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„Antimicrobial Properties of Eugenol Containing Essential Oils. An Update. “

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Abstract

For a long time essential oils have been investigated for their antimicrobial properties. Essential oils with high content of eugenol, like *Cinnamomum verum, Syzygium aromaticum, Laurus nobilis, Pimenta dioica* and *Ocimum basilicum* were shown to possess good antimicrobial properties in dependence of eugenol amount. Many studies reported on their strong inhibitory action against human pathogens and food spoilage microbes, implicating them as potential candidates for usage in food industry, cosmetic industry and pharmaceutical industry. This work gives an overview of the reports about antimicrobial properties of eugenol containing essential oils, published in the years of 2014 and 2016.

Zusammenfassung

Ätherische Öle werden schon seit langem für ihre antimikrobiellen Eigenschaften untersucht. Bei Pflanzen mit hohem Gehalt an Eugenol, wie *Cinnamomum verum, Syzygium aromaticum, Laurus nobilis, Pimenta dioica* und *Ocimum basilicum* wurde gezeigt, dass sie gute antimikrobielle Eigenschaften besitzen, in Abhängigkeit von der Eugenolkonzentration. Viele Studien berichten über ihre starke inhibitorische Wirkung gegen humanpathogene und den Verderb von Lebensmittel verursachenden Keimen, auch im Hinblick als potenzielle Kandidaten für den Einsatz in der Lebensmittelindustrie, der Kosmetikindustrie und Pharmaindustrie. Diese Arbeit gibt einen Überblick über die wissenschaftliche Publikationen zum Thema, über die antimikrobiellen Eigenschaften von Eugenol mit ätherischen Ölen, die in den Jahren 2014 und 2016 publiziert wurden.

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1. Introduction

In the last decade we have witnessed a dramatic increase in the proportion and absolute number of bacterial and fungal pathogens resistant to multiple antimicrobial agents. Multidrug-resistant microbes are currently considered as an emergent global disease and a major public health problem (Roca *et al*., 2015). In recent years, exists due to an increased antibiotic resistance of many microorganisms, an increased interest for antimicrobial substances of natural origins. The antimicrobial properties of essential oils have been known for many centuries. In the last few years, a large number of essential oils and their constituents have been investigated for their antimicrobial properties. Essential oils of spices and herbs like cinnamon, clove (Tarek *et al*., 2014), basil (Saharkhiz *et al*., 2014), allspice (Dharmadasa *et al*., 2015) and laurel (Safi *et al*., 2014) were found to possess the strongest antimicrobial properties among many tested. This paper gives an overview on the antimicrobial properties of containing essential oils, with eugenol as one of their main constituents, and the possibility of using them as a natural alternative to synthetic drugs.

1.1. Definition of essential oils

One can find various definitions of essential oils in the literature. Some definitions state that, essential or volatile oils are aromatic, lipophilic substances that give the plant its characteristic scent and thus are among most important molecules inside the plant. Essential oils are uniquely made up of around hundred different terpenes and phenyl propanes and some alkanes. These terpenes enable the plant to make unique biological activies, such as medical properties of that essential oil. Essential oils are extracted by different methods: cold

pressing, steam distillation, or some other more delicate methods (Hobbs, 1998). However, it is a bit naïve to think of essential oils as of the “soul of the plant”. Chemical composition of the plants that grow in their natural environment and chemical composition of the distilled essential oils are far from same. Only rare expressed oils, that have not met the fruit juice and that have been protected from aerial oxidation can be considered a true plant essential oil (Bașer and Buchbauer, 2010). To these days, around 3000 essential oils are known, of which about 300 are being comercially used in many different industries like food, cosmetic industy, pharmacy and medicine (National Association for Holistic Aromatherapy, 2015).

1.2. A brief history of essential oils

It was thought for a long time that essential oils were first used 3000 years ago in antient China, Egypt and India. But 1975 Paolo Rovesti found a distillation aparatus and perfume containers that were perfectly perserved and which were indicating that essential oils have been used for more than 5000 years of human history. In modern human history, Arabic perfumes were very famous across the 13th century Europe. In the 16th century Europe, essential oils were known by the the name „chymical oils“ and they could be bought from the apothecary. After the invention of the printing machine, there were many publications about many different herbals, as well as illustrations of tools that are used for extraction of essential oils. During the Renaissance, interest in essential oils was increased in the field of cosmetics, as well as in the field of medicine, as a protection from various epidemies. Essential oils fom cedar, cinnamon, frankincense, juniper, rose, rosemary, lavender and sage, but also essences like artemisia, cajeput, chervil, orange flower, valerian and pine were analysed and recorded (Lawless, 2001).

In 1883., French scientist M.J. Dumas was the first to investigate chemical components of essential oils. During the period from 1884-1914, Wallach wrote many articles about essential oils and later wrote a book *Terpene und Campher*. In 1910, he got a Nobel Prize for Chemistry “in recognition of his outstanding research in organic chemistry and especially in the field of alicyclic compounds”. In 1905, A von Baeyer got a Nobel Prize “in recognition of his contributions to

the development of Organic Chemistry and Industrial Chemistry, by his work on organic dyes and hydroaromatic compounds". In 1899., F.W. Semmler and G. Wagner investigated acyclic monoterpenes like geraniol, linalool and citral. F. Šorm et al. did infrared investigations in 1950 and suggested that caryophyllene has a 4- and 9-membered ring. W. Treibs isolated crystalline caryophyllene epoxide from the autoxidation products of clove oil in 1952. 1969, D. H. R. Barton confirmed Šorm's suggestions and got a Nobel Prize in Chemistry. 1956, J. Read, W. Huckel, H. Schmidt, W. Treibs, and V. Prelog figured out structures of menthols, carvomenthols, borneols, fenchols, and pinocampheols and the related ketones (Başer and Buchbauer, 2010). From then on, various researches are being done.

2.The main components of the essential oils

It is generally accepted that chemical constituents of essential oils depend on different factors, such as geographical origin, stage of development of the plant (Şenkal *et al*., 2014), climate and ecological conditions, analysis method (Said Al-Ahl *et al*., 2015), genetic factors, drying method, extraction method, harvest time, from which part of the plant the oil was extracted (Bajalan *et al*., 2015) and others. In their Handbook of Essential Oils : Science, Technology and Applications, Başer and Buchbauer (2010) said: "A number of important agronomic factors have to be considered before embarking on the production of essential oils, such as climate, soil type, influence of drought and water stress and stresses caused by insects and microorganisms, propagation (seed or clones), and cultivation practices. Other important factors include precise knowledge on which part of the biomass is to be used, location of the oil cells within the plant, timing of harvest, method of harvesting, storage, and preparation of the biomass prior to essential oil extraction". It is very important to take this into a considertion. Sometimes, experimental results of biological action of essential oils of the same species are different, suggesting that the chemical components of those essential oils cannot be the same. Generally, there are large varieties in the chemical constitution of essential oils, which will be shown in the following chapter.

Lauraceae:

Cinnamonum verum oil

In many publications cinnamaldehyde is declared as the main constituent of *Cinnamomum verum* essential oil and eugenol is either present in traces, or not present at all (Aminizare *et al.*, 2014; Mith *et al*., 2014; Evrendilek *et al*., 2015; Sienkiewicz *et al*., 2014; Perricone *et al*., 2015; Paparella *et al*., 2015). However, some publications report higher content of eugenol in this essential oil, in some cases reaching even over 80% (Oliveira *et al.*, 2014; Azeredo *et al.*, 2014).

Cinnamomum verum Blume leaves (Oliveira *et al.*, 2014):

Eugenol	**82.30 %**
Caryophyllene	3.33%
Benzyl benzoate	2.92%
Eugenyl acetate	2.01%
Linalool	1.92%
Cinnamaldehyde	1.04%

Cinnamomum verum Blume bark (Azeredo *et al.*, 2014):

Cinnamaldehyde	81.52%
Eugenol	**16.68 %**
Caryophyllene	1.19%
Cinnamyl acetate	0.01%
α-Humulene	0.12%

Cinnamomum cassia oil

Cinnamomum casia Blume bark (Tarek *et al.*, 2014):

trans-caryophyllene	17.18%
Eugenol	**14.67%**
Linalool	14.52%
trans-cinnamyl acetate	13.85%
Cymol	11.79%
Cinnamaldhyde	11.25%

Cinnamomum sintoc oil

Cinnamomum sintoc Blume bark (Sumiwi *et al.*, 2014):

Eugenol	**38.38%**
Myristicin	14.52%
Safrole	10.17%
Benzyl benzoate	4.66%
α-Terpineol	4.40%
Terpinen-4-ol	4.26%

Methyleugenol	4.14%

Cinnamomum zeylanicum oil

Cinnamomum zeylanicum Blume leaves (Santos *et al*., 2016):

Eugenol	**79.24%**
Benzyl benzoate	3.42%
trans-caryophyllene	3.20%
Linalool	2.42%
Eugenol acetate	2.02%
trans-Cinnamaldehyde	1.41%
α-Humulene	1.33%
p-Cymene	1.16%
Safrole	1.11%
α-Pinene	1.01%

Cinnamomum tamala oil

Cinnamomum tamala leaves, Himalaya (Heer *et al.*, 2016):

Eugenol	**52.54%**
Cinnamyl acetate	13.05%
Linalool	9.04%
α-Pinene	4.01%
Benzaldehyde	2.70%
Limonene	2.34%
β-Pinene	2.12%
Camphene	1.56%
Borneol	1.37%
Bornyl acetate	1.21%
α-Phellandrene	1.12%

Bay Leaf/ Laurel oil (*Laurus nobilis* L.)

Although in some earlier publications the eugenol content is very high- about 44% (Xu *et al*., 2013; Chudasama *et al*., 2012) , in many publications in the years of 2014 and 2015, eugenol is shown to be present in very low content, or not present et all (Shokoohinia *et al*., 2014).

Shokoohinia *et al*. (2014) investigated seasonal variations of volatile components of essential oils extracted from the leaves of *Laurus nobilis* in Iran, Isfahan and got the following results:

Laurus nobilis leaves, March (Shokoohinia *et al*., 2014):

1,8-Cineole	34.29%
δ-3-Carene	8.89%
α-Terpenyl acetate	6.14%
α-Pinene	5.81%
Sabinene	5.60%
Methyl eugenol	5.18%
γ-Terpinene	4.80%
Camphene	4.56%
Borneol	3.17%
α-Terpineol	2.92%
Eugenol	**2.88%**

Laurus nobilis leaves, June (Shokoohinia *et al*., 2014):

1,8-Cineole	40.25%
Camphor	7.80%
α-Terpenyl acetate	7.08%
α-Pinene	6.59%
Methyl eugenol	5.05%
Camphene	3.58%
Borneol	3.26%
δ-3-Carene	3.02%
Sabinene	2.33%
Eugenol	**2.25%**

Laurus nobilis leaves, September (Shokoohinia *et al*., 2014):

1,8-Cineole	37.32%
Camphene	10.22%
α-Terpenyl acetate	6.93%
Sabinene	5.87%
Methyl eugenol	4.28%
δ-3-Carene	3.97%
α-Pinene	3.00%
Borneol	2.94%
Eugenol	**2.74%**

Laurus nobilis leaves, December (Shokoohinia *et al*., 2014):

1,8-Cineole	30.80%
Sabinene	9.16%
γ-Terpinene	6.13%
α-Terpenyl acetate	5.85%
Camphor	5.69%
α-Pinene	5.31%
δ-3-Carene	4.59%

Methyl Eugenol	4.17%
ß-Pinene	2.87%
Camphene	2.56%
Borneol	2.12%
α-Terpineol	2.04%
Eugenol	**2.03%**

Myrtaceae:
Clove oil (*Syzygium aromaticum*)

According to Cortés-Rojas *et al*. (2014), the main component of clove essential oil is eugenol, attaining 89% of its chemical constituents. It also contains 5% to 15% of eugenol acetate and β-Caryophyllene. Another important compound found in clove essential oil is α-Humulene, about 2.1% (Cortés-Rojas *et al*., 2014).

Syzygium aromaticum, leaves (Baysal *et.al*., 2015):

Eugenol:	**67.03%**
β-Caryophyllene:	15.07%
α-Humulene:	13.00%

Syzygium aromaticum, buds (Razafimamonjison *et.al*., 2014):

Eugenol:	**82.36%**
β-Caryophyllene:	8.64%
α-Humulene:	1.04%

Syzygium aromaticum, stem (Razafimamonjison *et.al*., 2014):

Eugenol:	**96.65%**
β-Caryophyllene:	1.66%
α-Humulene:	0.22%

Syzygium aromaticum, buds (Khalilzadeh *et.al*., 2015):

Eugenol:	**54.86%**
β-Caryophyllene:	20.19%

Syzygium aromaticum (Sharma *et.al*., 2016):

Eugenol:	**75.41%**
β-Caryophyllene:	15.11%
α-Humulene:	3.78%
Caryophyllene oxyde:	1.13%

Syzygium aromaticum (Sameza *et.al*., 2015):

Eugenol:	**79.40%**
Eugenylacetate:	9.20%
Isocaryophyllene:	7.00%

Syzygium aromaticum flower buds (Ghoneem *et.al*., 2016):

Eugenol:	**81.60%**
Eugenol acetate:	9.00%
β-Caryophyllene oxyde:	1.70%
Nootkatone:	1.10%

Syzygium aromaticum buds (Xu *et.al*., 2016):

Eugenol:	**76.23%**
β-Caryophyllene:	11.54%
Caryophyllene oxyde:	4.29%
Eugenol acetate:	1.76%

Syzygium aromaticum dried buds (Xie *et.al*., 2015):

Eugenol:	**90.60%**
β-Caryophyllene:	9.40%

Syzygium aromaticum dried buds (Iwamatzu *et.al*., 2016):

Eugenol:	**89.80%**
β-Caryophyllene:	10.20%

Allspice oil (*Pimenta Dioica* (L.)Merr):

According to many scientists, allspice essential oil has eugenol as a major constituent with 70-80% of the total volume, 1,8-cineole, humulene or caryophyllene with 6-7% volume and some other constituents in trace amounts (Dharmadasa *et al*., 2015).

Pimenta dioica, leaves (Dharmadasa *et al*., 2015):

Eugenol:	**85.33%**
β-Caryophyllene:	4.36%
1,8-Cineole:	4.19%

Pimenta dioica, berries (Misharina *et al*., 2015):

Eugenol:	**35.42%**
Methyleugenol	28.02%
β-Caryophyllene:	8.66%
1,8-Cineole:	5.62%

Pimenta pseudocaryophyllus oil:

Pimenta pseudocaryophyllus, leaves (Suzuki *et al*., 2014):

Eugenol:	**88.06%**

β-Caryophyllene:	4.08%
1,8-Cineole:	0.07%

Lamiaceae:

Basil oil:
There are many differences in the publications, when it comes to the percentage of eugenol in the essential oils of basil. Reports of eugenol content in the basil essential oil vary in the publications from about 40% to about only 1%. As it was mentioned before, eugenol content depends on the species of basil, on the part of the plant the oil was extracted from, and on the developmental stage of the plant from which the oil was extracted, as it is represented in this chapter (Imeri *et al*., 2015; Saharkhiz *et al*., 2014).

Ocimum basilicum oil:

Ocimum basilicum L wide leaves (Imeri *et al.*, 2015):

Linalool	45.30%
Eugenol	**42.06%**
trans- Caryophyllene	7.58%
Carvacrol	4.31%
Caryophyllene oxide	3.50%
Spathulenol	2.78%

Ocimum basilicum L narrow leaves (Imeri *et al.*, 2015):

Linalool	48.00%
Eugenol	**36.09%**
trans- Caryophyllene	6.66%
Carvacrol	4.36%
Caryophyllene oxide	3.02%
Oxalic acid	2.01%

Ocimum basilicum L flower (Imeri *et al.*, 2015):

Farnesene	14.94%
Elemol	11.29%
Carvacrol	9.00%
Spathulenol	5.78%
Caryophyllene oxide	5.14%
γ-Cadinene	3.36%
Thymol	2.93%
Eugenol	**1.34%**

Ocimum basilicum leaf, flowering stage, India (Saran *et al.*, 2016):

Eugenol	**35.37%**
Methyl chavicol	22.34%
Linalylformate	13.93%
Naphthalene	7.63%
Eucalyptol	2.79%
α-cubebene	2.61%
Valencene	1.59%
Methyl eugenol	1.11%

Ocimum sanctum oil:

Ocimum sanctum vegetative stage (Saharkhiz *et al*., 2014):

β-Bisabolene	20.99%
1,8-Cineole	20.78%
Eugenol	**15.70%**
Estragol	11.49%
γ-Elemene	10.47%

Ocimum sanctum floral budding (Saharkhiz *et al*., 2014):

Eugenol	**37.15%**
1,8-Cineole	19.41%
β-Bisabolene	13.29%
Estragol	10.61%
γ-Elemene	7.70%

Ocimum sanctum full flowering (Saharkhiz *et al*., 2014):

Eugenol	**24.63%**
1,8-Cineole	20.45%
β-Bisabolene	18.76%
Estragol	11.40%
γ-Elemene	7.80%

Ocimum sanctum aerial parts (Hussain et al., 2016):

Eugenol	**22.0%**
β- Elemene	19.2%
β-caryophyllene	19.1%
Germacrene D	5.03%

Ocimum sanctum leaves, July, Egupt (Elsherbiny et al., 2016):

Estragol	55.95%
1.8-Cineole	10.56%
Eugenol	**10.09%**
Linalool	5.57%
(Z,E)- α -Farnesene	4.45%

Ocimum urticifolium oil:

Ocimum urticifolium leaves (Alemayehu et al., 2016):

α-Pinene	22.105%
Eugenol	**22.09%**
α-Cubebene	11.341%
α-Bisabolene	9.945%
α-Caryophyllene	7.709%
α-Caryophyllene oxide	5.754%
Copaene	3.594%

Ocimum grattissimum oil:

Ocimum gratissimum leaves (Silva et al., 2016):

Eugenol	72.26%
1,8-Cineole	9.69%
β-Selinene	4.25%
β-Caryophyllene	3.80%
Bicyclogermacrene	2.56%
Valencene	1.87%
Germacrene-D	1.73%
cis-Ocimene	1.49%
Linalyl propionate	0.94%
3-Hexen-1-ol	0.53%
γ-Muurolene	0.46%
Linalool	0.42%

3. Test methods

Possible *in vitro* methods for the investigation of antimicrobial activity of essential oils are presented below:

Broth dilution method

This is one of the earliest methods used for antimicrobial susceptibility testing. For this test method, liquid nutrient medium is mixed up with the antibiotic in increasing concentrations in several test tubes, where upon a suspension with microorganisms is added, mixed up and incubated at 37°C for 18-24h. After the incubation, tubes are examined to see the possible microbial growth. The tube with the lowest antibiotic concentration and turbidity absence at the same time is defined as minimal inhibitory concentration (MIC) (Mith *et.al.,* 2015).

Agar well diffusion assay method

In this method, melted agar is mixed up with a suspension with microorganisms and poured into a Petri plate. After the solidification, wells are punched into agar, filled with the antimicrobial solution and incubated. Upon incubation at 37°C overnight, the plate is reversed, because the zone of inhibition is measured through the glass plate (Al Yousef, *et. al.*, 2014).

Disc diffusion method

Disc diffusion method is simple, practical and well standardized. In this method, a layer of solution containing microorganims is spread on the agar plate. Afterwards, up to 12 commercially prepared antibiotic discs with the fixed concentration are placed on the agar, the plate is covered and incubated overnight at 35°C. The zones of growth inhibition around each antibiotic disc are measured. The results of this method (susceptible, intermediate, resistant) are “qualitative,” in that a category of susceptibility is derived from the test rather than an MIC (Sharma *et.al.,* 2014).

Vapor phase diffusion method

In this method, agar is melted and poured into a Petri plate. After a solidification, layer of microbial solution is spread over the agar. A small filter paper is dipped into essential oil and placed on the inside surface of the Petri plate cover. Cover is placed on the Petri plate, the plate is inverted and incubated for approximately 24 hours. After the incubation, the clear inhibiton zone on the agar is measured (Al Yousef *et.al.*, 2014)

Thin layer chromatography (TLC) - Direct bioautography

In thin layer chromatography the TLC plate is developed and then plunged into a suspension of microorganisms which are growing in a nutrient broth. After an incubation time in a humid atmosphere, microorganisms will grow directly on the surface of the TLC plate, except on the spots of antimicrobials. The TLC plate is sprayed with a tetrazolium salt which is converted into a purple formazan by dehydrogenases of living microorganisms. Zones of inhibition will appear on the place of antimicrobials, in the form of creamy spots against the purple background (Choma, Jesionek *et al*., 2015).

4. Antimicrobial activities

a. Antibacterial activities

The eugenol-containing essential oils have been investigated and reviewed that they possess strong antibacterial properties (Sharma *et al*., 2014). Although is not clearly known whether eugenol, as the major or one of the main components of those essential oils, is responsible for their antibacterial effects, or the antibacterial activity arises from the synergism between all molecules, several publications have reported that eugenol shows good antibacterial properties also when tested alone (Yap *et al*., 2014). From many studies, it can be concluded that eugenol-containing essential oils are generally shown to be more effective against Gram-positive than Gram-negative bacteria (Sharma *et al*., 2014; Yap *et al*., 2014; Hamedo *et al.*, 2015; Sleha *et al*., 2014; Biasi-Garbin *et al*., 2015; Yadav *et al*., 2015).

b. Antibacterial activity against human pathogens

Gram-positive bacteria

Basil essential oil (floral budding stage, by hydroditillation) containing 37.2% of eugenol is *in vitro* very effective against standard and clinical isolates of *Staphylococcus aureus* (MIC=0.5µg/mL; MMC=8 µg/mL), *Enterococcus faecalis* (MIC=4 µg/mL; MMC=8 µg/mL) and *Bacillus cereus* (MIC=0.5 µg/mL; MMC=4 µg/mL) (Saharkhiz *et al*., 2014). *S. aureus* can, for example, cause acne, boils, impetigo, food poisoning and toxic shock syndrome; *E. faecalis* causes infections of human gastro-intestinal tract and *B. cereus* causes foodborne illnesses, like diarrhea (Hogg, 2005). Basil oil also showed moderate activities against *Streptococcus sobrinus* (MIC=1000 µg/mL) and mild activities against *Streptococcus sanguinis* (MIC=2000 µg/mL) (Freires *et al*., 2015). *S. sobrinus* causes mainly problems with caries, while *S. sanguinis* can cause subacute bacterial endocarditis (Ryan *et al*., 2004). Laurel oil with a very low content of eugenol (0.5%) showed very mild antibacterial action on Gram-positive *Bacillus subtilis*, *Listeria innocua* and CNS (IZ=0.01mm- 7.5mm) and a bit stronger effect on *S. aureus* (IZ=2.6mm-10.8mm). On the other hand, 67.3% eugenol-containing clove oil exerted moderate antibacterial action against above mentioned bacteria, with inhibition zone ranging from 1mm to 14.8mm (Evrendilek, 2015). For instance, *B. subtilis* can sometimes cause food poisoning (Ryan *et al*., 2004) and *L. innocua* causes very dangerous invasive disease listeriosis (Carvalheira *et al*., 2010). Clove essential oil also showed very high antibacterial effect on *S. sobrinus* (MIC=200 µg/mL ; MBC=800 µg/mL), *S.sanguinis* (MIC=400 µg/mL; MBC=800 µg/mL) and *S. mutans* (MIC=125-600 µg/mL; MBC=125-800 µg/mL); when antibacterial effects of eugenol as its major constituent are analyzed alone, it scored very high (MIC=100 µg/mL) and indicated its responsibility for antibacterial properties of clove (Freires *et al*., 2015). *S. mutans* can destroy heart valve tissues and cause atheromatous plaque (Nakano *et al*., 2006). Cinnamon oil with eugenol content in only trace amounts showed very good results when its antibacterial properties were tested against *L. monocytogenes*, with zones of inhibition varying from 8.5mm to 34.1mm (Mith *et al*., 2014). High antibacterial action of 88.6% eugenol-containing allspice essential oil was reported, when tested on *S. epidermidis* (MIC=1000 µg/mL)

and *Corynebacterium xerosis* (MIC=1000 µg/mL; MBC=1000 µg/mL) (Suzuki *et al*., 2014). *S. epidermidis* is usually not pathogenic, sometimes it can be a cause for infection, but mainly both *S. epidermidis* and *C. xerosis* cause bad perspiration odor (Suzuki *et al*., 2014).

Xu *et al*., investigated antibacterial properties of clove essential oil against *S. aureus.* Clove essential oil showed strong antibacterial activity against *S. aureus* ATCC 25923 at a minimum inhibitory concentration (MIC) of 0.625 mg/ml. It also exerted a strong effect on growth rate of survival. This study also revealed that clove essential oil does not damage bacteria only on the physical, but also at the molecular level (Xu *et al*., 2016).

Gram-negative bacteria

The essential oils of leaves of *Cinnamomum verum* and *Cinnamomum cassia* were shown to be effective against *Pseudomonas fluorescens* (IZ=8.5mm- 23.6mm) which causes skin, ear, eye diseases and infections in cancer patients (Gershman *et al*., 2008), *E. coli* (IZ=7.9mm- 28.1mm) which mainly causes urinary infections (Ryan *et al*., 2004) and *Salmonella typhimurium* (IZ=8.5mm- 34.1mm) which is a cause of gastroenteritis (McCormic *et al*., 1995) (Mith *et al*., 2014). Essential oil of leaves of *C. verum*, as well as *Laurus nobilis* have shown a good antibacterial activity against *Brucella melitensis*, which causes brucellosis, with MIC value of *C. verum*=3.13µg/mL and MIC value of *L. nobilis*=6.2513µg/mL (Safi and Al-Mariri, 2014). Tarek *et al.* (2014) investigated antibacterial activities of many essential oils and came to result that leaf cinnamon essential oil (14.7% of eugenol) was highly effective against *P. aeruginosa* (MIC=<1µg/mL) and bud clove essential oil (84.1% of eugenol) was highly effective against *Salmonella typhi* (MIC=<1µg/mL). *P. aeruginosa* is known to infect wounds in humans and *S*. *typhi* is a cause of typhoid fever (Hogg *et al*., 2005). Also, cinnamon bark oil possesses high efficiency against both clinical and environmental strains of *Acinetobacter baumannii*, with MIC values ranging from 0.5 µg/mL to 2.5 µg/mL (Sienkiewicz *et al*., 2014). *A. baumannii* causes bacteremia, pneumonia, meningitis, urinary tract infections and wound infections (Maragakis and Perl, 2008). *Mycoplasma hominis* is a Gram-negative bacterium that causes bacterial vaginosis, pelvic inflammatory disease and pyelonephritis (Sleha *et al*., 2014). Cinnamon bark essential oil was found to be the most effective against this bacteria and totally inhibits their growth

with MIC=MBC=500 µg/mL (Sleha *et al*., 2014). Basil essential oil showed very high antibacterial effect on *E.coli* (MIC=1 µg/mL; MMC=2 µg/mL) and *Shigella flexneri* (MIC=0.25 µg/mL; MMC=0.5 µg/mL) (Saharkhiz *et al*., 2014).

c. Antibacterial activity against oral microbes

Khoudhi *et al*. (2015) investigated the potential use of natural products for the treatment of dental diseases and reported clove oil to be highly bactericidal, due to its main constituent eugenol. Also, it was reported that, due to other active constituents of clove oil (biflorin, kaempferol, rhamno-citrin, myricetin, gallic acid, ellagic acid and oleanoic acid), it is antibacterial against Gram-negative oral pathogens like Porphyromonas *gingivalis* and *Prevotella intermedia*. Clove oil again proved its antibacterial properties against oral microbata, when tested against *Streptococcus mutans*. With 67.5% of eugenol, clove oil inhibited growth of *S. mutans* from concentrations of 1000 up to 125 µg/mL (Rodrìgues *et al*., 2014).

5. Antifungal activities

Eugenol containing essential oils have shown very strong antifungal activities against many species, including *Aspergillus flavus*, *Aspergillus ochraceus*, *Eurotium amstelodami*, *Penicillium corylophilum* and *Rhizopus nigricans* (Mishra *et al*., 2015; Li *et al*., 2014; Hua *et al*., 2014), as shown in the following chapter.

a. Antifungal activity against molds

R. nigricans is commonly known as bread mold and is often found on spoiled bread and other spoiled food. It contains allergenic proteins and 31 allergens, which can cause problems in respiratory tract (Sridhara *et al*., 1990). Cinnamon oil inhibits growth of *R. nigricans* in a dose dependent manner. At MIC=0.1µg/mL of cinnamon oil, no *R. nigricans* growth was recorded, it was totally inhibited (Li *et al*., 2014). At MIC=500 µg/mL of cinnamon oil, inhibition of growth of *A. ochraceus* was permanent, without visible growth occurred. *A. ochraceus* is one of the most common producer of ochratoxin A, which contaminates food and drink products (Hua *et al*., 2014). Against *E. amstelodami*, *E. herbariorum*, *Elymus repens*, *E. rubrum*, *A. flavus*, *A.*

niger and *P. corylophilum* cinnamon leaf and bay leaf showed moderate antifungal activity at pH 6 and at different water activity (aw) levels (aw 80– aw 90), while clove oil exerted very high antifungal activities, especially when mixed with chemical preservatives (Mishra *et al*., 2015). When mixed with acetic acid, clove oil inhibited mycelial growth of *P. oxalicum* at the rate of 57.8%; when mixed with benzoic acid, clove oil inhibited mycelial growth of *P. oxalicum* at the rate of 73.7% and the growth of *A. flavus* at the rate of 26.3%; when mixed with citric acid it inhibited the growth of *P. oxalicum* at the rate of 10.5% and the growth of *A. flavus* at the rate of 31.0% and when mixed with lactic acid, clove oil inhibited the growth of *P. oxalicum* at the rate of 5.2%, while the inhibition of the *A. flavus* growth was not recorded. These results indicate that synergetic activity between clove essential oil and chemical preservatives improved antifungal activity of this essential oil (Mishra *et al*., 2015).

Sharma et al., investigated antimicrobial activities of various essential oils against *Fusarium oxysporum* f. sp. lycopersici 1322., a mold that causes diseases in plants and impedes agricultural production. Clove (Syzygium aromaticum), lemongrass (Cymbopogon citratus), mint (Mentha 3 piperita) and eucalyptus (Eucalyptus globulus) essential oils were evaluated against this mold. Even all the investigated essential oils were shown to be effective against *F. oxysporum,* clove essential oil was the most effective one. It completely inhibited mycelial growth and spore germination of *F. oxysporum* at 125 ppm with IC50 value of 18.2 and 0.3 ppm, respectively (Sharma *et al*., 2016).

b. Antifungal activity against yeasts

Tarek *et al*. (2014) reports high antifungal activity of cinnamon and clove oil against *C. albicans,* both with minimum inhibitory concentrations of <1µg/mL. Clove oil had 84.07% of eugenol as its major constituent, while cinnamon oil had 17.18% of trans-caryophyllene, followed by eugenol with 14.67% as major component (Tarek *et al*., 2014). As reported by the MedlinePlus (2015), *C. albicans* is a common cause of vaginal candidiasis.

6. Mechanisms of antimicrobial activity

According to some scientists, around 3000 essential oils are known so far and around 300 of them are widely used in food, pharmaceutical, cosmetic, perfume, agronomic and sanitary industries. A variety of

essential oils have been screened in order to spot their antimicrobial activity (Akhtar *et al*., 2014). Up to the present day, it is not clearly known how essential oils act against microbes, because each of them has a complex composition. It is supposed that antimicrobial activity comes from the complex interaction of not only one main compound but between many different components, which can act indifferent, additive, antagonistic or synergic (Reyes- Jurado *et al*., 2014).

a. Mechanisms of the antibacterial activities

There are variety of mechanisms by which essential oils act against bacteria, considering the diversity of chemical compounds present in essential oils. In general, thanks to the fact that essential oils are typical lipophils, their major target are bacterial cell walls and cell membranes. When they damage cell wall and membrane, it leads to leakage of macromolecules and lysis (Reyes- Jurado *et al*., 2014).

Jadav *et al.* (2015) investigated the effects of eugenol, as the main component of clove essential oil, against MRSA and MSSA cells. Results revealed that MIC values of eugenol for *S. aureus* ranged between 0.01% and 0.04%, while MBC value was approximately the double of the MIC. At low concentrations, decrease of biofilm formation depended on the dose of eugenol, while on higher concentrations eugenol showed complete inhibition of biofilm formation. Both eugenol-treated and non treated cells were analyzed under Scanning Electron Microscope (SEM). The SEM analysis showed cells, which received no eugenol treatment, being thick and well organized in 3 dimensional cell-to-cell knottings, while cells treated with eugenol were shrunken, with no cell-to-cell connections. It was apparent that eugenol disrupted normal morphology of the cells. Cell debrises or completely dead cells could have been identified by the pores in the cell membrane. This indicates that eugenol destroys the cell membrane and leads to cell lysis. Results of crystal violet absorption confirmed this. The uptake of crystal violet in the control cells was 42%, while in the eugenol treated cells increased to 90- 92%, which shows that eugenol alters the membrane permeability of *S. aureus*. Moreover, quantification of *icaD*, *sarA* and *seA* gene expression by Reverse transcription polymerase chain reaction (RT-PCR) was done and shown down

regulation of all three genes, which indicated that eugenol may be effective against bacteria by decreasing the expression of biofilm-related genes. The authors concluded that eugenol nossesses a great antibacterial activity, because it is able to disrupt cell-to-cell connections, lead to cell lysis by destroyment of cell membrane and decrease the biofilm-related genes (Jadav *et al.*, 2015).

This is in relation with the study of Biasi-Garbin *et al*., (2015) which investigated effects of eugenol as a natural component and major constituent of essential oils of many plants, against *Streptococcus agalactiae* and its synergistic interaction with biologically produced silver nanoparticles. The study showed almost the same results. Eugenol reduced number of bacterial cells approximately by 1 to 2 $\log_{10}$ CFU/mL (P <0.05) after one hour of incubation, while no cells or colony forming units were viable after 2, 4 and 10 hours of incubation. All control cells were green-fluorescent, while eugenol-treated cells were red-fluorescent, reflecting dead bacteria with damaged membranes. SEM analysis showed control cells cells with various morphological alterations, disruption of cell wall and decrease in electron density. The authors concluded that eugenol`s mechanism of antibacterial activity reflects itself by means of changes in the cell envelope, it induces cell lysis by protein and lipid leakage and leads to drawing of cytoplasmic content (Biasi-Garbin *et al*., 2015).

Suzuki *et al*. (2015) investigated the potential of the essential oil from *Pimenta pseudocaryophyllus* as an antimicrobial agent. It was concluded that this essential oil can be used as a personal care product, because it has high bacteriostatic actitvity against bacteria responsible for bad perspiration odor, due to its high content of eugenol (HR-GC showed that this essential oil contents 88.6% of eugenol). Microdilution assay revealed its high antibacterial activity with MIC values ranging from 500 to 1,000 μg mL–1. Further investigation with SEM showed rough surface morphology and shrinkage on bacterial cells treated with *P. pseudocaryophyllus* essential oil, when compared to healthy, nontreated ones. Eugenol treated cells also showed that eugenol destroyed the cell membrane, because cells had wrinkled abnormalities, cleft and pore formation on the cell membrane. Some cells showed other damages such as disruption or strange deformities in cell membranes, while some cells were even destroyed and dead, as it is shown below on Figure 1 and Figure 2 (Suzuki *et al*., 2015).

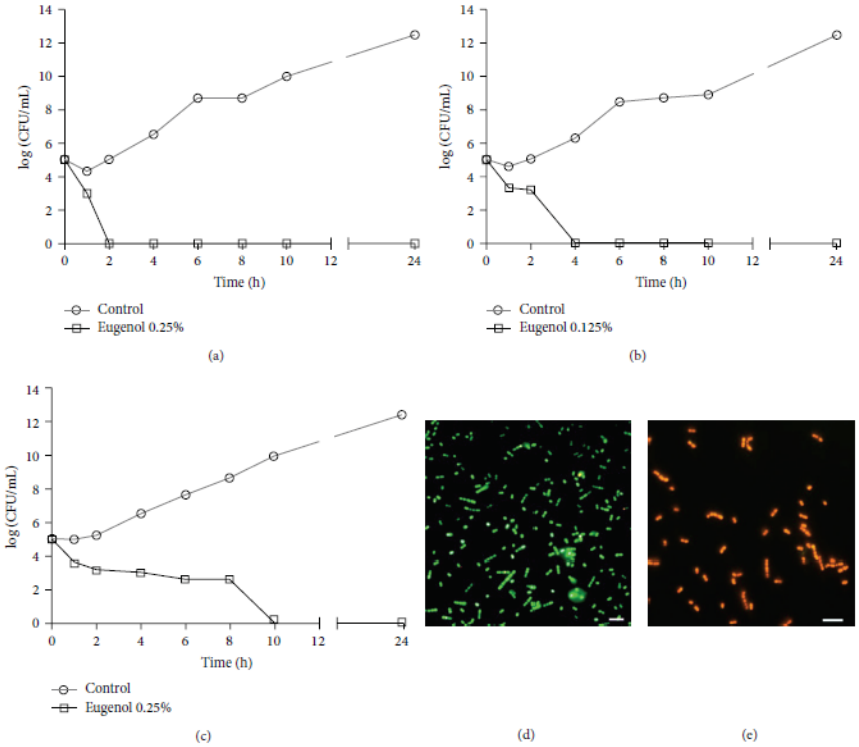

Figure 1: Effects of eugenol on growth of *Streptococcus agalactiae* and its vitality. Image a,b, and c show the time-kill curves of different strains of bacteria used during the experiment that were incubated with eugenol at MIC for 24 h at 37◦C and the CFU counts were determined at specified time points. D and e images show live-dead staining and GBSs with intact membranes that were green-fluorescent and eugenol-treated GBSs (at MIC) with damaged membranes that were red-fluorescent, in order to determine vitality of the cells (Suzuki *et al.,* 2015).

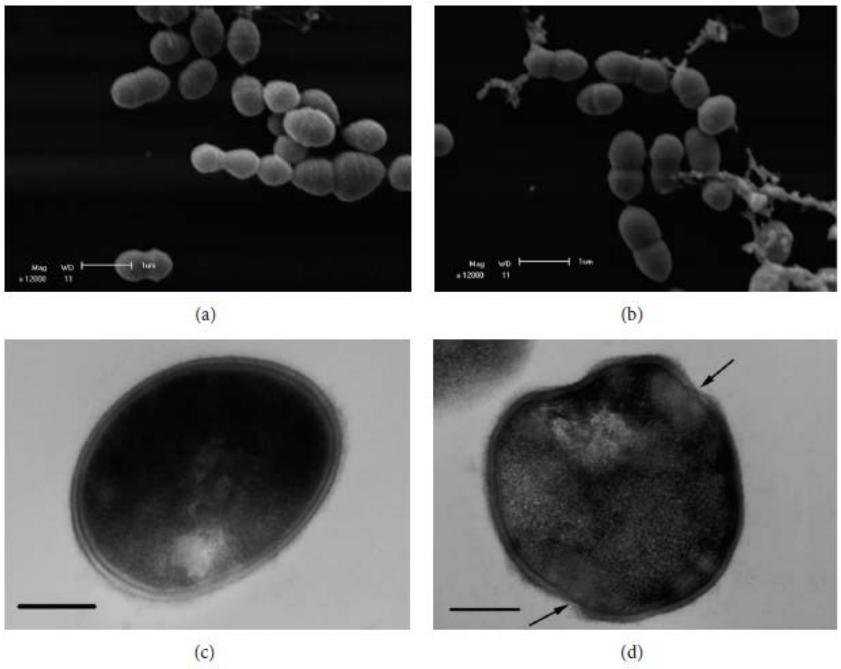

Figure 2: Effects of eugenol on morphology of Streptococcus agalactiae and its membranes are shown using SEM (a and b) and TEM (c and d). Images a and c show untreated, while images b and d show eugenol treated cells (Suzuki *et a*l., 2015).

As mentioned before, antibacterial activity of clove essential oil was investigated against *S. aureus* by Xu *et al*., and it exerted strong antibacterial properties. It completely distroyed cell wall and membranes and caused a loss of the most important inracellular materials. This resulted in the death of bacteria. Moreover, clove essential oil got through the cytoplasmic membrane, or after it distroyes a bacterial cell structure, it enters inside the cell and inhibits the normal DNA synthesis and synthesis of proteins that are required for bacterial growth. The authors concluded that the mechanism of antibacterial action of clove essential oil works not only to disrupt physically the bacterial cell, but also to destroy it on a molecular level (Xu *et al*.,2016).

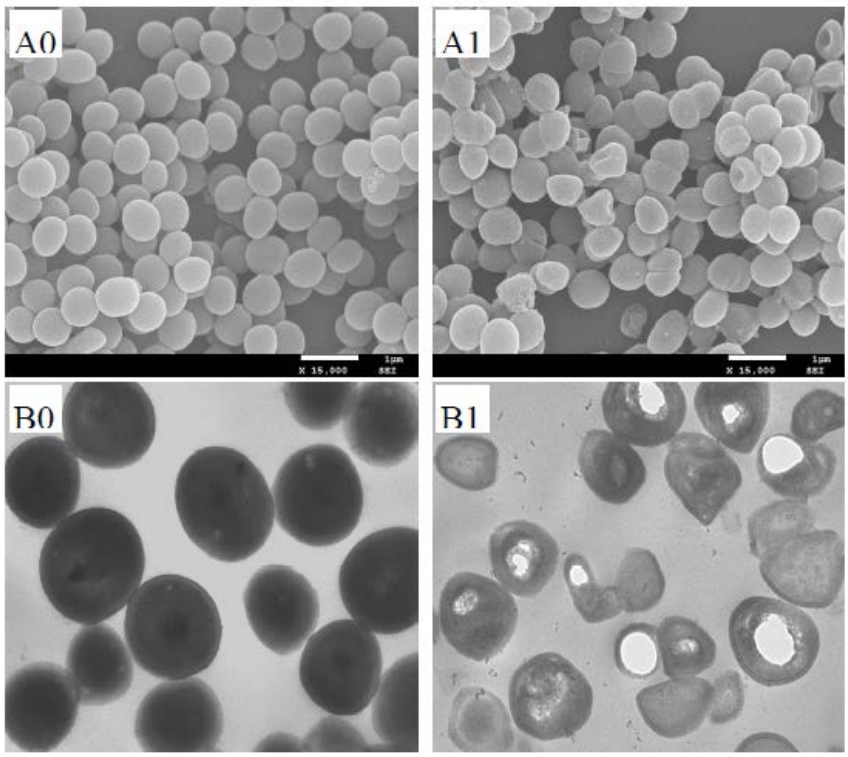

Figure 3: Scanning Electron Microscopy (A images) and Transmition Electron Microscopy (B images) images, showing morphological differences between untreated (A0 and B0) and clove essential oil treated (A1 and B1) *S. aureus* cells (Xu et al.,2016).

b. Mechanism of antifungal activity

The inhibitory effect of essential oils on *Aspergillus ochraceus* growth and ochratoxin A production were studied by Hua *et al.* in 2014. Cinnamon and clove oil showed to be very effective against *A. ochraceus.* Cinnamon oil completely inhibited *A. ochraceus* growth, while clove oil had slightly milder effect. When scanned with electron microscope, morphological changes are visible. Control mycelia showed regular, linear, homogenous and rough cell wall hyphae. But when treated with eugenol, the hyphae were disrupted: they became slender, shrank and winding and craters on the cell wall were visible. The condiophores and vesicles were also disrupted, with conidia dispersed in disorder. Eugenol also inhibited ergosterol production at 100-200µg/mL, with inhibiton rate of 45-85% and completely inhibited Ochratoxin A (OTA) production at 150-200µg/mL. The polyketide synthase gene (pks gene) is responsible for the synthesis of the polyketide dihydroisocoumarin and involved in the first steps of the OTA biosynthetic pathway. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) showed that eugenol completely inhibited transcription of pks gene at higher concentrations. It was concluded that cynamaldehyde from cinnamon oil and eugenol from clove oil was responsible for the morphological changes, disruption of

the cell walls and transcription inhibition of OTA production related gene, which lead to the decrease of fungal biomass (Hua *et al.*, 2014).
Also in 2014, Li *et al.* looked for possible mechanism of antifungal activity of cinnamon oil against *Rhizopus nigricans*, and found that cinnamon oil exhibits antifungal activity against growth of *R. nigricans* in a dose dependent manner. When analyzed with SEM, it was viable that cinnamon oil treated cells began to shrink, and the hyphae morphological changes were great: clear foam, dissolved cell walls and leakage of cytoplasmic matrix. Electric conductivity increased and $A_{260\,nm}$ of cinnamon oil treated culture media increased sharply. The authors concluded that cinnamon oil damages fungal cell membrane, leading to the leakage of cell content, which appears in the form of higher electric activity, and the amount of protein. They also said that their study can help in understanding the cinnamon oil as antifungal agent (Li *et al.*, 2014).

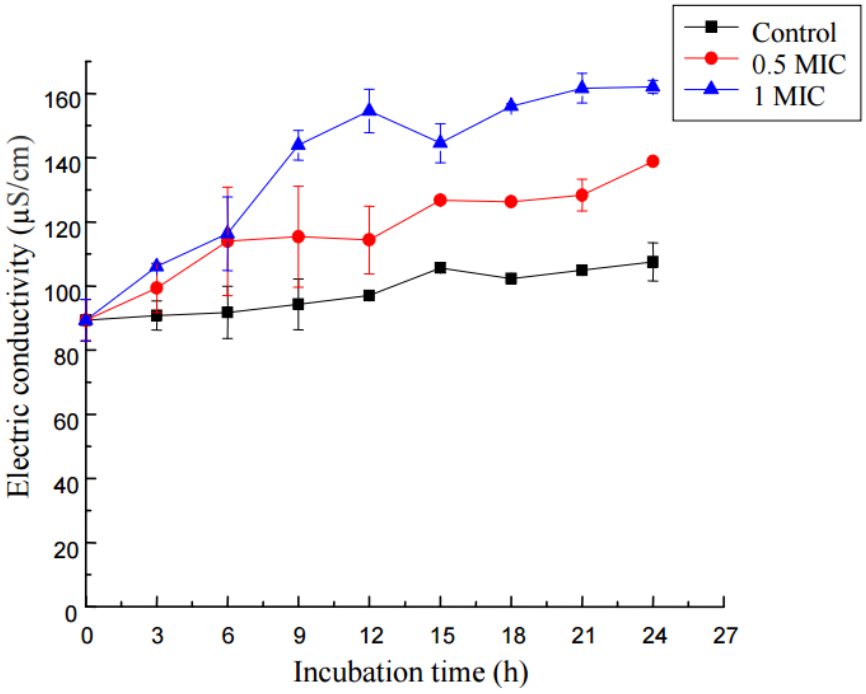

Figure 4: Effect of Cinnamon oil on electrical conductivity of *Rhizopus nigricans* (Li *et al*., 2014).

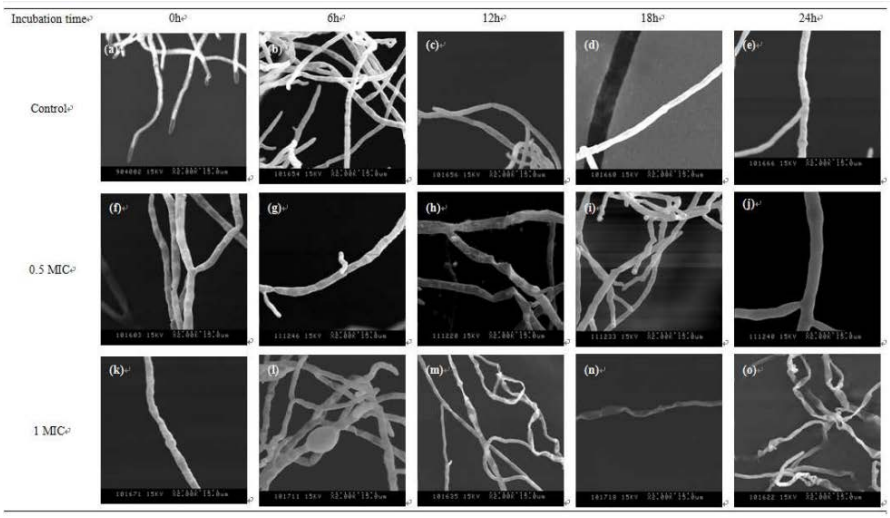

Figure 5: Scanning electron microscopy of *Rhizopus nigricans* treated with Cinnamon oil over time (Li *et al*., 2014).

As mentioned before, clove essential oil was proven to be the most effective agent against plant distrupting mold, *Fusarium oxysporum,* showing complete inhibition of mycelial growth and spore germination at relatively low concentration. When analyzed by gas chromatography-mass spectroscopy, the essential oil showed 31 different compounds. However its main constituents were the following: eugenol (75.41%), E-caryophyllene (15.11%), a-humulene (3.78%) and caryophyllene oxide (1.13%). When samples were studied by scanning electron microscopy (SEM) and atomic force microscopy (AFM), it was obvious that clove essential oil caused morphological damages on on hyphae. Due to the shrunken hyphae, fungal growth was supressed, showing a clear lack of cytoplasm and ditrupted cell wall, when compared to long, smooth and homogenous hyphae of control samples. The authors concluded that clove essential oil clearly acts as an antifungal agent, by fragmenting and distrupting characteristic curvilinear morphology of spores (Sharma *et al*., 2016).

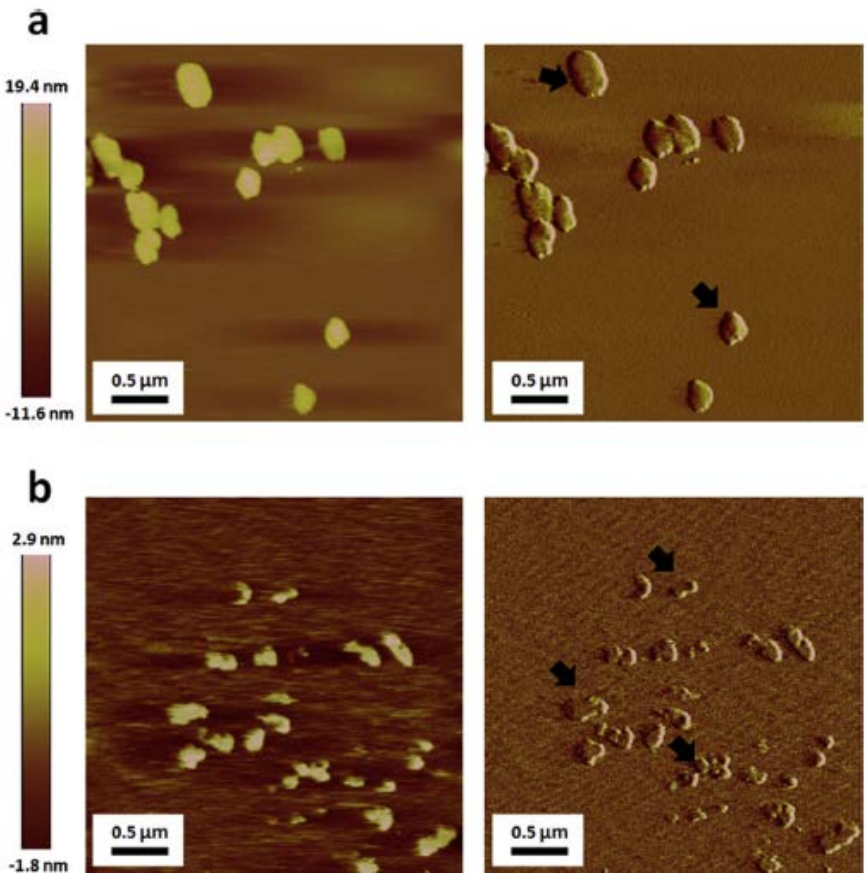

Figure 6: Atomic Force Microscopy images, showing control (a) and clove essential oil treated (b) samples of *F. oxysporum* spores. Considerable differences in both cases are observable (Sharma et al., 2016).

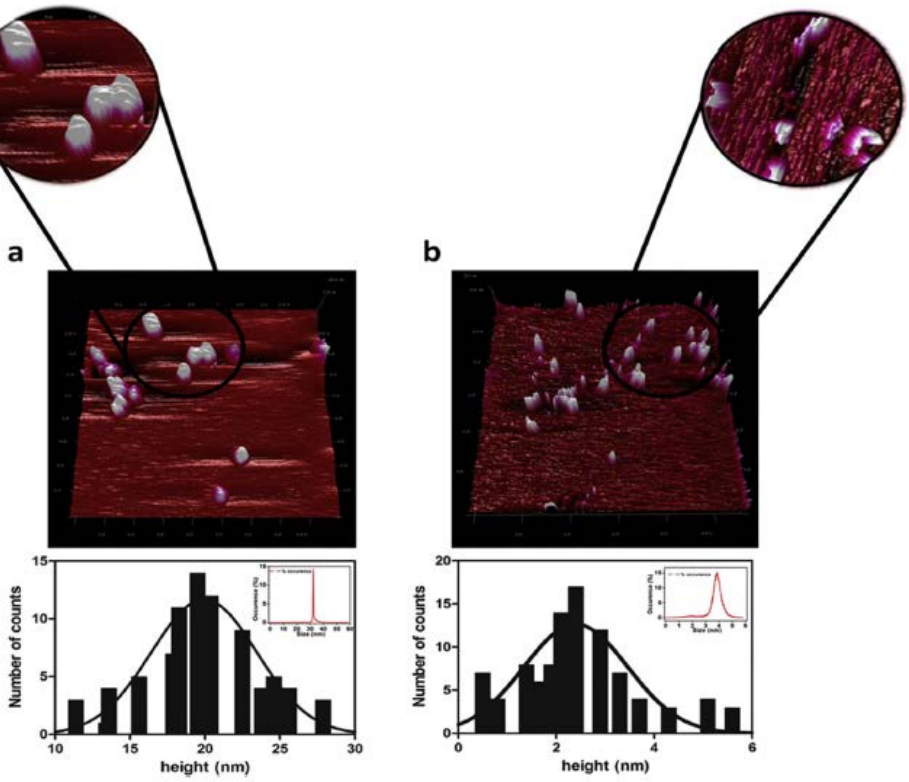

Figure 7: Atomic Force Microscopy three-dimensional images of (a) untreated and (b) clove essential oil treated *F. oxysporum* spores. In the zoomed pictures, crucial differenses in sizes and surface attributes are observable (Sharma et al., 2016).

7. Correlation between the constituents of the essential oils and their antimicrobial activity

Saharkhiz *et al*. (2015) investigated chemical composition and antimicrobial properties of essential oils of *Ocimum sanctum (Lamiaceae)* at different stages of the plant's development. The authors came to the result that the highest content of eugenol was in the essential oil from the floral budding stage of development with 37.2%, following full flowering stage with 24.6% and vegetative stage with 15.7%. When effects of these oils were tested against different microbes, the oil extracted from the floral budding stage with highest content of eugenol revealed to have the highest antimicrobial activity, with minimum inhibitory concentrations varying from 0.25µg/mL to 4µg/mL, except for one isolate of *P. aeruginosa* which had the highest MIC, even 64µg/mL. The authors proposed that these oils were very effective against almost all microbes tested, due to the high content of

eugenol, which destroys the cell membrane (Saharkhiz *et al*., 2015). Evrendilek (2015) found that among cinnamon, clove and bay leaf oils, that cinnamon oil, although containing no eugenol, showed the strongest antimicrobial activity against all microorganisms tested, with IZ values ranging from 21.2mm to 52.2mm. Clove oil with 67.3% eugenol content showed moderate (with IZ values from 11mm to 14.8mm), but much higher antibacterial activity than 0.5% eugenol-containing essential oil of bay leaf with IZ values varying from 6mm to 10.8mm. It was concluded that the antibacterial properties of these essential oils depend on their main chemical compounds present (Evrendilek *et al*., 2015). Freires *et al*. (2015) examined the antibacterial activity of essential oils and their isolated constituents against carcinogenic bacteria. According to their study, essential oils with eugenol as their main constituent possess promising antimicrobial activity against streptococci (Freires *et al*., 2015). Miht *et al*. (2014) reported that cinnamon leaves essential oils contain *(E)*-cinnamaldehyde as major component with 77.9% and clove buds essential oils to comprise eugenol as main component with 84.8%. However, cinnamon oil was shown to be more effective with IZ values varying from 8.5mm to 34.1mm, but the antimicrobial effectives of eugenol oil were also significant with IZ values from 7mm to 16.7mm. The authors suggest the high presence of highly bacteriostatic *(E)*-cinnamaldehyde in cinnamon oil as an explanation of this finding and high presence of eugenol in clove oil, which is known to be very antibacterial (Miht *et al*., 2014).

8. Synergistic- Interactions of Eugenol- tosylate and its Congeners with Fluconazole against *C. albicans*

Due to their high MIC many potential natural compounds are often overlooked. Based on previously observed antifungal properties of eugenol at a MIC value of 500 µg/ml against a variety of tested *C. albicans* strains, new derivatives of eugenol showed more potent *in vitro* activity. Fifteen clinical isolates of FLC (fluconazole) –susceptible and 9 FLC- resistant *C. albicans* strains were used to prove the antifungal property of eugenol tosylate and its congeners at a MIC value ranging from 1-63µg/ml. The results demonstrate that the

modification of eugenol decreases its MIC values significantly. The observations also confirmed the either synergistic, additive or indifferent effect of eugenol derivates in combination with fluconazole. These data are in agreement with the previous findings where the use of eugenol decreases the needed concentration of FLC in combination with specific antibiotics. The inhibition of the 14-α-demethylase, an enzyme which has importance in the ergosterol synthesis in fungi, was also confirmed by application of the eugenol derivatives. The incorporation of a sulfonyl group in place of the hydroxyl group of eugenol in the derivatives improves their activity probably because its entranced fitting into the active site of this enzyme.

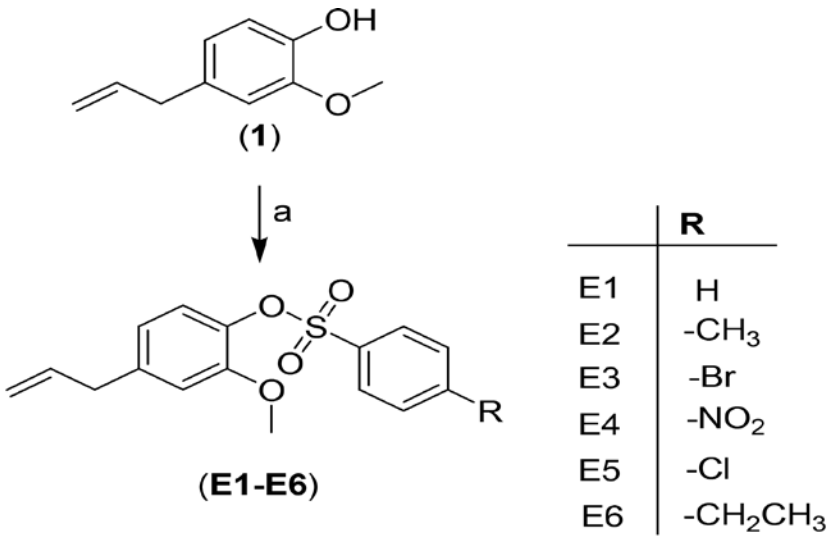

Figure 8: Synthesis of eugenol-tosylate and its congeners E1-E6 (Ahmad *et al*., 2015)

As the ERG 11 gene, a specific regulator for ergosterol biosynthesis in *C. albicans*, has an important role in azole resistance, it is worth mentioning that all eugenol derivatives down regulated this gene. Never the less, further studies are required to determine the exact mechanism of the antifungal properties and synergistic interactions of eugenol derivatives.

9. Anti-inflammatory effects in pulp tissues

Among a variety of biologic properties, eugenol is also known for its use as a capping material during pulp therapy in primary teeth in form of ZOE (eugenol mixed with zinc oxide powder).

To provide a better maintaining of the integrity of primary teeth until their exfoliation and to ovoid malocclusion or functional problems, the anti-inflammatory effect of this essential oil has been observed deliberately. Cultured fibroblast cells were used to explore the ability of eugenol to induce or inhibit the production of inflammatory cytokines and the expression of inflammatory related. The observation was made under and without inflammatory preconditions. The study results showed that low concentrated eugenol (13µM) inhibits gene expression of the proinflammatory NF-κB and TNF-α in the pulp tissue in inflamed state, where in healthy dental tissue it provides the expression of not only NF-κB or TNF-α, but also several other proinflammatory cytokines.

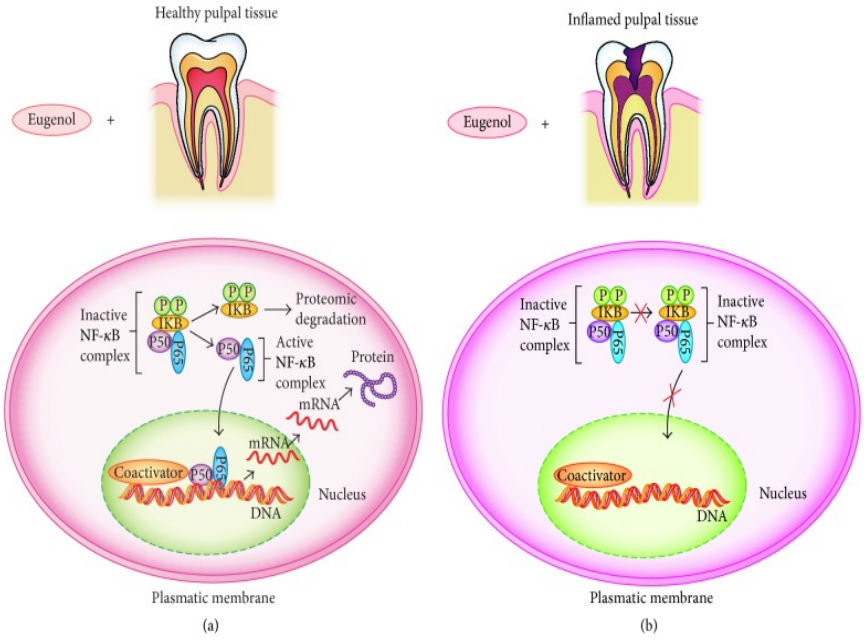

Figure 9: A possible translocation of NF-*κ*B for healthy pulp tissue (a) and inflamed pulpal tissue (b). (a) Healthy pulpal tissue(Herrera *et al*., 2016)

10. Application of essential oils in the food industry (against foodborne pathogens)

Eugenol, as a main component of some essential oils, is a very adaptable molecule, that is widely used as a functional compound of various products in pharmaceutical, agricultural, cosmetical and food industry (Biasi-Garbin *et.al*., 2015).

a. Essential oils in food preservation industry

Laurel oil (0.3-2.5% of eugenol) was shown to be good for the usage of prevention of the deterioration of food during storage, as it was very effective against *S. aureus*, *S. typhimurium* and *E. coli* and showed high antioxidant activity, because it showed the greatest Trolox Equivalent Antioxidant Capacity (TEAC) value obtained by hydrodistillation (HD) (Nehir El *et al*., 2014). Clove and cinnamon essential oils, with 84.1% eugenol content in clove and 14.7% in cinnamon have manifested high efficiency against food spoilage microbes as *P. aeruginosa, S. typhi , L. innocua* and *S. aureus*, with the MIC value for all tested microbes <1.0 ml/ml suggesting that these oils could be used for the development of the new techniques of food preservation (Tarek, *et al*., 2014). Again, cinnamon and clove oil showed antimicrobial properties against food borne pathogens, when tested on *L. monocytogenes* and *S. enterica*. Namely, clove oil with 77.8% of eugenol as its main component and cinnamon oil with 76.2% of trans-cinnamaldehyde as its main component, had lowest MIC values against these microbes: both cinnamon and clove with MIC=0.6-2.5µg/mL. The authors suggested these oils to be used as multifunctional biopreservatives, because they have both antibacterial and antioxidant activities (Mazzarinno *et al*., 2015). Cinnamon oil inhibits growth of *R. nigricans* at MIC=0.1µg/mL, where no *R. nigricans* growth was recorded, it was totally inhibited (Li *et al*., 2014). At MIC=500 µg/mL of cinnamon oil, inhibition of growth of *A. ochraceus* was permanent, without visible growth occurred (Hua *et al*., 2014). Against *Eurotium amstelodami*, *E. herbariorum*, *E. repens*, *E. rubrum*, *A. flavus*, *A. niger* and *Penicillium corylophilum* cinnamon leaf and bay leaf showed intermediate antifungal activity, while clove oil exerted very high antifungal activities, indicating their potential use in food industry as natural food preservants (Mishra *et al*., 2015).

11. Safety and toxicity

According to U.S. Food and Drug Administration (FDA, Code of Federal Regulations, Title 21, Volume 2, revised as of April, 1, 2014), essential oils extracted from roots, barks, leaves, flowers, berries, buds and similar parts of the spices like cinnamon, allspice, clove, basil and laurel are “Generally Recognized as Safe” (GRAS), if they are used for their indicated properties

12. Conclusion

Essential oils are of the natural origin. Therefore, their chemical composition may vary and it depends on the geographical region, climate conditions, stage of development of the plant, part of the plant from which the oil was extracted, mechanism of oil extraction and mechanism of evaluation of chemical constituents. Essential oils show good antimicrobial properties against many microorganisms tested. Tests have also shown that the chemical constitution of the essential oil is highly correlated with its antimicrobial properties. Eugenol containing essential oils have shown to posses the strongest antimicrobial action against many Gram-positive and Gram-negtive bacteria, human pathogens, oral microbes and food spoilage microorganisms. As they are all natural, with high antimicrobial properties and categorized as generally safe for use, eugenol containing essential oils can be used as a natural replacement for antibiotics (on which more and more bacteria are developing resistance), food preservants, compounds of cosmetical products and products for personal care.

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