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Evaluation of combinations of essential oils and essential oils with hydrosols on antimicrobial and antioxidant activities

[Evaluación de combinaciones de aceites esenciales y aceites esenciales con hidrosoles sobre actividades antimicrobianas y antioxidantes]

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Abstract

Context: Essential oils (EO) are commonly extracted from plants by steam distillation in which an aqueous phase called hydrosol (HD) is obtained. Unlike EO, hydrosol studies have been limited despite the interest of the food, cosmetic and phytotherapeutic industries to find natural preservative alternatives to synthetic ones.

Aims: To evaluate the *in vitro* antimicrobial and antioxidant efficacies of combinations of essential oils (EOs) and essential oils and hydrosols (HDs) of *Lippia alba*, *Rosmarinus officinalis*, and *Thymus vulgaris*.

Methods: The EOs and HDs were characterized by gas chromatography with flame ionization detector and gas chromatography coupled with mass spectrometry. Then, they were screened against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* using the microdilution method for the four first strains and the agar diffusion method for *Aspergillus niger*. Antioxidant capacity was evaluated using ABTS method.

Results: Interactions between essential oils, and essential oils and hydrosols were found to be as microbicide, and for the first time, antioxidant using the fractional inhibitory concentration. When compared with individual EOs, EO-EO combinations diminished the microbicide minimum concentration.

Conclusions: The *Thymus vulgaris* EO-HD combination, in comparison with individual extracts, diminishes by four times the MBC against *Escherichia coli* and decrease by half their antioxidant capacity.

Keywords: essential oil; fractional inhibitory indices; hydrosol; *Lippia alba*; *Rosmarinus officinalis*; *Thymus vulgaris*.

Resumen

Contexto: Los aceites esenciales (AEs) se extraen comúnmente de las plantas por destilación con arrastre de vapor en la que se obtiene una fase acuosa llamada hidrosol (HDs). A diferencia de los AE, los estudios de los HD han sido limitados a pesar del interés de las industrias alimentaria, cosmética y fitoterapéutica de encontrar alternativas naturales de conservación.

Objetivos: Evaluar la eficacia antimicrobiana y antioxidante *in vitro* de las combinaciones de AEs, AEs e HDs de *Lippia alba*, *Rosmarinus officinalis* y *Thymus vulgaris*.

Métodos: Los AEs e HDs fueron caracterizados por cromatografía de gases con detector de ionización en llama y cromatografía de gases acoplada a espectrometría de masas. Luego se realizaron pruebas de detección contra *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* y *Aspergillus niger* utilizando el método de microdilución para las cuatro primeras cepas y el método de difusión en agar para *Aspergillus niger*. La actividad antioxidante se evaluó utilizando el método ABTS.

Resultados: Se encontró que las interacciones entre los aceites esenciales, aceites esenciales e hidrosoles eran microbicidas y, por primera vez, antioxidantes usando la concentración inhibidora fraccionada. Cuando se compararon con AEs individuales, las combinaciones AE-AE disminuyeron la concentración microbicida mínima.

Conclusiones La combinación de tomillo AE-HD; en comparación con los extractos individuales, disminuye en cuatro veces el MBC contra *Escherichia coli* y disminuye a la mitad su actividad antioxidante.

Palabras Clave: aceite esencial; hidrosol; índices inhibición fraccionado; *Lippia alba*; *Rosmarinus officinalis*; *Thymus vulgaris*.

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INTRODUCTION

There is a pressing need to develop new, effective and eco-friendly fungicides and preservatives. As expressed by Kumar et al. (2008), although different synthetic chemicals such as fungicides or preservatives have enabled industry to limit losses, the use of these substances implicates issues such as residual toxicity, carcinogenic, hormonal imbalance, and spermatotoxicity that affect environmental and human health. Furthermore, the excessive use of these substances has caused some microorganisms to develop resistance mechanisms to most synthetic fungicides rendering them useless. Gholoum (2013) reported that commonly used antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are added to food to improve their shelf-life, make the food ingested toxic or carcinogenic by increasing the activity of microsomal enzymes (Gholoum, 2013).

Essential oils (EO) are commonly extracted from plants by steam distillation in which an aqueous phase called hydrosol (HD) is obtained. Unlike EO, hydrosol studies have been limited despite the interest of the food, cosmetic and phytotherapeutic industries to find natural preservative alternatives to synthetic ones (Tornuk et al., 2011; Hamedi et al., 2017).

Few studies report the evaluation of HD antioxidant capacity (Aazza, 2011; Djabou et al., 2014). However, this by-product of distillation can offer many benefits. Its use in products can be advantageous not only as a source of therapeutic principles, but also as a preservative, and especially, in phytotherapy, which includes aromatherapy (Price and Price, 2004).

On the other hand, numerous studies (Burt, 2004; Oussalah et al., 2007; Gutierrez et al., 2008b; Paparella et al., 2008; Nedorostova et al., 2009; Nowak et al., 2012; Miladi et al., 2013; Pandey et al., 2016) report on the antimicrobial efficacy of EO. The authors underscore the importance of limiting their fragrance and aroma to improve the organoleptic characteristics of food products and cosmetic scents. The use of combinations could minimize these sensory impacts, and, as reported by Gutierrez et al. (2009), control some bacteria known to develop resistance to natural antimicrobials like *Pseudomonas* spp. However, there are few studies that assess the antioxidant and antimicrobial activity of essential oil combinations, and none that evaluate the use of EO and HD combinations.

In this study, the positive interactions of EO and HD combinations on antimicrobial and antioxidant activities were validated and characterized. Two common European EO, *Thymus vulgaris* and *Rosmarinus officinalis* were selected because of the available information, and legal authorization for use in different industrial fields and defined their chemotypes, camphor and thymol, respectively. The antimicrobial and antioxidant capacity, and the hepatoprotective potential of the two EO have been evaluated and reported by various researchers (DiPasqua et al., 2005; Imelouane et al., 2009; Hajlaoui et al., 2010; Zaouali et al., 2010; Miladi et al., 2013; Raskovic et al., 2014; Gemeda et al., 2015; El-Newary et al., 2017).

In aromatherapy, rosemary (*Rosmarinus officinalis*) EO chemotype (CT) camphor is used to treat myalgia, muscular cramps, rheumatism, hypertension, and hepatomegaly as well as heart weakness. thyme (*Thymus vulgaris*) chemotype thymol is indicated to treat infectious pathologies and weariness (Franchomme et al., 2001).

White verbena (*Lippia alba*) CT carvone, the last species studied, known as *pronto alivio*, is native from Latin America. In traditional medicine, its leaves and flowers are used in infusions to treat gastrointestinal or digestive disorders, as well as spasms, biliary colic and as an expectorant, febrifuge and sudorific. Antispasmodic properties are also attributed to this oil (Blanco et al., 2013). The chemotype studied in this work is carvone. Results obtained by Vale et al. (1999) suggested that this EO has an anxiolytic effect. A Colombian group of researchers -using linoleic acid oxidation methods- reported that the antioxidant capacity of this EO had a similar effect to that of vitamin E (Stashenko et al., 2004).

This study aims to evaluate the *in vitro* antimicrobial and antioxidant efficacies of combinations of essential oils (EOs) and essential oils with hydrosols (HDs) of *Lippia alba*, *Rosmarinus officinalis*, and *Thymus vulgaris*.

MATERIAL AND METHODS

Plant material

Aerial parts of *Thymus vulgaris* and leaves and flowers of *Rosmarinus officinalis* were collected from the FARMAVERDE cooperative farm located in Usme, in the south of Bogota, Colombia (Latitude: 4°26'28.19"N; Longitude: -74°09'4.80"W). Leaves of *Lippia alba* were collected near Guaduas, in the department of Cundinamarca, Colombia (Latitude: 5°04'0.91"N; Longitude: -74°35'41.96"W).

Thymus vulgaris was compared with another specimen botanically identified in the National Herbarium of Colombia by Fernández AJL (COL 501873). *Rosmarinus officinalis* and *Lippia alba* were identified by Aguirre CJ with the next respective specimen codes; COL-518926 and COL-518927 in the National Herbarium of Colombia.

All plant materials cultivated under organic and ecologic conditions were collected during blooming, early in the morning, in sunny weather, and dried naturally in the shade on shelves. In this study were collected 71.0, 48.5 and 72.2 kg of fresh material of *Thymus vulgaris*, *Rosmarinus officinalis*, and *Lippia alba* and 21.0, 28.5 and 19 kg of dried material, respectively.

Essential oil and hydrosol isolation

Steam distillation to obtain essential oil and hydrosols from the dried plant material of the three-species listed previously was performed in FARMAVERDE cooperative (Bogota, Colombia), by mean of hydrodistillation. The first 13.5 L of HD from each distillation were recovered, and EO was dried using anhydrous sodium sulfate (Na_2SO_4). The HD and EO were kept refrigerated at 4°C until they were analyzed and tested. The EO and HD were selected based on aromatherapeutic safety and efficacy, and on antimicrobial and antioxidant properties as preservatives.

Gas chromatography analysis

The gas chromatography analyses of EO were carried out using a Varian 3900 Gas Chromatograph equipped with a flame ionization detector (GC-FID) (Varian, Nederland). The column used was an Elite 5 MS column (30 m x 0.25 mm x 0.25 µm) (Perkin Elmer, Nederland). The helium carrier gas flow rate was 1.0 mL/min. The oven temperature was initially set at 50°C for 2 min, then, 4°C/min up to 160°C, 8°C/min up to 220°C, and 15°C/min up to 280°C for 5 min. Injector and flame ionization detector temperatures were 250°C and 290°C, respectively. One µL of each EO was injected with a split ratio of 1:200.

Mass spectrometry analysis

Gas chromatography with mass spectrometry (GC-MS) analyses were carried out with an Agilent Technologies-6850 Series II Gas Chromatograph (GC) using a DB-5MS (5% phenyl methylpolysiloxane) column (60 m × 0.25 mm × 0.25 µm) (Agilent Tech., USA). The GC was equipped with an Agilent 5975B mass selective (MS) detector used in the full scan mode to monitor mass unit from 30 to 500 m/z with an electronic impact of 70 eV. The helium carrier gas had a flow rate of 1.0 mL/min. The injector and detector temperature were 250°C and 230°C, respectively. The quadrupole temperature was 150°C. The oven temperature settings used with the DB-5MS column were 100°C during the first 2 minutes, then, 4°C/min until 250°C, 35°C/min until 300°C and the last 5 minutes at 300°C. Preparation of the HD to be injected is explained previously. Simultaneous extraction and concentration of compounds from the vapour phase of the HD was carried out by head space solid-phase microextraction (HS-SPME) using a fused silica fiber coated with 65 µm thick PDMS/DVB acquired from Supelco (Bellefonte, PA, USA) (Stashenko et al., 2007). Chromatographic analysis was performed with the same chromatograph, columns and conditions used previously, except the injection that was performed in splitless mode using an SPME device (Bellefonte, PA, USA) for injection. Identification of EO and HD constituents was conducted by comparing the mass spectra with those reported in the NIST (2005) library and the retention index relative to n-alkanes with those reported by Adams (2007) and other literature data (Babushok et al., 2008; Stashenko et al., 2010).

GC-FID measurements were performed at the Laboratory of Agroindustrial Chemistry of the University of Toulouse (France) using a GC Varian 3900, and the column was an Elite 5MS (1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane) of

30 m x 0.25 mm x 0.25 µm. While, GC-MS measurements were performed in the Chromatography Laboratory of the Pontificia Universidad Javeriana (Colombia) using an Agilent Technologies-6850 Series II Gas Chromatograph (GC) equipped with an Agilent 5975B mass selective (MS), and the column was a DB-5MS (5% phenyl methylpolysiloxane) of 60 m × 0.25 mm × 0.25 µm.

Antimicrobial activity

All microbial strains were obtained from Pontificia Universidad Javeriana Microorganism Collection Bogotá Campus (CMPUJ Certification: National collection registry No. 148, WFFC and WDMC No.857) were used. A 20% glycerol bank was established and kept at -70°C, from which all antimicrobial assays were carried. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 25923 were used in this study. The cultures were kept at -70°C in 20% glycerol, in tryptic soy broth (TSB; Oxoid, Basingstoke, UK). The cultures were grown in TSB at 37°C for 24 h to obtain the sub-cultures, which were inoculated on tryptic soy agar (TSA; Oxoid, Basingstoke, UK) at 37°C for 24 h. Working cultures were prepared from sub-cultures in Mueller-Hinton broth at 37°C for 4 h and adjusted to the required concentration of $1.5x10^8$ UFC/mL to 0.5 Mac Farland standards (BioMérieux Inc., Craponne, France) (Schwalbe et al., 2007). *Candida albicans* CMPUJH022 was maintained at -70°C in 20% glycerol in malt extract broth (Oxoid, Basingstoke, Hampshire, England) and grown at 37°C for 24 h to obtain the sub-cultures, which were inoculated on Sabouraud agar (SA; Merck, Germany) at 37°C for 24 h. Working cultures were prepared from sub-cultures on SA at 37°C for 4h and adjusted to the required concentration of $1.5x10^8$ UFC/mL to 0.5 Mac Farland standards (Biomérieux Inc., Craponne, France). *Aspergillus niger* ATCC 16404 was grown on SA during five days at 25°C. Conidiospores were recovered from this culture using 2 mL of sterile saline solution that was aliquoted by 100 µL for later use. To obtain monosporic cultures, 100 µL of conidiospores solution were massively seeded on SA. Filter paper discs (5 mm) were then placed on the agar and incubated at 25°C for five days (Schwalbe et al., 2007).

Microwell dilution method

The antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* was determined by the microwell dilution method using a Bioscreen C microbiological growth analyser (Labsystems, Helsinki, Finland) as reported by Medina et al. (2012) and Hay et al. (2015). Optical density measures obtained by the Bioscreen represent the turbidity caused by cell growth. One hundred and fifty microliters of bacterial solution with Mueller-Hinton Broth (MHB) for bacteria or malt extract (Oxoid Ltd., Basingstoke, Hampshire, England) for *Candida albicans* were prepared with the working culture, obtained as described previously, before diluting 150 µL of each sample dilution with dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Three wells with 300 µL of MHB were used as negative controls and three as positive controls with gentamicin (Oxoid Ltd.) (100 µg/mL) for *Escherichia coli* and *Pseudomonas aeruginosa*, vancomycin (Oxoid Ltd.) (100 µg/mL) for *Staphylococcus aureus*, and amphotericin B (Sigma-Aldrich, St. Louis, USA) (1250 µg/mL) for *Candida albicans*. The concentration ranges used respectively for uncombined *Thymus vulgaris*, *Rosmarinus officinalis*, and *Lippia alba* EO against bacteria and fungi were 100 to 1600, 1874 to 10000, and 625 to 40000 µL/L, respectively. Concentrations used with uncombined HD were between 125000 and 500000 µL/L. Lastly, the minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) were confirmed by pouring 5 µL of each test solution into Petri dishes for *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* during 24 h at 37°C. The thiazolyl blue tetrazolium bromide [3-(4,5-dimethyl thiazol-2-yl-2,5-diphenyltetrazolium bromide] (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was applied for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococuus aureus* as reported by (Mosmann, 1983). Each experiment was performed three times in triplicate (Medina et al., 2012; Hay et al., 2015).

Agar dilution method

The minimum fungicidal concentrations (MFC) against *Aspergillus niger* of essential oils and hydro-

sols were determined by the agar dilution method as reported by Hammer et al. (1999), with modifications. Potato Dextrose Agar (PDA) media were prepared with serially diluted EO and HD at 30°C just before solidification, and poured into small Petri dishes. A disc containing monosporic cultures of *Aspergillus niger* prepared as described above was placed in the Petri dish center. The preparation was incubated at 25°C for 5 days. MFC was defined as the lowest concentration at which no growth was observed. Each assay was repeated three times in triplicate. Mean values were calculated for MFC. Three negative controls were prepared with PDA and three as positive controls on other media prepared with voriconazole at 10 µg/mL. The concentration ranges used were the same as those used for the microwell dilution method (Hammer et al., 1999).

Antioxidant capacity by ABTS method

The antioxidant capacity of all the combined and not combined EO and HD was assessed using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging capacity evaluation as reported by (Re et al. (1999) and modified by (Fitsiou et al., 2016; González et al., 2017). The ABTS, purchased from Sigma–Aldrich (St. Louis, MO, USA), was oxidized to the stable cation radical, $ABTS^{+\cdot}$ by the reaction of 20 mg/L ABTS mixed with 2.5 mg/L potassium persulphate (K_2SO_8) Sigma–Aldrich (St. Louis, MO, USA) in deionized water. It was kept in darkness for 16 hours at room temperature and its absorbance was adjusted to 0.73 at 740 nm. Initially, for each sample, six serials two-fold dilutions were prepared to define their optimal concentration range. Then, four dilutions of each sample were mixed with ethanol, and 10 µL from each one was added to 240 µL ABTS. The next four concentration ranges were defined for *Thymus vulgaris*, *Rosmarinus officinalis*, and *Lippia alba* EO at 0.75 to 6.0, 250 to 2000, and 20 to 160 µL/L, respectively. Concentrations used were between 1500 and 40000 µL/L with uncombined HD. This last concentration was the highest that can be used in these conditions. Each experiment was performed three times in triplicate. The absorbance at 740 nm was read at time 0 each 5 until 120 minutes. The inhibition percentage was calculated using the inhibition equation $\%I = [(A_0-A_t)/A_0] \times 100$ where A_0 is the absorbance of control (ABTS without sample), and at is the absorbance in the presence of sample at t time. The sample concentration required to reduce ABTS to 50% (IC_{50}) was obtained by calculating the inhibition percentage against sample concentrations. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) purchased from Sigma–Aldrich (St. Louis, MO, USA) was used as a synthetic antioxidant reference (Fitsiou et al., 2016; González et al., 2017).

The time required to reach the IC_{50} is defined by TIC_{50}, and depending on its value, the antioxidant will be categorized as fast (<5 min.), intermediate (5< TIC_{50} <30 min) or slow (>30 min.) (Sanchez-Moreno et al., 1998). The Sánchez-Moreno team defined a parameter to express antioxidant capacity, the antiradical efficiency (AE), which combines IC_{50} and TIC_{50} or AE = (1/ IC_{50}) x T IC_{50}, which considers not only the result, but also the time. Therefore, calculating the IC_{50} in the time corresponding to the steady phase beginning is important, taking into consideration the antioxidant characteristic of each extract studied.

Interactions study

The evaluation of antioxidant and antimicrobial activities for EO and HD combinations was performed as described above. ABTS free radical-scavenging capacity and antimicrobial activity on different strains were firstly evaluated with uncombined EO and HD. Then, for each combined extract, concentrations above and below IC_{50} for antioxidant capacity, MBC and MFC for antimicrobial activity were used. The interaction analysis of combinations on antimicrobial activity was assessed by calculating the fractional bactericidal concentration (FBC) and fractional fungicidal concentration (FFC) indices as reported by Gutierrez et al., (2008a) and (EUCAST, 2000), with modifications. The following equation was used for each activity considering FMC as the fractional microbicide concentration for bacteria or fungi: FMC = FMCA + FMCB. MMC was the minimum microbicide concentration (MBC for bacteria or MFC for fungi) of extracts A and B when combined or not combined. Therefore, FMCA and FMCB were respectively equal to MMCA combined/MMCA not combined and MMCB combined/MMCB not combined. A similar mathematical

method of fractional concentration indices explained above was adapted to the combinations antioxidant capacity to evaluate the fractional antioxidant concentration (FAC). Per the terminology relating to methods to determine the of susceptibility of bacteria to antimicrobial agents (EUCAST, 2000), the effects of the combinations were interpreted as follows, depending on the fractional inhibition concentration (FIC) value for each activity (FBC, FFC, and FAC): Synergy (S) = FIC ≤ 0.5; addition (A) = 0.5 < FIC ≤ 1.0; indifference (I) = 1.0 < FIC < 2.0; antagonism (Ag) = FIC ≥ 2.0

Statistical analysis

Variance analysis was conducted and differences between variables were assessed for significance by one-way ANOVA using an SPSS 11 (Statistical Package for the Social Sciences) program. Differences at $p<0.01$ were considered statistically significant (Ekstrom and Sørensen, 2014).

RESULTS AND DISCUSSION

Rosmarinus officinalis, and *Lippia alba* HD did not show significant antioxidant or antimicrobial activity. Consequently, only the *Thymus vulgaris* HD was used combined with the EO.

Chemical composition of EO and HD

The yields of *Lippia alba*, *Rosmarinus officinalis* and *Thymus vulgaris* EO obtained by distillation were 0.5, 0.3 and 0.1% respectively, based on the weight of fresh plant material. Table 1 shows the results of the relative composition of the EO and HD obtained by distillation of shade-dried plant materials. The assessment by GC-FID of the chemical composition analysis determined the relative percentage of chemotypes carvone, camphor and thymol for *Lippia alba*, *Rosmarinus officinalis* and *Thymus vulgaris* EO, respectively. The same main compounds were found in HD, which have a lower molecular diversity than EO. The results present only the molecules that had a concentration greater or equal to 1.0%. Six compounds representing 94.4% were identified in *Lippia alba* EO and four compounds in its HD. In *Rosmarinus officinalis* EO and HD, eleven and five compounds representing 89.0 and 98.0% were identified and in *Thymus vulgaris* EO and HD, nine and two compounds were identified representing 87.0 and 100.0%.

Table 1 shows some differences between the values of the linear retention index (LRI) calculated for the compounds present in the EO and in the HD. According to several authors, these differences correspond to the variability of the tests and the complexity of the samples (Peng, 2010; Stashenko et al., 2010). The variations in the interlaboratory results for the LRI values are due to the difference in the initial temperature of the column, the difference in the oven temperature setting, the difference in column length, phase polarity stationary, and column status (contamination, activity, retention capacity, and manufacturer).

For the GC-FID analysis, the data were obtained with a peak matching (>95%) between experimental mass spectra and data bases.

ABTS free radical scavenging capacity of individual EO and HD and their combination

The antioxidant capacity of individual and combined EO and HD was measured using the ABTS assay to evaluate their free radical scavenging capacity, which was expressed as IC_{50} in µL/L. The IC_{50} results for individual and combined EO and HD are shown in Table 2. For each combination, the two results were calculated according to the concentration range of the first and second extract to compare the extracts within the combination with the individual extracts.

IC_{50} was intentionally calculated at 20 and 120 min during the kinetic of antioxidant reaction. The first was used as a point of comparison with previous studies. The second was used because it corresponds to the steady phase beginning of the *Lippia alba* and *Rosmarinus officinalis* EO antioxidant reaction. Because of this, the difference between 20 and 120 min was significant for these extracts, especially for *Rosmarinus officinalis* EO, which showed 110.4 µL/L *versus* 74.8 µL/L for *Lippia alba* EO, and 3704.4 µL/L *versus* 1516.7 µL/L for *Rosmarinus officinalis* EO. Additionally, the standard deviation was more significant at t=20 min than at t=120 min; SD ± 13.2 *versus* SD ± 2.5 for *Lippia alba* EO and SD ± 251.3 *versus* SD ± 54.5 for *Rosmarinus officinalis* EO, respectively. The same difference is observed for *Thymus vulgaris* HD but in a lesser proportion;

however, its standard deviation was higher at 120 min than at 20 min. Fig. 1 shows the kinetics of antioxidant reactions of three EO, one HD and the Trolox as positive control used. The *Thymus vulgaris* EO and HD had similar behaviour; both absorbance levels decreased quickly reaching their steady phase before 10 minutes. *Lippia alba* and *Rosmarinus officinalis* EO had a slower kinetic reaction, which implies that almost 70 or 80 minutes was required to define the IC_{50}.

These variations mark notable differences in FIC values and consequently for interaction definitions as shown in Table 3 for all combinations except for the *Thymus vulgaris* EO-HD combination. *Lippia alba* EO and *Rosmarinus officinalis* EO combinations had an antagonism at 20 minutes and indifference at 120 minutes. Combinations of *Thymus vulgaris* EO with *Rosmarinus officinalis* and *Lippia alba* EO change their behaviour from indifference at 20 minutes to addition at 120 minutes.

One of the advantages of ABTS compared with 2,2-diphenyl-1-picrylhydrazyl (DPPH) is that ABTS can be solubilized in aqueous and organic media where the antioxidant capacity can be due to hydrophilic and lipophilic components (Arnao, 2000) contained by the EO. The results in this study show that *Lippia alba* and *Rosmarinus officinalis* EO are slow antioxidants. The difference of inhibition percentage between 20 and 100 minutes was, respectively 13 and 30%. These results were significant ($p<0.01$) for *Rosmarinus officinalis* EO and have an impact on IC_{50} results, as it was mentioned previously.

The IC_{50} (3.1 ± 0.24 µL/L) of *Thymus vulgaris* EO is almost three times lower than for Trolox (8.8 ± 0.72 µL/L), a difference which can be explained by its high thymol content, an important antioxidant molecule, which justifies the use of *Thymus vulgaris* EO thymol chemotype as an antioxidant standard (Sacchetti et al., 2005). *Thymus vulgaris* HD provided an IC_{50} of 3028.1 ± 118.78 µL/L, which was like that obtained by Aazza (2011) with the *Thymus vulgaris* HD chemotype, carvacrol. However, *Rosmarinus officinalis* and *Lippia alba* HD showed no activity below 40000 µL/L, the maximum concentration that could be used for HD to assess the antioxidant capacity at this method scale.

In combination with *Lippia alba* EO and *Rosmarinus officinalis* EO, *Thymus vulgaris* EO IC_{50} decreased significantly showing an addition interaction with the two EO at 120 minutes. However, when mixed, the *Lippia alba* and *Rosmarinus officinalis* EO combination showed indifference.

Antimicrobial activity of individual EO and HD and their combinations

Minimum bactericidal and fungicidal concentration results of individual and combined EO and HD are shown in Tables 4 and 5. The *Lippia alba* EO showed no antimicrobial activity until 40 µL/mL on *Escherichia coli*. Despite MTT test results showing a low cellular viability of 11.4 ± 0.5%, 24 hrs after incubation at 37°C, the bacteria exposed to this EO concentration showed a growth. *Rosmarinus officinalis* and *Lippia alba* EOs showed weak antifungal and bactericidal activities in comparison with *Thymus vulgaris* oil with its high concentration of thymol, a known antimicrobial and antifungal agent.

Lippia alba and *Rosmarinus officinalis* HD were inactive against bacteria and fungi. Thus, these HD were not evaluated in combination with *Thymus vulgaris* EO. *Thymus vulgaris* HD was inactive against *Escherichia coli* but showed microbicide activity on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*.

In this study, using the reproducible microdilution method with Bioscreen, the *Thymus vulgaris* EO chemotype thymol (36.1%) showed an MBC of 0.4 µL/mL; this was similar to the results obtained by Rota et al. (2008); 0.5 µL/mL using the tube dilution method with a *Thymus vulgaris* EO chemotype thymol (57.7%) against two strains of *Escherichia coli*. However, a study by Sacchetti et al. (2005) using the disc diffusion method with *Thymus vulgaris* EO characterized with the chemotype thymol (6.8%) obtained a MIC of 60 µg/mL with *Candida albicans*; with the present EO was obtained 0.2 µL/mL. This last difference can be explained by the difference of thymol contents in the two EO. This observation was verified by comparing the antimicrobial activity of the *Thymus vulgaris* EO in my study with another EO. The respective thymol and p-cymene content was 36.1% and 28.4% for the first EO and 27.3% and 37.7% for the second.

Table 1. Composition of essential oils and hydrosols (*Lippia alba*, *Rosmarinus officinalis*, and *Thymus vulgaris*)

Compounds	Essential oils [a]						Hydrosols [b]					
	L. alba		*R. officinalis*		*T. vulgaris*		*L. alba*		*R. officinalis*		*T. vulgaris*	
	RI [c]	% [d]	RI [c]	% [d]	RI [c]	% [d]	RI [c]	% [d]	RI [c]	% [d]	RI [c]	% [d]
Monoterpene hydrocarbons												
α-Pinene	-	-	935	10.1	933	1.2	-	-	-	-	-	-
Camphene	-	-	952	8.7	-	-	-	-	-	-	-	-
β-Pinene	-	-	980	7.1	-	-	-	-	-	-	-	-
α-Terpinene	-	-	-	-	1019	1.7	-	-	-	-	-	-
β-Myrcene	-	-	991	2.9	991	1.3	-	-	-	-	-	-
α-Phellandrene	-	-	1008	1.2	-	-	-	-	-	-	-	-
Limonene	1036	40.6	-	-	-	-	1090	1.6	-	-	-	-
γ-Terpinene	-	-	1060	1.4	1064	11.6	-	-	-	-	-	-
p-Cymene	-	-	-	-	1033	28.4	-	-	-	-	-	-
Oxygenated monoterpenes												
1,8-Cineole	-	-	1037	21.1	-	-	-	-	1100	38.2	-	-
Linalool	-	-	-	-	1107	3.2	-	-	-	-	-	-
Camphor	-	-	1156	29.2	-	-	1221	1.5	1220	51.9	-	-
Terpinen-4-ol	-	-	1179	4.0	1177	1.3	-	-	-	-	-	-
Verbenone	-	-	1213	2.1	-	-	-	-	1274	1.8	-	-
Geranial	1266	1.3	-	-	-	-	-	-	-	-	-	-
Bornyl acetate	-	-	1286	1.1	-	-	-	-	-	-	-	-
Borneol	-	-	-	-	-	-	-	-	-	-	-	-
Sesquiterpens hydrocarbons												
β-Bourbonene	1385	1.1	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene	-	-	-	-	1423	2.2	-	-	-	-	-	-
Germacrene D	1487	8.1	-	-	-	-	-	-	-	-	-	-
Aliphatic ketones												
3-Octanone	-	-	-	-	-	-	-	-	1026	2.1	-	-
Carvone	1259	41.6	-	-	-	-	1301	92.7	-	-	-	-
Piperitenone	1345	1.8	-	-	-	-	-	-	-	-	-	-
Eucarvone	-	-	-	-	-	-	1401	2.9	-	-	-	-
Aromatic compounds												
Thymol	-	-	-	-	1315	36.1	-	-	-	-	1318	98.1
Carvacrol	-	-	-	-	-	-	-	-	-	-	1331	1.9
Total, identified (%)		**94.4**		**89.0**		**87.0**		**98.7**		**98.0**		**100**

[a] Date obtained by GC-FID on an Elite-5 MS column. [b] Date obtained by GC-MS on a DB-5 MS column. Peakin matching (>95%) between experimental mass spectra and data bases. [c] RI: Retention indexes. [d] Percentage of area.

Table 2. Antioxidant capacity (ABTS Method) of EO and HD individually and combined.

EO / HD / Standard / Combinations	IC_{50} (µL/L) [a]			
	20 min		120 min	
EOs (individual)				
Lippia alba	110.4 ± 13.2		74.8 ± 2.5	
Rosmarinus officinalis	3704.4 ± 251.3		1516.7 ± 54.4	
Thymus vulgaris	3.1 ± 0.2		2.9 ± 0.2	
HDs (individual)				
Lippia alba	>>40000		>>40000	
Rosmarinus officinalis	>>40000		>>40000	
Thymus vulgaris	3420.9 ± 73.9		3028.1 ± 118.8	
Trolox (Standard)	8.8 ± 0.7		8.3 ± 0.8	
Combinations (E1 + E2) [b]	**E1**	**E2**	**E1**	**E2**
Thymus vulgaris EO + *Lippia alba* EO	1.8 ± 0.04	48.9 ± 1.1	1.4 ± 0.05	36.6 ± 1.2
Thymus vulgaris EO + *Rosmarinus officinalis* EO	2.6 ± 0.2	863.3 ± 65.5	1.6 ± 0.1	535.9 ± 51.1
Lippia alba EO + *Rosmarinus officinalis* EO	198.8 ± 29.8	2484.8 ± 372.4	75.7 ± 11.1	945.8 ± 138.4
Thymus vulgaris EO + *Thymus vulgaris* HD	2.05 ± 0.2	1027.4 ± 102.4	2.1 ± 0.3	1034.0 ± 145.9

[a] The results are presented as mean ± SD (n=3). Due to the limited sample numbers, student's t test tested the distribution normality. Assuming sample normality; statistically, the differences between E1 and E2 were significant ($p<0.01$). [b] E1 and E2 represent the first and second essential oil or hydrosol mentioned in the "combinations" field. Abbreviations: EO: Essential oil; HD: Hydrosol.

Table 3. Antioxidant FIC indices of EO and HD combinations.

Combinations	FIC 20	Int *	FIC 120	Int *
Rosmarinus officinalis EO + *Lippia alba* EO	2.45	AN	1.61	I
Thymus vulgaris EO + *Thymus vulgaris* HD	0.86	A	0.84	A
Thymus vulgaris EO + *Lippia alba* EO	1.03	I	0.96	A
Thymus vulgaris EO + *Rosmarinus officinalis* EO	1.07	I	0.91	A

* Interactions: Results are interpreted as synergy (S, FIC ≤ 0.5), addition (A, 0.5 < FIC ≤ 1.0), indifference (I, 1.0 < FIC < 2.0) or antagonism (AN, FIC ≥ 2.0). Abbreviations: EO: Essential oil; HD: Hydrosol. FIC: Fractional inhibition concentration; Int: Type of interaction.

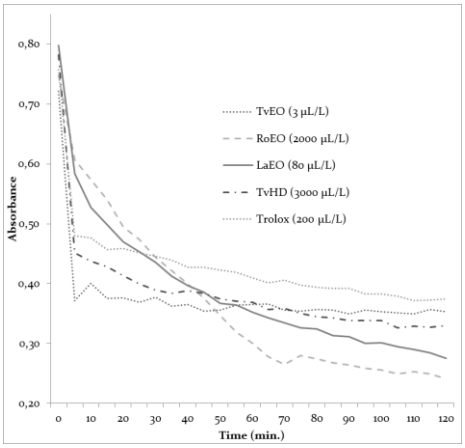

Figure 1. Reaction kinetics of three EOs, one HD and Trolox for 120 minutes.

Abbreviations: EO: Essential oil; HD: Hydrosol; Tv: *Thymus vulgaris*; Ro: *Rosmarinus officinalis*; La: *Lippia alba*.

Table 4. MBC (µL/mL) of individual and combined EO and HD on selected bacteria.

Essential oil and hydrosol	*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 9027		*Staphylococcus aureus* ATCC 25923	
EOs (individual)						
Lippia alba	>40		5.0		5.0	
Rosmarinus officinalis	7.5		7.5		3.7	
Thymus vulgaris	0.4		0.4		0.2	
HDs (individual)						
Lippia alba	>500		>500		>500	
Rosmarinus officinalis	>500		>500		>500	
Thymus vulgaris	>500		250		250	
Combinations (E1+E2) [a]	**E1**	**E2**	**E1**	**E2**	**E1**	**E2**
Thymus vulgaris EO + *Lippia alba* EO	0.2	2.5	0.2	2.5	0.2	1.2
Thymus vulgaris EO + *Rosmarinus officinalis* EO	0.2	3.7	0.2	3.7	0.2	1.9
Thymus vulgaris EO + *Thymus vulgaris* HD	0.1	125	0.1	125	0.1	125
Lippia alba EO + *Rosmarinus officinalis* EO	10.0	7.5	5.0	3.7	2.5	3.7

[a] E1 and E2 represent the first and second essential oil or hydrosol mentioned in the "combinations" field. Abbreviations: MBC: Minimum bactericidal concentration EO: Essential oil; HD: Hydrosol. The MBC value was obtained three times with triplicates by serial dilutions which showed the same value for each treatment (essential oil or hydrosol), giving a zero-standard deviation.

Table 5. MFC (µL/mL) of EOs and HDs not combined and its combinations on selected fungi.

Essential oil and Hydrosol	*Candida albicans* CMPUJH022		*Aspergillus niger* ATCC 16404	
EOs (individual)				
Lippia alba	1.2		5.0	
Rosmarinus officinalis	3.7		10.0	
Thymus vulgaris	0.2		0.4	
HDs (individual)				
Lippia alba	>500		>250	
Rosmarinus officinalis	>500		>250	
Thymus vulgaris	250		250	
Combinations (E1 + E2) [a]	**E1**	**E2**	**E1**	**E2**
Thymus vulgaris EO + *Lippia alba* EO	0.1	0.6	0.2	2.5
Thymus vulgaris EO + *Rosmarinus officinalis* EO	0.1	0.9	0.4	7.5
Thymus vulgaris EO + *Thymus vulgaris* HD	0.1	62.5	0.2	125.0
Lippia alba EO + *Rosmarinus officinalis* EO	0.6	0.9	2.5	7.5

[a] E1 and E2 represent respectively the first and second essential oil or hydrosol mentioned in combinations field. Abbreviations: MFC: Minimum fungicidal concentration EO: Essential oil; HD: Hydrosol. The MFC value was obtained three times with triplicates by serial dilutions which showed the same value for each treatment (essential oil or hydrosol), giving a zero-standard deviation of zero.

Table 6. FMC indices of EOs and HD combinations on bacteria and fungi selected.

Combinations	*Escherichia coli*	Int[a]	*Pseudomonas aeruginosa*	Int[a]	*Staphylococcus aureus*	Int[a]	*Candida albicans*	Int[a]	*Aspergillus niger*	Int[a]
Thymus vulgaris EO + *Lippia alba* EO	0.56	A	1.00	A	1.25	I	1.00	A	1.00	A
Thymus vulgaris EO + *Rosmarinus officinalis* EO	1.00	A	1.00	A	1.50	I	0.75	A	1.75	I
Thymus vulgaris EO + *Thymus vulgaris* HD	0.50	S	0.75	A	1.00	A	0.75	A	1.00	A
Lippia alba EO + *Rosmarinus officinalis* EO	1.25	I	1,.50	I	1.50	I	0.75	A	1.25	I

[a] Interaction: Results are interpreted as synergy (S, FMC ≤ 0.5), addition (A, 0.5 < FMC ≤ 1.0), indifference (I, 1.0 < FMC < 2.0) or antagonism (AN, FMC ≥ 2.0). Abbreviations: EO: Essential oil; HD: Hydrosol.

The antimicrobial activity of the first EO was two times higher against all strains (*Eschericihia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*); its IC_{50} with ABTS was 2.9 ± 0.3 µL/L. The second EO showed an IC_{50} of 3.9 ± 0.3 µL/L. These experiments were conducted with three different batches of EO and replicated three times.

Against *Aspergillus niger*, an inhibition diameter of 12 mm was obtained with a dilution 1/16 of *Thymus vulgaris* EO chemotype thymol (32.67%) (Moghtader, 2012). In a recent study by Miladi et al. (2013), using the microwell dilution method, they obtained an MBC for *Thymus vulgaris* EO chemotype thymol (41.33%) of 1.56 and 50 mg/mL against *Escherichia coli* ATCC 35218, 3.12 and 25 mg/mL against *Pseudomonas aeruginosa* ATCC 27853 and 3.12, and 25 mg/mL with *Staphylococcus aureus* ATCC 25923 for *Rosmarinus officinalis* EO chemotype cineol (24.10% *versus* 19.87% of camphor).

The same occurred with the *Rosmarinus officinalis* EO. The *Rosmarinus officinalis* EO chemotype camphor (31.8% *versus* 18.4% of 1.8 cineole) showed an MBC six times lower against *Escherichia coli* (7.5 µL/mL) and *Staphylococcus aureus* (3.75 µL/mL) and three times lower against *Pseudomonas aeruginosa* (7.5 µL/mL) than those obtained by Miladi et al. (2013). The use of Bioscreen absorbance, MTT and posterior inoculation to determine bacteria growth and its consequent MBC enabled to obtain a more precise reading than a visual assessment of turbidity. However, comparing EO activities from plant material grown in different conditions and test them on different strains is challenging.

The fractional microbicide indices (FMI) or fractional microbicide concentration (FMC) on the different strains tested and the type of interaction are presented in Table 6. *Thymus vulgaris* EO and HD combination were the only to show a synergy (FMI=0.5) against *Escherichia coli*. This last result was unanticipated. An EO-EO combination should be more effective than an EO-HD combination using concentrations ranges like those obtained for single oils and HD. However, the results obtained for *Thymus vulgaris* EO combination with *Lippia alba* and *Rosmarinus officinalis* EO presented a FIC of 0.56 and 1.0, respectively. These differences lead to further examination of the relationship between biological activity and responsible molecules. This relationship has been discussed in previous works and divided into two different opinions; (Farag et al., 1989) attribute it to the most dominant components, whereas Rota et al. (2008) attribute it to the contribution of minor components to the antimicrobial activity with a synergistic effect or influence on the EO.

As discussed by Gutierrez et al. (2008a), the mechanism of action and extract composition should be examined to explain why EO combinations with a strong individual antimicrobial efficacy fails to show synergism and why *Thymus vulgaris* HD, which has a low individual antimicrobial activity can enhance *Thymus vulgaris* EO antibacterial activity, especially on *Escherichia coli* and *Pseudomonas aeruginosa* when its MBC is divided by four (Gutierrez et al., 2008a).

The interaction between *Thymus vulgaris* and *Lippia alba* EO is thought provoking. *Lippia alba* EO

showed no bactericidal effect on *Escherichia coli* when used individually until 40 µL/mL; but, when mixed with *Thymus vulgaris* EO, its MBC decreases to 2.5 µL/mL. *Thymus vulgaris* EO MBC also decreased from 0.4 to 0.2 µL/mL. The previous implies that an additive effect is observed with an FMC=0.56 calculated with an MBC of *Lippia alba* EO equal to 40.0 µL/mL, but considering that this MBC is higher, this interaction should be a synergy. Moreover, individual *Lippia alba* EO showed an MBC of 5.0 µL/mL against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Aspergillus niger*, which was reduced by two, four and two, respectively, when mixed with *Thymus vulgaris* EO whose MBC was divided by two on *Pseudomonas aeruginosa*, and *Aspergillus niger* and did not change against *Staphylococcus aureus*. On *Candida albicans*, *Lippia alba* EO attained a higher MBC of 1.2 µL/mL, which was divided by two when mixed with *Thymus vulgaris* EO. Therefore, *Lippia alba* EO mixed with *Thymus vulgaris* EO showed an additive effect against *Escherichia coli* with a FMC index of 0.56 close to a synergic effect. This effect was emphasized using the isobologram method described by Altenburger et al. (1990).

Rosmarinus officinalis EO behaviour was different. In combination with *Thymus vulgaris* EO, its MBC was divided by two against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, by four against *Candida albicans* and decreased from 10 to 7.5 µL/mL against *Aspergillus niger*. It also showed additive effects against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* and indifference against *Staphylococcus aureus* and *Aspergillus niger*. The *Rosmarinus officinalis* EO and *Lippia alba* EO combination showed an additive antifungal activity against *Candida albicans* and an indifferent bacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Although *Rosmarinus officinalis* EO has a high camphor content, and is a molecule recognized as a stimulant of antimicrobial efficacy in terpenoids (Naigre et al., 1996), this effect was not observed.

As studied by Bassole and Juliani (2012), interpretation of interaction into combinations depends on the method used on one hand and parameters used to interpret the type of interaction on the other one. These authors present an interesting comparison between different FIC indexes used to determine the type of interaction reporting that for example, synergy can be concluded with a FIC inferior to 1 by some authors and inferior to 0.5 or 0.75 by others. Synergy with a FIC value inferior to 1 is the same interpretation than isobologram method would make. However, in this study it was decided to use the "correlation between FIC and FBC and the effect of the combination of antibacterial agents" presented by EUCAST as an official reference (EUCAST, 2000).

Thymus vulgaris and *Lippia alba* EOs combination has an addition effect, which could be explained, by a potentiating effect of carvone and limonene; *Lippia alba*'s major components which were previously reported to be active against a wide spectrum of human pathogenic fungi and bacteria tested in this study (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*) and others like *Trichophyton rubrum* responsible of onychomycosis (Aggarwal et al., 2002).

Except for *Staphylococcus aureus*, all combinations involving *Thymus vulgaris* oil had an additive effect related to its high content of thymol, which is facilitated by the swelling effect of p-cymene as reported before (Gutierrez et al., 2008b). Although MBC, MFC and antioxidant IC_{50} of *Thymus vulgaris* HD are very high in comparison with those of synthetic preservatives like parabens, if this HD is used at these concentrations as a therapeutic active principle it could ensure preservative effect in final product.

CONCLUSIONS

This work had two main achievements; assessing the antioxidant and antimicrobial activity of HDs and EOs not combined and their effect when combined. FIC index method - commonly used for the antimicrobial activity - was adapted to characterize the interaction of combinations on the antioxidant capacity. Although the IC_{50} of *Thymus vulgaris* HD obtained is very high compared with a synthetic or natural antioxidant used in the formulation of a cosmetic or phytopharmaceutical product, if it's used as therapeutic active principle, it could also allow the preservation of the product with its antioxidant efficacy. Finally, the results obtained with

Thymus vulgaris EO and HD combination or *Thymus vulgaris* and *Lippia alba* EO showed that these extracts mixed are good candidates in a finished product with their respective therapeutic indications to evaluate its preservative activity and ability to mitigate impacts on its odor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

Contribution	Hay YO	Abril-Sierra MA	Sequeda-Castañeda LG	Bonnafous C	Raynaud C
Concepts or ideas	X	X		X	X
Design	X		X	X	X
Definition of intellectual content					
Literature search	X		X		
Experimental studies	X	X	X		
Data acquisition	X				
Data analysis	X		X		
Statistical analysis	X		X		
Manuscript preparation	X		X		
Manuscript editing	X		X		
Manuscript review	X		X	X	X

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