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Antigenotoxic Effect Against Ultraviolet Radiation-induced DNA Damage of the Essential Oils from *Lippia* Species†

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ABSTRACT

The antigenotoxicity against ultraviolet radiation (UV)-induced DNA damage of essential oils (EO) from *Lippia* species was studied using SOS Chromotest. Based on the minimum concentration that significantly inhibits genotoxicity, the genoprotective potential of EO from highest to lowest was *Lippia graveolens*, thymol-RC ≈ *Lippia origanoides*, carvacrol-RC ≈ *L. origanoides*, thymol-RC > *Lippia alba*, citral-RC ≈ *Lippia citriodora*, citral-RC ≈ *Lippia micromera*, thymol-RC > *L. alba*, myrcenone-RC. EO from *L. alba*, carvone/limonene-RC, *L. origanoides*, α-phellandrene-RC and *L. dulcis*, trans-β-caryophyllene-RC did not reduce the UV genotoxicity at any of the doses tested. A gas chromatography with flame ionization detection analysis (GC-FID) was conducted to evaluate the solubility of the major EO constituents under our experimental conditions. GC-FID analysis showed that, at least partially, major EO constituents were water-soluble and therefore, they were related with the antigenotoxicity detected for EO. Constituents such as *p*-cymene, geraniol, carvacrol, thymol, citral and 1,8-cineole showed antigenotoxicity. The antioxidant activity of EO constituents was also determined using the oxygen radical antioxidant capacity (ORAC) assay. The results showed that the antigenotoxicity of the EO constituents was unconnected with their antioxidant activity. The antigenotoxicity to different constituent binary mixtures suggests that synergistic effects can occur in some of the studied EO.

INTRODUCTION

Skin cancer, especially malignant cutaneous melanoma, is increasing its morbidity and mortality incidence worldwide, becoming a public health problem (1–3). Solar radiation has been well established as the main risk factor for the development of these types of cancer, as UV, the most harmful and mutagenic component of the solar spectrum, can cause structural changes in DNA acting as an initiator and promoter of the carcinogenic process (4,5). In this sense, preventive protection strategies are successful to reduce the harmful effect of solar radiation in healthy human populations (6).

Commercial sunscreen formulations, especially those of broad spectrum, can protect against many of the acute and chronic effects of UV, including erythema, photoageing and skin cancer. Photoprotective compounds used in these formulations are inorganic and organic molecules that operate by different mechanisms, such as solar filtering agents absorbing photons directly, scavenging activities and strengthening the endogenous antioxidants (7–9). In addition, natural compounds used more recently in these formulations can also stimulate cellular DNA damage repair mechanisms and melanogenesis (9,10).

The ability of sunscreens to protect against UV is measured by their solar protection factor (SPF). SPF indicates how much longer a sunscreen increases the capacity of natural skin defense against erythema or redness before burn. However, this index can be overestimated when high doses of UV are used to test high SPF (11). In addition, biomolecule-sunscreen efficiency against UV has been generally determined *in vitro* by cosmetic methods, which have showed numerous limitations and are not well adapted for routine physiological and mechanistic studies (12). For this purpose, the use of DNA dosimeter has been proposed as a complement to improve the actual system that measures genoprotective efficacy of sunscreens (13).

Plants have metabolic pathways that naturally produce compounds to protect them against solar overexposure (14–16); therefore, they can be a source of genoprotective compounds that prevent cellular mutations involved in skin cancer and aging by regulating UV-induced mutability (10,17,18). The *Lippia* genus (Verbenaceae Fam.) has been widely used as seasoning and in traditional medicine for treatments of gastrointestinal, genitourinary and respiratory diseases, as well as anti-inflammatory and analgesic remedies (19,20). EO obtained from Colombian *Lippia* species exhibit a wide range of biological activities such as antioxidant (21), antiviral (22), antimicrobial (23), antiprotozoal (24) and antigenotoxic (25,26).

We are developing a project aimed to identify Colombian flora plants as potential sources of compounds with antigenotoxic activity against UV. The present work evaluated the antigenotoxic activity against UV-induced DNA damage of EO from Colombian *Lippia* species using the SOS Chromotest (27). This *in vitro* assay exploits the inducible SOS DNA repair and damage tolerance system present in *Escherichia coli*, which is activated in response to UV (28). In addition, we demonstrated that

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major EO constituents were responsible for the EO antigenotoxic activity and that this antigenotoxicity may result from synergistic effects between the constituents.

MATERIALS AND METHODS

Chemicals. Reference mutagens aflatoxin B1 (AFB1) and 4-nitroquinoline-1-oxide (4-NQO) and standard compounds carvacrol (98% purity), citral (95%), 1,8-cineole (99%), *p*-cymene (99%), epigallocatechin gallate, EGCG (95%), geraniol (98%), R-(+)-limonene (97%), resveratrol (99%), thymol (99.5%), Trolox® (97%) and α-tocopherol (97%) were obtained from Sigma-Aldrich Co. (St. Louis, Missouri). The 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). The S9 fraction of rat liver was obtained from Moltox (Boone, North Carolina).

Biological material and extracts. Plants of *Lippia* species, used in this study, were collected from experimental plots maintained at the Pilot Agroindustrial Complex of the National Center for Agroindustrialization of Aromatic and Medicinal Tropical Vegetal Species (CENIVAM). The species were identified at the Colombian National Herbarium, where their vouchers (COL; voucher number in parenthesis) were deposited as indicated in Table 1: *Lippia alba,* carvone-RC (COL480750); *L. alba,* citral-RC (COL512272); *L. alba,* myrcenone-RC (COL560257); *L. citriodora* (COL555845); *Lippia dulcis* (COL512079); *Lippia graveolens* (COL555839); *Lippia micromera* (COL516924); *Lippia origanoides,* carvacrol-RC (COL512075); *L. origanoides, p*-cymene/phellandrene-RC (COL519798) and *L. origanoides,* thymol-RC (COL519799). The EO extraction was performed by microwave-assisted hydrodistillation (MWHD), as described by Stashenko *et al.* (29,30).

Bacterial strain and culture. The *Escherichia coli* strain PQ37 [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 to detect carcinogens (27) was used. This strain has a fused copy of the structural LacZ gene coding for β-Galactosidase (βG) enzyme to *sfiA* (*sulA*), a SOS response gene responsible for temporarily inhibiting cell division following DNA damage. The lacZ reporter gene is expressed following DNA damage when RecA binds to ssDNA at stalled replication forks, cleaving LexA enabling transcription of the lacZ gene. The induced βG enzyme 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 damage induced. The cells were grown in LB broth (10 g tryptone L^{-1}, 5 g yeast extract L^{-1}, 10 g sodium chloride L^{-1}, pH 7.4), supplemented with 50 μg mL^{-1} of ampicillin and 17 μg mL^{-1} of tetracycline at 37°C with shaking at 100 rpm during 16 h. To obtain a culture in exponential phase, an overnight culture was 10-fold diluted in fresh media and grown until it reached an optical density of 0.4 at 600 nm ($OD_{600\ nm}$ = 0.4).

Cell irradiation. All irradiations were performed in darkness, and cell cultures (1 mL) in exponential phase (~2×10^8 cells mL^{-1}) were distributed uniformly into 5 cm diameter Petri plates, forming a layer of ~1 mm. The irradiation was carried out using a 0000502-G15WT8 germicidal lamp (Sylvania, England) with peak emission at 253.7 nm. The UV dose range used was between 1.4 and 27.0 J m^{-2}. The irradiation dose was calculated using the mathematical model proposed by Kowalski *et al.* (31). For antigenotoxicity assays, a dose of 20.3 J m^{-2} was used, which was determined experimentally by developing dose–response curves. This dose was selected because it induced adequately SOS functions without producing a significant ($P \leq 0.05$) constitutive alkaline phosphatase activity (CAPA) inhibition in PQ37 cells.

Genotoxicity assay. Genotoxicity of the EO and their major constituents were evaluated using the SOS Chromotest as previously described by Quillardet & Hofnung (32). As organic solvents can affect genotoxicity and antigenotoxicity estimates in the SOS Chromotest (33), we always used distilled water as dissolvent and ultrasonic bath E30H Elmasonic (Elma-Hans Schmidbauer GmbH & Co., Singen, Germany) to dissolve EO and pure compounds. Briefly, cultures in exponential phase ($DO_{600\ nm}$ = 0.4) were 10-fold diluted and dispensed into microcentrifuge tubes containing different EO dilutions (between 0.05 and 1.66%) or compounds at concentrations (between 10 and 2423 μg mL^{-1}) which varied depending on the solubility of each compound. The mixtures were incubated at 8°C for 30 min for the incorporation of the compounds into the cell and then for 2 h at 37°C with shaking at 300 rpm in Thermomixer apparatus (Eppendorf, Sao Paulo, Brazil) for cell recovery and SOS induction. Negative control (distilled water) and positive controls, AFB1 (16.68 μM) in the presence and 4-NQO (2.34 μM) in the absence of metabolic activation were always included in each assay. In all cases, a minimum of three independent experiments with four replicates were conducted.

After incubation, enzymatic assays were simultaneously developed using 96-well microplates (Brand GmbH, Wertheim, Germany). For βG activity, cell membranes were disrupted by mixing Z buffer (135 μL, 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 cell culture (15 μL) for 20 min at room temperature. The enzymatic reaction was started by adding ONPG (30 μL, 4 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0). After 40 min, the reaction was stopped by adding sodium carbonate (100 μL, 1 M). For alkaline phosphatase (AP) activity, cell membranes were disrupted by adding T buffer (135 μL, 1 M Tris HCl, pH 8.8, 0.1% sodium dodecyl sulfate SDS) to cell culture. The enzymatic reaction was started by adding PNPP solution (30 μL, 4 mg mL^{-1} in T buffer). After 40 min, the reaction was stopped by adding hydrochloric acid (50 μL, 2.5 M). After 5 min, Tris (50 μL, 2.0 M) was added to restore the color. The final absorbances of the enzymatic assays were measured at 420 nm using a Multiskan GO microplate reader (Thermo Scientific, MA).

The genotoxicity criterion used was the induction factor (*I*), whose value was calculated according to Quillardet & Hofnung (32). Enzyme Units (EU) = $(1000 \times A_{420})/t$, where A_{420} is the optical density reading at 420 nm and *t* is the incubation time with the substrate in minutes. The ratio of EU for βG and AP (R = EU-βG/EU-AP) reflects the induction of *sulA* gene, even when inhibition of protein synthesis occurs. The *I* represents the normalized induction data of the *sulA* gene in each treatment and, therefore, it is considered as an indirect measure of primary DNA damage (genotoxicity) induced by these treatments. This parameter is

Table 1. List of the specimens studied. For each plant's essential oils, the major constituents*, the minimal concentration that produce significant ($P \leq 0.05$) constitutive alkaline phosphatase synthesis inhibition (CAPSI) and the minimal concentration that produce significant ($P \leq 0.05$) genotoxicity inhibition (CGI) in PQ37 cells are shown.

Species names (CNH voucher)	Major constituents*	CAPSI (%, v/v)	CGI (%, v/v)
Lippia alba (COL 480750)	Carvone (38%)/Limonene (32%)	1.7 ± 0.0	NI
Lippia alba (COL 512272)	Neral (19%)/Geranial (23%)	NI	0.8 ± 0.0
Lippia alba (COL 560257)	Myrcenone (63%)	1.7 ± 0.0	1.7 ± 0.0
Lippia citriodora (COL 555845)	Neral (16%)/Geranial (19%)	0.8 ± 0.0	0.8 ± 0.0
Lippia dulcis (COL 512079)	δ-Cadinene (9%)/*trans*-β-Caryophyllene (10%)	1.7 ± 0.0	NI
Lippia graveolens (COL 555839)	Thymol (70%)/ρ-Cymene (9%)	0.2 ± 0.0	0.4 ± 0.0
Lippia micromera (COL 516924)	Thymol (29%)/Thymyl methyl ether (15%)/ρ-Cymene (13%)	NI	0.8 ± 0.0
Lippia origanoides (COL 512075)	Carvacrol (46%)/ρ-Cymene (11%)/γ-Terpinene (12%)	NI	0.4 ± 0.0
Lippia origanoides (COL 519798)	α-Phellandrene (13%)/ρ-Cymene (11%)/*trans*-β-Caryophyllene (11%)	1.7 ± 0.0	NI
Lippia origanoides (COL 519799)	Thymol (54%)/ρ-Cymene (10%)	NI	0.4 ± 0.0

CNH, Colombian National Herbarium; NI, No inhibition. *According to Stashenko *et al.* (30).

calculated as $I = R_t/R_{nt}$, where t and nt refer to treated and untreated cells, respectively. The interpretation of the results was performed considering the following: "nongenotoxic", if $I \leq 1.5$; "inconclusive", if $1.5 \leq I \leq 2.0$; and "genotoxic", if $I \geq 2.0$ with a clear dose–response relationship.

Cytotoxicity assay. Plant extract cytotoxicity can produce false-positive antigenotoxicity results with *in vitro* assays (34). To overcome this limitation, we considered the AP synthesis inhibition in the SOS Chromotest as cytotoxicity criteria. For each EO, the minimal concentration that produces significant ($P \leq 0.05$) constitutive alkaline phosphatase synthesis inhibition (CAPSI) in PQ37 cells was estimated. A minimum of four independent experiments per treatment with three replicates each were carried out.

Antigenotoxicity assay. The antigenotoxicity assay was carried out utilizing a co-incubation procedure (35). The procedure was developed basically as the genotoxicity assay but, in this case, cells were treated simultaneously with UV and different concentrations of *Lippia* EO, pure compounds and compound mixtures (CM). Three compound mixtures were prepared as follows: (i) CM1, thymol + carvacrol, (ii) CM2, thymol + *p*-cymene and (iii) CM3, carvacrol + *p*-cymene, in the 1 : 1 proportions. The compound concentrations used to prepare each CM were as indicated in the previous section. Briefly, the exponential phase culture was diluted five-fold in fresh LB medium at double strength and mixed (volume:volume) in a series of microcentrifuge tubes, containing different EO, compounds or CMs. The mixture was dispensed in Petri plates for irradiation, recovered in microcentrifuge tubes and, finally, incubated for enzymatic reactions as described above. In these assays, negative (nonirradiated cells) and positive (irradiated cells) controls were always included.

The criterion of antigenotoxicity used was the percentage of genotoxicity inhibition (%GI), which represents the ability of the EO, compound or CM to protect DNA, and is measured as a significant reduction of the I in co-treatments (EO, compound or CM + UV) compared to the positive control. This percentage is calculated as follows: $\%GI = 1 - (I_{ct} - I_{nt}/I_{UV} - I_{nt}) \times 100$, where I_{ct} represents the SOS induction factor in co-treatments, I_{nt} is the SOS induction factor to non-treated cell (negative control) and I_{UV} the induction factor in irradiated cells (positive control). Negative values of the %GI were considered as zero, so this indicator was defined for values between zero and 100%. The minimal concentration that produce significant ($P \leq 0.05$) genotoxicity inhibition (CGI) in PQ37 cells was used for comparison of genoprotective potential of tested samples.

Gas chromatography with flame ionization detection (GC-FID). The identification of EO water-dissolved constituents in our experimental conditions was performed using GC-FID by comparison with chromatographic peaks of the certified compounds (*p*-cymene, carvacrol, citral (geranial + neral), geraniol and thymol). The EO constituents were recovered from each aqueous matrix using liquid–liquid extraction. To accomplish that, each aqueous matrix (1 mL) was mixed with equal dichloromethane volume and the organic fraction was recovered. The procedure was repeated three more times to recover as many compounds as possible. Later, the volume was reduced to 1 mL with nitrogen flow. The estimates of constituent concentrations (μg mL^{-1}) in each aqueous matrix were determined using an external calibration method. Different standard compound concentrations were prepared in dichloromethane and analyzed by GC-FID. Using that information, calibration curves (area *vs* concentration) of each compound were built. The concentration of the EO constituents of interest was estimated by interpolation.

For GC-FID analysis, we used a 6890N gas chromatograph (Agilent Technologies Inc.). A DB-5MS (J&W Scientific, Folsom, CA) 60 m × 0.25 mm capillary column, coated with 5% phenyl poly(methylsiloxane) (0.25 μm film thickness), was used for quantification and linear retention indices determination. Injector and detector temperatures were 250 and 280°C, respectively. Oven temperature was programmed as follows: 45–150°C at 4°C min^{-1}, then at 5°C min^{-1} to 250°C (5 min), then at 10°C min^{-1} to 275°C. Helium (99.99%, gas AP, Linde, Bogotá, Colombia) at a volumetric flow rate of 1 mL min^{-1} was used as carrier gas.

Oxygen radical antioxidant capacity (ORAC) assay. The hydrophilic ORAC assay was performed as previously described (36,37) 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 was added. Fluorescence was measured in a Modulus™ II Microplate Multimode Reader (Turner Biosystems Inc., Sunnyvale, CA) 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 measuring. All measurements were made in triplicate, and results were expressed as average ± standard deviation. The ORAC values were calculated by plotting 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 micromol of Trolox® per gram (g) of triplicated samples.

Synergist or additive effect in compound mixtures. To determine, whether the genoprotective effect in compound mixtures was produced by synergistic or additive effect, the Bliss model (38) was used. This model used the probability theory of complete additive effect (39), where the observed (Y_o) and expected (Y_E) responses of mixtures were compared. Y_E assumes noncomponent interactions in the mixture and was calculated as follows: $Y_{E\ ab} = Y_a + Y_b - Y_aY_b$; where Y, in this case, is the photoprotective effect expressed as %IG, while **a** and **b** are the mixture components at a given dose. Component interaction in the mixture is declared synergistic, if $Y_o > Y_E$; antagonistic, if $Y_o < Y_E$; and independent, if $Y_o \approx Y_E$.

Statistical analysis. The *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* treatments were compared with controls using the Tukey test. Product–moment (Pearson) correlation analysis was used to examine dose–response relationship in genotoxic and antigenotoxic studies. For all statistical analyses, $P \leq 0.05$ was considered significant. The R program (40) was used for all analyses.

RESULTS

Induction kinetics of the *sulA* gene by UV

Table 2 shows the induction kinetics of the *sulA* gene in *E. coli* PQ37 following treatment with UVC. The extent and kinetics of *sulA* gene induction by UV varied with radiation dose. A significant induction of *sulA* gene was observed from doses of 3.4 J m^{-2}. The SOS induction level increased up to a dose of 13.5 J m^{-2} ($I = 11.7 \pm 2.5$) and then, this remains constant until 23.7 J m^{-2}. However, this value increased abruptly to 15.7 ± 5.2 at dose of 27.0 J m^{-2}. At this dose was detected a significant inhibitory effect on constitutive AP activity.

Table 2. Induction kinetics of the *sulA* gene in *Escherichia coli* PQ37 following treatment with UV. For each treatment, the constitutive alkaline phosphatase activity (CAPA) and the *I* values with their corresponding standard errors were calculated from a minimum of four independent experiments with four replicates each.

	SOS parameters	
UV doses (J m^{-2})	CAPA (%)	*I* values
0.0	100 ± 7	1.0 ± 0.0
1.4	131 ± 30 n.s	2.5 ± 0.6 n.s
3.4	124 ± 17 n.s	2.5 ± 0.3 *
6.8	118 ± 29 n.s	6.7 ± 1.0 *
10.1	119 ± 39 n.s	7.8 ± 2.1 *
13.5	114 ± 39 n.s	11.7 ± 2.5 *
16.9	118 ± 35 n.s	9.2 ± 1.7 *
20.3	108 ± 26 n.s	11.6 ± 2.0 *
23.7	121 ± 35 n.s	10.4 ± 1.1 *
27.0	86 ± 36 **	15.7 ± 5.2 *

n.s., no significant increase or reduction was found. *Significant increase ($P \leq 0.05$) respect to zero was found using Tukey test. **Significant reduction ($P \leq 0.05$) respect to zero was found using Tukey test.

EO cytotoxicity, genotoxicity and antigenotoxicity

The Table 1 shows cytotoxicity estimates for each EO during antigenotoxicity evaluation. Based on CAPSI values, the EO with cytotoxic effects were the following: *L. graveolens* COL 555839 (0.2%) > *L. citriodora* COL 555845 (0.8%) > *L. alba* COL 480750 (1.7%) = *L. alba* COL 560257 (1.7%) = *L. dulcis* COL 512079 (1.7%) = *L. origanoides* COL 519798 (1.7%). The results suggest that these plants possess antimicrobial activity against *E. coli* cells.

The genotoxicity of the *Lippia* EO was assayed before their antigenotoxic effect was investigated. At any tested doses, the EO increased the *I* values in PQ37 *E. coli* strain; therefore, this was considered as not genotoxic in *E. coli* cells (data not shown).

Based on the minimal concentration that produce significant ($P \leq 0.05$) genotoxicity inhibition (CGI), the EO antigenotoxic potential from the highest to the lowest was as follows: *L. graveolens* COL 555839- thymol-RC (0.4%) ≈ *L. origanoides* COL 512075- carvacrol-RC (0.4%) ≈ *L. origanoides* COL 519799- thymol-RC (0.4%) > *L. alba* COL 512272- citral-RC (0.8%) ≈ *L. citriodora* COL 555845- citral-RC (0.8%) ≈ *L. micromera* COL 516924- thymol-RC (0.8%) > *L. alba* COL 560257- myrcenone-RC (1.7%). EO from *L. alba* COL 480750- carvone/limonene-RC, *L. origanoides* COL 519798- *p*-cymene/α-phellandrene-RC, and *L. dulcis* COL 512079 β-caryophyllene-RC did not reduce the UV genotoxicity at any of the doses tested. The EO from *L. alba* COL 560257, *L. citriodora* COL 555845, and *L. graveolens* COL 555839 shown CAPSI ≤ CGI values suggesting that their antigenotoxicity was possibly associated to cytotoxicity.

EO from *L. alba* COL 512272- citral-RC, *L. micromera* COL 516924- thymol-RC, *L. origanoides* COL 512075- carvacrol-RC and *L. origanoides* COL 519799- thymol-RC showed reduction of UV genotoxicity at not cytotoxic concentrations lower than 1%, indicating their potential as source of natural compounds that protect the DNA against damage induced by UV. These EO showed a significant reduction of the UV genotoxicity from concentrations of 0.4 and 0.8% (Fig. 1). In general, high concentrations significantly inhibited genotoxicity in co-treated cells (EO + UV) relative to the positive control; low concentrations showed no inhibitory effect.

Determination of water-soluble EO constituents by GC-FID

Stashenko *et al.* (30) established that compounds present in the *Lippia* EO were mostly monoterpenes, oxygenated monoterpenes and sesquiterpenes. As terpenes have limited solubility in water (41), and we did not know which EO constituents were dissolved under our experimental conditions, the soluble constituents were recovered from each aqueous matrix using liquid–liquid extraction. Then, they were identified by comparison with those chromatographic peaks of the standard compounds analyzed by GC-FID.

In general, the major EO constituents previously identified by GC-MS (30) were also detected in the aqueous matrix analyzed by GC-FID (Fig. 2). This means that, at least partially, they were water-soluble and therefore can be related with UV antigenotoxicity detected for EO. The major constituents for each *Lippia* species were calculated as follows: (1) *L. alba* COL 512272: limonene (49.8 μg mL^{-1}), neral (451.4 μg mL^{-1}), geranial (729.8 μg mL^{-1}) and geraniol (233.7 μg mL^{-1}); (2) *L. citriodora* COL 555845: limonene (188.1 μg mL^{-1}), neral (792.4 μg mL^{-1}), geranial (1053.9 μg mL^{-1}) and geraniol (211.0 μg mL^{-1}); (3) *L. graveolens* COL 555839: thymol (78.6 μg mL^{-1}); (4) *L. micromera* COL 516924: thymol (83.4 μg mL^{-1}); (5) *L. origanoides* COL 512075: carvacrol (122.4 μg mL^{-1}) and thymol (20.5 μg mL^{-1}) and; (6) *L. origanoides* COL 519799: thymol (114.4 μg mL^{-1}). Other EO constituents as *p*-cymene and 1,8-cineole were also detected by GC-FID, but at lower quantities than those detected in calibration curves of each compound; therefore, their concentrations were not calculated. These

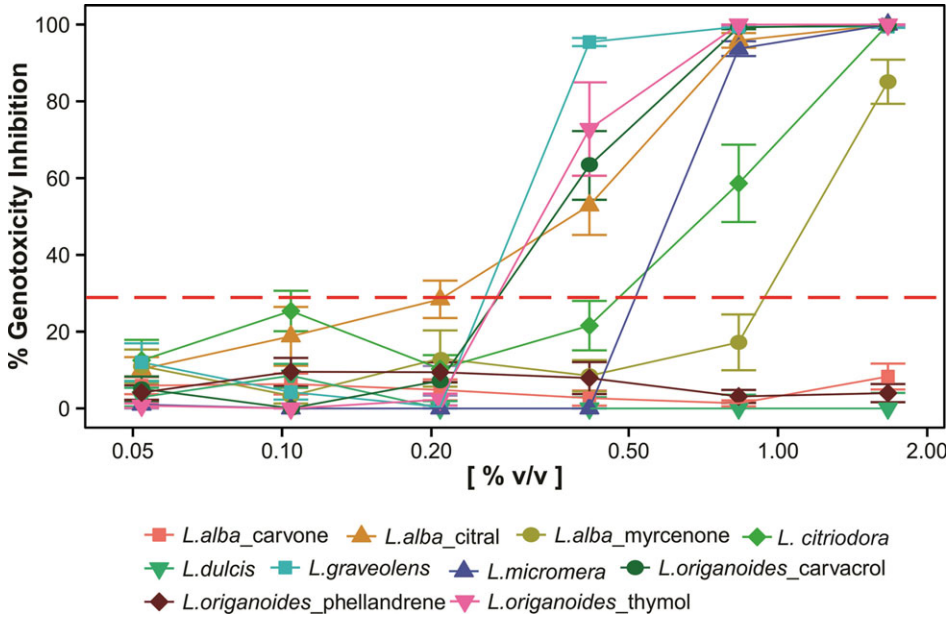

Figure 1. Antigenotoxic effect of *Lippia* EO against UV expressed as % GI. Above dotted line indicates significant inhibition of UV genotoxicity. Error bars indicate the standard error of the mean computed from four independent experiments ($n = 4$).

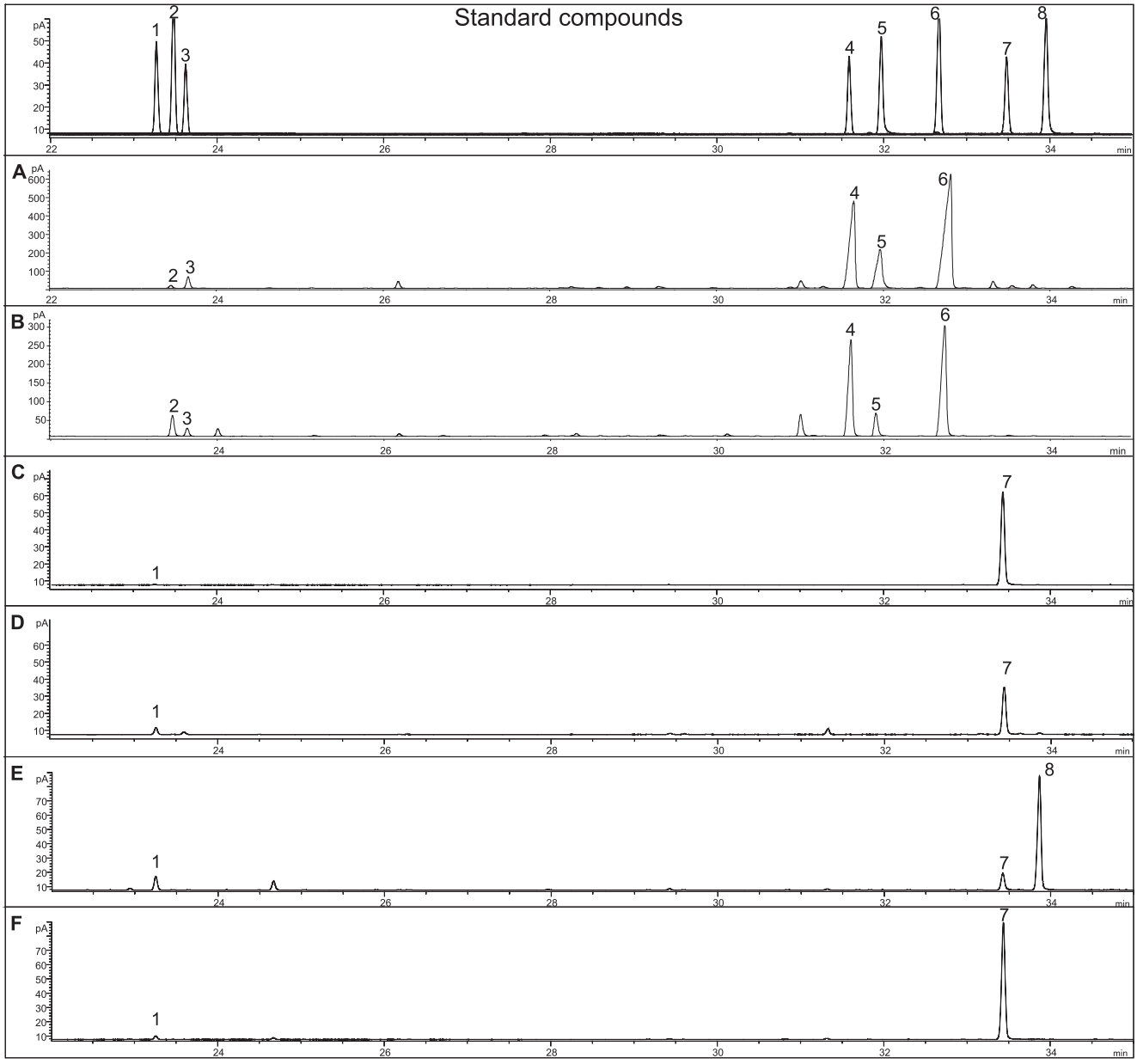

Figure 2. GC-FID profiles of EO soluble matrix constituents are as follows: (A) *Lippia alba* COL 512272, citral-RC, (B) *Lippia citriodora* COL 555845, (C) *Lippia graveolens* COL 555839, (D) *Lippia micromera* COL 516924, (E) *Lippia origanoides* COL 512075, carvacrol-RC and (F) *Lippia origanoides* COL 519799, thymol-RC. Standard compounds included for comparison are as follows: (1) *ρ*-cymene, (2) limonene, (3) 1,8-cineole, (4) neral, (5) geraniol, (6) geranial, (7) thymol and (8) carvacrol.

results indicate that the solubility of terpenes varied significantly from one compound to another and that this issue is critical to studying EO antigenotoxicity using SOS Chromotest.

EO major constituents antigenotoxicity

To determine constituents potentially responsible for EO genoprotective effects, we evaluated antigenotoxicity of the EO major constituents: *p*-cymene, carvacrol, citral, geraniol, limonene, and thymol. The antigenotoxic potency of these constituents was also compared with standard genoprotective compounds, such as EGCG, resveratrol, Trolox® and α-tocopherol. No one compound, except for limonene, resulted genotoxic in the SOS Chromotest. Limonene showed a significant increase in *I* values at a concentration range of 98.9–197.7 μg mL^{-1}. A positive correlation (R^2 = 0.975) between compound concentration and *I* was also found (data not shown); therefore, limonene was excluded from antigenotoxicity studies.

EO's compounds produced a significant decrease in the UV-induced DNA damage. Based on CGI of each constituent and standard compounds (Table 3), the relative genoprotective potential is described as follows: EGCG (49.4 μg mL^{-1}) > *p*-cymene (89.0 μg mL^{-1}) > geraniol (103.9 μg mL^{-1}) > carvacrol (140.1 μg mL^{-1}) > resveratrol (146.5 μg mL^{-1}) > thymol (202.0 μg mL^{-1}) > citral (267.3 μg mL^{-1}) > Trolox® (391.4 μg mL^{-1}) > 1,8-cineole (735.0 μg mL^{-1}) > α-tocopherol (2422.7 μg mL^{-1}). The constituents *p*-cymene, geraniol and carvacrol showed antigenotoxic potencies between EGCG and

Table 3. Genoprotective and antioxidant potentials of essential oil constituents and standard compounds measured with SOS Chromotest and with the oxygen radical antioxidant capacity (ORAC) method, respectively. The minimum concentration that significantly inhibits genotoxicity (CIG) and the ORAC values from a minimum of four independent experiments and the corresponding standard errors are shown.

Cell treatments	CIG values (μg mL^{-1})	ORAC values (μmol Trolox®/g substance)
EGCG	49.4	13200 ± 531
p-Cymene	89.0	219 ± 2
Geraniol	103.9	12300 ± 119
Carvacrol	140.1	3410 ± 50
Resveratrol	146.5	35300 ± 150
Thymol	202.0	2900 ± 103
Citral	267.3	990 ± 27
Trolox®	391.4	–
1,8-Cineole	735.0	299 ± 5
α-Tocopherol	2422.7	550 ± 13

resveratrol, two of the most effective photoprotective substances known.

The EO constituents showed a significant reduction of the UV genotoxicity from concentrations of 89 and 735 μg mL^{-1}. In general, high concentrations significantly inhibited genotoxicity in co-treated cells (terpenes + UV) relative to the positive control; low concentrations showed no inhibitory effect. The antigenotoxic properties of EO constituents studied are shown in Fig. 3.

EO major constituent antioxidant activity

In Table 3, the antioxidant activity of the EO major constituents was also shown. Based on ORAC values of each constituent and standard compounds, the relative antioxidant potential was as follows: resveratrol > EGCG > geraniol > carvacrol > thymol > citral > α-tocopherol > 1,8-cineole > *p*-cymene. Correlation analysis using CIG and ORAC data from these compounds showed no significant correlation coefficient ($R = -0.26$, n.s.), suggesting that the mechanisms by which these compounds exert their antigenotoxic and antioxidant activities are possibly different and unconnected.

Antigenotoxicity of mixtures of EO major constituents

As indicated above, the compound *p*-cymene found in several EO (Table 1), but detected at very low concentrations in aqueous matrices (Fig. 2), resulted with high genoprotective potency (Fig. 3). This suggests that EO antigenotoxic effects are possibly due to additive or synergistic effects between their constituents. To elucidate this hypothesis, we evaluated additive or synergistic effects among constituents such as carvacrol, *p*-cymene and thymol.

As indicated above, the CIG values for carvacrol, *p*-cymene and thymol were 140.1, 89.0 and 202.0 μg mL^{-1}, respectively. Here, we studied eighteen (CM1 to CM18) primary interactions among these terpenes, by combining different concentrations that could be equal or lower than CIG values of compounds (Table 3). Among them, seven combinations resulted with significant %GI values and four showed a clear synergist effect (Table 4). The %GI values for these four compound mixtures (CM2, CM3, CM8 and CM14) were higher than the corresponding values for pure compounds at concentrations used in the mixtures. Thus, CM2 (thymol 101.0 μg mL^{-1}/carvacrol 70.0 μg mL^{-1}) and CM14 (carvacrol 70.0 μg mL^{-1}/*p*-cymene 11.1 μg mL^{-1}) resulted the most genoprotective mixtures. According to the Bliss model, the Y_E value for CM2 and CM14 were calculated in 6.0 and 6.2%, while Y_o values for these CM were 100 and 99.0%, respectively. These results suggest that photoprotective effects in the mixtures were caused by synergistic component interactions.

DISCUSSION

This work studied the genoprotective properties of EO from Colombian *Lippia* species obtained by microwave-assisted hydrodistillation. We showed that EO from *L. alba*,

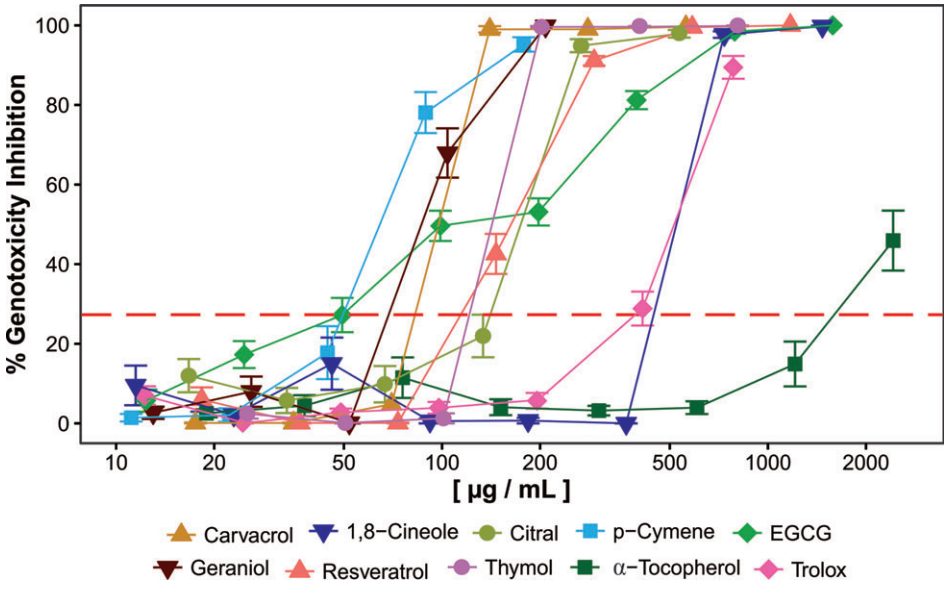

Figure 3. Antigenotoxic effect of *Lippia* EO major constituents against UV expressed as % GI. Above dotted line indicates significant inhibition of UV genotoxicity. Error bars indicate the standard error of the mean computed from four independent experiments ($n = 4$).

Table 4. Antigenotoxicity of the compound mixtures (CM) expressed as percentage of genotoxicity inhibition (% GI). The % GI mean values and the corresponding standard errors computed from four independent experiments ($n = 4$) are given.

No.	Thymol ($\mu g\ mL^{-1}$)	Carvacrol ($\mu g\ mL^{-1}$)	% GI values	No.	Thymol ($\mu g\ mL^{-1}$)	*p*-Cymene ($\mu g\ mL^{-1}$)	% GI values	No.	Carvacrol ($\mu g\ mL^{-1}$)	*p*-Cymene ($\mu g\ mL^{-1}$)	% GI values
	202.0	–	100 ± 0 *		202.0	–	100 ± 0 *		140.1	–	99 ± 0 *
	–	140.1	99 ± 0 *		–	22.3	1 ± 1 n.s		–	22.3	1 ± 1 n.s
CM1	202.0	140.1	100 ± 0 *	CM7	202.0	22.3	94 ± 1 *	CM13	140.1	22.3	99 ± 0 *
	101.0	–	1 ± 1 n.s		101.0	–	1 ± 1 n.s		70.0	–	0 ± 0 n.s
	–	70.0	0 ± 0 n.s		–	11.1	5 ± 3 n.s		–	11.1	5 ± 3 n.s
CM2	**101.0**	**70.0**	**100 ± 0 ***	**CM8**	**101.0**	**11.1**	**26 ± 6 ***	**CM14**	**70.0**	**11.1**	**99 ± 0 ***
	50.5	–	0 ± 0 n.s		50.5	–	0 ± 0 n.s		35.0	–	0 ± 0 n.s
	–	35.0	0 ± 0 n.s		–	5.6	0 ± 0 n.s		–	5.6	0 ± 0 n.s
CM3	**50.5**	**35.0**	**36 ± 14 ***	CM9	50.5	5.6	0 ± 0 n.s	CM15	35.0	5.6	0 ± 0 n.s
	25.2	–	0 ± 0 n.s		25.2	–	0 ± 0 n.s		17.5	–	0 ± 0 n.s
	–	17.5	0 ± 0 n.s		–	2.8	0 ± 0 n.s		–	2.8	0 ± 0 n.s
CM4	25.2	17.5	0 ± 0 n.s	CM10	25.2	2.8	0 ± 0 n.s	CM16	17.5	2.8	0 ± 0 n.s
	12.6	–	0 ± 0 n.s		12.6	–	0 ± 0 n.s		8.8	–	0 ± 0 n.s
	–	8.8	0 ± 0 n.s		–	1.4	0 ± 0 n.s		–	1.4	0 ± 0 n.s
CM5	12.6	8.8	0 ± 0 n.s	CM11	12.6	1.4	0 ± 0 n.s	CM17	8.8	1.4	0 ± 0 n.s
	6.3	–	0 ± 0 n.s		6.3	–	0 ± 0 n.s		4.4	–	0 ± 0 n.s
	–	4.4	0 ± 0 n.s		–	0.70	0 ± 0 n.s		–	0.70	0 ± 0 n.s
CM6	6.3	4.4	0 ± 0 n.s	CM12	6.3	0.70	0 ± 0 n.s	CM18	4.4	0.70	0 ± 0 n.s

Compound combination, which showed synergist effect appear in bold letters. n.s., no significant difference was found. *, significant increase ($P < 0.05$) of %IG values respect to positive control was found using Tukey test.

L. micromera and *L. origanoides* were promissory as sources of genoprotective compounds useful as sunscreen supplements for photoprotection and skin cancer prevention. These EO inhibited the UV-induced genotoxicity in the SOS Chromotest, effect that was directly related to their major constituents. Thus, carvacrol, citral, *p*-cymene, geraniol and thymol are potential antigenotoxic compounds against UV.

Previous studies have proved antigenotoxic properties of *Lippia* species' EO and their major constituents. For example, the *L. graveolens* EO has antimutagenic activity against 4-nitro-o-phenylenediamine (4-NPD) and sodium azide (42). *L. origanoides* and *L. alba* EO, and their major constituents (carvacrol, carvone, citral and thymol), have antigenotoxic activity against the clastogen bleomycin (25,26). Carvacrol and thymol as pure compounds also reduce the DNA strand breaks induced in human lymphocytes by 2-amino-3-methylimidazo[4,5-f]-quinoline, mitomycin-C (MMC) and H_2O_2 (43,44), as well as they protect DNA toward strand breaks induced by H_2O_2 in leukemic (K562), hepatoma (K562) and colonic (Caco-2) human cell lines (45,46). In addition, carvacrol inhibited the mutagenicity of urethane, 4-NPD and 2-aminofluorene in *Salmonella*/microsome assay (47,48), inhibited the induction of sister chromatid exchange formation by MMC in human lymphocytes (47) and has anticarcinogenic activity in human lung cancer cell line and mouse myoblast cells (49,50), while thymol showed radioprotection against gamma radiation (51).

Other terpenes such as citral, geraniol and *p*-cymene also have chemopreventive properties. Citral resulted antigenotoxic against bleomycin (26) and showed anticlastogenic activity against cyclophosphamide, MMC and nickel metal in mice (52,53). Geraniol showed anticancer activity in human colon (54) and pancreatic (55) cancer cells, and murine leukemia and melanoma cell lines (56), as well as antitumor activity in murine and rat hepatomas and melanomas (57). On the other hand, *p*-cymene showed antigenotoxic effect against cyclophosphamide−induced genotoxicity (58). All the works indicated above highlight the potential benefit of *Lippia* EO as dietary supplements with chemopreventive properties.

To know whether major constituents of EO exert their antigenotoxic activity by means of an antioxidant mechanism (43), we also studied their antioxidant activity. Overall, the antigenotoxic and antioxidant activities of these compounds were not related, suggesting that an antioxidant mechanism was not involved in their antigenotoxicity. We have previously shown that *E. coli* strain defective for OxyR regulon exposure to UV was as sensitive as wild-type phenotype (59). This supports previous findings that indicate cyclobutane pyrimidine dimers (CPD) and 6-4-pyrimidine pyrimidone photoproducts (6-4PP), instead of oxidative DNA damage, as mainly responsible for the genotoxic effects caused by all types of UV radiation (60). Some phenolic monoterpenes as carvacrol and thymol can bind to the major and minor groove of DNA (61), which could interfere with photoproduct formation. In addition, the *Salvia officinalis* EO constituents, camphor, cineole and thujone act as bioantimutagens against the UV modulating DNA repair process (62); however, additional studies are necessary to clarify terpene photoprotective mechanisms.

We have hypothesized and supported that *Lippia* EO antigenotoxic effects are caused by synergistic constituent interactions. Synergism is a common event in EO and extracts constituents, which can have practical significance (63,64). Here, we showed that CM produced DNA protection against UV at compound concentrations lower than with pure compounds. This is a practical advantage as terpenes at high concentrations can be cytotoxic (43,44,65). An expert panel to Cosmetic Ingredient Review has indicated that the use of carvacrol and thymol in cosmetics is safe at concentrations below 0.5% (66). However, the synergistic effect detected with these CM corresponds only to primary interactions between constituents. It will be necessary to study secondary and tertiary interactions. Since the Bliss model does not account for response variability (39), the hypothesis of synergism for the constituents of *Lippia* EO could be demonstrated with major details in further studies.

This work supports the use of the SOS Chromotest for screening and studying of antigenotoxic compounds that could be used as supplement in sunscreen formulation. The SOS Chromotest

exploits the inducible SOS DNA repair and damage tolerance system present in *E. coli*, which is activated in response to UV (28). The PQ37 strain used the expression of β-galactosidase to assay the functional transcription of the *sulA* gene, whose product is responsible for cellular division blocked in *E. coli*. Since photoproducts are an obstacle for *E. coli* chromosome replication, a postirradiation reduction of *sulA* gene expression in the presence of genoprotective compounds can be considered indicative of DNA photoproduct repair and cellular division recovery (59). Therefore, we assume that the antigenotoxic effect detected for *Lippia* EO against UV occurs by reducing photoproducts formation or stimulating their repair on the DNA molecule.

Despite the obvious genetic differences between bacteria and human cells, there are at least two theoretical reasons that support the use of SOS Chromotest to identify antigenotoxic compounds with potential photoprotective activity in humans. First, a DNA damage-inducible system like bacterial SOS response that involves photoproduct (i.e. CPD, 6-4PP) DNA repair, cell cycle arrest and increased antioxidant defenses and melanogenesis has been described in human skin cells (67). Therefore, predictive capability to photoprotection in human skin cells could be expected with SOS Chromotest because this assay is highly sensitive to UV (28). Second, the suppression of SOS repair in *E. coli* comprises a possible mechanism of antimutagenicity and protective effects of common vegetables (68). In addition to this, the SOS Chromotest is a validated genotoxicity assay (69) that compared to other methods allows greater throughput screening (70).

Based on literature data (71,72), *Lippia* EO and their major constituents have very broad-ranging therapeutic potential. The antigenotoxic properties against UV (present work) widen their potential as source of compounds with possible application in photoprotection and skin cancer chemoprevention. Our results demonstrate that *Lippia* EO are sources of genoprotective compounds against UV-induced DNA damage. This antigenotoxic effect is closely linked to EO major constituents and influenced by the solubility of these compounds. Based on antigenotoxicity data of compound mixtures assays, we suggested that EO antigenotoxic effects are possibly due to synergistic interactions between their major constituents. Harmonized studies on genotoxicity of these compounds using a battery of *in vivo* assays that evaluates different levels of DNA damage expression will be required for practical use of these compounds in photoprotection.

CONCLUDING REMARKS

In conclusion, the SOS Chromotest provided useful information about cytotoxic and antigenotoxic effects of plant EO. The EO from *L. alba* COL 512272*, L. micromera* COL 516924 and *L. origanoides* COL 512075 and COL 519799 have antigenotoxic effect against UV. This effect was linked to EO major constituents such as citral, carvacrol, ρ-cymene, geraniol and thymol, which were somewhat soluble in water fraction of EO. The antigenotoxicity of these EO constituents was unconnected with their antioxidant activity. Inside the EO, these terpenes act synergistically for its antigenotoxicity, which suggest a combined more than a single mode of action for these EO constituents. The work provides useful information to design sunscreens based on EO. However, the role of antigenotoxic compounds in the etiology of skin cancer is very complex and involves several action modes (9,10), and our results concern only *in vitro* experiments with a bacterial assay; therefore, additional studies with skin human cell and animal models should be addressed to clarify the protection mode of action of *Lippia* EO constituents and support their use in humans.

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