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Research of antifungal effects on some essential oils with tube dilution

Pesquisa de efeitos antifúngicos em alguns óleos essenciais com diluição em tubo

Rukiye Colak Sasmazer[1*]; Mihriban Korukluoglu[2]

Highlights

Essential oils (eugenol, limonene and cinnamic acid) have antimicrobial effects
Cinnamic acid has the most inhibition effect
M.fructicola and *K.apiculata* are the most resistant yeast against the cinnamic acid
The most effective yeast against eugenol and cinnamic acid is *M.fructicola*

Abstract

The aim of study to investigate inhibition effects of cinnamic acid (volatile compound of cinnamon), limonene (essential oil of lemon) and eugenol (essential oil of clove, cinnamon) on *Metschnikowia fructicola, Candida oleophila, Schisosaccharomyces pombe, Saccharomyces uvarum* and *Kloeckera apiculata.* In this study, tube dilution method was used. Among eugenol, limonene and cinnamic acid, it was found that cinnamic acid has the most inhibition effect with low concentrations (%2.8, %3.84, %4.36, %5,4) at tube dilution methods. Also it was found that test yeast have different resistance against test materials.
Key words: Antifungal effect. Essential oil. Spices.

Resumo

O objetivo do estudo foi investigar os efeitos de inibição do ácido cinâmico (composto volátil da canela), limoneno (óleo essencial de limão) e eugenol (óleo essencial de cravo, canela) sobre *Metschnikowia fructicola, Candida oleophila, Schisosaccharomyces pombe, Saccharomyces uvarum* e *Kloeckera apiculata*. Neste estudo, foi utilizado o método de diluição em tubo. Entre eugenol, limoneno e ácido cinâmico, verificou-se que o ácido cinâmico tem o maior efeito de inibição com baixas concentrações (% 2,8,% 3,84,% 4,36,% 5,4) nos métodos de diluição em tubo. Também foi descoberto que a levedura testada tem diferentes resistências contra os materiais de teste.
Palavras-chave: Efeito antifúngico. Especiarias. óleo Essencial.

[1] Student of Ph.D., at Food Science, Department of Food Engineering, University of Uludag, Bursa, Turkey. E-mail: rukiyecolak82@gmail.com
[2] Prof. Dr., Department of Food Engineering, University of Uludag, Bursa, Turkey. E-mail: mihriban@uludag.edu.tr
* Author for correspondence

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Introduction

Medicinal plants have been used by people for various purposes since ancient times, without knowing the presence of biologically active compounds in them. Studies have increased in recent years due to the interest in medicinal plants and the active substances derived from them. Because, easy and cheap treatment is possible by using plants. In addition, the dangerous side effects seen in some of the new synthetic substances introduced into the treatment area can only be fully understood after being used and cause damages that cannot be repaired (Pandey, Sonker, & Singh, 2016; Pandey, Singh, Palni & Tripathi, 2014).

The Turkish people are closely related to wild plants, as the majority live in the countryside. Folk uses some of the wild plants as food, spice, dyestuff or medicine. Some plants are important for public and animal health, as they contain toxic compounds. The use of wild plants as food and spice is quite common in Anatolia. Above ground part or roots of many wild plants are consumed as vegetables. Among the wild plants used as food, among the people, it can be counted as kiger, evelik, mımakak, mallow, ciris, çüzük (Baytop, 1999).

The most important features of aromatic plants are their pleasant smells and flavors. They owe these features to the essential oils they carry. It has a wide use in the preparation of aromatic drugs and essential oils obtained from them, in the perfumery and cosmetics industry, in the fields of medicine and pharmacy (Tak & Isman, 2017; Baytop, 1999).

In this study, the effects of 3 different essential oils (limonene, eugenol and cinnamic acid), 5 yeasts (*Candida oleophila, Kloeckera apiculata, Metschnikowia fructicola, Saccharomyces uvarum* and *Schizosaccharomyces pombe*) with the minimum effects of inhibitors on the purpose of investigating the antimicrobial properties of biologically active substances concentrations (MIC) were tried to be determined.

Material and Methods

Pure yeast cultures in Uludağ University Faculty of Agriculture, Department of Food Engineering were used as materials. The species selected in the research were *Saccharomyces uvarum and Schizosaccaharomyces pombe U.Ü., Candida oleophila, Metschnikowia fructicola* and *Kloeckera apiculata U.Ü.* from the Faculty of Agriculture, Department of Food Engineering. They were obtained from the Plant Protection Department of the Faculty of Agriculture. As the active ingredient, cinnamic acid, eugenol and limonene obtained from Aromsa company were used.

Malt Extract liquid and solid media were used in the experiment. Various concentrations of cinnamic acid, eugenol and limonene diluted in Dimethylsulfoxide (DMSO, Sigma-Aldrich) were used to prevent yeast growth (Reyes et al., 2017). While working, the unit was studied and the results were converted to concentration.

The yeasts were renewed and kept in Malt Extract Agar (MEA Merck) every two months during the research. During the trial phase, 1 loopful of culture was taken

from the flat agar and inoculated into tubes containing 10 mL of sterile Malt Extract liquid (MEB-Merck). Young cultures to be used for cultivation were prepared by leaving the tubes for 24 h incubation at 30 ° C (Reyes et al., 2017).

Determination of antimicrobial property

Tube dilution methods were used to determine the antimicrobial properties of eugenol, cinnamic acid and lemonen.

Tube dilution method

Tubes containing 2.5 mL Malt Extract Broth were inoculated with 0.5 mL of culture and 10 µL of eugenol, cinnamic acid or lemone dissolved in alcohol in various concentrations in Dimethylsulfoxide (DMSO, Sigma-Aldrich). Vaccination was carried out in 4 replications. After the inoculated tubes were left to incubate at 30 ° C for 24-48 hours, yeast development at the end of 24-48 hours was observed by sowing to the petri dishes poured with the malt Extract Agar (Reyes et al., 2017).

Evaluation of results

In liquid culture trials, it was observed whether there was yeast development at the end of 24-48 hours by cultivating in petri poured from the tubes with Malt Extract Agar.

Statistical analysis

Cluster analysis in determining the effects of eugenol, limonene and cinnamic acid on yeasts test were carried out using Özdamar (8) SPSS 10.0 package program.

Results and Discussions

Determination of microorganism number

Table 1 shows the average values of the count results of the test microorganisms used in the experiment. The fact that the counts made at the end of each inoculation are close to the values, eliminated the errors that may arise from the numerical difference of microorganisms in the results obtained (Reyes et al., 2017).

Table 1
Initial microorganism count averages

Name of Microorganism	Count (Log)
Candida oleophila	7.46
Kloeckera apiculata	7.85
Metschnikowia fructicola	7.48
Saccharomyces uvarum	6.67
Schizosaccharomyces pombe	6.88

Results of tube dilution method

Eugenol results

In Table 2, minimum concentrations of inhibition of eugenol of different concentrations dissolved in Dimethylsulfoxide (DMSO, Sigma-Aldrich) on test yeast are given.

Table 2
Minimum inhibition of eugenol on test microorganisms concentrations

Concentrations g/100mL	*C.oleophila*	*K.apiculata*	*M.fructicola*	*S.uvarum*	*S.pombe*
106,70	-	-	-	-	-
53,35	+	+	+	+	+
71,13	-	-	-	-	-
66,69	-	-	*	-	*
62,76	-	-	*	-	*
59,28	-	-	*	-	*
54,72	-	-	*	-	*
54,44	+	+	*	-	*
54,16	*	*	*	+	+
53,89	*	*	*	*	+

-: Microorganism development is not observed.
+: Microorganism development is observed.
*: There is no trial at this concentration.
n = 4 (4 replicates worked).

In the experiments conducted with the tube dilution method, it was determined that the most resistant microorganism was *M.fructicola* with a value of 71.13%. *K.apiculata* and *C.oleophila* followed *M.fructicola* with equal resistance (54.72%). In the trials made with the tube dilution method, even if the lemon was used pure, no test yeast was tried in different concentrations since it did not prevent development in any test yeast.

Results of cinnamic acid

M.fructicola and *K.apiculata* were found to be the most resistant yeast to the cinnamic acid with a value of 5.4%. C.oleophila follows this and the minimum inhibition concentration is 4.36%. The most resistant microorganisms were *S.pombe* and *S.uvarum.* Their minimum inhibition concentration was determined as 3.84%.

Table 3
Minimum of the test microorganisms of cinnamic acid inhibition concentrations

Concentrations g/100mL	*C.oleophila*	*K.apiculata*	*M.fructicola*	*S.uvarum*	*S.pombe*
6,00	-	-	-	*	*
5,40	*	-	-	*	*
5,20	*	+	+	*	*
4,90	*	+	+	-	*
4,80	-	+	+	*	*
4,60	-	*	*	*	-
4,40	-	*	*	*	*
4,36	-	*	*	*	*
4,32	+	*	*	*	*
3,84	*	-	*	-	*
3,80	*	+	*	+	-
2,40	*	+	*	+	+
0,40	+	+	+	+	+
0,20	+	+	+	+	+
0,08	+	+	+	+	+
0,004	+	+	+	+	+

-: Microorganism development is not observed.
+: Microorganism development is observed.
*: There is no trial at this concentration.
n = 4 (4 replicates worked).

In a study on how these effects of essential oils on microorganisms are, it was determined that essential oil damaged mitochondrial DNA. It is stated that the damage is caused by the combination of 2 genes in DNA. One of these genes has been reported to be RNR3 (the gene involved in DNA metabolism) and the other is RAD51 (the gene involved in repairing DNA). In this study, other damages of essential oils were found to cause mutation in the cytoplasm membrane and impaired mitochondrial structures and functions. It was stated that the result obtained from this study on *S.cerevisiae* may also be valid for all yeasts (Bakkali, Averbeck, Averbeck, Zhırı, & Idaomar, 2005). In another study on 4 Candida species (*C.albicans, C.krusei, C.parapsilosis, C.tropicalis, C.albicans* ATCC 10231) essential oil formed a special wall around the yeast cell, then the yeast cell wall shrunk towards the center and It was determined that large vacuoles were formed in the space between them. Then, it was stated that the cell wall was completely deformed (Nakamura, Ishida, Faccin, Dias, Cortez, Rozental, 2004).

There are many studies on the antimicrobial activity of eugenol. One of them is Eugenol exhibited a wide range of antibacterial activity with maximum zones of inhibition against Campylobacter jejuni (36.33 + 1.53 mm) and Helicobacter pylori (34.00 ± 1.00 mm). The smallest amount of

activity was recorded against Pseudomonas aeruginosa (10.00 + 1.00 mm) followed by Listeria monocytogenes, Bacillus subtilis and Sreptococcus pyogenes with zones of inhibition corresponding to 17.33, 21.00 and 20.33 mm, respectively (Ebenezer Jeyakumar & Lawrence, 2021).

Several other studies have shown the antibacterial properties of eugenol against L. monocytogenes, B. cereus and C. jejuni (Friedman, Henika, & Mandrell, 2002; Hao, Brackett, & Doyle, 1998; Kim, Marshall, & Wei, 1995; Thoroski, Blank, & Biliaderis, 1989).

Eugenol has also been reported to inhibit several multi drug resistant human pathogenic bacteria including E. coli, Staphylococcus, Proteus, Klebsiella, Enterobacter, and Pseudomonas (Shashidar, 2002; Suresh, Ingle, & Vijayalakshmi, 1992).

Dhara and Tripathi, (2020) reported that eugenol de monstrated minimum inhibitory concentration (MIC) of 63-999 µg/mL among maximum numbers of K. pneumoniae.

In our study the effect of eugenol concentrations on test yeast can be seen in Figure 1.

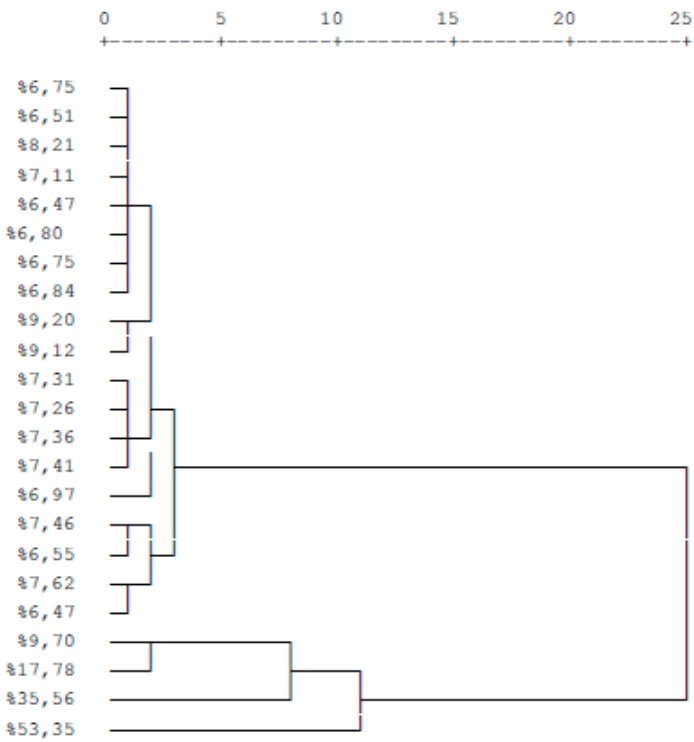

Figure 1. Effect diagram of eugenol concentrations on test yeast.

When the graph of eugenol was examined, it was observed that the grouping in the tree diagram obtained by cluster analysis was basically 3. The first group is the group closest to the zero line and concentrations without trial and the lowest concentration values are included in this group. While concentrations of 9.70% and 17.78% in group 2 had similar effects on test yeasts, 35.56% concentration was in the same group despite

higher inhibition effect. In the third group, there was a 53.35% concentration, which gave the most effective result on test yeasts.

The sensitivity of test yeasts to eugenol can be seen in Figure 2.

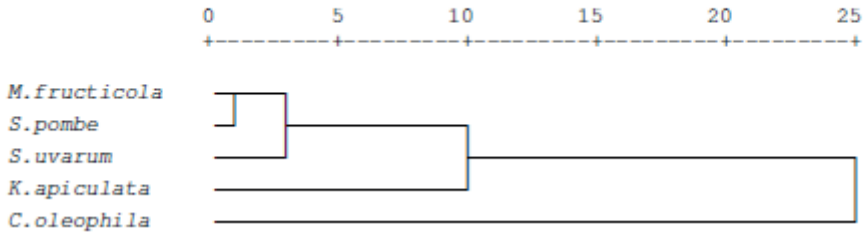

Figure 2. Test yeast susceptibility diagram to eugenol.

When the resistance of test yeasts against eugenol was examined, it was observed that *M.fructicola, S.pombe, S.uvarum* had a close effect, and it was observed that eugenol was only inhibited at high concentrations. *K.apiculata,* on the other hand, was less resistant than these three yeasts and is in the 2nd group. It was determined that eugenol was the most effective test yeast *C.oleophila.* The effect of limonene concentrations on test yeast can be seen in Figure 3.

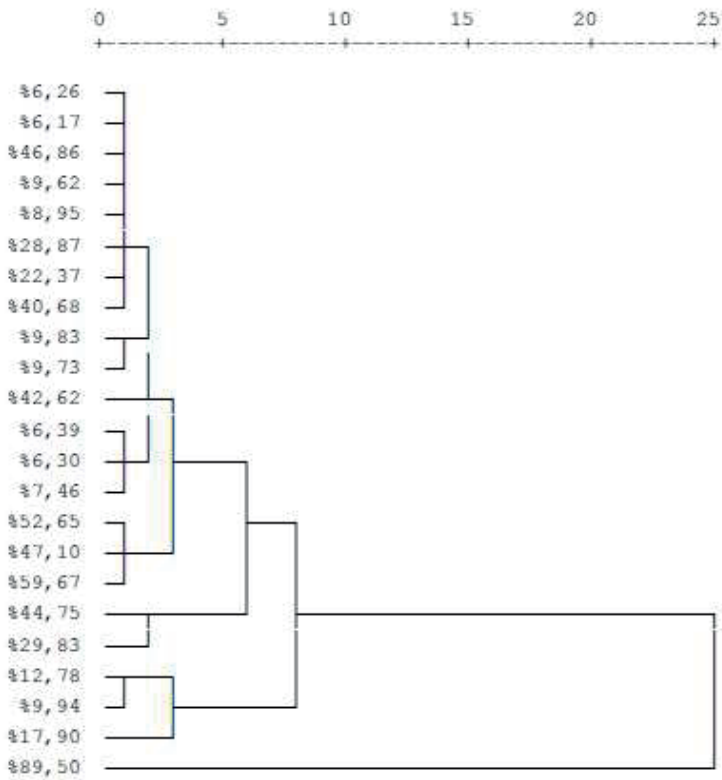

Figure 3. Effect of limonene concentrations on test yeast diagram.

In a study, influence of limonene, which is the major constituent of citrus essential oils on Langmuir films mimicking bacterial membrane was studied. Citrus essential oils, apart from antimicrobial activity, were found to exhibit also antioxidant, anticancer, anti-inflammatory and insecticidal properties, while limonene alone is of strong antibacterial and antifungal effect also against food borne pathogens (Jing et al., 2014).

In the diagram of the effect of the limonene concentrations on the test yeast, 3 distinct groups are seen. The first group is the group closest to the zero line and non-trial concentrations and the lowest concentration values are included in this group. In the second group, there are concentrations of 52.65%, 6.30% and 9.94%. While the highest effect is the concentration of 89.50%, the closest value is the concentration of 44.75% and it is in the 3rd group. The sensitivity of test yeasts against limonene can be seen in Figure 4.

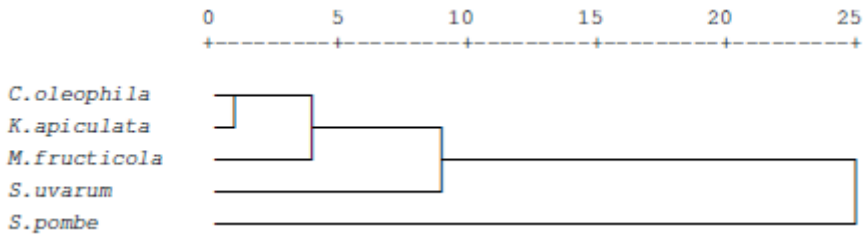

Figure 4. Sensitivity diagram of test yeasts against limonene.

When the resistance of test yeasts against limonene was examined, it was observed that *C.oleophila, K.apiculata,* and *M.fructicola* had the closest effect and formed the first group. This group is the most resistant group against limonene. *S.pombe* is the most sensitive yeast and is in the 3rd group. The effect of cinnamic acid concentrations on test yeast can be seen in Figure 5.

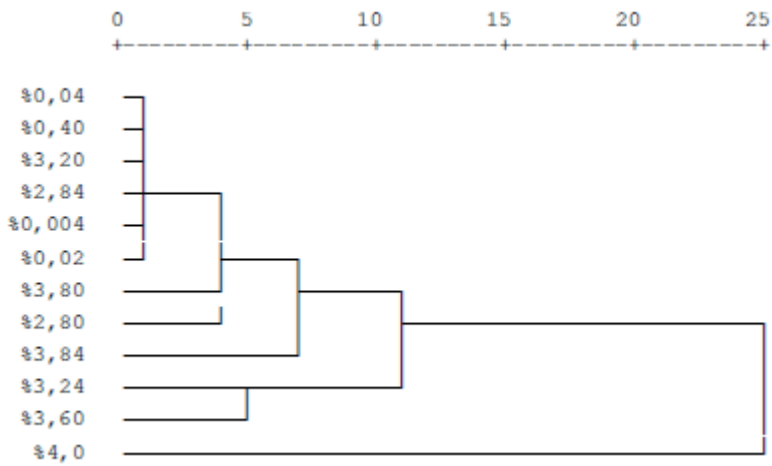

Figure 5. Effect diagram of cinnamic acid concentrations on test yeast.

When the resistance of test yeasts against cinnamic acid was examined; It has been observed that *C.oleophila* and *M.fructicola,* which are the most resistant yeasts, have very close effects and *K.apiculata* is less resistant. It was determined that the effect of *S.pombe* and *S.uvarum* are very close and most sensitive yeasts. The sensitivity of test yeasts against cinnamic acid can be seen in Figure 6.

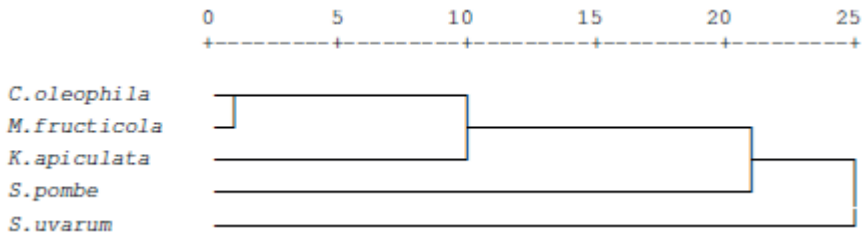

Figure 6. Sensitivity diagram of cinnamic acid concentrations on test yeast.

In the diagram of the effect of cinnamic acid concentrations on the test yeast, 3 distinct groups are seen. The 1st group is the closest to the zero line, the concentrations that are not tested and the lowest concentration values are in this group. In the second group, 3.84%, 3.80%, 3.60%, 3.24% and 2.80% concentrations were in the same group with similar effects. The concentration that shows the greatest effect is 4% concentration and is in the third group. The sensitivity of test yeasts to cinnamic acid can be seen in Figure 6.

In a recent study (Malheiro et al., 2016), cinnamaldehyde and cinnamic acid demonstrated significant antibacterial and dispersal activities. Cinnamaldehyde had a MIC (3 and 5 mM) and MBC (10 and 12 mM) against *E. coli* and *S. aureus* while cinnamic acid was able to completely remove the adhered bacteria after their exposure to the phytochemical for 1 h. This knowledge was taken into consideration for the selection of the 15 chemicals used in this study.

The mechanism of antibacterial and antifungal properties of the essential oil components is connected with the ability of these hydrophobic substances to incorporate into the membrane. It is thus clear that antimicrobial effect of the essential oils is determined by lipid composition of the pathogen cellular membrane as well as the structure of the active compound affecting membrane lipid/essential oil interactions. However, from the point of view of practical application of the essential oils in food industry also the factors resulting from the properties of food (e.g. the presence of salt or fats) as well as the external conditions are highly important (Anees, Srinivas, & Pramod, 2015; Scollard, Francis, & O'Beirne, 2009; Tongnuanchan & Benjakul, 2014).

Conclusion

With this study, antimicrobial effects of 5 test yeasts (*M.fructicola, S.uvarum, S.pombe, C.oleophila, K.apiculata*) on 3 active substances (eugenol, cinnamic acid and limonene) were determined:

Cinnamic acid is found to be the most effective active ingredient in 3 active substances, which are trial materials, by showing inhibition effects at lower concentrations in tube dilution method.

Limonene has no effect in the tube dilution method.

Eugenol is quite high in tube dilution method (71.13%, 54.72%, 54.44%, 54.16%).

In tube dilution method, the most effective yeast against eugenol and cinnamic acid was *M.fructicola.*

In general, no parallelism was observed between the resistance of yeasts to different active substances. As a result, it is known that there are many published articles about the research of antimicrobial properties of herbal extracts and essential oils.

Recommendations

In these studies, various essential oils were tested against test microorganisms and their antimicrobial effects were tried to be determined. Thus, it is thought that these biologically active substances may be an alternative to use as an alternative to chemical preservatives, and it is recommended to investigate their effects on a large number of microorganisms. This study is a part of my master's thesis.

References

Anees, A. M., Srinivas, R., & Pramod, G. (2015). Studies on antimicrobial activity of spices and effect of temperature and Ph on its antimicrobial properties. *IOSR Journal of Pharmacy and Biological Sciences, 10*, 99-102. doi: 10.9790/3008-101299102

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A., & Idaomar, M. (2005). Cytotoxicity and gene induction by some essential oils in the yeast *Saccharomyces cerevisiae. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 585*(1-2), 1-13. doi: 10.1016/j.mrgentox.2005.03.013

Baytop, T. (1999). *From today to the past treatment with plants in Turkey* (2nd ed.). Istanbul: Nobel Press.

Dhara, L., & Tripathi, A. (2020). Antimicrobial activity of eugenol and cinnamaldehyde against extended spectrum beta lactamase producing enterobacteriaceae by in vitro and molecular docking analysis. *European Journal of Integrative Medicine, 5*(2013), 527-536. doi: 10.1016/j.eujim.2013.08.005

Friedman, M., Henika, P. R., & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against Campylobacter jejuni, *Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica. Journal Food Protection, 65,* 1545-1560. doi: 12380738

Hao, Y. Y., Brackett, R. E., & Doyle, M. P. (1998). Inhibition of Listeria monocytogenes and Aeromonas hydrophila by plant extracts in refrigerated cooked beef. *Journal Food Protection, 61,* 307-312. doi: 10.4315/0362-028x-61.3.307

Jing, L., Zhentian, L., Ligai, L., Rangjin, X., Wanpeng, X., Yu, G.,... Zhiqin, Z. (2014). Antifungal activity of citrus essential oils. *Journal of Agricultural and Food Chemistry, 62,* 3011-3033. doi: 10.1021/jf500 6148

Kim, J., Marshall, M. R., & Wei, C. (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. *Journal of Agricultural and Food Chemistry, 43,* 2839-2845. doi: 10.1021/jf00059a013

Malheiro, J., Gomes, I., Borges, A., Bastos, M. M. S. M., Maillard, J. Y., Borges, F., & Simões, M. (2016). Phytochemical profiling as a solution to palliate disinfectant limitations. *Biofouling, 32*(9), 1007-1016. doi: 10.1080/08927014.2016.1220550

Nakamura, C. V., Ishida, K., Faccin, L. C., Dias, B. P., Fº., Cortez, D. A. G., Rozental, S.,... Ueda-Nakamura, T. (2004). In vitro activity of essential oil from Ocimum gratissimum L. against four Candida species. *Research in Microbiology, 155*(7), 579-586. doi: 10.1016/j.resmic.2004.04.004

Pandey, A. K., Singh, P., Palni, U. T., & Tripathi, N. N. (2014). In vivo evaluation of two essential oil based botanical formulations (EOBBFs) for the use against stored product pathogens and pests, Aspergillus species and Callosobruchus species (Coleoptera: Bruchidae). *Journal of Stored Products Research, 59,* 285-291. doi: 10.1016/j.jspr.2014.09.001

Pandey, A. K., Sonker, N., & Singh, P. (2016). Efficacy of some essential oils against Aspergillus flavus with special reference to Lippia alba oil an inhibitor of fungal proliferation and aflatoxin B1 production in green gram seeds during storage. *Journal of Food Science, 81*(4), M928-M934. doi: 10.1111/1750-38 41.13254

Reyes, R. G., Umagat, M. R., Garcia, B. L., Barza, A. J. J., Sumi, R., Mori, N.,... Eguchi, F. (2017). A new record of the mycoparasitic habit of Collybia reinakeana RGR-FE-NSC strain against Aspergillus flavus, Fusarium oxysporum and Cladosporium sphaerospermum. *International Journal of Pharmaceutical Research and Allied Sciences, 6*(3), 29-32.

Scollard, J., Francis, G. A., & O'Beirne, D. (2009). Effects of essential oil treatment, gas atmosphere, and storage temperature on Listeria monocytogenes in a model vegetable system. *Journal of Food Protection, 72*(6), 1209-1215. doi: 10.4315/0362-028X-72.6.1209

Shashidar, N. S. (2002). *Studies on bioactive natural compounds for their Antimicrobial and antioxidant properties.* Ph.D. thesis, Department of Microbiology, Osmania University, Hyderabad.

Suresh, P., Ingle, V. K., & Vijayalakshmi, V. (1992). Antibacterial activity of eugenol in comparison with other antibodies. *Journal of Food Science and Technology, 29*(4), 254-256.

Tak, J. H., & Isman, M. B. (2017). Acaricidal and repellent activity of plant essential oil-derived terpenes and the effect of binary mixtures against Tetranychus urticae Koch (Acari: Tetranychidae). *Industrial Crops and Products, 108,* 786-792. doi: 10.1016/j.indcrop.2017.08.003

Thoroski, J., Blank, G., & Biliaderis, C. (1989). Eugenol induced inhibition of extracellular enzyme production by Bacillus subtilis. *Journal Food Protection, 52,* 399-403. doi: 10.4315/0362-028X-52.6.399

Tongnuanchan, P., & Benjakul, S. (2014). Essential oils: extraction, bioactivities, and their uses for food preservation. *Journal of Food Sciences, 79*(7), R1231-1249. doi: 10.1111/1750-3841.12492