Genetics and Molecular Biology, 34, 3, 479-488 (2011)

www.sbg.org.br

Research Article

Chemical composition and antigenotoxic properties of *Lippia alba* essential oils

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Abstract

The present work evaluated the chemical composition and the DNA protective effect of the essential oils (EOs) from *Lippia alba* against bleomycin-induced genotoxicity. EO constituents were determined by Gas Chromatography/Mass Spectrometric (GC-MS) analysis. The major compounds encountered being citral (33% geranial and 25% neral), geraniol (7%) and *trans*-β-caryophyllene (7%) for *L. alba* specimen COL512077, and carvone (38%), limonene (33%) and bicyclosesquiphellandrene (8%) for the other, COL512078. The genotoxicity and antigenotoxicity of EO and the compounds citral, carvone and limonene, were assayed using the SOS Chromotest in *Escherichia coli*. The EOs were not genotoxic in the SOS chromotest, but one of the major compound (limonene) showed genotoxicity at doses between 97 and 1549 mM. Both EO**s** protected bacterial cells against bleomycin-induced genotoxicity. Antigenotoxicity in the two *L. alba* chemotypes was related to the major compounds, citral and carvone, respectively. The results were discussed in relation to the chemopreventive potential of *L. alba* EOs and its major compounds.

Key words: Lippia alba, essential oil, antigenotoxicity, bleomycin, SOS chromotest.

Received: September 30, 2010; Accepted: March 23, 2011.

Introduction

Lippia alba (Mill.) N.E. Brown (Verbenaceae), an aromatic shrub reaching 1.7 m high, is distributed throughout the Caribbean, South and Central America and Tropical Africa. The species is mainly used in folk medicine against digestive and respiratory ailments, but also as a sedative, analgesic, anti-inflammatory, antipyretic and antihypertensive remedy (Pascual *et al.*, 2001a; Hennebelle *et al.*, 2008a). In Colombia it is popularly known as "Orégano de cerro" (Hill oregano), "Pronto alivio" (ready-relief) and "Curatodo" (all-round cure) depending on the region (Stashenko *et al.*, 2003).

The species *L. alba* is characterized by variability in the chemical composition of the essential oils, depending on the origin of plant material, as well as the stage of the plant and the part selected for distillation of the oil (Zoghbi *et al.*, 1998). Various chemotypes have been proposed (Hennebelle *et al.*, 2006; Oliveira *et al.*, 2006). Based on both the composition and the possible common biosynthetic pathways among the different oils, the existence of at least seven has been indicated (Hennebelle *et al.*, 2008a). These are: chemotype I (citral, linalool and β-caryophyllene, as the main constituents), chemotype II (tagetenone), chemotype III (limonene and carvone or related monoterpenic ketones), chemotype IV (myrcene), chemotype V (γ-terpinene), chemotype VI (camphor-1,8-cineole) and chemotype VII (estragole). In Colombia, *L. alba* chemotypes I and III, and a combined (I/III) form, not previously reported, have been found.

Various studies on *L. alba* bioactivities bolster their use in traditional medicine. The essential oils (EOs), either extracts or their constituents, have revealed antiviral, antibacterial, antifungal and antiparasitic activities (Pino-Alea *et al.*, 1996; Abad *et al.*, 1997; Andrighetti-Fröhner *et al.*, 2005; Teixeira-Duarte *et al.*, 2005; de Carvalho and da Fonseca, 2006; Sena-Filho *et al.*, 2006; Paduch *et al.*, 2007; Ara *et al.*, 2009; Arruda *et al.*, 2009; Mesa-Arango *et al.*, 2009; Shukla *et al.*, 2009), thus sustaining their use in the treatment of diseases of microbial origin. Currently, several major compounds from *L. alba* EOs are used to control food pathogens (Burt, 2004; Rojas-Graü *et al.*, 2007; Teixeira-Duarte *et al.*, 2007; du Plooy *et al.*, 2009; Linde *et al.*, 2009). Moreover, analgesic, anti-inflammatory and sedative effects in mammalian models have been related to cer-

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tain EO major constituents, such as citral, myrcene and limonene (Viana *et al.*, 1998, 2000; Vale *et al.*, 1999). Sedative effects, attributed to non-volatile flavonoids and iridoids (Zétola *et al.*, 2002, Hennebelle *et al.*, 2008b), were also encountered with *L. alba* ethanol extracts. Furthermore, aqueous extracts also reduced cardiac rate and gastric ulceration induced by indomethacin in rats (Pascual *et al.*, 2001b; Gazola *et al.*, 2004).

In a previous work (Vicuña *et al.*, 2010), the importance of EOs as sources of antitumor, anti-carcinogenic and chemopreventive agents, was emphasized. Although the chemopreventive properties of *L. alba* terpenoids, such as carvone, geraniol, limonene, and perillyl alcohol have been well-documented (He *et al.*, 1997; Crowell, 1999; Uedo *et al.*, 1999; de Carvalho and da Fonseca, 2006; Paduch *et al.*, 2007; Patil *et al.*, 2009; Rabi and Bishayee, 2009), little is known about the chemopreventive potential of other *L. alba* EO constituents, for example, citral (Connor, 1991; Nakamura *et al.*, 2003; Seo *et al.*, 2008).

After determining the EOs composition of the two *L. alba* specimens by GC-MS analysis, their specific antigenotoxic activity against the clastogenic mutagen, bleomycin, was evaluated by using the SOS Chromotest (Quillardet *et al.*, 1982). The antigenotoxic properties of the major EO constituents (citral, carvone and limonene) were also studied and their activity compared with the antigenotoxic standard compound Trolox. Our work provides new insights into chemoprevention by *L. alba* EO major compounds.

Materials and Methods

Chemicals

Sodium sulfate and dichloromethane were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). High purity gases (helium, nitrogen, hydrogen and air) for chromatography were obtained from AGA-Fano S.A. (Bucaramanga, Colombia). Different standard compounds (*n*-tetradecane, *n*-alkanes (C_8-C_{25}), citral (40:60 neral:geranial), S(+)-carvone and S(–)-limonene), Luria-Bertani (LB) media, and antibiotics (ampicillin, bleomycin and tetracycline) were obtained from Sigma-Aldrich Co. Inc. (Milwaukee, WI, USA). The standard antioxidant compound 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchased from Fluka (Steinheim, Germany). The substrates for β-galactosidase (ortho-nitrophenyl-β-d-galactopyranoside) and alkaline phosphatase (*p*-nitrophenylphosphate) were purchased from Merck (Darmstadt, Germany).

Plant material

L. alba plants were collected from the experimental gardens at CENIVAM Agroindustrial Pilot Complex, located at the Universidad Industrial de Santander campus (Bucaramanga, Colombia). Plant growing conditions were as indicated by Stashenko *et al.*, (2008). Taxonomic identification was undertaken by Dr. José Luis Fernández Alonso (National University, Bogotá, Colombia). The two *L. alba* specimens (COL512077 and COL512078) were stored at the Colombian National Herbarium.

EO extraction and chromatographic analysis

Fresh leaves and flowers from *L. alba* plants were used for EO extraction using the microwave-assisted hydrodistillation method, as described by Stashenko *et al.*, (2004). Briefly, a Clevenger-type hydro-distillation apparatus was placed inside a domestic microwave oven (LG, 1100 W, 2.45 GHz) with a side orifice, through which an external glass condenser linked the 2 l-round flask with the plant material (ca. 300 g) and water (ca. 0.5 l) inside the oven. The oven was operated for 40 min (4 x 10 min) at full power, which caused water to boil vigorously and reflux. Essential oil was decanted from the condensate, and then dried with anhydrous sodium sulfate. For chromatographic analysis, neat essential oil (50 μL) and *n*-tetradecane (0.5 μL) were dissolved in 1 mL of dichloromethane (Chromatography-grade reagent, Merck, Darmstadt, Germany). EO compound identification was based on chromatographic/spectroscopic analysis, as previously indicated by Vicuña *et al.*, (2010).

Bacterial strains and culture

The *Escherichia coli* PQ37 strain, as proposed by Quillardet *et al.* (1982) for detecting genotoxic carcinogens, was used. The cells, grown overnight at 37 °C, were stired at 100 rpm in Luria-Bertani (LB) medium (10 g tryptone/L, 5 g yeast extract/L, 10 g sodium chloride/L, pH 7.4), supplemented with 50 μg/mL ampicillin and 17 μg/mL tetracycline.

Genotoxicity assay

The SOS Chromotest, as indicated by Quillardet *et al.*, (1982), was used for genotoxicity assaying. Briefly, overnight-cultures were grown in fresh LB medium (indicated above) until reaching an optical density of OD_{600nm} = 0.4. They were then diluted 10-fold in double-strength LB medium, and mixed (v/v) with a specific substance for identification (EO, citral, carvone and limonene). Pure EOs (density of 900 mg/mL determined with a BRAND picnometer, Wertheim, Germany) were diluted in distilled water by vigorously stirring to a concentration ranging between 1.7 and 450.0 mg/mL, this including the antioxidant dose as previously indicated (Stashenko *et al.*, 2004). Negative (distilled water) and positive (1 μg/mL of bleomycin) controls were always included in each assay. Cells were exposed to substances during 30 min at 8 °C, and then cultured during 2 h at 37 °C. The assays for β-galactosidase and alkaline phosphatase activities were according to Vicuña *et al.* (2010).

The genotoxicity criterion applied was the Induction Factor (IF), which, by representing fold induction of the *sulA* gene in each treatment (EO, mutagen, etc), could be considered as an indirect measure of induced primary DNA damage. The IF was calculated as: IF = (β-galactosidase/alkaline phosphatase)$_t$ / (β-galactosidase/alkaline phosphatase)$_{nt}$, where *t* and *nt* are the treated and non-treated cells, respectively.

Antigenotoxicity assay

Antigenotoxicity was assayed using the co-incubation procedure, as indicated by Fuentes *et al.*, (2006). Although the procedure was basically the same as that of the genotoxicity protocol, the cells were simultaneously co-treated with different concentrations of the tested substances (EO, citral, carvone and limonene) and the mutagen (1 μg/mL of bleomycin). Antigenotoxicity, *i.e.*, the DNA-protective capacity of the tested substance, was measured as a significant reduction in IF in the combined treatments (substance + bleomycin), and expressed as a percentage of genotoxicity inhibition:

$$\%GI = 1 - \frac{IF_{co} - IF_{basal}}{IF_{bleo} - IF_{basal}} \times 100$$

where IF_{co} is the SOS induction factor in co-treated cells (substance + bleomycin), IF_{basal} the basal SOS induction factor, and IF_{bleo} the SOS induction factor in bleomycin-treated cells.

Statistical analysis

The average values of alkaline phosphatase and IF and the corresponding standard errors were calculated. Normality of the data was tested using the Kolmogorov-Smirnov test. Variance homogeneity and analysis of variance (ANOVA) tests were also conducted. Mean values were compared using Student's *t*-test. Product-moment (Pearson) correlation analysis was applied for examining dose-response relationships in genotoxicity studies. In all statistical analyses, $p < 0.05$ was considered significant. The STATISTICA software package (Version 6.0, StatSoft Inc (2003), Tulsa, OK, USA) was used for all analyses.

Results

EO chemical analysis

L. alba EO compounds, as defined by GC-MS analysis, are listed in Table 1. Essential oil chemical composition in the two *L. alba* specimens was different. In specimen COL512077, oxygenated monoterpenes (70.5%) were predominant, followed by sesquiterpenes (13.6%) and monoterpenes (3.5%). In specimen COL512078, there were high percentages of oxygenated monoterpenes (49.4%) and monoterpenes (36.0%), followed by sesquiterpenes (13.6%). The major compounds in specimen COL512077 were citral (geranial 33% and neral 25%), geraniol (7%) and *trans*-β-caryophyllene (7%), whereas specimen COL512078 was characterized by a high proportion of carvone (38%), limonene (33%) and bicyclosesquiphellandrene (8%) (Figure 1). Based on EO densities and the percentage of chromatogram area for major compounds, compound concentrations were thus estimated: neral (231 mg/mL), geranial (302 mg/mL), geraniol (65 mg/mL), *trans*-β-caryophyllene (59 mg/mL), carvone (345 mg/mL), limonene (301 mg/mL) and bicyclosesquiphellandrene (70 mg/mL). The *L. alba* EOs studied here were classified as citral (COL512077) and carvone/limonene (COL512078) chemotypes.

Genotoxic and antigenotoxic effects of *L. alba* EOs

The genotoxicity of *L. alba* EO was assayed before the antigenotoxic effect was investigated. Oils did not increased the IF values in PQ37 *Escherichia coli* strain indicating that they do not induce the SOS response in *E. coli* cells (Table 2). Interestingly, a stimulating effect on protein synthesis, measured as alkaline phosphatase activity, was observed with increased EO concentration in the case of

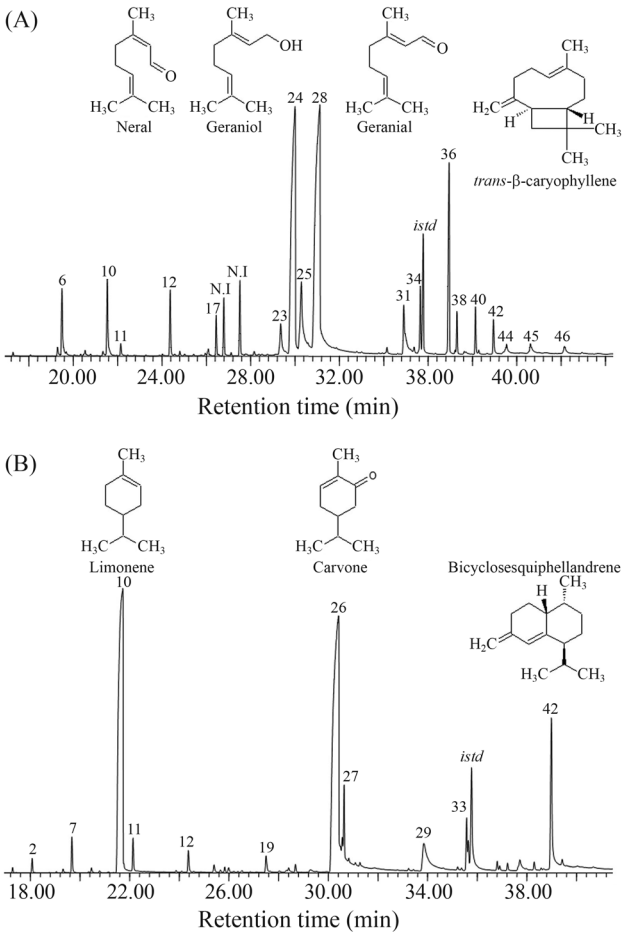

Figure 1 - GC-MS profiles of EO from *L. alba* specimens, COL512077 (A) and COL512078 (B). Major EO constituents were numbered according to elution order on DB-5MS column indicated in Table 1.

Table 1 - Chemical composition of the *Lippia alba* essential oils obtained by microwave-assisted hydrodistillation of each specimen or chemotype.

			Relative amount (%)	
No.	Compounds	I_K	COL512077 (citral)	COL512078 (carvone/limonene)
1	α-pinene	937	0.1	0.1
2	Camphene	954	-	0.4
3	Verbenene	967	-	0.2
4	1-octen-3-ol	981	0.3	-
5	β-pinene-1-octen-3-ol	982	-	0.1
6	6-methyl-5-hepten-2-one	986	2.2	-
7	β-Myrcene	990	0.2	1.0
8	α-phellandrene	1010	0.2	-
9	ρ-cymene	1028	0.2	-
10	Limonene	1035	2.5	**33.2**
11	*trans*-β-ocimene	1048	0.4	1.0
12	Linalool	1101	1.8	0.7
13	*trans-p*-mentha-2,8-diene-1-ol	1127	-	0.3
14	*cis*-limonene oxide	1138	-	0.1
15	Hexenyl *cis*-3-isobutanoate	1140	0.1	-
16	*cis-p*-mentha-2,8-diene-1-ol	1141	-	0.2
17	Citronellal	1153	1.0	-
18	Rosefuran epoxide	1171	0.1	-
19	Borneol	1180	-	0.6
20	*cis*-dihydrocarvone	1203	-	0.3
21	*trans*-dihydrocarvone	1210	-	0.2
22	*trans*-carveol	1227	-	0.3
23	Nerol	1228	2.0	-
24	Neral	1246	**25.4**	-
25	Geraniol	1254	7.1	-
26	Carvone	1257	-	**38.1**
27	Piperitone	1263	-	4.4
28	Geranial	1276	**33.1**	-
29	Piperitenone	1349	-	4.3
30	Neryl acetate	1357	0.2	-
31	Geranyl acetate	1376	2.7	-
32	α-copaene	1384	-	0.1
33	β-bourbonene + β-Elemene	1394	-	2.2
34	β-elemene	1397	1.9	1.2
35	β-ylangene	1429	-	0.3
36	*trans*-β-caryophyllene	1432	**6.6**	0.2
37	β-gurjunene	1441	-	0.3
38	α-guaiene	1444	1.2	-
39	*trans*-β-farnesene	1456	0.2	0.8
40	α-humulene	1469	1.3	-
41	Caryophyllene-9-epi-E	1473	0.1	0.3
42	Bicyclosesquiphellandrene	1493	1.2	**7.7**
43	Bicyclogermacrene	1507	-	0.5
44	α-bulnesene	1511	0.6	-
45	*cis*-α-bisabolene	1547	0.6	-
46	Caryophyllene oxide	1599	0.5	-
	Monoterpene hydrocarbons		3.5	36.0
	Oxygen containing monoterpenes		70.5	49.4
	Sesquiterpene hydrocarbons		13.6	13.6
	Oxygen containing sesquiterpenes		0.5	0.0
	Other not identified		5.4	0.0
	Total		93.5	99.1

No., Order of elution is given in DB-5MScolumn, I_K, Values of retention index (Kovats, 1965) calculated from a minimum of three independent chromatograms.

citral chemotype. Since this did not occur with a water soluble EO fraction (data not shown), apolar compounds in the EO mix are possibly involved.

The antigenotoxic properties of *L. alba* EO are shown in Table 3. As previously indicated (Vicuña *et al.*, 2010), a dose of 1 μg/mL bleomycin was used for antigenotoxicity assaying. EOs produced a significant decrease in bleomycin-induced genotoxicity (IF values) at doses between 28.1 and 450 mg/mL, though insignificant at those lower. Complete inhibition occurred with both citral and carvone/limonene chemotypes at doses higher than 56.2 mg/mL.

Genotoxic and antigenotoxic effects of *L. alba* EO major constituents

Genotoxicity of the major EO constituents (citral, carvone and limonene) was also assayed (Table 4). Citral

Table 2 - Genotoxicity study of the *L. alba* EO measured by the SOS chromotest.

	COL512077 (citral)		COL512078 (carvone/limonene)	
Treatments	AP	IF	AP	IF
Distilled water (negative control)	0.015 ± 0.006	1.0 ± 0.3	0.032 ± 0.008	0.9 ± 0.6
Bleomycin (positive control)	0.011 ± 0.004 n.s	6.2 ± 2.7 *	0.03 ± 0.012 n.s	7.5 ± 0.4 *
EO (0.9 mg/mL)	0.015 ± 0.005 n.s	0.8 ± 0.6 n.s	0.022 ± 0.009 n.s	0.7 ± 0.5 n.s
EO (1.7 mg/mL)	0.05 ± 0.016 *	0.4 ± 0.3 n.s	0.05 ± 0.013 *	0.3 ± 0.2 n.s
EO (3.5 mg/mL)	0.16 ± 0.039 *	0.2 ± 0.1 n.s	0.11 ± 0.043 *	0.4 ± 0.2 n.s
EO (7.0 mg/mL)	0.17 ± 0.052 *	0.3 ± 0.1 n.s	0.22 ± 0.080 *	0.5 ± 0.3 n.s
EO (14.1 mg/mL)	0.14 ± 0.023 *	0.4 ± 0.1 n.s	0.33 ± 0.059 *	0.7 ± 0.3 n.s
EO (28.1 mg/mL)	0.14 ± 0.034 *	0.6 ± 0.2 n.s	0.29 ± 0.055 *	0.7 ± 0.2 n.s
EO (56.2 mg/mL)	0.22 ± 0.052 *	0.3 ± 0.1 n.s	0.25 ± 0.073 *	0.6 ± 0.2 n.s
EO (112.5 mg/mL)	0.27 ± 0.060 *	0.2 ± 0.1 n.s	0.18 ± 0.062 *	0.6 ± 0.3 n.s
EO (225.0 mg/mL)	0.228 ± 0.073 *	0.5 ± 0.3 n.s	0.10 ± 0.043 *	1.0 ± 0.7 n.s
EO (450.0 mg/mL)	0.141 ± 0.030 *	0.5 ± 0.1 n.s	0.07 ± 0.027 *	0.7 ± 0.3 n.s

Bleomycin dosage was 1 μg/mL. Densities of essential oils were estimated in 900 mg/mL using a 9.814 mL BRAND picnometer (Wertheim, Germany).†, Average values for direct absorbance measurement of alkaline phosphatase (AP) activity and SOS Induction Factor (IF), from a minimum of three independent experiments with three replicates each, as well as the corresponding standard error, are given. *, The significant increase ($p < 0.05$) in negative control was found by Student *t*-testing. n.s., no significant differences were found.

Table 3 - Antigenotoxic effect of *L. alba* EO against bleomycin-induced DNA damage in PQ37 *Escherichia coli* cells.

	IF† (%GI)	
Cell treatments	COL512077 (citral)	COL512078 (carvone/limonene)
Distilled water (negative control)	1.0 ± 0.1	1.0 ± 0.0
Bleomycin (positive control)	5.8 ± 1.2	5.4 ± 1.4
EO (450.0 mg/mL)	0.5 ± 0.1	0.7 ± 0.3
EO (450.0 mg/mL) + Bleomycin	0.4 ± 0.1 (100%) *	0.4 ± 0.1 (100%) *
EO (225.0 mg/mL) + Bleomycin	0.5 ± 0.1 (100%) *	0.6 ± 0.2 (100%) *
EO (112.5 mg/mL) + Bleomycin	0.9 ± 0.3 (100%) *	0.5 ± 0.2 (100%) *
EO (56.2 mg/mL) + Bleomycin	0.9 ± 0.5 (100%) *	0.9 ± 0.6 (100%) *
EO (28.1 mg/mL) + Bleomycin	1.9 ± 0.5 (81%) *	2.4 ± 1.6 (68%) *
EO (14.1 mg/mL) + Bleomycin	5.0 ± 1.2 (17%) n.s	5.0 ± 2.3 (9%) n.s
EO (7.0 mg/mL) + Bleomycin	5.0 ± 1.2 (17%) n.s	6.5 ± 2.2 (0%) n.s
EO (3.5 mg/mL) + Bleomycin	5.1 ± 1.2 (15%) n.s	6.6 ± 1.1 (0%) n.s
EO (1.7 mg/mL) + Bleomycin	5.5 ± 2.1 (6%) n.s	5.6 ± 0.8 (0%) n.s

Bleomycin dosage was1 μg/mL. The densities of essential oils were estimated in 900 mg/mL using a 9.814 mL BRAND picnometer (Wertheim, Germany).†, SOS Induction Factor (IF) averages from a minimum of three independent experiments with three replicates each, as well as the corresponding standard errors, are given. Percentages of genotoxicity inhibition (%GI) were calculated as indicated in Materials and Methods. *, significant reduction ($p < 0.05$) in positive control was found by Student *t*-testing. n.s., no significant reduction was found.

and carvone did not increase the IF values in PQ37 *Escherichia coli* strain, indicating that these compounds do not induce the SOS response in *E. coli* cells. Limonene significantly increased IF values for a dose range between 97 and 1549 mM, but no response association was observed by means of Product-moment correlation analysis (R = -0,06, n.s) and, therefore, results were considered as non conclusive.

Citral induced a significant reduction in bleomycin-induced genotoxicity at a dose of 182 mM. Percentages of genotoxicity inhibition (% GI) increased with citral doses suggesting a direct mode of action for antigenotoxicity of this compound mixture and supporting the results observed with the EO. Carvone and limonene were also antigenotoxic. S(+)-carvone was significantly active only from a dose of 798 mM on, as also very similarly its isomer (R(-)-carvone) (data not shown). Limonene was antigenotoxic from a relatively lower dose (97 mM) on, although GI percentages were always consistently lower than those observed with citral (Table 4). Thus, citral was considered of higher antigenotoxic potential.

Data on antigenotoxicity of positive standard Trolox were also presented for comparison with citral, carvone and limonene. Assayed doses were determined experimentally, since no previous reports on this standard compound and SOS Chromotest were available in the literature. Trolox produced a significant decrease in bleomycin-induced genotoxicity from a dose of 586 μM, onwards, thus comparatively nearly 90, 398 and 1548 times lower than those of citral, carvone and limonene, respectively.

Discussion

The chemical composition and genotoxic and antigenotoxic properties of *L. alba* EOs obtained by microwave-assisted hydrodistillation, were evaluated. Citral, geraniol, *trans*-β-caryophyllene, carvone, limonene and bycyclosesquiphellandrene were identified as the principal components. According to Hennebelle *et al.* (2008a), there are at least seven chemotypes: I (Citral, linalool and β-caryophyllene, as the main constituents), II (tagetenone), III (limonene and carvone or related monoterpenic ketones), IV (myrcene), V (γ-terpinene), VI (camphor-1,8-cineole) and VII (estragole). So, the two EOs studied here were classified as citral and carvone/limonene chemotypes, thus corresponding to chemotypes I and III, respectively.

Apparently, this is the first report on the genotoxic and antigenotoxic properties of *L. alba* EOs. Under the experimental conditions assayed here (absence of exogenous metabolic activation), the *L. alba* chemotypes (citral and carvone/limonene) did not induce DNA primary damage in the SOS Chromotest. In addition, EO major constituents as citral and carvone were not genotoxic in the SOS Chromotest. This was in accordance with previous studies using SOS Chromotest, *Salmonella*/microsome and *Drosophila melanogaster* SMART assays (Franzios *et al.*, 1997; Gomes-Carneiro *et al.*, 1998; Stammati *et al.*, 1999). For limonene, IF increased at doses between 194 and 774 mM, thereby contrasting with the results obtained with EO of the carvone/limonene chemotype. This limonene-effect was possibly masked in the EO by interaction with other constituents, perhaps even carvone itself. Nevertheless, this presumption needs to be tested. A previous study (Vukovic-Gacic *et al.*, 2006) indicated non-mutagenic effects for limonene using *Salmonella*/microsome assay. As the results so far have been inconclusive, harmonized studies on the genotoxicity of these compounds are now underway in our laboratory.

The antigenotoxic potential of *L. alba* EO was also shown. Although both the citral and carvone/limonene chemotypes were antigenotoxic against the clastogen bleomycin, citral appears as the most promising source of chemopreventive compounds, apparent by the antigenotoxicity observed in the major constituents (Table 4). The order of antigenotoxic activity for these compounds was found to be citral > carvone > limonene, indicating that citral was the most active compound. Although the chemopreventive properties of *L. alba* terpenoids, as carvone, geraniol, limonene and perillyl alcohol, have already been well-documented (He *et al.*, 1997; Crowell, 1999; Uedo *et al.*, 1999; de Carvalho and da Fonseca, 2006; Paduch *et al.*, 2007; Patil *et al.*, 2009; Rabi and Bishayee, 2009), little is really known as regards citral. Connor (1991) was the first to indicate citral chemopreventive potentiality against skin chemical carcinogenesis in mice. Further experimental evidence lent supported that citral has an ability to suppress oxidative stress, possibly through the induction of endogenous antioxidant proteins, such as phase II xenobiotic metabolizing enzymes, as well as glutatione S-transferase (Nakamura *et al.*, 2003). In addition, it has been recently demonstrated that citral strongly inhibited the CYP2B60 hydroxylase activity (Seo *et al.*, 2008) involved, not only in xenobiotic activation of a wide variety of pro-mutagens, but also in the synthesis of Aflatoxin B_1 mycotoxin (Shukla *et al.*, 2009), involved in gastric carcinogenesis. The present work provides new insights into citral and carvone chemoprevention. Since bleomycin genotoxicity involves the generation of radicals in the DNA molecule, which thus induce DNA-strand breakages (Claussen and Long, 1999), it can be expected that the antigenotoxic effect of citral and carvone against bleomycin occurs through radical scavenging mechanisms within the molecule. In fact, Stashenko *et al.* (2004) have previously demonstrated antioxidant properties for the *L. alba* carvone/limonene chemotype.

On considering the importance of oxidative damage in carcinogenesis, the antioxidant effect of citral and carvone can be explored as cancer chemopreventive agents against inflammation-related disorders, such as skin and colon cancers. Since carvone and limonene are natural enhancers of transdermal drug delivery by increasing per-

Table 4 - The genotoxic† effects of citral, carvone and limonene, and respective antigenotoxicity†† against bleomycin-induced DNA damage in PQ37 *Escherichia coli* cells. Antigenotoxic data on standard compound Trolox are also shown.

Genotoxicity		Antigenotoxicity	
Cell treatments	IF†	Cell treatments	IF†† (% GI)
Distilled water (negative control)	1.0 ± 0.1	Distilled water (negative control)	1.0 ± 0.3
Bleomycin (positive control)	7.7 ± 1.6 *	Bleomycin (positive control)	12.5 ± 4.4
Citral (2915 mM)	0.6 ± 0.1 n.s	Citral (2915 mM) + Bleomycin	0.5 ± 0.1 (100%) **
Citral (1457 mM)	0.6 ± 0.1 n.s	Citral (1457 mM) + Bleomycin	0.6 ± 0.2 (100%) **
Citral (729 mM)	0.8 ± 0.2 n.s	Citral (729 mM) + Bleomycin	1.0 ± 0.2 (100%) **
Citral (364 mM)	1.3 ± 0.2 n.s	Citral (364 mM) + Bleomycin	1.5 ± 0.3 (96%) **
Citral (182 mM)	1.2 ± 0.2 n.s	Citral (182 mM) + Bleomycin	1.5 ± 0.3 (96%) **
Citral (91 mM)	1.2 ± 0.2 n.s	Citral (91 mM) + Bleomycin	6.9 ± 2.4 (49%) n.s
Citral (45 mM)	1.2 ± 0.2 n.s	Citral (45 mM) + Bleomycin	17.1 ± 5.6 (0%) n.s
Citral (23 mM)	1.2 ± 0.2 n.s	Citral (23 mM) + Bleomycin	17.2 ± 6.8 (0%) n.s
Citral (12 mM)	1.0 ± 0.3 n.s	Citral (12 mM) + Bleomycin	13.9 ± 6.0 (0%) n.s
Distilled water (negative control)	1.0 ± 0.1	Distilled water (negative control)	0.9 ± 0.1
Bleomycin (positive control)	5.8 ± 0.9 *	Bleomycin (positive control)	9.5 ± 2.8
Carvone (3192 mM)	1.2 ± 0.3 n.s	Carvone (3192 mM) + Bleomycin	2.2 ± 0.5 (85%) **
Carvone (1596 mM)	1.0 ± 0.2 n.s	Carvone (1596 mM) + Bleomycin	1.7 ± 0.6 (84%) **
Carvone (798 mM)	1.3 ± 0.4 n.s	Carvone (798 mM) + Bleomycin	5.2 ± 1.5 (50%) **
Carvone (399 mM)	1.4 ± 0.3 n.s	Carvone (399 mM) + Bleomycin	7.6 ± 3.1 (50%) n.s
Carvone (199 mM)	1.3 ± 0.1 n.s	Carvone (199 mM) + Bleomycin	11.4 ± 2.5 (22%) n.s
Carvone (100 mM)	1.2 ± 0.2 n.s	Carvone (100 mM) + Bleomycin	11.0 ± 4.3 (0%) n.s
Carvone (50 mM)	1.3 ± 0.3 n.s	Carvone (50 mM) + Bleomycin	9.6 ± 1.8 (0%) n.s
Carvone (25 mM)	1.1 ± 0.2 n.s	Carvone (25 mM) + Bleomycin	9.7 ± 2.9 (0%) n.s
Carvone (12 mM)	0.9 ± 0.2 n.s	Carvone (12 mM) + Bleomycin	9.8 ± 3.6 (0%) n.s
Distilled water (negative control)	1.0 ± 0.1	Distilled water (negative control)	0.9 ± 0.2
Bleomycin (positive control)	8.9 ± 1.1 *	Bleomycin (positive control)	7.2 ± 1.4
Limonene (3098 mM)	0.9 ± 0.3 n.s	Limonene (3098 mM) + Bleomycin	2.0 ± 0.9 (82%) **
Limonene (1549 mM)	2.0 ± 0.9 *	Limonene (1549 mM) + Bleomycin	2.0 ± 0.6 (82%) **
Limonene (774 mM)	5.7 ± 1.2 *	Limonene (774 mM) + Bleomycin	4.6 ± 1.5 (41%) **
Limonene (387 mM)	4.7 ± 1.0 *	Limonene (387 mM) + Bleomycin	4.6 ± 1.5 (41%) **
Limonene (194 mM)	2.0 ± 0.3 *	Limonene (194 mM) + Bleomycin	4.7 ± 1.6 (40%) **
Limonene (97 mM)	1.6 ± 0.2 *	Limonene (97 mM) + Bleomycin	4.8 ± 1.5 (38%) **
Limonene (48 mM)	1.4 ± 0.3 n.s	Limonene (48 mM) + Bleomycin	5.0 ± 1.6 (35%) n.s
Limonene (24 mM)	1.4 ± 0.2 n.s	Limonene (24 mM) + Bleomycin	5.6 ± 1.4 (25%) n.s
Limonene (12 mM)	1.3 ± 0.3 n.s	Limonene (12 mM) + Bleomycin	6.5 ± 1.2 (11%) n.s
Distilled water (negative control)	-	Distilled water (negative control)	1.1 ± 0.1
Bleomycin (positive control)	-	Bleomycin (positive control)	9.9 ± 2.1
Trolox (4687 μM)	-	Trolox (4687 μM) + Bleomycin	0.9 ± 0.3 (100%) **
Trolox (2344 μM)	-	Trolox (2344 μM) + Bleomycin	1.1 ± 0.3 (100%) **
Trolox (1172 μM)	-	Trolox (1172 μM) + Bleomycin	0.8 ± 0.4 (100%) **
Trolox (586 μM)	-	Trolox (586 μM) + Bleomycin	2.0 ± 1.0 (90%) **
Trolox (293 μM)	-	Trolox (293 μM) + Bleomycin	7.7 ± 2.1 (25%) n.s
Trolox (146 μM)	-	Trolox (146 μM) + Bleomycin	12.9 ± 2.9 (0%) n.s
Trolox (73 μM)	-	Trolox (73 μM) + Bleomycin	14.3 ± 3.0 (0%) n.s
Trolox (37 μM)	-	Trolox (37 μM) + Bleomycin	10.8 ± 2.6 (0%) n.s
Trolox (18 μM)	-	Trolox (18 μM) + Bleomycin	10.8 ± 2.1 (0%) n.s

Bleomycin was always used at a dose of 1 μg/mL. According to technical product data (Sigma-Aldrich Co, St. Louis, Missouri, USA), citral, carvone and limonene densities were 0.888, 0.959 and 0.844 g/mL, respectively. Major compound dose ranges were estimated based on their amount (%) in the chromatogram and oil density, as indicated in Tables 1 and 2. Average IF values for genotoxicity† and antigenotoxicity†† from a minimum of three independent experiments with four replicates each, and the corresponding standard error, are given. A substance is classified as nongenotoxic if IF remains < 1.5, nonconclusive if IF is between 1.5 and 2.0, and genotoxic if IF exceeds 2.0 and a dose-response relationship is observed. Percentage of genotoxicity inhibition (% GI) was calculated as indicated in Materials and Methods. *, a significant increase ($p < 0.05$) in negative control was found using the Student *t*-test. **, a significant reduction ($p < 0.05$) in positive control was found using Student *t*-test. n.s., no significant differences were found.

cutaneous permeation (Aqil *et al.*, 2007; Sapra *et al.*, 2008), the simultaneous use of either of these compounds together with citral in gel preparation, should be an effective approach for skin chemoprotection. However, cytotoxicity in human fibroblast cells has been reported at concentrations higher than 1% of citral (Hayes and Marcovic, 2002). Moreover, lemongrass (*Cymbopogon citratus*) EO with a high citral content (70%-90%) induced phototoxic effects in murine fibroblastic cell-line 3T3 and rabbit cornea derived cell-line SIRC (Dijoux *et al.*, 2006). Thus, further studies on skin sensitization and phototoxicity with terpenoids are required.

Based on data from the literature (Pino-Alea *et al.*, 1996; Abad *et al.*, 1997; Viana *et al.*, 1998, 2000; Vale *et al.*, 1999; Pascual *et al.*, 2001b; Zétola *et al.*, 2002, Gazola *et al.*, 2004; Andrighetti-Fröhner *et al.*, 2005; Teixeira-Duarte *et al.*, 2005; de Carvalho and da Fonseca, 2006; Sena-Filho *et al.*, 2006; Paduch *et al.*, 2007; Hennebelle *et al.*, 2008a,b; Ara *et al.*, 2009; Arruda *et al.*, 2009; Mesa-Arango *et al.*, 2009), *L. alba* clearly has a wide-ranging therapeutic potential, even further amplified by the antigenotoxic properties against bleomycin, as a source of compounds with application in cancer chemoprevention. As indicated above, citral, carvone and limonene have shown protective properties *in vitro* and *in vivo* (Connor, 1991; He *et al.*, 1997; Crowell, 1999; Uedo *et al.*, 1999; Nakamura *et al.*, 2003; de Carvalho and da Fonseca, 2006; Paduch *et al.*, 2007; Seo *et al.*, 2008; Patil *et al.*, 2009; Rabi and Bishayee, 2009; Shukla *et al.*, 2009), all of which highlight the potential benefit of *L. alba* and its major components, citral, carvone and limonene, as dietary supplements with chemopreventive and/or antioxidant properties.

In conclusion, this study showed the antigenotoxic properties of *L. alba* EO, citral, carvone and limonene against the drug bleomycin, lending support to the potential of the oils and compounds in chemoprevention and cancer therapy. Since the role of chemopreventive agents in the etiology of cancer is very complex, and involves several modes of action, and our results concern only *in vitro* experiments with a bacterial assay, additional animal and human studies involving different endpoints should be addressed in order to clarify the antimutagenic potential of *L. alba* EOs and their major constituents. In addition, harmonized studies on the genotoxicity of citral, carvone and limonene, using a battery of *in vivo* assays that evaluate different levels of DNA damage expression, are required, prior to the practical use of these compounds in chemoprevention.

Acknowledgments

The authors wish to thank Dr. José Luis Fernández Alonso for botanical identification of specimens and Dr. Montserrat Llagostera Casal from the Universidad Autónoma de Barcelona for gently supplying the PQ37 *E. coli* strain. This work was supported by the Vice-Rectory for Scientific Research at the Universidad Industrial de Santander (UIS, Grant 5154), and by the Colombian Institute of Science and Technology, CENIVAM-COLCIENCIAS (Grant RC-432-2004).

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Associate Editor: Catarina S. Takahashi