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Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*

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ABSTRACT: The aims of this work were to study the antimicrobial activity of nine monoterpenes and the synergistic or antagonistic associations between them, and to relate water solubility, H-bonding and *p*Ka values with antimicrobial activity. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) were determined. The MIC of carvacrol against *S. aureus* was 3.2 g/l and of thymol was 7.5 g/l. *E. coli* was resistant. Carvacrol and thymol were bactericidal. The associations geraniol/menthol against *S. aureus* and *B. cereus* and thymol/menthol against *B. cereus* were totally synergistic. Eugenol/geraniol displayed partial synergism against *B. cereus*. The other groups did not show any synergistic effect. Eugenol had the lowest *p*Ka, followed by thymol and carvacrol. Eugenol had the highest total area and polar area and intermolecular and intramolecular hydrogen-bonding capacity, while carvacrol and thymol only had intermolecular hydrogen-bonding capacity. The terpenes alone and in combination were effective against microorganisms. Phenolic compounds were the most active terpenes. Associations between terpenes were related to the chemical structure. Studies on the antimicrobial activity of associations of terpenes will advance the search for new alternatives for food preservation. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: essential oils; terpenes; antibacterial activity; synergism; antagonism; hydrogen-bonding capacity

Introduction

Essential oils (EOs) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots), chemically constituted by variable mixtures of terpenoids, mainly monoterpenes (C_{10}) and sesquiterpenes (C_{15}). They can be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for the production of EOs.[1,2] These plant secondary metabolites have their greatest use in food as flavourings, in perfumes as fragrances, in aftershaves and in pharmaceuticals for their functional properties. It has long been recognized that some EOs have antimicrobial, antitoxigenic, antiparasitic and insecticidal properties.[1–3] Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of EOs.[4] Several studies have described the major terpenic components as responsible for the biological activity of EOs.[5,6] Nowadays, however, there is evidence that minor components have a critical part to play in antimicrobial activity, possibly by producing a synergistic effect between other components. This has been found to be the case for sage, certain species of *Origanum* and the genus *Thymus*. Most of the antimicrobial activity in EOs appears to derive from oxygenated terpenoids as alcoholic and phenolic terpenes, while other constituents are believed to contribute little to the antimicrobial effect.[1,3,7–9]. The poor activity of hydrocarbons and esters is associated with their low aqueous solubility, while the formation of hydrogen bonding is thought to be associated with high antimicrobial activity.[3,10] Purified compounds derived from essential oils, such as carvacrol, eugenol, linalool, cinnamic aldehyde and thymol, are able to inhibit a wide variety of microorganisms.[3] Carvacrol and thymol were able to inhibit the growth of bacteria such as *Bacillus cereus, Salmonella* spp., *Shigella sonnei* and phylamentous fungi.[7] These hydrophobic compounds are isomers and they are likely to dissolve in the hydrophobic domain of the cytoplasmic membrane of bacterial cells, between the lipid acyl chains. Thymol disintegrates the outer membrane and increases the permeability of the cytoplasmic membrane to ATP of *Escherichia coli* and *Salmonella typhimurium* cells.[11]

Individual essential oils may contain complex mixtures of compounds. Interactions between these terpenes may lead to additive, synergistic or antagonistic effects. The synergistic rationale for using combinations of products seems to be the obtaining of

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a dynamic product that has multiple modes of action, respecting the principle that the action of the combined product is greater than the sum of individual chemical components.[9,12] The application of essential oils or their components, either alone or in combination with other preservative strategies, could control the growth of food-borne bacteria and other pathogenic microorganisms.[9,13–15]

The pharmaceutical and food industries have expressed the desire to reduce the use of antibiotics and synthetic chemicals for food preservation. Common culinary herbs, spices and aromatic plants that exhibit antimicrobial activity could provide sources of acceptable, natural alternatives. The combination of chemically different terpenes, oxygenated and non-oxygenated, may cause synergistic effects against microorganisms.[3–16]

The aims of this study were to examine the antimicrobial activity of terpenes against three microorganisms of food industry and clinical relevance, to study the possible synergistic or antagonistic effects of the combination of active and inactive terpenes, and to relate water solubility, H-bonding and *p*Ka values of terpenes with antimicrobial activity.

Materials and Methods

Terpenes

The terpenes used were: carvacrol, 2-methyl-5-(1-methylethyl) phenol; carvone, 2-methyl-5-(prop-1-en-2-yl)cyclohex-2-enone; citronellol, 3,7-dimethyloct-6-en-1-ol; eugenol, 2-methoxy-4-(2-propenyl) phenol; geraniol, 3,7-dimethyl-2,6-octadien-1-ol; menthol, 2-isopropyl-5-methylcyclohexanol; menthone, 2-isopropyl-5-methylcyclohexanone; myrcene, 7-methyl-3-methylene-1,6-octadiene; and thymol, 5-methyl-2-(1-methylethyl) phenol. They were obtained from natural sources and the level of purity was 99.0%, tested by gas chromatography–mass spectrometry (GC–MS) at the Cátedra de Química Orgánica, Universidad Nacional de Córdoba, Argentina.

Reagents

Resazurin was used as a visual redox indicator to determine the minimum inhibitory concentration (MIC). A solution of resazurine redox indicator (Riedel/de Haën/Sigma-Aldrich) was prepared to 0.01% w/v in sterile distilled water. The terpenes were emulsified in sloppy agar, prepared with 0.15% agar-agar (Britannia), which was dissolved, sterilized by autoclaving and cooled to room temperature before use.[17]

Microorganisms

The activity of terpenes was tested against the following microorganisms: *E. coli* (isolated from water), *B. cereus* (isolated from rice) and *Staphylococcus aureus* ATCC 21212.

Culture Methods

Tubes containing Müeller–Hinton Broth (MHB; Britannia) with 0.15% w/v agar were prepared at pH 7.0, inoculated with each microorganism and incubated overnight (18 h) at 37°C. Optical densities were measured at 620 nm in a spectrometer. Cell densities were estimated from standard curves and confirmed by the viable plate count on tryptic soy agar (Britannia).

First, the cell concentration necessary to cause reduction of resazurin within 2 h was determined for each of the test microorganisms. Serial 10-fold dilutions of the overnight culture were prepared in MHB. Aliquots (170 µl) of the inocula were dispensed into microplates containing 20 µl agar solution (0.15% w/v) and 10 µl resazurin solution; then they were incubated for 2 h at 37°C. The appropriate dilution to work was the one unable to reduce resazurin (blue), which was tested by the plate count method. Resazurin is a redox indicator that is blue in its oxidized form and pink in its reduced form.[17]

Determination of the MIC of Terpenes

The antimicrobial activity was determined by the broth microdilution method described by Mann and Markham (1998).

Resazurin MIC assay. Serial two-fold dilutions of each terpene were prepared by vortexing in sloppy agar at room temperature. The resazurin assay medium, MHB, was inoculated with the test organism to yield a final cell density ca. 1 log cycle lower than the cell density required to reduce resazurin (usually 10^6 cfu/ml). The inoculum density was confirmed by plate count. A sterile 96-well microtitre tray was set up with each of the tested bacteria as follows: columns 1–9, 170 µl inoculum + 20 µl terpene dilution; column 10, 170 µl inoculum + 20 µl terpene diluent (positive control); columns 11 and 12, sterile resazurin assay medium plus 20 µl terpene diluent (negative controls). The well contents were thoroughly mixed. Two trays were prepared for each organism and incubated at 37°C for 3 h. After incubation, 10 µl resazurin solution was added to all except columns 11 and 12, to which 10 µl distilled water was added. After a second incubation for 2 h at 37°C, the wells were assessed visually for colour change, with the highest dilution remaining blue, indicating the MIC. After this, absorbance was measured at 600 nm.[17]

Determination of MBC of Terpenes

The MBC was determined for those terpenes that presented a MIC value. 100 µl of the dilution belonging to the MIC and the two previous dilutions were inoculated in Mueller–Hinton Agar (MHA) and incubated at 37°C for 24 h. The MBC was considered to be the last dilution that did not show cell growth.[18]

Determination of Associations between Terpenes

The antimicrobial activities of mixed terpenes were determined by a modification of the checkerboard assay described by Dydri *et al.* (1993).[19]The modification consisted in combining this technique with the one described by Mann and Markham (1998).[17]

Briefly, increasing two-fold dilutions of terpenes were made in 0.15% agar, beginning from the pure compounds. In the columns of a microplate, 10 µl of the dilutions of compound A were added and in the rows, 10 µl of dilutions of compound B were added. 170 µl bacterial suspensions, with an appropriate density unable to reduce the resazurine, were then added to the wells. These microplates were incubated for 3.5 h at 37°C, then 10 µl 001% resazurine solution was added. The plates were incubated for 2 h at 37°C and the results were assessed visually for colour change.

In the first column and the first row of the microplate, the MIC control of each terpene was made, following the methodology described above. The fractionary inhibitory concentration (FIC) index was then calculated as follows:

$$FIC = (A/MIC\ A) + (B/MIC\ B)$$

where *A* is the concentration of compound A in the minimum inhibiting association, *B* is the concentration of compound B in the minimum inhibiting association, and *MIC A* and *MIC B* are MIC values obtained as described above. The values were interpreted as follows: total synergism, FIC ≤ 0.50; partial synergism, 0.50 < FIC ≤ 0.75; indifference, 0.75 < FIC ≤ 2; antagonism, FIC > 2.

Estimation of *p*Ka Values

Titrations were carried out using a Herrman Model 8010 pH meter. The measurements were made in water:ethanol solutions and the pH electrode was calibrated against three buffers. Phenols solution (1×10^{-2} M) were prepared in water:ethanol (2:1). An aliquot (10 ml) of phenols solution was titrated against standardized aqueous sodium hydroxide (ca. 10^{-2} M) and pH was determinated. Equilibrium constants were calculated with the aid of the Origin 7.0 SRO programme, which employs a non-linear least-squares Gauss–Newton–Marquardt algorithm.

Molecular Modelling and Calculation of Molecular Parameters

Compounds were constructed using the molecular modelling programme PC Model v.6 and the lowest energy 3D configurations of the molecules were determined using the energy minimization function of the programme. After energy minimization, the following molecular parameters were calculated for each molecule, molecular volume (cubic Angstroms, $Å^3$), polarity and hydrogen-bonding capacity (the tendency of a molecule to form hydrogen bonds). *p*Ka values were calculated using the ACD Labs online programme.

Results

Antimicrobial Activity of Terpenes

MIC of terpenes. The antimicrobial activity of terpenes was tested on three different microorganisms, *S. aureus*, *B. cereus* and *E. coli*. The terpenes that showed antimicrobial activity against *S. aureus* were carvacrol, carvone, eugenol, geraniol and thymol. Carvacrol was the most effective terpene, with an MIC value of 3.8 g/l, while thymol and carvone showed good antimicrobial activity with MIC values of 7.5 g/l. Eugenol and geraniol were also active against *S. aureus*; however, their activities were not so remarkable compared to carvacrol, thymol and carvone (Table 1).

B. cereus was strongly inhibited by carvacrol (MIC = 3.8 g/l) and thymol (MIC = 7.5 g/l). This microorganism was also inhibited by eugenol (MIC = 66.8 g/l) and geraniol (MIC = 222.2 g/l) (Table 1). The terpenes citronellol, menthol, menthone and myrcene did not show antimicrobial activity against any of the microorganisms analysed (Table 1).

MBC of terpenes. The MBC was determined for those terpenes that showed antimicrobial activity. Carvacrol and thymol showed bactericidal activity against all the strains analysed (Table 2). The MBC of thymol against *E. coli* and *S. aureus* was 60.3 g/l. In contrast, a higher concentration of this compound was necessary to achieve a bactericidal effect on *B. cereus* (MBC = 120.6 g/l). Carvacrol was more effective than thymol, showing a bactericidal effect over all microorganisms at lower concentrations (MBC = 15.2 g/l). Eugenol and geraniol did not show MBC values, so they were considered bacteriostatic (Table 2).

Interactions between Terpenes

The following associations were investigated in order to evaluate whether synergistic, antagonistic or indifference associations took place between terpenes:

- *Terpenes with antimicrobial activity*: thymol/eugenol; thymol/geraniol; eugenol/geraniol; carvacrol/thymol; carvacrol/eugenol.
- *Terpenes with antimicrobial activity with terpenes without antimicrobial activity*: thymol/menthol; eugenol/menthol; geraniol/menthol; carvacrol/myrcene.
- *Terpenes without antimicrobial activity*: myrcene/citronellol.

The associations of terpenes with antimicrobial activity showed different results. The combinations thymol/eugenol and eugenol/geraniol were partially synergistic against *B. cereus* (FIC = 0.75 and FIC = 0.65, respectively). The same associations were indifferent when they were tested against *S. aureus* and *E. coli*. The mixture

Table 1. Minimum inhibitory concentration (MIC) of terpenes (g/l)

Terpene	Concentration range (●/●)	Microorganisms *S. aureus*	*B. cereus*	*E. coli*
Carvacrol	1.91–980	3.81	3.81	7.62
Carvone	1.87–960	7.54	NI	NI
Citronellol	1.66– 855	NI	NI	NI
Eugenol	2.08–1070	33.43	66.87	66.87
Geraniol	1.71–880	222.25	222.25	222.25
Menthol	1.73–890	NI	NI	NI
Menthone	1.74–895	NI	NI	NI
Myrcene	1.54–790	NI	NI	NI
Thymol	1.88–965	7.53	7.53	15.07

NI, no inhibition.

Table 2. Bacteriostatic or bactericidal effect of terpenes (g/l)

Microorganisms	Terpene Carvacrol	Thymol	Eugenol	Geraniol
S. aureus	15.25 (bactericidal)	60.31 (bactericidal)	Bacteriostatic	Bacteriostatic
B. cereus	15.25 (bactericidal)	120.62 (bactericidal)	Bacteriostatic	Bacteriostatic
E. coli	15.25 (bactericidal)	60.31 (bactericidal)	Bacteriostatic	Bacteriostatic

Table 3. Fractional inhibitory concentration and interaction between terpenes

Association	*S. aureus*		*B. cereus*		*E. coli*	
	FIC	Interaction	FIC	Interaction	FIC	Interaction
Thymol/eugenol	2	I	0.75	PS	11	I
Thymol/geraniol	2	I	0.99	I	1.12	I
Eugenol/geraniol	1.12	I	0.65	PS	1.03	I
Carvacrol/thymol	3.99	A	3.99	A	4	A
Carvacrol/eugenol	4	A	4	A	4	A
Thymol/menthol	>2	A	0.25	TS	>2	A
Eugenol/menthol	2	I	2	I	2	I
Geraniol/menthol	0.5	TS	0.061	TS	2	I
Carvacrol/myrcene	>2	A	>2	A	>2	A
Citronellol/myrcene	NI		NI		NI	

NI, no inhibition; I, indifference; PS, partial synergism; TS, total synergism; A, antagonism.

Table 4. Physicochemical constants

Components	*p*Ka values			Total area (Å^3)	Polar area (%)	Hydrogen-bonding capacity
	Measured	Calculated[1]	Literature[2]			
Carvacrol	9.07	10.38	11.02	216	12.08	Intermolecular
Thymol	8.81	10.59	11.11	216	11.98	Intermolecular
Eugenol	8.55	10.29	10.72	217	15.42	Inter- and intramolecular

[1] *p*Ka values calculated by ACD Labs online programme.
[2] *p*Ka values reported by Reiner *et al.* (2009).[34]

thymol/geraniol showed indifference with the three tested microorganisms; carvacrol/thymol and carvacrol/eugenol were antagonistic, with FIC values > 2. The combination thymol/menthol was totally synergistic against *B. cereus* (FIC = 0.25) and the mixture geraniol/menthol showed total synergism against *S. aureus* (FIC = 0.5) and *B. cereus* (FIC = 0.061). The association geraniol/menthol was found to be indifferent with the three tested microorganisms, whereas carvacrol/myrcene displayed antagonism for these microorganisms. The combination between inactive compounds, citronellol/myrcene, did not demonstrate antimicrobial activity against any of the three microorganisms analysed (Table 3).

Estimation of *p*Ka Values

The *p*Ka values of the monoterpenes are summarized in Table 4. The *p*Ka values of the phenols evaluated were different, owing to their involvement in the prototropic equilibrium. Eugenol had the lowest *p*Ka (8.55), followed by thymol (8.81) and carvacrol (9.07). Despite the fact that the *p*Ka values measured in this study differed from those reported in the literature, the relative values were in agreement with them.

Molecular Modelling and Calculation of Molecular Parameters

The phenols polar area (%) and hydrogen-bonding capacity was calculated and these values are summarized in Table 4. Carvacrol and thymol had the same total area (216 Å^3); however, the polar area of carvacrol (12.08%) was greater than that of thymol (11.98%). Eugenol had the highest total and polar areas of the terpenes evaluated. Moreover, eugenol had intermolecular and intramolecular hydrogen-bonding capacity, while carvacrol and thymol only had intermolecular hydrogen-bonding capacity.

Discussion

Antimicrobial Activity of Terpenes

The activity of terpenes would be expected to relate to the structural configuration and to their functional groups. An attempt to correlate the antimicrobial activity of the compounds tested with their chemical structure, functional groups and configuration was made in this study.

The terpenes carvacrol and thymol showed the best antimicrobial activity against the tested microorganisms. Previously, the antimicrobial activity of these terpenes has been described by other authors.[7,16,20] Despite the fact that eugenol also showed activity against each microorganism, its activity was lower than that shown by carvacrol and thymol. Thus, the importance of the hydroxyl group in antibacterial activity has been confirmed, and the significance of the aromatic ring was demostrated by the lack of activity of menthol (Table 1). However, we report that carvacrol was more active than thymol and eugenol (Table 1). Therefore, the relative position of the hydroxyl group on the phenolic ring appeared to strongly influence the degree of antibacterial activity.

The components with phenolic structures, such as carvacrol, thymol and eugenol, are known to be microbial inhibitory agents, with the bactericidal or bacteriostatic effect depending upon the concentration used. These compounds were strongly active despite their relatively low capacity to dissolve in water. The importance of the hydroxyl group in the phenolic structure was

confirmed in terms of activity when carvacrol was compared to its methyl ether. Furthermore, the position of the hydroxyl group in the ring exerted an influence upon the effectiveness of the components, as they showed differences in the activity of carvacrol and thymol against Gram-negative and Gram-positive bacteria.[4–21]

The relative antimicrobial activity of monoterpenes is associated with its aqueous solubility and the hydrogen-bonding capacity.[3–10] The aqueous solubilities of carvacrol, thymol and eugenol were 830, 846 and 2406 ppm, respectively.[10] Although eugenol had the greatest aqueous solubility, its antimicrobial activity was lower. It has been suggested that phenolic compounds destabilize the cytoplasmic membrane and, in addition, act as proton exchangers, thereby reducing the pH gradients across the cytoplasmic membrane. The resulting collapse of the proton motive force and depletion of the ATP pool eventually leads to cell death.[3] Thus, the antimicrobial activities of phenolic compounds appeared to be explained by the *p*Ka values. A ranking of the antimicrobial activities of phenolic compounds related to *p*Ka values has been obtained from our results (Tables 1, 4): carvacrol (*p*Ka = 9.07) > thymol (*p*Ka = 8.81) > eugenol (*p*Ka = 8.55). From our results, when phenolic components were acting as good proton exchangers their bioactivity was disminished, because their ability to make hydrogen bonding was lower. Moreover, the position and nature of the neighbouring functional group was found to affect the molecular properties of the components, such as a intramolecular hydrogen-bonding capacity. Eugenol showed lower activity against microorganisms than carvacrol and thymol, due to its capacity to make intramolecular hydrogen bonding with the neighbouring ether groups; therefore, it has diminished ability to make intermolecular hydrogen bonding. Moreover, the para position of the electron-donating alkyl group made the intramolecular hydrogen bonding easy to break, as in eugenol.[22]

Carvone was active only toward *S. aureus*, while the remaining microorganisms tested did not show any antimicrobial activity. However, the inhibitory activity of (4*R*)-carvone over *Enterococcus faecium, E. coli* and *A. niger* has been described.[23]

Several reports on the antimicrobial activity of monoterpenes have shown that the number of double bonds in a structure and the acyclic, monocyclic and/or bicyclic structure have no significant influence on its activity, although higher inhibitory activity is seen in aromatic compounds such as carvacrol, thymol and eugenol.[3–10] However, oxygenated terpenoids show characteristic and distinct activity patterns towards microorganisms; those terpenoids that contain alcohols possess higher activity than the corresponding carbonyl compounds,[3–9] work with Cilantro fractions deficient in phenolic compounds and show strong antimicrobial activity, an observation contrary to the assumption that these chemicals are responsible for most of the antimicrobial activity in essential oils. Fractions rich in long-chain (C_6–C_{10}) alcohols and aldehydes were particularly active against Gram-positive bacteria. In addition the antimicrobial properties of alcohols are known to increase with molecular weight.[9]

The Gram-positive bacteria *S. aureus* and *B. cereus* were strongly inhibited by carvacrol, thymol, eugenol and geraniol, showing more sensitivity than the Gram-negative bacterium *E. coli*. The Gram-positive rod *Bacillus cereus* is a facultative anaerobe and is spore-forming. It has been related to raw and processed meat, vegetables, rice and dairy products. *B. cereus* is associated with two kinds of food-borne illnesses, a diarrhoeal and an emetic type, caused by two distinct toxins. Consequently, it is becoming one of the most important causes of food poisoning in the industrialized world.[24]

S. aureus is a major human pathogen that causes a wide spectrum of infections, ranging from superficial wound infections to life-threatening septicaemia and toxic-shock syndrome.[25] Methicillin-sensitive *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have created major problems for burns units and intensive care units. Alternative therapies are being sought for the treatment of MRSA and one area of interest is the use of essential oils and their constituents.[26] It is also a major food-poisoning bacterium, posing a great risk to consumer health, mainly through its production of heat-stable enterotoxins.[25]

E. coli is a Gram-negative rod, a facultative anaerobe that normally lives in human and animal intestinal tracts. However, some strains cause severe diarrheas, e.g. enteropathogenic, enterotoxigenic, enteroinvasive, enteroadherent, enteroaggregative and enterohaemorrhagic.[27] EOs with activity against *E. coli* could be considered for the treatment of these illnesses. Gram-negative bacteria are known to be more resistant to a wide number of antimicrobial agents than Gram-positive bacteria.[9] However, in this study, *E. coli* showed sensitivity to carvacrol, thymol, eugenol and geraniol but was resistant to carvone. The resistance of this bacterium could be attributed to the presence of the outer membrane, characteristic of Gram-negative microorganisms. The outer membrane functions as a molecular sieve through which molecules with molecular mass >600–1000 Da cannot penetrate. Despite the presence of porins with low specificity, the outer membrane shows very low permeability toward hydrophobic compounds, which has been ascribed to the presence of the lipophilic LPS. However, it has been demostrated that highly lipophilic compounds such as steroids penetrate relatively easily though the outer membranes of several bacteria.[28,29]

The mode of action of essential oils and terpenes is still unknown. Other investigations have found that carvone was ineffective on the outer membrane of *E. coli* and *Salmonella typhimurium* and did not affect the intracellular ATP pool.[28] These could explain the results obtained with *E. coli* in this study.

It has been proposed that, as a result of the lipophilic character of the essential oils and their monoterpenoid components, cyclic monoterpenes will preferentially partition from an aqueous phase into membrane structures. This results in membrane expansion, increased membrane fluidity and inhibition of a membrane-embedded enzyme. It has been reported that the effects of different essential oil components on outer membrane permeability in Gram-negative bacteria include induced damage to cell membrane structure, accompanied by a decline in viability.[28–30]

Interactions between Terpenes

The components that constitute essential oils are those that confer their biological properties to them.[5] The terpenes of essential oils have antimicrobial activity by themselves; however, this activity does not always agree with that of the complete essential oil. This could suggest that complex mixtures of these terpenes determine the synergistic or antagonistic relations between them. The increase of the inhibitory capacity of essential oils and terpene mixtures caused by different combinations between them has been described by several investigative groups, as well as antagonistic reactions.[19–31]

Synergism could be defined as a combination of substances (terpenes, EOs) that results in a dynamic product that has

multiple modes of action, respecting the principle that the action of combined substances is greater than the sum of known and unknown chemical components. Antagonism could be seen as a diminution in the biological activity of a mixture of components, compared with the individual activity of each component alone.[12]

Different terpene components can interact in an aqueous dispersion to either reduce or increase antimicrobial efficacy, depending upon their relative concentrations and the overall susceptibility of the target microorganism.[8] In this study the combination of menthol/thymol and menthol/geraniol showed synergistic effects on the growth inhibiton of *S. aureus* and *B. cereus*, while eugenol/geraniol and thymol/eugenol were partially synergistic (Table 3). However, the terpene menthol did not show an antimicrobial effect when it was tested independently (Table 2).

Delgado *et al.*[11] proposed an explanation for the synergism between thymol and cymene. The two compounds have almost the same structure, although cymene lacks the hydroxyl group, which is present in thymol and results in an increase of antibacterial activity. Because the two compounds are hydrophobic, both are expected to partition preferentially in the membranes of cells, and then the action of one compound may facilitate the uptake of the other in the lipid bilayer of the cytoplasmic membrane. This could explain the synergism found in this study. These behaviours are supported by other reports that terpenes act as effective penetration enhancers for both hydrophilic and lipophilic components. It has been recognized that hydrophilic terpenes capable of hydrogen binding, such as fenchone and thymol, are more active towards promoting the permeation of hydrophilic components, whereas hydrocarbon terpenes, such as limonene, provide higher enhancing activity for the lipophilic components.[32] The combination between terpenes and antibiotics has been studied, looking for an enhancement in the biological activity of the antibiotic. The lipidic nature of terpenes could allow a best transport of drugs until they reach its bacterial cell target.[12] Combinations between terpenes and antibiotics could be considered promising chemotherapics for the treatment of diseases produced by these microorganisms.

On the other hand, an example of antagonism between the components of oils is the mixture of thymol and carvacrol, terpenes present in the essential oil of oregano, which demonstrate less biological capacity together than when tested separately.[11,19,31] The combination of carvacrol with other monoterpenes was demostrated to have antagonist effects on the growth inhibition of microorganisms (Table 3). An antagonistic effect between *p*-cymene, thymol and carvacrol was reported in the oil of *Lippia*.[3] Non-oxygenated monoterpene hydrocarbons, such as myrcene, appeared to create an antagonistic effect with the most active component, carvacrol or geraniol, by lowering its aqueous solubity. Furthermore, γ Terpinene and *p*-cymene, both non-oxygenated monoterpene hydrocarbons, produce antagonistic effects against more tolerant microorganisms. The most probable explanation for this antagonism is that the non-aqueous monoterpene hydrocarbon phase reduces aqueous terpene solubility and, therefore, the microbial availability of the active components. Such effects may have significant implications with regard to the efficacy of formulations made with these components.[8]

In summary, these results showed that terpenes alone and the multiple combinations between them were effective against microorganisms of clinical and food industry interest. A major advantage of combinations of terpenes is that they inactivate microorganisms without the need for other severe treatments.

The application of these combined natural antimicrobials on foods would produce minimal damage to the nutritive and organoleptic characteristics of some types of food products. In this way, the quality of treated products will be markedly superior to that of conventionally processed products and, therefore, will accomplish the current food manufacturers' and consumers' demands.[11]

The clinical importance of the presence of synergy or antagonism in a combination of terpenes could be justified by the fact that minor interactions *in vitro* may not only result in significant synergism *in vivo* but also make a difference to the duration of an effective drug level *in vivo*. It is important to select an active synergistic mixture with the optimum therapeutic properties in order for it to be used as a good antimicrobial.[33]

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