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Study of the potential synergistic antibacterial activity of essential oil components using the thiazolyl blue tetrazolium bromide (MTT) assay

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Abstract

The thiazolyl blue tetrazolium bromide (MTT) assay was used to study the potential interactions between several active compounds from plant essential oils (carvacrol, eugenol, cinnamaldehyde, thymol and eucalyptol) when used as antibacterial agents against *Escherichia coli* and *Listeria innocua*. The minimum inhibitory concentration (MIC) of each active compound and the fractional inhibitory concentration (FIC) index for the binary combinations of essential oil compounds were determined. According to FIC index values, some of the compound binary combinations showed an additive effect, but others, such as carvacrol-eugenol and carvacrol-cinnamaldehyde exhibited a synergistic effect against *L. innocua* and *E. coli*, which was affected by the compound ratios. Some eugenol-cinnamaldehyde ratios exhibit an antagonistic effect against *E. coli*, but a synergistic effect against *L. innocua*. The most remarkable synergistic effect was observed for carvacrol-cinnamaldehyde blends for both *E. coli* and *L. innocua*, but using different compound ratios (1:0.1 and 0.5:4 respectively for each bacteria).

Keywords: antimicrobial synergy, *Listeria innocua*, *Escherichia coli*, MIC, FIC index

1. Introduction

Foodborne pathogens and spoilage bacteria are the major concerns of food companies, since they produce a large amount of food waste with the consequent economic losses, as well as causing important foodborne illnesses, which is one of the major global health preoccupations (Ghabraie, Vu, Tata, Salmieri, & Lacroix, 2016). Synthetic preservatives have been widely used for decades to maintain quality, extend the shelf life and ensure the safety of foodstuffs (Jaiswal & Jaiswal, 2015). However, their repeated applications have led to chemical residue accumulation in the food chain and the development of microbial resistance and side effects for human health (Akinyemi, Oluwa, & Omomigbehin, 2006). For these reasons, consumer preferences are changing toward safer, natural food preservatives. In this context, essential oils (EOs) and several of their constituents represent a natural, safe alternative to chemical food preservatives, due to their capacity to inhibit the growth of a wide variety of pathogenic and food-spoiling microorganisms including bacteria, fungi and yeasts (Conner &Beuchat, 1984; Ghabraie et al., 2016; Wilson, Solar, El Ghaouth, & Wisniewski, 1997). Thus, carvacrol, which is the main compound of oregano EO, has been effective at inhibiting the growth and survival of several foodborne and spoilage bacteria, such as *Listeria monocytogenes*, *Aeromonas hydrophila*, *Pseudomonas fluorescens* (de Sousa et al., 2012) and different strains of *Escherichia coli* (Stratakos et al., 2018), as well as some important foodborne fungal pathogens (Abbaszadeh, Sharifzadeh, Shokri, Khosravi, & Abbaszadeh, 2014). Carvacrol is also present in thyme EO, where thymol is the most abundant active compound. Several *in vivo* studies demonstrated that thymol exhibits antimicrobial activity against a broad spectrum of Gram negative or Gram-positive bacteria (Moon & Rhee, 2016) and fungi (Abbaszadeh et al., 2014). Eugenol is the main compound of cinnamon leaf EO (70-95%), which also contains cinnamaldehyde in a proportion of 1 to 5% (Vangalapati, Satya Prakash & Avanigadda, 2012). Both active compounds have exhibited significant antimicrobial effects in *in vitro* tests against different foodborne pathogens, such as *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp. *Enterobacter* sp. (Moleyar, & Narasimham, 1992), *Escherichia coli* (Pei, Zhou, Ji, & Xu,

2009) and *Helicobacter pylori* (Ali et al., 2005). Eucalyptol, which occurs in different active aromatic plants such as oregano, rosemary, thyme and ginger, also has proven broad-spectrum antimicrobial activity that includes the inhibition of both Gram-positive (*Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus and Enterococcus faecalis*) and Gram-negative bacteria (*E. coli, Aeromonas hydrophila, Pseudomonas aeruginosa and fluorescens, Klebsiella pneumoniae and Moraxella catarrhalis*) (de Oliveira et al., 2015; Van Vuuren & Viljoen, 2007).

However, the concentrations of the EOs or their constituents required to inhibit bacterial growth in foods can modify the taste or exceed the acceptable flavour threshold of food products (Gutierrez, Barry-Ryan & Bourke, 2008). In this sense, the potential synergistic activity of these EO compounds has appeared as an alternative means of reducing the active doses needed to achieve antimicrobial effects in food, since several authors have demonstrated some synergistic interactions against several foodborne pathogens in *in vitro* studies combining carvacrol, thymol, eugenol, cinnamaldehyde and eucalyptol (de Sousa et al., 2012; Guarda, Rubilar, Miltz, & Galotto, 2011; Hill, Gomes, & Taylor, 2013; Pei et al.,2009; Van Vuuren & Viljoen, 2007).

Nevertheless, it is very difficult to compare the published results for the same EO compounds, since there are several factors that influence their antimicrobial effects. The most important variable is the antimicrobial test method, including incubation temperature, inoculum size and test microorganisms (Lambert, & Pearson, 2000; Nostro, Germano, D'Angelo, Marino, & Cannatelli, 2000). Therefore, it is necessary to standardize the antimicrobial activity assessment in order to obtain comparable and reproducible results.

Diffusion methods (agar disk diffusion and agar well diffusion) have been widely used to screen the antimicrobial activity of EOs and their main compounds (Huang, Chen, Hung, & Kao, 2012; Stratakos et al., 2018); however, these tests do not permit the quantification of their bioactivity in terms of minimum inhibitory concentration (MIC), since they are qualitative tests (Ncube, Afolayan, & Okoh, 2008). Likewise, methods in vapour-phase, such as the disk volatilization assay, have been used in many studies for the antimicrobial evaluation of EOs in vapour-phase, but they only allow us to identify the most effective compound from several active ingredients (Bueno,

2015; Houdkova, Rondevaldova, Doskocil, & Kokoska, 2017). Moreover, these tests are not able to perform a large-scale screening with many different active compounds at different concentrations. Some other methods used to determine the EO compounds' antimicrobial activity, such as the agar-plate method for total microbial count, are resource-intensive and time-consuming (Clark, 1965), and more sophisticated studies, such as flow cytometry or tests based on absorbance measurement, require special equipment which is not commonly available (Gunasekera, Attfield, & Veal, 2000; Krepker et al., 2017).

The thiazolyl blue tetrazolium bromide (MTT) colorimetric assay is one of the most useful methods for the evaluation of *in vitro* cell viability using microtiter plate design or the broth microdilution method (Houdkova et al, 2017), which has been used to study EO antimicrobial susceptibility (Houdkova et al, 2017; Ye, Shen, Xu, Lin, Yuan, & Jones, 2013), as well as drug interactions against bacteria (Rondevaldova, Novy, Urban, & Kokoska, 2017) and fungi (Te Dorsthorst, Verweij, Meis, Punt, & Mouton, 2002). This checkerboard experiment avoids the need for culturing procedures and could allow for distinguish between bacteriostatic and bactericidal effects and, therefore, obtain an easy and rapid quantitative determination of the MIC of large numbers of samples (Ncube et al., 2008), unlike the antimicrobial susceptibility methods based on colony counting by decimal dilution and agar plating, which are not able to check many different active compounds and concentrations within a short time (Pei et al., 2009). Moreover, the MTT assay is an inexpensive and reproducible test, which can be used for a wide variety of microorganisms, since the use of the MTT reagent as a colorimetric indicator avoids the need for a spectrophotometric plate reader. Nonetheless, EO compounds can alter the results of microplate toxicity assays, due to their volatile nature (Novy et al., 2014). Thus, it is advisable to use an effective vapour barrier, such as a sealer mat made of non-reactive rubber, to avoid vapour transmission between adjacent wells (Houdkova et al., 2017; Rondevaldova et al., 2017).

To the best of our knowledge, the potential use of the MTT assay as a tool with which to determine the possible interactions between different active compounds of essential oils at controlling microbial growth has been little explored. The aim of this study was to analyse the

potential synergistic activity of the most effective antimicrobial compounds from EOs (carvacrol, eugenol, cinnamaldehyde, thymol and eucalyptol) against *E. coli* and *L. innocua* using MTT assay. *E. coli* was chosen as model for pathogenic Gram-negative bacteria (Sondi & Salopek-Sondi, 2004), whereas *L. innocua* was selected as representative strain of *L. monocytogenes* (model Gram-positive bacteria), because of its non-pathogenicity to humans (Coma, Sebti, Pardon, Deschamps, & Pichavant, 2001) and similar sensitivity to EO compounds (Teixeira et al., 2013.).

2. Materials and methods

2.1. Reagents and bacterial strains

Carvacrol, eugenol, cinnamaldehyde, thymol, eucalyptol and MTT reagent were supplied by Sigma-Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO) was purchased from Panreac (Barcelona, Spain), whereas sterile Phosfate Buffered Saline (PBS), Tryptone Soy Broth (TSB) and Tryptone Soy Agar (TSA) were supplied by Scharlab (Barcelona, España).

Listeria innocua (CECT 910) and *Escherichia coli* (CETC 101) lyophilized strains were supplied by the Spanish Type Culture Collection (CECT, Universitat de València, Spain), and stored at −40 °C with 30% glycerol. Active cultures were regenerated by inoculating the microbial stock suspensions into TSB followed by their incubation at 37 °C for 24 h. The inoculums were properly diluted to obtain bacterial suspensions of 10^5 CFU/mL.

2.2. MIC assessment and combined antimicrobial effects

A MTT colorimetric assay was carried out by using a 96-well disposable sterile microtiter plate design in order to determine the MIC of the different EO compounds (Figure 1). Stock solutions of each EO compound (10 mg/mL) were obtained using DMSO as emulsifier. Diluted EO solutions were prepared from stock solutions using TSB broth medium as solvent and aliquots of 100 µL of each dilution were placed in their corresponding wells, thus obtaining EO concentrations from 0.05 to 2.5 mg/mL. To this end, the concentration of each EO compound was increased 0.05 mg/mL between two consecutive wells. Then, plates were inoculated with 100 µL of the 10^5

CFU/mL bacterial suspension and covered with an autoclavable sterile sealer mat as an effective vapour barrier to prevent the volatile compounds from contaminating the adjoining wells. Sterility and bacterial growth control were also prepared with non-inoculated and inoculated culture media, whereas the outer wells were left empty to prevent edge effect. All experiments were carried out in sterile conditions within a laminar flow cabinet and all culture media were properly autoclaved.

After 24 h incubation at 37 ^{o}C, 10 µl of MTT reconstituted in PBS at 5 mg/mL were added to each well and incubated for 4h at 37 ^{o}C. MTT is a yellow tetrazolium salt, which is reduced to a purple formazan by dehydrogenases of a live cell. Thus, the formazan amount produced is directly proportional to the number of live cells and the MIC of the EO compounds can be assessed by the naked eye (Ye et al., 2013). In this way, the MIC values were determined as the lowest concentration of active compound at which no purple colour was observed. All the experiments were carried out in duplicate.

The potential synergistic effects of binary combinations of the different EO compounds were also tested by the chequerboard method. EO compound stock solutions were prepared in DMSO and properly diluted in TSB to obtain binary combinations with final concentrations of each active compound that ranged from the MIC values to 1:100 dilution below the corresponding MIC. The microtiter plate design allowed the concentrations of each antimicrobial to be varied along the different axes, thus ensuring that each well of the plate represents a different combination (Figure 2a). The antimicrobial effects of each binary combination were evaluated by calculating the fractional inhibitory concentration (FIC) index, following Eq. (1). As shown in the theoretical isobologram (Figure 2b), it was considered to be a synergistic action when the FIC index was lower than 1, additivity when the FIC was 1, and an antagonistic effect when the FIC was higher than 1 (Bell, 2005; Krepker et al., 2017; Pei et al., 2009). All the concentrations were tested in duplicate.

$$FIC_{index} = FIC_A + FIC_B \quad (1)$$

where

$FIC_A = MIC_{A \text{ in presence of } B} / MIC_{A \text{ alone}}$

$FIC_B = MIC_{B \text{ in presence of } A} / MIC_{B \text{ alone}}$.

3. Results and discussion

3.1. Minimum inhibitory concentration

All the active components evaluated exhibited antibacterial activity against *E. coli* and *L. innocua*, with values of MIC ranging from 0.5 to 1.75 mg/mL (Table 1). Cinnamaldehyde was the most effective at inhibiting the growth (lowest MIC) of both bacteria, and the reported MIC was similar to that found by other authors (Hill et al., 2013; Ye et al., 2013). As reported de Sousa et al. (2012) and Van Vuuren & Viljoen (2007) for *L. monocytogenes* and *E. coli*, respectively, eucalyptol was the least effective at inhibiting bacterial growth, *E. coli* being more resistant. Likewise, in accordance with the MIC reported by Pei et al. (2009) and Hill et al. (2013) for *E. coli* and *L. innocua,* respectively, eugenol showed lower values as compared to cinnamaldehyde, carvacrol and thymol, being more effective against *L. innocua*.

Carvacrol and thymol, with very similar molecular structures (Table 1), showed similar MIC values for both bacteria, *E. coli* being more affected than *L. innocua*. This coincides with that obtained in previous studies, although the MIC values were slightly lower (Guarda et al., 2011; Du et al., 2015). The differences in terms of the MIC values for the same active component and bacterial strain can be explained by the different methodology applied, the culture media used, inoculum size, pH, incubation time and temperature (Pei et al., 2009).

3.2. Interactions between components in binary active compound mixtures

The potential synergistic antibacterial effect of all binary combinations of the compounds were determined quickly and easily by calculating the fractional inhibitory concentration (FIC) index, thus obtaining the isobolograms for the different active binary mixtures against *L. innocua* (Figure

3) and *E. coli* (Figure 4). It was considered to be a synergistic action when the FIC index was lower than 1, additivity when the FIC index was 1, and an antagonistic effect when the FIC index was higher than 1 (Bell, 2005; Krepker et al., 2017; Pei et al., 2009). The binary combinations that exhibit a synergistic effect with the lowest FIC index values are given in Table 2 for both *E. coli* and *L. innocua*.

Santiesteban-Lopez et al. (2007) reported some generally accepted mechanisms for synergistic action of the antimicrobial combinations: the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, combinations of cell wall active agents, or the action of cell wall active agents to enhance the uptake of other antimicrobials. Likewise, there are mechanisms that produce antagonism for the antimicrobial combinations. Although these are less known, generally they include the combinations of bactericidal and bacteriostatic agents, the use of compounds that act on the same target of the microorganism, or chemical interactions among the active compounds (Goñi et al., 2009).

Carvacrol/cinnamaldehyde combinations exhibited a synergistic effect against *E. coli* for almost all combination ratios (Figure 4), but the synergistic effect against *L. innocua* (Figure 3) was only observed when cinnamaldehyde was the major component in the mixture. Ye el al. (2013) also reported strong synergistic activity for carvacrol/cinnamaldehyde combinations against 7 kinds of bacteria, including *E. coli*. In contrast, almost all the eugenol/cinnamaldehyde combinations exhibited an antagonistic effect against *E. coli* and *L. innocua*. On the contrary, Pei et al. (2009) found a synergistic action between eugenol and cinnamaldehyde against *E. coli* and only an additive effect between carvacrol and cinnamaldehyde for the same bacteria. Wendakoon and Sakaguchi (1993) hypothesized that the hydroxyl group on eugenol might combine with proteins, preventing enzyme action while the carbonyl group on cinnamaldehyde might adhere to proteins to prevent the action of amino acid decarboxylases.

Every ratio of eugenol/carvacrol combinations showed an antagonistic effect against *L. innocua*, whereas either synergistic or additive effects were observed against *E. coli,* depending on the

212 ratio of both components. Similarly, no synergistic effects were observed for different ratios of
213 eugenol/carvacrol combinations against *L. innocua* by García-García, López-Malo, & Palou (2011).
214 Carvacrol and thymol were hydrophobic and prone to disturb the outer membrane of Gram-
215 negative bacteria, releasing lipopolysaccharides, and increasing the permeability of the
216 cytoplasmic membrane to ATP (Helander and others 1998; Lambert and others 2001). Based
217 these previous studies, the synergistic effects of eugenol/carvacrol and eugenol/thymol might be
218 associated with the fact that carvacrol and thymol can disintegrate the outer membrane of E. coli,
219 making it easier for eugenol to enter the cytoplasm and combine with proteins.

220 As concerns carvacrol/thymol combinations, an antagonistic effect was observed for *E. coli* at
221 every ratio while a mild synergistic action was detected for *L. innocua* at the highest carvacrol
222 ratio (Table 2). In contrast, Pei et al. (2003) observed a synergistic activity of these compounds
223 *against E. coli*. However, other authors (Gallucci et al., 2009; Rivas et al., 2010) did not find that
224 positive interactions between these compounds improved their antibacterial action. The
225 occurrence of an additive or indifferent interaction between carvacrol and thymol could be
226 related to the similarity in their molecular structures (they are isomers), suggesting a similar
227 mechanism of action.

228 Despite the different molecular structure of carvacrol and eucalyptol, which could promote a
229 different mechanism of action, antimicrobial activity was not promoted in the
230 carvacrol/eucalyptol mixtures, in contrast with that reported by de Sousa et al. (2012) and de
231 Oliveira et al. (2015). In fact, binary combinations with eucalyptol were the least effective in most
232 cases, in line with its higher MIC value for both bacteria. So, its antibacterial activity was the
233 lowest, both alone or combined with other, more active compounds. Only when combined with a
234 small proportion of thymol, was the FIC index value lower than 1 for *L. innocua* (Table 2).

235

Compound combinations, given in Table 2, allow for greater antibacterial action than that achieved with the respective, pure compounds, using a lower total amount of actives. It is remarkable that wider synergistic spectrum was obtained for *L. innocua* than for *E. coli*, which could be related with the different bacteria cell envelope of Gram-positive and Gram-negative bacteria. Gram-positive bacteria surrounded by layers of peptidoglycan, many times thicker than is found in *E. coli,* could be more sensitive to the combined action of different compounds that are able to interact with the bacteria cell envelope to a different extent. The compound combination that was best at controlling the growth of *E. coli* was carvacrol/cinnamaldehyde (1:0.1 ratio), whose MIC value was 0.55 mg/mL. This combination was also the most effective against *L. innocua* (MIC value 0.45 mg/mL), but when using a caravacrol/cinnamaldehyde ratio of 0.5:4.

4. Conclusions

The MTT method was effective at evaluating the potential synergistic antibacterial effect simply and quickly through the FIC index assessment of blends of active components from essential oils, which can be easily standardized. This method provided reliable MIC values of the active compounds, as well as the FIC index value of their binary combinations over a wide concentration range below the respective MICs. The most remarkable synergistic effect was observed for carvacrol/cinnamaldehyde blends for both *E. coli* and *L. innocua*, but using different compound ratios (1:0.1 and 0.5:4 respectively for each bacteria). In general, the obtained results concerning the synergistic effects of the EO components agree with those reported by other authors, although some discrepancies were obtained that are attributable to the antimicrobial susceptibility method used (temperature, culture media, pH, bacterial strain, …). Likewise, the MTT method allows for a wide range of concentrations to be tested, which better permits the estimation of the optimal ratio of active compounds with which to obtain the maximum synergy. The synergistic effect was more notable in *Listeria innocua* than in *Escherichia coli*. The obtained

261 results allowed the dose of active compounds used for food application purposes to be optimized,
262 thus minimizing their sensory impact.
263
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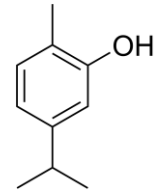
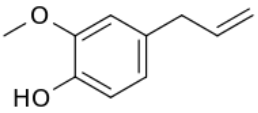
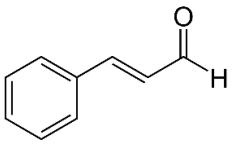
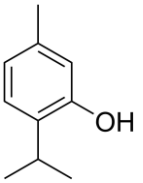
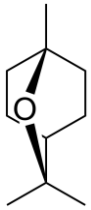
386 cinnamaldehyde in combination with carvacrol against food-borne bacteria. *Food*
387 *Control,* 34(2), 619-623. This reference has been followed for the assessment of the
388 antimicrobial activity of EO compounds, as well as the synergistic interactions between
389 them by using the MTT assay.
390

391 **Table 1.** Minimum inhibitory concentration (MIC) of the different active compounds tested

392 against *E. coli* and *L. innocua*

		MIC (mg/mL)	
Active compound	**Molecular structure**	***E. coli***	***L. innocua***
Carvacrol		0.70	0.75
Eugenol		1.35	1.05
Cinnamaldehyde		0.50	0.50
Thymol		0.65	0.70
Eucalyptol		1.75	1.25

393

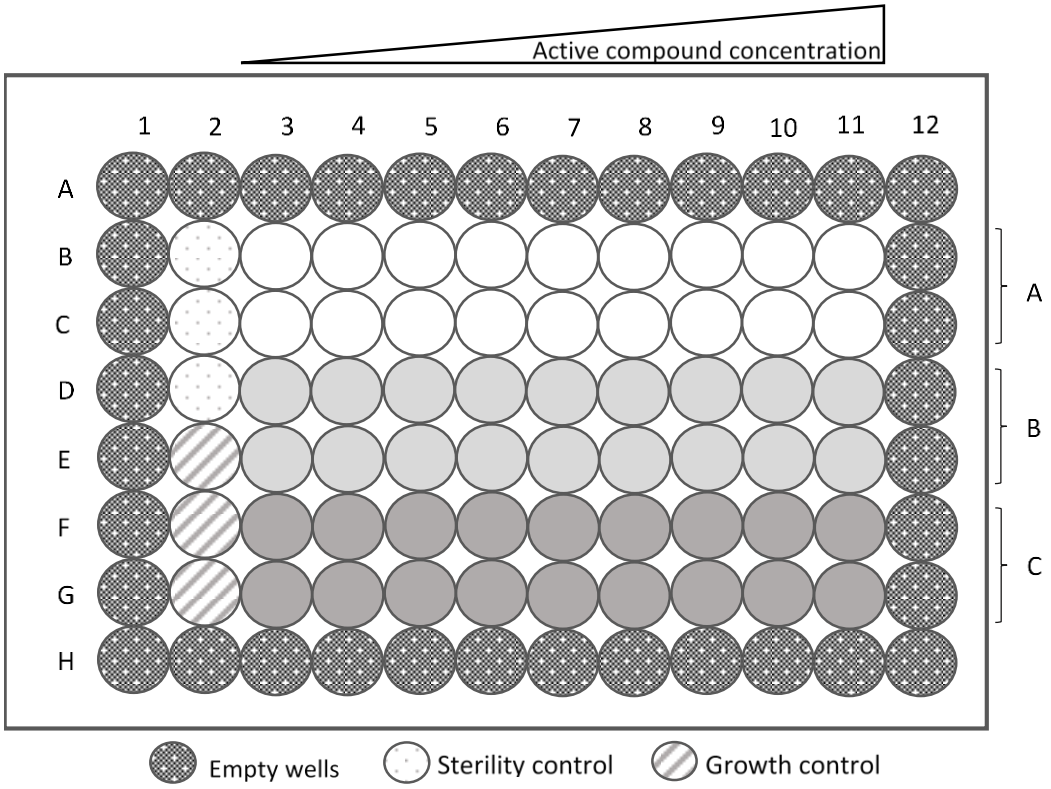
394

Table 2. Binary combinations with the highest synergistic effect (lowest FIC index) against *L. innocua* and *E. coli.*

	E. coli		*L. innocua*	
Synergistic combination (A/B)	**A (mg/mL)**	**B (mg/mL)**	**A (mg/mL)**	**B (mg/mL)**
Carvacrol/Cinnamaldehyde	0.50	0.05	0.05	0.40
Carvacrol/Thymol	nf	nf	0.60	0.10
Eugenol/Carvacrol	0.40	0.45	0.90	0.10
Eugenol/Cinnamaldehyde	0.80	0.10	0.20	0.40
Eugenol/Thymol	nf	nf	0.90	0.05
Eucalyptol/Thymol	nf	nf	1.00	0.05
Eucalyptol/Cinnamaldehyde	nf	nf	1.00	0.10
Thymol/Cinnamaldehyde	nf	nf	0.45	0.10

nf: No synergistic combination found

399



400

401

402 **Figure 1.** Experimental design for the determination of the minimum inhibitory concentration
403 (MIC) of three different active compounds (A, B and C), with their respective duplicates. Sterility
404 and growth control were prepared with non-inoculated and inoculated culture media, whereas
405 the outer wells were left empty to avoid edge effect. Wells in the same position

406

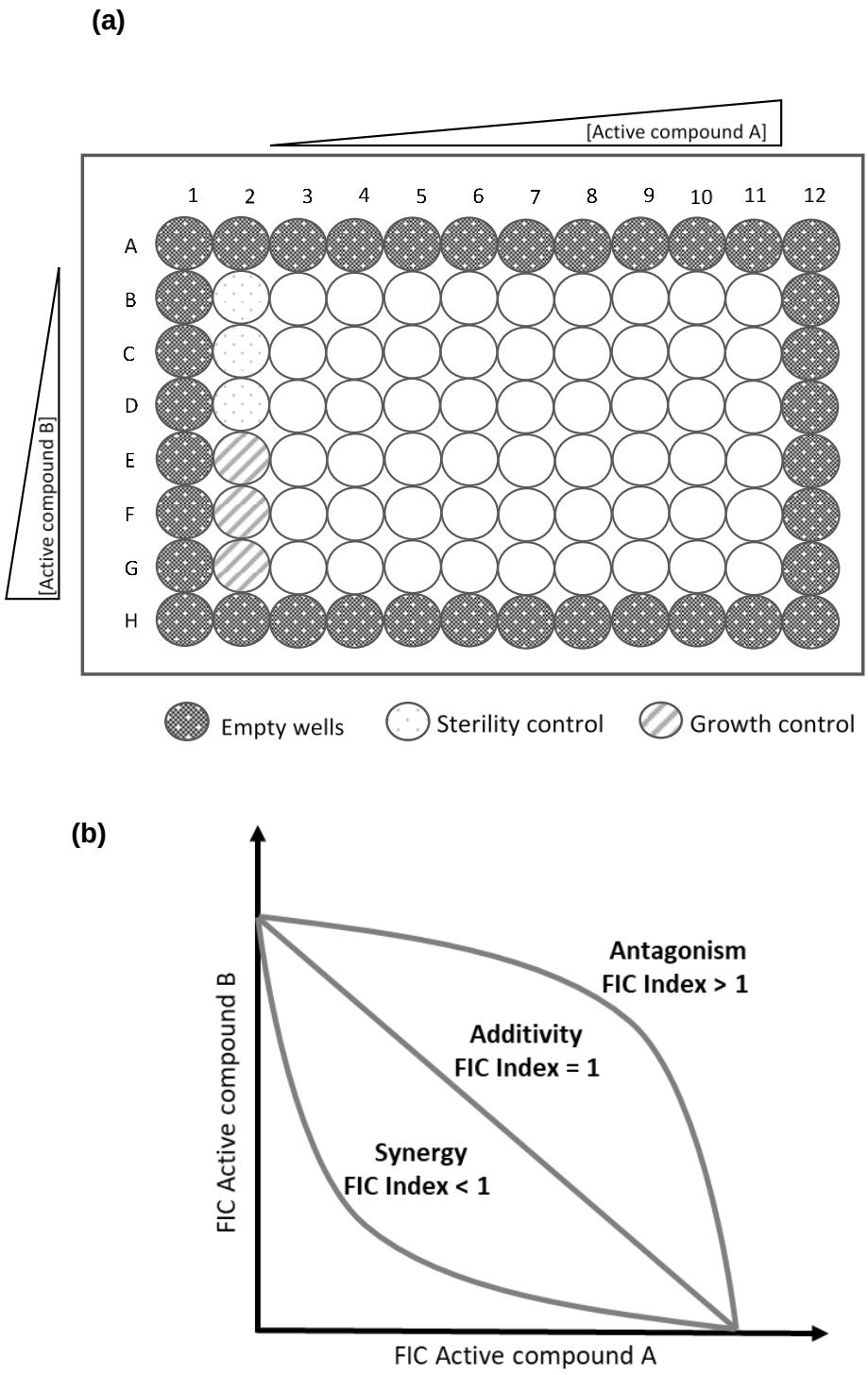
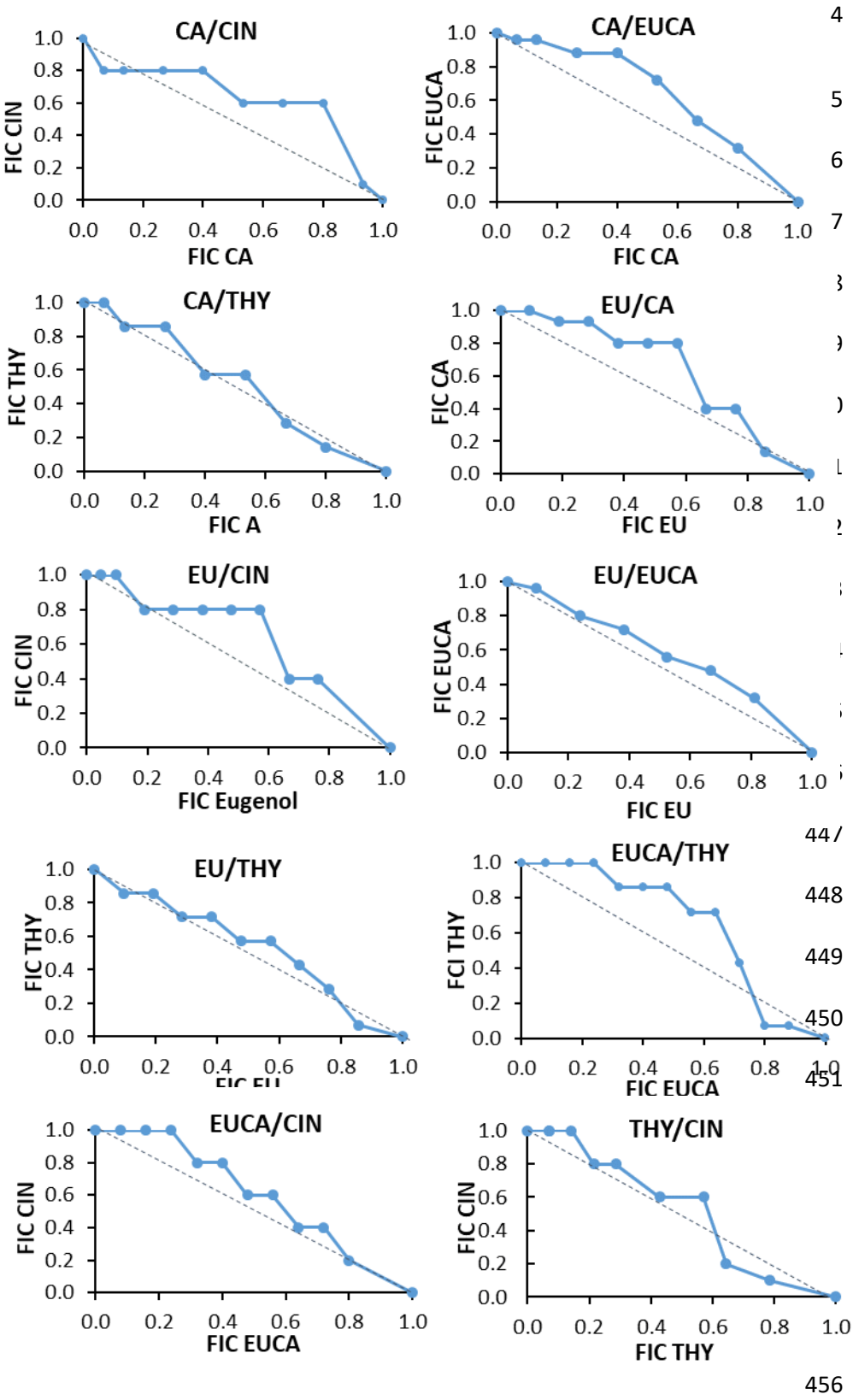
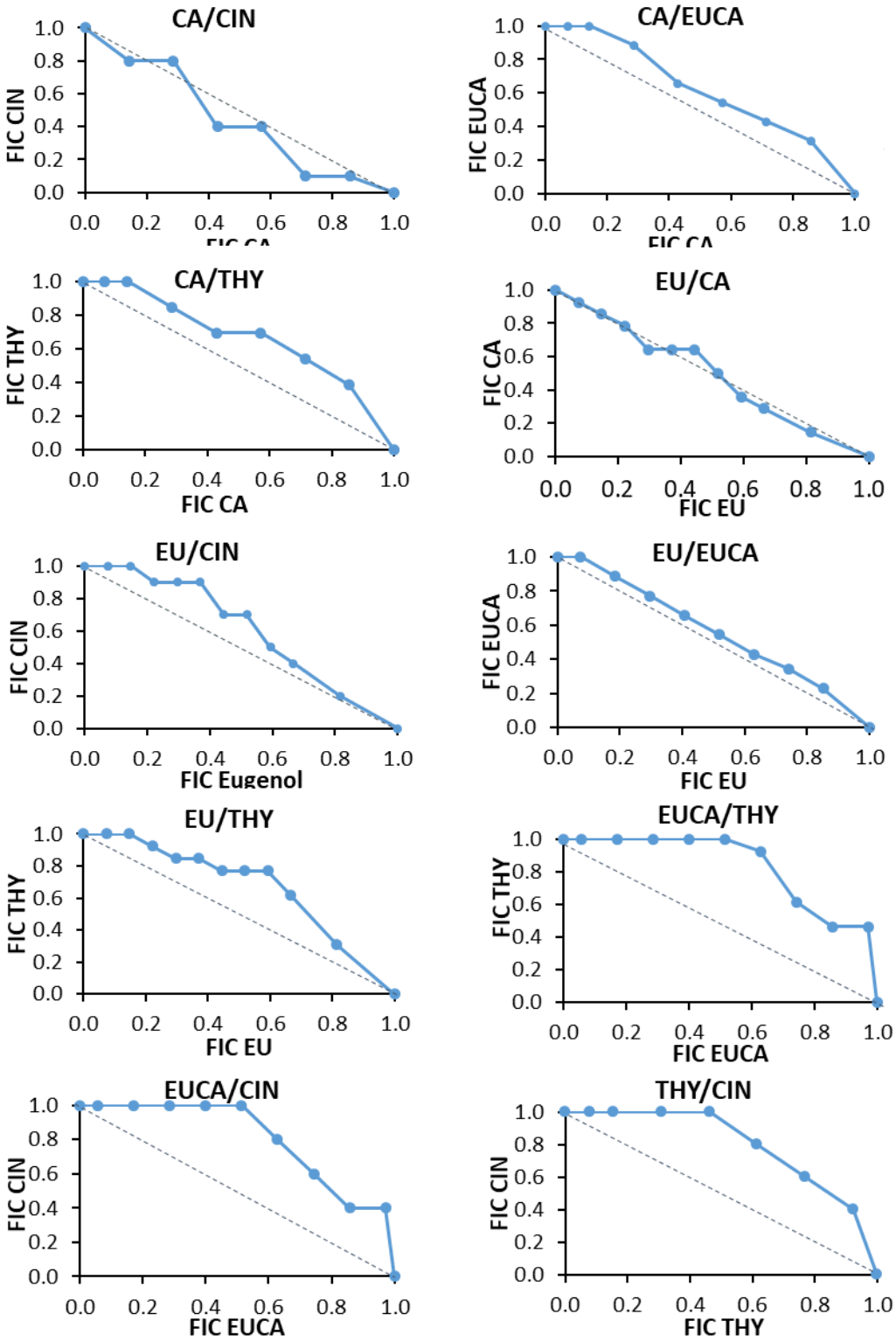


Figure 2. (a) Experimental design for the determination of the fractional inhibitory concentration (FIC) for each active compound in a binary mixture. Sterility and growth control were prepared with non-inoculated and inoculated culture media, whereas the outer wells were left empty to avoid edge effect. (b) Theoretical isobolograms displaying the three types of possible effects (additivity, antagonism and synergy), according to the FIC index values.



457 **Figure 3.** Isobolograms showing the fractional inhibitory concentration (FIC) for the binary
458 combinations of the active compounds (carvacrol (CA), eugenol (EU), cinnamaldehyde (CIN),
459 thymol (THY), eucalyptol (EUCA)) against *L. innocua.*



490 **Figure 4.** Isobolograms showing the fractional inhibitory concentration (FIC) for the binary
491 combinations of the active compounds (carvacrol (CA), eugenol (EU), cinnamaldehyde (CIN),
492 thymol (THY), eucalyptol (EUCA)) against *E. coli.*

493

Figure captions

Figure 1. Experimental design for the determination of the minimum inhibitory concentration (MIC) of three different active compounds (A, B and C). Sterility and growth control were prepared with non-inoculated and inoculated culture media, whereas the outer wells were left empty to avoid edge effect.

Figure 2. (a) Experimental design for the determination of the fractional inhibitory concentration (FIC) for each active compound in a binary mixture. Sterility and growth control were prepared with non-inoculated and inoculated culture media, whereas the outer wells were left empty to avoid edge effect. (b) Theoretical isobolograms displaying the three types of possible effects (additivity, antagonism and synergy), according to the FIC index values.

Figure 3. Isobolograms showing the fractional inhibitory concentration (FIC) for the binary combinations of the active compounds (carvacrol (CA), eugenol (EU), cinnamaldehyde (CIN), thymol (THY), eucalyptol (EUCA)) against *L. innocua.*

Figure 4. Isobolograms showing the fractional inhibitory concentration (FIC) for the binary combinations of the active compounds (carvacrol (CA), eugenol (EU), cinnamaldehyde (CIN), thymol (THY), eucalyptol (EUCA)) against *E. coli.*