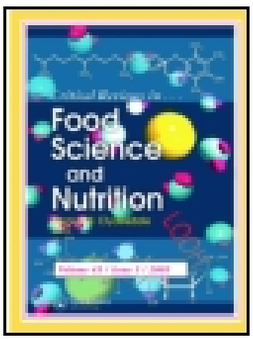

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A Review on Influencing Factors on the Minimum Inhibitory Concentration of Essential Oils

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ABSTRACT

With growing interest in essential oils as natural preservatives in the food industry, the literature is expanding enormously. To understand the antimicrobial activity of essential oils, the antimicrobial mechanism of individual essential oil (EO) compounds, and their minimum inhibitory concentrations (MICs), are interesting starting points for research. Therefore, and to get insight into the factors influencing their antimicrobial activities, the Web of Science was searched for MICs of EO compounds (1995-2016). Many MICs for individual EO compounds have already been reported in the literature, but there is large variability in these data, even for the MIC of the same compound against the same species.

No correlation was found between the tested structural parameters of EO compounds (polarity, water solubility, dissociation constant, molecular weight and molecular complexity) and their MICs against all microorganisms, Gram-negative bacteria, Gram-positive bacteria and fungi. Few clear differences in sensitivity between microorganisms could be found. Based on this

review it is clear that different incubation conditions, culture media and the use of emulsifiers/solvents have an influence on the MIC, causing big variance. This review points out the need for a good international standard method to assess the antimicrobial activity of EO compounds for better comparability between studies.

KEYWORDS

essential oil compounds, minimum inhibitory concentration, molecular structure, experimental design, emulsion

INTRODUCTION

Essential oils (EOs), produced by the plants' secondary metabolism, are volatile aromatic extracts, liquid at room temperature (Hyldgaard et al. 2012). They are known for their beneficial biological activities, such as bactericidal, virucidal, fungicidal, antiparasitic, insecticidal and antioxidative effects (Bakkali et al. 2008). Furthermore, EOs work at the level of multiple targets preventing the development of resistance in microorganisms (Nazzaro et al. 2013; Leite de Souza 2016). In recent years, more interest has been devoted to the application of natural EOs in cosmetics and pharmaceuticals, as well as as flavorings and preservatives for foods, likely due to growing negative perception of synthetic additives by consumers (Hyldgaard et al. 2012).

EOs are composed of terpenes, terpenoids, phenylpropane-derived aromatic compounds and aliphatic compounds (Nazzaro et al. 2013). They are complex mixtures that can contain many different compounds (Zuzarte and Salgueiro 2015). Even more than 220 different compounds were found in Indian rose-scented geranium oil (Rao 2009). The composition (compounds and their respective concentrations) of EOs can vary significantly, depending on the extraction method, the plant organ used, the harvest season and the origin of the plant (Figueiredo et al. 2008; Yuan et al. 2016). The antimicrobial activity of an essential oil (EO) is dependent on the antimicrobial effect of each of the individual EO compounds and some synergistic, additive and antagonistic interactions between them (Hyldgaard et al. 2012; Nazzaro et al. 2013). Therefore, the varying composition of EOs makes it difficult to compare their antimicrobial activities. However, knowledge of the specific antimicrobial action of the individual EO compounds could help to understand the antimicrobial activity of the EOs and give insight into their application

potentials. Hence, by looking only at the antimicrobial effect of individual EO compounds instead of the EOs, variation in composition of EOs is ruled out. Furthermore, studies investigating the synergistic or additive effects of different combinations of EO compounds can give more insight into how the composition of EOs affects their antimicrobial activities. Knowledge of the antimicrobial activity of individual compounds of EOs can lead to applications of individual EO compounds themselves, but it can also be a basis for a reasoned application of specific combinations of EOs.

In this review the minimum inhibitory concentration (MIC) was used as a measure for antimicrobial activity. Several studies have been conducted to prove the influence of different parameters (chemistry of the EO compound, experimental parameters, microorganisms) on the antimicrobial activity of EOs and their compounds, but to our knowledge, no reviews have been published on all parameters influencing the MIC of EO compounds. Janssen et al. (1987) reviewed some aspects of test methods for antimicrobial activity (assay techniques, growth media and microorganisms). However, they only focused on EOs instead of their individual compounds. Kalemba and Kunicka (2003) reviewed the antimicrobial activity of both the EOs and their compounds with the main focus on different test methods. Lang and Buchbauer (2012) reviewed the antimicrobial activity of EOs in general and only briefly discussed the influence of different test methods. Bassolé and Juliani (2012) wrote that there were only 3 articles that tested the effect of different parameters (NaCl concentration, vapor phase and temperature) on the synergy/antagonism between EO compounds. So, currently, no review is available summarizing the possible antimicrobial effects of the different individual EO compounds present in the EOs and relating their activity with different influencing factors.

Therefore, the aim of this systematic review was to analyze the published data related to the antimicrobial activity of different EO compounds. The review aims to link the observed MIC of individual EO compounds with the chemistry of the EO compound (polarity/solubility, molecular size, functional groups, vapor pressure, stereochemistry and stability), the microorganism (domain, species and strain) and the experimental conditions (method, microbial medium, atmosphere, incubation time, incubation temperature, inoculum size, inoculum preparation, shaking in contrast to static incubation and emulsifier/solvent use).

DATA COLLECTION AND STATISTICAL ANALYSIS

The Web of Science was searched for articles (from 1995 to 2016) reporting the MIC of EO compounds against all possible investigated microorganisms. The included EO compounds are reported in EO compositions in the literature. Therefore, the following searching criteria were used: the name of the EO compound combined with minimum inhibitory concentration, antimicrobial, antifungal, antibacterial, bactericidal, antiviral and antiparasitic, present in the title. Only those papers that reported minimum inhibitory concentrations (MICs) in µM or that could be recalculated to µM were used, so comparison between different studies was possible. The included EO compounds are: farnesol, ocimene, myrcene, nerol, citronellol, geraniol, linalool, lavandulol, nerolidol, tagetone, geranyl acetone, citral, farnesal, linalyl acetate, geranyl acetate, citronellyl acetate, farnesyl acetate, geranyl formate, linalyl propionate, citronellal, geranic acid, bisabolene, elemene, phellandrene, terpinene, limonene, terpineol, perillaldehyde, carveol, terpinen-4-ol, perillol, carvone, piperitone, iso-piperitone, menthone, pulegone, menthol, menthyl acetate, terpinyl acetate, curcumene, cymene, thymol, carvacrol, tyrosol, eugenol,

anisole, anethole, estragole, methyl eugenol, methyl salicylate, salicylaldehyde, benzaldehyde, cuminaldehyde, anisaldehyde, vanillin, cinnamaldehyde, cinnamyl alcohol, chavicol, hinokitiol, asarone, paeonol, camphene, carene, pinene, sabinene, thujone, camphor, fenchone, verbenone, pinocamphone, pinocarvone, eucalyptol, ascaridole, borneol, fenchol, verbenol, myrtenol, myrtanol, chrysanthenol, myrtenal, bornyl acetate, caryophyllene, aromadendrene, menthofuran, safrole, piperine, cadinene, guaiazulene and nootkatone. The search resulted in 347 usable research papers and includes MIC values against 353 different species belonging to 147 different genera (67 bacteria (42 Gram-negative and 25 Gram-positive), 79 fungi and 4 parasites). All data were gathered including the following information: MIC of the EO compound against microorganisms for each reference reported as single values, ranges or cutoffs and experimental conditions. Before performing statistical analysis, all entries where a cutoff for the MIC was reported (MIC >, <, ≥ or ≤ x) were removed. If ranges were reported they were replaced by their mean value. All statistics were performed using R 3.2.3 (R Development Core Team 2008).

The water solubility of the selected EO compounds was searched in the Human metabolome database (HMDB) (Wishart et al. 2013). If an experimental value was reported, the experimental value was chosen, otherwise, as for most (n = 63 out of 87) of the EO compounds, the predicted solubility was used (ALOGPS) (Tetko and Tanchuk 2002).

To assess the polarity of the EO compounds, the XLogP3 was retrieved from PubChem (Cheng et al. 2007; NCBI 2016). To test correlations between physicochemical parameters of the EO compounds and their antimicrobial activity, the Pearson correlation coefficient (r) was calculated in R, using the rcorr function (Harrell and Dupont 2016).

FACTORS THAT INFLUENCE THE MIC OF EO COMPOUNDS

1. Structure-function relationships

There is an enormous variability in the MIC of EO compounds reported in the literature. In the full dataset, independent of the EO compound or the microorganism tested, an average (SD) and median of respectively 2.5 x 10^4 (1.1 x 10^5) and 3.6 x 10^3 µM, were obtained. The different structural factors (water solubility and polarity, dissociation constants, molecular size, isomers, functional groups and chemical stability), that might have an influence on the MIC of an EO compound, are further discussed here.

Water solubility

The water solubility of an EO compound is linked to its polarity. Elemene (XlogP3 = 6.1) is the most hydrophobic EO compound included in the dataset, and tyrosol (XlogP3 = 0.4) is the least hydrophobic one. The median XlogP3 of the selected EO compounds is 2.9.

Looking at the whole generated dataset (Figure 1a), there is a no correlation between the MIC of an EO compound and its XlogP3 (polarity). The same is true for the subsets of Gram-negative bacteria (Figure 1b), Gram-positive bacteria (Figure 1c) and fungi (Figure 1d). This means that the polarity of the EO compound only explains a small part of the variability of the MIC of the included EO compounds.

Andrade-Ochoa et al. (2015) found, with mathematical modelling, that the Moriguchi octanol-water partitioning coefficient (MLogP) shows a high contribution to the antimicrobial activity of EO compounds against *Mycobacterium tuberculosis*, while the hydrophobicity negatively

contributed to the antimicrobial activity of EO compounds against *Mycobacterium bovis*. This indicates that the influence of hydrophobicity on the antimicrobial activity of EO compounds is species dependent. The polarity of an EO compound influences its ability to travel across and/or perturb membranes. It is, therefore, possible that the more hydrophobic EO compounds (membrane disruption), have different cellular targets than less hydrophobic ones (interaction with proteins). For some EO compounds, important mechanisms, other than membrane disruption, have already been discovered. For example, cinnamaldehyde was found to inhibit cell wall synthesis enzymes (β-(1,3)-glucan synthase and chitin synthase 1) (Bang et al. 2000). Thymol and carvacrol, on the other hand, interfere with ergosterol biosynthesis leading to changes in membrane fluidity next to their direct membrane disrupting activity (Ahmad et al. 2011).

Griffin et al. (1999) determined the MICs of 60 terpenoids against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* and clustered the EO compounds according to the MICs. Five groups of terpenoids were distinguished regarding their MIC, and the logP of the EO compounds was only a distinguishing factor between two of these groups (Griffin et al. 1999). The first group had a high antimicrobial activity against all tested organisms and a mean LogP of 3.05 while the other group had a low antimicrobial activity against all tested organisms and a mean LogP of 3.68. However, this difference in polarity is small and the authors concluded that the water solubility and the hydrogen bonding capacity contributed more to the difference between the groups than the LogP (both actors are discussed in more detail below) (Griffin et al. 1999).

Due to their high hydrophobicity, EO compounds have a limited solubility in water. The lowest solubility among the selected EO compounds, 5.2×10^{-1} µM, was found for cadinene while the highest solubility, 1.8×10^{5} µM, was found for tyrosol. The median solubility of the selected EO compounds is 3.2×10^{3} µM. There is no correlation between the water solubility of the selected EO compounds and their MIC against all microorganisms (Figure 2a), as well as within the microbial groups: Gram-negative bacteria (Figure 2b), Gram-positive bacteria (Figure 2c) and fungi (Figure 2d). So, the same conclusions can be drawn for the solubility as for the XLogP3 regarding the antimicrobial activity. However, it should be considered that the real maximum aqueous concentration of the EO compounds in the antimicrobial test medium can differ from the intrinsic water solubility of the EO compound that we used for the calculations. Particularly, the solubility of EO compounds is influenced by some experimental parameters, such as pH, temperature, salt concentration and emulsifier/solvent.

In contrast with the lack of correlation discussed above, Griffin et al. (1999) found that the water solubility was the most important factor for determining the MIC of terpenoids after H-bonding capacity. They suggest that the thin water layer that surrounds microorganisms might form a barrier for less soluble terpenoids, preventing the action of these EO compounds, even if they are dispersed evenly in an emulsion. Cox et al. (2001) showed that the antimicrobial activity of terpinen-4-ol was reduced when the concentration of γ-terpinene was increased. The increased concentration of γ-terpinene reduced the aqueous solubility of terpinene-4-ol. This also suggests that the EO compounds should be dissolved to be active. In conclusion, it might be important for the antimicrobial activity that the EO compounds are dissolved in the aqueous phase although

their hydrophobicity is part of their mode of action. However, more research in this area is needed.

Dissociation constants

The dissociation constants of EO compounds may also play an important role in their antimicrobial activity, because they determine their polarity and water solubility at a given pH. Terpinen-4-ol (pKa = 20) had the highest pKa of the selected EO compounds, while hydrocarbons are not acidic or basic at all. There is no correlation between the pKa of the included EO compounds and their MIC against all microorganisms (Figure 3a), as well as within the microbial groups: Gram-negative bacteria (Figure 3b), Gram-positive bacteria (Figure 3c) and fungi (Figure 3d).

Ultee et al. (2002) found that the antimicrobial effect of carvacrol against *Bacillus cereus* was caused by the disruption of the membrane and by the fact that carvacrol was acting as a proton exchanger, thereby destroying the proton gradient over the microbial membrane and inhibiting ATP production. EO compounds with a pKa between the pH inside the cell (microorganism dependent (Krulwich et al. 2011)) and outside the cell (growth medium dependent) are ideal to function as proton exchangers, because they can take up a proton at one side of the membrane and release it on the other side. Gallucci et al. (2009) determined the antimicrobial activity of eugenol (pKa = 8.55), thymol (pKa = 8.81), carvacrol (pKa = 9.07), geraniol (pKa = 16.33 (Wishart et al. 2013)) and carvone (pKa ≈ 25 (Matthews et al. 1975)) against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (Gallucci et al. 2009). Carvacrol had the lowest MIC against the three species (MIC: 5.1×10^4, 2.5×10^4 and 2.5×10^4 µM respectively), followed by

thymol (MIC: 1.0 x 10^5, 5.0 x 10^4 and 5.0 x 10^4 µM respectively), eugenol (MIC: 4.1 x 10^5, 2.0 x 10^5, 4.1 x 10^5 µM respectively) and geraniol (1.4 x 10^6 µM against all). Carvone, which is unlikely to act as a proton exchanger, showed only antimicrobial activity against *Staphylococcus aureus* (MIC: 5.0 x 10^4 µM) (Gallucci et al. 2009). Geraniol, which has an intermediate pKa, also has an intermediate MIC against the tested organisms. The pKa of carvacrol, thymol and eugenol is in the same range, near the physiological conditions inside and outside the cells. Therefore, the EO compounds can act as proton exchangers. However, there is still some variation in MIC between them, suggesting that other mechanisms might also play a role in their antimicrobial activity (hydrogen bonding) (Gallucci et al. 2009).

Molecular size

Next to the polarity of an EO compound, the ability of the EO compound to cross membranes also depends on the molecular size of the EO compound. The molecular weight of an EO compound gives an easy indication of the molecular size. Benzaldehyde (106 g/mol) is the EO compound with the lowest molecular weight included in the dataset and piperine (285 g/mol) was the one with the highest molecular weight. The median molecular weight of the included EO compounds is 154 g/mol. There is no correlation between the molecular weight of the selected EO compounds and their MIC against all microorganisms (Figure 4a), as well as within the microbial groups: Gram-negative bacteria (Figure 4b), Gram-positive bacteria (Figure 4c) and fungi (Figure 4d).

The molecular weight alone does not give complete information about the molecular size. The molecular complexity of an EO compound is a parameter that is less convenient to calculate or

interpret. It is however of great importance to molecular docking and can therefore, also have an influence on the antimicrobial activity (Hann et al. 2001). Different calculations can be used to determine the molecular complexity. Each of them takes into account some aspects of molecular complexity such as the elements present in the EO compound, the symmetry and stereochemistry of the EO compound (Hendrickson et al. 1987; Ruijter et al. 2011). The measure used here, the Bertz, Hendrickson and Ihlenfeldt formula, is the most commonly used one (Hendrickson et al. 1987). It takes into account the skeletal complexity, diversity of elements and symmetry and is reported in PubChem (NCBI 2017). The median Bertz, Hendrickson and Ihlenfeldt complexity of the included EO compounds is 185. No correlation between the Bertz, Hendrickson and Ihlenfeldt complexity of an EO compound and its MIC was found (Figure 5). But both parameters, molecular weight and Bertz, Hendrickson and Ihlenfeldt complexity respectively, are very highly correlated ($r=0.82$; $p<0.001$; Figure 6). The absence of their correlation with the MIC means that the molecular weight and complexity of an EO compound only explain a small part of the variability in the MIC data. Andrade-Ochoa et al. 2015 found, with mathematical modelling, that the molar volume of EO compounds is important for their antimicrobial activity against both *Mycobacterium tuberculosis* and *Mycobacterium bovis*. They linked this to the involvement of the compounds in the solute-transfer process across the cytoplasmic membrane.

Isomers

Different isomers of EO compounds can occur with different antimicrobial activity. Both isomers of pinene (α and β) differ in the position of a double bond. Yousefzadi et al. (2008) found that α-pinene was more active against *Bacillus subtilis* (MIC=2.8 x 10^4 µM) and

Escherichia coli (MIC=1.1 x 10^5 µM) than β-pinene (MIC=1.1 x 10^5 µM and >1.1 x 10^5 µM respectively). Also, Ngassapa et al. (2016) found 2- up to more than 5-fold lower MICs of α-pinene than β-pinene (against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Owolabi et al. (2010) found that the most active pinene isomer was species dependent: α-pinene had the lowest MIC against *Escherichia coli* (2.3 x 10^3 in contrast to 4.6 x 10^3 µM for β-pinene), *Pseudomonas aeruginosa* (4.6 x 10^3 in contrast to 9.1 x 10^3 µM for β-pinene) and *Candida albicans* (1.1 x 10^3 in contrast to 4.6 x 10^3 µM for β-pinene), while β-pinene had the lowest MIC against *Aspergillus niger* (4.6 x 10^3 in contrast to 1.1 x 10^3 µM against α-pinene) and they both had the same MIC against *Bacillus cereus* (2.3 x 10^3 µM) and *Staphylococcus aureus* (4.6 x 10^3 µM). In contrast, Tampieri et al. (2005) found a higher MIC of α-pinene (>7.3 x 10^3 µM) than β-pinene (7.3 x 10^2 µM) against *Candida albicans*. The difference in results between both studies can be attributed to the use of a different strain of *Candida albicans* (ATCC No.90028 in contrast to a non-specified strain from human origin) or different incubation conditions (inoculum size of 7.5 x 10^7 CFU/mL in contrast to approximately 10^3 CFU/mL; 48 in contrast to 24h; 37 in contrast to 25°C; yeast-mold broth in contrast to Sabouraud Dextrose Broth; 1% stock in DMSO in contrast to 0.5% agar and 0.1% Tween-20 added to the medium; 96-well plate in contrast to tubes). The MIC of o-cymene (3.7 x 10^3 µM) against *Candida albicans* was different from that of p-cymene (7.5 x 10^2 µM). Both isomers differ in the position of a methyl group on the aromatic ring. Two tested isomers of terpinene (α and γ) had the same MIC (7.3 x 10^2 µM) against *Candida albicans*. EO compounds can also contain chiral atoms and can thus have different stereoisomers, which might result in a different antimicrobial activity. Aggarwal et al.

(2002) found that (R)-(+)-carvone was more active against both bacteria and fungi than (S)-(-)-carvone and (S)-(-)-limonene had little antimicrobial activity compared to (R)-(+)-limonene. In contrast, Mahumane et al. (2016) found the same MIC for (S)-(-)-limonene and (R)-(+)-limonene against *Lactobacillus acidophilus, Streptococcus pyogenes* and *Streptococcus pneumoniae*. Against *Streptococcus mutans* and *Streptococcus agalactiae*, the MIC of (S)-(-)-limonene was slightly higher (2.5×10^3 in contrast to 1.6×10^3 µM and 4.9×10^3 in contrast to 4.1×10^3 µM respectively). The effectivity of the stereoisomers of α-pinene is microbial species and strain dependent (Lis-Balcnin et al. 1999). Both stereoisomers of pinocamphone (R and S) were inactive against *Candida albicans* (MIC > 6.6×10^3 µM) (Tampieri et al. 2005). In conclusion, the antimicrobial activity of isomers of EO compounds can differ depending on the microbial strain, EO compound and possibly the experimental conditions.

Functional group

The functional groups of EO compounds affect their antimicrobial activity on multiple levels. They play a role in the polarity, solubility, hydrogen bonding capacity and pKa of the EO compounds, which all might influence the antimicrobial activity. Kalemba and Kunicka (2003) noted that phenols had the highest antimicrobial activity, followed by aldehydes, ketones, alcohols, ethers and hydrocarbons. This was confirmed using a mathematical modeling approach by Andrade-Ochoa et al. (2015). They found that, out of the parameter set: number of ring tertiary C, number of total quaternary C, number of ring quaternary C, nerber of terminal primary C, number of phenolic groups, number of ketone groups energy of the lowest unoccupied molecular orbital, Moriguchi octanol-water partition coefficient, molar volume, sum of atomic

Sanderson electronegativities, total absolute charge and electron affinity, the number of phenolic groups was the most positively related (1.05% contribution) with the antimicrobial activity against *Mycobacterium tuberculosis*. The number of ketone groups is negatively related (-1.06% contribution) with this antimicrobial activity. Also for the antimicrobial activity against *Mycobacterium bovis*, the number of phenol groups was the most important parameter out of the set: number of terminal primary C, number of non-aromatic conjugated C, number of phenolic groups, number of aliphatic tertiary C, number of acceptor atoms for H-bonds, unipolarity,Ghose-Crippen octanol-water partition coefficient, energy of the highest occupied molecular orbital and dipole moment.

By comparing the antimicrobial activity of EO compounds that are structurally similar, it is possible to hypothesize about the importance of certain functional groups and about the mechanism of action. In the selected groups (Figure 7-12; structures Table 1-3), the order of activity reported by Kalemba and Kunicka (2003) cannot be distinguished, probably because of the large variability in MICs of EO compounds found in the literature. Veldhuizen et al. (2006) compared the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* of carvacrol with that of structurally related EO compounds (3-isopropylphenol, p-cymene, o-cresol, 3,4-dimethylcumene and 2-amino-p-cymene) (Figure 13). The hydrocarbon EO compounds were not active (MIC > 3.6×10^4 µM) and insoluble (< 5.0×10 µM). The removal of the methyl group or the isopropyl group of carvacrol has little effect on its antimicrobial activity indicating that the hydroxyl group is the most important. The substitution of the hydroxyl group with an amino group reduces the antimicrobial activity but does not affect its solubility. This may be explained by i) the phenolic hydroxyl group is more acidic rendering it a

better proton exchanger and explaining its involvement in the disruption of the proton gradient over the microbial membrane; ii) the hydroxyl group has a higher hydrogen bonding capacity than the amino group. Hydrogen bonding with phosphate or ester linkages of phospholipids could influence the membrane disrupting activity of EO compounds. Also Griffin et al. (1999) already identified the hydrogen-bonding capacity as the most important parameter for antimicrobial activity of terpenoids. Gallucci et al. (2009) also attributed the difference in antimicrobial activity between the two phenolic terpenoids carvacrol and thymol and the phenylpropane-derived eugenol to the hydrogen bonding capacity. Eugenol can form intramolecular hydrogen bonds rendering it less able to form intermolecular hydrogen bonds, diminishing its antimicrobial activity.

The first example of a set of EO compounds with similar structure are the limonene-related EO compounds. Limonene is a monocyclic monoterpene (Figure 7 and 8; Table 1). The other EO compounds in Table 1 have the same basic structure but have additional functional groups. Overall carveol has the lowest median MIC against Gram-negative bacteria (9.9×10^2 µM), carvone against Gram-positive bacteria (9.3 x 10 µM) and perillol against fungi (5.0 x 10 µM) (Figure 7). Carveol and perillol have an alcohol function while carvone has a ketone function (structures Table 1). Carveol and perillol are both the least active against Gram-positive bacteria, followed by Gram-negative bacteria and fungi. Their median MIC against Gram-positive bacteria (3.4×10^3 and 4.1×10^3 µM respectively) are approximately the same indicating that the position of the alcohol function is less important for their antimicrobial activity against this group. Their median MIC against fungi (5.9×10^2 and 5.0 x 10 µM respectively) however, differs more. Perillaldehyde and perillol are especially active against fungi. Comparing carvone

with camphor indicates the importance of the degree of cyclization for the antimicrobial activity. Both EO compounds have a ketone function and the same molecular weight but differ in their degree of cyclization. Figure 8 shows that the biggest difference in antimicrobial activity between carvone and camphor is against Gram-positive bacteria (> 1 log difference). This is, possibly, because the spatial area of the bicyclic camphor makes it more difficult to penetrate the thick peptidoglycan layer.

A second example of a set of structurally related EO compounds are the EO compounds that are structurally related to the linear monoterpenes myrcene and ocimene (Figure 9 and 10; Table 2). Overall farnesol had the lowest median MIC against Gram-negative bacteria (1.0×10^2 µM) and Gram-positive bacteria (5.8 x 10 µM) and citronellol against fungi (8.0×10^2 µM) (Figure 9). The pKa of farnesol is 16.33, which is the same as for geraniol and nerol, having a similar structure but are only 10 carbon units long. Since geraniol (median MIC=2.8×10^3 µM) and nerol (median MIC=3.6×10^3 µM) are less active, the length of farnesol (median MIC=1.3×10^2 µM) might be responsible for its higher antimicrobial activity. A longer chain might be better for membrane disruption. However, the MICs of farnesol are more diverse than geraniol and nerol. In individual studies, the MIC of farnesol was not always lower than geraniol and nerol. Geraniol and nerol are more active against the Gram-negative bacteria *Chromobacterium violaceum* (MIC=8.1×10^2 and 1.6×10^3 µM respectively in contrast to >2.2×10^3 µM for farnesol) (Ahmad et al. 2015), *Salmonella* Typhimurium (MIC= 2.6×10^3 µM for geraniol in contrast to >4.4×10^4 µM for farnesol) (Nagaki et al. 2011), *Escherichia coli* (MIC: 2.9×10^3 and 4.0×10^3 µM respectively in contrast to >4.4×10^4 µM for farnesol) (Nagaki et al. 2011), *Proteus mirabiiis* (MIC=2.6×10^3 µM for geraniol in contrast to >4.4×10^4 µM for farnesol) (Nagaki et

al. 2011), *Shigella sonnei* (MIC=2.9 x 10^3 and 2.6 x 10^3 µM respectively in contrast to >4.4 x 10^4 µM for farnesol) (Nagaki et al. 2011) and *Serratia marcescens* (MIC: 2.7 x 10^3 µM for geraniol in contrast to >4.4 x 10^4 µM for farnesol) (Nagaki et al. 2011) while farnesol was more active against the Gram-positive bacteria *Staphylococcus aureus* (MIC: 8.3 x 10^2 µM for farnesol in contrast to 3.5 x 103 for geraniol and 2.6-3.6 x 10^3 µM for nerol) (Nagaki et al. 2011) and *Listeria monocytogenes* (MIC: 6.0 x 10^2 µM for farnesol in contrast to 2.5 x 10^3 µM for geraniol) (Nagaki et al. 2011). Farnesol is more active against fungi (*Trichophyton sp.* (Nagaki et al. 2011), *Colletotrichum graminicola* (Yang et al. 2011), *Fusarium graminearum* (Yang et al. 2011) and *Candida albicans* (Nagaki et al. 2011)) than geraniol when ethanol or DMSO is used to dissolve the EO compounds. But, when Tween-20 or Tween-80 is used to emulsify the EO compounds, geraniol and nerol are more active against fungi (*Candida albicans* (Marcos-Arias et al. 2011; Tampieri et al. 2005), *Candida tropicalis* (Tampieri et al. 2005), *Candida krusei* (Tampieri et al. 2005), *Candida parapsilosis* (Tampieri et al. 2005), *Candida glabrata* (Tampieri et al. 2005), *Candida guillermondii* (Tampieri et al. 2005) and *Candida dubliniensis*(Tampieri et al. 2005)).

The last example of a set of structurally related EO compounds are the cymene related aromatic EO compounds (Figure 11 and 12, Table 3). Overall estragole has the lowest median MIC against Gram-negative bacteria (6.4 x 10^2 µM), anethole against Gram-positive bacteria (6.7 x 10^2 µM) and cinnamaldehyde against fungi (6.1 x 10^2 µM) (Figure 11). Cymene (median MIC=8.3 x 10^3 µM) and cinnamyl alcohol (median MIC=8.0 x 10^3 µM) are less active than the other compounds in Table 3 (median MIC=6.1 x 10^2 - 3.8 x 10^3 µM). This indicates that phenols

act mainly as a proton donor/acceptor, while cymene (hydrocarbon) and cinnamyl alcohol (pKa=15.6) are the least likely to do so.

In conclusion, not much difference can be observed in the literature between EO compounds with different functional groups regarding their MICs because of the large variability in experimental conditions and microbial strains. Aldehydes and some alcohols seem to be potentially active against fungi.

Chemical stability

EO compounds can be chemically unstable due to chemical reactions (that is isomerization, oxidation, dehydrogenation and/or polymerization reactions and/or thermal rearrangements) altering their structure when exposed to heat, light and/or oxygen (Turek and Stintzing 2013). These processes depend on the EO compound used and the experimental conditions. Inouye et al. (2001a) incubated blood agar overnight at 37°C in an atmosphere with a dose of 1.5 x 10^3 µM in air of d-limonene. Afterward next to limonene (1.1 x 10 µM), different degradation products of limonene were found: oxygenated limonene (4.1 x 10 µM), p-menthane derivative (5.8 x 10 µM), trans-carveol (4.7 x 10 µM) and cis-carveol (2.2 x 10 µM) in the blood agar. When a dose of 2.9 x 10^3 µM α-pinene in air was used in the same setup, α-pinene (1.0 x 10 µM), dehydro-α-pinene (1.6 x 10 µM), oxygenated α-pinene (5.1 x 10 µM), verbenone (5.6 x 10 µM), hydroxy-ether derivative (4.2 mg/L) and t-sobrerol (1.9 x 10 µM) was found in the blood agar (Inouye, Takizawa, et al. 2001). Avonto et al. (2016) showed that terpinolene and α-terpinene levels in tea tree oil dropped to 50% after 2 and 13 days under elevated oxygen levels respectively. The other tested compounds (γ-terpinene, sabinene and terpinene-4-ol) remained stable or slightly

increased (p-cymene and α-terpineol). Further research is needed to see to what extent the stability of EO compounds affects susceptibility testing experiments. The limited stability of EO compounds should be considered in experimental setups that use these compounds.

2. Sensitivity of different microorganisms for EO compounds

Even for individual EO compounds, there is still an enormous variability in the MICs reported in the literature. The factors that determine the sensitivity of microorganisms for EO compounds are discussed here on different levels (domain, species and strain).

Domain dependency of the MIC of EO compounds

Microorganisms can be found in the three domains of life: Bacteria, Archaea and Eukarya. Viruses are non-cellular life forms not fitting in these three domains. No MICs of EO compounds against viruses were found in the literature. But there is evidence that EO compounds have antiviral activity. Gilling et al. (2014) found that 1.6 x 10^4 µM carvacrol reduced *Murine norovirus* with 4.5 log after 24 h while 3.3 x 10^4 µM was sufficient to completely inhibit the virus. They proved that 3.3 x 10^4 µM carvacrol evoked an expansion of the virus particles leading to capsid disintegration followed by destruction of the viral RNA. Pilau et al. (2011) determined the half effective concentration (EC_{50}) and selectivity index (SI_{50}) of carvacrol against different viruses when it was applied only before infection in contrast to only after infection. Carvacrol was active against *Human herpes virus 1* (not active before; $SI_{50,after}$=5.1; $EC_{50,after}$=3.2 x 10^2 µM), acyclovir-resistant *Human herpes virus 1* ($SI_{50,before}$=0.2; $EC_{50,before}$=6.4 x 10^3 µM; $SI_{50,after}$=8.7; $EC_{50,after}$=1.9 x 10^2 µM), *Bovine herpesvirus 2* ($SI_{50,before}$=0.1; $EC_{50,before}$=1.2 x 10^4 µM; $SI_{50,after}$=0.3; $EC_{50,after}$=4.3 x 10^3 µM), *Human respiratory syncytial*

virus ($SI_{50,before}$=0.01; $EC_{50,before}$=8.4 x 10^4 µM; $SI_{50,after}$=4.15; $EC_{50,after}$=4.0 x 10^2 µM), *Human Rotavirus* ($SI_{50,before}$=1.7; $EC_{50,before}$=3.4 x 10^3 µM; $SI_{50,after}$=33; $EC_{50,after}$=1.8 x 10^2 µM), *Bovine viral diarrhea virus* ($SI_{50,before}$=1.8; $EC_{50,before}$=7.6 x 10^2 µM; $SI_{50,after}$=4.2; $EC_{50,after}$=3.3 x 10^2 µM). The activity of camphor against *Influenza A* (H1N1) virus is limited (SI_{50}=2; ED_{50}=1.6 x 10^3 µM) compared to camphor imine derivatives (SI_{50}=1.9-503; ED_{50}=2.0-8.4 x 10 µM) (Sokolova et al. 2015). Shadyro et al. (2016) showed the antiviral activity of salicylaldehyde (SI_{50}=18.3; EC_{50}=2.2 x 10 µM) against *Human herpesvirus 1* while it did not inhibit *Influenza A* (H7N1). Tolstorozhev et al. (2012) attributed the effectivity of vanillin and salicylaldehyde (compared to ortho-anisaldehyde) against herpes virus to their ability to form H-bonds. Krenn et al. (2009) related the effectivity of hinokitiol against *Human Rhinovirus*, *Coxsackievirus* and *Mengovirus* with its ability to chelate Zn^{2+} ions and increase intracellular Zn^{2+} levels. Müller et al. (2016) showed that 1,8-cineole (1-2 x 10^2 µM) induced the IRF3-mediated antiviral response in human stem cells and reduced NF-κB activity.

Only three studies reported the MIC of EO compounds against parasites (Eukarya). The MIC of tyrosol against *Cryptosporidium parvum* is higher than 1.8 x 10^3 µM (Teichmann et al. 2016). The MIC of estragole against *Plasmodium ovale* (4.2 x 10^2 µM) is in the same range as against bacteria and fungi (Andrade et al. 2015). Also the MIC of benzaldehyde against *Phytophthora capsici* (8.0 x 10^3 µM) is in the same range as against bacteria and fungi (Ullah et al. 2015).

A lot of information can be found about the antimicrobial activity of EO compounds against bacteria (Bacteria) and fungi (Eukarya), but many different EO compounds are tested against many different microorganisms using different experimental setups. Figure 14 shows that EO

compounds are active against both bacteria and fungi, with a lower median MIC against fungi (2.0×10^3 µM) than against bacteria (4.4×10^3 µM). Differences in MIC could be explained by their difference in cell wall charge and/or composition. As the cell wall of bacteria is negatively charged mainly due to teichoic acid in Gram-positive bacteria and Lipopolysaccharides (LPS) in Gram-negative bacteria while the cell wall of fungi is more or less neutral (Malanovic and Lohner 2016), the hydrophobic EO compounds are repelled less by fungi. Furthermore, the lipid composition of the membranes is also important for the antimicrobial activity of EO compounds (Trombetta et al. 2005). Fungi differ from bacteria, as fungal membranes typically contain phosphatidylcholine while bacteria contain phosphatidylglycerol and cardiolipin (Trombetta et al. 2005). These lipids might interact differently with EO compounds. The cell wall of fungi also contains chitin which is a polysaccharide that forms a narrow cross-linked network (together with glycoproteins and glucans) that provides rigidity to the cell (Bowman and Free 2006). Some EO compounds interact with cell wall related enzymes (chitin synthase, chitinase, glucanases) (Bang et al. 2000; Giweli et al. 2013). For example, cinnamaldehyde inhibits the cell wall synthesizing enzymes β-(1,3)-glucan synthase and chitin synthase of *Saccharomyces cerevisiae* at 50% inhibitory concentrations (IC50) of 8.4×10^2 µM and 1.4×10^3 µM respectively (Bang et al. 2000).

Figures 7, 9 and 11 show boxplots of all MICs of individually selected EO compounds against Gram-negative bacteria, Gram-positive bacteria and fungi for individual EO compounds. Fungi are more sensitive than bacteria for most EO compounds (perillaldehyde, perillol, citral, citronellal, geraniol, nerol, citronellol, geranyl acetate, cymene, cinnamaldehyde and eugenol). A compound was considered more active against fungi than bacteria when the box of the MICs

against Gram-negative as well as Gram-positive bacteria did not include the median MIC against fungi. The MICs of the rest of the compounds in figures 7, 9 and 11 are in the same range against bacteria and fungi. In general, it can be concluded that fungi are more or equally susceptible to EO compounds as bacteria.

In contrast to bacteria and fungi, not much information can be found in the literature about the antimicrobial activity of EO compounds against Archaea. Tachibana et al. (1996) found that 5.0 µM of farnesol reduced the growth of *Haloferax volcani* in rich medium after two days to 15% of the control (Tachibana et al. 1996). Geraniol (5.0×10^2 µM) could not reduce its growth (Tachibana et al. 1996). Ohene-Adjei et al. (2008) found a small but statistically insignificant reduction of the archaeal population after supplementation of the diet of lambs with 1.5×10^2 µmol/kg cinnamaldehyde (Ohene-Adjei et al. 2008). A denaturing gradient gel electrophoresis showed also some shifts in the diversity of the archaeal community (Ohene-Adjei et al. 2008). A few studies (Busquet et al. 2006; Ohene-Adjei et al. 2008; Patra and Yu 2012) indicated that EO can have some inhibitory effect against Archaea. Based on the knowledge that antibiotics inhibiting bacteria/fungi are also inhibitory for Archaea, and on the previous conclusion that individual EO compounds are active against bacteria/fungi, one can hypothesize that individual EO compounds can also have some activity against Archaea (Dridi et al. 2011). However, further research is needed to support this hypothesis.

Species dependency of the MIC of EO compounds

Due to differences in cell wall charge and composition Gram-positive bacteria are said to be more sensitive to EO compounds than Gram-negative bacteria. The gathered data do not support

this hypothesis. Figure 14 shows that there is no difference in MICs between Gram-negative (median MIC=4.0 x 10^3 µM) and Gram-positive (median MIC=4.8 x 10^3 µM) bacteria if the data of all included EO compounds are pooled. Besides for most of the individual EO compounds, no clear difference in susceptibility can be observed between Gram-positive and Gram-negative bacteria (Figures 7, 9 & 11). Some EO compounds are more effective against Gram-positive bacteria (e.g. myrcene and linalyl acetate) while others against Gram-negative bacteria (e.g. carveol, perillol and nerol). So, it seems that linear hydrophobic EO compounds are more effective against Gram-positive bacteria while alcohols are particularly effective against Gram-negative bacteria. The fact that for most EO compounds, no differences were observed between Gram-negative and Gram-positive bacteria can be explained on multiple levels. First, although the cell wall composition is different between Gram-negative and Gram-positive bacteria, most Gram-positive bacteria have a negative charge like Gram-negative bacteria (Dickson and Koohmaraie 1989, Mamo 1989, Ukuku and Fett 2002). Furthermore, there are variations within both groups with regard to cell wall architecture and other EO compound targets. There is also large variability in mechanisms of action of EO compounds. In conclusion, the MIC of EO compounds reported in the literature against Gram-positive and Gram-negative bacteria are in the same order of magnitude for most EO compounds.

As *Staphylococcus aureus* (136 studies of 346 in this review; median MIC over all compounds: 4.8 x 10^3 µM) and *Escherichia coli* (134 studies of 346 in this review; median MIC over all compounds: 4.1 x 10^3 µM) are the most used model organisms representing Gram-positive and Gram-negative bacteria respectively, their susceptibility against EO compounds is discussed more in detail. The MIC of the individual EO compounds against *Staphylococcus aureus* and

Escherichia coli (Figure 8, 10 and 12), do not necessarily follow the same trend as seen in all Gram-positive and Gram-negative bacteria together (Figures 7, 9 and 11). So looking at only the susceptibility of *Staphylococcus aureus* and *Escherichia coli* for EO compounds is not always representative for all Gram-positive and Gram-negative bacteria, and thus clear variability between species exists.

The gram-negative species *Pseudomonas aeruginosa*, is also a microorganism that is often used in susceptibility studies (64 studies of 346 in total in this review). *Pseudomonas aeruginosa* (median MIC over all compounds: 6.0 x 10^3 µM) is often less susceptible for EO compounds than other species included in this review (median MIC over all compounds: 3.6 x 10^3 µM). Multiple explanations for this difference in susceptibility exist. Mann et al. (2000) showed that the outer membrane of *Pseudomonas aeruginosa* NCTC 6749 is involved in its resistance against tea tree oil, p-cymene and γ-terpinene but they also suggest that other mechanisms like active efflux of EO compounds, inhibition of porin production and changes of the membrane structure might play a role. These other mechanisms have been reviewed by Isken and De Bont (1998) for organic solvents but the same mechanisms might work against EO compounds. However, not all EO compounds showed this difference (Figures 4, 6 and 8), indicating different modes of action. Some bacteria are also capable of modifying EO compounds to make them less toxic. Hahn et al. (2013) showed the oxidation of thymol to form less toxic products by *Nocardia cyriacigeorgica* and *Mycobacterium neoaurum*. In conclusion, different species often have a different susceptibility for a certain EO compound. These differences can often be related to differences in cell wall structure as discussed above. Also active mechanisms of protection exist (e. g. efflux pumps).

Strain dependence of the MIC of EO compounds

In the literature 20 research papers were found that reported the MIC value of different EO compounds for different strains of the same bacterial, yeast and/or mold species. Together they comprised 22 different species including 7 Gram-negative bacteria, 7 Gram-positive bacteria, 6 yeasts and 2 molds. Over the different research papers, 32 different EO compounds were assessed. Some EO compounds were only assessed in one research paper but others were assessed in up to 6 studies. In 62 reported cases, there was no difference between strains. In 45 cases, there was only one twofold dilution difference. In the other 50 cases, there was a bigger difference. In conclusion, the variation in susceptibility between different strains of the same microbial species is generally limited.

Even within a strain variations in susceptibility can occur. Karatzas et al. (2000) saw that cells from the stationary phase of *Listeria monocytogenes* were not susceptible to S-carvone. They hypothesize that this is because of the physiological changes that occur with the transition to the stationary state (decreased membrane fluidity/permeability and expression of stress proteins). Walsh et al. (2003) showed that it is possible to generate increased tolerance to thymol and eugenol in *Escherichia coli*, however, only to a limited extent (<2 up to 14 times higher MIC). Gomes Neto et al. (2015) showed the involvement of gene regulation by specific sigma factors in tolerance of *Escherichia coli* against two EOs. Until now, we have only limited knowledge of mechanisms that lead to tolerance against EOs. It also remains unclear to what extent tolerance and/or resistance against EO compounds can occur in microorganisms.

3. Experimental design for antimicrobial activity testing of EO compounds

The MIC is defined as the lowest concentration of an antimicrobial agent to completely inhibit growth (bacteriostatic) of a microorganism within a certain time (mostly 24 h) and under specific conditions (normally the optimal growth conditions of the microorganism). MICs were used independently of the definition of the author. The different experimental factors (MIC determination methods, growth medium, incubation conditions and use of emulsifiers/solvents) that might have an influence on the MIC of an EO compound against a certain microorganism are further discussed here.

MIC determination methods

Different methods exist to determine the MIC of chemical compounds, such as methods based on diffusion (agar) and methods based on dilution (agar or broth) (Reyes-Jurado et al. 2014). The agar diffusion methods generate data in the form of inhibition zones. Broth and agar dilution methods directly generate the MICs. These MICs are easier to compare between studies than inhibition zones but still they depend on many different experimental parameters like the incubation conditions and growth phase of the microorganism. Agar dilution seems to give higher or equal MICs than broth dilution methods (Inouye, Tsuruoka, et al. 2001 ; Kim et al. 2011 ; Shih et al. 2013). Although using tea tree oil as such, Hammer et al. (1999) determined the MIC of this EO oil against different microbial species using the broth dilution as well as the agar dilution method, incubated under aerobic and anaerobic conditions. They found a higher difference between both methods under aerobic conditions than under anaerobic conditions. Therefore, the differences between both methods might be mainly attributed to differences in

oxygen availability. Microorganisms grown on solid medium (agar) can readily access the oxygen present in the atmosphere while microorganisms grown in broth depend on the oxygen that dissolves in the broth. Overall no clear link between the use of broth dilution or agar dilution and the MIC could be found in the literature. Therefore, both broth dilution and agar dilution can be used to determine the MIC of EO compounds.

Growth medium

Compounds in the microbial medium can influence the antimicrobial activity of EO and their compounds. First, proteins are known to interfere with the antimicrobial activity of EO compounds. Juven et al. (1994) found that the addition of bovine serum albumin (BSA) (9 mg/mL) to nutrient agar and minimal medium plates completely counteracted the antimicrobial activity of 1.2 x 10^3 µM thymol against *Salmonella* Typhimurium. Veldhuizen et al. (2007) showed that BSA (0-1%$_{w/v}$) and egg yolk (0-2%$_{w/v}$) counteracted the antimicrobial activity of 2.5 x 10^3 µM carvacrol against *Listeria monocytogenes* in tryptone soya broth (TSB) in a concentration-dependent way. The inhibitory effect of proteins on the antimicrobial activity of EO compounds, seems to be related to the binding of the EO compound to the protein. The binding of carvacrol on BSA was shown. Adding ethanol partially reversed the binding of carvacrol to BSA as it reduces the hydrophobic interactions between both (Veldhuizen et al. 2007).

Also carbohydrates present in the growth medium can have an effect on the antimicrobial activity of EOs and their compounds. To our knowledge, no studies are available with EO compounds as such and the interaction with carbohydrates. Gutierrez et al. (2008) found an

inhibitory effect of starch on the antimicrobial activity of 2 EOs (oregano and thyme oil) against *Listeria monocytogenes*, while monosaccharides (e.g. glucose) did not interfere with the EO antimicrobial activity. Therefore, they suggest using growth media containing simple sugars rather than complex sugars to study the antimicrobial activity of EOs (Gutierrez et al. 2009).

Also, the presence of lipids in the medium can influence the antimicrobial activity of EOs and their compounds. Also here, no studies with pure EO compounds are available. But Gutierrez et al. (2008) found that high concentrations of sunflower oil (up to 10%) had a negative effect on the antimicrobial activity of oregano and thyme oil. The negative effect of lipids on the antimicrobial activity of EO compounds has also been discussed in studies about the antimicrobial activity of EOs on food products (S. Burt 2004, Perricone et al. 2015; Weiss et al. 2015). In general, the antimicrobial activity of EOs and their compounds is lower in food products with higher fat content (Owen and Palombo 2005; Singh et al. 2003; Smith-Palmer et al. 2001). This effect is contributed to partitioning effects (S. Burt 2004; Perricone et al. 2015; Weiss et al. 2015). The hydrophobic EO compounds will preferentially dissolve in the lipid fraction of the food products limiting their contact with microorganisms that are generally present in the aqueous fraction of the food products or on the surface. Weiss et al. (2015) argue that at very high oil concentrations (more than 80%) water-in-oil emulsions are formed instead of oil-in-water emulsions, resulting in a decline of bacterial motility (Brocklehurst et al. 1995). This leads to longer diffusional path lengths for compounds limiting the efficacy of EO compounds but also reducing the nutrient delivery and thereby limiting the growth of bacteria (Weiss et al. 2015).

Another element that can influence the antimicrobial activity of EO compounds is the salt concentration. Higher salt concentrations improve the antimicrobial activity of EOs and their compounds (S. Burt 2004, Wendakoon and Sakaguchi 1993). Wendakoon and Sakaguchi (1993) attribute the synergy between clove EO and NaCl to the interaction between eugenol and NaCl. They suggest that eugenol increases the permeability of the bacterial membrane, allowing NaCl to inhibit the intracellular enzymes. Ultee et al. (2000), on the other hand, showed that 0.125% NaCl counteracted the antimicrobial activity of 2.0 x 10^3 µM carvacrol whether or not combined with 2.0 x 10^3 µM cymene.

The pH of the medium can also influence the antimicrobial activity of EO compounds. Vande Maele et al. (2016) found a higher MIC at pH 7.5 than pH 6 of carvacrol (1.3 x 10^3 in contrast to 6.3 x 10^2 µM), thymol (1.3 x 10^3 in contrast to 6.3 x 10^2 µM) and cinnamaldehyde (3.1 x 10^2 in contrast to 1.6 x 10^2 µM). The MIC of eugenol (2.5 x 10^2 µM) was the same under both conditions. At low pH some EO compounds are more hydrophobic and therefore, can dissolve more easily in the hydrophobic part of the microbial membranes (Holley and Patel 2005; Juven et al. 1994), resulting in an increased antimicrobial activity. Juven et al. (1994) showed that the antimicrobial activity of 9.3 x 10^2 µM thymol was higher at pH 5.5 than at pH 6.5. However, this pH effect was counteracted by Tween-80. This might be because of partitioning effects that will be discussed more in detail later. The fact that Tween-80 improves the solubility of thymol in H_2O, makes less of the thymol available to interact with the membrane. Therefore, the antimicrobial activity will be lower and the increase in hydrophobicity of thymol due to lower pH will not be as effective as in the absence of Tween-80. However, the effect of Tween-80 and other emulsifiers/solvents in combination with pH should be further investigated.

Incubation conditions

Next to the composition of the growth medium, also the incubation conditions such as temperature and time are important. The incubation time is an important factor in the MIC determination of EO compounds because EO compounds can also change the growth kinetics of microorganisms (Zwietering et al. 1994). Valero and Giner (2006) showed that EO compounds (borneol, carvacrol, cinnamaldehyde, eugenol, menthol, thymol and vanillin) can have an effect on the lag time, exponential growth rate and Log10 maximum number of *Bacillus cereus*. However, the effect on the Log10 maximum number was limited (maximum 1.3) at the tested concentrations of the EO compounds.

The effect of temperature is more complicated. The incubation temperature influences the growth kinetics of the microorganisms in a species-dependent way and thus also the MIC of EO compounds against the microorganism. Furthermore, temperature can also influence the physicochemical characteristics (vapor pressure, viscosity, density, chemical stability) of the EO compounds and/or the used emulsifier/solvent. Veldhuizen et al. (2007) showed that although 2.5 x 10^3 µM carvacrol had a bactericidal effect on *Listeria moncytogenes* at 30°C, the effect at 10 and 20°C was bacteriostatic. This might be because the bacteria are less metabolically active at low temperatures and therefore less susceptible to EO compounds. Also the membrane fluidity and composition can be different depending on the temperature altering the interactions of the EO compound with the membrane (Chintalapati et al. 2004). Di Pasqua et al. (2006) showed that sublethal concentrations of EO compounds (thymol, carvacrol, limonene, cinnamaldehyde and eugenol) also induced changes in the fatty acid composition of the membranes of 5 bacterial

species. This could be a form of adaptation to the EO compounds. Manrique et al. (2016) showed that the adaptation of bacteria to eugenol is species dependent. *Pseudomonas fluorescens* showed the most adaptation followed by *Staphylococcus carnosus*, *Listeria innocua* and *Escherichia coli K12*. Conner and Beuchat (1984) showed that heat treated yeast cells (20 minutes at 44 to 54°C) formed smaller colonies after treatment with EOs (allspice, cinnamon, clove, garlic, onion, oregano, savory and thyme oil). They attributed this effect to the fact that EOs inhibit the repair of sublethal injuries after heat treatment. Burt and Reinders (2003b) showed that the effect of the incubation temperature on the antimicrobial activity of EO is depending on the emulsifier/stabilizer used. Without the use of an emulsifier/stabilizer there was a higher activity of red thyme oil at 37°C than at 10 and 20°C (S. A. Burt and Reinders 2003). However, when soy lecithin was used as an emulsifier, the activity was higher at 20 and 37°C than at 10°C, while using 0.05% agar as a stabilizer, the activity was the highest at 10°C, followed by 20°C and 37°C. The fact that agar improves the antimicrobial activity of the EO at low temperatures is attributed to the fact that the agar matrix is firmer at low temperatures leading to a slower phase separation (S. A. Burt and Reinders 2003). All these findings show that the incubation temperature (and time) are important factors influencing the results of a MIC determination and should always be mentioned when MICs are determined.

Oxygen levels can affect the antimicrobial activity of EO compounds in multiple ways. First, the oxygen levels affect the physiological state of the microorganisms and this is, as discussed under "Strain dependence of the MIC of EO compounds", a determining factor in the antimicrobial activity of EO compounds against the microorganism. The cell wall structure of *Staphylococcus aureus*, for instance, is different under anaerobic than aerobic conditions. Under anaerobic

conditions, it is thicker and more sensitive to bacteriolytic enzymes indicating that the peptidoglycan network is less condense (O'Brien and Kennedy 1971). The changes in cell wall composition can also alter the susceptibility of the microorganism against EO compounds. Second, under aerobic conditions, autoxidation of EO compounds can occur depending on the chemical structure, oxygen concentration, energy and available reaction partners (Turek and Stintzing 2013). This leads to the formation of other compounds that might have a higher or lower antimicrobial activity than the original EO compound. Chalchat et al. (2000) showed that the hydroperoxides formed by photosensitized oxidation of the (+) and (-) isomers of α-pinene, β-pinene and limonene had higher antimicrobial activity that the original EO compounds against five bacterial species. In contrast, Juven et al. (1994) found that thymol had a higher antimicrobial activity against *Staphylococcus aureus* and *Salmonella* Typhimurium under anaerobic conditions than under aerobic conditions. However, they could not confirm the autoxidation hypothesis. They attributed the higher activity under anaerobic conditions to the lower energy yields of bacterial metabolism.

Chueca et al. (2014a; 2014b) showed that citral, carvacrol and (+)-limonene had different modes of action against *Escherichia coli* under aerobic and anaerobic conditions. They showed that under aerobic conditions the generation of reactive oxygen species (ROS) played a role in the antimicrobial activity (Chueca et al. 2014a). However, under anaerobic conditions the antimicrobial activity was higher than under aerobic conditions despite the absence of ROS (Chueca et al. 2014a). The fact that these EO compounds increase the concentration of ROS in bacteria is in contradiction with the fact that they are potent antioxidants (Ruberto and Baratta

2000). The effect of oxygen levels on the antimicrobial activity is thus complex and should be further investigated.

Use of emulsifiers/solvents

EO compounds have limited solubility in water, with a median solubility among the included EO compounds of 3.2×10^3 µM. The lowest solubility among the included EO compounds, 5.2×10^{-1} µM, was found for cadinene, while the highest solubility, 1.8×10^5 µM, was found for tyrosol. Therefore, it is necessary to add solvents and/or emulsifiers to solubilize these EO compounds in a water-based broth, considering that solvents and emulsifiers preferably do not have an antimicrobial activity on their own. Even if these chemicals do not exhibit an antimicrobial effect, it is still possible that they alter the antimicrobial effect of the tested EO compound. Indeed, the effect on the antimicrobial activity of EOs depends on the type of emulsifying/stabilizing agent used (Remmal et al. 1993). Si et al. (2006) tested five different gums (fenugreek gum, xanthan gum, Arabic gum and yellow mustard gum) or surfactants (Tween-80), all at a concentration of 0.05%, for the emulsification of five EO compounds (cinnamaldehyde, carvacrol, thymol, geraniol and eugenol) at their MIC against Salmonella Typhimurium. They found that overall Tween-80 had the most reducing effect on the antimicrobial activity of all the EO compounds (52.3% (1.7×10^3 µM geraniol) up to 98.4% (1.6×10^3 µM cinnamaldehyde) of the growth inhibition that was observed without addition of Tween-80) except for cinnamaldehyde that was counteracted most by yellow mustard gum (97.7% of the growth inhibition that was observed without addition of yellow mustard gum). Xanthan gum even had an increasing effect on the antimicrobial effect of cinnamaldehyde,

carvacrol, thymol and eugenol (Si et al. 2006). The biggest effect of all emulsifiers on growth inhibition was with geraniol (52.3% (Tween-80) up to 97.8% (fenugreek gum) of the growth inhibition that was observed without addition of Tween-80) (Si et al. 2006). Shaaban and Edris (2015) compared the antimicrobial activity of a 1% carvacrol microemulsions formulated with a non-ionic surfactant (Tween-20) with that of a 1% carvacrol microemulsions formulated with a cationic surfactant (cetylpyridinium chloride) using the disk diffusion method. The antimicrobial effect of the microemulsion with the cationic surfactant was the highest but the surfactant itself also showed some antimicrobial activity. Low concentrations of agar (e.g. 0.15%) can also be used to stabilize emulsions of EOs or EO compounds so that no or less emulsifier has to be used (Mann and Markham 1998). To our knowledge the use of agar has no or limited effect on the antimicrobial activity of EO compounds as discussed earlier in the comparison between broth dilution and agar dilution methods. The protein sodium caseinate can also be used as a dispersant. Pan et al. (2014) showed that nanodispersions of thymol with sodium caseinate were more active against *Listeria monocytogenes* than free thymol. Encapsulation in liposomes is another way of solubilizing EO compounds. Liolios et al. (2009) found that carvacrol, a mixture of carvacrol and thymol (6:1) and a mixture of carvacrol and γ-terpinene (3:1) had bigger inhibition zones against *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus mutans, Staphylococcus viridans, Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Candida albicans, Candida tropicalis, Candida glabrata* and *Listeria monocytogenes* when they were incorporated in liposomes (5 mg/mL phosphatidyl choline per 1 mg/mL cholesterol) than when applied purely. Molecular encapsulation can also be used to solubilize EO compounds and inhibits evaporation of volatile EO compounds (Marques

2010). Tao et al. (2014a) showed that β-cyclodextrin inclusion complexes of thymol could have a higher antimicrobial effect against *Escherichia coli* K12. The effect on the antimicrobial activity depended on the method of production of the inclusion complexes. Freeze-dried inclusion complexes improved the antimicrobial activity of thymol while kneaded inclusion complexes decreased it. The difference in antimicrobial activity between the two production methods was attributed to differences in steric conformation of the thymol and its release rate from the cavity of the inclusion complex. Wattanasatcha et al. (2012) showed that thymol encapsulated in submicron sized ethylcellulose/methylcellulose spheres kept its antimicrobial activity in a cosmetic lotion for more than 3 months while free thymol was only active for 2-4 weeks. The authors attributed this to reduction of the volatilization of thymol. Yegin et al. (2016) found that 300 nm nanoparticles of geraniol in the stabilizing polymer Pluronic F-127 had a lower MIC than unencapsulated geraniol. The MICs of the nanoparticles against *Escherichia coli* O157:H7 and *Salmonella* were 1.3×10^4 µM and 1.6×10^4 µM respectively while those of unencapsulated geraniol were 2.6×10^4 µM and 4.5×10^4 µM. They found that the fluorescent dye Nile Red, mixed with geraniol, could be found in the cytoplasm of *Escherichia coli* cells when co-encapsulated in nanoparticles but not when the mixture was not encapsulated. In the latter case the dye was localized in droplets outside the cells. Hili et al. (1997) showed that the solubilizer dimethylsulfoxide (DMSO) limited the antimicrobial effect of cinnamon oil against *Saccharomyces cerevisiae*. This effect was associated to partitioning of cinnamon oil between the different phases.

Tween-80 is most often used as emulsifier for the solubilization of EO compounds. The antimicrobial effect of thymol (9×10^2 µM) against *Salmonella* Typhimurium, for example was

lower when higher concentrations of Tween-80 (0.0125-0.100 µL/L) were used (Juven et al. 1994). Ma et al. (2016) tested the antimicrobial effect of eugenol (3.8×10^3 µM) against a cocktail of *Listeria monocytogenes* using different mass ratios of Tween-80:eugenol (1:1-1:0) at 32°C for 24 h. Pure eugenol resulted in 0.26 log CFU/mL reduction compared to 2.97 log CFU/mL growth without treatment. When Tween-80 was added there was no antimicrobial effect of eugenol (2.99-3.07 log CFU/mL growth). Nielsen et al. (2016) showed that 0.1% Tween-80 also increased the minimum biofilm eradication concentration of iso-eugenol against *Staphylococcus aureus* from 9 mM to 37 mM. Also Inouye et al. (2001b) found that 0.5% Tween-80 increased the MIC of eight EO compounds.

Nielsen et al. (2016) showed that Tween-80 itself had a species-dependent effect on microbial growth. They gave three possible explanations for these effects of Tween-80. First they mentioned the change in availability of nutrients. Tween-80 can reduce the diameter of nutrient particles resulting in a higher surface to volume ratio and leading to a higher nutrient availability (Nielsen et al. 2016). Secondly, Tween-80 itself can be used as nutrient source (Howe and Ward 1976; Mizuno and Tsukamura 1978; Nielsen et al. 2016; Tang et al. 2009). Finally, they also showed that Tween-80 can alter the permeability of membranes (Nielsen et al. 2016). Tang et al. (2009) proved that Tween-80 also promotes O_2 uptake by limiting aggregate formation of *Mycobacterium smegmatis*. The EO compounds solubilized in micelles might also be physically separated from the microorganisms (steric hindrance) (Inouye, Tsuruoka, et al. 2001; Juven et al. 1994). However, these mechanisms should be further investigated. The effect of Tween-80 on growth was species dependent. Tween-80 increased the growth of *Staphylococcus aureus*, decreased the growth of *Pseudomonas fluorescens* and did not alter the growth of *Listeria*

monocytogenes. The species (and strain) dependency was explained by differences in cell wall composition determining the degree of membrane permeability induced by Tween-80. Also the expression of a secreted lipase that can degrade Tween-80 (at its ester site) to oleic acid, and polyethylene is important for the effect of Tween-80 on the growth of the microorganisms (Nielsen et al. 2016; Toutain-kidd et al. 2009). In conclusion, the use of emulsifiers/solvents can alter the growth of the microorganisms as well as the antimicrobial activity of EO compounds. Therefore, the use of emulsifiers/solvents in antimicrobial experiments should be well-considered and the effects should be further researched.

Next to the type of emulsifier used also the droplet size of the EO compound emulsion might be important for its antimicrobial activity. Uribe and Pena (1990) already related the antimicrobial activity of 1 mM of β-pinene and limonene against *Saccharomyces cerevisiae* to the droplet size of the emulsion using different solubilizing agents. The antimicrobial activity was best for DMSO and dimethylformamide (DMF), followed by ethanol and dioxane. The droplets in the emulsion with DMSO were the biggest and the smallest in the one with dioxane. They hypothesize that in the big droplets the concentration of the EO compounds is very high resulting in high antimicrobial activity if there is contact between the droplet and microbial membranes. This trend of bigger droplets having higher antimicrobial activity is also confirmed by the findings that higher Tween-80 (0-1%) concentrations resulted in less antimicrobial activity (Inouye, Tsuruoka, et al. 2001; Juven et al. 1994; Ma et al. 2016; Nielsen et al. 2016), as higher emulsifier concentrations result in smaller droplets (Roldan-Cruz et al. 2016). Terjung et al. (2011) found the same trend for emulsions of eugenol and carvacrol in Miglyol 812N with 2% Tween-80 generated with different homogenization pressures. They attribute this to the fact that

there is less eugenol/carvacrol present in the aqueous phase if the droplets are smaller. They suggest that this might be because of the sequestering of the EO compounds to the droplet interface and/or the fact that there is less Tween-80 left in solution to solubilize the EO compounds (Terjung et al. 2011).

The droplet size can also influence bacterial growth. Brocklehurst et al. (1995) showed that the growth form (only planktonic (25 µm), film (15-25 µm), colonies (2-15 µm)) of *Listeria monocytogenes* and *Yersinia enterocolitica* depends on the droplet size of the emulsion that it grows in. Differences in droplet size can thus be responsible for the difference in antimicrobial activity because microorganisms in films and colonies are less accessible for EO compounds.

To conclude, there are many different substances that can be used to stabilize solutions, emulsions or dispersions of EOs and their compounds. However, these substances can interfere with the antimicrobial activity of the EOs and their compounds in a substance, concentration, droplet size, compound and species-dependent way. The mechanisms behind this interference are yet to be unraveled, although some hypotheses have been proposed. These hypotheses include i) the protection of EO compounds by encapsulation against evaporation, oxidation and heat degradation ii) steric hindrance effects iii) pro- and antibacterial activity of the emulsifiers/solvents themselves. These aspects of emulsifiers, dispersants and solvents should be kept into account when conducting antimicrobial experiments with EO compounds because they can significantly influence the results.

CONCLUSION

In the gathered dataset of MICs, no clear differences between different EO compounds could be found. There were also no clear differences in sensitivity of the tested microbial species. This is mainly due to the large variation in experimental conditions used by researchers leading to a big variation in MICs, even for specific EO compound/microorganism pairs. Although standard methods exist for the MIC determination of water soluble antibiotics, these are not sufficient for EO compounds, as EO compounds have a limited solubility in water, are volatile and can have limited chemical stability. So a need for a standard method for the determination of the MIC of EO compounds emerges, as it is important to have a mechanistic understanding of why certain differences in antimicrobial activity between EO compounds occur. Knowledge of the chemical stability of EO compounds is limited and should be elaborated to decide if measures have to be taken to limit or to take into account degradation. The use of emulsifiers/solvents can limit or increase the antimicrobial activity of EO compounds but is needed for practical reasons. Further research is needed to have a better understanding of the interaction of emulsifiers/solvents on the antimicrobial activity of EO compounds.

To conclude, it is important that researchers elaborately report their experimental conditions. Next to the MIC definition, information about emulsifier/solvent use, recipient and sealing are particularly important for EO compounds. Although a lot of research is available on the antimicrobial activity of EO, the mechanistic insight into how the EO compounds work as antimicrobial compounds is still lacking.

AUTHOR CONTRIBUTIONS

Elien Van de Vel is a PhD student under the supervision of Imca Sampers and Katleen Raes. Elien Van de Vel has searched the literature to write the review and prepared a first draft. Together with her supervisors Imca Sampers and Katleen Raes the manuscript was further revised and written.

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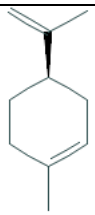
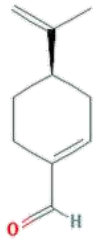
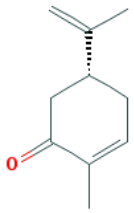
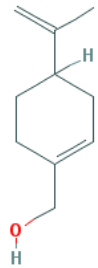
Table 1 Structure and XLogP3 of limonene related EO compounds

EO compound	**Structure**	**Class**	**Molecular weight (g/mol)**	**XLogP3**(Cheng et al. 2007)
limonene		monocyclic monoterpene	136.23	3.4
perillaldehyde		monocyclic terpenoid with an aldehyde group	150.22	2.6
carvone		monocyclic terpenoid with a ketone group	150.22	2.4
carveol		monocyclic terpenoid with an alcohol group	152.23	2.1

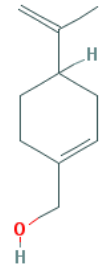
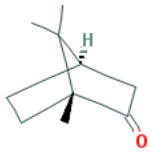
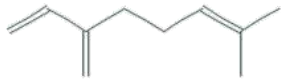
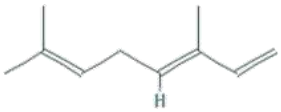
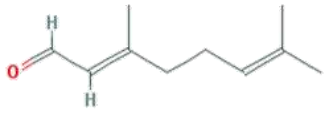
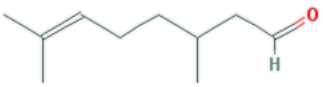
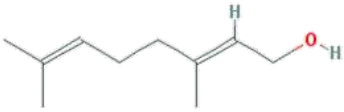
perillol		monocyclic terpenoid with an alcohol group	152.23	2.1
camphor		bicyclic terpenoid with a ketone group	152.23	2.2

Table 2 Structure and XLogP3 of myrcene related EO compounds

EO compound	**Structure**	**Class**	**Molecular weight (g/mol)**	**XLogP3**(Cheng et al. 2007)
Myrcene		acyclic monoterpene	136.23	4.3
Ocimene		acyclic monoterpene	136.23	4.3
Citral		terpenoid with an aldehyde group	152.23	3.0
Citronellal		terpenoid with an aldehyde group	154.25	3.0
Geraniol		terpenoid with an alcohol group	154.25	2.9

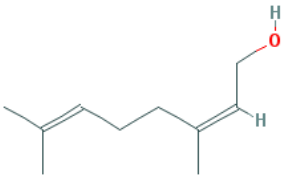
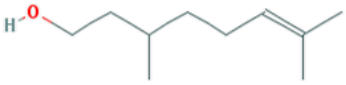
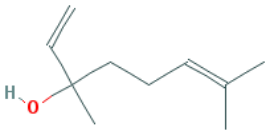
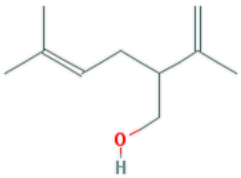
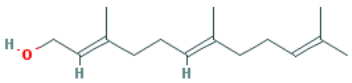
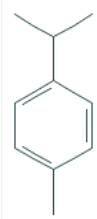
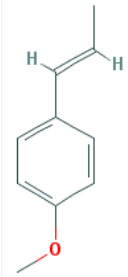
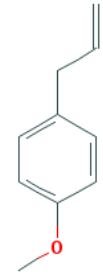
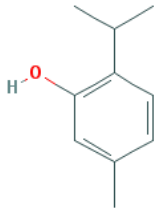
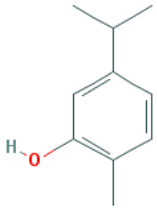
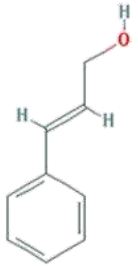
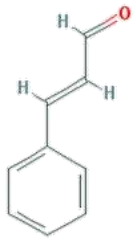
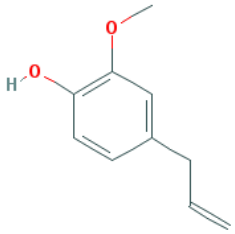
Nerol		terpenoid with an alcohol group	154.25	2.9
Citronellol		terpenoid with an alcohol group	156.27	3.2
Linalool		terpenoid with an alcohol group	154.25	2.7
Lavandulol		terpenoid with an alcohol group	154.25	3
Farnesol		terpenoid with an alcohol group	222.37	4.8

Table 3 Structure and XLogP3 of cymene related EO compounds

EO compound	**Structure**	**Class**	**Molecular weight (g/mol)**	**XLogP3**(Cheng et al. 2007)
Cymene		aromatic monocyclic monoterpene	134.22	4.1
Anethole		aromatic terpenoid with an ether group	148.20	3.3
Estragole		aromatic terpenoid with an ether group	148.20	3.4
Thymol		aromatic terpenoid with an alcohol group	150.22	3.3

Carvacrol		aromatic terpenoid with an alcohol group	150.22	3.1
Cinnamyl alcohol		aromatic terpenoid with an alcohol group	134.18	1.9
EO compound	**Structure**	**Class**	**Molecular weight (g/mol)**	**XLogP3**(Cheng et al. 2007)
Cinnamaldehyde		aromatic terpenoid with an aldehyde group	132.16	1.9
Eugenol		aromatic terpenoid with an alcohol group and an ether group	164.20	2

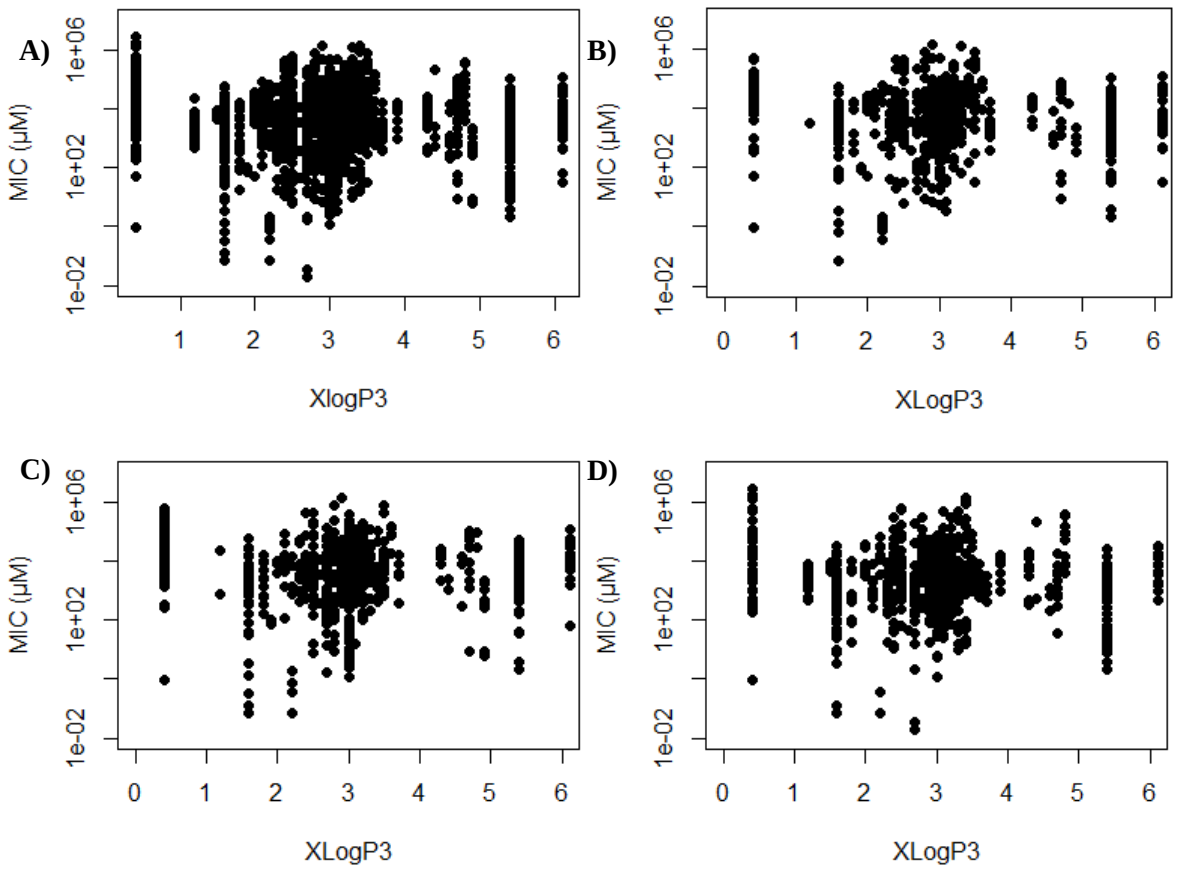


Figure 1 Scatterplots of the MIC value of the tested EO compounds against their polarity (XlogP3) with A) against all bacteria and fungi; B) against Gram-negative bacteria; C) against Gram-positive bacteria & D) against fungi

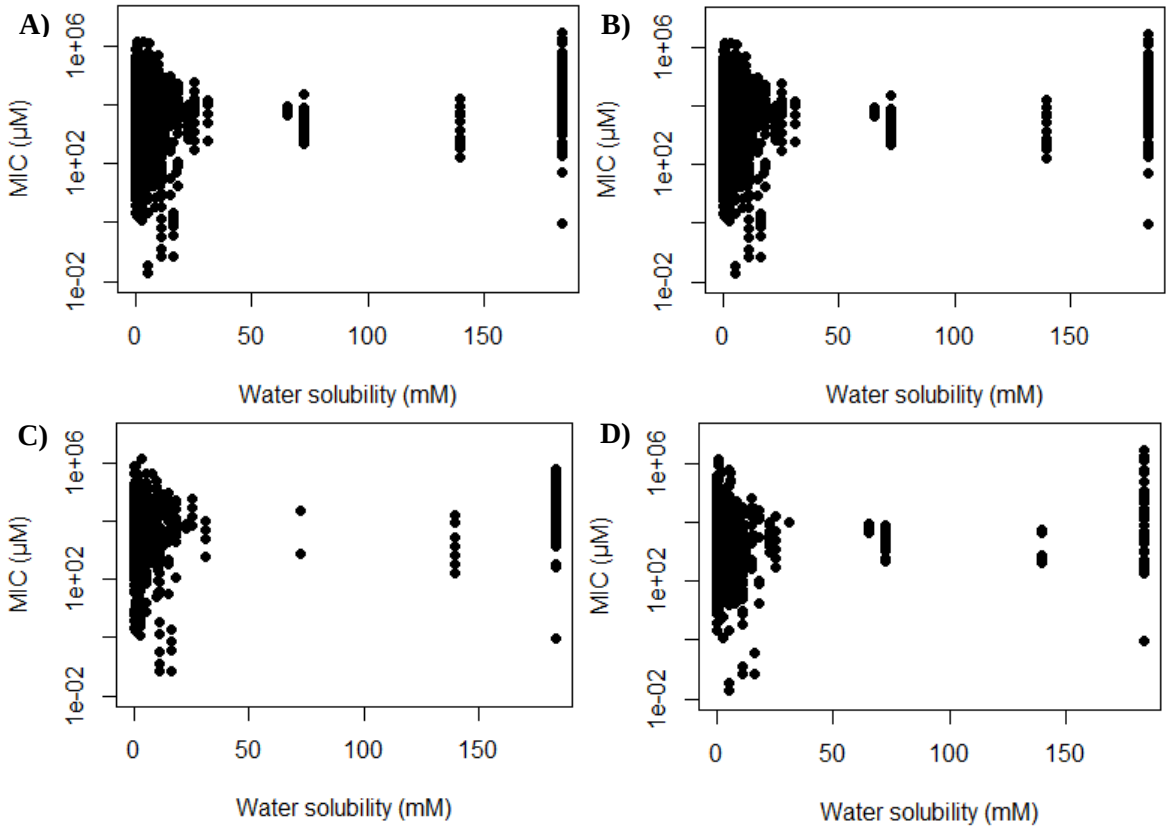


Figure 2 Scatterplots of the MIC (µM) value of the tested EO compounds against their water solubility (mM) with A) against all bacteria and fungi; B) against Gram-negative bacteria; C) against Gram-positive bacteria; D) against fungi

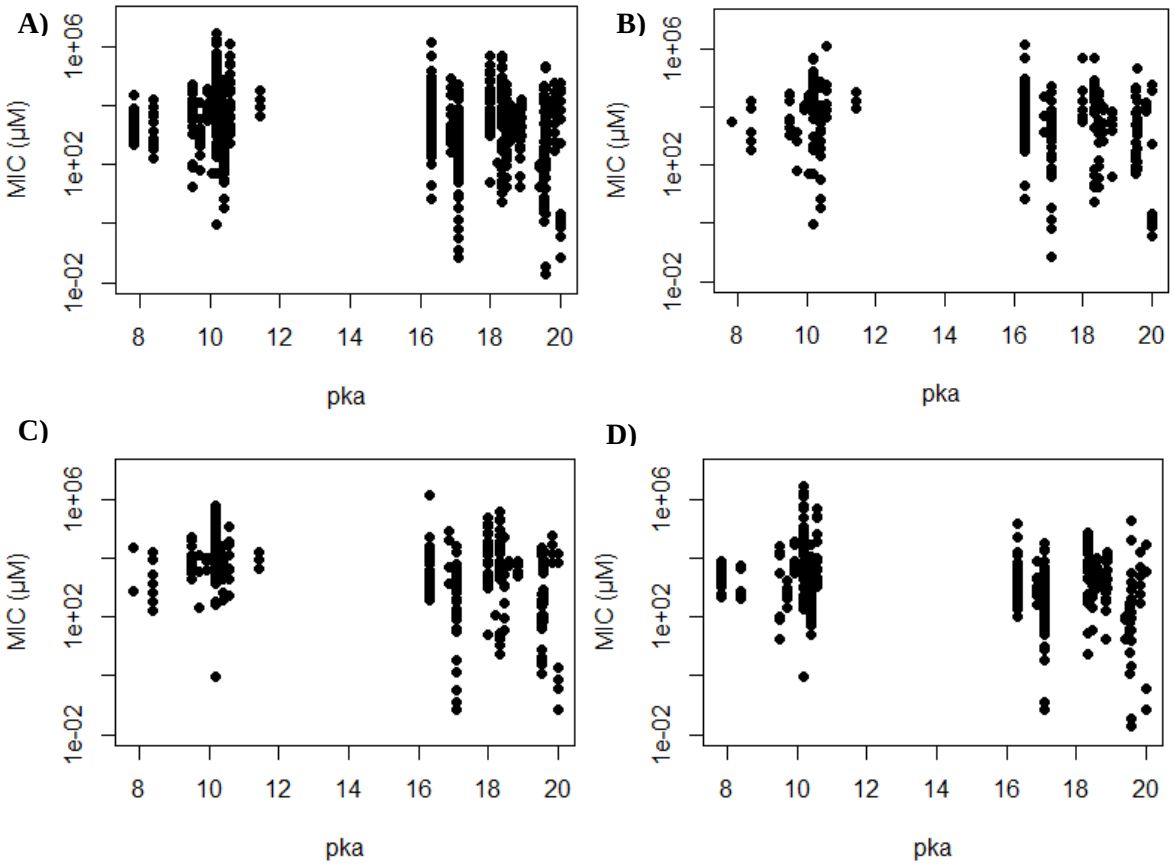


Figure 3 Scatterplots of the MIC (µM) value of the tested EO compounds against their pKa with A) against all bacteria and fungi; B) against Gram-negative bacteria; C) against Gram-positive bacteria; D) against fungi

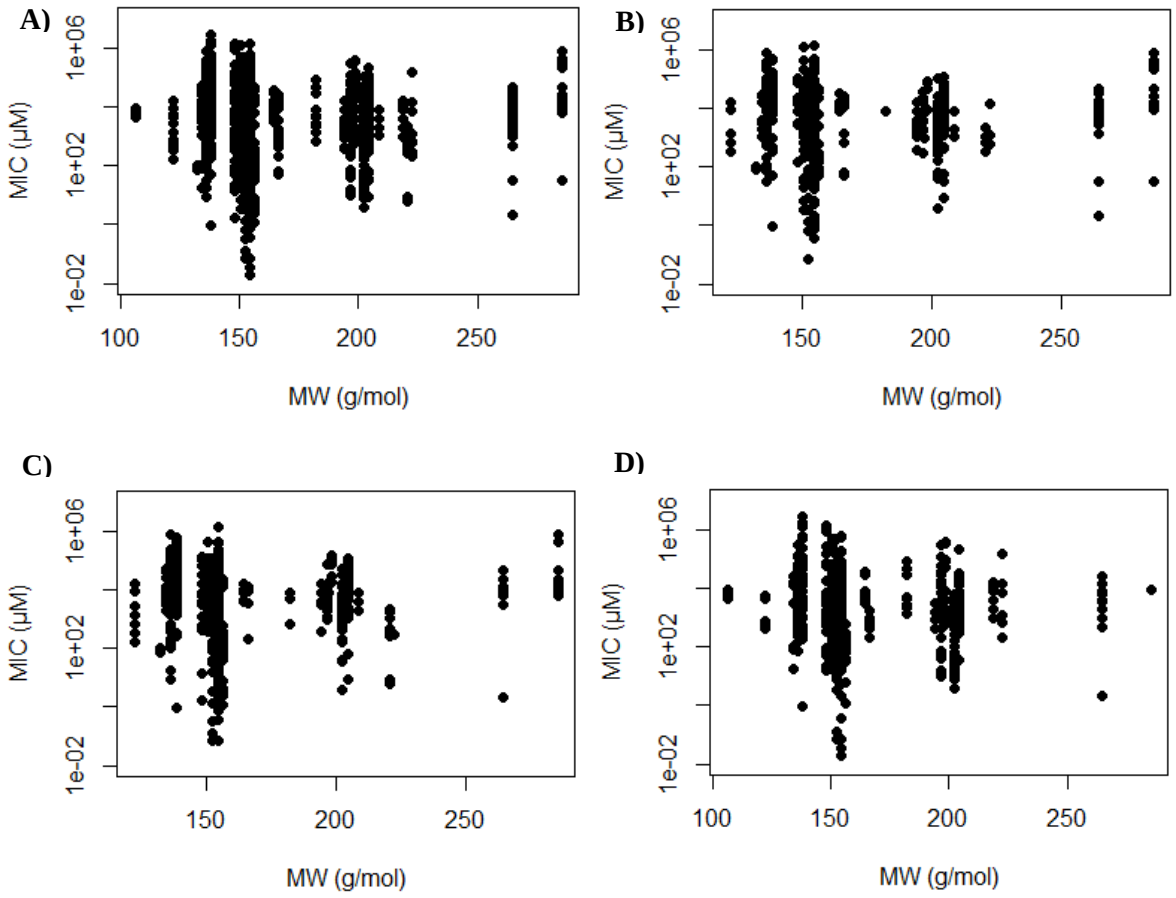


Figure 4 Scatterplots of the MIC (µM) value of the tested EO compounds against their molecular weight (MW (g/mol)) with A) against all bacteria and fungi; B) against Gram-negative bacteria; C) against Gram-positive bacteria; D) against fungi

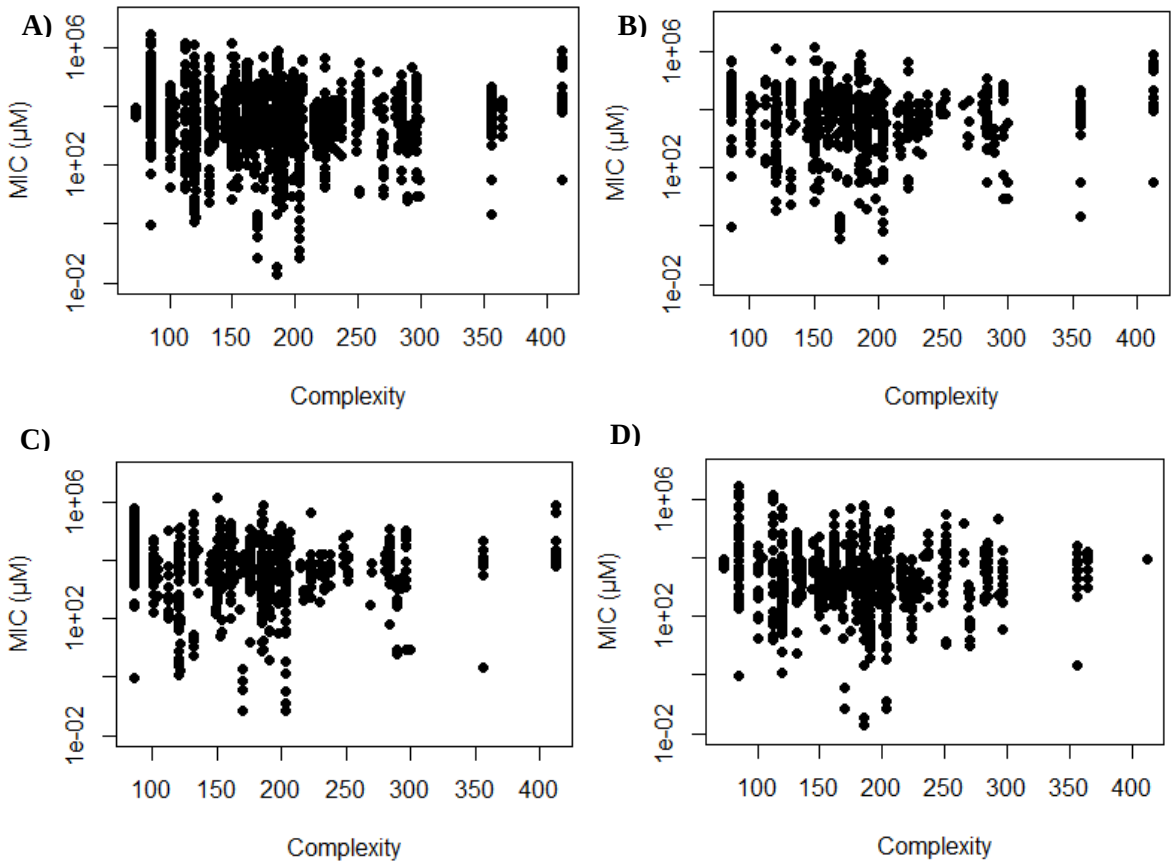


Figure 5 Scatterplots of the MIC (µM) value of the tested EO compounds against their Bertz, Hendrickson and Ihlenfeldt complexity with A) against all bacteria and fungi; B) against Gram-negative bacteria; C) against Gram-positive bacteria; D) against fungi

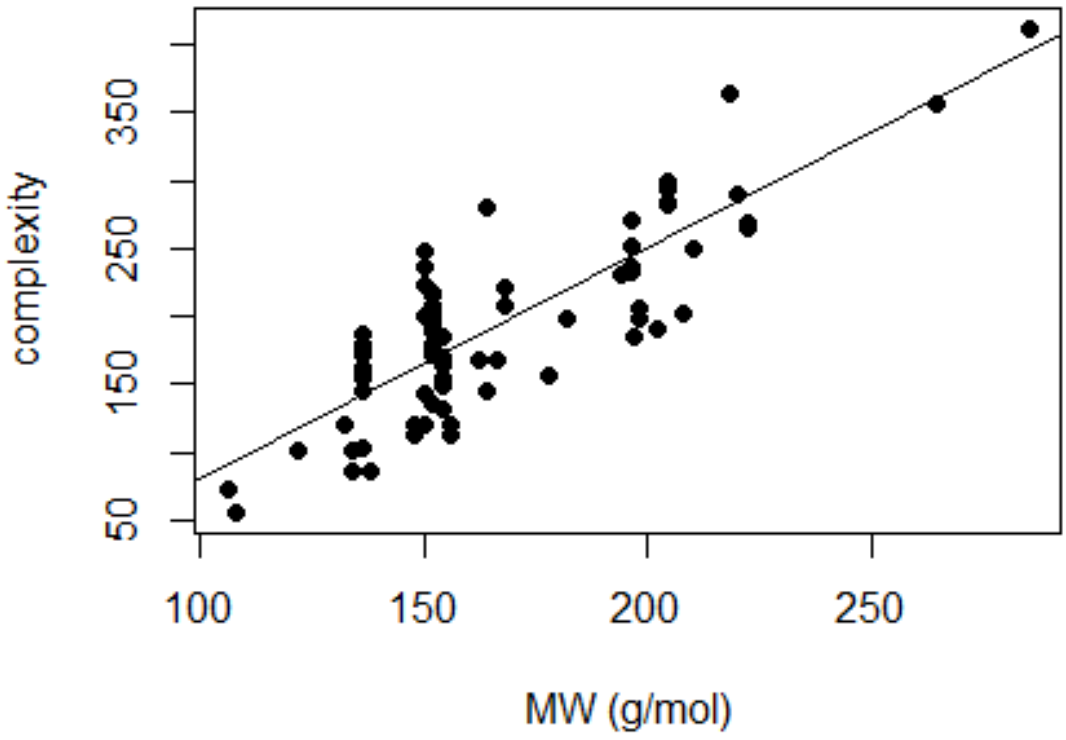


Figure 6 Bertz, Hendrickson and Ihlenfeldt complexity of the included EO compounds against their molecular weight (MW (g/mol))

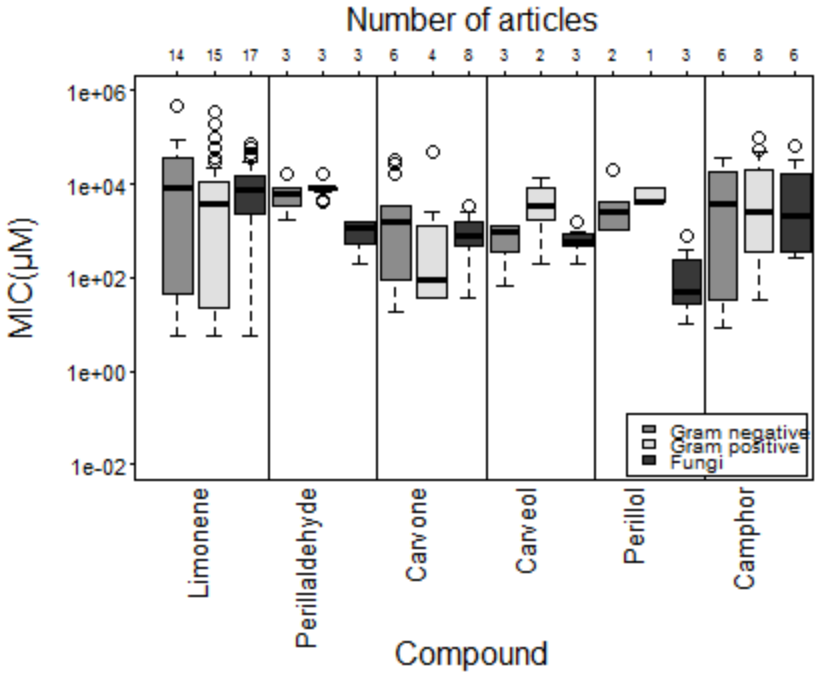


Figure 7 MIC of limonene related EO compounds against Gram negative bacteria, Gram positive bacteria and fungi

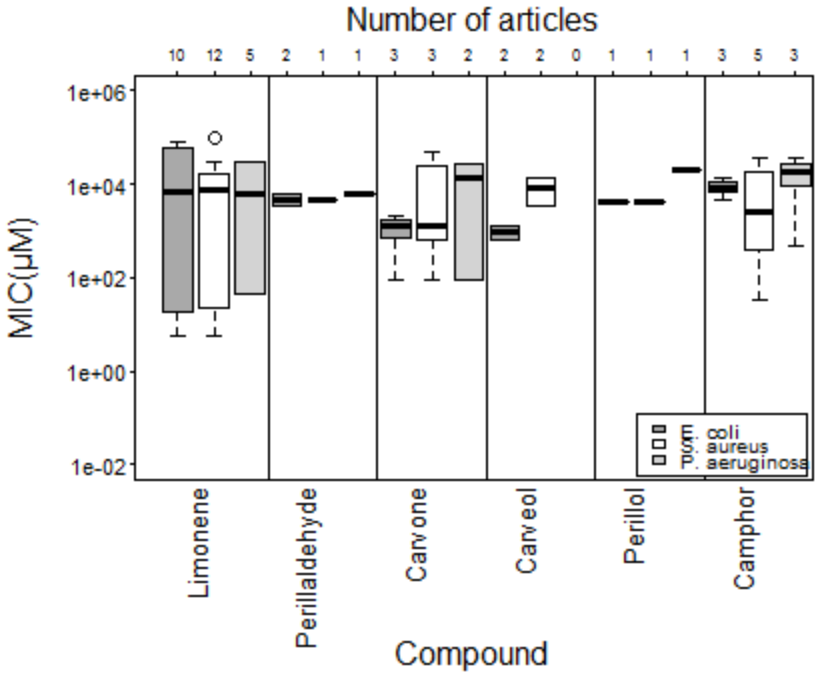


Figure 8 MIC of limonene related EO compounds against E. coli, S. aureus and P. aeruginosa

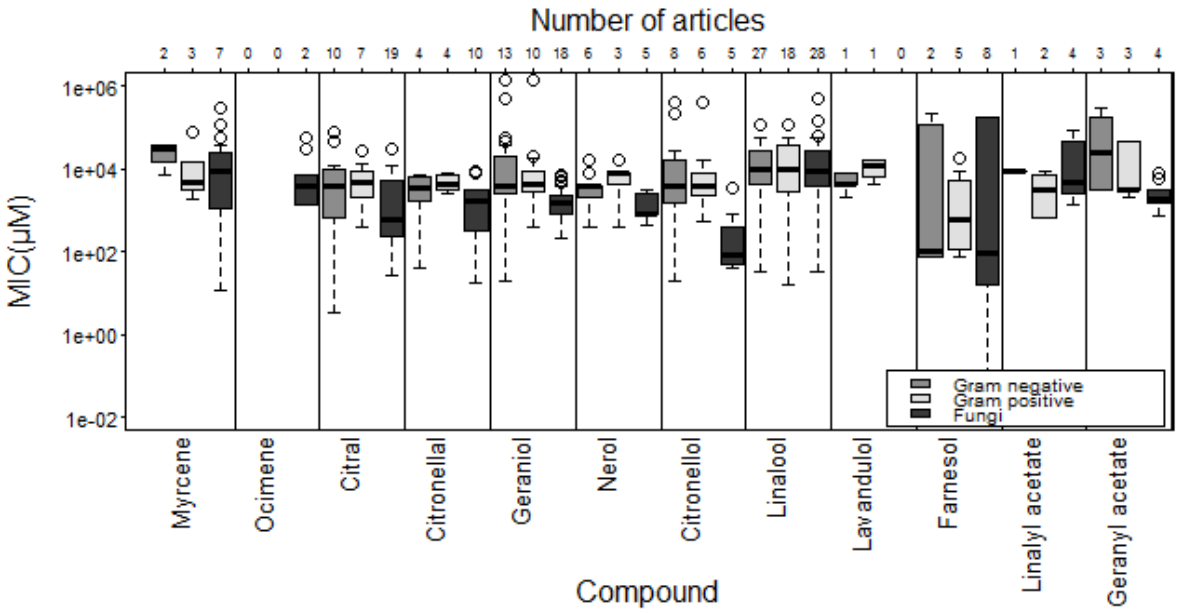


Figure 9 MIC of linear EO compounds against Gram negative bacteria, Gram positive bacteria and fungi

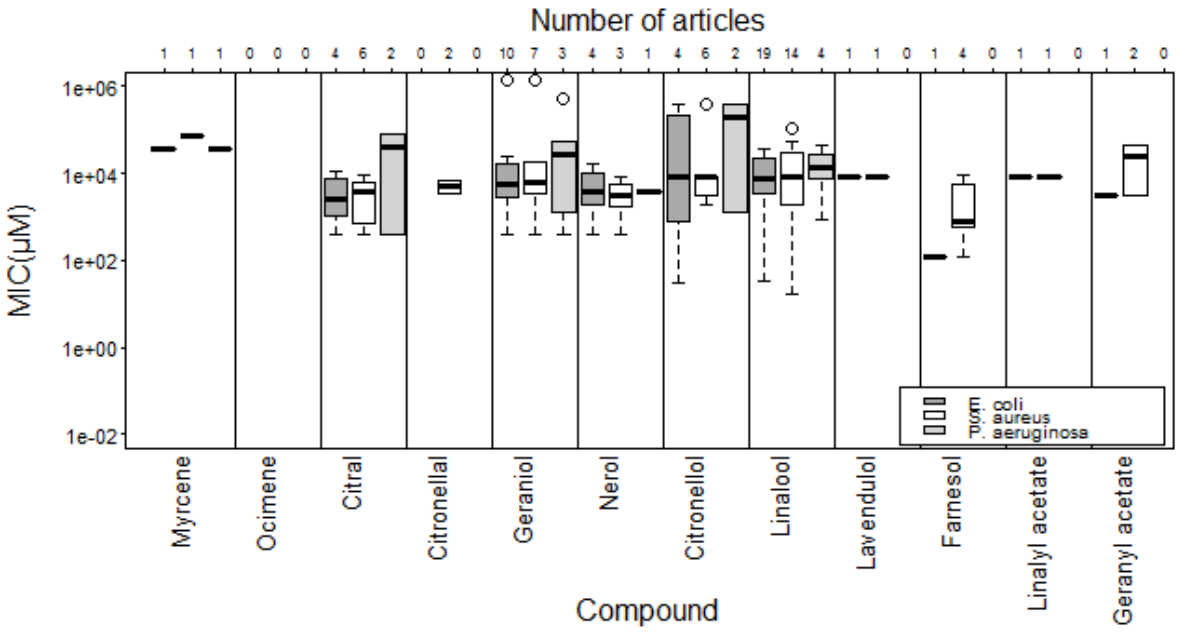


Figure 10 MIC of linear EO compounds against E. coli, S. aureus and P. aeruginosa

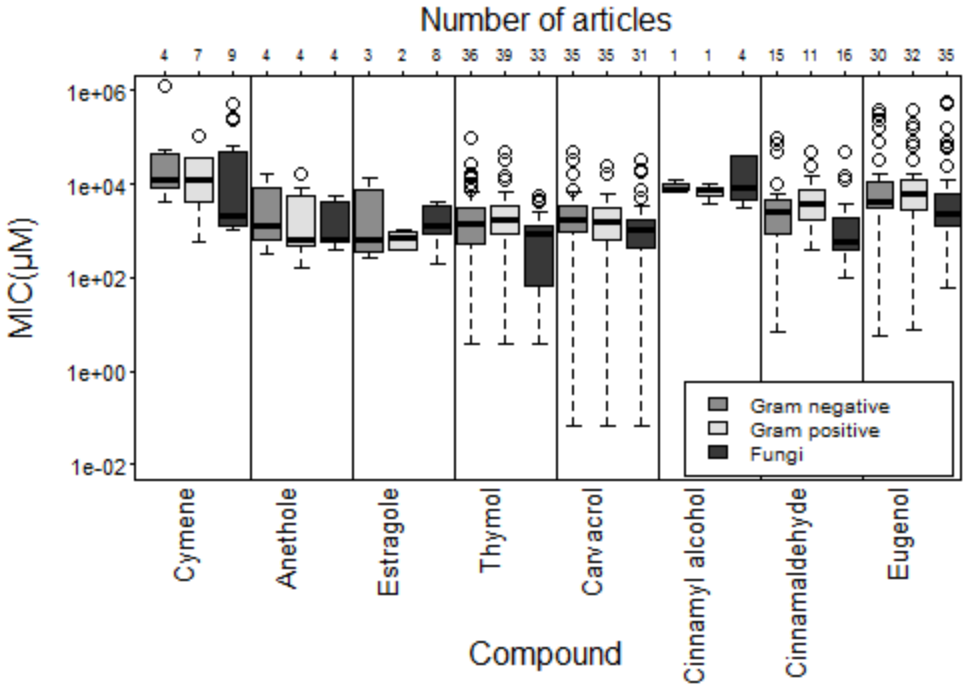


Figure 11 The MIC of aromatic EO compounds against Gram negative bacteria, Gram positive bacteria and fungi

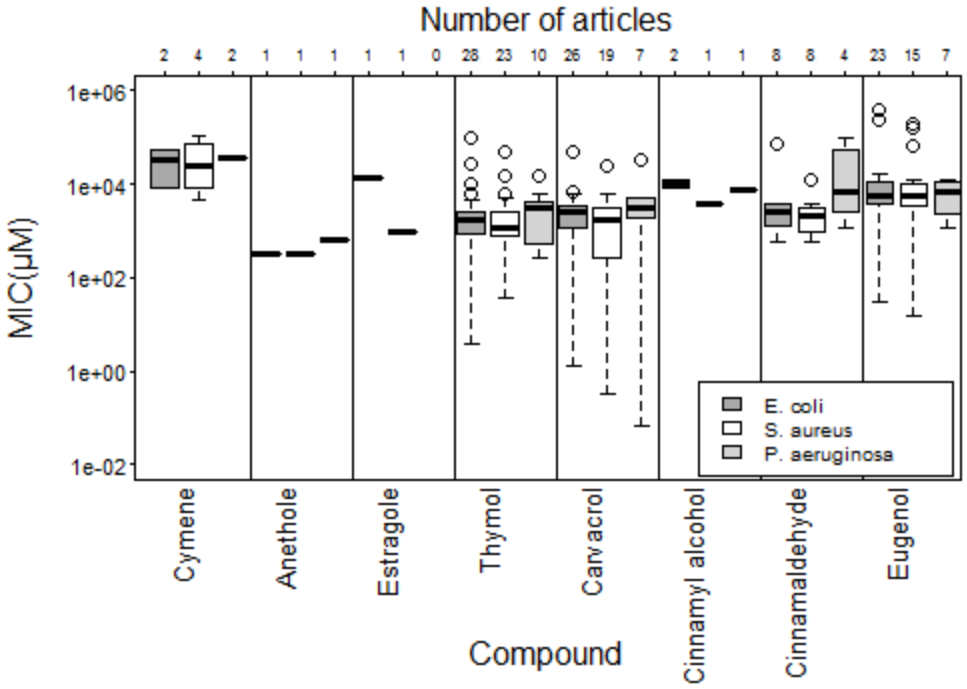


Figure 12 MIC of aromatic EO compounds against E. coli, S. aureus and P. aeruginosa

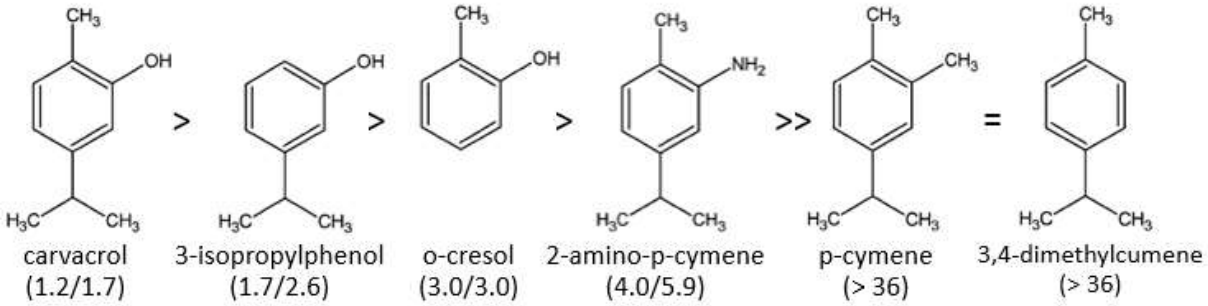


Figure 13 Order of activity of carvacrol related EO compounds with the MIC (mM) against E. coli/S. aureus between brackets (Veldhuizen et al. 2006)

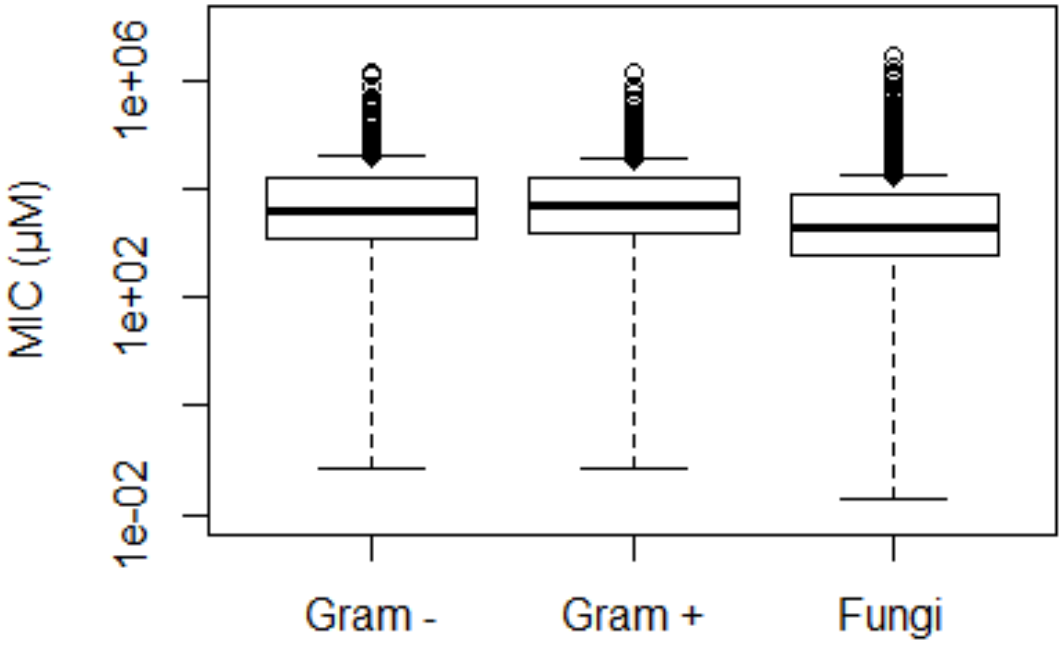


Figure 14 MIC pooled over the included EO compounds against Gram-negative bacteria, Gram-positive bacteria and fungi