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Inactivation of *Bacillus subtilis* and *Candida tropicalis* by *Wisteria sinensis* maceration oil

Willeria sinensis maserasyon yağı ile *Bacillus subtilis* ve *Candida tropicalis* inaktivasyonu

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Abstract
In this study, the antimicrobial effect of maceration oil obtained from *W. sinensis* flowers oil soaked in olive oil on *B. subtilis* and *C. tropicalis* was investigated. In addition, the efficacy of *W. sinensis* maceration oil on inactivation of the strains of *B. subtilis* and *C. tropicalis* inoculated bulgur (pounded wheat) was investigated using dip incubation method. The components of *W. sinensis* macerate were analyzed by GC-MS and found the main components as olealdehyde (38.03%), oleic acid (29.13%), 9-octadecenoic acid (15.09%), (*Z*)-9,17-octadecadienal (7.87%) and palmitic acid (5.97%). Broth Microdilution and Agar Well Diffusion Method for antimicrobial activity of *W. sinensis* and also Modified TDtest for persistent/tolerant levels of microorganisms were used. Minimum Inhibitory Concentrations (MICs) of *W. sinensis* were 10.3 mg/mL and 9.6 mg/mL for *B. subtilis* and *C. tropicalis* while the inhibition zones were 2.23 mm and 2.07 mm, respectively. In TDTest which was made persistent/tolerant screening of microorganisms in *W. sinensis* condition, both of microrganisms were persistent sensitive. *W. sinensis* at 50 µL, 100 µL and 150 µL caused an almost 2-log reduction on the number of *B. subtilis* and *C. tropicalis* on bulgur.

Özet
Bu çalışmada, zeytinyağına batırılmış *W. sinensis* çiçeklerinden elde edilen maserasyon yağının *B. subtilis* ve *C. tropicalis* üzerindeki antimikrobiyal etkisi araştırıldı. Ek olarak, *W. sinensis* maserasyon yağının, bulgura (dövülmüş buğday) aşılanmış *B. subtilis* ve *C. tropicalis* suşlarının inaktivasyonu üzerindeki etkinliği daldırma inkübasyon metodu kullanılarak araştırıldı. *W. sinensis* maserat bileşenleri GC-MS ile analiz edildi ve ana bileşenleri olealdehit (%38.03), oleik asit (%29.13), (*Z*)-9,17-oktadekadienal asit (%15.09), (*Z*)-9,17-oktadekadienal (%7.87) ve palmitik asit (%5.97) olarak bulundu. *W. sinensis*'in antimikrobiyal aktivitesi için Sıvı Mikrodilüsyon ve Agar Kuyucuk Difüzyon Yöntemi ve ayrıca kalıcı/toleranslı mikroorganizma seviyeleri için Modifiye TDtest kullanıldı. *W sinensis*'in Minimum İnhibitör Konsantrasyonları (MİK), *B. subtilis ve C. tropicalis* için 10.3 mg/mL ve 9.6 mg/mL iken inhibisyon zonları sırasıyla 2.23 mm ve 2.07 mm idi. *W. sinensis* varlığında mikroorganizmaların kalıcı/toleranslı taranması yapılan TDTest'te her iki mikroorganizmanın da kalıcı duyarlılığı saptandı. *W. sinensis*'in 50 µL, 100 µL ve 150 µL'si, bulgur üzerindeki *B. subtilis* ve *C. tropicalis* sayısında neredeyse 2 log azalmaya neden oldu.

INTRODUCTION

Foodborne diseases are increasing day by day in the world and are a major problem for both human health and the food industry. The most effective way to prevent these diseases is to use antimicrobial agents that will prevent the development of harmful microorganisms transmitted to food by air, water or human hands. The use of herbs in the fight against infection for centuries is still up to date (Şengün and Öztürk 2018). Many microorganisms (*Bacillus subtilis*, *Candida tropicalis, Candida glabrata, Staphylococcus aureus*, *Candida parapsilosis, Escherichia coli*), and mycotoxin-producing fungi are classified as food-borne pathogens that are responsible for infection and food poisoning (Jacques and Casaregola 2008, Loureiro and Malfeito-Ferreira 2003, Chen et al. 2016).

While, many *Bacillus* species such as *B. subtilis, B. licheniformis, B. cereus,* are well known as a cause of food poisoning and food-associated illness (Logan 2012), among yeast, in the past two decades, infections have been reported to increase due to non-albicans species

(Fidel et al. 1999, Miguel et al. 2005). Some species of *Candida* genus among yeasts such as *C. parapsilosis, C. glabrata*, and *C. tropicalis* with those species cause high morbidity and mortality rates because of nosocomial bloodstream infections (Wisplinghoff et al. 2004, Yapar 2014, Pappas et al. 2016). In this study, we studied two important species among known pathogens; *B. subtilis* and *C. tropicalis*. Because, *B. subtilis* and *C. tropicalis* microorganisms have been shown to be isolated from cereal products such as flour and bulgur. Therefore, these microorganisms can cause serious problems in the food industry (Yurdakul et al. 2017, Çetinkaya 2019).

Antimicrobials obtained from plants such as secondary metabolites are significant sources of novel therapeutics and have been used in conventional medicine for years. Today, although these microbes are struggled with synthetic drugs, plant agents and especially their secondary metabolites are still up to date in development of new antimicrobial drugs (Nascimento et al. 2000, Preethi et al. 2010). Among medicinal plants, *W. sinensis*, belongs to Fabaceae family, is known as perennial shrub-like climbing vine plant. It is commonly distributed in natural forests, riparian zones and ruderal areas because of the fast growth, long lifespan and hardiness (Cook et al. 2015, Li et al. 2017, Jiang et al. 2011, Jiang et al. 2011). Wisteria is a plant native to the Eastern United States and East Asian countries of Korea, China, and Japan is widely used as an ornamental plant. Pharmacologically, it has been reported that some doctors use Wisteria extracts in the treatment of stomach cancer and treat rheumatoid arthritis patients. Antimicrobial activities of various species have been determined (Mohamed et al. 2011). In addition, there are several studies showing that phenylpropanoids and-chromenes and some triterpenes have been detected from Wisteria extracts in previous studies (Konoshima et al. 1989, Joulain and Tabacchi 1994). As far as we know, *W. sinensis* studies on food borne pathogens are very few. Therefore, with this study, it was first investigated the antimicrobial activity on *B. subtilis* and *C. albicans* of *W. sinensis* macerate. Then, it was reported inhibition performance of *W. sinensis* against *B. subtilis* and *C. tropicalis* on bulgur.

MATERIAL AND METHODS

Materials and instruments

Triptic Soy Broth (TSB), Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB) were supplied from Merck (Darmstadt, Germany). *B subtilis* and *C. tropicalis* were taken from Refik Saydam Hıfzıssıhha Centre (Ankara/Turkey). Bulgur was purchased from the spice store in Mersin/TURKEY (2020).

Preparing the maceration oil and GC-MS analysis

500 g fresh *W. sinensis* flowers were kept in 300 g olive oil for 21 days in the sun. At the end of the 21 days, the flowers were drained, the filtrate was obtained as *W. sinensis* macerate. The components of macerated *W. sinensis*, were analysed by GC-MS 7890A-(5975C inert MSD) instrument equipped with column (Agilent 19091S-433; 30m X 250 µm film X 0.25 µm thickness) with helium carrier gas. The oil was eluted for 64 minutes of retention time using initial temperature of 60°C for 5 min and temperature was gradually raised to 150°C by an increase of 3°C/min for 2 min, by 3°C/min to 200°C and by 4°C/min to 240°C. The characterization of the components of *W. sinensis* macerated were performed based on the mass spectra library (Wiley Registry 9th/NIST 2011 database, W9N11.L) (Yabalak 2018).

Antimicrobial screening

The inoculums of *B. subtilis* and *C. tropicalis* were prepared in 4 mL TSB and 4 mL SDB, respectively, and incubated at 37°C, overnight. Then, the pathogen suspensions were adjusted to 0.5 McFarland Standard and stored at +4°C until experiments.

Broth Microdilution Method

The two-fold serial dilutions of 50 µL *W. sinensis* macerate (0.2 µL in DMSO 10%) was made into 96-well plates which was previously added 50 µL of MHB medium along from 2^{nd} to 10^{th} columns. The column 11 and 12 were used as negative control (only MHB and microbe). Then, 5 µL of microorganism culture were inoculated on the wells except negative control and were incubated at 37°C for

24 hours. As Positive controls, Ampicillin and Fluconazole were used for bacteria and yeast, respectively. MIC was determined as the lowest concentration where no visible turbidity was observed in the each row of the 96-well plate (Sıcak and Erdoğan 2019).

Agar Well Diffusion Method and Modified TDtest

To determine of inhibition zone of *W. sinensis* on *B. subtilis* and *C. tropicalis* were used well diffusion method. For this, the microbe cultures at stationary phase were spread onto MHA plates and 6mm diameter wells were drilled into the middle of petri. The 50 µL of *W. sinensis* oil placed in the wells and incubated at 37°C for 24 h, calculated clear zones. To evaluate tolerance or persistence levels in *B. subtilis* and *C. tropicalis* against *W. sinensis* were used by TDtest (Tolerance Disc Test) originated Kirby-Bauer disk diffusion method. TDtest was created by expanding the disc diffusion with a few simple techniques. This method consists of two steps: First: MIC values of *W. sinensis* were used for well difusion method. Second: 50 µL glucose solution (10%) was placed in the well which discharged because of the diffusion of the oil into the agar. The alteration in the zone regions of the petri dishes re-incubated during 37°C for 24 h were measured and compared with the clear zone in the primary step. According to the method, it is interpreted as susceptible strain (S) if inhibition zone were found around the well after glucose addition and tolerant strain if colonies inside the clear zone after glucose (Gefen et al. 2017).

Inactivation method of pathogens on bulgur by *Wisteria sinensis* macerate

The inoculation of *B. subtilis* and *C. albicans* to bulgur was made using dip inoculation method (Singh et al. 2002). According this method, 0.03 g sample of bulgur was dipped into 500 µL of inoculum (approximately 10^8 cfu/mL) prepared before and then shaken gently using an shaker incubator at 120 rpm for 1 min at room temperature to ensure an even distribution of organisms. At the end of each treatment, bulgur were drained and washed immediately with 500 µL of sterile saline (0.9 %) with agitation (120 rpm) for 1 min to remove residual oil. The number of *B. subtilis* and *C. albicans* on bulgur inactivated by *W. sinensis* macerate using were logarithmically calculated. For enumeration of microorganisms, bulgur were transferred into eppendorfs added previously 500 µL 0.9% saline by sterile spatula. The eppendorfs were mixed during 2 min and serially diluted (10^{-6}) in 9 mL of sterile 0.9% salin solution and was spread-plated on MHA. After incubated of plates for 24 h at 37°C, the colonies counted and logaritmic reduction were measured. The negative control was bulgur without inoculation and aqueous treatment.

Statistical analysis

Statistical analyses of MICs and IZ were measured by Tukey test in one way analysis of variance using ANOVA SPSS 25 ($p \leq 0.05$).

RESULTS AND DISCUSSION

Chemical composition of macerated *W. sinensis*

The components of *W. sinensis* were detected by comparing the relative RI (retention index) and mass spectra from data library. The data of the chemical composition of *W. sinensis* were presented in Table 1. *W. sinensis* contained predominanly olealdehyde (38.03%), oleic acid (29.13%), 9-octadecenoic acid (15.09%), (*Z*)-9,17-octadecadienal (7.87%) and palmitic acid (5.97%). The other components were farnesol (2.20%) and cyclooctene (1.16%). The resemblance of these components with olive oil is due to the fact that maceration is made in olive oil. It was common known that the main component in olive oil were palmitic, oleic and oleic derivates (Andjelkovic et al. 2009; Erdoğan 2020).

Antimicrobial activity and response of microorganisms according to TDTest

The results showed that *W. sinensis* was effective against *B. subtilis and C. tropicalis* by Broth Microdilution and Agar Well Diffusion Method (Table 2). There was no statistically significant difference between the MICs of the oil against the pathogens. The MICs of *W. sinensis* on *B. subtilis and C. tropicalis* were 10.3 mg/mL and 9.6

mg/mL, respectively. Also, it wasn't seen any statistically significant difference between inhibition zones at the end of the 24 h incubation, they were 2.23 mm and 2.07 mm for *B. subtilis and C. tropicalis*, respectively.

Table 1. Chemical composition of macerated *W. sinensis*

	Compound	% [b]RA
54.811	Palmitic acid	5.97
62.361	Oleic acid	29.13
63.121	9-Octadecenoic acid	15.09
68.089	Olealdehyde	38.03
71.341	Cyclooctene	1.61
72.967	(*Z*)-9,17-octadecadienal	7.87
74.042	Farnesol	2.20
	Total	**99.9**

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

In this study, it was applied the TDtest with *W. sinensis* oil and *B. subtilis and C. tropicalis* were sensitive at the end of the 48 h incubation (p<0.05). It is clear that the oil had persistent antimicrobial against *B. subtilis and C. tropicalis* (Figure 1). According to this study, the reason for the antimicrobial effect is due to the components in the maceration. The interaction of olive oil and *W. sinensis* may have caused the antimicrobial effect to be strong and permanent. Many studies have reported that various components of plant oils interact with another antimicrobial agents to changes antimicrobial effect and may lead to new approaches in treatment infectious (Hammer et al. 2012, Kon and Rai 2012, Erdoğan 2020).

Inactivation method of pathogens on bulgur by *Wisteria sinensis* macerate

Inactivation of *B. subtilis* and *C. tropicalis* inoculated to bulgur by *W. sinensis* macerate 50 µL, 100 µL and 150 µL were investigated by dip inoculation method (Table 3). *W. sinensis* oil at 50 µL, 100 µL and 150 µL caused an almost 2-log reduction in each two pathogens.

The number of B. subtilis colony, at 50 µL, 100 µL and 150 µL of concentration, was changed between $2x10^6$ and $2.3x10^6$, respectively. In *C. tropicalis*, it was between $1.3 x10^6$ and $2.2 x10^6$.

Table 2: Minimal Inhibition Concentration and Inhibition zone (mm) of macerated *W. sinensis* against *B. subtilis and C. tropicalis.* Res: Response of microorganisms in step 2 according to TDTest, S: Susceptible strain.

	MIC (mg/mL)	IZ (24 h)	IZ (48 h)-Res
Mac. for *B. subtilis*	10.3[a] ±0.01	2.23[a]±0.03	1.76[a]±0.08-**S**
Mac. for *C. tropicalis*	9.6 [a] ±0.02	2.07[a]±0.02	1.76[a]±0.02-**S**
Ant. for *B. subtilis* (64 µg/mL)	32±0.11	15.9±0.31	15.8±0.03-**S**
Ant. for *C. tropicalis* (64 µg/mL)	64±0.12	10.2±0.13	10.2±0.10-**S**

The average MICs were expressed with the standard deviation (±) and significance level (ANOVA, 25; 0.05, Tukey test). "a": not differ statistically at the 0.05 level.Ant: antibiotic, Mac: macerated *W. sinensis.*

Table 3: Log reduction in *B. subtilis* and *C. tropicalis* on bulgur by *W. sinensis*

	Log reduction (CFU/mg)					
	B. subtilis			***C. tropicalis***		
	50µL	**100µL**	**150 µL**	**50µL**	**100µL**	**150 µL**
	$2.3x10^6$	$2.07 x10^6$	$2 x10^6$	$2.2 x10^6$	$1.4 x10^6$	$1.3 x10^6$
Log reduction	~2 log	~2 log	~2 log	~2 log	~2 log	~2 log
Control*		~$1.5 x 10^8$			~$1.5 x 10^8$	

*Starting population

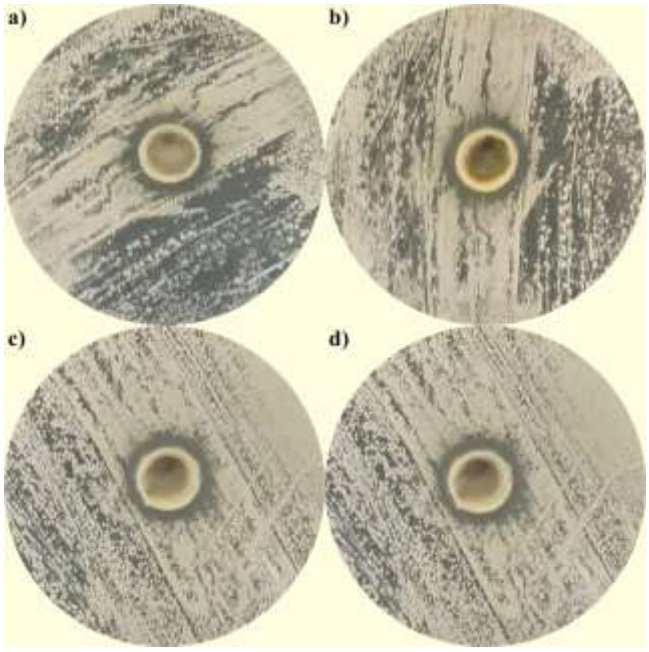

Figure 1: The images of tolerance and sensitivity levels of *B. subtilis* (a,b) and *C. tropicalis* (c,d) in exposure to *W. sinensis*. The first (a, c) and second (b, d) step of TDtest.

With this study, it was showed that *W. sinensis* extract can be used in the inhibition of pathogens found on surfaces of dry foods. It is clear that the number of *B. subtilis* and *C. tropicalis* on bulgur was been reduced by applying *W. sinensis* maceration oil. There are no studies in the literature regarding the industrial use of *W. sinensis* essential oil. However, its antimicrobial efficacy can be improved with more extraction methods. Furthermore, it is possible to use *W. sinensis* oil components by using microcapsulation technologies. The use of essential oils in increasing the shelf life of food because of their high antimicrobial properties is quite important for food industry. However, because of their instability, new technologies such as microcapsulation is needed to take advantage of its antimicrobial properties for longer (Jackson and Lee 1991, Beristain et al. 2001). Herbal sources with natural antimicrobial effects that can be used in the food industry are considered to be more reliable than many other antimicrobial products (Tajkarimi et al. 2010). Therefore, *W. sinensis* can be considered that natural antimicrobial agents can be used on food. In addition, it is encouraged the use of natural antimicrobials because of increasing concerns about synthetic substances leaving hazardous waste on foods (Al-Haq et al. 2005).

CONCLUSIONS

This study indicates that the fatty acid of macerated *W. sinensis* have antimicrobial effect on *B. subtilis* and *C. tropicalis*, moreover as persistent. At the same time, the bulgur infected with these pathogens could be reduced to a certain extent. To reduce the risk of these pathogens multiplying in food, *W. sinensis* macerate may be an alternative. In future, more detailed studies should be done for use in the food field.

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