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Determination of time dependent antibacterial activities of curcumin, carvacrol and styrax liquidus on *Salmonella* Enteritidis

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Abstract: *Salmonella* Enteritidis is amongst the most common causes of foodborne salmonellosis. Multi-drug resistant *Salmonella* strains has been associated with treatment failures. Plant-derived phytochemicals may be an alternative to antibiotics in combating these bacteria. The purpose of this study is to investigate the antibacterial activity of curcumin, carvacrol and styrax liquidus on *S.* Enteritidis and *S.* Enteritidis PT4. Minimum inhibitory concentration (MIC) values of these substances were detected at 1.5, 3, 7.5 and 24 h by broth microdilution method to evaluate their time-dependent antibacterial activities. The findings of the present study showed that MIC values of carvacrol, curcumin and styrax liquids for both *S.* Enteritidis and *S.* Enteritidis PT4 were 125.0 µg/mL, 132.5 µg/mL, 31.3 mg/mL for 24 h, respectively. Also, a time-dependent change was observed in the MIC values of curcumin. Carvacrol, curcumin and styrax liquidus can be used to provide antimicrobial effect on *S.* Enteritidis and *S.* Enteritidis PT4 in food applications, taking into account the MIC values and contact times.
Keywords: Carvacrol, curcumin, MIC, *Salmonella* Enteritidis, styrax liquidus.

Kurkumin, karvakrol ve sığla yağının *Salmonella* Enteritidis üzerine zamana bağlı antibakteriyel aktivitesinin belirlenmesi

Özet: *Salmonella* Enteritidis, gıda kaynaklı salmonellozisin en yaygın nedenleri arasındadır. Çoklu antibiyotiklere dirençli *Salmonella* suşları, tedavide başarısızlıklara neden olmaktadır. Bitki kaynaklı fitokimyasallar, bu bakterilerle mücadelede antibiyotiklere bir alternatif olabilir. Bu çalışmanın amacı, kurkumin, karvakrol ve sığla yağının *S.* Enteritidis ve *S.* Enteritidis PT4 üzerindeki antibakteriyel aktivitesini araştırmaktır. Bu maddelerin minimum inhibitör konsantrasyon (MİK) değerleri, zamana bağlı antibakteriyel aktivitelerini değerlendirmek için broth mikrodilüsyon yöntemi ile 1,5, 3, 7,5 ve 24. saatlerde belirlenmiştir. Çalışmanın bulgularında, hem *S.* Enteritidis hem de *S.* Enteritidis PT4 için karvakrol, kurkumin ve sığla yağının MİK değerlerinin 24 saat boyunca sırasıyla 125,0 µg/mL, 132,5 µg/mL, 31,3 mg/mL olarak tespit edilmiştir. Ayrıca, kurkuminin MİK değerlerinde zamana bağlı bir değişiklik de görülmüştür. MİK değerleri ve temas süreleri dikkate alındığında karvakrol, kurkumin ve sığla yağının, gıda uygulamalarında *S.* Enteritidis ve *S.* Enteritidis PT4 üzerinde antimikrobiyal etki sağlamak için kullanılabileceği sonucuna varılmıştır.
Anahtar sözcükler: Karvakrol, kurkumin, MİK, *Salmonella* Enteritidis, sığla yağı.

Introduction

Foodborne diseases resulting from ingestion of contaminated food by a defined list of microbes, parasites and chemicals are cause of morbidity and mortality worldwide (4). The World Health Organization (WHO) estimated that they affect 600 million people and 420,000 deaths occur annually in 2010, resulting in the loss of 33 million healthy life years. *Salmonella* species are responsible for a quarter of 550 million diarrheal illness worldwide each year (48). It is estimated that 155,000

deaths occur in the world each year due to bacteria *Salmonella* spp. (10). *Salmonella* spp. reside in the gastrointestinal tract of different domestic animals and are usually present in stool exerted by healthy animals and may contaminate fruits and vegetables and foods of animal origin (27, 46). Thus, *Salmonella* can be spread between human and animals, and cause disease. *Salmonella* Enteritidis and *S.* Enteritidis PT4 are most commonly isolated serotypes from foods of animal origin and are important causes of infections associated with these foods in humans (18, 47).

Antimicrobials play an important role in the control of bacterial foodborne infections. However, misuse and overuse of antibiotics in the management of human and animal diseases encourage the bacteria to develop resistance. The rapid emergence of antimicrobial resistance has been a global problem for managing the health care of people and animals (30). It has been reported that there is a link between the use of antimicrobials in livestock and the emergence of antimicrobial resistance in pathogenic bacteria (13, 27). Spread of resistant bacteria from animals to humans may occur through foods, environment, or direct interaction with animals and leads to great challenges in infection control (19). The emergence and spread of resistance to multiple antibiotics as well as a lack of new drug development by the pharmaceutical industry has led to an increase interest in medicinal plants. Various crude extracts or individual compounds and essential oils of the medicinal plants could serve as an alternative source of new antimicrobials due to a broad range of secondary metabolites (15, 23).

Several plant-derived compounds including carvacrol and curcumin have attracted the attention of the scientific community for their antimicrobial properties. Carvacrol, the main active ingredient of essential oils, are known by its broad-spectrum antimicrobial and antioxidant activities (6, 11, 33). Curcumin, a polyphenolic natural ingredient derived from *Curcuma longa* roots, is known to exert antimicrobial activity against a variety of bacterial species (2, 26). Styrax liquidus, locally named as “sığla yağı” is a resinous exudate (balsam) obtained from the incision trunk of *Liquidambar orientalis* Miller tree which is an endemic species in Türkiye. It has been used for the treatment of peptic ulcer in Turkish folk medicine (16). Styrax liquidus consists of resin, essential oils, styrene and cinnamic acid (16, 20). There are few studies investigated the antibacterial activity of styrax liquidus (35).

There is no research investigating antibacterial activity of these substances, becoming popular in the world and Türkiye, on *Salmonella* species. Hence, it was aimed to investigate of antibacterial activities of carvacrol, curcumin and styrax liquidus on *S*. Enteritidis and *S.* Enteritidis PT4 at different time parameters (1.5, 3, 7.5 and 24 h) in the present study.

Materials and Methods

Compounds and Materials: Carvacrol (Sigma, 282197) and curcumin (C1386, Sigma, ≥65%) were purchased from Sigma-Aldrich (Madrid, Spain), Styrax liquidus (Sweetgum) was purchased from local producers. Carvacrol and curcumin were dissolved in dimethyl sulfoxide (DMSO, Aldrich, 99.5%) to prepare stock solutions of 500 mg/mL for carvacrol (w/v, 1/1) and 3.6 mg/mL for curcumin (curcumin/DMSO, w/v, 8.33 mg/1.5 mL). Sweetgum was dissolved in absolute ethanol (Sigma, 1.02428) to prepare stock solution of 500 mg/mL (v/v, 1/1). Mueller Hinton broth (MHB, CM0405) and Mueller Hinton agar (MHA, CM0337) were purchased from Oxoid (Oxoid Ltd., Basingstoke, England).

Bacterial Strain: American Type Culture Collection (ATCC) standard *S.* Enteritidis (ATCC: 13076) and National Collection of Type Cultures standard *S.* Enteritidis PT4 (NCTC: 13349) were used for the screening antibacterial activities of sweetgum, carvacrol and curcumin. The strains were incubated at 37°C for 24 hours to evaluate the antibacterial activity of plant-derived compounds.

Evaluation of Antibacterial Activity: The MIC values of carvacrol, curcumin and styrax liquidus against *S.* Enteritidis and *S.* Enteritidis PT4 were determined according to the microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (8). Briefly, the MICs of the carvacrol, curcumin and styrax liquidus were investigated at eight different concentrations ranged from 7.813 µg/mL to 1000 µg/mL, 5.07 µg/mL to 650 µg/mL and 1.95 mg/mL to 250 mg/mL, respectively. Serial 2-fold dilutions of carvacrol, curcumin and styrax liquidus were prepared in MHB. Two hundred microliters of each different concentrations of carvacrol, curcumin and styrax liquidus were added into the wells of a 96-well plate as separate study groups. Then, 20 µL containing 0.5 McFarland cell/mL bacteria was added each well (9). The well plates were incubated at 37°C under aerobic conditions for 24 h. The MIC was defined as the lowest concentration of the substance where bacterial growth was not detected. All trial groups were carried out in triplicate. Absorbance was measured at 600 nm (OD600) to monitor the microbial growth by using a microplate reader (Epoch, BioTek, USA) at 1.5, 3, 7.5 and 24 h of incubation. In the evaluation of optical density values, the average of the trials of each bacterium in triplicate were taken. It was found by taking the difference of the values given as "blank" from the reproductive optical density values of the relevant bacteria. The lowest substance concentration with no microbial growth and OD600 value of ≤0.1 was accepted as the MIC value (21). The effects of solvents used on bacterial growth was examined using the MIC method at the final concentration of solvents in each well. It was observed that the final concentration of the solvents in each well did not show any inhibitory effect against bacterial growth.

Results

The broth microdilution test was carried out to assess the antimicrobial activities of carvacrol, curcumin and styrax liquidus on *S.* Enteritidis and *S.* Enteritidis PT4 at 1.5, 3, 7.5 and 24 h. All MIC values are given in Table 1. Since bacterial growth could not be observed clearly, the MIC values of curcumin and styrax liquidus at 1.5 and 3 h could not be determined. Similarly, the MIC values of carvacrol could not be determined at 1.5 h. According to the results of the study, although there was no time-dependent change in the MIC values of carvacrol and styrax liquidus, a time-dependent change was observed in the MIC values of curcumin. Curcumin exhibited the excellent antimicrobial activity against these bacteria in 24 h of treatment. After 7.5 h of application, the MIC value of curcumin against *S.* Enteritidis was 650 µg/mL while it was 325 µg/mL against *S.* Enteritidis PT4. However, in 24-h application, curcumin presented similar MIC values against both bacteria (Figure 1).

According to the MIC values, Although *S.* Enteritidis and *S.* Enteritidis PT4 were found to be less susceptible to styrax liquidus (MIC value: 31.3 mg/mL), they were more susceptible to carvacrol (125 µg/mL). In the wells treated with curcumin, optical densities up to 0.25 (OD600 nm) were observed in the measurements made at 1.5 and 3 h. In this study, the solvents (Ethanol and DMSO) used to dissolve the substances did not show any antibacterial activity at the application doses.

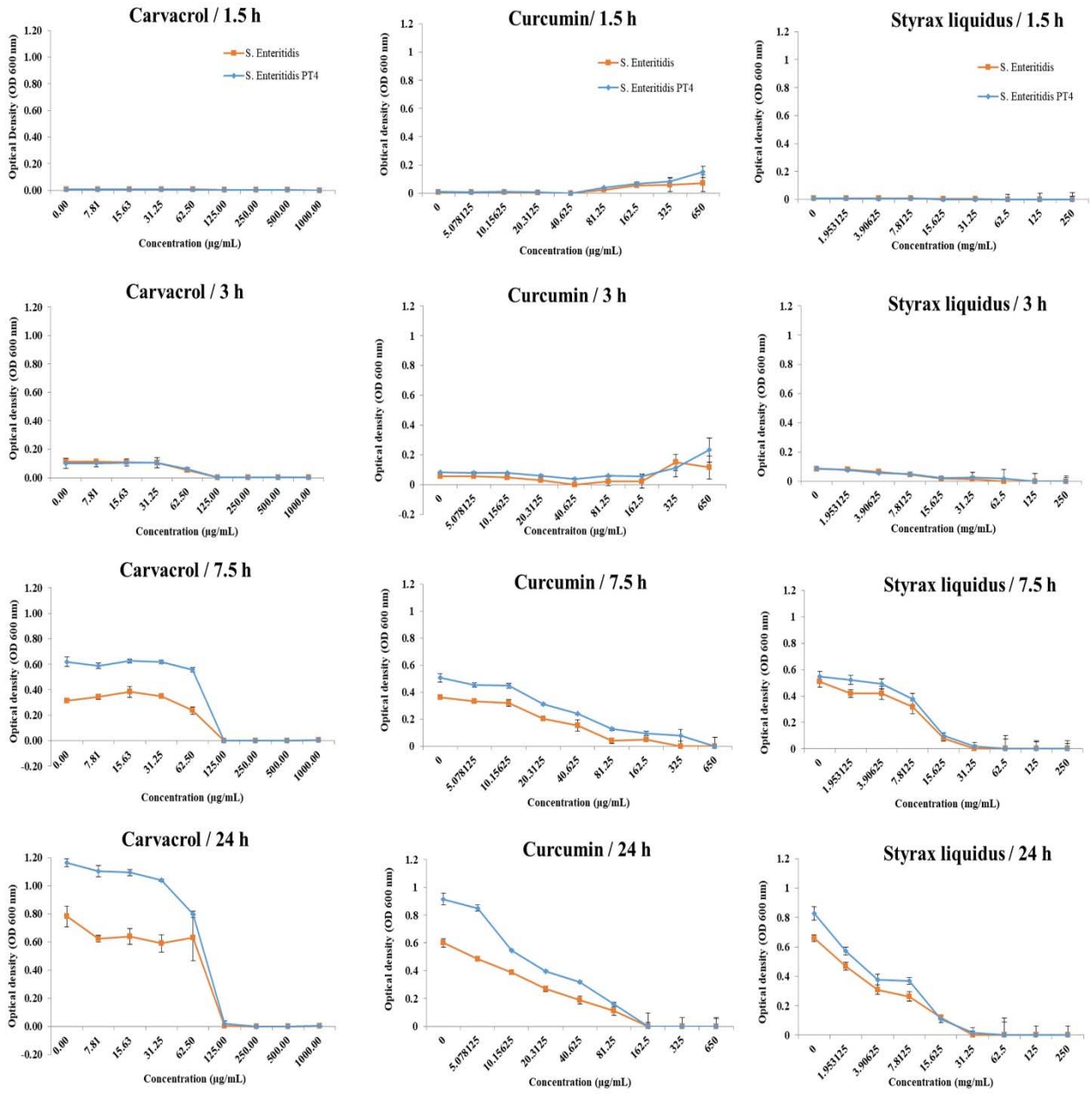

Figure 1. The growth rates of *S.* Enteritidis and *S.* Enteritidis PT4 according to exposure time and concentration of substances.

Table 1. MIC values of Carvacrol, Curcumin and Styrax Liquidus at 37ºC for 1.5, 3, 7.5 and 24 h.

Times	Trials	Carvacrol (µg/mL)		Curcumin (µg/mL)		Styrax Liquidus (mg/mL)	
		S. Enteritidis	*S.* Enteritidis PT4	*S.* Enteritidis	*S.* Enteritidis PT4	*S.* Enteritidis	*S.* Enteritidis PT4
1.5 h	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
	3	*	*	*	*	*	*
3 h	1	125.0	125.0	*	*	*	*
	2	125.0	125.0	*	*	*	*
	3	125.0	125.0	*	*	*	*
7.5 h	1	125.0	125.0	650.0	325	31.3	31.3
	2	125.0	125.0	650.0	325	31.3	31.3
	3	125.0	125.0	650.0	325	31.3	31.3
24 h	1	125.0	125.0	162.5	162.5	31.3	31.3
	2	125.0	125.0	162.5	162.5	31.3	31.3
	3	125.0	125.0	162.5	162.5	31.3	31.3

Discussion and Conclusion

Antibacterial (antibiotic) drug resistance is a growing global problem and the number of new approved drugs is declining. Hence, the need for new antimicrobials becomes more pressing in bacterial infections (22, 31). Plant extracts and essential oils are known as a good source of antimicrobial substance effective on foodborne pathogens as they have antibacterial, antifungal, antiparasitic and antiviral properties (40, 41). There is a great number of natural compounds isolated from plants, a part of them has an important role in food, cosmetics, sanitary fields and oral-dental treatments (32, 37). Recently, there has been an increased interest in the assessment of the antimicrobial potential of natural plant compounds such as curcumin and carvacrol against pathogen bacteria (7, 23, 24).

Carvacrol has been reported as antibacterial agent. However, the reported values of MICs are widely divergent. Several studies have reported that carvacrol shows antibacterial activity against *S.* Enteritidis with MIC values ranging 156 to 331 µg/mL (5, 6, 11, 33). The result of this study showed a MIC value for carvacrol of 125 µg/mL for *S.* Enteritidis and *S.* Enteritidis PT4. The MIC and above concentrations of carvacrol inhibited the growth of *S.* Enteritidis and *S.* Enteritidis PT4 at 3, 7.5 and 24 h. The antibacterial activity of carvacrol has been attributed to its hydrophobic property that influences the fluidity and permeability of the bacterial cell membrane by changing the lipid fraction (34). Also, carvacrol leads to the leakage and loss of ATP from bacterial cells (44, 45). The membrane fluidity has been found to play an important role in the bactericidal activity of the carvacrol against *Bacillus cereus* (44). In this study, at concentration of 125 µg/mL and above, total inhibition of the growth was observed and carvacrol may be bactericidal towards *S.* Enteritidis and *S.* Enteritidis PT4. The bactericidal activity against these bacteria may be due to affecting the fluidity and permeability of bacterial cell membrane.

Curcumin, naturally found as a constituent of dietary species called turmeric (*Curcuma longa*), has been the subject of intensive investigation on its various activities including antiviral, antibacterial and anticancer (39). Contrary of these, there is an evidence that curcumin increases the resistance of *S.* Typhimurium resistance to antimicrobial agents such as antimicrobial peptides, reactive oxygen and nitrogen species. The tolerance developed may be attributed to the up-regulation of genes involved in resistance to some antimicrobial peptides and genes with antioxidant function (28). Adamczak et al. (1) reported that curcumin exhibit a significantly larger variation in the its antibacterial activity (MICs ranged from 31.25 to 5000 µg/mL against over 100 strains of pathogens belonging to 19 species) and suggested that curcumin can be considered as a promising antibacterial agent but, with a very selective activity. Several studies have reported that curcumin shows strong antimicrobial activity against Gram-positive than Gram-negative bacteria with MIC values ranged from 62.5 µg/mL to 5000 µg/mL (1, 14, 36, 38). Further detailed studies are needed to investigate its antibacterial activity. There are few studies investigating the antibacterial activity of carvacrol on *Salmonella* spp. A study reported that MIC value for curcumin were found to be 250 µg/mL for *S.* Typhimurium (36). In the current study, even though curcumin exhibited the excellent antimicrobial activity against both *S.* Enteritidis and *S.* Enteritidis PT4 (MIC value of 125 µg/mL) in 24 h exposure, the MIC values of curcumin against *S.* Enteritidis and *S.* Enteritidis PT4 were 650 µg/mL and 325 µg/mL, respectively, at 7.5 h of exposure. As the exposure time of curcumin to these

bacteria increased, its antibacterial activity strengthened. Curcumin shows it antibacterial activity through various mechanisms, including inhibiting bacterial DNA replication, altering gene expression and disrupting the bacterial cell membrane hence it can affect the cell division and proliferation of bacteria (1, 42). Increased antibacterial activity over time in this study might be attributed to its mechanism of action. Also, it was thought that the high OD observed at high concentration of curcumin (325 and 659 µg/mL) at 1.5 and 3 h measurements might be due to its coloring properties, low water solubility and poor chemical stability (25).

Styrax liquidus which is a resinous exudate obtained from the wounded barks of *Liquidambar orientalis* mainly consists of acid, ester, alcohol, phenolic and volatile compounds. Its main components are cinnamic acid, styracin, styrol, stoyrone, storesinol, storesin, cinnamyl cinnamate, 3-phenylpropyl cinnamate, benzyl cinnamate, styrene, trans cinnamyl alcohol, hydrocinnamyl and vanillin. Its composition may vary widely depending upon a number of factors such as collection site, processing and storage conditions (3, 16). It has been used against parasitic infections, for treatment of peptic ulcers and burns, and as antiseptic in Turkish traditional medicine (12, 17, 43). In an *in vitro* study investigating antibacterial activity against 20 different strains of bacteria using an agar diffusion method, the results showed that styrax liquidus inhibited completely the growth of 13 bacteria at a 10% concentration and did not inhibit the growth of any bacteria at a 0.1% concentration. All treatment concentration (10%-0.1%) were inactive against 7 bacteria (35). There is literature on the antimicrobial activity of Styrax liquidus on *Salmonella* spp. Our study showed that it has an inhibitory effect on *S.* Enteritidis and *S.* Enteritidis PT4 at high concentrations (MIC values of 31.2 mg/mL for both). Its antimicrobial activity may be attributed to the presence of substances with antimicrobial activity, such as cinnamic acid, in its composition.

In conclusion, carvacrol and curcumin have stronger antibacterial activities than styrax liquidus. While curcumin exhibit its strongest antibacterial effect at 24 h, carvacrol and styrax liquidus showed at 7.5 h. The antibacterial effect of carvacrol and styrax liquidus started at seven and a half hours of administration. Carvacrol and styrax liquidus can be used to provide antimicrobial effect on *S.* Enteritidis and *S.* Enteritidis PT4 in food applications at lower exposure times.

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Ethical Statement

This study does not present any ethical concerns.

Conflicts of interest

The authors declare no conflict of interest.

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