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ORIGINAL ARTICLE

Inhibition of Mevalonate Pathway and Synthesis of the Storage Lipids in Human Liver-Derived and Non-liver Cell Lines by *Lippia alba* Essential Oils

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Abstract The essential oils (EOs) of *Lippia alba*, an herb extensively used as a folk medicine in Latin America, are today promoted as an effective means of eliminating problems caused by hyperlipemia. We hypothesized that *L.alba* EOs inhibited cholesterol and triacylglycerols synthesis and decreased the intracellular depots of those lipids (lipid droplets), mechanisms involving the induction of a hypolipidemic response. Our aim was, therefore, to evaluate the hypolipogenic capability of the EOs of four *L. alba* chemotypes on liver-derived (HepG2) and non-liver (A549) human cell lines and to identify the potential biochemical targets of those chemotypes, particularly within the mevalonate pathway (MP). [14C]Acetate was used as radioactive precursor for assays. Lipid analyses were performed by thin-layer and capillary gas chromatography, lipid droplets analyzed by fluorescence microscopy, and HMGCR levels determined by Western blot. In both cell lines, all four chemotypes exerted hypocholesterogenic effects within a concentration range of 3.2–32 µg/mL. Nonsaponifiable lipids manifested a decrease in incorporation of [14C]acetate into squalene, lanosterol, lathosterol, and cholesterol, but not into ubiquinone, thus suggesting an inhibition of enzymes in the MP downstream from farnesyl pyrophosphate. The tagetenone chemotype, the most efficacious hypocholesterogenic *L. alba* EO, lowered HMGCR protein levels; inhibited triacylglycerols, cholesteryl esters, and phospholipids synthesis; and diminished lipid droplets in size and volume. These results revealed that *L. alba* EOs inhibited different lipogenic pathways and such lipid-lowering effects could prove essential to prevent cardiovascular diseases.

Abbreviations

C	Cholesterol
CE	Cholesteryl esters
ChoGpl	Choline glycerophospholipids
EOs	Essential oils
EtnGpl	Ethanolamine glycerophospholipids
HMGCR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
LaEO	*Lippia alba* essential oil
LaEOca	*Lippia alba* essential oil carvone chemotype
LaEOci	*Lippia alba* essential oil citral chemotype
LaEOpi	*Lippia alba* essential oil piperitone chemotype
LaEOta	*Lippia alba* essential oil tagetenone chemotype
LD	Lipid droplet
PL	Phospholipids
PtdSer	Phosphatidylserines
TAG	Triacylglycerols

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Introduction

For centuries plants have played a role in maintaining human health, as well as being essential pharmaceutical sources. *Lippia alba* (Miller) N.E. Brown is an aromatic shrub in the Verbenaceae family widely available in the Americas and extensively used in traditional medicine

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because of plant's antimicrobial, antispasmodic [1], expectorant, anti-inflammatory [2], analgesic [3], anti-convulsive [4], antioxidant [5], digestive [6], and hypotensive properties [7], among others. Today *L. alba* is itself highly touted as a plant that lowers cholesterol levels and helps to reduce body-fat mass [8]. The essential oil of *L. alba* (LaEO) has a high chemical variability that is related to the geographical origin of the plant and determines different chemotypes (individuals of the same botanical species morphologically identical, but with different chemical compositions of volatile secondary metabolites), with some seven having been reported so far [9]. The carvone, citral, and piperitone chemotypes have been the ones most widely studied. The tagetenone chemotype (LaEOta)—composed of tagetenone (a mixture of mircenone and ocimenone) and cineole as the major components [10]—is widely distributed in Mexico, Guatemala [11], Costa Rica [12], and Argentina [13], but the biologic properties of that chemotype have yet to be studied. Furthermore, none of those chemotypes has been assessed yet for the potentiality of regulating the levels of cholesterol and triacylglycerols, an essential means of preventing not only cardiovascular disease, but also inflammatory processes. The mevalonate pathway (MP) is a central metabolic sequence playing a key role in multiple cellular processes through the synthesis of cholesterol, dolichol, heme-A, isopentenyl tRNA, and ubiquinone, with 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) being the rate-limiting enzyme of that biochemical scheme. While the MP has been extensively studied with respect to cholesterol synthesis and the relationship of that sequence to atherogenesis and the development of cardiovascular disease, in recent years cholesterogenesis has also been investigated as a promising therapeutic target in other areas of ongoing research—e.g., oncology, autoimmune disorders, and Alzheimer's disease [14].

We hypothesized that *L.alba* EOs would decrease serum-cholesterol and -triacylglycerols levels through synthesis inhibition of those lipids along with their intracellular storage, both mechanisms being usually associated with a hypolipidemic response. In order to test these premises, the present work was designed in a human-liver tumor-cell line (HepG2) that expresses most of the lipid metabolism characteristic of a normal hepatocyte and in a human-lung tumor-cell line (A549) as a model of a non-hepatic cell. Our main objectives were to evaluate the mechanisms of action of LaEOs on lipid metabolism in general and on cholesterogenesis in particular through biochemical and cytological analyses and to investigate the LaEOs' potential hypolipidemic effects.

Methods and Materials

Reagents

Solvents were obtained from Carlo Erba (Milan, Italy, [^{14}C]acetate (56.8 Ci/mol) from Perkin Elmer Life Science, Inc. (Boston, MA, USA), and streptomycin from Richet (Argentina). The sodium salt of simvastatin was prepared by dissolving the drug in ethanol at 60 °C, adding equimolar amounts of NaOH, and incubating at 60 °C for 1 h. The ethanol was then evaporated under nitrogen and the salt dissolved in distilled water at a final concentration of 5 mM. 4′,6-Diamidino-2-phenylindole (DAPI) and BODIPY (boron dipyrromethene) were obtained from Invitrogen (Carlsbad, CA, USA).

Essential Oils: Plant Material, Extraction, and Analysis

Leaves were collected from four *L. alba* chemotypes (carvone, piperitone, tagetenone, and citral) cultivated in Costa Rica. The *L. alba* carvone (originally from Talamanca, province of Limón, Costa Rica) and piperitone (originally from Argentina) chemotype plants were cultivated in the wet tropic of Bouganvillea S.A., in Baltimore, Matina, Province of Limón, while the *L. alba* tagetenone and citral chemotype plants were grown in a private garden near San José in the Central Valley, Province of San José, Costa Rica. The plants were identified by R. Ocampo and J. F. Cicció, University of Costa Rica. Voucher specimens were deposited in the Herbarium of the University of Costa Rica (*L. alba* carvone chemotype: USJ 70741, *L. alba* piperitone chemotype: USJ 93918, *L. alba* tagetenone chemotype: USJ 70698, and *L. alba* citral chemotype: USJ 100626). The extraction and chemical composition analysis of the *L. alba* essential oils (LaEOs) were conducted according to Chaverri and Cicció [15]; which results for the LaEOs carvone (ca), piperitone (pi), tagetenone (ta) and citral (ci) chemotypes have been reported by Montero Villegas *et al*. (unpublished work). Table 1 itemizes the major components of these four chemotypes.

Cell Cultures and Treatments

The HepG2 human-hepatoma cells were purchased from the American Type Culture Collection and the A549 human-alveolar-adenocarcinoma cells kindly provided by Dr. Amada Segal-Eiras (CINIBA, UNLP. Argentina). The cells were maintained in 75-cm^2 flasks in filter-steriled Eagle's Minimum Essential Medium (MEM; Gibco, Invitrogen Corporation) supplemented with 10% (v/v) fetal-bovine serum plus 0.1 mg L^{-1} streptomycin (Natocor,

Table 1 Partial chemical composition of the essential oils from the four chemotypes of *Lippia alba* (Miller) N.E. Brown cultivated in Costa Rica

Compound	Percent composition			
	LaEOca	LaEOpi	LaEOta	LaEOci
Sabinene	–	–	4.3	–
6-Methyl-5-hepten-2-one	–	–	–	12.5
Myrcene	0.2	0.1	11.0	t
Limonene	13.6	9.0	2.0	0.4
1,8-Cineole	–	–	21.4	–
Myrcenone	–	–	30.4	–
Citronellal	–	–	0.8	–
Neral	–	–	–	14.1
Carvone	58.6	0.2	–	–
Piperitone	1.5	52.4	1.9	–
Geranial	–	–	–	22.9
Germacrene-D	10.1	1.9	3.2	4.2
Germacrene D-4-ol	0.4	8.5	0.4	–
Geraniol	–	–	–	17.6

T traces (<0.05%)

Córdoba, Argentina) in a humidified incubator at 5% (v/v) CO_2/air and 37 °C.

For assays, cultures were incubated in serum-containing MEM until they reached logarithmic growth, then incubated (according to the experiment) in serum-free MEM zinc option (IMEM-Zo) or in serum-containing MEM supplemented with different concentrations of LaEO previously dissolved in dimethyl sulfoxide at a final concentration of the vehicle of 0.2% (v/v). That same concentration of dimethyl sulfoxide was, therefore, added to the parallel control cultures. The simvastatin sodium salt (5 mM) was added at the appropriate concentration in aqueous solution.

Lipid Extraction and Analysis

Total lipids from the cells were extracted with methanol/chloroform (2:1, v/v) and partitioned with distilled water (5:1, v/v) according to Folch *et al.* [16]. An aliquot was used to separate free cholesterol and cholesteryl esters, triacylglycerols, and phospholipids by thin-layer chromatography (TLC) on silica gel G developed in hexane/diethylether/acetic acid (80:20:1, v/v/v). The polar lipids and triacylglycerols fractions were then removed from the plate for quantification. Triacylglycerols was measured with a commercial kit (TG color GPO/PAP AA, Wiener Laboratories, Buenos Aires, Argentina) following the kit instructions and the phosphorus from the phospholipids fraction quantified as described in Chen *et al.* [17]. The plate was sprayed with an acidic ferric-chloride solution and then heated at 120 °C in order to quantify the rest of lipids by means of

a curve constructed with pure standards that had been run on the same plate. The spots were analyzed by the Image J program. Another aliquot was saponified with 10% (w/v) KOH in methanol at 85 °C for 45 min. The nonsaponifiable lipids were extracted with petroleum ether (boiling point, 30–40 °C) and the total fatty acids from the methanolic phase with light petroleum ether (boiling point, 30–40 °C) after acidification with HCl. Capillary-gas-chromatography analyses of the fatty-acid methyl esters were performed on a Hewlett-Packard 6890 gas chromatograph. Samples were analyzed on a 30-m × 0.32-mm–I.D. 0.5-mm–stationary-phase Omega Wax 250 capillary column (Alltech Associates, Arlington Heights, IL, USA). The temperature of the oven was programmed from 175 to 220/°C at a rate of 3 °C/min, then held at 220 °C for 15 min.

Incorporation of [14C]Acetate

For radioactivity uptake studies with [14C]acetate, cultures were treated with different concentrations of LaEO in serum-containing MEM for 24 h. Then the medium was removed, the cells washed three times with PBS, and the cultures incubated with LaEO in serum-free MEM Zinc option (IMEMZo) for another 24 h. [14C]Acetate (1 µCi/mL of culture medium) was added over the final 3 h of treatment; the cultures were then harvested and the lipids extracted with hexane/isopropanol (3:1, v/v). Cholesterol and other MP nonsaponifiable metabolites were partitioned by TLC upon development in 100% chloroform, the neutral lipids in hexane/diethylether/acetic acid (80:20:1, v/v/v), and the phospholipids in chloroform/methanol/acetic acid/water (50:37.5:7.5:2, v/v/v/v) as the mobile phases. Lipid fractions were visualized by autoradiography in a Storage Phosphor Screen (GE Healthcare, Amersham, UK). Quantitative densitometric analyses were performed by means of the Image J program. All lipid classes were identified by comparison with standard mixtures added to the same plate or to their respective retention factors. Aliquots from all the above lipid fractions were used to determine the incorporation of radioactivity by liquid-scintillation counting in a Wallac 1214 RackBeta counter (Pharmacia, Turku, Finland).

Western Blot

HepG2 cells were incubated in serum-containing MEM supplemented with LaEOta for 48 h. The cells were harvested by scraping in 2× Laemmli lysis buffer (Tris–HCl, 62.5 mM; pH 6.8; 25% (v/v) glycerol; 2% (w/v) sodium dodecyl sulfate [SDS]; 0.01% (v/v) Bromophenol Blue). The samples were separated on 12.5% (w/v) SDS–polyacrylamide gels and adsorbed to polyvinylidene difluoride membranes (GE Healthcare, Amersham, UK) by semidry transfer at 10 V for 1 h. Nonspecific protein-binding sites

were blocked by incubation in PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 5% (v/v) skimmed milk. The membrane was incubated with rabbit anti-HMGCR diluted 1/200 in antibody-dilution buffer [2% (v/v) skimmed milk in PBS plus 0.1% (v/v) Tween 20] for 1 h.; horseradish-peroxidase–conjugated goat anti(rabbit IgG) antibodies were then added to the membrane for 1 h. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The subsequent immunoreactive bands were detected by enhanced chemiluminescence Western blot–detection reagents (GE Healthcare, Amersham, UK) and processed through the use of common X-ray film developers and fixers. The band intensity was quantified by the Image J software.

Lipid Droplets

HepG2 cells grown on coverslips were incubated as described in serum-containing MEM supplemented with LaEOta for 48 h and the samples processed according to Layerenza [18]. The procedure stated in brief: cells were kept on ice and fixed by an overnight incubation in 4% (w/v) paraformaldehyde in PBS. Cell permeabilization was effected by incubation with 0.08% (v/v) Triton X-100 in PBS followed by staining with DAPI (final concentration, 1 μg/mL) and BODIPY 493/503 (final concentration, 1 μg/mL) in PBS supplemented with 3% (w/v) BSA. The fluorescence-microscopical observations were performed with an Olympus BX51 microscope (Tokyo, Japan); and the cells, photographed with an Olympus DP70 digital camera,

were analyzed by means of the ImagePro Plus (IPP™) v. 5.1 software (Media Cynernetics, Silver Spring, MD, USA).

Statistical Analysis

The Kolmogoro–Smirnov test with Lilliefors corrections was used to test the normality of the data. Parametrical statistical analyses were performed through the use of the one-way analysis of variance (ANOVA) and either the Tukey–Kramer multiple-comparisons test or the unpaired Student t test (GraphPad inStat program). The IC_{50} values for cholesterol synthesis (IC_{50CS}) were calculated by nonlinear-regression curves (SigmaPlot software; Systat Software, Inc., Point Richmond, CA, USA). Nonparametric statistical analysis was performed through the use of Kruskal–Wallis test, the Chi-squared test, and the post hoc Bonferroni method. The significance level was set at $p < 0.05$ for all the statistical analyses.

Results

Effect of LaEO Chemotypes on the Cellular MP and Cholesterol Content

HepG2 and A549 cells were treated with increasing concentrations of the four LaEO chemotypes and then radiolabelled with [14C]acetate. The nonsaponifiable lipids separated by TLC (Fig. 1) showed that in the control cultures, most of the
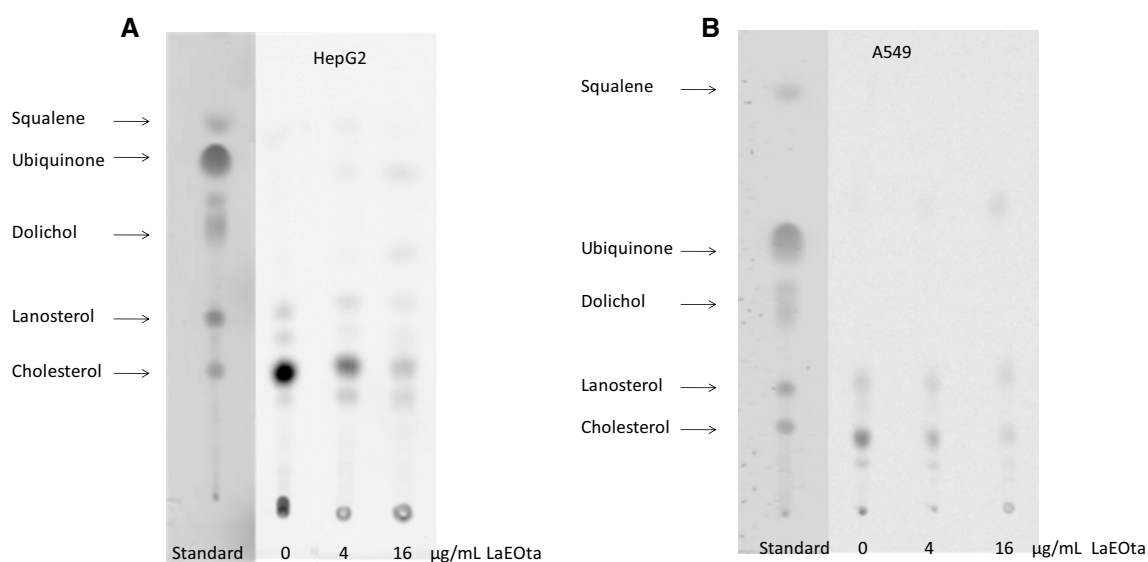

Fig. 1 Thin-layer chromatographic profiles of HepG2 (**a**) and A549 (**b**) nonsaponifiable lipids developed in hexane (100%). The cultured cells were treated with two concentrations of the *Lippia alba* chemotype tagetenone (LaEOta) (4 and 16 μg/mL) for 48 h, with [14C]

acetate being added during the last 3 h. The incorporation of radioactivity into the cellular lipid fractions was finally visualized by autoradiography

radioactivity (60–70%) was incorporated into cholesterol (the main final product of the MP). Treatment with all four LaEO chemotypes significantly suppressed cholesterogenesis in both cell lines in a concentration-dependent manner (Fig. 2). The incorporation of radioactivity into ubiquinone (another final product of the MP) was increased in both cell lines treated with all four LaEO chemotypes (Fig. 2). [^{14}C] Acetate incorporation into certain specific intermediates of the MP specifically involved in cholesterol synthesis could be also identified and quantified (e.g., lathosterol and lanosterol in both cell lines and squalene in only the HepG2 cells). The radioactivity in most of these intermediates decreased in cells treated with the LaEO chemotypes, with the corresponding curves of percent inhibition of radioactivity incorporation vs. concentration being similar to those of cholesterol (cf. the Fig. 2, inserts).

Table 2 summarizes the concentrations of the LaEO chemotypes and simvastatin that inhibited cholesterol synthesis by 50% (the IC$_{50CS}$ values) in both cell lines, calculated from dose–response curves obtained by nonlinear regression (cf. the Fig. 3). Simvastatin was used as a positive control for the inhibition of cholesterogenesis. The LaEOta and LaEOci chemotypes exhibited a more efficient inhibition of cholesterogenesis than the others in both cell lines (Table 2). In the HepG2 cells the IC$_{50CS}$ values of both those chemotypes were similar, but the value corresponding to LaEOci proved to be more twice than that of LaEOta in the A549 cells. In cells treated with LaEOta in particular the maximum inhibition of cholesterogenesis was reached at a concentration close to 10 μg/mL, above which value no further decreases were recorded (Fig. 3). The results (Fig. 4) revealed that 10 μg/mL of LaEOta caused an inhibition of nearly 80% of cholesterogenesis in the two cell lines, whereas the remaining three chemotypes produced significantly lower inhibitions in both.

In order to evaluate the impact of the cholesterol-synthesis inhibition produced by the LaEOs on the content of cholesterol in the cells, HepG2 and A549 cultures were treated with IC$_{50sc}$ concentrations of each of the four chemotypes, and then the cellular free- and esterified cholesterol content was determined (Table 3). LaEOta significantly decreased the free and the esterified cholesterol content in A549 and HepG2 cells, respectively, but was the only chemotype that produced such an inhibition: no significant differences were obtained when either cell line was treated with the other three EO chemotypes.

Effect of LaEOta on Lipid Metabolism in HepG2 Cells

Since LaEOta was the most efficacious chemotype to inhibit cholesterol synthesis, and considering that HepG2 cells retain many of the metabolic characteristics of normal hepatocytes (which cells play a central role in maintaining lipid homeostasis [19]), we performed further studies with this cell line aimed at characterizing the effect of LaEO on lipid metabolism. LaEOta treatment caused a decrease in acetate incorporation into total (saponifiable and nonsaponifiable) lipids (Fig. 5). This decrease was dose-dependent for the nonsaponifiable lipids, whereas the decrease in the saponifiable fraction was similar with both concentrations tested (4 and 16 μg/mL). In contrast, the radioactivity incorporated into total fatty acids (Fig. 5) and distribution of label between saturated and unsaturated fatty acids (data not shown) were not significantly modified by treatment with LaEOta. Figure 6a shows the incorporation of [^{14}C] acetate into neutral lipids (cholesterol, triacylglycerols, and cholesteryl esters) and phospholipids. In HepG2 cells treated with either concentration of LaEOta, the incorporation of radioactivity decreased by 22–28% in the major saponifiable lipids (i.e., phospholipids and triacylglycerols), while a greater and dose-dependent inhibition of incorporation into cholesterol and cholesteryl esters was observed. In cells treated with 4 μg/mL of LaEOta, the incorporation of acetate into cholesterol and cholesteryl esters decreased by 57 and 65%, respectively, whereas 16 μg/mL produced a greater decline of 87% in cholesterol and 80% in cholesteryl esters. Phospholipids showed a modest decrease in the radioactivity incorporated into ethanolamine glycerphospholipids, choline glycerophospholipids, and phosphatidylserine (Fig. 6b) akin to that produced in the total phospholipids (Fig. 6a), as it would be expected. Otherwise, LaEOta treatment significantly decreased the amount of triacylglycerols, phospholipids, and cholesteryl esters, but caused no change in the free cholesterol content (Fig. 7). In contrast, the decrease in the amount of saponifiable lipids was not accompanied by a change in their composition (Table 4). The fatty acid profile obtained by capillary gas chromatography contained four major fatty acids: palmitic (16:0) and oleic (18:1n-9), together constituting 55.4% of the total fatty acids, followed by vaccenic (18:1n-7) and stearic (18:0)—with those two together comprising 27.6% of the total—along with minor components ranging from 16 to 22 carbons. This pattern remained essentially unchanged for cells incubated either with or without LaEOta (Table 4).

HepG2 cells incubated with LaEOta (4 and 16 μg/mL) manifested significant differences in the lipid droplets (LDs) diameters from those of the control cultures and even between the two experimental treatments (Figs. 8, 9a). On the basis of the quartiles calculated for the diameters of the control-cell LDs, three LDs groups could be defined according to size: small (S) at ≤0.51 μm, medium (M) at between 0.51 and 1.02 μm, and large (L) at >1.02 and significant differences in the proportions of these three groups between the treatments could be demonstrated. LaEOta-treated cells contained a higher proportion of small LDs and also fewer
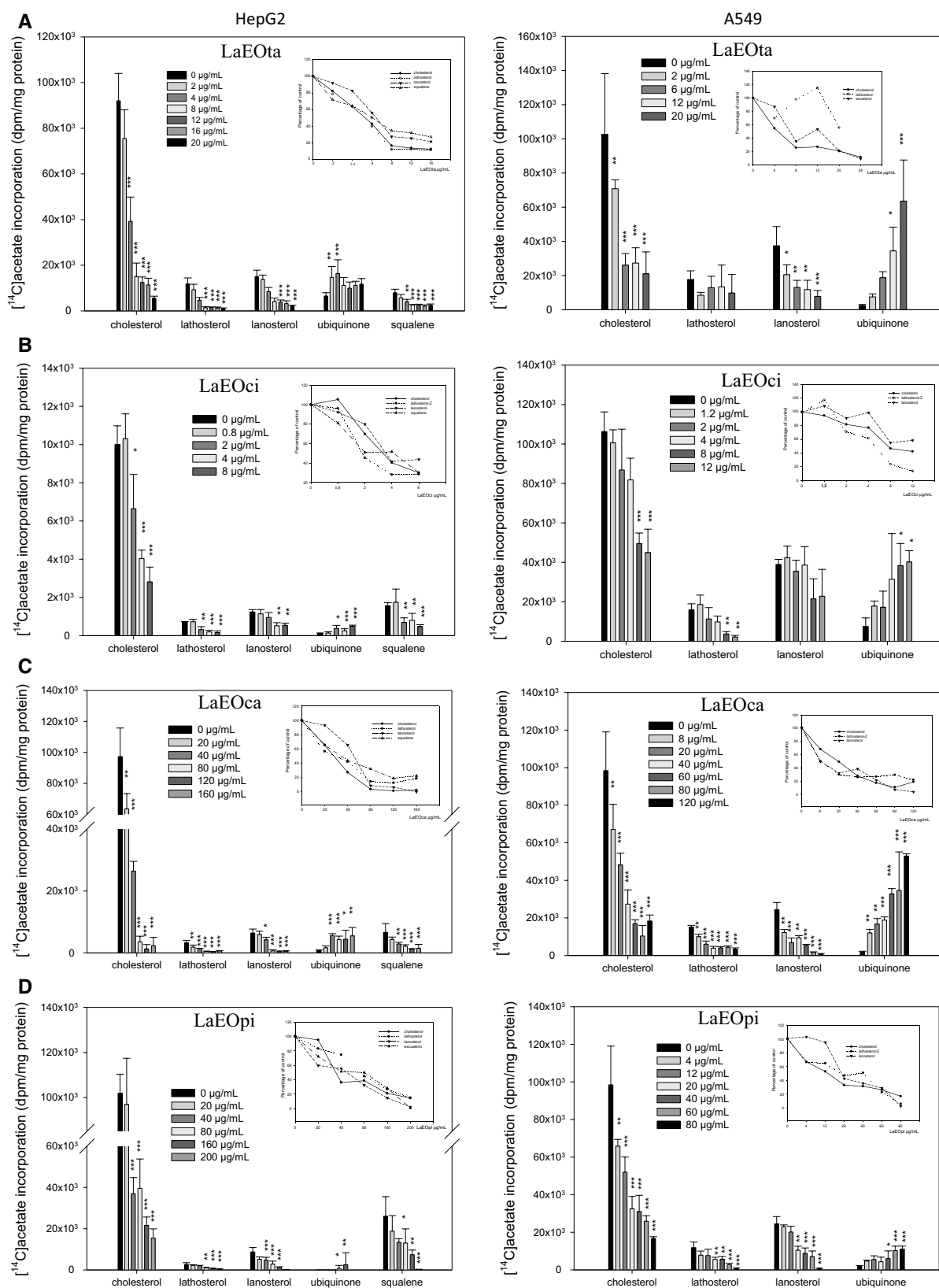
Fig. 2 [^{14}C]Acetate incorporation into cholesterol and other nonsaponifiable lipids of the mevalonate pathway in HepG2 and A549 cells. Cells were treated with increasing concentrations of *Lippia alba* (Miller) N.E. Brown essential oils (LaEOs) chemotypes tagetenone (LaEOta; **a**), cit-

ral (LaEOci; **b**), carvone (LaEOca; **c**), and piperitone (LaEOpi; **d**). The data are the mean ± SD of three independent experiments performed in quadruplicate; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Insets* dose–response curves of the metabolic intermediates of the mevalonate pathway

Table 2 Anticholesterogenic activity of *Lippia alba* (Miller) N.E. Brown chemotypes on HepG2 and A549 cells

	IC$_{50CS}$ (µg/mL)*	
	HepG2	A549
LaEOta**	3.5	3.8
LaEOci	3.2	8.0
LaEOca	28	20
LaEOpi	32	12
Simvastatin	0.13	0.88

* IC$_{50CS}$: the concentration of the *Lippia alba* essential oil (LaEO) that inhibited cholesterol synthesis by 50%

** Key to the LaEO chemotypes: tagetenone (LaEOta), citral (LaE-Oci), carvone (LaEOca), and piperitone (LaEOpi)

LDs defined as large, than the control cells (Fig. 9b). The number and total volume (considering the LDs as spheres) of the LDs per cell were also calculated. The total number of LDs per cell was similar among the three culture conditions, but the volume of the LDs became significantly decreased in cells incubated with 16 µg/mL of LaEOta compared to the size distribution in the controls (Fig. 10).

The evaluation by Western blots for the levels of HMGCR demonstrated that the protein levels of the enzyme did not change after incubating the cells with 4 µg/mL of LaEOta, but then decreased significantly at 16 µg/mL of that EO chemotype (Fig. 11). In contrast, after incubating cells with the competitive inhibitor of the enzyme simvastatin, a significant increase in the levels of the HMGCR protein was observed.

Discussion

Lippia alba (Mill) N.E. Brown is a shrub from the American humid subtropical and tropical forests. Plants of this species have a high chemical variability, especially in the
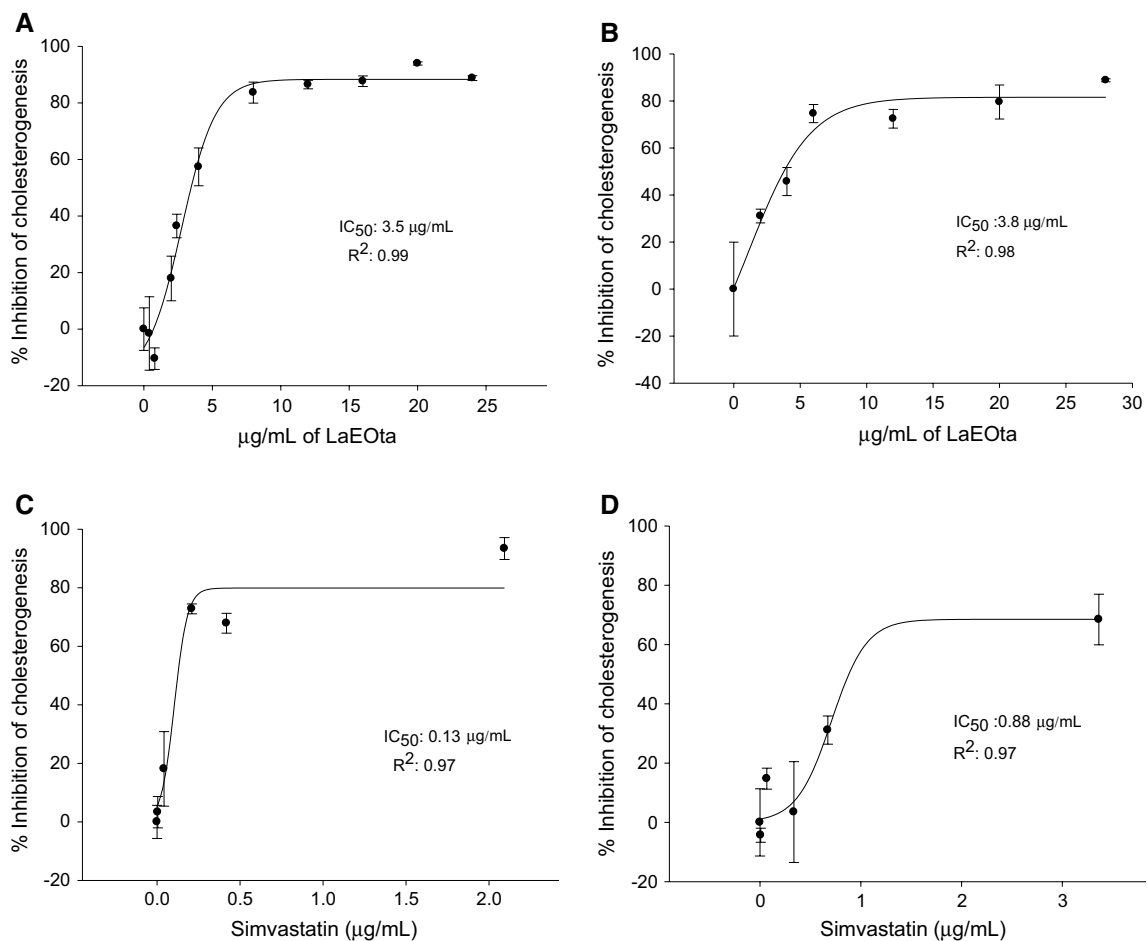

Fig. 3 Dose–response *curves* of HepG2 (**a**, **c**) and A549 (**b**, **d**) cells incubated, respectively, with the *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (LaEOta) and simvastatin with respect to cholesterogenesis. Each *point* on the *curve* was calculated from the mean value ± SD from four replicate experiments
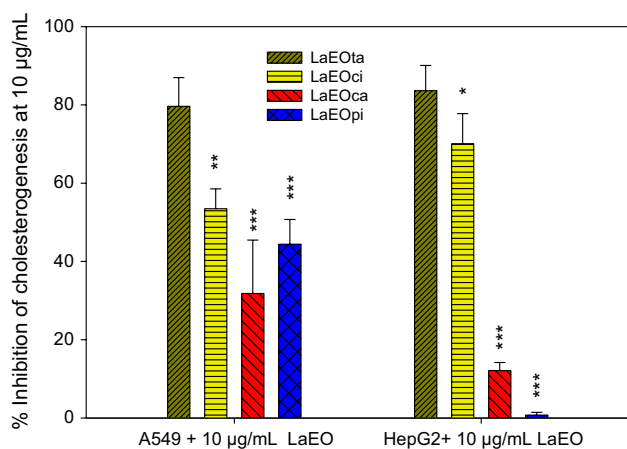
Fig. 4 Percentage of inhibition of [^{14}C]acetate incorporation into cholesterol in HepG2 and A549 cells treated with 10 µg/mL of *Lippia alba* (Miller) N.E. Brown essential-oil (LaEO) chemotypes tagetenone (LaEOta), citral (LaEOci), carvone (LaEOca), and piperitone (LaEOpi). The data are the mean ± SD of three independent experiments performed in quadruplicate; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

composition of their essential oils [12]. *L. alba* has an notable therapeutic record in traditional medicine [20–22] and is nowadays also popularly promoted as an effective means of losing weight and eliminating problems caused by cholesterolemia [8, 21, 22]. Up to the present, however, almost no reports have shown effects of the plant or its products on lipid metabolism or have pointed to a potential healing action on dyslipidemias or cardiovascular diseases. Indeed, thus far only Olivero-Verbel *et al.* [23] has reported effects of the *L. alba* LaEOci on the expression of certain genes related to lipid metabolism. The present report thus constitutes the first documentation of the effects of four *L. alba* chemotypes on the synthesis of cholesterol, triacylglycerols, and phospholipids in cultured tumor cells from two types of human tissues, liver, and lung.

We have herein demonstrated that all the LaEOs tested decreased the synthesis of cholesterol and of the intermediates of the MP related to cholesterogenesis (i.e., squalene, lathosterol, and lanosterol) and at the same time increased acetate incorporation into ubiquinone. These
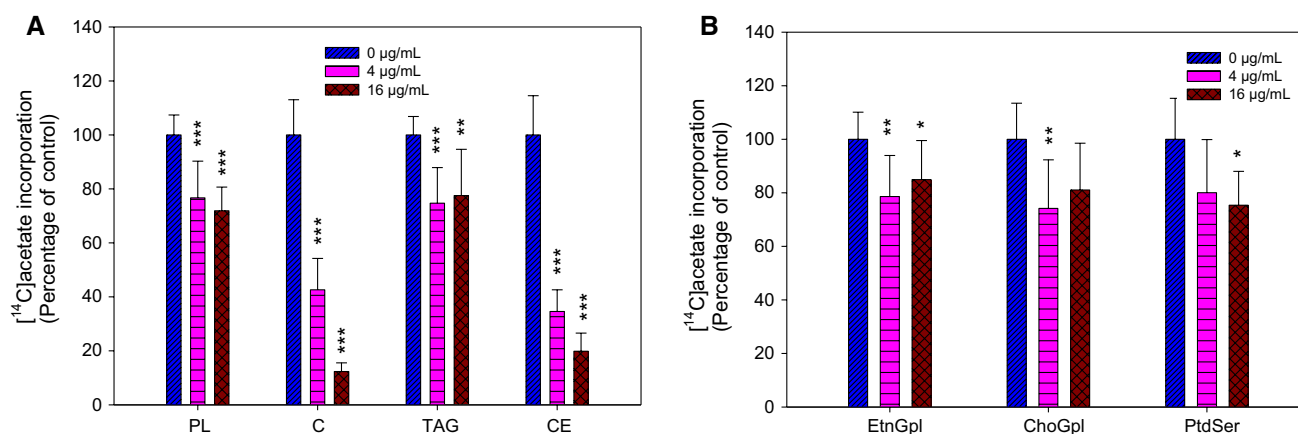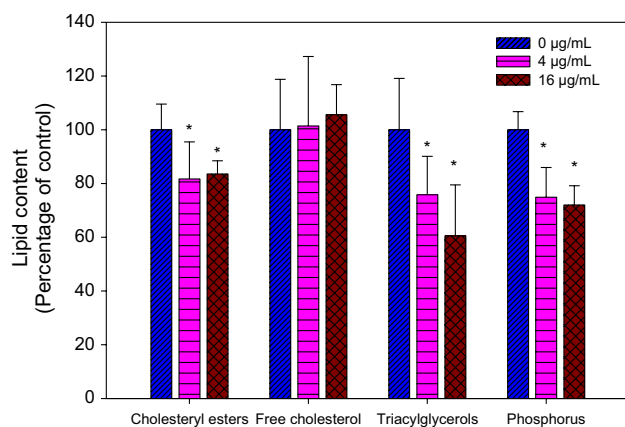
Table 3 Effect of *Lippia alba* (Miller) N.E. Brown chemotypes on the cholesterol content of the HepG2 and A549 cells

Cholesterol (µg/mg TCP)	Cell line	Control	LaEOta	LaEOca	LaEOci	LaEOpi
Free	HepG2	14.5 ± 2.7	13.6 ± 2.8	15.9 ± 2.2	15.2 ± 1.5	15.1 ± 3.3
	A549	10.5 ± 0.8	8.2 ± 0.6*	10.1 ± 0.9	8.6 ± 0.1	10.5 ± 1.8
Esterified	HepG2	4.3 ± 0.4	3.5 ± 0.5*	4.3 ± 0.8	4.7 ± 0.7	5.3 ± 1.6
	A549	2.7 ± 0.4	2.1 ± 0.5	2.3 ± 0.5	1.9 ± 0.4	2.2 ± 0.3

The values were obtained from cell cultures incubated with the concentration of the *Lippia alba* essential oil (LaEO) that inhibited cholesterol synthesis by 50% (IC$_{50CS}$). LaEO chemotypes: carvone (LaEOca), piperitone (LaEOpi), tagetenone (LaEOta), and citral (LaEOci). The data are the mean ± SD of four independent experiments performed in quadruplicate

TCP total cellular protein

* $p < 0.05$

Fig. 5 [^{14}C]Acetate incorporation into cellular lipid classes. HepG2 cells were treated with *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (4 and 16 µg/mL). The data are the mean ± SD of three independent experiments performed in quadruplicate; ***$p < 0.001$

A

B

Fig. 6 [^{14}C]Acetate incorporation into **a** the neutral lipids cholesterol (C), triacylglycerols (TAG), cholesteryl esters (CE), and the total phospholipids (PL), as well as into **b** the polar-lipid species ethanolamine glycerophospholipids (EtnGpl) ethanolamine, choline glycerophospholipids (ChoGpl), and phosphatidylserines (PtdSer). HepG2 cells were treated with *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (4 and 16 μg/mL). The data are the mean ± SD of three independent experiments performed in quadruplicate; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Fig. 7 Lipid content of HepG2 cells treated with *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (4 and 16 μg/mL). The data are the mean ± SD of three independent experiments performed in quadruplicate; *$p < 0.05$

Table 4 Effect of the *Lippia alba* (Miller) N.E. Brown tagetenone (LaEOta) chemotype on the fatty acid composition of HepG2 cells

Fatty acid	LaEOta (μg/mL)		
	0	4.0	16.0
16:0	30.7 ± 5.7	29.3 ± 5.1	29.3 ± 2.6
16:1	8.7 ± 3.5	8.0 ± 0.1	8.8 ± 0.7
18:0	13.2 ± 1.3	15.8 ± 2.9	17.5 ± 0.9
18:1n-9	24.7 ± 0.1	25.2 ± 3.8	23.5 ± 0.8
18:1n-7	14.4 ± 0.1	12.7 ± 2.4	13.7 ± 0.8
18:2n-6	3.0 ± 1.2	4.0 ± 0.6	3.1 ± 0.6
20:4	4.9 ± 2.3	4.8 ± 1.3	3.9 ± 1.0
22:6	5.6 ± 3.7	2.7 ± 1.2	4.6 ± 2.1

The values are the mean ± SD ($n = 4$)

results suggest an inhibition of squalene synthetase that redirects the intermediate farnesyl pyrophosphate towards the synthesis of other end products of that branched pathway, such as ubiquinone. Apart from this effect, all four LaEOs decreased cholesterol synthesis, but did not modify cellular cholesterol content. On the basis of these results, we propose that the decline in cholesterol caused by the inhibition of its synthesis is likely offset by an increase in the uptake of extracellular cholesterol, in this instance from the culture medium. Whereas we have found no reports on the cholesterol-lowering effect of *L. alba* EOs, studies in murine animal models in our laboratory, as well as certain data from the literature have indicated that monoterpenes (major components of these EOs), in addition to decreasing cholesterogenesis, produce an increase in the expression of low-density lipoprotein receptors (those responsible for the uptake of exogenous cholesterol) and a decrease in plasma cholesterol levels [24–27].

Although the four chemotypes exhibited similar effects on the cells in all the aspects evaluated, LaEOta was the most effective one in having the lowest IC$_{50}$ values for cholesterol synthesis in both cell lines (Table 2). Otherwise, the concentration of LaEOta that inhibited cholesterol synthesis by more than 80% (16 μg/mL) did not decrease the content of free cholesterol, a structural component of membranes, but did decrease the amount of cholesteryl esters (Fig. 7), those being one of the major components of the LDs. LDs are intracellular lipid-storage structures with a core of esterified lipids (mostly triacylglycerols and cholesteryl esters) encased by a phospholipid monolayer and a coat of specific proteins [28, 29]. Hepatocytes use LDs to synthesize the circulating lipoproteins that carry
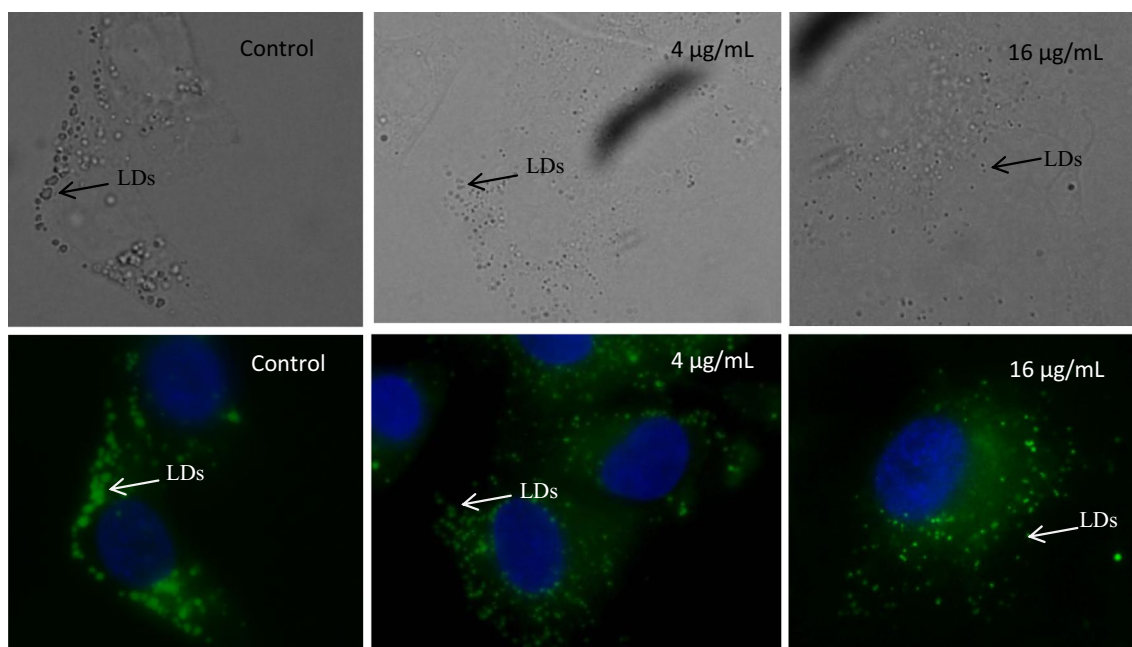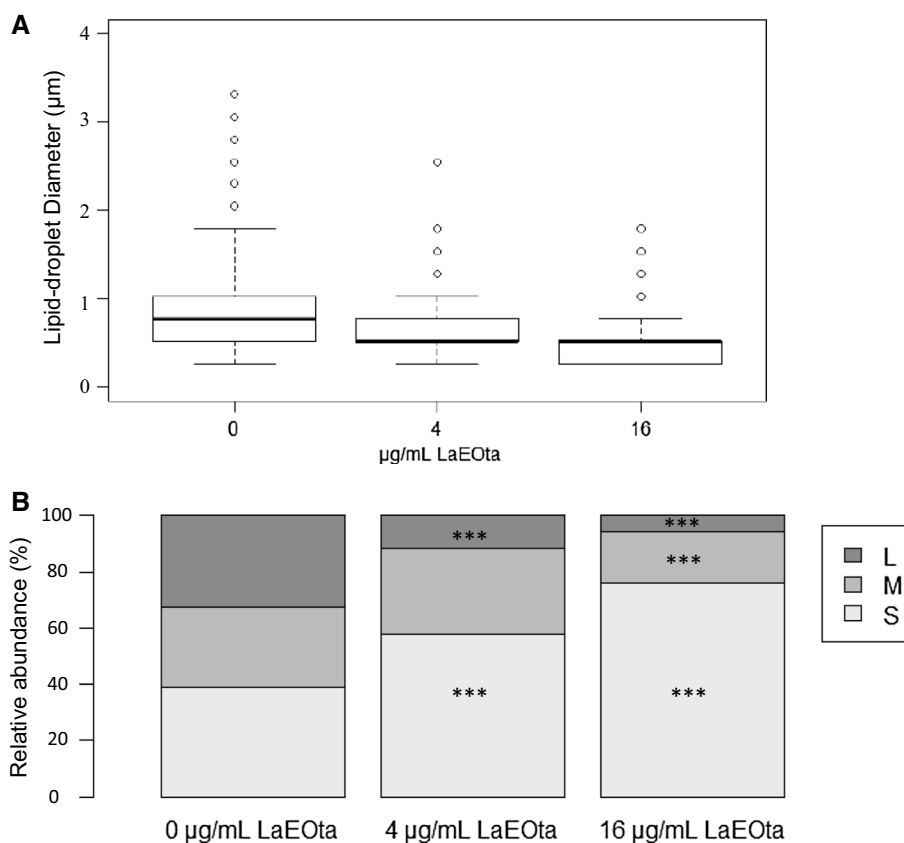
Fig. 8 Lipid droplets (LDs) in HepG2 cells incubated with the *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (4 and 16 µg/mL) compared to controls. Cells were visualized under bright-field (*upper panels*) and fluorescence (*lower panels*) microscopy. Nuclei and lipid droplets were stained with DAPI (*blue*) and BODIPY 493/503 (*green*), respectively. The photographs correspond to representative observations. Magnification: ×100 (color figure online)

Fig. 9 Lipid droplet (LDs) diameters in HepG2 cells incubated with or without the *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (4 and 16 µg/mL). **a** *Box plots* representing LDs diameters, indicating significant differences between all three groups ($p < 2.2 \times 10^{-16}$) and between the treated cells and the controls. The data are from 2000 LDs measured under each experimental condition. **b** Percent abundance of the three LD size classes defined as small (S) at ≤0.51 µm, medium (M) at between 0.51 and 1.02 µm, and large (L) at >1.02 µm. Each LD size category for a given treatment was compared with the corresponding control size range, ***$p < 0.001$
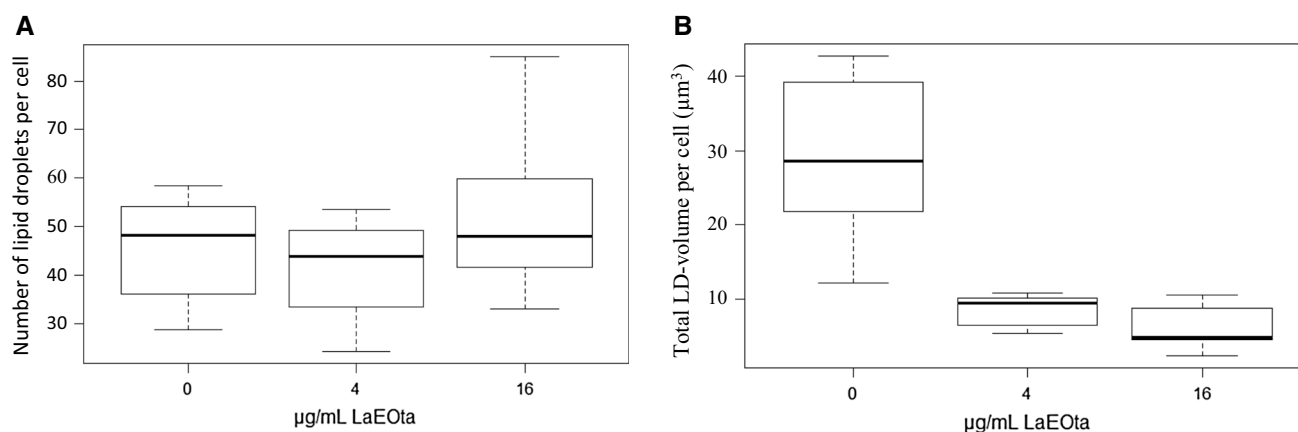
Fig. 10 Total number (**a**) and volume (**b**) of the lipid droplets (LDs) populations in HepG2 cells incubated with or without the *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (LaEOta) (4 and 16 μg/mL). LDs volume in cells incubated with 16 μg/mL of LaEOta were significantly different from controls ($p < 0.05$). The data of the three experimental conditions were obtained from the analyses of the LDs in cells imaged in seven microscopical fields with a mean number of eight cells each

Fig. 11 Effect of the *Lippia alba* (Miller) N.E. Brown chemotype tagetenone (LaEOta) on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) levels in HepG2 cells. Simvastatin was employed as a positive control for increased HMGCR levels. *Lower panel* bands obtained by immunoblotting. β-actin was used as a loading control. The data are expressed as the mean ± SD of three independent experiments. *IQ* intensity quantified
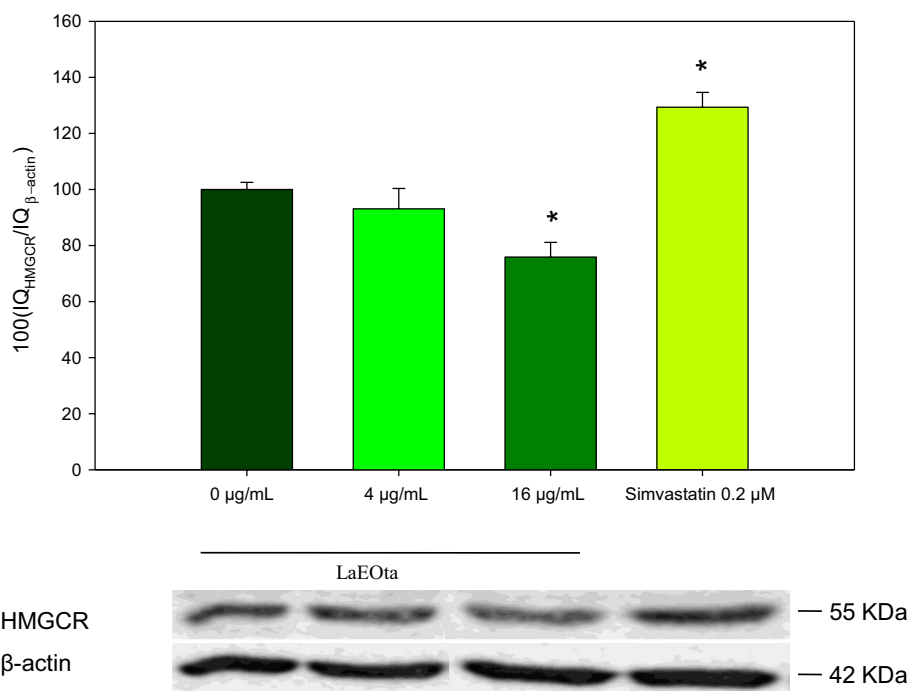
triacylglycerols in the blood (i.e., the VLDLs). In the present work, we demonstrated that the amount of triacylglycerols, cholesteryl esters, and phospholipids (Fig. 7), as well as the sizes and total volume of the LDs (Fig. 9), decreased in hepatic cells treated with LaEOta.

These results have demonstrated that LaEOta possesses both a hypocholesterogenic and a hypolipogenic capability. This conclusion is reinforced by the results obtained with simvastatin, a drug widely used as a hypocholesterolemic agent that decreases cholesterol synthesis, but also increases the synthesis of triacylglycerols and

phospholipids (data not shown). The hypocholesterogenic effect of simvastatin results from a competitive inhibition of HMGCR catalysis with a consequent compensatory increase occurring in the enzyme-protein levels [30]. In contrast, hypocholesterogenic concentrations LaEOta very likely also inhibited HMGCR activity at as low as 2 μg/mL—a concentration that was seen to decrease cholesterol synthesis (Fig. 2a)—but instead of increasing enzyme-protein levels, at 16 μg/mL this EO chemotype significantly decreased the amount of HMGCR protein (Fig. 11). In addition when cells were treated with LaEOta, the

incorporation of [^{14}C]acetate into cholesterol (Fig. 2a) decreased to a greater extent than did the radiolabelling of nonsaponifiable lipids (Fig. 5), with those effects being accompanied by a redistribution of carbon into ubiquinone, thus suggesting an inhibition of other enzymes in the MP downstream from farnesyl pyrophosphate. Therefore, the mechanism by which this *L. alba* chemotype decreases cholesterol synthesis is different from that of simvastatin, in that with the latter drug concentrations, which significantly inhibit cholesterol synthesis also decrease ubiquinone synthesis as one of the adverse medical side effects [31]. This work also demonstrated that *L. alba* EOs decreased cholesterol and triacylglycerols synthesis, as well as accumulated intracellular storage lipids, two cellular events usually associated with a hypolipidemic response.

The data herein reported consequently contribute to a more detailed understanding of the action of *L. alba* EOs and suggest that, if used for the treatment of cardiovascular diseases, these oils could provide more extensive health benefits while being hampered by fewer adverse side effects than the statins.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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