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ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

PROTECTION OF STORED PRODUCTS

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M. Sc. Thesis

Integrated Master's in Bioengineering – Biological Engineering

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Abstract

Essential oils have a primary role in plants´ protection. Such function points them as candidates to be used in future in biopreservation systems directed to minimal processed foods and non-perishable food products. A review of the methods used to assess antibacterial activity of essential oils and the perspectives and current applications in food systems are also addressed.

The antimicrobial activity of clove bud (*Syzygium aromaticum)* and pennyroyal (*Mentha pulegium*) essential oils and of their major components, eugenol and pulegone, against standard bacteria-and phytobacteria was assessed using different diffusion methods. The results showed that phytobacteria are extremely susceptible to these phytochemicals, which suggest that they may be effective in a biopreservation system applied to stored products. Nonetheless, the activity of the substances tested revealed a good spectrum of action. Clove bud essential oil seems to be the substance having better performance. Improvement of growth inhibition was notorious as the type of diffusion changed from agar to broth and from this type to vapour diffusion.

Keywords: Essential oils; Antibacterial activity; Biopreservatives; Phytobacteria.

Resumo

Os óleos essenciais têm um papel primário na proteção das plantas. Tal função faz dos mesmos candidatos para serem utlizados em sistemas de biopreservação direcionados a alimentos de processamento mínimo e não-perecíveis. Uma revisão dos métodos utilizados para determinar a atividade antimicrobiana dos óleos essenciais é realizada e as perspetivas e aplicações correntes em sistemas alimentares são também apresentadas.

A atividade antimicrobiana do óleo de cravinho (*Syzygium aromaticum*) e de menta-poejo (*Mentha pulegium*) e dos seus principais componentes, o eugenol e a pulegone contra bactérias *standard* e fitobactérias, foi determinada utilizando diferentes métodos de difusão. Os resultados revelaram que as fitobactérias são extremamente suscetíveis aos fitoquímicos utilizados, o que sugere que poderão ser eficientes em sistemas de biopreservação para produtos armazenados. Não obstante, a atividade das substâncias testadas revelou bom espectro de ação. Deve ainda ser salientado que o óleo do cravinho parece ser a substância que tem melhor performance. Por outro lado, à medida que o tipo de difusão foi alterado de agar para meio líquido e deste para vapor, a melhoria da inibição de crescimento foi notória.

Palavras-chave: Óleos essenciais; Atividade antimicrobiana; Biopreservantes; Fitobactérias.

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Glossary

ATCC – American Type Culture Collection
BHI – Brain Heart Infusion Medium
CB-EO – Clove Bud Oil
CCUG – Culture Collection, University of Göteborg
CFU – Colony-Forming Unit
CLSI – Clinical and Laboratory Standards Institute
$d_{Inhibition}$ - Inhibition Radius
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
$d_{Petri\ dish}$ - Petri Dish Radius
DSM – Culture Collection, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EC – European Commission
EO – Essential Oil
FDA - Food and Drug Administration (FDA)
GRAS – Generally Recognized as Safe
HPTLC - High Performance Thin-Layer Chromatography
INT – *p*-iodonitotetrazolium violet
LB – Luria Broth
LMG – Culture Collection, Laboratorium voor Microbiologie Universiteit Gent
MBC – Minimum Bactericidal Concentration
MEA – Malte Extract Agar
MHA – Müller-Hinton Agar Medium
MIC – Minimum Inhibitory Concentration
MID - Minimum Inhibitory Dose
MTT – 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide
OD – Optical Density
OPLC – Overpressured-Layer Chromatography
PEC – Planar Electrochromatography
PCA – Plate Count Agar
PDA – Potato Dextrose Agar
PR-EO – Pennyroyal Oil
REACH – Registration, Evaluation, Authorisation and Restriction of Chemicals System
SEM – Scanning Electron Microscope
TEM – Transmission Electron Microscopy
TLC – Thin-Layer Chromatography
TTC – 2,3,5-triphenyltetrazolium chloride

1. Introduction

Essential oils (EOs) are formed as secondary metabolites by plants and they have always been used in traditional medicine due to their antibacterial, antiviral, antifungal and insecticidal activities. Such functions enable EOs to fulfil their primary role, which is plants´ protection. Moreover, herbs of those plants are used as spices in kitchens worldwide (Lang & Buchbauer 2012; Bakkali et al. 2008).

Nowadays EOs became once again popular since the available synthetic drugs are often related with unpleasant side effects and drug resistance may occur (Lang & Buchbauer 2012; Bakkali et al. 2008). Additionally, research has been made in order to apply these substances in the agricultural domain. In fact, diseases caused by plant pathogenic bacteria are an emergent concern of food safety. These bacteria can cause not only considerable losses in productivity and quality of harvests but also harm to the ones who ingest infected products. The management of plant disease is difficult and the complexity increases due to the large number of phytopathogenic bacteria and their easiness to spread along large distances through infected seeds. Despite such problems in disease control, there is a lack of antimicrobial agents likely to be applied in agriculture. The bactericides currently available in the market have high toxicity and are not biodegradable. The two main groups of bactericides existent are antibiotics and cooper compounds. Antibiotics are forbidden in most countries due to possible production of resistant strains. On the other hand, cooper agents are strictly controlled by the European Union due to their toxicity and environmental impact (Bajpai et al. 2011; Lo Cantore et al. 2009). Also, the green consumerism trends impel the development of new food products, especially the ones derived from plants since consumers prefer natural substance rather than synthetic ones (Lang & Buchbauer 2012; Lo Cantore et al. 2009).

Hence, the development of a biopreservation system capable to act against plant disease is a nowadays' concern. Developing countries have particular interest to use this technology in order to protect minimal processed foods. Non-perishable food products preservation is a major interest since most of them are generally used not only as food but also as crop seeds. Thereby, spoilage of this product would prejudice populations both in the present and in the future.

The high volatility and low water solubility of EOs are, probably, the main barriers to the implementation of these natural antimicrobials in food preservation (Lang & Buchbauer

2012). To overcome these problems, special storage conditions are required to avoid volatilization and solubility can be enhanced using solvents or emulsifiers (which should not decrease antibacterial activity and must be suitable for food systems) (Burt 2004).

1.1. Objectives

In this Thesis the antimicrobial activity of pennyroyal (*Mentha pulegium*) and clove bud (*Syzygium aromaticum*) essential oils and their major components pulegone and eugenol was studied in order to analyse whether this substances would be suitable as biopreservatives for stored products. Contrary to the major part of the investigations made in the literature, both standard microorganisms and phytopathogenic ones were herein studied. The core of this work is directed to the study of *Staphylococcus aureus* and *Xanthomonas campestris*. Not only direct contact tests (as disc diffusion and broth microdilution) but also vapour phase assays were performed. A simulation of seed environment and an investigation using food matrix (maize) was attempted.

2. State of the art

2.1. Essential oils composition

Essential Oils (EOs) are liquid, volatile and rarely coloured lipid soluble substances with density normally lower than the one of water. They are synthetized in all plant organs such as flowers, herbs, buds, leaves, fruits, roots and others. Further, these compounds can be obtained by different methods: extraction, fermentation, steam distillation or expression (Solórzano-Santos & Miranda-Novales 2012; Bakkali et al. 2008). Nevertheless, concerning food and pharmaceutical applications, extraction by steam distillation or by expression are preferred (Bakkali et al. 2008).

Several works report antimicrobial activity of EOs dependence on different factors. Some of them can be the producing-part of plant under analysis, the stage of plant development, harvesting time, climatic and ecological conditions, and extraction methods used (Burt 2004; Fisher & Phillips 2008; Solórzano-Santos & Miranda-Novales 2012; Bakkali et al. 2008; Cosge et al. 2009; Lang & Buchbauer 2012; Ennajar et al. 2009; Ennajar et al. 2010; Hayouni et al. 2008; Bourgou et al. 2012).

Additionally, EOs are complex mixtures and may comprise from twenty to sixty individual components. Normally they have two major components in higher concentrations (20-70%) and several others in trace amounts. Even if the major constituents would determine the biological properties, typically the whole EO has greater antimicrobial activity than the pure constituents by themselves. Thereby the minor components may play a significant role in antibacterial activity suggesting that synergism must occur (Ahmadi et al. 2010; Bosnić et al. 2006; Bouhdid et al. 2009; Cosge et al. 2009; Dung et al. 2008; Mkaddem et al. 2009; Ennajar et al. 2010; Lopes-Lutz et al. 2008; Betoni et al. 2006; Burt 2004; Bakkali et al. 2008; Bourgou et al. 2012).

In the present work the EOs from the plants *Syzygium aromaticum* and *Mentha pulegium* and their major components (pulegone and eugenol, respectively) are of special interest. These substances are known to have antifungal, insecticidal and/or antibacterial activity, which would allow a satisfactory performance in the protection of stored products.

Additionally, such EOs are already used in food as flavourings, each means that approval for food application as biopreservatives would be easier to obtain (Hyldgaard et al. 2012).

In the Appendix A, the composition of *Syzygium aromaticum* and *Mentha pulegium* EOs are displayed. Several differences among samples can be perceived due to the several factors interfering in the EOs composition such as producing-part of plant under analysis, the stage of plant development and others already enumerated (Mahboubi & Haghi 2008).

2.1.1. Clove bud essential oil

Syzygium aromaticum is an evergreen tree belonging to the family Myrtaceae (Srivastava et al. 2005; Aneja & Joshi 2010). *S. aromaticum* oil (also known as clove bud oil, CB-EO) has been used for a long time in dentistry due to its analgesic, antiseptic and anti-inflammatory properties. Further, CB-EO has antibacterial, anthelmintic, antifungal, antiviral and anticarcinogenic properties (Srivastava et al. 2005; Chaieb et al. 2007; Lee et al. 2009; Aneja & Joshi 2010).

Several authors reported CB-EO composition (Srivastava et al. 2005; Aneja & Joshi 2010; Chaieb et al. 2007; Burt 2004; Lee et al. 2009; Edris & Malone 2012). The major constituent is eugenol which can be present in a concentration of 70 to 89% (see Table A.1 in the Appendix). Besides, the presence of other phenylpropanoids such as carvacrol, thymol, and cinnamaldehyde is usually reported (Srivastava et al. 2005; Aneja & Joshi 2010; Chaieb et al. 2007). Eugenol by itself reveals antioxidant and insecticidal properties (Chaieb et al. 2007) and it is strongly active in spite its low capacity to dissolve in water (Dorman & Deans 2000). Moreover, eugenol was shown to have antimicrobial activity also in the vapour phase (Goñi et al. 2009). Despite the recognized activity of eugenol by itself, there is some known synergism of the several components constituting the CB-EO, contributing to its antimicrobial activity (Lee et al. 2009). CB-EO is known to be effective against various types of bacteria, exhibiting a board spectrum of action (Lee et al. 2009; Aneja & Joshi 2010; Betoni et al. 2006), being effective against Gram-positive and Gram-negative bacteria (Dorman & Deans 2000) and as well against food spoilage bacteria (Hamed et al. 2012). In particular, high activity against *S. aureus* was demonstrated (Chaieb et al. 2007), whereas some resistance regarding *Pseudomonas aeruginosa* is reported (Lee et al. 2009; Hamed et al. 2012).

2.1.2. Pennyroyal essential oil

Mentha pulegium is also referred to as European pennyroyal and belongs to the family Lamiaceae. Since a long time *M. pulegium* EO (also known as pennyroyal oil, PR-EO) has been used in traditional medicine and aromatherapy. Moreover, it can also be found in foodstuff and cosmetics (Teixeira et al. 2012; Mahboubi & Haghi 2008). Nevertheless, only few papers (Teixeira et al. 2012; Franzios et al. 1997) reviewed *M. pulegium* EO composition, in opposition to the other EO herein addressed. The main constituent of *M. pulegium* EO is

known to be pulegone achieving concentrations from 23 to 76% as shown in Table A.2 from Appendix (Teixeira et al. 2012; Franzios et al. 1997).

The antimicrobial activity of this essential oil is normally associated to the presence of pulegone and other compounds as menthone and neo-menthol (Teixeira et al. 2012; Mkaddem et al. 2009). Additionally, the presence of high concentrations of piperitone is also recognized to contribute to the EO activity (Mahboubi & Haghi 2008). PR-EO is normally associated to its insecticidal activity and pulegone is known to have the same effectiveness or even higher (Franzios et al. 1997; Duru et al. 2004).

Concerning the spectrum of action, Mkaddem and coworkers (2009) claimed that EOs from mint species do not usually exhibit antimicrobial activity based on cell wall differences. Low susceptibility of Gram-negative bacteria has been reported as well (Teixeira et al. 2012; Mahboubi & Haghi 2008; Lang & Buchbauer 2012).

2.2. Chemical structure of essential oils

The chemical structure of EOs components is also a crucial characteristic since it affects the precise mode of action. There are two main chemical groups responsible for antimicrobial activity of EOs: terpenes and terpenoids, and aromatic and aliphatic constituents (Bakkali et al. 2008; Burt 2004). It should be noticed that the biosynthetic route of terpenes is independent of that of aromatic compounds. Even though, these two types of substances might coexist (Bakkali et al. 2008).

2.2.1. Terpenes and terpenoids

Terpenes are the most usual constituents of EOs, produced by a wide range of plants and they consist in combination of isoprene units (five-carbon-base units, C5) (Bakkali et al. 2008; Laciar et al. 2009). The two main terpenes types prevailing in EOs are monoterpenes (C10) and sesquiterpenes (C15), coupling two and three isoprene units, respectively (Bakkali et al. 2008). Terpenoids, also known as isoprenoids, can be described as terpenes with oxygen atoms as substituents. The nomenclature of terpenoids is similar to that of terpenes (Burt 2004).

Terpenoids and terpenes act as function of their lipophilic properties and thereby phospholipid bilayer appears to be the main target. Thus the central effects are related with inhibition of electron transport, protein translocation and enzyme-dependent reactions, as for instance phosphorylation (Dorman & Deans 2000; Laciar et al. 2009). Due to the outer membrane of Gram-negative bacteria, which comprises hydrophilic lipopolysaccharides, a barrier is created offering additional tolerance to hydrophobic antimicrobial compounds. Thereby, Gram-positive, which do not possess such barrier, would be more susceptible to

terpenoids-containing EOs (Dung et al. 2008; Mkaddem et al. 2009; Laciar et al. 2009; Ennajar et al. 2009).

2.2.2. Aromatic and aliphatic compounds

Aromatic compounds are derived from the phenylpropane pathway and EOs containing phenolic compounds are the strongest antimicrobials (Burt 2004; Bajpai et al. 2009; Cosge et al. 2009). For these EOs, the mechanism of action is similar to the one reported for other phenolic compounds and consequently the most notorious effects are in cellular membranes, similarly to terpenes and terpenoids. Phenolics not only attack membrane integrity, increasing permeability and release of intracellular molecules, but additionally they affect membrane function (as electron transportation, nutrient uptake, enzyme activity) and also protein and nucleic acid synthesis (Burt 2004; Bajpai et al. 2009).

Among the phenolic compounds, the presence of hydroxyl groups is determinant and can be proven by comparison of the activity of the phenolic carvacrol and the lack of activity of the non-phenolic menthol (see Figure 2). Additionally, the relative positions of these groups appear to influence the EO effectiveness. This is proven by carvacrol and thymol (see Figure 2) activity against Gram-positive and Gram-negative. Thymol, with a –OH group at the adjacent carbon to isopropyl group, (–$CH(CH_3)_2$), seems to be preferentially active against Gram-positive bacteria (Burt 2004; Dorman & Deans 2000), showing, for instance, low inhibition of Gram-negative bacteria such as *Pseudomonas aeruginosa* (Dorman & Deans 2000).

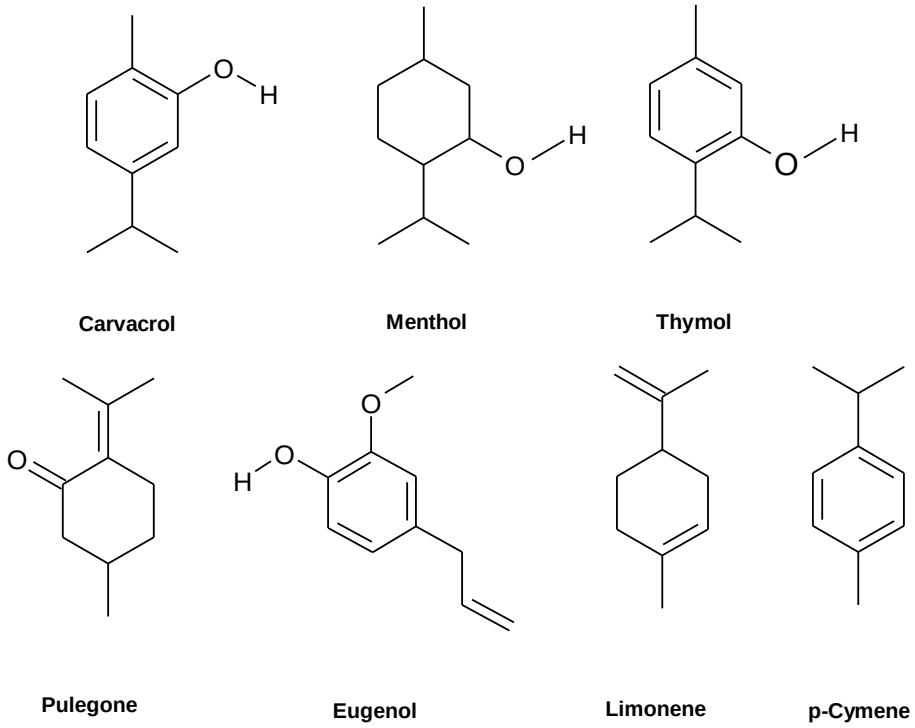

Figure 2 – Chemical structure of several metabolites: carvacrol, menthol and thymol, pulegone, eugenol, limonene and *p*-cymene.

Concerning non-phenolic compounds, the structure of alkyl groups seems to affect antimicrobial activity. The existence of double bonds seems to favour activity since compounds having alkenyl groups exhibit enhanced activity when compared to others possessing alkyl groups. The presence of alkyl groups interferes in the partition coefficient, reducing the surface tension and modifying selectivity. The lack of activity of *p*-cymene (with alkyl group) *versus* the activity of limonene (with alkenyl group) confirms this condition (Burt 2004; Dorman & Deans 2000).

Regarding the two substances of special interest (Figure 2), pulegone can be classified as a cycle monoterpene with ketone function and eugenol as a phenolic aromatic compound (Bakkali et al. 2008).

2.3. Mode of antibacterial action of essential oils

Regarding the antibacterial mode of action of EOs, additional research is needed since some doubts remain about the mechanisms involved. Nevertheless, several mechanisms have been proposed (Figure 3) and it is settled that EOs antibacterial activity is most-likely resultant from a combination of actions with several targets in the cell (Burt 2004).

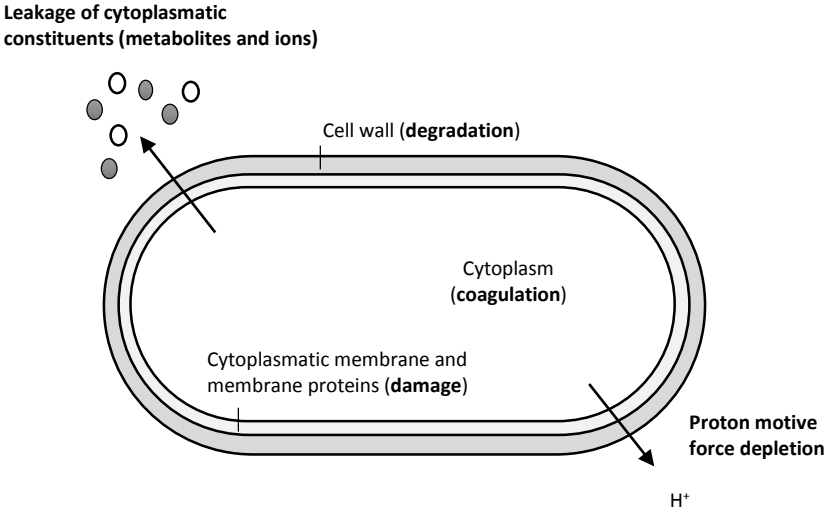

Figure 3 – Overview of locations and mode of EOs action: degradation of cell wall, damage of cytoplasmic membrane and of membrane proteins, leakage of cell contents, coagulation of cytoplasm and depletion of the proton motive force. Adapted from (Burt 2004).

Different authors used scanning electron microscopy (SEM) (Khan & Ahmad 2012; Dung et al. 2008; Bajpai et al. 2009) and transmission electron microscopy (TEM) (Fisher & Phillips 2008; Bouhdid et al. 2009) to observe morphological changes in the cell when contacting with different EOs. In Figure 4 electron micrographs clearly show that in the absence of EO the cell

surface is smooth and regular contrary to cells in the presence of *Cleistocalys operculatus* essential oil. Hereby, EOs appear to deteriorate the morphology of cell membrane which starts to display several clefts, ending in disruption and lysis (Dung et al. 2008; Bakkali et al. 2008).

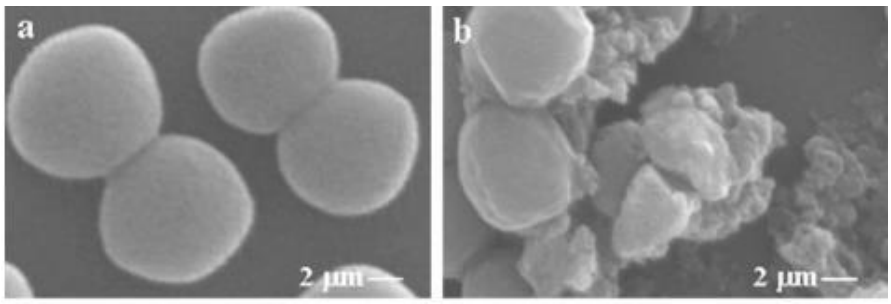

Figure 4 - Scanning electron micrographs of *S. aureus*. At the left (a) the bacteria were not exposed to EOs and at the right (b) exposition effects can be observed (*Cleistocalys operculatus* buds EO at its MIC concentration). Adapted from (Dung et al. 2008).

The most important characteristic that enables the EOs action is their hydrophobicity. As hydrophobic compounds, EOs are able to partition into the lipids of cell membranes (e.g. bacterial cell membrane and mitochondria). Such action disturbs these structures which become more permeable, allowing the leakage of ions and metabolites. Even if up to a certain point leakage does not imply loss of cell viability, further leakage can be lethal (Nedorostova et al. 2009; Burt 2004; Solórzano-Santos & Miranda-Novales 2012; Fisher & Phillips 2008). In addition, a decrease of pH due to cell membrane disruption has been reported. This fact suggests that the control of cellular processes as for instance DNA transcription, protein synthesis or enzyme activity is lost (Fisher & Phillips 2008). Lipids and protein are damaged and cytoplasm might coagulate (Bakkali et al. 2008). Moreover respiration processes is known to be affected (Bouhdid et al. 2009; Pavithra et al. 2009).

Further, EOs appear to act on cell proteins from the cytoplasmatic membrane. There are two main possible mechanisms. The EO lipophilic molecules might accumulate in the lipid bilayer and disturb lipid-protein interactions or the lipidic compounds can directly interact with the protein hydrophobic parts (Burt 2004).

Even though the mechanisms of action are poorly understood, there are some evidences indicating that EOs mode of action is different from that of common antibiotics since, for example, methicillin-resistant strains were susceptible to EOs action (Mulyaningsih et al. 2010; Aneja & Joshi 2010; Chaieb et al. 2007).

2.3.1. Gram positive versus Gram negative susceptibility

Concerning susceptibility, it is often reported in the literature that Gram-positive bacteria seem to be slightly more sensitive to EOs action than Gram-negative ones (Laouer et al. 2009; Al-Reza et al. 2009; Bosnić et al. 2006; Bouhdid et al. 2009; Busatta et al. 2008;

Cosge et al. 2009; Ennajar et al. 2009; Ennajar et al. 2010; Hamed et al. 2012; Magina et al. 2009; Mahboubi & Haghi 2008; Mulyaningsih et al. 2010; Oyedeji et al. 2009; Shahat et al. 2008; Zarai et al. 2012; Nedorostova et al. 2009; Wang et al. 2012; Burt 2004; Fisher & Phillips 2008; Solórzano-Santos & Miranda-Novales 2012; Laciar et al. 2009; Chaieb et al. 2007). Usual explanations are related with the fact that Gram-negative are owners of an outer membrane surrounding the cell wall in opposition to Gram-positive. Thereby they offer higher resistance to EOs action, not allowing an easy diffusion through the lipopolysaccharide membrane and avoiding the accumulation of EOs in the cell (Burt 2004; Solórzano-Santos & Miranda-Novales 2012; Fisher & Phillips 2008; Bajpai et al. 2009; Busatta et al. 2008; Ennajar et al. 2009; Laciar et al. 2009; Magina et al. 2009; Mahboubi & Haghi 2008; Mulyaningsih et al. 2010; Teixeira et al. 2012).

However a reduced number of papers claim that there is no difference among Gram-positive and Gram-negative bacteria (Dorman & Deans 2000; Dung et al. 2008; Goñi et al. 2009; Mkaddem et al. 2009). Additionally, some authors (Burt 2004; Lang & Buchbauer 2012) report only a delay in EOs action concerning Gram-negative organisms. On the other hand, concerning antimicrobial vapour activity of EOs, the opinions seem to be consistent and it is reported that there is no difference among Gram-positive and Gram-negative bacteria (Bajpai et al. 2011; Lopez et al. 2005).

In the last decade, it was proposed that the degree of activity against organisms with different cell wall structure is due to individual components of EOs. This would explain the preferential activity of some EOs against certain type of bacteria. Given there are several factors affecting EOs composition, the susceptibility would differ in that extent (Burt 2004; Lang & Buchbauer 2012; Bajpai et al. 2009). Different studies (Ahmadi et al. 2010; Bosnić et al. 2006; Bouhdid et al. 2009; Cosge et al. 2009; Dung et al. 2008; Mkaddem et al. 2009; Ennajar et al. 2010; Lopes-Lutz et al. 2008; Betoni et al. 2006; Bourgou et al. 2012) support that the activity of the EO is due to additive, synergetic or antagonistic effects of individual components in specific quantities and thereby the same EO activity cannot be easily replicated due to the dependence on its composition and on different external factors. Further, the antimicrobial activity would be affected by EOs plant origin, composition and concentration (Lang & Buchbauer 2012; Bakkali et al. 2008; Cosge et al. 2009; Ennajar et al. 2009; Ennajar et al. 2010; Hayouni et al. 2008).

In the present study, *Staphylococcus aureus* and *Xanthomonas campestris* were used to assess the antimicrobial activity of CB-EO and PRO-EO and of their major constituents. *S. aureus* is a standard microorganism widely used in antibacterial assays and its popularity started due to their propensity to develop multi-drug-resistance (Solórzano-Santos & Miranda-Novales 2012). Today it is also used in antibacterial assays involving EO, allowing some degree of comparison with the results in literature. *X. campestris* is a phytopathogenic bacteria. Given it is intended to proceed to assays in food matrix using non-perishable products as maize, it is interesting to study a bacteria that normally spoilages products like this when stored.

2.3.1.1. *Staphylococcus aureus*

Staphylococcus aureus comprise Gram-positive bacteria commonly found in the human skin (Lang & Buchbauer 2012). These bacteria are often in the origin of food-borne diseases and skin infections (Solórzano-Santos & Miranda-Novales 2012; Ahmadi et al. 2010). Hence, elimination and control of *S. aureus* are main objectives of different industries, specially food-related ones (Ahmadi et al. 2010).

Staphylococcus genus, and specially *S. aureus*, is constantly reported in the literature as one of the most susceptible bacteria to EOs action (Laouer et al. 2009; Aneja & Joshi 2010; Bajpai et al. 2009; Betoni et al. 2006; Bosnić et al. 2006; Çetin et al. 2009; Ennajar et al. 2009; Goñi et al. 2009; Hamed et al. 2012; Mahboubi & Haghi 2008; Shahat et al. 2008; Zarai et al. 2012; Saeed et al. 2013; Nedorostova et al. 2009; Fatope et al. 2008; Bourgou et al. 2012; Chaieb et al. 2007). Actually, from the survey made, only one work out of forty nine (Hayouni et al. 2008) reports lack of antibacterial activity of *Salvia officinalis* L. and *Schinus molle* L. EOs against *S. aureus*.

2.3.1.2. *Xanthomonas campestris*

The genus *Xanthomonas,* comprising Gram-negative rods, causes diseases in several plants including cereals, affecting a wide range of plant parts. Even in developed countries, this fact make *Xanthomonas* one of the major problems in agriculture domain. The diseases control is extremely difficult and expensive as they can be easily transmitted from infected plants through different mechanisms as for instance rain waters, wind, birds or insects (Bajpai et al. 2011). Despite very few publications report the activity of EOs against *Xanthomonas,* some applications comprising EOs have been effective in inhibiting *Xanthomonas* growth (Bajpai et al. 2009; Inouye 2003). Additionally, along with other bacterial species (as for instance *Pseudomonas syringae or Clavibacter michiganensis*) *Xanthomonas campestris* is one of the major phytopathogenic bacteria type (Bajpai et al. 2011).

2.4. Overview of the methodologies

A wide range of assays to *in vitro* assessment of antimicrobial activity can be used. Such methods are usually classified as diffusion, dilution or bioautographic methods, and the most used are the first two (Burt 2004; Rios et al. 1988; Horváth et al. 2010; Lang & Buchbauer 2012).

The agar disc diffusion method is often employed and is frequently used as screening assay before further studies. Its application is useful for selection of antimicrobial substances

to be used. However, the comparison of different data concerning this method is difficult due to differences in operation conditions applied by different authors (Burt 2004).

On the other hand, antimicrobial activity assays are traditionally established and standardized for the utilization of antibiotics as active substances. As referred to above, essential oils and their components are volatile and complex viscous substance. Further, these substances are hydrophobic in opposition to antibiotics, which are generally hydrophilic. Hence the above mentioned methods were modified in order to be adapted to the utilization of EOs as active substance (Burt 2004; Horváth et al. 2010).

Concerning EOs antimicrobial activity study, different factors might bias the results. One of the major problems is their volatility, since substances with higher volatility evaporate faster and the EO activity would not be properly assessed. To avoid this problem, dilution tests are sometimes preferred. Nevertheless, hydrophobicity, which demands the use of surfactants or solvents, can distort the result. Each solvent has different properties which consequently may influence the EO activity in a different way (Lang & Buchbauer 2012). Also extraction methods of the EO can be a variable since the difference in organoleptic profile indicates differences in the composition of oils obtained by different extraction methods, which might influence the antimicrobial properties of the EO (Burt 2004). Further, there is a high difficulty in the comparison of published data, since individual modifications in the procedure conditions (even simple as volume of inoculum) can extremely influence the final result (Lang & Buchbauer 2012; Burt 2004).

Therefore, even if the different methods are described in the literature, no standardized method to evaluate antimicrobial activity of EOs against food-related microorganisms can be found in the literature (Burt 2004) even though several authors (Ebrahimabadi et al. 2010; Dung et al. 2008; Mahboubi & Haghi 2008; Hayouni et al. 2008; Saeed et al. 2013; Laciar et al. 2009; Hammer et al. 1999; Bajpai et al. 2009; El-Baroty et al. 2010; Mulyaningsih et al. 2010; Teixeira et al. 2012; Betoni et al. 2006; Wang & Liu 2010; Zarai et al. 2012) have been adapting Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility tests, designed for antibiotics, to EOs testing.

2.4.1. Diffusion methods

Agar diffusion is often used either as well diffusion, disc diffusion, cylinder diffusion or vapour diffusion. In the first case small holes are punched in the inoculated agar plate using simple devices such as a sterile Pasteur pipettes. The well is then filled with the EO which would diffuse into the agar.

Disc diffusion requires the placement of small paper discs impregnated with the EO onto the inoculated agar surface. The disc can be directly placed onto the agar after impregnation or EO can be allowed to dry in the disc before placement (Lang & Buchbauer 2012; Burt 2004).

Cylinder diffusion is a technique not so often used, as remarked by the lack of reference to it in several reviews about antimicrobial assays methodologies. This method is quite similar to well diffusion. Here, stainless steel or porcelain cylinders are placed on a previous inoculated Petri dish. The cylinders are filled with the antimicrobial substances and the Petri dishes are incubated. After that period the cylinders are removed and results evaluated (Choma & Grzelak 2011; Rios et al. 1988).

All these techniques result in an inhibition zone (halo) surrounding the area where the EO was placed. The size of this halo gives the strength of the active substance in a relative manner since enables the comparison between different substance activities (Lang & Buchbauer 2012; Burt 2004). It has been proposed in the literature (Fu et al. 2007) that inhibition diameters lower than 10 mm should be considered as weak antimicrobial activity and higher than 10 mm should be considered as satisfactory. As stated above, the main problem of this type of assay is the EOs low solubility in water which sometimes can lead to a false low antimicrobial activity. For instance, some EO present low activity in the agar diffusion but higher antimicrobial activity in dilution assays (Lang & Buchbauer 2012).

Still regarding diffusion assays, vapour phase methods are also applied, even though, less often (Lang & Buchbauer 2012; Inouye 2003). There are two variations of this technique nominated slow and fast evaporation. The most usual is fast evaporation (or inverted Petri dish method), in which a seeded agar plate is placed upside down onto a reservoir. A sterile filter paper impregnated with the volatile oil is placed bellow the seeded plate in order to allow vapour diffusion. Slow evaporation consists in a agar plate inoculated and a filter paper or glass dish containing EO placed inside a sealed container, which would allow the simulation of a closed system where the EO will slowly evaporate and exert its antibacterial action (Inouye 2003; Goñi et al. 2009; Fisher & Phillips 2008). In both cases, inhibition zone is measured and determination of minimum inhibitory doses (MID) is possible. The principal handicap of this technique relays also on the inexistence of a standardized method. Nevertheless, this type of assay takes advantage of EOs high volatility, overcoming the lack of solubility of these substances which is notorious in other type of assays (Lang & Buchbauer 2012; Inouye 2003).

2.4.2. Dilution tests

Dilution methods can use as support solid medium (agar) or liquid medium (broth). Both cases consider a concentration gradient of the substance and therefore a dilution series is made. The creation of a saturated moistened atmosphere is significantly useful to adjust the volatility (Lang & Buchbauer 2012). If agar is used, after dilution it is allowed to solidify onto the Petri plates (Rios et al. 1988).

Within broth dilution type, broth microdilution is often applied. This variant may comprise the growth of the microorganisms in a microwells plate and uses lower volumes (Choma & Grzelak 2011).

As mentioned above, the major advantage of dilution methods is the possibility to apply it to the study of water-soluble or insoluble compounds, thus being especially useful to EOs (Rios et al. 1988; Lang & Buchbauer 2012). Nevertheless it takes laborious handling and high costs (Lang & Buchbauer 2012).

Dilution methods allow the determination of the minimal inhibitory concentration (MIC) and, consequently, the determination of the EO strength against certain microorganism (Lang & Buchbauer 2012; Burt 2004; Fisher & Phillips 2008). In order to generalize the classification of antimicrobial activity of these substances it has been proposed the following classification: strong in the range 0.05-0.5 mg/ml, moderate for 0.6-1.5 mg/ml and weak if MIC is higher than 1.5 mg/ml (Sartoratto et al. 2004; Aligiannis et al. 2001; Magina et al. 2009).

2.4.3. Other methods

As stated, other often used method is bioautography which relays on the diffusion methods principle although the diffusion occurs from the chromatographic layer to the agar medium (Choma & Grzelak 2011). Therefore, it requires the execution of a previous EO characterization using chromatography and it can be efficaciously combined with different layer liquid chromatography methods (Rios et al. 1988; Horváth et al. 2010; Choma & Grzelak 2011). Moreover, bioautography can be subdivided in contact, direct or immersion types.

Besides, there are less typical methods. The determination of quickness and duration of antibacterial activity can be made using time-kill analysis by plotting the survival curves (number of viable cells in the broth after EO addition) versus time. Thereby, the measurement of optical density and plating out onto solid medium is required (Burt 2004; Lang & Buchbauer 2012). Other technique is the air-washer coupled with air-sampler which allows the study of the effect of EOs in air-born microorganisms (Lang & Buchbauer 2012). Further, the physical effects of antibacterial activity can be as well made using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). These methods allow the assessment of damage of the bacterial cell wall and cytoplasm such as swelling, vacuolations or leakage (Burt 2004; Bouhdid et al. 2009; Fisher & Phillips 2008; Bakkali et al. 2008).

2.4.4. Incidence in the literature

According with a review of several publications, the most used method to assess EO antimicrobial activity is the disc diffusion assay soon followed by broth microdilution. The distribution of the several methods found can be seen in Figure 5. Moreover, concerning bioautography only the direct type was found. Within the literature assessed cylinder diffusion technique was not found.

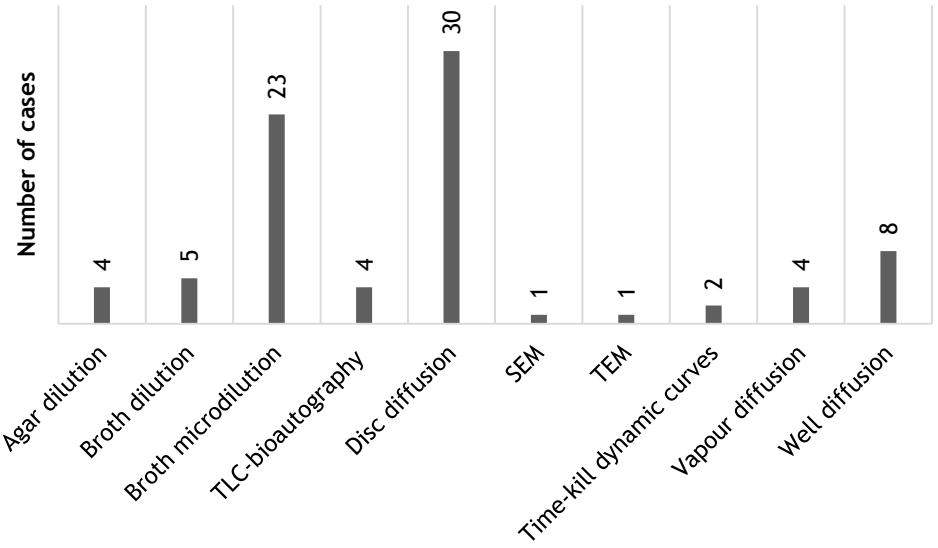

Figure 5 - Distribution of the different antimicrobial activity assays in the literature consulted. The survey was made from a total of 49 publications and some of them apply more than one method. Detailed information can be accessed in Appendix B.

2.4.5. Growth indicators

Antibacterial assays are occasionally coupled with bacterial growth indicators in order to easily analyze the effect of EOs growth inhibition. Examples are 2,3,5-triphenyltetrazolium chloride (TTC) (Sartoratto et al. 2004; O'Bryan et al. 2008; Laciar et al. 2009), 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) (Zarai et al. 2012; Horváth et al. 2010), *p*-iodonitotetrazolium violet (INT) (Oyedeji et al. 2009; El-Baroty et al. 2010) or 7-Hydroxy-3H-phenoxazin-3-one 10-oxide (Alamar Blue or resazurin) (Bouhdid et al. 2009; Valgas & Souza 2007).

Additionally, growth indicators capable of detect dehydrogenase activity (such as tetrazolium salts) are quite often in bioautography methods (Choma & Grzelak 2011).

2.4.6. Minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) is normally used as a measure of the antibacterial performance of EOs. Nevertheless, MIC definition diverges between publications, which is an additional problem concerning data comparison. Some studies report as well, the minimum bactericidal concentration (MBC) whose definition can also diverge. An overview of the most usual definitions for both MIC and MBC is presented in Table 1. Figure 6 displays a schematic definition of both MIC and MBC. As stated, MIC and MBC are determined using dilution methods.

It should be noticed that despite the variations among the protocols and considerations taken by different authors, MICs determined by agar dilution are generally in the same range of magnitude (Burt 2004; Busatta et al. 2008) whereas broth dilution methods are not. This

can be explained by the utilization of different techniques to determine the dilution assay end-point. The most frequent methods to do so are optical density (turbidity) and enumeration of colonies by viable count (after incubation in solid medium). Other methods can be listed such as visible growth (macroscopic evaluation), absorbance, colourimetry or conductivity (Burt 2004).

Table 1 - Overview of the most usual definitions in the literature of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Term	Definition	Reference
MIC	The lowest concentration inhibiting visible growth…	(Wang et al. 2012; Goñi et al. 2009; Adiguzel et al. 2009; Hammer et al. 1999; Kloucek et al. 2012; Betoni et al. 2006; Ahmadi et al. 2010; Oyedeji et al. 2009; Ebrahimabadi et al. 2010; Teixeira et al. 2012; CLSI 2012a)
	…after macroscopic evaluation.	(Al-Reza et al. 2009; Dung et al. 2008; Magina et al. 2009; Wang & Liu 2010)
	…after 24h incubation period.	(Pavithra et al. 2009)
	…after 24h incubation period at 37 °C.	(El-Baroty et al. 2010)
	…24h for bacteria and 48h for fungi.	(Mahboubi & Haghi 2008)
	…indicated by the staining agent.	(Sartoratto et al. 2004; Zarai et al. 2012; Bouhdid et al. 2009; Laciar et al. 2009; O'Bryan et al. 2008)
	…indicated by no visible turbidity.	(Joshi et al. 2008)
	…indicated by no visible turbidity after 24h incubation period.	(Lee et al. 2009)
	…indicated by the presence of a white "pellet" on the well bottom.	(Hayouni et al. 2008)
	…resulting in a clearly visible inhibition zone.	(Nedorostova et al. 2009; Aneja & Joshi 2010; Shahat et al. 2008)
	…resulting in 80% reduction in visible growth when compared with that for substance-free sample.	(Havlik et al. 2009)
MBC	The lowest concentration resulting in 99% absence of growth.	(Magina et al. 2009)
	The lowest concentration resulting in absence of growth…	(Pavithra et al. 2009)
	…on the solid media surface.	(Dung et al. 2008; Mahboubi & Haghi 2008)
	…on the solid media surface, determined by seeding 10 µL from each well on a plate which was then incubated for further 24 h at 37 °C.	(Wang et al. 2012)

Moreover, it is also possible to determine the minimum inhibitory dose (MID), generally defined as the minimum dose of the gaseous state to inhibit growth. Even so, definition can vary. The determination of MID is similar to that for MIC but instead of dilution methods it demands vapour diffusion techniques. There are two main problems related with MID determination. First, the incubation temperature can have an effect in the inhibition once

MID is dependent on the evaporation of the EO volatile components. Second, loss of vapour can occur due to absorption into the media (Fisher & Phillips 2008; Goñi et al. 2009).

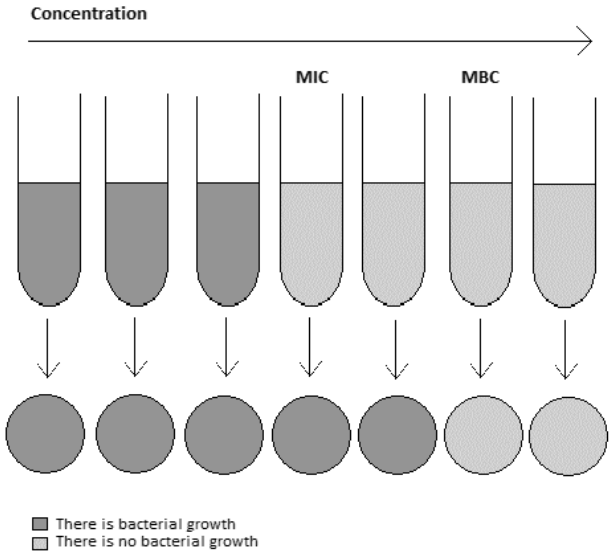

Figure 6 - Schematic representation of MIC and MBC. With the increasing of the concentration of antimicrobial solutions, the biomass present will decrease until the achievement of MIC and MBC.

2.4.7. Usual solvents

In the literature it is often reported the utilization of different solvents to allow a better incorporation of the EO into the medium when using bacterial systems (Burt 2004). The importance of it rises when applying diffusion methods due to the water insolubility problems previously mentioned. However, after literature review several are the authors who do not report the utilization of any solvent when using diffusion techniques (Park et al. 2010; Pavithra et al. 2009; Hayouni et al. 2008; O'Bryan et al. 2008; Çetin et al. 2009; Mulyaningsih et al. 2010; Goñi et al. 2009; Busatta et al. 2008; Mkaddem et al. 2009; Joshi et al. 2008; Fatope et al. 2008; Nedorostova et al. 2009; Saeed et al. 2013; Bourgou et al. 2012; Ennajar et al. 2010; El-Baroty et al. 2010; Fatope et al. 2008).

It is notorious a preference in the application of DMSO as solvent in different method types (El-Baroty et al. 2010; Pavithra et al. 2009; Aneja & Joshi 2010; Mahboubi & Haghi 2008; Hayouni et al. 2008; Joshi et al. 2010; Laciar et al. 2009; Çetin et al. 2009; Ahmadi et al. 2010; Lopes-Lutz et al. 2008; Mulyaningsih et al. 2010; Dung et al. 2008; Adiguzel et al. 2009; Laouer et al. 2009; Magina et al. 2009; Shahat et al. 2008; Oyedeji et al. 2009; Wang & Liu 2010; Ebrahimabadi et al. 2010; Teixeira et al. 2012). In addition, other solvents reported are: acetone (Maggi et al. 2010), agar (O'Bryan et al. 2008; Bouhdid et al. 2009), Tween-80 (Ennajar et al. 2010; Wang & Liu 2010; Fu et al. 2007), Tween-20 (Wang et al. 2012; Hammer et al. 1999), ethanol (Zarai et al. 2012; Horváth et al. 2010; Teixeira et al. 2012), methanol (Lee et al. 2009; Adiguzel et al. 2009; Bajpai et al. 2009; Saeed et al. 2013), ethyl acetate

(Sartoratto et al. 2004; Kloucek et al. 2012; Bajpai et al. 2009), ethyl ether (Goñi et al. 2009; Lopez et al. 2005), *n*-hexane (Bajpai et al. 2009; Ahmadi et al. 2010), water (Teixeira et al. 2012; Cosge et al. 2009; Saeed et al. 2013) and chloroform (Bajpai et al. 2009). Tween-20 was reported as more suitable for solubilization than Tween-80. Both compounds are normally attractive due to their low toxicity and safety regarding food and pharmaceutical applications (Edris & Malone 2012). In some cases, combination of two solvents are found. From the consulted publications, Aneja and Joshi (2010) used a combination of DMSO and Tween-20, Laciar et al. (2009) used *n*-hexane with ethyl acetate and Havlik et al. (2009) used agar and Tween-80. Figure 7 illustrates the incidence of different solvents used.

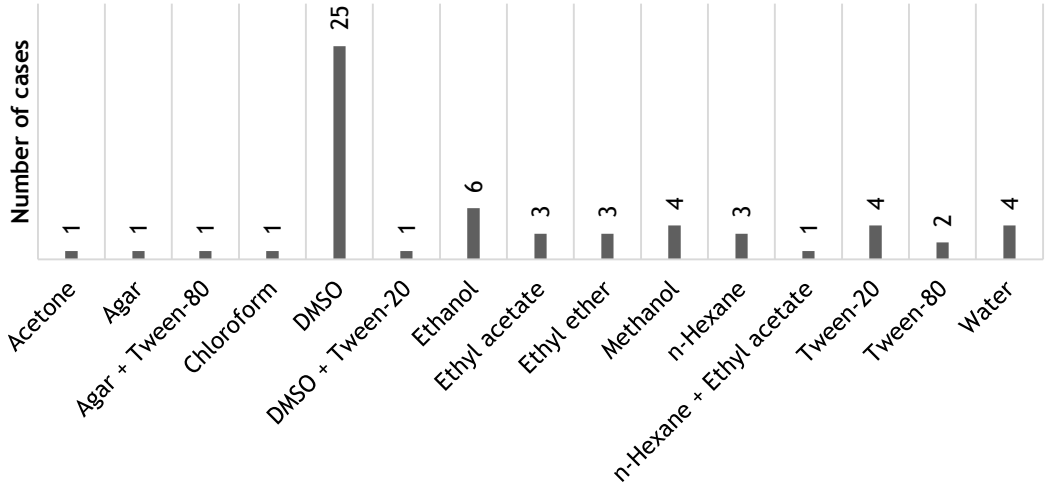

Figure 7 - Distribution of the different solvents used in the literature. The survey was made from a total of 49 publications and some of them apply more than one solvent. Detailed information can be accessed in Appendix B.

Additionally, it was previously reported that the utilization of substances to solubilize the EO can decrease its antibacterial effect. An evidence of that is for instance the utilization of Tween-80 as neutralizer of some disinfectants (Burt 2004). On the opposite, it was shown that agar at 0.05% (w/v) improves the antibacterial effect of some types of EOs by enhancement of the dispersion in water (Burt et al. 2005). In addition, 0.2% (w/v) agar was proven to enable an homogeneous dispersion of the EOs with better performance than the one obtained with Tween-80 or ethanol (Burt et al. 2005; Burt 2004).

Also regarding Tween, some studies reported the utilization of this substances as carbon source especially for *P. aeruginosa*, since the members of genus *Pseudomonas* are typically capable of use several exogenous substrates as carbon source (Howe & Ward 1976). Tween-80 is most frequently used as carbon source than Tween-20. Both compounds derive from fatty acids which justify their utilization as carbon source for some application such as lipase or medium-chain-length polyhydroxyalkanoates production (Zouaoui & Bouziane 2011; Chan et al. 2006). Therefore, despite its advantages for food and pharmaceutical applications, Tween do not seem to be the best choice as solvent, in particular Tween-80.

Even if in one work DMSO is reported as prejudicial to antimicrobial activity (Hili et al. 1997), in the works where DMSO is used as negative control, no inhibition zone for DMSO is shown, and thus, no activity can be seen (Bosnić et al. 2006; Aneja & Joshi 2010). Thereby, DMSO seems to be the most advantageous solvent to be applied.

2.5. Applications of essential oils as preservatives in food systems

Since food systems are environments prone to microorganisms' proliferation, food spoilage is usual. Food-borne diseases are caused due to ingestion of food contaminated with pathogenic microorganisms and/or their toxins (Solórzano-Santos & Miranda-Novales 2012).

Thereby, EOs have potential to be applied in package coating containing the antimicrobial compound or even by development of biodegradable coating films (Hamed et al. 2012; Havlik et al. 2009). Besides, EOs are able to reduce bacterial population when applied directly in the soil and this will, consequently, avoid spoilage of fresh organic products cultivated in that soil (Solórzano-Santos & Miranda-Novales 2012).

It should be highlighted that, when handling with antibacterial compounds in food systems, an improved incorporation is a main concern. Often, lack of water solubility of EOs reduces availability for antimicrobial action. Moreover, comparing food systems tests with *in vitro* tests, food systems have greater availability of nutrients which may allow bacteria to repair damaged cells faster and thus, be less susceptible. This leads to the use of higher concentrations than the ones used in *in vitro*. Such fact may be a problem, since exceeding the acceptable flavour and/or odour thresholds would imply a stronger herbal aroma and thus consumer's rejection once the original food aroma is changed (Shah et al. 2013; Velázquez-Nuñez et al. 2013).

Moreover, different parameters of the foodstuff are of great concern. Both intrinsic (as fat, protein, water content; antioxidants; preservatives; pH; salt and additives) and extrinsic properties (temperature, packing in vacuum, gas or air; characteristics of the microorganism) are indeed important and can affect antimicrobial activity of EOs (Burt 2004; Havlik et al. 2009; Hayouni et al. 2008). Actually, it is known that the affinity of food components (as proteins and fat) can bind and solubilize phenolic compounds reducing their availability to act against bacteria (Hayouni et al. 2008; Shah et al. 2013; O'Bryan et al. 2008). Moreover, in food systems, MIC must be achieved in all points of the food to ensure protection and thereby concentration would be higher (Havlik et al. 2009).

As stated before, different studies concerning application of EOs as biopresevatives have been accomplished. The EOs act by reducing or eliminating the pathogens. Food quality

can, thus, be improved and to do so EOs effectiveness was already assessed for some food products (Solórzano-Santos & Miranda-Novales 2012). Indeed, eugenol was already tested as seed disinfectant, showing promising results (Lang & Buchbauer 2012; Lo Cantore et al. 2009). Actually, Lo Cantore research group (2009) was able to lower the *X. campestris* bacterial population of infected been seeds from $2.6x10^6$ to $7.0x10^2$ CFU/ml using eugenol emulsions. Despite the major components of the EO could be used isolated, it is more sustainable to use the whole EO. Furthermore there is the additional advantage of the synergetic effects of the minor components which normally increase antimicrobial activity (Burt 2004).

Several topics must to be taken in consideration and in the next sub-section different methods of application of this technology are summarized (Solórzano-Santos & Miranda-Novales 2012; Fisher & Phillips 2008).

2.5.1. Available technologies for implementation of EOs as biopreservatives

Notwithstanding lack of investigation about implementation of EOs as biopreservatives in foods, there are some works reporting a few solutions.

Several works have demonstrated the potential of microencapsulation using either proteins or polysaccharides (Shah et al. 2013; Hayouni et al. 2008). Recently, some other studies (Shah et al. 2013; Edris & Malone 2012; Hamed et al. 2012) describe microencapsulation by the utilization of synthetic surfactants which would assemble in oil-water (O/W) micelles at the critical micelle concentration (CMC), forming micro or nanoemulsions. In the interior of the micelles, a hydrophobic environment is provided allowing EOs solubilization (Rodrigues et al. 2013). These methods aim to reduce toxicity, to protect the active substances from deterioration and to avoid the alteration of organoleptic characteristics of the foodstuff (Hayouni et al. 2008).

The use of EOs vapour diffusion can be a safer alternative by eliminating organoleptic issues since EOs would not contact directly with foodstuff (Dorman & Deans 2000; Ennajar et al. 2010; Fisher & Phillips 2008). Other greater advantage of this approach is the vehicle. When in solution, EOs tend to form micelles which would suppress the complete attachment of the EO to the microorganism. On the other hand, vapour phase dispersion allows free attachment (Fisher & Phillips 2008). Actually, when comparing MIDs and MICs, the first ones seem to be lower. This represents an advantage since foodstuff properties would be protected by the utilization of lower doses. Even so, dispersion of the oils as vapours instead of the natural evaporation must be analysed, as heating the oils to increase evaporation can affect the oils antimicrobial properties (Solórzano-Santos & Miranda-Novales 2012; Fisher & Phillips 2008).

2.5.2. Legal aspects

Regarding legal aspects, European Commission (EC) allows the utilization of some EOs in foods as flavourings (Burt 2004). United States Food and Drug Administration (FDA) has classified these compounds as generally recognized as safe (GRAS) or as approved food additives (Burt 2004; Fisher & Phillips 2008). Nevertheless, some EO components can cause irritation and toxicity (Burt 2004). Even so, pulegone and eugenol (principal components of the EOs studied in the present work) are approved by the above mentioned lists (Burt 2004; FDA 2012; EC 2002; Kollanoor Johny et al. 2010). Besides, the organic solvents commonly used to enhance solubility involve dosing restrictions (Edris & Malone 2012) and some cautions must be taken since high doses of EOs (>0.05% v/v) are proven to have cytotoxic effects (Fisher & Phillips 2008). Despite these facts, most of these oils are available for purchase as a whole or contained in pharmaceutical or cosmetic products, which indicates that possible toxic properties do not prohibit their commercialization and utilization (Hammer et al. 1999).

The use of EOs in food is regulated by other directive concerning to flavourings for use in foodstuffs, the directive 88/388/CEE. This regulation allows the utilization with restriction in foodstuffs of some substances forbidden for direct application (among them there is pulegone). Further, recently the REACH system (registration, evaluation, authorization and restriction of chemicals, EC regulation 1907/2006) was implemented. The target substances are the ones produced in amounts of more than one ton per year. Therefore, until the present, EOs were not submitted to this regulation and probably would not be due to the lack of patents applicable to plant extracts (Vigan 2010).

3. Materials and methods

3.1. Reagents

Dimethyl-sulfoxide (DMSO) (≥ 99.5%), (R)-(+)-pulegone (≥ 85%), Triton x-100 and Tween-20 were obtained from Sigma-Aldrich. Eugenol (99%) and stabilized (+)-limonene (96%) were purchased from Acros Organics. Clove bud oil (CB-EO) and pennyroyal oil (PR-EO) were acquired from Inovia International. Müller-Hinton Agar (MHA) and Brain Heart Infusion Agar (BHI) were obtained from Oxoid and Müller-Hinton Broth (MHB) from HiMedia. Plate Count Agar (PCA), Potato Dextrose Agar (PDA) and Luria Broth (LB) were acquired from Liofilchem and Malte Extract Agar (MEA) from Merk. The fungicides Benlate, Previcur N and Baycor 300 were obtained from commercial brands.

3.2. Microorganisms, culture and stock conditions

The standard bacteria *Staphylococcus aureus* DSM 2569 and *Pseudomonas aeruginosa* DSM 1117 and the phytopathogenic bacteria *Xanthomonas campestris* LGM 568, *Clavibacter michiganensis* DSM 46364, and *Rathayibacter tritici* DSM 7486 were used during the assays.

Bacteria stocks were made in LB with 50% glycerol and maintained at -80ºC. BHI was used to transfer the bacteria from the freezing medium since this is the recommended media by CLSI standards (CLSI 2012a; CLSI 2012b; Inouye 2003). The incubation time was 24 h for standard bacteria and 48 h for phytopathogenic bacteria.

MHA and MHB were used for the antimicrobial assays since these are the most suitable media to such purpose (CLSI 2012a; CLSI 2012b; Inouye 2003). Preliminary assays revealed difficulties when *S. aureus* and *X. campestris* were used in suspension since in the absence of dispersants aggregation of cells seemed to occur. Thus, the surfactant Triton x-100 was used

as dispersant and was added to the liquid medium (MHB) at a concentration of 0.001% (Manuel 2007).

3.3. Antimicrobial stock solutions

Due to the low solubility in water of the compounds to be tested, preliminary tests using 10% DMSO or 5% Tween-20 in sterile water were made. These concentrations were chosen since they are the most subscribed in the literature (Hammer et al. 1999; Adiguzel et al. 2009; Mahboubi & Haghi 2008; Ebrahimabadi et al. 2010; Valgas & Souza 2007; Ahmadi et al. 2010). DMSO was found to be the best solvent and thereby it was used to disperse the antimicrobials in all the assays carried out.

3.4. Disc diffusion assay

Disc diffusion method was used as screening assay in order to evaluate the relative antimicrobial activity of eugenol, limonene, pulegone, PR-EO and CB-EO at the concentrations listed in Table 8. The antimicrobial activity was tested against bacteria also listed in Table 8 (Results and Discussion section). The protocol employed was an adaptation of CLSI (CLSI 2012b) normally used for antibiotics antimicrobial activity tests since there is a lack of established and standardized protocols for EOs testing (Burt 2004). CLSI methods were chosen to be used as guideline since the more standardized the method, the more reproducible it is (Hammer et al. 1999; CLSI 2012a; CLSI 2012b).

For each organism, a suspension was prepared in MHB with 0.001% Triton x-100 and its optical density (OD) was adjusted to 0.5 McFarland standard (absorbance around 0.08-0.1 at 600 nm). MHA was used to spread on plate the suspension with a sterile swab and 6 mm sterile filter paper discs were place over the inoculated plate. Then, 3 µl of each antimicrobial solution were used to impregnate the paper discs (20-25 µm porosity). This volume was chosen in order to prevent leakage of the compounds to the area around the disc. Phytopathogenic bacteria were allowed to incubate during 48 h at 30 ºC. *S. aureus* and *P. aeruginosa* incubated during 24 h at 37 ºC. Inhibition halos were then measured.

The antimicrobial solutions were constituted by the non-diluted substances or by dispersion of those using sterile water with 10% DMSO. The concentrations tested were chosen based in the range of the expected minimum inhibitory concentration determined by preliminary results of broth microdilution assays. Sterile water and solvent were used as negative controls.

3.5. Minimum inhibitory concentration (MIC) determination

MIC was determined by broth microdilution assay. Once again, CLSI (CLSI 2012b) methods were chosen to be used as guideline. According with the CLSI standards, broth microdilution assays should start from a OD representing around 10^5 CFU/ml (CLSI 2012a; CLSI 2012b). Such requirement led to the need of calibration curves figuring OD vs colony forming units per ml (CFU/ml) for the microorganisms to be used in broth microdilution assays. The calibration curve for *X. campestris* and *S. aureus* can be seen in Appendix C.

The inoculum was prepared in MHB with 0.001% Triton x-100 using individual colonies previously grown in BHI. OD was adjusted in order to obtain $1x10^7$ CFU/ml. A 96-well plate was used. At each well, 10 µL of the suspension was mixed with 100 µL of MHB, and with 100 µL of antimicrobial solution. Thus, it is possible to achieve an initial cell density of about 10^5 CFU/ml. The range of concentration of each antimicrobial tested is displayed in Table 2. In negative controls, sterile water and solvent were used instead of inoculum. Positive controls were made using sterile water and solvent instead of the antimicrobials solutions (Table 3). Each assay was performed in triplicate.

Table 2 – Microorganisms and concentration ranges of the antimicrobials used in MIC determination.

Microorganism	Substance	Concentration range (mg/ml)
S. aureus **DSM 2569**	Eugenol	0.5-10
	Pulegone	5-50
	Clove Bud essential oil	0.01-1
	Pennyroyal essential oil	0.01-10
X. campestris **LGM 568**	Eugenol	0.01-50
	Pulegone	1-50
	Clove Bud essential oil	0.5-0.6
	Pennyroyal essential oil	0.01-10

Table 3 - Summary of wells content in the broth microdilution assay.

Sample	Content
Antimicrobial solutions	10 µL of bacterial suspension + 100 µL of MHB + 100 µL of antimicrobial solution
Positive controls	10 µL of bacterial suspension + 100 µL of MHB + 100 µL of sterile water 10 µL of bacterial suspension + 100 µL of MHB + 100 µL of solvent
Negative controls	10 µL of sterile water+ 100 µL of MHB + 100 µL of sterile water 10 µL of solvent + 100 µL of MHB + 100 µL of solvent

Absorbance readings were made at the time zero and after the incubation period of 20 h for *S. aureus* or 48 for *X. campestris*. MIC was defined as the lowest concentration inhibiting visible growth indicated by no significant changes in absorbance readings after the incubation period and verified by spread plate results. The colonies counting was made by seeding 100 µL from each well on a Petri dish (90 mm diameter) which incubated for 20 h at 37 °C for *S. aureus* or 48 h at 30ºC for *X. campestris*. Further, minimum bactericidal concentration (MBC) was defined as the lowest concentration resulting in absence of growth on the solid media surface.

3.6. Minimum inhibitory doses (MID) determination

MID was determined by inversed diffusion assay trough fast rate evaporation. Due to the lack of standard described protocols, the one applied is an adaptation of Nedorostova et al. (2009). Inoculum was prepared in MHB with 0.001% Triton x-100 using individual colonies previously grown in BHI. OD was adjusted in order to obtain about $1x10^5$ CFU/ml. A sterile swab was used to inoculate uniformly the 55 mm diameter Petri dishes. These Petri dishes were filled with 7 ml of MHA in the bottom part and 2 ml in the lid (Figure 8). The layer of MHA on the lid allows a better sealing of the Petri dish and prevents adsorption of the volatile compounds to the plastic (Nedorostova et al. 2009; Goñi et al. 2009; Kloucek et al. 2012). A sterile filter paper (20-25 µm porosity) with 55 mm diameter was placed in the lid over the MHA layer and 300 µl of antimicrobial solutions were used to impregnate the disc. The Petri dish was immediately close in order to prevent the loss of volatile compounds and additional sealing was made using Parafilm tape. Paper filter with the same diameter as the Petri dish was proved to be crucial to achieve uniform composition in the headspace of the Petri dish, since it allows observation of more precise and uniform inhibition zones (Kloucek et al. 2012).

Since at this point MIC was known, dilution series of the four substances dispersed in sterile water with 10% DMSO was made using MIC (µg/ml) as starting point. The range of concentrations of each antimicrobial tested is displayed in Table 4. Detailed calculations can be seen in Appendix D.

Sterile water and solvent were used as positive controls and non-inoculated MHA was used as abiotic control (negative control) (Table 5). Incubation time was maintained in accordance with dilution assays: *X. campestris* was allowed to incubate during 48 h at 30 ºC and *S. aureus* incubated during 24 at 37 ºC. Inhibition halos were then measured. All the assays were made in triplicate.

Table 4 - Antimicrobial concentration ranges used for MID determination.

Microorganism	Substance	Concentration range (mg/cm^3 air)
***S. aureus* DSM 2569**	Eugenol	0.25-2
	Pulegone	1.25-14
	Clove Bud essential oil	0.075-0.6
	Pennyroyal essential oil	0.141-9
***X. campestris* LGM 568**	Eugenol	0.063-0.5
	Pulegone	0.5-14
	Clove Bud essential oil	0.119-0.475
	Pennyroyal essential oil	1-14

Table 5 – Overview of the controls used in MID determination.

Controls	Content
Positive	Disc impregnated with sterile water + MHA inoculated with *S. aureus*
	Disc impregnated with sterile water + MHA inoculated with *X. campestris*
	Disc impregnated with solvent + MHA inoculated with *S. aureus*
	Disc impregnated with solvent + MHA inoculated with *X. campestris*
Negative	Disc impregnated with sterile water + not inoculated MHA
	Disc impregnated with solvent + not inoculated MHA

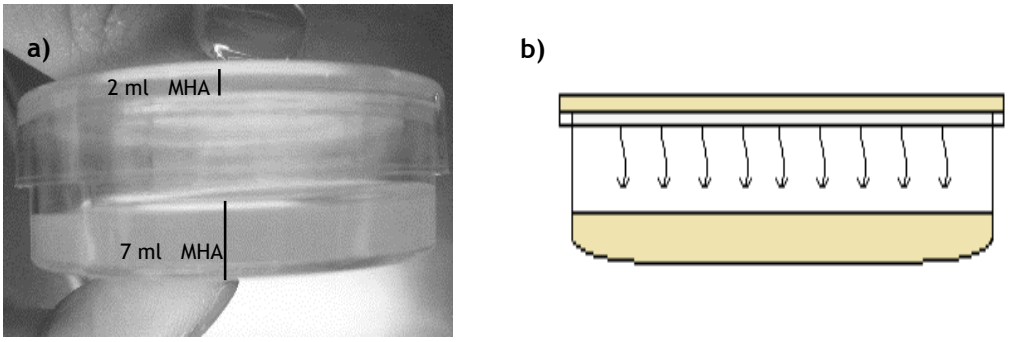

Figure 8- Representation of the Petri dishes used in vapour diffusion assays with 7 ml MHA in the bottom and 2 ml in the upper part: a) example of a Petri dish used in the assays; b) representative scheme of vapour diffusion process in this type of systems.

Minimum inhibitory doses (MID) was defined as the minimal concentration of substance per unit space resulting in total inhibition of growth (Inouye 2003) (no biomass present in the whole perimeter of the Petri dish). The concentration corresponding to absence of inhibition was also determined. Several intermediate concentrations were also evaluated and registered.

3.7. Glass beads assay

Glass beads were used as susceptibility screening test to simulate the geometry of the maize or other type of cereals. The protocol used was similar to the one for MID determination. Inversed diffusion assay trough fast rate evaporation took place using a Petri dish with 2 ml of cultivation medium (MHA) in the lid, where a 55 mm filter paper (20-25 µm porosity) was placed. Glass beads with approximately 5 mm diameter immersed in cellular suspension in MHB of *X. campestris* or *S. aureus* in order to achieve about 10^5 CFU/ml. Six glass beads where then placed in each Petri dish and the sterile paper discs were impregnated with the solutions at their MID concentrations. The positive control was made with paper discs impregnated with water or solvent using the beads inoculated with each microorganism. Negative control (abiotic) was made with paper discs impregnated with water or solvent and glass beads immersed in sterile water. The assay occurred in triplicate.

Table 6 – Overview of the controls used in glass beads assay.

Controls	Content
Positive	Disc impregnated with sterile water + beads inoculated with *S. aureus*
	Disc impregnated with sterile water + beads inoculated with *X. campestris*
	Disc impregnated with solvent + beads inoculated with *S. aureus*
	Disc impregnated with solvent + beads inoculated with *X. campestris*
Negative	Disc impregnated with sterile water + not inoculated beads
	Disc impregnated with solvent + not inoculated beads

Incubation period of 20 h was chosen since a longer one could compromise cells viability due to the lack of nutrients. After such incubation period, 10 ml of saline solution was added to each Petri dish with the purpose of achieve a suspension with about 10^4 CFU/ml. Successive dilutions in saline solution were made until reaching 10^2 CFU/ml. And 100 µl of each dilution was spread onto plate (90 mm diameter Petri dishes with MHA) so that the colonies could be enumerated after 48 h for *X. campestris* and 20 h for *S. aureus*.

3.8. Preliminary *in vivo* assay

In vivo antimicrobial assays using food matrix are of major interest. In order to allow the implementation of these assays, the food matrix to be used must be sterile so one be able to inoculate it at a known CFU/ml concentration using a bacterial suspension. Thereby, treatments using UV, bleach and commercially available fungicides were carried out. The

chosen food matrix was maize harvested, without application of any chemical, at *Quinta Experimental* from *Instituto Nacional de Investigação Agrária e Veterinária* in October, 2012.

3.8.1. UV treatment

Firstly, two UV cycles of 20 minutes each were used in an attempt to sterilize the maize seeds. The media PDA, MEA, PCA, MHA, BHI and sterile filter paper impregnated with sterile water were used as substrate. Negative control was made using sterile glass beads and positive control was made using maize without treatment. The assay occurred in triplicated.

3.8.2. Bleach treatment

Maize was subjected to the action of 5% or 10% bleach during a 30 or 60 minutes incubation period. Sterile filter paper impregnated with sterile water was used as substrate (agar was not used as the main objective was to assess if fungi could develop after treatment and if the maize remained capable to germinate). Negative control was made using sterile glass beads and positive control was made using maize without treatment. The assay occurred in triplicated.

3.8.3. Fungicide treatment

MHA medium supplemented with the fungicides Previcur N, Baycor 300 or Benlate at their average MIC value (Table 7) were used to prevent fungi growth. In order to proceed to assays in food matrix the growth of the bacteria in study is needed. Thereby *S. aureus* and *X. campestris* were seeded in such media to analyse viability of the bacteria. Negative control was made using sterile glass beads and positive control was made using maize in MHA without fungicide. The assay was carried out in triplicate.

Table 7 - Composition and average MIC value for the fungicides used.

Fungicide	Composition	Average MIC Value	Reference
Previcur N	Propyl-3 {3-dimethylamino)propyl}-carbamate monohydrochloride	3.75 µl/ml	(Bayer CropScience 2013c; Bayer CropScience 2013d; Bayer CropScience 2012)
Baycor 300	Bitertanol (27.7%) dissolved in N-Methyl-2-pyrrolidone (50-55%) and other non-hazardous ingredients	4.90 µl/ml	(Bayer CropScience 2013a; Bayer CropScience 2013b)
Benlate	Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate	0.22 mg/ml	(Masabni 2007; DuPont 2001)

Due to the lack of time it was impossible to proceed to further *in vivo* assays using the maize as food matrix.

3.9. Statistical analysis

The MIC, MBC and MID values were compared using the ANOVA approach using the *R-project* software version 3.1.0 from Institute for Statistics and Mathematics of Wirtschaftsuniversität Wien. Moreover, MIC and MBC results were subjected to the outliers' detection using the Modified Tomphson Tau (Garcia 2012). In all cases, results were considered significant when $p<0.05$.

4. Results and discussion

4.1. Disc diffusion

The disc diffusion assays showed, in general, reasonable antimicrobial activity of all the substances tested (Table 8). However, only the non-diluted substances presented activity. Among them, the EOs were more effective than their respective major components, as reported previously (Burt 2004). Substances diluted in 10% DMSO resulted in absence of inhibition. Even though, such concentrations were able to inhibit growth in liquid medium (see next subsection). This confirms that minimum inhibitory concentrations in solid medium are higher than in liquid medium due to the much lower diffusivity of EOs components (Burt 2004; Fisher & Phillips 2008).

Table 8 - Disc diffusion assay results (n = 2).

Sample		Inhibition halo diameter (mm)				
		S. aureus DSM 2569	*P. aeruginosa* DSM 1117	*C. michiganensis* DSM 46364	*X. campestris* LMG 568	*R. tritici* DSM 7486
Eugenol	Pure	22	10	60	55	54
	40 mg/ml	0	0	0	0	0
Pulegone	Pure	10	0	19	35	13
	50 mg/ml	0	0	0	0	0
Limonene	Pure	8	0	12	0	14
	50 mg/ml	0	0	0	0	0
CB-EO	Pure	26	24	55	45	42
	20 mg/ml	0	0	0	0	0
PR-EO	Pure	30	8	19	33	45
	10 mg/ml	0	0	0	0	0
DMSO	Pure	0	0	0	0	0
	10% DMSO	0	0	0	0	0

Standard deviation is not presented as n = 2. It should be noticed that according with the normal standards, inhibition diameter higher than 10 mm represents satisfactory inhibition and lower than that characterizes weak activity (Fu et al. 2007).

Despite the exact composition of the CB-EO used (and consequently the respective concentration of eugenol) is unknown, similar results were obtained for both substances, enforcing the role of eugenol on the CB-EO antimicrobial activity. Thereby, CB-EO and eugenol were shown to be the most efficacious substances exhibiting satisfactory antimicrobial activity (inhibition diameter of 10 mm or higher) against all the microorganisms tested. Moreover, PR-EO and pulegone showed as well identical results for the microorganism tested, with the exception of *P. aeruginosa*. This bacteria demonstrated lack of susceptibility to pulegone and merely weak susceptibility (inhibition diameter lower than 10 mm) to PR-EO. On the other hand, PR-EO and pulegone exhibited satisfactory antimicrobial activity against all the other bacteria tested. Limonene was the weaker antimicrobial herein tested, exerting antimicrobial activity only against the Gram-positive bacteria studied (*R. tritici*, *C. michiganensis*, and *S. aureus*). Therefore the use of this substance was discharged in further assays.

Gram-positive bacteria are described as slightly more sensitive to EOs action than Gram-negative ones. This is explained by the outer membrane surrounding the cell wall of Gram-negative, offering higher resistance to EOs action, not allowing an easy diffusion through the lipopolysaccharide membrane (Burt 2004; Fisher & Phillips 2008). The Gram-positive *S. aureus* is frequently reported as the most susceptible microorganism to EOs (Burt 2004; Fisher & Phillips 2008). Interestingly, this study revealed that not only the Gram-positive *R. tritici* and *C. michiganensis* but also the Gram-negative phytobacteria *X. campestris* were equally susceptible to EOs.

Additionally, only the phytobacteria present satisfactory susceptibility (>10 mm inhibition diameter) to all the substances tested, with the exception of *X. campestris* regarding limonene. Indeed, limonene is the most apolar substance tested and this characteristic would be expected to allow a better penetration in the also apolar lipopolysaccharide membrane of Gram-negative bacteria. Even so, it is possible that limonene is so apolar that would be retained and accumulated in the outer membrane due to interaction with membrane components, or hampered to rich the membrane due to the presence of hydrophilic sugar residues of lipopolysaccharides (Bajpai et al. 2009). This fact may explain the lack of activity against the two Gram-negative bacteria tested (*X.* campestris and *P. aeruginosa*). Nevertheless other unknown causes may be involved since some *in vitro* tests in the literature reported that terpenes as limonene are inefficient as antimicrobials when used as pure substance (Fisher & Phillips 2008).

Lo Cantore (2009) research group studied the activity of eugenol and limonene against *X. campestris* and *C. michiganensis* and both species showed susceptibility against both substances. Although this relations were confirmed in our investigation for eugenol, as stated, no activity of limonene could be recorded against *X. campestris*.

No data reporting the susceptibility of *R. tritici* to the studied substances could be found in the literature reviewed.

P. aeruginosa was the most resistant bacteria tested, being only inhibited by eugenol, CB-EO and weakly by PR-EO. Indeed, *P. aeruginosa* seems to have intrinsic resistance to a wide range of antimicrobial compounds, including EOs (Burt 2004). Its Gram-negative nature cannot be the only explanation to its lack or weak susceptibility against the substances tested since the other Gram-negative herein tested (*X. campestris*) exhibited significant susceptibility to four out five compounds tested with the exception of limonene (which, as explained above, might be related with its apolar nature).

Additionally, the control disc using non-diluted DMSO and 10% DMSO in sterile water revealed no inhibitory effect, which allows the utilization of this solvent in subsequent assays.

The results herein presented allowed to obtain a preliminary snapshot of the EOs activity against standard and phytopathogenic bacteria. However, this method provides qualitative data only due to the hydrophobic nature of the substances used which do not allow uniform diffusion through the agar surface.

4.2. Minimum inhibitory concentration (MIC) determination

Based on disc diffusion assay results, the MIC of CB-EO and PR-EO and their major components were determined for *S. aureus* and *X. campestris*. These microorganisms were proven to be susceptible to all the substances in investigation. Furthermore it enables the utilization of a standard bacteria and a phytopathogenic bacteria along with the use of Gram-positive and Gram-negative types. Even if several attempts were made to use as well *P. aeruginosa* and *C. michiganensis* (data not shown), the intrinsic resistance of *P. aeruginosa* and difficult dispersion of *C. michiganensis* in broth lead to the rejection of such microorganisms.

DMSO was used to promote more uniform diffusion of the compounds trough the broth. Despite that, in disc diffusion assay pure DMSO or 10% DMSO showed no harm to the microorganisms, some preliminary assays in liquid medium revealed some susceptibility when using a final concentration of 10% DMSO (data not shown). Other authors (Basch & Gadebusch 1968) determined a DMSO MIC of 8%, 20%, and 30% (v/v) for 3 different strains of *S. aureus*. In the broth microdilution assay, when the antimicrobial solutions are dispersed in 10% DMSO in sterile water, the final concentration in the well is ≈4.8%, which is lower than the MICs reported in the literature. Actually, comparison of control with and without DMSO using this concentration revealed that *S. aureus* and *X. campestris* are not susceptible to ≈4.8% DMSO.

The broth microdilution assays showed satisfactory antimicrobial activity of all the substances tested (Table 9). As it was not always possible to define a single value for

minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC), when necessary, the values were given in a range format. As the assay was repeated tree or more times, the resultant data was validated with an outlier's detection by the Modified Thompson Tau which corresponds to a confidence interval of 95% (p<0.05) (Garcia 2012). This method allows to determine whether there are statistically significant difference within the given ranges. Regarding the usual classification of MIC for EOs (Sartoratto et al. 2004; Aligiannis et al. 2001; Magina et al. 2009), CB-EO is the only substance with strong antimicrobial effect (0.05—0.5 mg/ml) for both microorganisms. On the other hand, eugenol exerts as well strong activity but only against *X. campestris*, while against *S. aureus* can be classified as having weak activity (>1.5 mg/ml).

Table 9 – Minimum inhibitory (MIC) and bactericidal (MBC) concentrations.

Microorganism	*S. aureus* DSM 2569		*X. campestris* LMG 568	
Substance	MIC	MBC	MIC	MBC
	mg ml^{-1}/mM	mg ml^{-1}/mM	mg ml^{-1}/mM	mg ml^{-1}/mM
Eugenol	2/12	2-3.5/12-21	0.5/3	1/6
Pulegone	15-19/99-125	18-20/118-131	4/26	4/26
CB-EO	0.5-0.6/-	0.6/-	0.5/-	0.5/-
PR-EO	9/-	≥10/-	4/-	6-8/-

Standard deviation is not presented since when it is different than zero results are presented in a range format (n ≥ 3). Massic and molar concentrations are presented. When molar concentration is not valid the sign "-" is displayed. It should be noticed that it has been proposed the following classification: strong in the range 0.05-0.5 mg/ml, moderate for 0.6-1.5 mg/ml and weak if MIC is higher than 1.5 mg/ml (Sartoratto et al. 2004; Aligiannis et al. 2001; Magina et al. 2009).

It should be remarked that due to weak solubility in water of PR-EO, even when in 10% DMSO, concentrations above 10 mg/ml were not tested. Thereby it is only possible to state that MBC for *S. aureus* should be ≥10 mg/ml.

Contrary to disc assay, substances diluted in 10% DMSO resulted in inhibition, confirming higher susceptibility in broth and better sensibility of diffusion method variant well (Valgas & Souza 2007). CB-EO revealed to be far better antimicrobial than PR-EO, showing lower MICs (p<0.05). The same is true for eugenol when compared to pulegone, which was expected, as these are the major components of the mentioned EOs. Globally, *X. campestris* exhibit higher susceptibility than *S. aureus*, supporting the results of disc assay. Even so, for CB-EO there is no statistical significance among the MICs for both microorganisms (p>0.05). Concerning the PR-EO MIC of *X. campestris* and *S. aureus*, the value is about 1/2 times higher for *S. aureus* (p<0.05). For eugenol and pulegone, *X. campestris* revealed about 1/4 less resistance than *S. aureus* (p<0.05). Further, the results obtained for *X. campestris* revealed that MIC range for CB-EO *versus* eugenol and PR-EO *versus* pulegone are identical (p>0.05). In opposite, for *S. aureus* EOs presented significantly higher (p<0.05) antimicrobial activity than their major components. Additionally, MBC values were slightly higher than MIC.

Although, the statistical analysis revealed that this difference only has significance for pulegone regarding *S. aureus* and for PR-EO concerning *X. campestris* ($p<0.05$).

As stated before, there is a deficiency of reported values in the literature and those that exist cannot be considered as standard values since factors such as methodologies applied and considerations taken by different authors, utilization of different techniques to determine the dilution assay end-point, substance origin and composition or emulsifier used lead to divergences in the results at least in magnitude (Lang & Buchbauer 2012; Burt 2004; Busatta et al. 2008; Teixeira et al. 2012). Even so, it should be noticed that usually the presence or absence of activity of certain substance is transversal to the different authors. Hence, some MICs values found for *S. aureus* are 10 to 10000 times lower in magnitude (Mahboubi & Haghi 2008; Hammer et al. 1999; Morris et al. 1979; Sonboli et al. 2006) than the ones obtained herein and others are 10 to 100 times higher (Fu et al. 2007; Aneja & Joshi 2010). The major deviations concern to the EOs and not their major constituents which is understandable since the proprerties and composition of the EOs change easily as fully explained before. Due to the phytophatogenic nature of *X. campestris*, reported values are not so often found since standard microorganims are normally used as test strains. However, as in the disc diffusion assay, is interesting to notice that the phytobacteria is more suceptible despite of being a Gram-negative.

Besides the lack of comparable results there is another limitation related with the aqueous solubility of the hydrophobic compounds tested, which has been suggested as an issue that limits the extent to which these compounds can accumulate to lethal levels in cells, which can lead to erroneous results (Goñi et al. 2009; Hili et al. 1997).

Finally, it should be highlited that the absorvance results for the MIC assays (data not shown) were not completely reliable once they led mostly to false positives in especial for pulegone and eugenol. One possible explanation may be related with active substance preciptation even if wells‘ content was homogenized before each absorvance reading. Thereby, as proposed in Teixeira *et al*. (2012), bacterial counts are most trustful than absorvance or visual inspection to determine not only the dilution assay end-point but also MIC and MBC value, which was confirmed experimentally in this work.

4.3. Minimum inhibitory doses (MID) determination

MIC values obtained were used as start point for MID determination and, as described in Materials and Methods section, serial dilutions were made in order to assess the degree of susceptibility of *S. aureus* and *X. campestris*. The MID values obtained are listed in Table 10.

For both microorganisms the MIDs for eugenol and CB-EO are comparable to the MICs obtained (see Table 9) (p>0.05). On the other hand, the MIDs for pulegone and PR-EO are lower than the MICs presented in Table 9 (p<0.05). In the literature some authors (Fisher & Phillips 2008) report that MIDs are normally lower than MICs. However, it would depend on the definition adopted. Indeed, according to the detailed results presented in Appendix D, is possible to observe that in vapour diffusion there is growth inhibition at lower concentrations than in broth microdilution assay. Thereby, one can state that susceptibility in vapour phase seems to be higher than in liquid phase.

Table 10 - Minimum inhibitory doses (MID) results (n = 3).

Microorganism	***S. aureus* DSM 2569**	***X. campestris* LMG 568**
Substance	**MID**	**MID**
	mg cm^{-3} air/mM	**mg cm^{-3} air/mM**
Eugenol	2/12	0.5/3
Pulegone	2.5/16	2/13
CB-EO	0.5/-	0.5/-
PR-EO	1/-	2/-

Massic and molar concentrations are presented. When molar concentration is not valid the sign "-" is displayed.

Further, pulegone is a monoterpene and this type of compounds are known to have higher antimicrobial activity in vapour phase than in direct contact (Velázquez-Nuñez et al. 2013) The same would be true for PR-EO since it is mainly constituted by pulegone, explaining why pulegone and PR-EO exhibit MIDs not only lower than MICs but also lower than MBCs for both bacteria (p<0.05). Moreover, PR-EO and pulegone when dispersed in the solvent formed an instable dispersion. Thereby, when applied to broth in the microdilution assay, some molecules remained in the surface of the well content not allowing a full contact between the bacteria and the molecules, which could increase the MIC value obtained.

Moreover, the comparison of both species regarding CB-EO and eugenol shows that there are no statistical significant differences between CB-EO and eugenol intra and interspecies (p>0.05). Actually, it has been reported in the literature that the previously denoted differences in susceptibility among different bacteria types are reduced when in vapour diffusion assays (Bajpai et al. 2011; Lopez et al. 2005). Analysing these results this fact is confirmed for all the substances tested (p>0.05).

Further comparison with published results is problematic due to the divergences in protocols and due to the lack of scientific data related with phytobacteria.

The lower concentrations tested are, as referred above, detailed in Appendix D. According with the inhibition halos measured, a linearization of the data using the logarithmic concentration *versus* percentage of inhibition was attempted but, unluckily, the linearization was not able to adjust conveniently to additional data. Indeed, the susceptibility do not seem to vary linearly with the concentration of the compounds.

Additionally, there are some problems concerning MID determination. The first is the incubation temperature that can affect the inhibition once MID depends on the evaporation of the substances tested and correspondent rate of evaporation (Fisher & Phillips 2008; Goñi et al. 2009; Hili et al. 1997). Even so, as *S. aureus* incubated at 37 °C during 20h and *X. campestris* incubated for larger time (48h) and lower temperature (30 °C) in a sealed container, the temperature influence might be diminished. Other problem is loss of vapour due to media absorption (Fisher & Phillips 2008; Goñi et al. 2009). This issue could not be investigated with any of the assays performed.

Another barrier of this assay is the technique of diffusion applied herein. Despite being the most used, fast evaporation is convenient for qualitative analysis but cannot be used for an accurate quantitative comparison (Inouye 2003). Even so, this method allows a fast and not excessive laborious screening of vapour antimicrobial activity using easily accessible materials (Kloucek et al. 2012).

It would be interesting to access if the concentrations used are cidal or only bacteriostatic. Lopez et al. (2005) determine the cidal nature of the compounds by observing whether the inhibition remained after removal of the antimicrobial atmosphere. Nevertheless, with the protocol used herein such determination would not be possible once even with removal of the antimicrobial atmosphere, it is known that part of the substance would still be absorbed into the agar media and thus the antibacterial effect would persist (at least partially). Instead, the Petri dishes were maintained in incubation for 30 days to monitor possible changes, which were absence as growth inhibition was maintained across time.

4.4. Glass beads assay

Glass beads with a spherical shape covered with a thin layer of substrate were used as model to allow the simulation of a stored product (as for instance, maize). The treatment of the glass beads with EOs and their major compounds vapours at their MID concentration was reasonable. As can be seen in Table 11, all the substances completely inhibited the growing of *X. campestris*. Concerning *S. aureus,* only PR-EO did not fully inhibited the bacteria development. Despite that, growth was reduced about 1000 times which is statistically comparable to total absence of growth (p>0.05).

Moreover, both bacteria were inoculated with ≈10^5 CFU/ml and the control using H_2O revealed a value 10 times lower for *X. campestris*. Such phenomena can be related with the shorter incubation period of this microorganism that was 20h instead of the usual 48h. As stated in Materials and Methods section, this period of time was chosen once a longer one could compromise cells viability due to the lack of nutrients.

Table 11 - Estimated cell density in the beads resultant of cell counts (*n* = 3).

Microorganism	Sample	Concentration (mg/cm^3)	CFU/ml
***S. aureus* DSM 2569**	Eugenol	2	0
	Pulegone	2.5	0
	CB-EO	0.5	0
	PR-EO	1	$4x10^2$
	H_2O	-	$2x10^5$
***X. campestris* LMG 568**	Eugenol	0.5	0
	Pulegone	2	0
	CB-EO	0.5	0
	PR-EO	2	0
	H_2O	-	$1x10^4$
Abiotic	-	-	0

These results allowed to obtain a preliminary snapshot of the activity of the EOs in a different matrix which can be useful for future work in this area. Even though, *in vivo* studies using real food matrix are always required in order to validate this results and this type of treatment. Hence, with the aim of prepare *in vivo* studies, some preliminary work with maize was made. Unfortunately the attempt to sterilize of maize to be used as matrix failed. In Appendix F, the several efforts with different treatments are reported. Thereby, due to the lack of time no further advances could be made in this subject. After maize sterilization, the aim would be the inoculation of these grains instead of glass beads in order to represent a natural environment for bacteria growth.

Moreover, this assay allowed to determine that the substances tested seem to have cidal effect and not only static, which is in accordance with dilution assays where MBC was possible to be determine for both species.

Several *in vitro* studies have been reported to access the antimicrobial or antifungal action of EOs and their major components. However, *in vivo* studies or simulations comparable to the presented herein are less usual (Bajpai et al. 2011; Lopez et al. 2005). Lo Cantore research group (2009) was able to lower the *X. campestris* bacterial population of infected bean seeds from $2.6x10^6$ to $7.0x10^2$ CFU/ml using eugenol emulsions, which along with the present results, allows optimistic perspectives for future work in this area.

4.5. Global discussion

The antimicrobial activity of the substances tested revealed to be satisfactory. CB-EO seems to be the substance exerting better performance, reflected by activity against both Gram-positive and Gram-negative bacteria including *P. aeruginosa* in disc diffusion assay. Moreover, the MIC and MID values of CB-EO were the lowest for both *X. campestris* and *S.*

aureus. The main component of CB-EO, eugenol, had, as well, notorious activity. On the other hand, while PR-EO showed reasonable activity, its main constituent, pulegone, had the weakest performance among all the substances tested. Such results might be related with the fact that as eugenol and, consequently the respective EO (CB-EO), is less lipophilic and thus more hydrophilic than pulegone and PR-EO. Hence, eugenol would not be retained and accumulated in the external membrane of Gram-negative bacteria in the same extend as pulegone, which is a more lipophilic molecule. Besides, in liquid medium assays, higher hydrophilicity would allow a better dispersion in the solvent used and, thereby, a closer contact with the bacteria in the suspension.

The fact that whole EO shows better performance than the individual components suggests that synergism must occur. Regarding bacteria types, the phytobacteria seem to be more susceptible to the phytocompounds tested.

Furthermore, improvement of the antimicrobials activity and growth inhibition was notorious as the type of diffusion changed: agar<broth<vapour. Actually, when in solid medium, the contact between the antimicrobial substance and the inoculated media takes place on the surface, which simulates better the interaction with food products than the well microdilution approach (Lopez et al. 2005). Nevertheless, as *in* vivo assays are still in their infancy, there is no certain about the vehicle to be used in order to preserve stored food products properties at its best. However, there is a clear assumption that the antimicrobial substances must be added to these products directly to better performance rather than to the packaging (Lopez et al. 2005). Nonetheless, even if in fresh food a problem related with alteration of the organopletic characteristics may emerge, this does not seem to be a major problem considering products as maize or other type of grains. In addition, as a considerable degree of inhibition in vapour phase has been demonstrated, the creation of an antimicrobial atmosphere within the package using these substances seems to be an interesting alternative to other preservation methods, which deserves to be considered in future. Thereby, there is a clear need to perform vapour diffusion assays. Even so, this type assays only provide qualitative data whereas traditional direct contact tests in liquid medium allow quantitative results. Also, the broth dilution assays are the most standardized across the literature. Hence, this assays are the only ones allowing reliable comparison of results since even if results may vary in magnitude, their positive or negative antimicrobial activity is often coherent. Despite these facts, good correlations between the results of the several assays were found.

5. Conclusions

Essential oils have a primary role in plants´ protection. Such function points them as candidates to be used in future in biopreservation systems directed to minimal processed foods and non-perishable food products. Moreover, due to the low costs involved and due to the ease of obtaining, the implementation of this type of food processing in developing countries is of great interest. The high volatility and low water solubility of essential oils are, probably, the main barriers to implement these natural antimicrobials with satisfactory performance in food preservation systems, as dilution tests are always required to determine the range of action of the substances in study.

The antimicrobial activity of clove bud oil (Syzygium *aromaticum*), pennyroyal oil (*Mentha pulegium*) and of their major components, eugenol and pulegone, against standard and phytobacteria was assessed using different diffusion methods. The results showed that phytobacteria are extremely susceptible to these phytochemicals, which suggest that they may be effective in a biopreservation system. Nonetheless, the activity of the substances tested revealed to be satisfactory and exhibited good spectrum of action. Among the complex and individual substances tested, CB-EO was the substance presenting best performance.

Additionally, it was possible to confirm that when tested in vapour diffusion assays, some divergences between bacteria types may be diminished. The glass beads assay was of extremely importance, since showed worthy perspectives to the future work in this area.

5.1. Limitations and future work

Concerning the methodologies applied several limitation can be pointed out. Firstly, the lack of comparable results as a consequence of the absence of standard methods difficult the establishment of concentration ranges to be tested leading to laborious and time-consuming work concerning the determination of minimum inhibitory concentrations.

Furthermore, the hydrophobicity of the compounds tested lead to the utilization of DMSO as solvent, which can have an additive or antagonist effect to the antimicrobial activity when conjugated with these substances.

The greatest obstacle in the present work was the impossibility of sterilize the food matrix (maize) on time. Consequently *in vivo* assays could not be performed. Thus, the results descending from the simulation of stored products using the glass beads could not be confirmed with real food matrix.

In future work *in vivo* tests must be performed. Further, as there are substances with considerable lower activity in liquid medium than in vapour phase, the final application of this technology will determine the best assay to be executed in order to investigate antimicrobial susceptibility. Additionally, toxicity and safety studies must be taken in consideration. Also, the determination of the exact composition of the essential oils using analytical methods would also be interesting.

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Appendix

A. Overview of the composition of *S. aromaticum* and *M. pulegium*

Table A.1 - Composition of *S. aromaticum* essential oil.

Compound	Bud oil India (Srivastava et al. 2005)	Bud oil Madagascar (Srivastava et al. 2005)	(Aneja & Joshi 2010)	(Edris & Malone 2012)	Bud oil (Burt 2004)
(E)-Nerolidol	0.1	0.4	-	-	-
(E)-*α*-Bergamotene	1.3	-	-	-	-
allo-Aromadendrene	0.3	0.1	-	-	-
Anethole	t	-	-	-	-
Calamenene	0.1	0.1	0.10	-	-
Carvone	0.1	0.2	-	-	-
Caryophyllene	19.5[c]	7.2[b]	7.2[b]	5.15[a]-	-
Caryphyllene oxide	0.4	0.3	-	-	-
Chavicol	t	0.1	-	-	-
Eugenol	70	82.6	88.58	85.29	75-85
Germacrene D	0.1	-	-	-	-
Humulene epoxide II	0.1	t	-	-	-
iso-Eugenol-I	0.8	0.1	-	-	-
Linallol	0.1	t	-	-	-
Lynalyl acetate	t	0.1	-	-	-
Methyl salicylate	0.3	0.1	-	-	-
m-methyl acetophenone	t	0.1	-	-	-
n-Buthylbenzoate	1.3	-	-	-	-
Nerol	t	0.1	-	-	-
t-Cadinol	0.1	0.1	-	-	-
Vanillin	t	-	-	-	-
α-Cadinol	0.1	0.1	-	-	-
epi-α-Cadinol	-	0.1	-	-	-
α-Copaene	0.1	0.1	-	-	-
Copaene	-	-	-	-	-
α-Humulene	1.9	0.8	0.19	-	-
α-Selinene	0.2	0.3	-	-	-
Eugenyl acetate	2.1	6	5.62	6.91	15-25
γ-Cadinene	0.8	0.2	-	-	-
n-Octamne	-	0.1	-	-	-
α-Pinene	-	0.1	-	-	-
Cubenol	-	0.1	-	-	-
n-Heptadecane	-	0.1	-	-	-
(E)-*β*-Ocimene	-	t	-	-	-
Methyl benzoate	-	t	-	-	-

α-Cubene	-	t	-	-	-
Methyl eugenol	-	t	-	-	-
α-Ylangene	-	t	-	-	-
t-Muurolol	-	t	-	-	-
2-Heptanone	-	-	0.93	-	-
Ethyl hexanoate	-	-	0.66	-	-
Humulenol	-	-	0.27	-	-
Calacorene	-	-	0.11	-	-
Methylhydrazone 2-Propanone	-	-		-	-
Methyl-cyclopentane	-	-		-	-
Tetrahydro-3-methyl-furan	-	-		-	-
2-Buthyl-1-methyl Pyrrolidine	-	-		-	-
Tetrahydro-6,6-dimethyl 2H-Pyran-2-one	-	-		-	-

Compounds present in trace amounts are identified with "t". **Subtitle:** [a] α isomer; [b] β isomer; [c] configuration not specified.

Table A.2 - Composition of *M. pulegium* essential oil according with different authors.

Compounds	Composition (%)	
	(Franzios et al. 1997)	(Teixeira et al. 2012)
Pulegone	75.7	23.2
Menthone	10.1	35.9
Neo-menthol	-	9.2
8-Hydroxy-*δ*-4(5)-p-menthen-3-one	-	2.1
3-Menthene	-	3.6
trans-5-Methyl-2-(1-methylethenyl)-cyclohexanone	-	1.1
2-Cyclohexen-1-ol	-	1.1
2,6-Dimethoxytoluene	-	0.9
4-Hydroxy-*δ*-8-p-menthen-3-one	-	0.9
2-Amino-6-chloro-4-(2-ethenylamino) pyrimidine	-	0.1
1-Ethyl-3-methyl-2-(2-methylpropylidene) imidazolidine	-	0.8
3-Octanol	-	0.7
1-Methyl-3,5-dimethoxy-1H-pyrazole	-	0.6
3-(2-Oxocyclopentyl) propanal ethylene ketal	-	0.5
Caryophyllene oxide	-	0.5
Eucalyptol	-	0.5
β-(3-Thienyl)acrylic acid	-	0.4
(1RS,4SR)-8-hydroxy-p-menthan-3-one	-	0.4
Piperitone	-	0.5
Piperitenone	-	0.6
6-Diethyl-2-methoxypyrimidine	-	0.3
α-Pinene	-	0.2
3-Methyl-hexanedioic acid	-	0.2
Palustrol	-	0.2
Epimanoyl oxide	-	0.1
3-Octyl acetate	-	0.2
2-α-Pinene	-	0.2
1-Menthene	-	0.2
Mint furanone 1	-	0.2
Methyl eugenol	-	0.2
L-Limonene	-	0.2
Carene	-	0.2
ß-Bourbonene	-	0.1
Linalool	-	0.1
n-Cymene	-	0.1
Elemicin	-	0.1
Veridiflorol	-	0.1
3-Methyl-cyclopentanone	-	0.1
5-Methyl-3-heptanone	-	0.1
Muurolol	-	0.1
α-Cadinol	-	0.1
Mint furanone 2	-	0.1
(−)-Allo-spathulenol	-	0.1
2-tert-butyl-4-methylphenol	-	0.1
Hexadecanoic acid	-	0.1
Menthol	-	0.1
3-*p*-Menthanol	-	0.1
α-Terpineol	-	0.1
Berbenone	-	0.1
(+)-trans-Carveol	-	0.1
Camphene	-	t
Sabinene	-	t
α-Thujene	-	t

Compounds present in trace amounts are identified with “t”.

B. Survey from the methodologies in the literature

Table B.1 – Survey from the methodologies applied in the literature in a total of 49 papers.

Reference	Substances origin	Microrganisms tested	Minimum concentrations measured	Methods	Solvent	Growth indicator
Bajpai *et al.*, 2009	*Metasequoia glyptostroboides Miki ex Hu* (essential oil and extracts of hexane, chloroform, ethyl acetate and methanol)	*Pseudomonas aeruginosa* KCTC2004, *Staphylococcus aureus* ATCC6538, *S. aureus* KCTC1916	MIC	Disc diffusion	Hexane, chloroform, ethylacetate, methanol	-
				Broth microdilution	n.s.	
El-Baroty *et al.*, 2010	*Cinnamomum zeylanicum, Zingiber officinale* (essential oil)	*Bacillus subtilis* ATCC 6633, *B.s cereus* ATCC 14579, *S. aureus* ATCC 27840, *Micrococcus luteus* ATCC 4698, *Klebsiella pneumoniae* ATCC 13883, *Serratia marcescens* ATCC 13880	MIC	Disc diffusion	DMSO	-
				Direct TLC-bioautography	None	INT
Horváth *et al*., 2010	*Thymus vulgaris* L., *Lavandula angustifolia Mill., Eucalytus globulus Labill. Mentha spicata C. zeylanicum Presl.* (essential oils)	*P. syringae* pv. *phaseolicola* Burkholder) *Xanthomonas campestris* pv. *vesicatoria* Doidge, S.*s epidermidis, S. saprophyticus, S. aureus* HNCMB 112002, methicillin-resistant *S. aureus*	-	Direct TLC-bioautography	Ethanol	MTT
Park *et al*., 2010	*Chamaecyparis obtusa* (essential oil and fractions), Terpinen-4-ol (99% v/v)	*Klebseilla pneumoniae* KCTC 2241, *Listeria. monocytogenes* KCTC 3569, *Salmonella typhimurium* KCTC 12401, *S. aureus* KCCM 11764, *Escherichia coli* O157:H7 KCCM 40406, *Legionella pneumophila* ATCC 33216, methicillin-resistant *S. aureus* CCARM 3089	MIC	Disc diffusion	None	-
				Agar dilution	None	
Pavithra *et al*., 2009	*Pamburus missionis* (essential oil)	*B. subtilis* NCIM 2718, *S. aureus* ATCC 25923, *P. aurginosa* ATCC 27853, *K. pneumonia* ATCC 70063, *E. coli* ATCC 25922	MIC, MBC	Disc diffusion	None	-
				Broth microdilution	DMSO	
Sartoratto *et al.*, 2004	*Mentha piperita, M. spicata, T. vulgaris, Origanum vulgare, O. applii, Aloysia triphylla, Ocimum gratissimum, O. Basilicum* (essential oils)	*P. aeruginosa* ATCC13388, *Salmonella choleraesuis* CCT4296, *Rhodococcus equi* CCT0541, *Micrococcus luteus* CCT2692, *S. aureus* CCT2740, *S. epidermides* ATCC12228, *E. coli* CCT0547, *B. subtilis* Cohn CCT2576, *Enterococcus faecium* ATCC10541, *E. faecium* CCT5079, *Candida albicans* Berkhout ATCC 10231	MIC	Broth microdilution	Ethyl acetate	TTC
				Direct TLC-bioautography		

Wang *et al*., 2012	*Rosmarinus officinalis* L. (essential oil)	*B.subtilis, S. aureus, S. epidermidis, E. coli, P. aeruginosa*	MIC, MBC	Broth microdilution	Tween-80	-
				Time-kill dynamic curves	n.s.	
Zarai *et al*., 2012	*Ricinus communis* L. (essential oil)	*S. aureus* 1327*, S. epidermidis, Micrococcus luteus, E. faecalis, Enterobacter cloacae, S. aureus* 25923*, B. subtilis, B. cereus, P. aeruginosa 27853, Klebsiella pneumoniae* WHO24*, E. coli* 25922*, Botrytis cinerea, Fusarium solani, Penicillium digitatum, Aspergillus niger*	MIC	Well diffusion	Ethanol	-
				Broth microdilution	Ethanol	MTT
				Disc diffusion	Ethanol	-
Aneja and Joshi, 2010	*Syzygium aromaticum* (essential oil and extracts of acetone, methanol, ethanol, and cold and hot water)	*Streptococcus mutans* MTCC 497, *S. aureus* MTCC 740, *Lactobacillus acidophilus* MTCC 447, *C. albicans* MTCC 227, *Saccharomyces cerevisiae* MTCC 170	MIC	Well diffusion	DMSO + Tween-20	-
Hammer *et al*., 1999	*Aniba rosaeodora, Boswellia carterii, Cananga odorata, Commiphora myrrha, Cymbopogon citratus, Cymbopogon martinii, Cymbopogon nardus,Juniperus communis, Lavandula angustifolia*(Tasmanian)*, Macadamia integrifolia, Mentha x piperita,Oenothera biennis, Pimenta racemosa, Pogostemon patchouli, Prunus dulcis, Santalum album, S. aromaticum, Thymus vulgaris, Vetiveria zizanioides, Z. officinale* (essential oils)	*Acinetobacter baumanii* NCTC 7844, *Aeromonas veronii biogroup sobria* ATCC 9071, *C. albicans* ATCC 10231, *E. faecalis* NCTC 8213, *E. coli* NCTC 10418, *K. pneumoniae* NCTC 11228, *P. aeruginosa* NCTC 10662, *S. enterica* subsp. *enterica* serotype *typhimurium* ATCC 13311, *Serratia marcescens* NCTC 1377, *S. aureus* NCTC 6571	MIC	Agar dilution	Tween-20	-
				Broth microdilution	Tween-20	
Lee *et al*., 2009	*S. aromaticum* (essential oil)	*E. coli* ATCC 25922, *Citrobacter freundii* ATCC 8090, *Aeromonas hydrophila* ATCC 49140, *P. aeruginosa* ATCC 35032, *Streptococcus agalactiae* ATCC13813, *Edwardsiella tarda* ATCC 15947, *Yersinia enterocolitica* ATCC 23715	MIC	Broth microdilution	Methanol	-
Mahboubi and Haghi, 2008	*Mentha pulegium* (essential oil)	*S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *B. cereus* ATCC 1247, *L. monocytogenes* ATCC 7644, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Vibrio cholera* Inaba, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231	MIC, MBC	Disc diffusion	DMSO	-
				Broth microdilution	DMSO	
Hayouni *et al*., 2008	*Salvia officinalis* L., *Schinus molle* L. (essential oils)	*S. aureus* ATCC 6539 and ATCC 25923, *P. aeruginosa* ATCC 27853 and ATCC 9027, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *C. albicans* ATCC 10239, plus *L. monocytogenes Pseudomonas morgani, Klebsiella pneumoni, S. enteritidis* (2 strains), *Salmonella anatum*, *E. coli* (2 strains) obtained from the Laboratory of Bacteriology at Habib Thameur Hospital	MIC	Disc diffusion	None	-
				Broth microdilution	DMSO	

Joshi *et al*., 2010	*Neolitsea pallens, Lindera pulcherrima, Dodecadenia grandiflora, Persea duthiei, Persea odoratissima, Persea gamblei, Phoebe lanceolata* (essential oils)	*E. c*oli MTCC 443, *S. enterica enterica* MTCC 3223, *Pasturella multocida* MTCC 1148, *S. aureus* MTCC 737	MIC	Disc diffusion	DMSO	-
				Broth dilution	DMSO	
Laciar *et al*., 2009	*Artemisia echegarayi* (essential oil)	*L. monocytes* CLIP 74903 and CLIP 74904, *B. cereus, S. aureus, E. coli, S. enterica serovar enteritidis, S. enterica serovar Typhimurium, Proteus mirabilis*	MIC	Disc diffusion	Dried	-
				Broth microdilution	DMSO	TTC
				Direct TLC-bioautography	n-hexane + ethyl acetate	
O'Bryan *et al*., 2008	*Citrus sensis* Valencia (extracts and essential oil)	Salmonella strains	-	Disc diffusion	None	-
				Broth dilution	Agar	TTC
Çetin *et al*., 2009	*Artemisia incana* L. (essential oil)	*S. aureus* KCTC1916, *B. subtilis* ATCC6633, *S. aureus* ATCC6538, *P. aeruginosa* KCTC2004, *E. coli* 0157:H7 ATCC43888, *E. coli* ATCC8739, *E. coli* O/51 Human, *E. aerogenes* KCTC2190, *S. typhimurium* KCTC2515, *S. enteritidis* KCTC2021	MIC	Disc diffusion	None	-
				Agar dilution	Tween-20	
				Broth microdilution	DMSO	
Ahmadi *et al.*, 2010	*Hymenocrater longiflorus* Benth (essential oil and extract of methanol)	*E. feacalis* ATCC 29122, *S. aureus* ATCC 11522, *K. pneumonia* ATCC 13183, *P. aeruginosa* ATCC 27853, *Shigella flexneri* 2a (LS3), *Salmonella thphimurium* ATCC 19430, *E. coli* ATCC 11522	MIC	Disc diffusion	n-Hexane	-
				Broth microdilution	DMSO	
Lopes-Lutz *et al*., 2008	*A. absinthium* L., *A. biennis* Willd*., A. cana* Pursh, *A. dracunculus* L*., A. frigida* Willd*., A. longifolia* Nutt., *A. ludoviciana* Nutt (essential oils)	*C. albicans* Serotype B ATCC 36802, *Cryptococcus neoformans* T1-444 Serotype A, *A. niger, Trichophyton rubrum* T544, *Microsporum canis, M. gypseum, E. coli, S. epidermidis, S. aureus* MRSA BMB9393	-	Disc diffusion	DMSO	-
Mulyaningsih *et al.*, 2010	*Kadsura longipedunculata* (essential oil)	*B. subtilis* ATCC 6051, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 14990, *S. saprophyticus* ATCC 15305, *S. pyogenes* ATCC 12344, *S. agalactiae* ATCC 27956, methicillinresistant *S. aureus* NCTC 10442, *E. faecalis* ATCC 29212, vancomycin-resistant *E. faecalis* ATCC 51299, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 90028, *C. glabrata* ATCC MYA 2950, C. *parasilopsis* ATCC 22019	MIC, MBC	Well diffusion	None	-
				Broth microdilution	DMSO	
Dung *et al*., 2008	*Cleistocalyx operculatus* (essential oil)	*B. subtilis* ATCC6633, *P. aeruginosa* KCTC2004, *S. aureus* ATCC6538, *S. aureus* KCTC1916, *L. monocytogenes* ATCC19166, *Enterobacter aerogenes* KCTC2190, *S. typhymurium* KCTC, *S. enteritidis* KCCM 12021, *E. coli* ATCC8739, *E. coli* O157:H7 human, *E. coli* O157:H7 ATCC43888; *S. aureus* KCTC1621, *S. epidermidis* KCTC1917, *E. coli* KCTC1039; *C. albicans* KCTC7965, methicillin-resistant *S. aureus* (P227, P254, P249) , vancomycin-resistant *E. faeccium* (A93, B2332, U914) , *Acinetobacter baumannii* 05KA007, *A. baumannii* 05 KA31, *E. coli* 02K 276, *E. coli* 04K717, *E. cloacae* 04K453, *E. cloacae* 04K908, *Klebsialla pneumoniae* 05K279, *K. pneumoniae* 05K406, *P.*	MIC, MBC	Disc diffusion	DMSO	-
				Broth dilution	DMSO	
				SEM	n.a.	n.a.

		aeruginosa 03K442, *P. aeruginosa* 03K711, *Serratia marcescens* 03K188 and *S. mar cescens* 03K201, *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853, E. *coli* ATCC25922				
Nedorostova *et al*., 2009	*Allium sativum* L.*, Achillea millefolium* L.*, Armoracia rusticana* Mey. & Scherb., *A. absinthium* L.*, A. dracunculus* L., *A. vulgaris* L., *Calamintha nepeta* Savi, *Caryopteris x clandonensis* Hort., *Foeniculum vulgare* Mill., *Hypericum perforatum* L., *Hyssopus officinalis* L., *Lavandula angustifolia* Mill., *Mentha x piperita* L., *Mentha x villosa* Huds., *Nepeta grandiflora* Benth., *Nepeta x faassenii* Bergmans, *O. basilicum* var. Grant verte L., *O. basilicum* var. purple L., *O. majorana* L., *O. vulgare* L., *Perovskia atriplicifolia* Benth., *Ruta montana* Mill., *Salvia officinalis* L., *Satureja montana* L., *T. pulegioides* L., *T. serpyllum* L., *T. vulgaris* L. (essential oils)	*L. monocytogenes* ATCC 7644, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enteritidis* ATCC 13076	MIC	Vapour diffusion	None	-
Goñi *et al.*, 2009	*C. zeylanicum, S. aromaticum,* mixture of *C. zeylanicum* and *S. aromaticum* (essential oils)	*E. coli* ATCC 29252, *Y. enterocolitica* CECT 4315, *S. choleraesuis* CECT 4000, *P. aeruginosa* ATCC 27853, *B. cereus* CECT 495, *L. monocytogenes* ATCC 7644, *E. faecalis* ATCC 29212, S. *aureus* ATCC 29213	MIC	Disc diffusion Vapour diffusion	None Ethyl ether	-
Kloucek *et al.*, 2012	*Armoracia rusticana, Allium sativum* L.,*C. aromaticum Nees, Cymbopogon citratus* Stapf , *C. flexosus* Nees ex Steud. Will. Watson, *Eugenia caryophyllata* Thunb., *Ocimum citriodorum* Vis., *Origanum compactum* Benth., *O. vulgare* L., *P. dioica* L. Merr., *Pimenta racemosa* Mill. J.W.Moore, *Thymus satureoides* Coss. & Balansa, *T. serpyllum* L., *T. vulgaris* L., *Abies siberica* Lindl., *Acorus calamus* L., *Amyris balsamifera* L., *Anthemis nobilis* L., *A. absinthium* L., *Betula lenta* L., *Cannabis sativa* L., *Citrus aurantium* L., *C. aurantium* L., *Citrus bergamia* Risso, *Citrus limonum* L. Burm.f., *Coriandrum sativum* L., *Daucus carota* L., *Erigeron canadensis* L., *Eucalyptus globulus* Labill., *Juniperus communis* L., *J. communis* L., *J. virginiana* L., *Laurus nobilis* L., *Lavandula angustifolia* Mill., *L. latifolia* Medik., *Melaleuca alternifolia* Cheel, *M. quinquenervia* Cav. S.T.Blake, *Mentha arvensis* L., *M. citrata* Ehrh., *M. pulegium* L., *M. spicata* L., *Nepeta cataria* L., *Ocimum basilicum* L., O. *basilicum* L., *Origanum majorana* L., *O. majorana* L., *Pelargonium graveolens* L'Hér., *P. roseum* Willd., *P. roseum* Willd., *Pogostemon cablin* Bemth., *Ravensara aromatica* Sonn.,	*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *S. enteritidis* ATCC 13076, *Alternaria alternata* 8326, *A. niger* ATCC 6275, *Penicillium digitatum* F-382	MIC	Disc diffusion	Ethyl acetate	-

	Rosmarinus officinalis L., *R. officinalis* L., *Salvia officinalis* L., *S. sclarea* L., *Santalum album* L., *Styrax bentan* L., *Tagetes bipinata* L., *Tanacetum annum* L., *T. vulgare* L., *Tarachonanthus camphoratus L. Thuja occidentalis* L., *Thymus mastichina* L., *Tsuga canadensis* Carrière, *Zigiber cassumunar* Roxb., *Z. officinale* Roscoe (essential oils)					
Adiguzel *et al*., 2009	*Nepeta cataria* (essential oil and extract of methanol)	*Bacillus megaterium* A59,*Burkholdria cepacia* A225, *Clavibacter michiganensis* A227, *Enterobacter cloacae* A135, *K. pneumoniae* A137, *P. syringae* pv. tomato A35, *X. campestris* A235, *Acinetobacter baumanii* A8, *B. subtilis* ATCC 6633, *B. subtilis* A57, *Brucella abortus* A77, *B. macerans* A199, *E. faecalis* ATCC 29122, *E. coli* A1, *Proteus vulgaris* A161, *P. vulgaris* KUKEM 1329, *P. aeruginosa* ATCC 9027, *P.s aeruginosa* ATCC 27859, *S. enteritidis* ATCC 13076, *S. aureus* A215, *S. aureus* ATCC 29213, *S. epidermis*A233, *S. pyogenes* ATCC 176, *S. pyogenes* KUKEM 676, *C. albicans* A117, *A. alternate, A. flavus, Fusarium acuminatu, F. oxysporum, F. solani, Moniliania fructicola, Penicillum spp., Rhizopus spp., Rhizoctonia solani, Sclorotinia minor, Sclorotinia sclerotiorum, Trichophyton rubrum, T. mentagrophytes*	MIC	Disc diffusion	Methanol	-
				Broth microdilution	DMSO	
Laouer *et al*., 2009	*Carum montanum* (essentil oil)	*S. aureus* subsp. *aureus* ATCC 6538, *S. epidermitis* CIP 10464, *S. saprophyticus* subsp. *saprophyticus* CIP 10464, *S. simulans* CIP 81.64, *S. lugdunensis* CIP 103584, *E. faecalis* 14C1104, *P. aeruginosa* 13C3104, *E. coli* ATCC 9738, *Candida tropicalis* ATCC 66029	-	Disc diffusion	DMSO	-
Betoni *et al*., 2006	*A. sativum, Baccharis trimera, Cymbopogon citratus, Mikania glomerata, Psidium guajava, S. aromaticum, Zingiber oddicinale, M. peperita* (extracts of methanol)	32 *S. aureus* strains	MIC	Agar dilution	-	-
Magina *et al*., 2009	*Eugenia brasiliensis, E. beaurepaireana, E. umbelliflora* (essential oils)	*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922	MIC, MBC	Broth microdilution	DMSO	-
Shahat *et al*., 2008	*Enterolobium contortisiliquum* (essential oil)	*B. subtilis, B. cereus, S. aureus, M. luteus, K. pneumoniae, Serratia marcescencs*	MIC	Disc diffusion	DMSO	-
Bouhdid *et al*., 2009	*O. compactum* (essential oil)	*P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213	MIC	Broth microdilution	Agar	Resazurin
				TEM	Agar	Resazurin

Busatta *et al*., 2008	*O. majorana* L. (essential oil)	*Aeromonas* sp., *B. subtilis, E. faecalis, E. coli, K. pneumoniae, Salmonela choleraensius, Serratia* sp., *Shigella flexneri, S. aureus, S. mutans*	MIC	Disc diffusion	None	-
				Broth dilution	None	
Oyedeji *et al.*, 2009	*Callistemon citrinus, C. viminalis* (essential oils)	*B. cereus* ATCC 10702, *B. pumilus* ATCC 14884, *S. aureus* ATCC 3983, *S. aureus* ATCC 6538, *S. faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, *E. coli* ATCC 4983, *K. pneumoniae* ATCC 2983, *Proteus vulgaris* ATCC 6830, *P. vulgaris* CSIR 0030, *P. aeruginosa* ATCC19582, *S. marcescens* ATCC 9986	MIC	Disc diffusion	DMSO	-
				Broth microdilution	DMSO	INT
Ennajar *et al.*, 2009	*Juniperus phoenicea L.* (essential oil and extracts of methanol, ethanol, dichloromethane and ethyl acetate)	*B. subtillis* ATCC 6633, *S. aureus* CIP7625, *L.monocytogenes* Scott A 724, *P. aeruginosa* CIPA22, *E. coli* ATCC10536, *K. pneumoniae* CIP8291, *S. cerevisiae* ATCC 4226 A, *Mucor ramamnianus* ATCC 9314, *Aspergillus westerdijkiae*	-	Well diffusion	Tween-80	-
Mkaddem *et al*., 2009	*Mentha longifolia* L., *M. viridis* (essentila oils)	*S. aureus* CIP7625, *L. monocytogenes* Scott A 724, *E. coli* ATCC10536, *K. pneumoniae* CIP8291, *S. cerevisiae* ATCC 4226 A, *C. albicans* IPA 200, *M. ramamnianus* ATCC 9314, *A. ochraceus* NRRL 3174	-	Well diffusion	None	-
Wang and Liu, 2010	*Litsea cubeba* (essential oil and fractions)	*B. subtilis* ATCC 6501, *E. faecalis* ATCC 15753, *E. coli* ATCC 25922, *Monilia albicans* ATCC 64548, *P. aeruginosa* ATCC27852, *S. aureus* ATCC 25923	MIC	Disc diffusion	DMSO (essential oil), Tween-80 (fractions)	-
				Broth microdilution	DMSO (essential oil), Tween-80 (fractions)	
Ebrahimabadi *et al*., 2010	*Salvia eremophila* (essential oil and extract of methanol)	*P. aeruginosa* ATCC 27853, *E. coli* ATCC 10536, *B. subtilis* ATCC 6633, *S. aureus* ATCC 29737, *K. pneumoniae* ATCC 10031, *S. epidermidis* ATCC 12228, *Shigella dysenteriae* PTCC 1188, *P. vulgaris* PTCC 1182, *Salmonella paratyphi* A serotype ATCC 5702, *C. albicans* ATCC 10231, *A. niger* ATCC 16404	MIC	Disc diffusion	DMSO	-
				Broth microdilution	DMSO	
Joshi *et al*., 2008	*Hedychium aurantiacum, H. ellipticum, H. coronarium, H. spicatum* (essential oils)	*Pasteurella multocida* MTCC 1148, *E. coli* MTCC 443, *S. enterica enterica* MTCC 3223, *Shigella flexneri* MTCC 1457, S. *aureus* MTCC 737	MIC	Disc diffusion	None	-
				Broth dilution	None	
Maggi *et al*., 2009	*Ferula glauca* L. (essential oil)	*S. aureus* ATCC 25923, *B. subtilis* ATCC6633, *E. faecalis* ATCC 29212, *E. coli* ATCC 13706, *C. albicans* ATCC 14053, *S. mutans* DSM 20523	MIC	Broth microdilution	Acetone	-
Havlik *et al*., 2009	*Rhaponticum carthamoides* (essential oil)	*E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *S. pyogenes* ATCC 19615, *C. albicans* ATCC 10231	MIC	Broth microdilution	Tween-80 + agar	-

Fatope *et al.*, 2008	*O. basilicum* L., *O. forskolei* Benth (essential oils)	*E. coli* ATCC 9637, *K. pneumoniae* ATCC 10031, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *S. choleraesuis* ATCC 14028, clinical isolates *B. subtilis*, *C. albicans* ATCC 10231	-	Well diffusion	None	-
Ennajar *et al.*, 2010	*Juniperus phoenicea* L. (essential oil)	*B. subtilis* ATCC 6633, *B. cereus* ATCC 14579, *S. aureus* CIP 7625, *L. monocytogenes* CIP 82110, *E. coli* ATCC 10536, *P. aeruginosa* CIP A22, *K. pneumoniae* CIP 8291, *S. cerevisiae* ATCC 4226A, *C. albicans* IPA 200, *A. ochraceus* NRRL 3174, *A. carbonarius* NRRL 3174, *Mucor ramamnianus* ATCC 9314	-	Well diffusion	None	-
Cosge *et al.*, 2009	*Origanum acutidens* (extracts of hot water)	*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S typhimurium* ATCC 14028, *S. marcescens* ATCC 8100, *P. vulgaris* ATCC 13315, *E. cloacae* ATCC 23355, *K. pneumoniae* ATCC13883, *S. pyogenes* ATCC 19615, *S. aureus* ATTC 25923, *S. epidermidis* ATCC 12228	-	Disc diffusion	Water	-
Saeed *et al.*, 2013	*S. aromaticum* (extracts of water and methanol)	Spoiled bread samples	MIC	Disc diffusion	Water, methanol	-
				Well diffusion	none	
Bourgou *et al.*, 2011	*Citrus aurantium, C. sinensis, C. limon, C. reticulate*(essential oils)	*S. aureus* ATCC 25923, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853	-	Disc diffusion	none	-
Chaieb *et al.*, 2007	*S. aromaticum* L. (extracts)	*Campylobacter jejuni, S. enteritidis, E. coli and S. aureus*	-	Disc diffusion	n.s.	n.s
Teixeira *et al.*, 2012	*M. pulegium* (essentail oil and extracts of water and ethanol)	*Brochothrix thermosphacta* CECT847, *E. coli* ATCC25922, *Listeria innocua* CECT910, *L. monocytogenes* CECT5873, *Pseudomonas putida* CECT7005, *S. typhimurium* ATCC14028, *Shewanella putrefaciens* CECT5346	MIC	Disc diffusion	DMSO, ethanol, water	-
				Broth microdilution	DMSO, ethanol, water	-
López *et. al.* 2005	*Cinnamon zeylanicum, S. aromaticum, O. basilicum, Rosmarinus officinalis, Anethum graVeolens* and *Z. officinalis* (essential oils)	*S. aureus* ATCC 29213, *B. cereus* CECT 495, *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 7644, *E. coli* ATCC 29252, *Y. enterocolitica* CECT 4315, *Salmonella choleraesuis* CECT 4000, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 64550, *Penicillium islandicum* CECT 2762NT, *Aspergillus flavus* CECT 2687	-	Disc diffusion	none	-
				Vapour diffusion	ethyl ether	
Fu *et al.,* 2007	Clove and rosemary (essential oils)	*S. epidermidis* ATCC 12228, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *P. vulgaris* ATCC 49132, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 8739, *A. niger* ATCC 16404, *C. albicans* ATCC 10231	MIC, MBC	Broth microdilution	Tween-80	-
			-	Time-kill dynamic curves	-	
Inouye *et al.*, 2003	*Cinnamomum verum, Citrus junos, Lavandula stoechas, L. angustifolia, Cymbopogen citratus, Perilla fructescens var. crispa, Tea tree oil, Melaleuca alternifolia* and *Thymus serpyllum* (essential oils)	*Trichophyton mentagrophytes* TIMM 1189, *Aspergillus fumigatus* IFM41392, *S. pyogenes* ATCC 12344, *S. aureus* ATCC 6538P, *E. coli* ATCC 11775	-	Vapour diffusion	-	-

C. Calibration curves for *S. aureus* and *X. campestris*

C.1. Method

A calibration curves (OD *versus* CFU/ml) for both *S. aureus* and *X. campestris* was made. A suspension with OD of approximately 0.9 at 610 nm was prepared in MHB. This initial suspension was posteriorly diluted in MHB in order to achieve OD of about 0.7, 0.5, 0.3 and 0.1. Then, each of suspensions was submitted to series dilution in 9 ml of saline solution until 10^{-8}. This dilutions were spread onto solid media (MHA) and the cell counting was made after an incubation period of 20 h for *S. aureus* and 48h for *X. campestris*. Thus, the number of CFU/ml in for each OD was determined.

C.2. Results

Below the calibration curves obtained by linear regression can be seen.

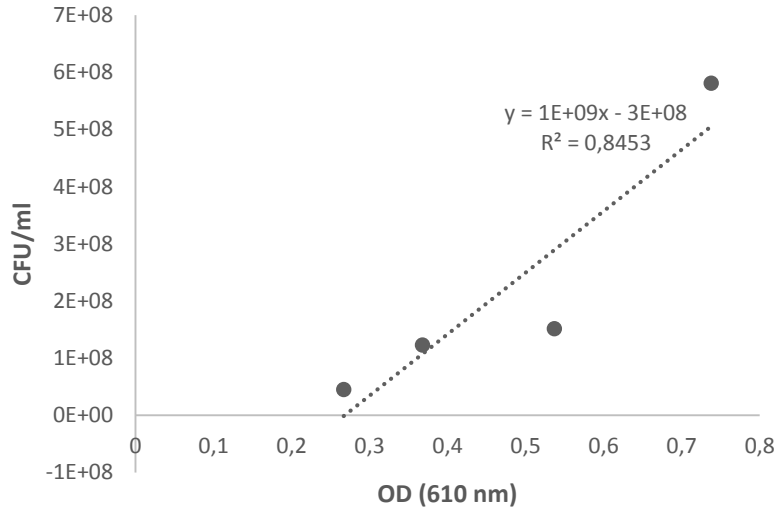

Figure C.1 - *S. aureus* calibration curve.

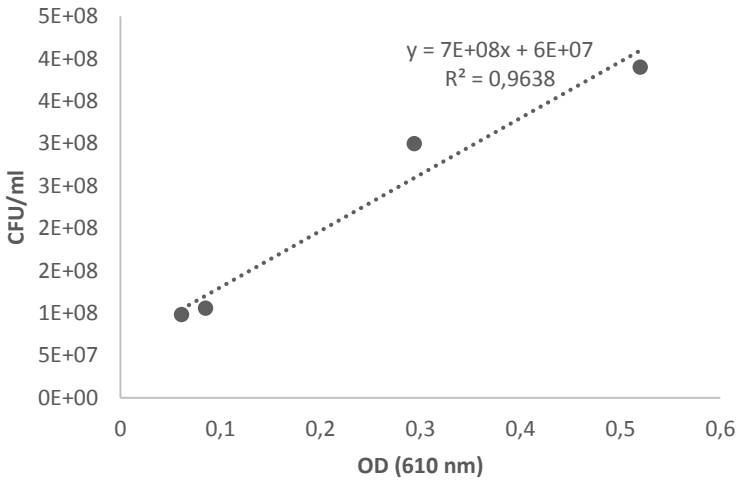

Figure C.2 - *X. campestris* calibration curve.

D. Minimum inhibitory doses (MID) determination

D.1. Concentration calculation

The diameter of the Petri dish (55 mm) and height (15 mm) was known and therefore theoretical volume was calculated (36 cm^3). Even so, filling the Petri dish with water revealed that it can only bear around 30 cm^3. Also, 55 mm diameter corresponds to the Petri dish lid and the inner part is slightly smaller, thus 30 cm^3 was the value considered as total volume. Thus, as the Petri dish is filled with 2+7 ml of MHA, the remaining empty volume is 21 cm^3. Hence, the solutions were prepared taking in consideration that once the 300 µl impregnate the filter paper a kind of dilution is made, in which the final volume is 21 cm^3.

D.2. Detailed results

Inhibition percentage was calculated considering the Petri diameter (55 mm) which is fully covered of cells in the controls *versus* the inhibition diameters measured (mm) in each triplicate as in the equation bellow, followed by average calculation:

$$\%\ Inhibition = 100 - \frac{\pi d_{Petri\ dish} - \pi d_{Inhibition}}{\pi d_{Petri\ dish}} \qquad \text{Equation (1)}$$

Where, $d_{Petri\ dish}$ is the Petri dish radius and $d_{Inhibition}$ is the inhibition diameter.

Figures D.1 and D.2 summarize the results obtained regarding *S. aureus* and *X. campestris*, respectively.

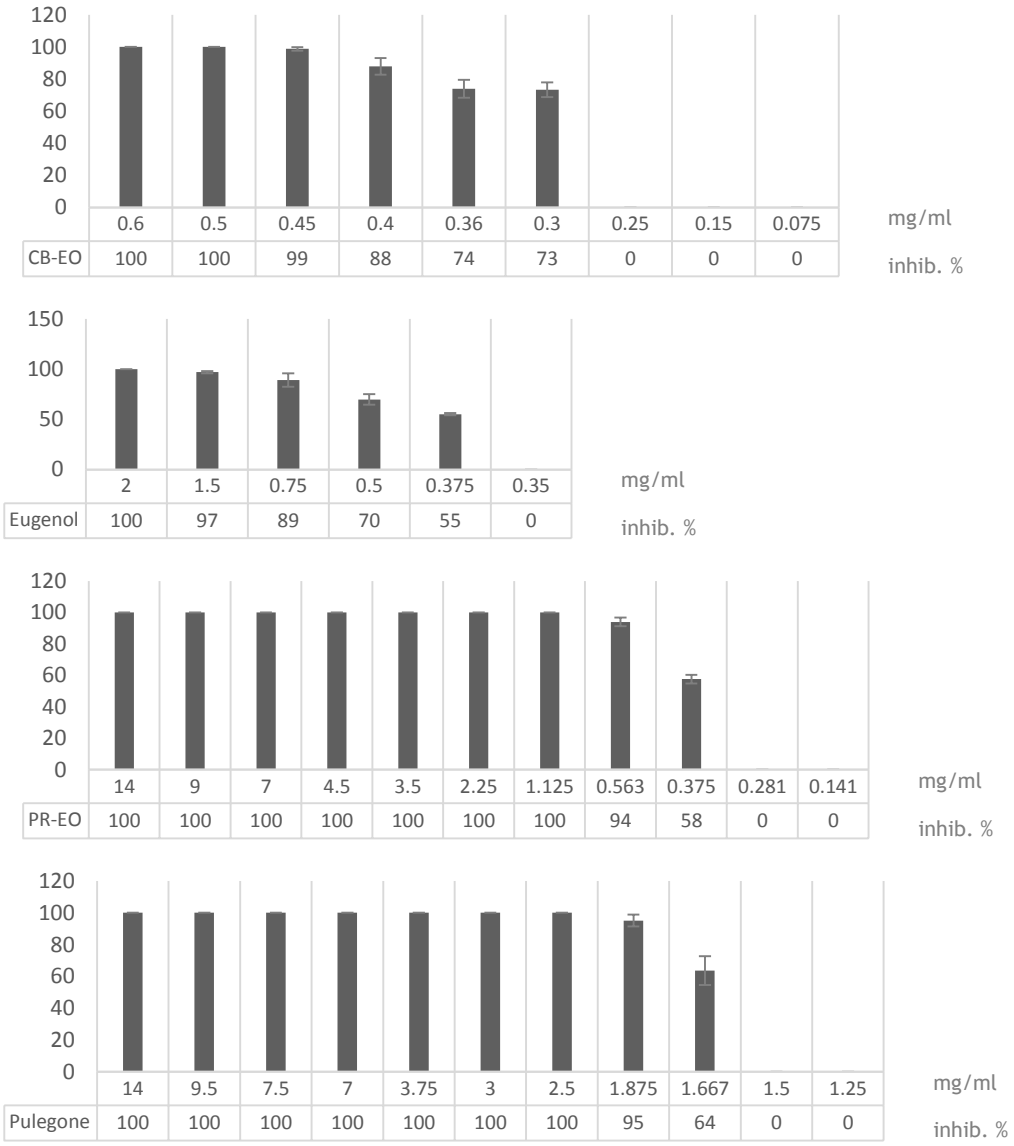

Figure D.1 - *S. aureus* detailed results of inhibition zone percentage in vapour phase assay: Clove Bud oil (CB-EO), Pennyroyal oil (PR-EO), eugenol and pulegone (*n* =3).

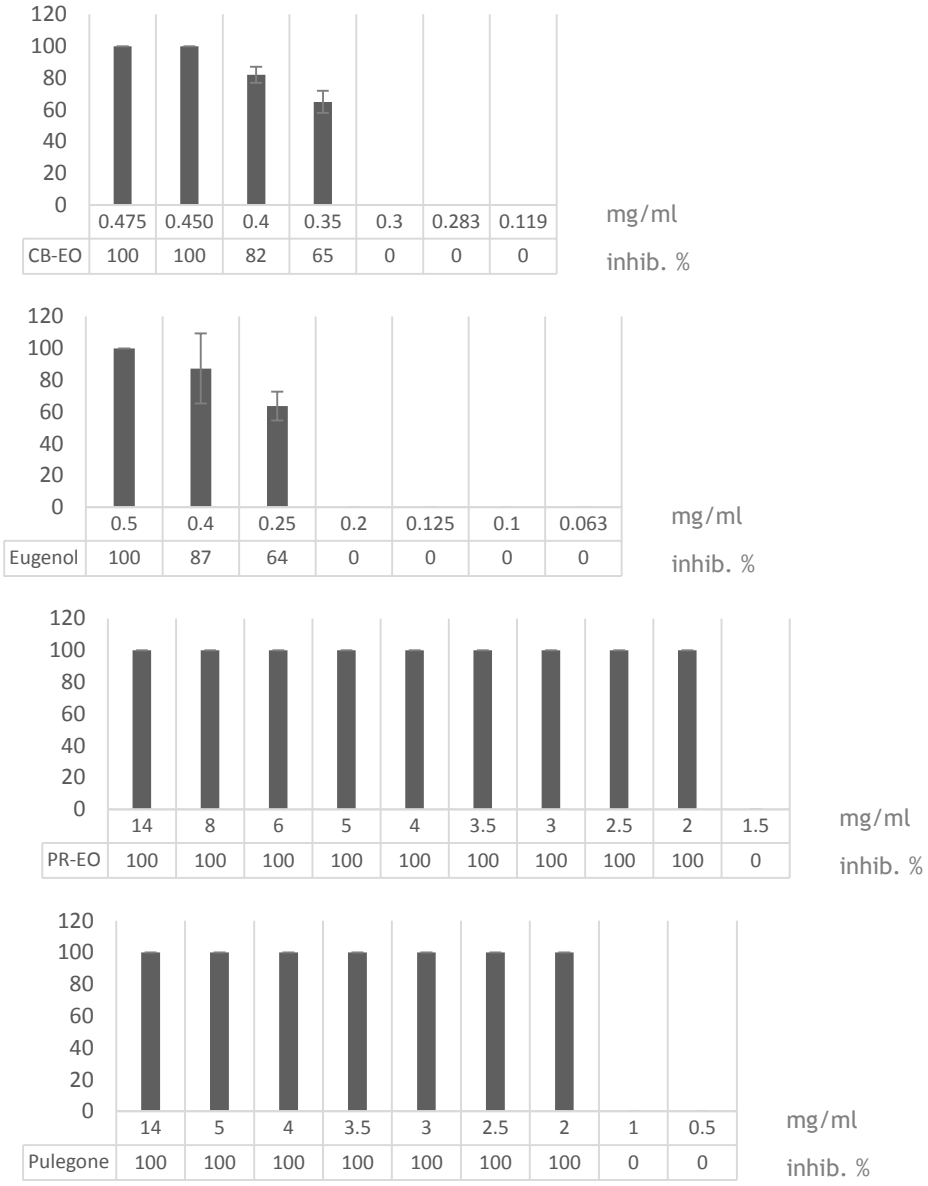

Figure D.2 - *X. campestris* detailed results of of inhibition zone percentage in vapour phase assay: Clove Bud oil (CB-EO), Pennyroyal oil (PR-EO), eugenol and pulegone (*n* =3).

F. Maize sterilization results

F.1. Bleach treatment

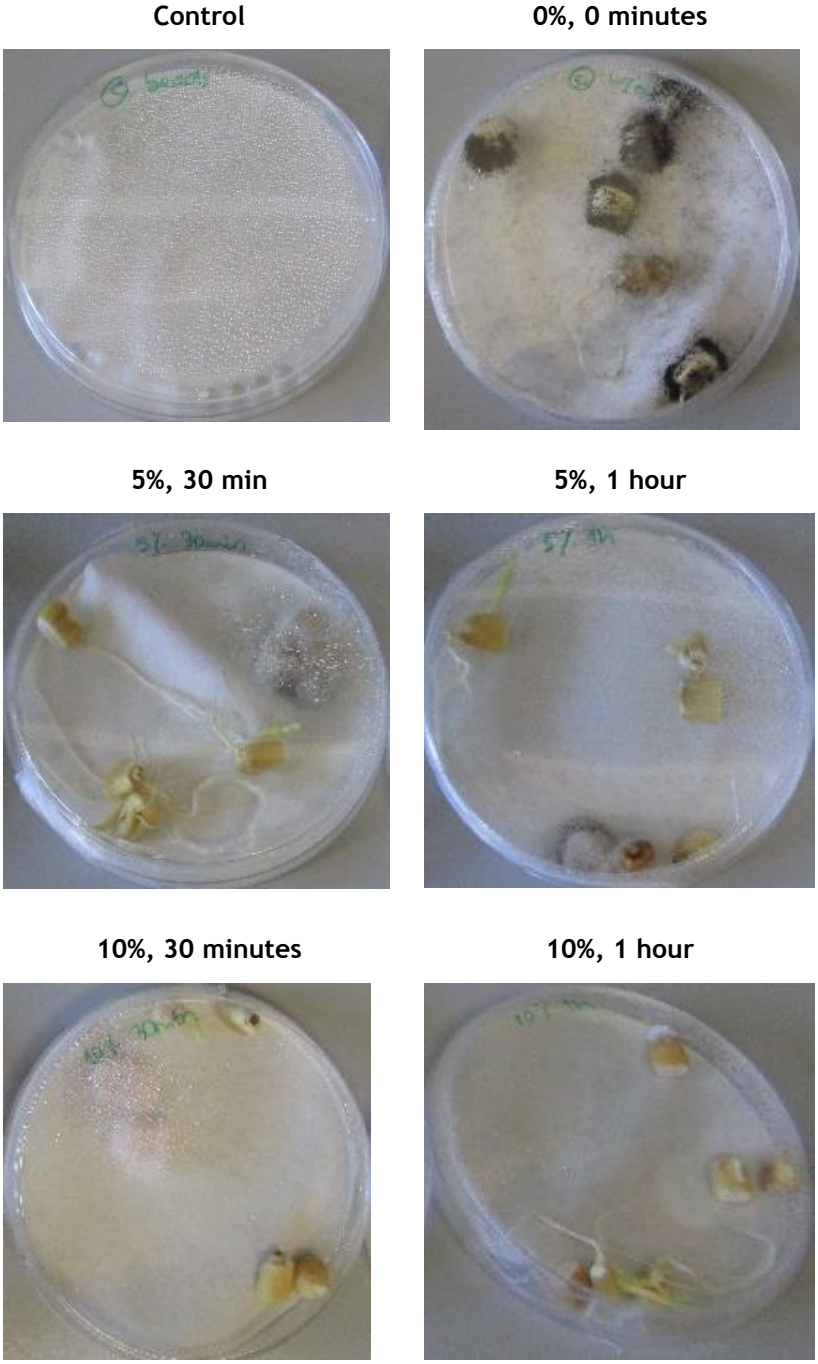

Figure F.9 - Appearance of beads control solution and maize treated with diluted bleach after 1 week (168 h) incubation at 30 °C. Only one plate per sample is shown (*n* = 3).

F.2. UV treatment

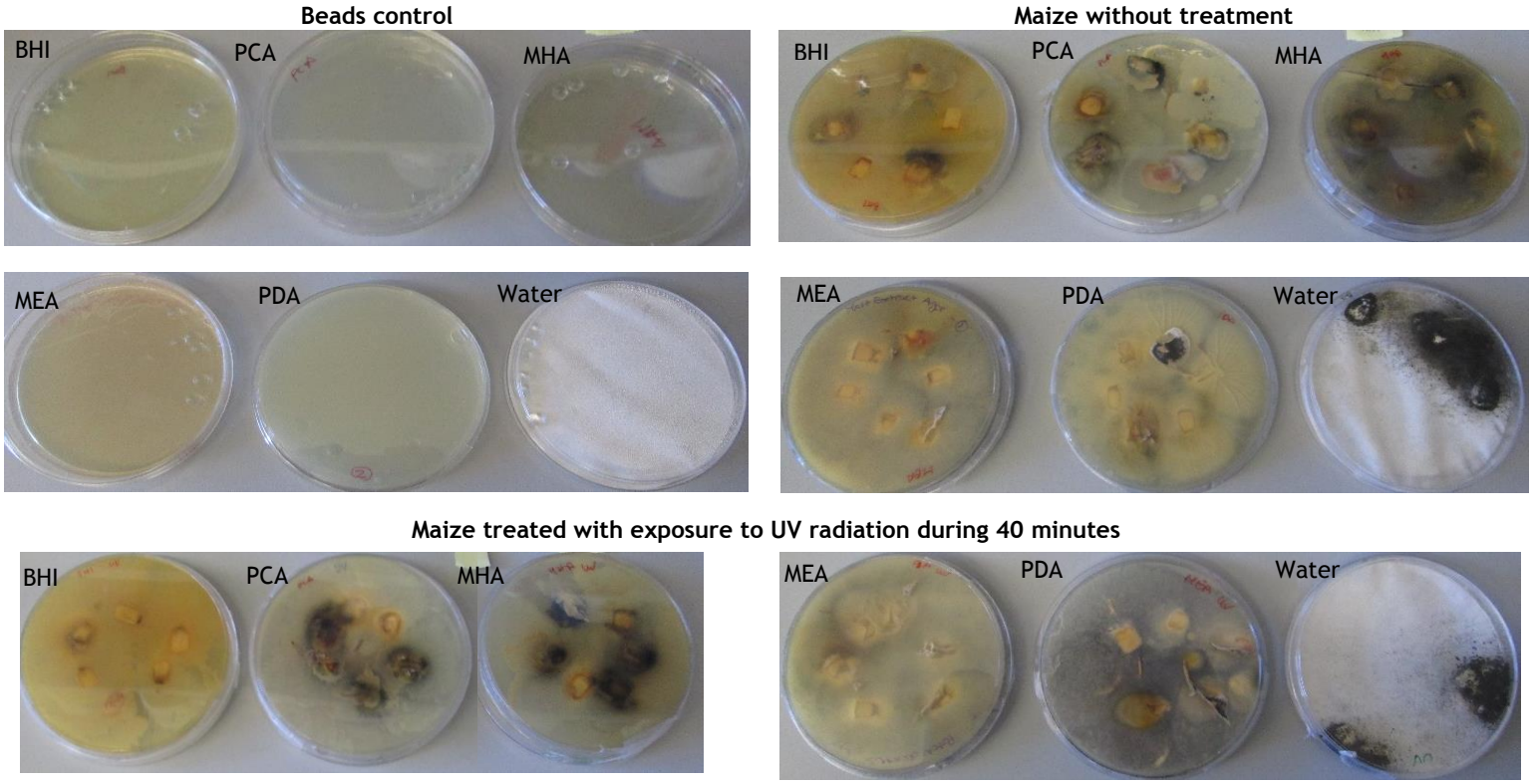

Figure F.2 - Appearance of beads control and maize treated with UV cycle after 1 week (168 h) incubation at 30 °C. Only one plate per sample is shown (*n* = 3).

F.3. Fungicides treatment

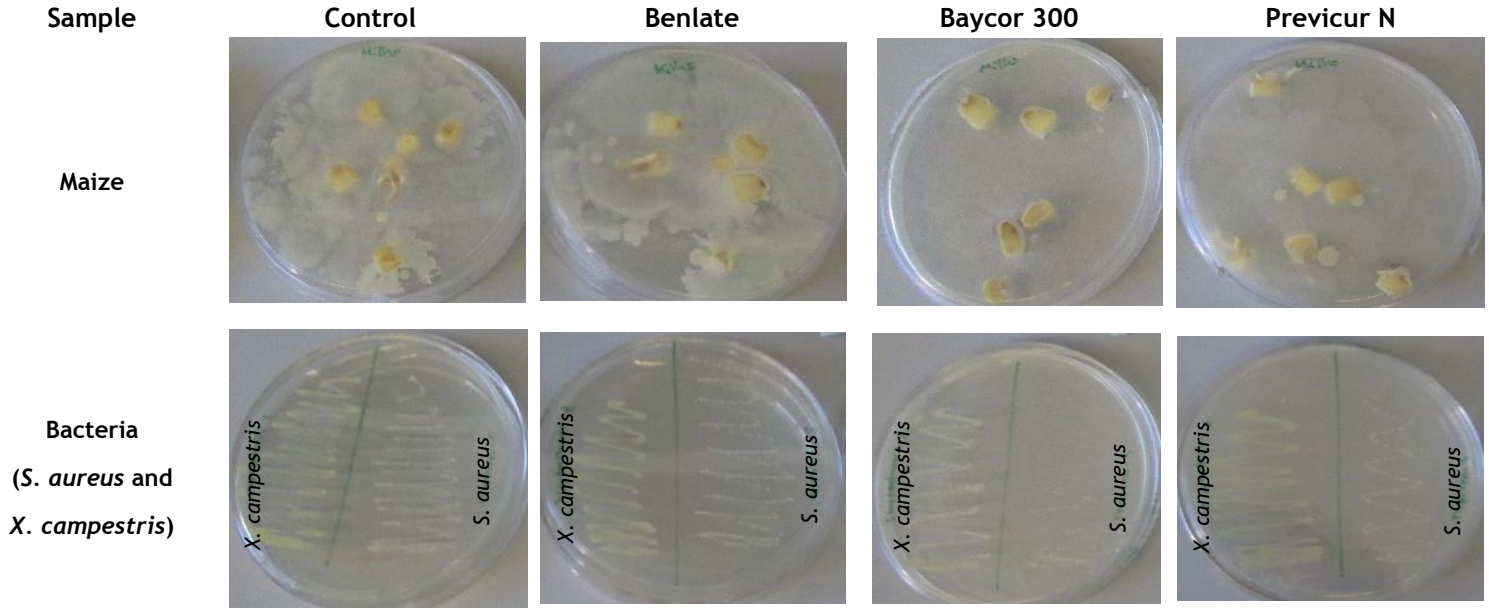

Figure F.3 - Appearance of maize and bacteria seeded in medium with different fungicides after 48h incubation at 30 °C. Only one plate per sample is shown (*n* = 3).