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ORIGINAL PAPER

Chemical variability of essential oils of *Protium colombianum* from two tropical life zones and their in vitro activity against isolates of *Fusarium*

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Abstract There is considerable interest in finding replacements for the synthetic fungicides and preservatives that are currently used to control fungal pathogens; however, any alternative compounds must be safe and must prevent the development of microbial resistance. In this regard, essential oils have received special attention. *Protium colombianum* Cuatrecasas is an endemic tree found in tropical rainforests and possesses aromatic and resinous characteristics. To date, there have been no reports concerning the chemistry of this species, which belongs to a genus that represents an interesting source of essential oils that are occasionally used as antimicrobial agents. Therefore, the chemical composition of the essential oils found in *P. colombianum* collected from two life zones over a five-month period was analyzed using gas-chromatography/mass-spectrometry. A total of 92 components, comprising 97.7–99.9 % of the total composition, were identified based on their mass spectra and the retention indices. Precedence markers were identified for each life zone, and seasonality affected the samples from one life zone when rainfall was maximum. The in vitro activity against *Fusarium oxysporum* isolates from carnation and chrysanthemum, as well as a *Fusarium solani* isolate from chrysanthemum, was tested by determining the minimum inhibitory concentration. Assays using essential oils and control substances showed that terpenoid and phenolic compounds are mainly responsible for the observed anti-fungal activity. These results suggest that *P. colombianum* essential oils exhibit cytotoxicity against *Fusarium* isolates and that the search for new sources of anti-fungal essential oils should include sources that contain different ratios of terpenoid and phenolic compounds.

Keywords Anti-fungal activity · *Fusarium* spp. · *Protium colombianum* essential oil · Seasonal variation

Key message

- Essential oils of *P. colombianum* from two life zones were chemically characterized and activity against *Fusarium* isolates was tested using metabolites and controls.
- Origin markers were established for biogeographical life zones during 5 months and chemistry revealed that alkyl-benzene derivatives and phenolics were deterministic of bioactivity.
- The results suggest that sources containing different ratios of alkyl-benzene derivatives and phenolics should be included in the search for new sources of anti-fungal EO and design of future agrotechnological products.

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Introduction

Synthetic pesticides and preservatives comprise the principal strategy for controlling fungal pathogens in the field and in storage. The emergence of resistant microorganisms, strict regulations, and the need for safe and biodegradable alternatives provide the principal motivation to replace fungicides that are not considered safe for human health (Daferera et al. 2003; Bakkali et al. 2008; Naeini et al. 2010; Cardiet et al. 2011; Tak et al. 2015). Different strategies are being used to identify new antimicrobial agents—one strategy involves the development and use of so-called generally recognized as safe materials by the FDA (2014). This group includes different types of naturally occurring substances such as essential oils (EO), which are aromatic oily liquids that are obtained from plant material responsible of the odor of a plant. EO are composed of a complex mixture of monoterpenes, sesquiterpenes, phenylpropanes, and low-molecular-weight aliphatic compounds and may represent an alternative to the currently used control agents. Since they constitute a rich source of bioactive compounds, EO can act on several targets at the same time, and no particular resistance or adaption to EO has been described thus far (Bakkali et al. 2008; Faleiro and Miguel 2013; Sivakumar and Bautista-Baños 2014).

The genus *Fusarium* is found in crop plants worldwide; several species from this genus are economically relevant because of their ability to infect the vascular system and cause wilt diseases in important edible and ornamental crops such as tomato, wheat, corn, carnation, chrysanthemum, and others. *Fusarium* species also produce mycotoxins in the field and in storage, which are noxious products to animals and humans (Bai and Shaner 2004; Hashem et al. 2010). Therefore, it is important to search for new antimicrobial agents, mainly by examining plant extracts, with the goal of controlling these pathogens. In this sense, several groups have reported on the activities of EO rich in cuminaldehyde, β-pinene, terpinolene, thymol, and carvacrol against *Fusarium solani* (Mart.) Sacc. and *Fusarium oxysporum* Schlecht. (Daferera et al. 2003; Hashem et al's 2010); however, toxicity varies wide between isolates and volatile nuclei even within the same species, thus spurring research into new sources of mixtures of active compounds in EO (Naeini et al. 2010; Hashem et al. 2010).

Species of the *Protium* genus are characterized by the production of EO and resins, which are used in folk medicine for the treatment of wounds and ulcers (Rüdiger et al. 2007) and commercially for other purposes (Siani et al. 2012). Menthane-type monoterpenes are the principal compounds found in volatile fractions of this genus, and EO with high *p*-cymen-8-ol, piperitenone, thymol, and spathulenol content have shown activity against microorganisms of medical interest (*Staphylococcus aureus* Rosenbach., *Mycobacterium smegmatis* Trevisan. and *Bacillus subtilis* Ehrenberg.). Nevertheless, there are no reports on the activity *Protium* genus EO toward microorganisms with agroindustrial relevance. In addition, the aerial parts of some species from this genus have shown seasonal variation in terms of their EO composition, and different chemical profiles are observed in different life zones (Machado et al. 2003; Siani et al. 2011; Tafurt-garc and Muñoz-acevedo 2012; Martins de Moraes et al. 2013). These data indicate that plant species from this genus should be thoroughly investigated. Therefore, *Protium colombianum* Cuatrecasas, an endemic tropical species found in rainforests from 0 to 1500 m above sea level in the province of Chocó and the mountain basins of Colombia, Panamá and Ecuador, deserves attention. To date, there are no reports concerning the chemistry or bioactivity of *P. colombianum.* The purpose of the present study was to examine the chemical and biological potential of *P. colombianum* by characterizing fruit EO from two different growth zones and to evaluate their minimum inhibitory concentration (MIC) against *Fusarium* isolates from carnation and chrysanthemum. Pure substances and aromatic plant oils with different volatile profiles were included in the study as bioactivity controls to determine which components were responsible for the inhibitory effects.

Materials and methods

Chemicals

Anhydrous sodium sulfate, dihydrogen potassium phosphate, and hydrogen dipotassium phosphate were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was of reactive grade and was purchased from Sigma (St. Louis, MO). A mix of alkanes, C7–C40, was obtained from Supelco (Bellefonte, PA). Italian mandarin oil (*Citrus reticulata* Fluka W265713), eugenol (98 %), thymol (99.5 %), and carvacrol (98 %) were purchased from Sigma-Aldrich (Milwaukee, WI). *Trans*-cinamaldehyde (98 %) was purchased from Alfa Aesar (Ward Hill, MA). Sabouraud's glucose broth was obtained from Merck (Darmstadt, Germany). Potato dextrose agar was purchased from OXOID (Cambridge, England).

Plant material and essential oil extraction

The fruits of *P. colombianum* were collected in Cocorná (PC CO) and San Luis (PC SL), which are 70 and 130 km

northeast of Medellín-Antioquia, respectively. The principal characteristics of both zones are summarized in Table 1. The fruits were collected monthly during the first 5 days of October, November, and December of 2010 and January and February of 2011 to monitor variations over time. Each sample was collected in duplicate for comparisons. Rainfall registers were obtained from two meteorological stations of the Institute of Hydrology and Meteorology (IDEAM) at the sampling time: Station 23080750, located 6.15 km from the Cocorná sampling zone, and Station 23070020, located 7.88 km from the San Luis Sampling zone. The voucher specimens were deposited in the JAUM herbarium (Medellín Botanical Garden—Joaquin Antonio Uribe Medellín) under no. 60224 and 60220. The two specimens were determined to be adult females based on flower observations. Other species were used as controls. Therefore, lemongrass leaves (*Cymbopogon citratus* (DC.) Stapf), clove buds (*Syzygium aromaticum* (L.) Merr. & L.M. Perry), origanum shrubs (*Origanum vulgare* L.), cinnamon bark (*Cinnamomum triplinerve* (Ruiz & Pav.) Kosterm.), rosemary leaves (*Rosmarinus officinalis* L.), palosanto bark (*Bursera graveolens* (Kunth) Triana & Planch.), and cardamom seeds (*Elettaria cardamomum* (L.) Maton) were obtained from local markets. A sample of Bulgarian lavender (*Lavandula vera* DC.) was obtained from a local producer in the province of Stara Zagora. All material was submitted to hydrodistillation for 2 h using a Clevenger-type apparatus; the temperature was constant over the extraction. After separation from water, the samples were dried with anhydrous sodium sulfate and stored in 10-mL vials at −20 °C.

Volatile analysis

Gas chromatography–mass spectrometry (GC/MS)

GC/MS analysis was carried out on an Agilent 7890 GC apparatus (Wilmington, DE) equipped with mass selective detector (MSD) 5975C and automatic liquid sampler (ALS) 7683B using two capillary columns: HP-1MS 1 % and HP-5MS 5 % phenylpolymethylsiloxane (PMS) (30 m, 0.25 mm i.d., film thickness = 0.25 μm, J&W Scientific, Folsom, CA). The oven temperature was set at 40 °C (5 min), raised to 200 °C at 5 °C/min, held isothermally at 200 °C for 1 min and then raised to 270 °C at 5 °C/min and held isothermally for 5 min. The injector temperature was maintained at 260 °C. Injections (1 μL) were made in splitless mode using helium as the carrier gas (1 mL/min). The MSD temperatures of the ionization chamber and the MS Quad were set at 230 and 150 °C, respectively. Mass spectra and total ion currents (TIC chromatograms) were obtained by automatic scanning at 4.74 $scan^{-1}$ with energy ionization 70 eV, in the mass range of 40–300 m/z. Chromatographic peaks were checked for homogeneity with the aid of the extracted ions of characteristic fragments to optimize resolution and peak symmetry.

Quantification and identification of volatile chemicals

Components were identified via three methods. Method (a) compared the GC retention index (RI) on nonpolar columns with a series of *n*-alkanes (C7–C40) by linear interpolation. Method (b) was based on computer matching with commercial mass spectral libraries (NIST/EPA/NIH, 2008) (Adams 2001). Method (c) was based on comparisons with spectra from our laboratory library. To calculate RI, we used Automatic Mass Spectral Deconvolution and Identification System software (AMDIS 2.68) compared with standard compounds or databases (http://www.pherobase.com/database/kovats/kovats-index.php). The use of both capillary columns with different polarities of stationary phases allowed (a) the resolution of overlapping compounds that have the same retention times and (b) better component identification by calculating two retention indices for some compounds. *P. colombianum* EO were reported with a minimum content of 0.01 %. A lower quantity was considered as trace and was not quantified. For the composition of aromatic plants, the ten principal detectable components were reported to use them as controls. The proportion of each individual compound in the EO could be expressed as the percentage relative to the total compounds (Tables of composition). The areas of the GC/MS peaks depend not only on the concentrations of the corresponding compounds but also on the intensities of their mass spectral fragmentation. Therefore, the data given in composition tables (Supplementary Material, Tables 3, 4, and 5) do not express a real quantification, although they can be used to compare the samples, which was the objective of this work.

Table 1 Identification, precedence, and environment of *Protium colombianum* trees

Specimen code	JAUM voucher	Precedence	Rainfall (mm/year)	Altitude (m.a.s.l.)	Latitude	Longitude	Holdridge life zone	Environment
PC CO	60220	Cocorná	>4000	1118	6°1′1.72″	75°8′58.50″	Premontane moist forest	Clear open field
PC SL	60224	San Luis	1900–4000	706	5°56′30.4″	74°51′31.8″	Tropical wet forest	Crowded population

Pathogen isolates

The isolate of *F. oxysporum* from chrysanthemum was kindly donated by Dr. Bertha Miryam Gaviria Gutierrez from Universidad Católica de Oriente (Antioquia-Colombia). The isolate of *F. oxysporum* from carnation and the isolate of *F. solani* from chrysanthemum were obtained from Universidad EAFIT ceparium (Medellín-Colombia). All strains were purified and preserved at 23 °C on Potato dextrose Agar.

In vitro assays

MIC values corresponding to growth inhibition were determined using the broth dilution method as described previously (Wiegand et al. 2008). A total of 8 mg of individual EO and pure substances, mixed with 40 µL of DMSO and 160 µL of phosphate buffer pH 7.3, was incorporated into the fungal growth medium at concentrations of 5000, 2500, 1250, 625, 313, and 156 µg/mL. After setting, the medium was inoculated with 25 µL spore suspensions (300 spores/mL) and incubated at room temperature (23 °C) for 5 days. Three replicates were prepared for each oil concentration and each isolate. Absolute controls containing only broth media and fungal suspensions, negative controls containing broth media, EO and DMSO, and positive controls containing broth media, DMSO and thymol (5 mg/mL) were prepared and incubated under the same conditions. The MIC was defined as the lowest EO concentration that did not permit any visible fungal growth.

Results

Essential oil characterization and principal component analysis (PCA)

Fruit development occurred between October 2010 and February 2011; therefore, fruits were collected during this time to sample complete state of development and ripening. EO isolated from *P. colombianum* showed yields between 1.42 and 3.73 % across the full sampling time (Table 2). These are remarkable yields in comparison to the EO from fruits of other species of this genus (Pontes et al. 2007; Sutthanont et al. 2010). During the observation period, Cocorná EO had an average yield of 2.78 ± 1.69 % and a relative standard deviation (%RSD) of 24.86, indicating a wide dispersion in the samples and a significant variability in metabolic aspects. The highest average of EO was obtained for the samples co4a and co4b, with an average of 3.66 %, whereas the lowest was for the samples co3a and co3b, with an average yield of 1.85 %. Meanwhile, EO yields in San Luis presented an average of 2.64 ± 0.18 % and a %RSD of 6.72, indicating no significant variations in metabolism.

The chemical composition did not vary significantly over time but presented significant variations according to the origin zone. A total of 92 components were identified, which comprised 97.73–99.95 % of the total composition (Supplementary Material, Tables 3 and 4) for the sample group. The population from Cocorná showed sabinene (51.81–70.59 %) as the major compound, and the population from San Luis showed α-thujene (9.37–20.36 %), α-pinene (17.51–25.47 %), sabinene (7.73–15.04 %), and limonene (21.49–32.65 %) as the major compounds. Of all identified compounds, monoterpenes constituted the highest percentage of all components (75.62–95.64 %), followed by oxygenated monoterpenes (1.59–16.01 %), alkylbenzene (AB) derivatives (1.18–7.64 %), phenolic compounds (0.03–3.44 %), sesquiterpenes (0.40–1.22 %), and oxygenated sesquiterpenes (0.00–0.40 %). Principal component analysis (PCA) was used to identify possible relationships between volatile components and origin zone or sampling month. Two principal population groups were identified based on chemical composition (Fig. 1). Sabinene is the principal compound that contributes to population clustering of the plants from Cocorná. The population from San Luis was characterized by the α-thujene, α-pinene, and limonene content, thus establishing these components as origin zone indicators. The PCA results explained 99.1 % of the variability in the samples.

On the other hand, aromatic plants showed differentiated profiles in the chemical composition of their EO (Supplementary Material, Table 5). *C. citratus* was characterized by relatively high citronellol and geranial content; limonene was the main component in *B. graveolens* and *C. reticulata*, along with α-terpineol and γ-terpinene, respectively. Eugenol was the main component in *S. aromaticum*, along with eugenol acetate and was also found together with *trans*-cinamaldehyde in *C. triplinerve*. *O. vulgare* was characterized by high γ-terpinene and carvacrol content, whereas linalool was found with eucalyptol in *E. cardamomum* and with linalyl acetate in *L. vera*. *R. officinalis* was characterized by high limonene and eucalyptol contents. These profiles were similar to the previously reported EO compositions for these plants (Tisserand and Young 2014) and determined their bioactivity toward *Fusarium.*

Anti-fungal activity

The EO from the *P. colombianum* samples were tested for antagonistic activity against *Fusarium* isolates (Supplementary Material Table 3). EO from Cocorná did not show any action against fungal isolates. Meanwhile, EO sl3a from San Luis displayed remarkable activity against *F.*

Table 2 Rainfall of life zones and yield of isolated essential oils of *Protium colombianum*

Specimen code/life zone	Month of sampling	Rainfall (mm/month)	Oil code	Yield	Specimen code/life zone	Month of sampling	Rainfall (mm/month)	Oil code	Yield
PC CO/premontane moist forest (Cocorná)	Oct-10	461.00	co1a	2.56	PC SL/tropical wet forest (San Luis)	Oct-10	606.90	sl1a	2.85
			co1b	3.42				sl1b	2.85
	Nov-10	670.00	co2a	2.85		Nov-10	704.10	sl2a	2.56
			co2b	2.85				sl2b	2.56
	Dec-10	445.00	co3a	1.42		Dec-10	379.00	sl3a	2.56
			co3b	2.28				sl3b	2.85
	Jan-11	162.50	co4a	3.73		Jan-11	82.10	sl4a	2.63
			co4b	3.59				sl4b	2.77
	Feb-11	204.50	co5a	2.40		Feb-11	124.90	sl5a	2.40
			co5b	2.66				sl5b	2.40
	Yield average	SD	%RSD			Yield average	SD	%RSD	
	2.78	0.69	24.86			2.64	0.18	6.72	

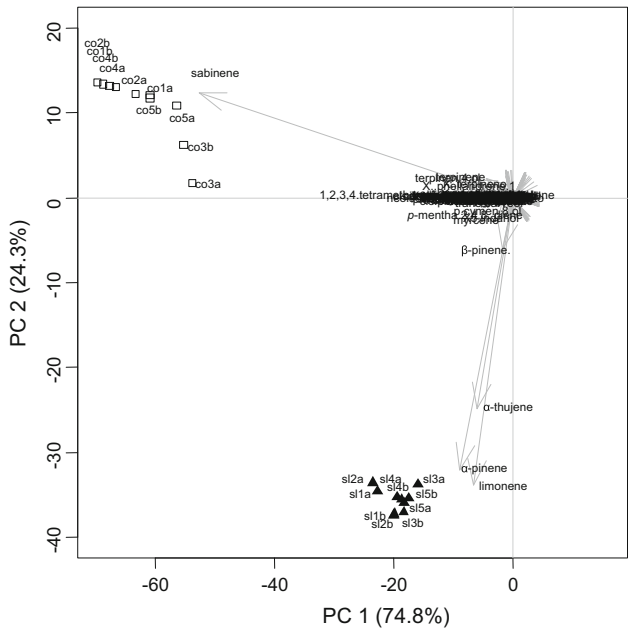

Fig. 1 Principal component analysis of essential oils isolated from *Protium colombianum* from two life zones. *Filled triangle* samples from San Luis. *Open square* samples from Cocorná

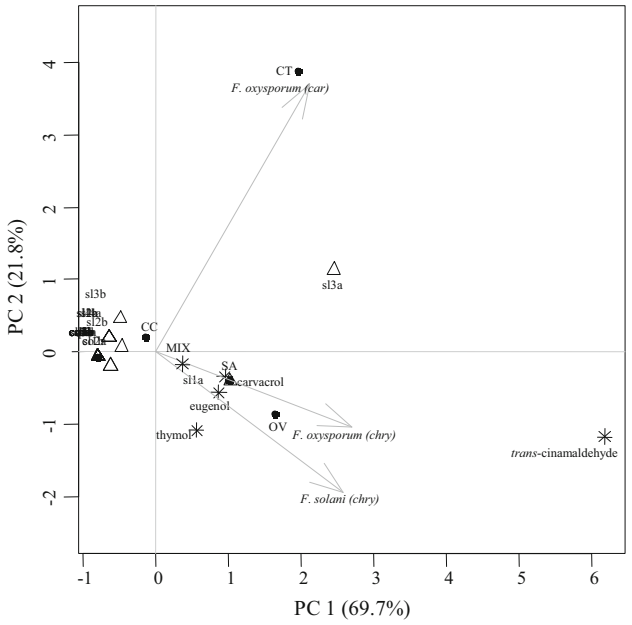

Fig. 2 Principal component analysis of bioactive samples and controls on *Fusarium oxysporum* (chrysanthemum isolate), *Fusarium oxysporum* (carnation isolate), and *Fusarium solani* (chrysanthemum isolate). *Open triangle P. colombianum* essential oils, *filled circle* aromatic plants essential oils. *Pure substances; SA, *Syzygium aromaticum*; CC, *Cymbopogon citratus*; OV, *Origanum vulgare*; CT, *Cinamonum triplinerve*; MIX, equal parts mixture of thymol, carvacrol, *trans*-cinamaldehyde and eugenol

oxysporum (car) and weak activity against *F. oxysporum* (chry). Sample sl1a has low activity against the pathogens and has a moderate effect on *F. solani* (chry) (Supplementary Material Table 4; Fig. 2). EO of *C. triplinerve*, *O. vulgare*, and *S. aromaticum* showed potent anti-fungal activities (Supplementary Material Table 5). Thymol, carvacrol, *trans*-cinamaldehyde, and eugenol were highly active in the experiments; therefore, an mixture of equal parts of these compounds was prepared to establish their possible effects on bioactivity. The samples sl1a, sl3b, sl3a, *O. vulgare*, *C. triplinerve*, and *S. aromaticum* presented 4.8, 5.3, 11.0, 39.5 84.3, and 98.3 % of AB derivatives and phenolic compounds (ABD + Ph), respectively, and showed activity against *Fusarium* isolates. Other samples

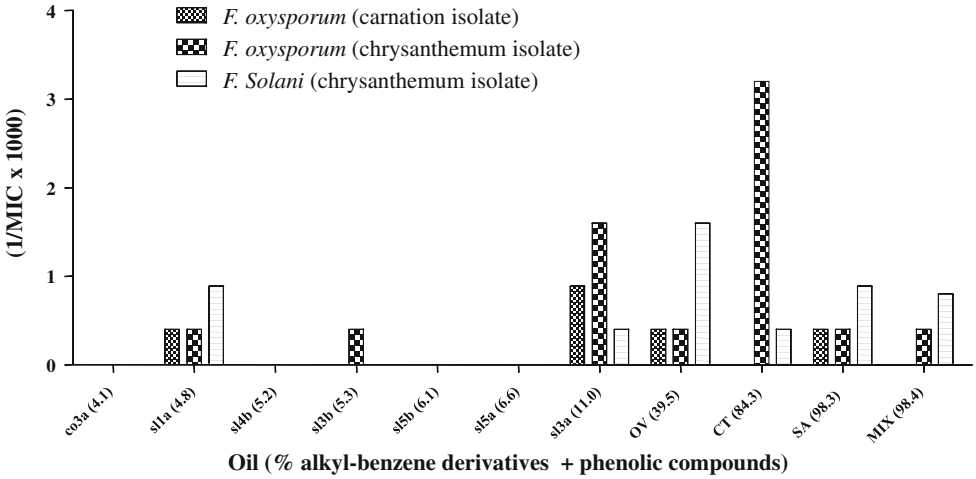

Fig. 3 Relationship between aromatic and phenolic compounds and growth inhibition in *Fusarium* isolates. SA, *Syzygium aromaticum*; OV, *Origanum vulgare*; CT, *Cinamonum triplinerve*; MIX, equal parts mixture of thymol, carvacrol, *trans*-cinamaldehyde and eugenol. For other samples, see the oil code in Table 2

containing similar levels of ABD + Ph had no effect on *Fusarium* isolates. In general, bioactivity was observed to increase with increasing levels of ABD + Ph compounds in some of the proven substances.

Discussion

Essential oil characterization

Samples co3a and co3b were significantly separated from the Cocorná cluster in the PCA (Fig. 1) due to their levels of sabinene, which were 51.81 and 53.97 %, respectively. The metabolic alterations observed in the chemical and yield variability of these samples can be explained by the rainfall found in the sampled months (Table 2), where the maximum rainfall for both studied zones was found in Nov-10, and the value reported corresponds to the accumulated millimeters for the month, causing a possible alteration in the following month's samples. Sample sl3a showed a decrease in the majority of compounds except for limonene, which increased. The components in sample sl3b, however, remained constant, showing no alteration over the sampled months (Table 2). Due to variations in the extraction process, samples sl3a and sl3b showed significant variations, which generated the only remarkable active sample of the isolated EO of *P. colombianum*. Changes in soil water availability represent changes in conditions that affect metabolism, as observed in some studies of the seasonal variability of volatile components (Machado et al. 2003; Ben Jemâa et al. 2012). These previous reports observed variations in the composition after short periods of rain or drought. Hence, it could be suggested that there is a direct relationship between rainfall increases and decreased production of major components in the oils from Cocorná.

Anti-fungal activity

The EO sl3a showed higher levels of AB derivatives (7.64 %) and phenolic (3.44 %) compounds, and EO that contain aldehydes and phenolic nuclei compounds as the main constituents have been shown to be the most active substances in microbial control. These nuclei can interact with membrane proteins and disrupt the permeability barrier of microbial membrane structures, causing malformations, injuries, and lysis (Inouye et al. 2000; Hashem et al. 2010; Faleiro and Miguel 2013; Kon and Rai 2013). All these mechanisms affect membrane integrity, the chemical composition of the cell and metabolic processes, which affect mycelia growth and spore germination in fungal pathogens (Sivakumar and Bautista-Baños 2014). Therefore, our results are in accordance with other authors results, showing biological differentiation between EO and two fungal pathogen isolates from the same species (Naeini et al. 2010; Hashem et al. 2010). Levels of aldehydes and phenolic compounds present in EO are also relevant to understanding their efficacy. Compounds such as *p*-cymene (AB derivative) are known to induce the swelling of fungal cell membranes and to allow transportation of other active compounds, which suggests a synergistic effect between AB derivatives and phenolic compounds (Nguefack et al. 2012). Therefore, the correlation between the levels of ABD + Ph compounds and growth inhibition in *Fusarium* isolates was analyzed (Fig. 3).

The potent anti-fungal activities of the EO of *C. triplinerve*, *O. vulgare*, and *S. aromaticum* (Supplementary Material Table 5) could thus be attributed to the high content of these compounds. However, a quaternary mixture of pure substances showed lower activity than the individual components, thereby demonstrating a possible competitive effect between structurally similar agonists, as in the case of the more active substances. The EO of *C.*

triplinerve was highly active against *F. oxysporum* (car) and weakly active against *F. solani* (chry); however, *trans*-cinamaldehyde exhibited a strong effect against all pathogens, especially against *F. solani* (chry). Carvacrol and *O. vulgare* EO showed little action against *F. oxysporum* isolates, but the EO was more active on *F. solani* (chry) than carvacrol. Eugenol exhibited a greater effect on *F. solani* (chry) than *S. aromaticum* EO (Supplementary Material Table 5; Fig. 2). These results are in accordance with reports showing that EO with high carvacrol, thymol, eugenol, and *trans*-cinamaldehyde content have marked anti-fungal activities (Nakatsu et al. 2000; Isman 2000; Faramarzi et al. 2009).

The anti-fungal and antimicrobial properties of EO from aromatic plants have in some cases been explained in terms of their main constituents, but in other cases, it was found that the activity of EO cannot easily be correlated with any individual component, rather by a mixture of compounds, which reflect the higher antimicrobial activity of the whole EO than the major constituents do when tested separately (Burt 2004). In these cases, minor components could at least exert a synergistic effect with active components, if not a direct activity (Nakatsu et al. 2000; Ranasinghe et al. 2002; Pitarokili et al. 2002; Faleiro et al. 2003; Combrinck et al. 2011). These results suggest that in research into EO for the development of new anti-fungal agents, potential candidates could be enriched in ABD + Ph compounds to achieve the different profiles responsible for the growth inhibition of economically important microorganisms. The EO showed weak anti-fungal activity; however, the findings of the present study suggest that EO rich in active compounds such as carvacrol, thymol, eugenol, and *trans*-cinamaldehyde may have potential applications in controlling fungal pathogens. In addition, our experiments established that the nominal content of volatile ABD + Ph compounds is not sufficient to explain the observed efficacy in the anti-fungal model used. Therefore, the qualitative composition of these fractions could better define their bioactivity profiles. According to our results, effectiveness was reduced when positive control substances were grouped, suggesting that in research into anti-fungal EO, it may be possible to achieve higher efficacy using simple mixtures and low numbers of ABD + Ph-type structural analogs. Finally, further studies are needed to validate the proposed mechanisms and the toxicity against specific molecular targets in these organisms to guide rational studies of biological activity.

Author contribution

DC and RA conceived and designed research. DC conducted experiments. EO contributed new reagents and/or analytical tools. DC, RA, and EO analyzed data. DC wrote the manuscript. All authors read and approved the manuscript.

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