercetin (95%), resveratrol (99%), squalene (98%), α-tocopherol (97%), thymol (99.5%), and Trolox® (97%) were obtained from Sigma Aldrich Co. (St Louis, Missouri, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Luria–Bertani (LB) and the substrates for β-galactosidase (2-nitrophenyl-β-D-galactopyranoside, ONPG) and alkaline phosphatase (4-nitrophenyl phosphate, PNPP) were purchased from Amresco (Solon, Ohio, USA). The other reagents were obtained from commercial houses J. T. Baker (Phillipsburg, NJ, USA) or Merck (Kenilworth, NJ, USA).

Plant material and extracts

Fifty extracts were obtained from thirty-five Colombian plant species collected in different collection sites (Table 1), which were taxonomically identified by Dr José Luis Fernández Alonso at Colombian National Herbarium (National University, Bogotá, Colombia). Plant species collection during botanical expeditions was supported by the official collection permit granted by the Colombian Ministry of Housing and Environment (Agreement No. 101, Resolution No. 0812). For each specimen, fresh leaves and flowers were used to obtain the extract using supercritical fluid (CO_2) extraction.[33] The extractions were developed at a pressure of 500 bar, while the

Table 1 List of the studied plants and collection sites is shown. For each plant extract, the minimal concentration that produces significant ($p \leq 0.05$) constitutive alkaline phosphatase synthesis inhibition (CAPSI) and the minimal concentration that produces significant ($p \leq 0.05$) genotoxicity inhibition (CGI) in PQ37 cells are shown. Only CAPSI and CGI values ≤100 μg mL^{-1} were considered as biologically relevant cytotoxic and antigenotoxic concentrations, respectively.[34] The samples with relevant cytotoxic and antigenotoxic activities appear in bold letters

Family	Species name (CNH voucher)	Collection site	CAPSI (μg mL^{-1})	CGI (μg mL^{-1})
Asteraceae	*Achyrocline satureioides* (COL 579420)	Zapatoca (Santander)	1000 ± 0	125 ± 0
	Ageratina popayanensis (COL 579422)	Zapatoca (Santander)	250 ± 0	500 ± 0
	Ambrosia peruviana (COL 579246)	Zapatoca (Santander)	1000 ± 0	500 ± 0
	Austroeupatorium inulifolium (COL 559438)	Curití (Santander)	500 ± 0	500 ± 0
	Baccharis nítida (COL 559436)	Villanueva (Santander)	NI	**16 ± 0**
	Chromolaena pellia (COL 559437)	Los Santos (Santander)	NI	125 ± 0
	Lourteigia ssp. (COL 559444)	Los Santos (Santander)	250 ± 0	250 ± 0
	Tagetes caracasana (COL 559441)	Zapatoca (Santander)	1000 ± 0	125 ± 0
	Wedelia calycina (COL 559439)	Barichara (Santander)	**4 ± 0**	**16 ± 0**
	Wedelia calycina (COL 578353)	Zapatoca (Santander)	1000 ± 0	250 ± 0
Burseraceae	*Bursera tomentosa* (COL 560236)	Los Santos (Santander)	**4 ± 0**	NI
Capparaceae	*Capparis flexuosa* (COL 574225)-F1	Puerto Colombia (Atlántico)	NI	500 ± 0
Ericaceae	*Bejaria aestuans* (COL 560990)	Los Santos (Santander)	NI	1000 ± 0
Escalloniaceae	*Escallonia pendula* (COL 560238)	Curití (Santander)	**63 ± 0**	1000 ± 0
Labiatae	*Hyptis brachiata* (COL 582531)-F1	Tame (Arauca)	NI	1000 ± 0
	Hyptis brachiata (COL 582531)-F2	Tame (Arauca)	500 ± 0	1000 ± 0
	Hyptis sinuata (COL 578965)-F1	Tame (Arauca)	NI	1000 ± 0
	Hyptis sinuata (COL 578965)-F2	Tame (Arauca)	NI	1000 ± 0
	Hyptis suaveolens (COL 560241)	Umpalá (Santander)	NI	**31 ± 0**
	Hyptis suaveolens (COL 578964)-F1	Tame (Arauca)	1000 ± 0	250 ± 0
	Minthostachys septentrionalis (COL 560244)	Curití (Santander)	**4 ± 0**	500 ± 0
	Plectranthus amboinicus (COL 560245)	Umpalá (Santander)	NI	500 ± 0
	Salvia aratocensis (COL 560246)	Los Santos (Santander)	NI	125 ± 0
	Satureja ssp. (COL 560973)	Jordán (Santander)	**8 ± 0**	500 ± 0
Lauraceae	*Persea caerulea* (COL 560247)	Charalá (Santander)	250 ± 0	**31 ± 0**
Malvaceae	*Sida aggregate* (COL 560249)	Los Santos (Santander)	NI	1000 ± 0
Myrtaceae	*Calycolpus* ssp. (COL 560976)	Los Santos (Santander)	250 ± 0	250 ± 0
	Calycolpus moritzianus (COL 578360)	Zapatoca (Santander)	1000 ± 0	250 ± 0
	Eugenia punicifolia (COL 560250)	Los Santos (Santander)	**63 ± 0**	1000 ± 0
	Psidium sartorianum (COL 578359)	Zapatoca (Santander)	NI	125 ± 0
Piperaceae	*Piper cumanense* (COL 578977)	Zapatoca (Santander)	1000 ± 0	250 ± 0
	Piper dilatatifolium (COL 578975)	Zapatoca (Santander)	250 ± 0	125 ± 0
	Piper eriopodon (COL 578364)	Zapatoca (Santander)	NI	125 ± 0
	Piper eriopodon (COL 578974)-F2	Tame (Arauca)	500 ± 0	125 ± 0
	Piper subflavum (COL 578976)	Zapatoca (Santander)	125 ± 0	500 ± 0
	Piper subflavum (COL 582361)-F2	Dagua (Valle del Cauca)	500 ± 0	1000 ± 0
Solanaceae	*Solanum crotonifolium* (COL 560253)	Umpalá (Santander)	NI	**16 ± 0**
Turneraceae	*Turnera aff. diffusa* (COL 560255)	Los Santos (Santander)	NI	250 ± 0
	Turnera diffusa (COL 578361)	Girón (Santander)	250 ± 0	500 ± 0
	Turnera diffusa (COL 578361)-F1	Girón (Santander)	NI	125 ± 0
	Turnera diffusa (COL 578361)-F2	Girón (Santander)	1000 ± 0	500 ± 0
Verbenaceae	*Lippia americana* (COL 578970)	Zapatoca (Santander)	1000 ± 0	1000 ± 0
	Lippia cannescens (COL 578969)	Zapatoca (Santander)	1000 ± 0	NI
	Lippia micromera (COL 578971)	Zapatoca (Santander)	NI	500 ± 0
	Lippia origanoides (COL 560267)	Umpalá (Santander)	125 ± 0	250 ± 0
	Lippia origanoides (COL 560259)	Los Santos (Santander)	NI	**62 ± 0**
	Lippia origanoides (COL 560260)	Barichara (Santander)	NI	250 ± 0
	Lippia origanoides (COL 560980)	Los Santos (Santander)	**4 ± 0**	250 ± 0
	Lippia origanoides (COL 582599)-F1	Tame (Arauca)	1000 ± 0	1000 ± 0
	Lippia origanoides (COL 582599)-F2	Tame (Arauca)	1000 ± 0	1000 ± 0

CNH: Colombian National Herbarium, NI: no inhibition.

collection of F1 and F2 fractions was done at pressures of 40 and 80 bar, respectively.

Bacterial strains and culture

Escherichia coli PQ37 strain [F$^-$ *thr leu his-4 pyrD thi galE galK or galT lacΔU169 srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc*$^+$ *sfiA::Mud(Ap,lac)ts*], proposed for detecting genotoxic carcinogens,[20] was used. This strain carried the *sulA::lacZ* gene fusion on the chromosome as a reporter of the primary DNA damage induced during the SOS response. The cells were grown overnight at 37 °C and were shaken at 100 rpm in Luria–Bertani (LB) media (10 g tryptone per L, 5 g yeast extract per L, 10 g sodium chloride per L, pH 7.4), supplemented with ampicillin (50 μg mL^{-1}) and tetracycline (17 μg mL^{-1}).

Cell irradiation

Cultures (1 mL) with an optical density (OD_{600}) of 0.4 (~2×10^8 cells per mL) were distributed into Petri plates with 5 cm-

diameter for irradiation during assays. For UVA (320–400 nm) and UVB (280–315 nm), the Petri plates with the cells were irradiated in darkness using a UVA/UVB irradiation chamber BS-02 equipped with a radiation controller UV-MAT (Opsytec Dr Groebel, Ettlingen, Baden-Würtemberg, Germany). This radiation controller continuously measured the irradiance, calculated the irradiation dose and switched the lamps after reaching the target dose. The radiation dose used for the antigenotoxicity assay was 10 J m^{-2}, which induces adequately SOS functions in *E. coli* PQ37 cells without significantly affecting their survival.[28]

Genotoxicity assay

Genotoxicity was evaluated using the SOS Chromotest as previously described.[30] Since organic solvents can affect genotoxicity and antigenotoxicity estimates in the SOS Chromotest,[35] we always used distilled water as the dissolvent and an ultrasonic bath E30H Elmasonic (Elma-Hans Schmidbauer GmbH & Co., Singen, Germany) to dissolve the extract and pure compounds. Overnight cultures were grown in LB medium to an optical density ($OD_{600\ nm}$) of 0.4, diluted 10-fold in fresh LB medium and distributed (150 μL) into a series of Eppendorf tubes containing 5 μL of the extract or compound to be tested. The bacteria were exposed for 30 min at 4 °C to different extract or compound concentrations, and then were cultured for 2 h at 37 °C with shaking at 300 rpm in a Termomixer apparatus (Eppendorf, Sao Paulo, Brazil). Negative (distilled water) and positive (2.34 μM of 4-NQO) controls were always included in each assay. A minimum of four independent experiments per treatment with two replicates each were carried out.

β-Galactosidase (βG) and alkaline phosphatase (AP) activities were assayed in 96-well plates (Brand GMBH, Germany). For βG activity, cell membranes were disrupted by mixing 135 μL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 0.1% SDS, and 40 mM β-mercaptoethanol, pH 7.0) with 15 μL of cell culture for 20 min at room temperature. The reaction was started by adding 30 μL of ONPG (4 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0). After 40 min, the enzymatic reaction was stopped by adding 100 μL of Na_2CO_3 (1 M). For AP activity, cell membranes were disrupted by adding 135 μL of T buffer (0.1% sodium dodecyl sulfate, 1 M Tris HCl, pH 8.8) to 15 μL of the cell culture. The enzyme reaction was started by adding 30 μL of PNPP solution (4 mg mL^{-1} in T buffer). After 40 min, the reaction was stopped by adding 50 μL of HCl (2.5 M). After 5 min, 50 μL of Tris (2 M) were added to restore the color. The final absorbances of the βG and AP assays were measured at λ = 420 nm, using a microplate reader Multiskan GO (Thermo Scientific, MA, USA). βG and AP activities were calculated using the relationship: enzyme units = $(1000 \times A_{420})/t$, where A_{420} is the optical density at λ = 420 nm and t is the length of incubation (min) with the substrate (ONPG or PNPP). The ratio of βG units to AP units (R = βG/AP) reflects the induction of the *sulA* gene, even when there is some inhibition of protein synthesis. The genotoxicity criterion used was the SOS induction factor (I) that represents the normalized induction data of the *sulA* gene in each treatment (extract or compound) and was therefore considered to be an indirect measure of the primary DNA damage (genotoxicity) induced by the treatments. This parameter was calculated as $I = R_t/R_{nt}$, where t and nt were the treated and non-treated cells, respectively. The samples were classified as not genotoxic, if I was < 1.5, inconclusive, if I was between 1.5 and 2.0, and genotoxic, if I was >2.0, and a clear concentration–response relationship was observed.

Cytotoxicity assay

As we indicated above, plant extract cytotoxicity can produce false positive antigenotoxicity results with the SOS Chromotest.[29] In order to overcome this limitation, we considered the protein (AP) synthesis inhibition as a cytotoxicity criterion during screening. For each extract or compound, the concentration that produces significant ($p \leq 0.05$) AP synthesis inhibition (CAPSI) in PQ37 cells was estimated. CAPSI values ≤100 μg mL^{-1} for extracts and ≤25 μM for compounds were considered as biologically relevant cytotoxic concentrations.[34] A minimum of four independent experiments per treatment with two replicates were carried out.

Antigenotoxicity assay

Antigenotoxicity was assayed using a co-incubation procedure as previously described.[36] The cells were simultaneously treated with the extract or compound at different concentrations and 10 J m^{-2} of UVB, and then were cultured for 2 h at 37 °C with shaking at 300 rpm in a Termomixer apparatus (Eppendorf, Sao Paulo, Brazil). Negative (distilled water) and positive (10 J m^{-2} of UVB) controls were always included in each assay. A minimum of four independent experiments per treatment with two replicates were carried out. The βG and AP activities were assayed as indicated above. Antigenotoxicity (the ability of the test extract or compound to protect against UV-induced genotoxicity) was measured as a significant reduction in the induction factor (I) in the co-incubation procedure and expressed as a percentage of the genotoxicity inhibition: $\%GI = 1 - (I_{ct} - I_{nt}/I_{UVB} - I_{nt}) \times 100$, where I_{ct} is the SOS induction factor in the co-incubation procedure; I_{nt} is the SOS induction factor in non-treated cells, and I_{UVB} is the SOS induction factor in UVB-treated cells. Negative values of %GI were considered as zero; therefore, this parameter ranged from a minimum of 0% to a maximum of 100%. The minimal concentration that produces significant ($p \leq 0.05$) genotoxicity inhibition (CGI) in PQ37 cells was used for comparison of the genoprotective potential of the tested samples.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed as previously described[37,38] with minor modifications. Pure compounds were prepared in potassium phosphate buffer (75 mM, pH 7.4), mixed with fluorescein prepared at 8.16 μM in a 96-well microplate (Brand GmbH, Wertheim, Germany), and the mixture was incubated at 37 °C for 18 min. After that, an AAPH solution prepared at 153 mM in potassium phosphate buffer was added.

Fluorescence was measured on a Modulus™ II Microplate Multimode Reader (Turner Biosystems Inc., Sunnyvale, CA, USA), equipped with absorbance (UV-Vis) and fluorescence modules. An excitation band (λ = 490 nm) and emission range λ from 510 to 579 nm were used for fluorescence measurement. All measurements were done in triplicate and their results were expressed as average ± standard deviation. The ORAC values were calculated by plotting the Trolox® concentration (μM) *versus* the remaining area under the fluorescence curve (AUC) for each compound. The remaining AUC was calculated subtracting the blank's AUC from the AUC of the sample. The results were expressed as micromoles of Trolox® per gram (g) (n = 3).

Statistical analysis

The βG, AP, *I*, %GI, CGI, CAPSI, and ORAC average values and the corresponding standard errors were calculated. The normality of the data was tested using the Kolmogorov–Smirnov test. Variance homogeneity and analysis of variance (ANOVA) tests were also conducted. Average values *per* treatment were compared with controls using a *t*-test for independent samples. Product–moment (Pearson) correlation analysis was used to examine the dose–response relationship in genotoxic and antigenotoxic studies and between the CGI and ORAC values. For all statistical analyses, $p < 0.05$ was considered significant. The R program[39] was used for all analyses.

Live subject statement

The project RC-0572-2012 was approved by the Operational Research and Extension Committee (Record No. 36-2011, File No. 1110) from UIS. The experiments and the chemical management were done according to the National law (Resolution No. 008430-1993) from the Ministry of Health of Colombia and Institutional Manual of Integrated Management and Processes (PGIR-PGGA.05).

Results

Induction of the *sulA* gene by UV

Table 2 shows the induction of the *sulA* gene in *E. coli* PQ37 following treatment with UVB (280–315 nm) and UVA (320–400 nm).

The extent of *sulA* gene induction by UV varied with the radiation type and the dose. A significant increase of *I* values was observed from doses 10 (UVB) and 5000 (UVA) J m^{-2}, respectively; although, smaller doses were not evaluated. UVB showed a biphasic induction of the *sulA* gene. Doses from 10 to 100 J m^{-2} showed *I* values between 8.7 and 9.8 and then, this value increased abruptly to 21.6 at a dose of 280 J m^{-2}. At this dose, a significant inhibitory effect on protein synthesis was detected. Finally, UVA reached the highest induction level (I = 10) at a dose of 20.000 J m^{-2} at the expense of a significant inhibition of protein synthesis.

Table 2 Induction of the sulA gene in *E. coli* PQ37 after treatment with UVB and UVA. For each treatment, the βG, AP and *I* values were calculated from a minimum of four independent experiments with four replicates and the corresponding standard errors are given

	SOS induction parameters		
UV doses (J m^{-2})	βG value	AP value	*I* values
UVB			
0.0	2.7 ± 0.2	2.9 ± 0.3	1.0 ± 0.0
10.0	22 ± 1.6*	2.8 ± 0.1 n.s	8.7 ± 0.1*
20.0	22 ± 2.8*	2.8 ± 0.1 n.s	8.4 ± 0.6*
40.0	24 ± 2.8*	2.7 ± 0.1 n.s	9.2 ± 0.6*
50.0	23 ± 3.9*	2.7 ± 0.1 n.s	9.0 ± 0.7*
100.0	23 ± 1.3*	2.6 ± 0.1 n.s	9.8 ± 0.3*
180.0	28 ± 4.8*	1.6 ± 0.7**	19 ± 2.3*
280.0	24 ± 4.3*	1.1 ± 0.4**	22 ± 5.4*
390.0	17 ± 3.4*	1.2 ± 0.5**	16 ± 2.9*
500.0	16 ± 1.7*	1.0 ± 0.4**	16 ± 1.9*
UVA			
0.0	2.4 ± 0.3	1.6 ± 0.3	1.0 ± 0.0
5000.0	8.9 ± 0.3*	1.4 ± 0.2 n.s	4.0 ± 0.5*
10 000.0	11 ± 0.5*	1.3 ± 0.1 n.s	6 ± 1.0*
15 000.0	10 ± 0.4*	0.9 ± 0.1**	8 ± 1.5*
20 000.0	10 ± 1.0*	0.7 ± 0.0**	10 ± 2.0*
40 000.0	1.2 ± 0.1 n.s	0.2 ± 0.0**	4.1 ± 0.8*
60 000.0	1.2 ± 0.0 n.s	0.2 ± 0.0**	4.2 ± 0.8*

*Significant increase ($p < 0.05$) with respect to zero was found using the *t*-test. **Significant reduction ($p < 0.05$) with respect to zero was found using the *t*-test. n.s. – no significant increase or reduction was found.

Plant extract cytotoxicity, genotoxicity and antigenotoxicity against UV

Table 1 shows cytotoxicity estimates for each plant extract during antigenotoxicity screening. Based on CAPSI values, the plant extracts with relevant cytotoxic effects in *E. coli* (CAPSI values ≤ 100 μg mL^{-1})[35] were as follows: *W. calycina* COL559439 (4 μg mL^{-1}) = *B. tomentosa* COL560236 (4 μg mL^{-1}) = *M. septentrionalis* COL560244 (4 μg mL^{-1}) = *C. moritzianus* COL560976 (4 μg mL^{-1}) = L. *origanoides* COL560980 (4 μg mL^{-1}) > *Satureja* ssp. COL560973 (8 μg mL^{-1}) > *E. pendula* COL560238 (63 μg mL^{-1}) = *E. punicifolia* COL560250 (63 μg mL^{-1}). The results suggest that these plants possess antimicrobial activity against *E. coli* cells.

The extract direct genotoxicity was assayed before its antigenotoxic effect was investigated. At tested doses, the extracts did not increase the *I* values in PQ37 *E. coli* strain; therefore, they were considered not genotoxic in *E. coli* cells (data not shown).

Table 1 shows the CGI values for each plant extract. Based on CGI values, the plant extracts with relevant antigenotoxic effects (CGI values ≤100 μg mL^{-1}) were as follows: *B. nítida* COL559436 (16 μg mL^{-1}) = *S. crotonifolium* COL560253 (16 μg mL^{-1}) = *W. calycina* COL559439 (16 μg mL^{-1}) > *H. suaveolens* COL560241 (31 μg mL^{-1}) = *P. caerulea* COL560247 (31 μg mL^{-1}) > *L. origanoides* COL560259 (62 μg mL^{-1}). The *W. calycina* (COL559439) extract showed CAPSI ≤ CGI values, which suggested that its antigenotoxicity was possibly associated with the inhibition of protein synthesis. Thus, *B. nítida*

COL559436, *S. crotonifolium* COL560253, *H. suaveolens* COL560241, *P. caerulea* COL560247 and *L. origanoides* COL560259 are not cytotoxic extracts that significantly reduce UV-induced genotoxicity depending on the concentration (Fig. 2).

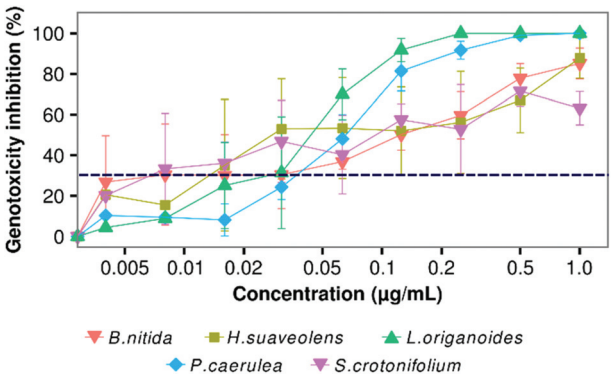

Fig. 2 Antigenotoxic effect of promissory plant extracts against UV expressed as %GI. Values above the dotted line indicate significant inhibition of UV-genotoxicity. Error bars indicate the standard error of the mean computed from four independent experiments (n = 4).

Plant constituent cytotoxicity, genotoxicity and antigenotoxicity against UV

Table 3 shows cytotoxicity estimates for plant constituents and standard compounds during antigenotoxicity screening.

Based on CAPSI values of each constituent and standard compounds, the relative inhibitory effect was as follows: naringenin (38 µM) > pinocembrin (62 µM) > resveratrol (80 µM) > EGCG (108 µM) > thymol (523 µM) > geraniol (22 454 µM) > carvacrol (25 414 µM) > citral (47 625 µM). The remaining compounds did not inhibit protein synthesis. Since only compounds with CAPSI values ≤25 µM are considered as relevantly cytotoxic;[35] none of the compounds studied was cytotoxic in the SOS Chromotest.

Table 3 Promissory plant constituents and their CAPSI and CGI values in PQ37 cells

Plant constituents	CAPSI (µM)	CGI (µM)	ORAC values (µmol Trolox® per g substance)
Apigenin	NI	7 ± 0	24 500 ± 615
Carvacrol	25 414 ± 0	25 414 ± 0	3410 ± 50
β-Caryophyllene	NI	1911 ± 0	2800 ± 109
1,8-Cineol	NI	94 679 ± 0	299 ± 5
Citral	47 625 ± 0	23 813 ± 0	2900 ± 103
Curcumin	NI	33 ± 0	4350 ± 77
p-Cymene	6509 ± 0	104 151 ± 0	219 ± 2
EGCG	108 ± 0	108 ± 0	13 200 ± 531
Geraniol	22 454 ± 0	89 816 ± 0	12 300 ± 119
Naringenin	38 ± 0	38 ± 0	27 600 ± 429
Pinocembrin	62 ± 0	15 ± 0	20 500 ± 128
Quercetin	NI	26 ± 0	34 000 ± 1531
Resveratrol	80 ± 0	642 ± 0	35 300 ± 150
Squalene	NI	3220 ± 0	0 ± 0
Thymol	523 ± 0	8373 ± 0	2900 ± 103
α-Tocopherol	NI	NI	550 ± 13
Trolox®	NI	3130 ± 0	—

NI: no significant inhibition.

The genotoxicity of plant constituents was also assayed before their antigenotoxic effect had been investigated. At tested doses, the compounds did not increase I values in PQ37 *E. coli* strain; therefore, these were considered as not genotoxic in *E. coli* cells (data not shown).

Table 3 also shows the CGI values estimated for each plant constituent and standard compound. In general, flavonoids showed CGI values lower than terpene compounds.

Based on CGI values, the relative antigenotoxic potential of compounds was as follows: apigenin (7 µM) > pinocembrin (15 µM) > quercetin (26 µM) > curcumin (33 µM) > naringenin (38 µM) > EGCG (108 µM) > resveratrol (642 µM) > β-caryophyllene (1911 µM) > Trolox® (3130 µM) > thymol (8373 µM) > citral (23 813 µM) > carvacrol (25 414 µM) > geraniol (89 816 µM) > 1,8-cineol (94 679 µM) > squalene (102 925 µM) > *p*-cymene (104 151 µM). The compounds EGCG, naringenin and resveratrol showed CAPSI values lower than or equal to the CGI values, suggesting that its antigenotoxicity was possibly associated with the inhibitory effect of protein synthesis. Considering CGI values, the compounds with the highest antigenotoxic potential were apigenin, pinocembrin, and quercetin (CGI values ≤ 25 µM).

Plant constituent antioxidant activity

In Table 3, the antioxidant activity of plant constituents is also shown. Based on ORAC values (µmol Trolox® per g substance) of each constituent and standard compound, the relative antioxidant potential was as follows: resveratrol (35 300 ± 150) > quercetin (34 000 ± 1531) > naringenin (27 600 ± 429) > apigenin (24 500 ± 615) > pinocembrin (20 500 ± 128) > EGCG (13 200 ± 531) > geraniol (12 300 ± 119) > carvacrol (3410 ± 50) > thymol (2900 ± 103) = citral (2900 ± 103) > β-caryophyllene (2800 ± 109) > α-tocopherol (550 ± 13) > 1,8-cineol (299 ± 5) > *p*-cymene (219 ± 2). Product–moment correlation analysis using CGI and ORAC data from these compounds showed moderate and negative correlation (R = −0.55, p < 0.009), suggesting that the mechanisms by which these compounds exert their antigenotoxic and antioxidant activities are possibly different and unconnected.

Discussion

We showed that the SOS Chromotest was highly sensitive to UV-induced DNA damage. The dose detection limits depended on the radiation type as follows: UVB (10 J m^{-2}) > UVA (5000 J m^{-2}). Previous works showed the differences in dose detection limits and they depend on the UV dosimeter and radiation type used. Using the SOS Chromotest, Quillardet and Hofnung[25] showed significant SOS induction with 0.02 J m^{-2} of UVC. Differences found in this study could be explained based on the different energies of UVC, UVB and UVA radiation. Using a DNA dosimeter,[40] detection of UVB-induced

photoproducts occurs at a dose of 400 J m^{-2}; while the Comet assay detected UVC- and UVA-induced DNA damage at doses of 1000 and 50 000 J m^{-2}, respectively.[41,42] Recently, Schuch and Menck[43] showed that a DNA dosimeter system using bacteriophage T4 endonuclease V was sensitive to the detection of CPDs at doses of 50 J m^{-2} (UVC), 2000 J m^{-2} (UVB) and 50 000 J m^{-2} (UVA). All these results indicate that the SOS Chromotest stands among the most sensitive UV biological dosimeters currently known.

In this work, we showed that a proper use of the SOS Chromotest can provide useful information on the cytotoxic and antigenotoxic activities of plant extracts and their constituents. As we showed, extracts with high antigenotoxic activity were not cytotoxic with the only exception of the *W. calycina* COL 559439 extract. In this way, our study supports the use of the SOS Chromotest for antigenotoxic compound screening that might be used as a supplement in sunscreen formulation.

As indicated in the Introduction section, compounds used as the active principle of sunscreens are selected mainly based on their UV absorbance capability to be used as solar filters.[17] Sunscreen efficacy has been extensively evaluated using *in vivo* measuring MED indices.[15] However, UV genotoxicity can occur at UV doses lower than MED.[16] Thus, cell and DNA dosimeter assays have been proposed as an alternative to overcome limitations of sunscreen efficacy testing on humans.[18,44] The SOS Chromotest exploits the inducible DNA replication–cell division coupling system (SOS response) present in *E. coli*. Since photoproducts such as CPD and 6-4PP are an obstacle for chromosome replication, detection of antigenotoxic effects with this assay could be linked to the inhibition of CPD or 6-4PP formation, photoproducts DNA repair, and cell division restoration events. These modes of action have been described for several photoprotective agents (Table 4). Since PQ37 strain (*uvrA*) is defective for nucleotide excision repair and the *sulA* gene in this strain was not turned off after UV treatment,[28] we believe that the antigenotoxicity of extracts detected with PQ37 strain was linked to the inhibition of CPD or 6-4PP formation due to their UV absorbance capability. However, the stimulation of the recombination pathway, which is also involved in CPD repair,[28] cannot be discarded as a possible DNA protection mechanism of these plant extracts. The extracts or compounds with such properties could be a useful supplement in sunscreen formulation, because they can prevent mutations that lead to skin cancer.

Although we proposed the SOS Chromotest for bioprospecting of plant genoprotective agents, the simultaneous use of isogenic strains with defective DNA repair functions and wild types with this assay could provide preliminary information about the protection mechanisms by which compounds act against UV-induced genotoxicity as previously showed.[54,58,61] The SOS Chromotest can also overcome some limitations of the cell-based assays. For example, many plant compounds do not penetrate through cytoplasmic membranes in cell-based assays.[29] To overcome this limitation, the SOS Chromotest contains a *rfa* mutation that alters outer membrane lipopolysaccharides allowing better diffusion of compounds into the cell.[30] In addition, plant extract cytotoxicity can produce false positive antigenotoxicity results with an *in vitro* assay.[29] The SOS Chromotest overcomes this limitation measuring AP activity in PQ37 cells, which reflects constitutive protein synthesis and bacteria cell density.[30] Thus, samples that showed the UV-genotoxicity inhibitory effect related to their cytotoxic activity can be discarded during screening. Here, the measurements of AP activity inhibition provide additional and preliminary information about the antimicrobial potential of plant extracts against *E. coli* cells.

However, some critical points must be taken into account when using the SOS Chromotest for antigenotoxicity studies. Secondary interactions between the solvent used to dissolve plant extracts or compounds may result in synergistic effects in the cells; this interference can cause antigenotoxicity overestimates with the SOS chromotest.[34] The relevance in humans of the tested concentrations with *in vitro* cell-based assay is little known. Testing antimicrobial activity, Cos *et al.*[34] indicated IC_{50} values below 100 μg mL^{-1} as biologically relevant for extracts and below 25 μM for pure compounds. However, the relevance of this criterion when the cell-based bioassays use different endpoints to measure bioactivities is unknown. Since the SOS Chromotest is an *in vitro* cell-based assay, additional studies using skin human cell and animal models should be addressed to clarify the protection mode of action of extract constituents and to support their use for sunscreen formulation.

The present work evidenced that Colombia flora contains plants useful as a source of antigenotoxic compounds against UV. We showed for the first time that extracts from *B. nítida*, *S. crotonifolium*, *H. suaveolens*, *P. caerulea*, and *L. origanoides* have high genoprotective potential against UV. A preliminary chemical characterization study of these extracts using gas chromatography–mass spectrometry (data not shown) showed the major constituents (relative amount higher than 10%) as follows: *B. nítida* (17% α-eudesmol, 56% non-identified flavonoid compounds), *H. suaveolens* (14% fenchone, 13% caryophyllene oxide, 52% non-identified flavonoid compounds), *P. caerulea* (13% α-copaene, 14% β-caryophyllene, 33% non-identified flavonoid compounds) and *L. origanoides* (11% β-caryophyllene, 55% pinocembrin). In addition, using high performance liquid chromatography (HPLC) coupled with a diode-array detector (DAD), Stashenko *et al.*[33] identified as the major constituents of *Lippia* extracts the flavonoid compounds apigenin, naringenin, pinocembrin and quercetin; the flavonoids naringenin and pinocembrin being the major constituents in *L. origanoides* extract. We evaluated several of these plant constituents as pure compounds and these resulted in antigenotoxicity against UV (Table 3). Interestingly, the antigenotoxicity of these compounds was not directly co-related to their antioxidant activity, which suggests different mechanisms for these two bioactivity types. Our results also suggested that flavonoids were better antigenotoxic compounds than terpenes, showing always the lowest CGI values among all the compounds studied.

We have recently revealed that the antigenotoxic effects against UV of the essential oils from *Lippia* species depend on

Table 4 Some plant extracts or constituents with the reported antigenotoxic effect against UV-induced DNA damage

Plan extracts (constituents)	Models (assays used)	Effect detected [sources]
Araucaria angustifolia (biflavonoid fraction)	Calf thymus DNA (8-OHdG and CPD HPLC detection)	Inhibition of 8-OHdG and CPD formation[45]
Artemisia herba alba	*S. cerevisiae* (reversion and gene conversion assays)	Inhibition of UV mutagenesis[46]
Camellia sinensis (EGCG polyphenols)	Human skin cell DNA (^{32}P postlabeling technique)	Inhibition of UV-induced DNA damage (CPD) and stimulate nucleotide excision repair involving interleukin (IL)-12 [13,47–52]
	Mice (CPD immunodetection)	
	Human skin cells (CPD immunodetection)	
Bixa orellana (norbixin)	*E. coli* (SOS Chromotest)	Inhibition of SOS functions[53]
Cinnamomum camphora	*S. cerevisiae* (reversion and gene conversion assays)	Inhibition of UV mutagenesis[46]
Colobanthus quitensis (flavonoid and carotenoids)	*S. cerevisiae* (reversion assay)	Prevent CPD formation and stimulate DNA repair processes[54]
	Hamster V79 fibroblasts cells (comet assay)	
	H. aspersas (comet assay)	
Deschampsia antarctica (flavonoid and carotenoids)	*S. cerevisiae* (reversion assay)	Prevent CPD formation and stimulate DNA repair processes[54]
	Hamster fibroblasts (comet assay)	
	H. aspersas (comet assay)	
Fragaria ananassa (anthocyanins)	Human fibroblasts (comet assay)	Inhibition of UV-induced DNA damage[55]
Jatropha curcas	Human lymphocytes (comet assay)	Inhibition of UV-induced DNA damage[56]
Lycopodium clavatum (apigenin)	Human keratinocytes (comet assay)	Inhibition of UV-induced DNA damage and stimulate nucleotide excision repair[57]
	Mice (chromosome aberrations and micronuclei assays)	
	Mice skin cells (DNA repair qPCR expression analysis)	
Origanum compactum	*S. cerevisiae* (reversion and gene conversion assays)	Inhibition of UV mutagenesis[46]
Phyllanthus orbicularis (polyphenols)	(flow cytometry analysis) Human skin cells (CPD immunodetection)	Remove UV-induced CPD modulating DNA repair[58]
Polytrichum juniperinum (flavonoids and carotenoids)	*S. cerevisiae* (reversion assay)	Prevent CPD formation and stimulate DNA repair processes[54]
	Hamster fibroblasts (comet assay)	
	H. aspersas (comet assay)	
Romanian propolis (*p*-cumaric acid, ferulic acid)	Mice (CPD immunodetection)	Inhibition of UV-induced CPD formation[59]
Prunella vulgaris (rosmarinic acid)	Human keratinocytes (comet assay)	Inhibition of UV-induced DNA damage[60]
Salvia officinalis (EO, thujone, cineole, camphor)	*E. coli* (reversion assay)	Inhibition of UV mutagenesis[61–63]
	S. typhimurium (reversion assay)	
	S. cerevisiae (reversion assay)	
Silybum marianum (silymarin)	Human keratinocytes and fibroblasts (comet assay, CPD immunodetection, DNA repair qPCR expression analysis)	Inhibition of UV-induced CPD formation and stimulate DNA repair processes[64]
Undaria crenata	Human keratinocytes (comet assay)	Decrease UV-induced DNA fragmentation[65]
Vitis vinifera (proanthocyanidins)	Human fibroblasts (CPD immunodetection)	Inhibition of UV-induced CPD and stimulate nucleotide excision repair involving interleukin (IL)-12[66]

CPD: cyclobutane pyrimidine dimer. 8-OHdG: 8-hydroxy-20-deoxyguanosine. HPLC: high performance liquid chromatography.

specimen chemotypes,[67] indicating that chemical characterization is critical for studying plant extract antigenotoxicity. Therefore, broad chemical characterization of promissory plant extracts from *B. nítida*, *S. crotonifolium*, *H. suaveolens* and *P. caerulea* is necessary to identify the major constituents and evaluate their antigenotoxicity against UV to support this hypothesis.

At least one of these promissory plant species, *i.e.*, *L. origanoides* (COL560259), have shown very broad-ranging therapeutic potential[68] and its extracts have been well characterized exhibiting pinocembrin and naringenin as the major compounds.[33] The genoprotective properties against UV (present work) widen its potential as a source of compounds (*i.e.*, pinocembrin and naringenin) with possible application in photoprotection and skin cancer chemoprevention. In addition, we showed several plant extracts that inhibited constitutive AP synthesis (*i.e.*, *W. calycina*, *B. tomentosa*, *M. septentrionalis*, *C. moritzianus*, *L. origanoides*, *Satureja* ssp., *E. pendula*, and *E. punicifolia*). The antimicrobial potential of these plants against *E. coli* is under investigation.

Concluding remarks

Our results demonstrated that the SOS Chromotest was highly sensitive to UV-induced DNA damage. This assay provided

useful information about the cytotoxic and antigenotoxic effects of plant extracts and their constituents. Therefore, this assay can be used for bio-prospecting of plant antigenotoxic agents. The extracts from *B. nítida*, *S. crotonifolium*, *H. suaveolens*, *P. caerulea*, and *L. origanoides* have antigenotoxic properties against UV. The *L. origanoides* (COL560259) extract antigenotoxicity was linked to its flavonoid constituents, pinocembrin and naringenin.

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