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Inactivation of Some Pathogens Inoculated to Noodle by *Syzygium aromaticum* (L.) Merr. & L.M.Perry Essential Oil

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ABSTRACT: In this study, it was aimed the the efficacy of *Syzygium aromaticum* essential oil (EO) on inactivation of *Eschericha coli* (ATCC 25293), *Klebsiella pneumoniae* (ATCC 10031), *Bacillus subtilis* (ATCC 6633), and *Staphylococcus aureus* (ATCC 25925) inoculated to noodle was investigated by dip incubation method. The components of *S. aromaticum* EO were analyzed by GC-MS and found the main components as eugenol, (*Z*)-9,17-octadecadienal (25.96%), (-)-caryophyllene (5.29%), and aceteugenol. Broth Microdilution and Agar Well Diffusion Method were used for antibacterial activity of *S. aromaticum* EO. Minimum Inhibitory Concentrations (MICs) of *S. aromaticum* were 14.8 mg mL^{-1} for *E. coli*, 10.98 mg mL^{-1} for *K. pneumoniae*, 9.29 mg mL^{-1} for *B. subtilis*, and 9.8 mg mL^{-1} for *S. aureus*, while the inhibition zones were between 3 mm and 7.1 mm for the pathogens. In the study, 100 µL of clove oil have 100% inhibition effect on *E. coli*, *K. pneumoniae*, and *S. aureus* attached to the noodle. However, *B. subtilis* was found to be quite resistant. As a result, the essential oil appears to be effective agains both Gram-negative and Gram-positive bacteria.

Keywords: *Syzygium aromaticum***,** essential oil, antimicrobial, food pathogens, noodle

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INTRODUCTION

Foodborne infections pose a significant danger to consumers. Therefore, one of the most important issues in the food industry is hygiene and sanitation. Various chemicals are used for pathogenic microorganisms carried by food, but antimicrobial agent research has gained continuity due to the development of resistance of microorganisms against them (Stermitz et. al., 2000; Shan et al., 2007). The indicator microorganisms used in relation to hygiene practices are *S. aureus* and *Bacillus* strains (Pamuk et al., 2018) in the food industry and *Escherichia coli* and *Klebsiella pneumoniae* found in the industrial wastewater (Guan and Holey, 2003; Cabral et al., 2010). Contamination of ready-to-eat foods with *S. aureus* is common during slicing, cooking or packaging of food and the its resistance to dry and stressful environments poses a threat to human health (Chaibenjawong and Foster, 2011; Syne et al., 2013). Another bacterial group important for food contamination is *Bacillus* species. Many species such as *B. cereus, B. subtilis, B. licheniformis* are well known as a cause of food-associated illness (Logan, 2012). In present study, we studied four important food pathogens and they can cause serious problems in the food industry. Previously, they have been shown to be isolated from cereal products such as, noodles (Rong and Xu-Hui, 2012), flour and bulgur (Yurdakul et al. 2017, Çetinkaya 2019).

Many herbs that have medicinal uses and that we use as spices have important pharmacological components due to their aromatic properties. Year by year, interest in active ingredients obtained from plants and their antimicrobial activities have increased due to the problem of bacteria gaining resistance to antimicrobial agents (Notermans et al., 1991; Shan et al., 2007). Among medicinal plants, *S. aromaticum* (synonym: *Eugenia caryophylata*), known as the clove tree and belongs to Myrtaceae family, is common in many countries such as East Indonesia, Sri Langka, Brazil, Jamaica, Turkey (Burt and Reinders 2003; Sohilait, 2015). Clove plant have been used commonly in fragrance and flavouring industries besides cooking as spice. Medically, it has the effect of relieve pain and promote healing because of significant biological components (Chaieb et al., 2017). Furthermore, its bioactive compounds have antimicrobial (Pundir et al., 2010), anti-inflammatory (Ozturk and Ozbek, 2005) and antioxidant (Jirovetz et al., 2006) properties.

As far as we know, paper related to the inactivation of pathogens contaminated with any food with clove oil is very few (Omidbeygi et al., 2007; Mytle et al., 2006). With this study, it was first investigated the inhibition performance of *S. aromaticum* against *E. coli, K. pneumoniae, B. subtilis*, and *S. aureus* on noodle.

MATERIAL AND METHODS

Preparing the *S. aromaticum* Essential Oil and GC-MS Analysis

S. aromaticum was collected from Köyceğiz/Muğla in 2018. Approximately, 500 grams of *S. aromaticum* seed sample was used for the essential oil extraction process. Solvent-extraction method was performed using a hydrodistillation with pure water for 2 hours. The mixture added to hexane. After liqiud-liqiud extraction, the aqua in organic phase was dried over anhydrous $MgSO_4$. The essential oil (EO) was concentrated after the solvent of organic phase was evaporated using the rotary evaporator under vacuum. Obtained essential oil was kept in desiccator. It was protected from sunlight until analysis. The components of *S. aromaticum* EO were analysed by GC-MS 7890A-(5975C inert MSD) instrument equipped with 30m X 250 μm film X 0.25 μm-thickness column (Agilent 19091S-433) with helium carrier gas. The EO was eluted for 64 minutes of retention time using initial temperature of 60°C for 5 min and temperature was raised to 150°C by an increase of 3°C for 2 min, by 3°C min^{-1} to 200°C, by 4°C min^{-1} to 240°C. The components of *S. aromaticum* EO were performed based on the mass spectra library (Wiley Registry 9th/NIST 2011 database, W9N11.L) (Yabalak, 2018).

Antibacterial Screening

To the antimicrobial tests, *E. coli* (ATCC 25293), *K. pneumoniae* (ATCC 10031), *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 25923) were taken from Refik Saydam Hıfzıssıhha Centre (Ankara/Turkey). The inoculums of *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* were prepared in 4 mL Triptic Soy Broth and incubated at 37°C, overnight. Then, the bacteria suspensions were adjusted to 0.5 McFarland Standard Turbidity and stored at +4°C until experiments (Sıcak and Erdogan Eliuz, 2019).

Broth Microdilution Method and Well Diffusion Method

According to Broth Microdilution, the two-fold serial dilutions of 50 µL *S. aromaticum* EO (1.160 g mL^{-1} in DMSO 10%) was performed into 96-well plates which was previously added 50 µL of MHB (Mueller Hinton Broth) medium along from 2nd to 10th columns. There are only MHB and microbe in 11 and 12 well as as negative control. Then, 5 µL culture of bacteria were inoculated in all wells except negative control. The plates were incubated at 37°C for 24 h and MIC (Minimum Inhibitory Concentration) was calculated as the lowest concentration where no visible turbidity was observed in each row of the 96-well plate (Patton et al., 2006; Sıcak and Erdogan, 2019).

To determine of inhibition zone of *S. aromaticum* on *E. coli, S. aureus, K. pneumoniae,* and *B. subtilis* were used Well Diffusion Method. For this, the bacteria cultures at stationary phase were spread onto Mueller Hinton Agar plates (MHA) and 6 mm-wells were drilled into the middle of petri. The 50 µL of *S. aromaticum* EO placed in the wells and incubated at 37°C during 24 h, calculated clear zones (Sıcak and Erdogan Eliuz, 2019). As positive control, Ampicillin was used for bacteria and the experiments were repeated three times.

Inactivation Method of Pathogens on Noodle by *S. aromaticum* EO

The inoculation of *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* to noodle was made using dip inoculation method (Singh et al., 2002). According this method, 0.02 g sample of noodle was dipped into 500 µL of inoculum (approximately 10^8 CFU mL^{-1}) prepared before and then shaken gently using a shaker incubator at 120 rpm for 1 min at 25°C to homogeneous distribution of organisms. At the end of each treatment, noodle was drained and washed immediately with 500 µL of sterile saline (0.9%) with agitation (120 rpm) for 1 min to remove residual oil. Then, three different amounts of *S. aromaticum* EO (50 µL, 1 mL and 1.5 mL) were added to the eppendorf with noodle and shaked (120 rpm) for 1 min. Then, noodles were transferred by sterile spatula into the eppendorf which is added previously 1 mL 0.9% saline. The eppendorf was mixed during 2 min and noodle was removed. Then, serial dilution (10^{-6}) of the liquid in the eppendorf was made with sterile 0.9% salin solution. To enumeration of surviving microorganisms, 0.1 ml sample was spread-plated on MHA. After the incubation of bacteria at 37°C and 24 h, the colonies were counted and logaritmic reduction were measured. The negative control was noodle without inoculation and the aqueous treatment.

Statistical Analysis

Statistical analyses and significance of MICs and Inhibition zone (IZ) were measured by Tukey test in one-way analysis of variance using SPSS 25 ($p \leq 0.05$). The experiments were repeated three times.

RESULTS AND DISCUSSION

Chemical Composition of *S. aromaticum* EO

The components of *S. aromaticum* were detected by GC-MS. The results of the chemical composition of *S. aromaticum* were presented in Table 1. *S. aromaticum* EO contained predominanly

eugenol (60.34%), (*Z*)-9,17-octadecadienal (25.96%), (-)-caryophyllene (5.29%), and aceteugenol (4.99%). The other components were eucalyptol, *p*-menthan-3-one, β-selinene, calamenene, (-)-caryophyllene oxide.

Table 1. Chemical composition of *S. aromaticum* EO

[a]RI	Compound	% [b]RA
1027	Eucalyptol	0.07
1152	*p*-Menthan-3-one	0.29
1370	Eugenol	60.34
1416	Caryophyllene	5.29
1451	β-Selinene	0.60
1521	Calamenene	0.18
1531	Aceteugenol	4.99
1581	(-)-Caryophyllene oxide	0.29
2476	(Z)-9,17-Octadecadienal	25.96

[a]Retention Index. [b]Relative area (peak area relative to the total peak area).

Antimicrobial Activity of *S. aromaticum* EO

The results showed that *S. aromaticum* EO was effective against *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* by broth microdilution and well diffusion method (Table 2). Any statistically significant difference did not see between the MICs of the oil against the pathogens. The MICs of *S. aromaticum* on *E. coli*, *K. pneumoniae*, *B. subtilis* and *S. aureus* were 14.8, 10.98, 9.29, 9.8 mg mL^{-1}, respectively. According to well diffusion method, IZs of the oil were 3.01 mm for *E. coli*, 5.02 mm for *K. pneumoniae*, 3.80 mm for *B. subtilis* and 7.1 mm for *S. aureus*. Statistically, there was a significant difference between *E. coli* (3.01 mm) and *S. aureus* (7.1 mm) IZs (Figure 1).

Many papers in different countries have shown that the main components are eugenol, *β*-caryophyllene and caryophyllene oxide although the minor components of clove oil differs, as in our work (Bhuiyan et al., 2010; Razafimamonjison et al., 2014; Sohilait, 2015). For instance, the major compounds in EO of *Eugenia caryophylata* was identified as eugenol (78.72%) and *β*-caryophylene (17.49%) and the antimicrobial performances of these two components have been previously reported (Marchese et al., 2017). *Syzygium polyanthum*, contained cis-4-decanal, 1-decyl aldehyde, and capryl aldehyde, and *Syzygium polyanthum*, contained *p*-eugenol and *β*-caryophyllene, were not inhibited *E. coli*. However, *B. subtilis* and *S. aureus* were inhibited with between MIC of 31.25 and 250 µg mL^{-1} (Hamad et al., 2017). In this study, all three bacteria were strongly inhibited with MIC of >9 mg mL^{-1} and IZ>3 mm by *S. aromaticum* EO that contain mostly eugenol and 9,17-octadecadienal. In another study, MICs of *Eugenia caryophyllata* were reported to be 1 µl mL^{-1} for *S. aureus*, *K. pneumoniae* and *B. cereus*, while it was 2 µl mL^{-1} for *E. coli*. In the same study, IZs were between 17 mm and 30 mm for these pathogens (Mahboubi and Mahboubi, 2015). In addition, infusion, decoction and oil of clove were reported that inhibits Gram negative bacteria including *E. coli* and *K. penumoniae* 8 mm and 23.75 mm (Saeed and Tariq, 2008). Differences in MIC and IZ levels may be caused by interactions with minor compounds other than eugenol.

In our study, the pathogens were almost susceptible against *S. aromaticum* as in some studies, while *Eugenia caryophylata* extract inactivated *B. cereus*, *S. aureus* and *E. coli* (Shan et al., 2007). The essential oil of clove essential oil was found to be quite effective against *B. cereus* and *E. coli*, However, *E. coli* O 157 and *K. pneumoniae* were moderately sensitive to the oil (Badhe et al., 2013). Mostafa et

al. (2018), showed that *B. cereus*, *S. aureus* and *E. coli* were susceptible with >11.9 mm IZs against *S. aromaticum*.

Table 2: MIC and IZ of *S. aromaticum* EO against *E. coli*, *K. pneumoniae*, *S. aureus* and *B. subtilis*.

	EO-MIC (mg mL^{-1})	A-MIC (µg mL^{-1})	EO-IZ (mm)	A-IZ (mm)
***E. coli* Gram (-)**	14.8 ^{a} ±0.12	16 ±0.22	3.01^{a}±0.04	10.1±0.03
***K. pneumoniae* Gram (-)**	10.98 ^{a} ±0.11	16 ±0.08	5.02 ^{ab} ±0.03	12.04 ±0.04
***B. subtilis* Gram (+)**	9.29 ^{a} ±0.09	32 ±0.01	3.80 ^{ab} ±0.02	4.3 ±0.01
***S. aureus* Gram (+)**	9.8 ^{a} ±0.23	32 ±0.03	7.1 ^{b} ±0.02	7.7 ±0.02

The mean MICs and IZs were expressed with the standard deviation (±) and significance level (ANOVA, 25; 0.05, Tukey test). In the same column, values marked with different exponential letters differ statistically at 0.05 level. A: Ampicillin: 16 µg/mL

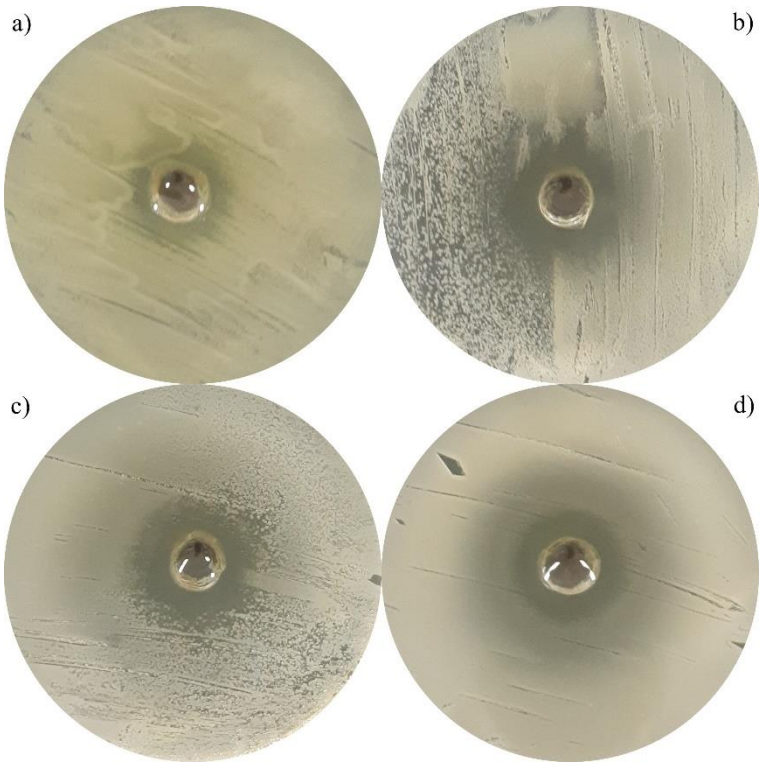

Figure 1: The images of tolerance and sensitivity levels of *E. coli* (a), *K. pneumoniae* (b), *B. subtilis* (c) and *S. aureus* (d) in exposure to *S. aromaticum.*

In this paper, the MICs of *S. aromaticum* EO (<15 mg mL^{-1}) were found to be higher than the MICs of Sethi et al. (2013), study which methanol extract of *S. aromaticum* were researched (MIC> 75 mg mL^{-1}) against bacteria. In this case, it can be said that its essential oil is more effective.

Inactivation Method of Pathogens on Noodle by *S. aromaticum* EO

Inactivation of *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* inoculated to noodle by *S. aromaticum* EO at 50 µL, 100 µL and 150 µL were investigated by dip inoculation method (Table 3). *S. aromaticum* EO at 50 µL caused between 0.4 and 8.0 log reduction all pathogens. Log reduction of *E. coli*, *K. pneumoniae* and *S. aureus* colony, at 50 µL and 100 µL of the EO, were 8.0 CFU mL^{-1}. However, log reduction of *B. subtilis* at 50 µL, 100 µL and 150 µL of the EO were 0.4, 0.8 and 0.9 CFU mg^{-1}.

Table 3: Log reduction in *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* on noodle by *S. aromaticum* EO

Log reduction (CFU mg^{-1})			
	S. aromaticum EO (g mL^{-1})		
	50µL	**100µL**	**150 µL**
E. coli	1.7	8.0	8.0
K. pneumoniae	1.5	8.0	8.0
S. aureus	8.0	8.0	8.0
B. subtilis	0.4	0.8	0.9
Control*		~1.5 x 10^8	

*Starting population

With this study, it was shown that the number of *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus* on noodle can be reduced by applying *S. aromaticum* EO. In the study, after 100 µL of clove oil, we can say about a 100% effect on *E. coli*, *K. pneumoniae*, and *S. aureus*. However, *B. subtilis* attached to the noodle was found to be quite resistant.

CONCLUSIONS

The oil appears to be rather effective against both Gram-positive and Gram-negative bacteria. This study indicates that the essential oil of *S. aromaticum* has antibacterial effect on *E. coli*, *K. pneumoniae*, *B. subtilis*, and *S. aureus.* At the same time, to reduce the risk of these pathogens multiplying on noodle, *S. aromaticum* EO components may be an alternative.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contiıbutions

The authors declare that they have contributed equally to the article.

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