Türk Hijyen ve Deneysel Biyoloji Dergisi

In vitro examination of toothpastes with *Cinnamomum cassia* methanolic extract

Cinnamomum cassia metanolik ekstraktı ilave edilen diş macunlarının *in vitro* incelenmesi

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ABSTRACT

Objective: In recent years, it has developed resistance to antibiotics and antibacterial chemicals used in the treatment of a large number of bacterial diseases. For this reason, today, an alternative product has been searched for instead of synthetic chemicals. Phytochemicals in traditional medicine and isolated from plants are seen as a good alternative to synthetic chemicals. The purpose of this study; to investigate the antibacterial activity of toothpastes of different contents and toothpastes with *Cinnamomum cassia* methanolic extract added against *Staphylococcus aureus* bacteria.

Methods: In the study, the experimental set up of six different toothpastes, a positive control group of a flüoride free toothpaste and a distilled water negative control group. The content of *C. cassia* methanolic extract was determined by GC/MS. Anti-*S. aureus* activities of toothpastes alone and with *C. cassia* methanol extract were determined according to the agar disc diffusion method and n = 3 repeats. Inhibition zone diameters were determined using digital calipers and all results were evaluated statistically.

Results: According to the GC/MS, cinnamaldehyde, one of the main substances of *C. cassia* methanolic extract was found to be higher than other components.

ÖZET

Amaç: Son yıllarda, çok sayıda bakteriyel hastalığın tedavisinde kullanılan antibiyotik ve antibakteriyel kimyasallara karşı direnç gelişmiştir. Bu nedenle, günümüzde sentetik kimyasalların yerine alternatif bir ürün arayışına girilmiştir. Geleneksel tıpta yer alan ve bitkilerden izole edilen fitokimyasallar, sentetik kimyasallara karşı iyi bir alternatif olarak görülmektedir. Bu çalışmanın amacı; farklı içerikteki diş macunlarının ve *Cinnamomum cassia* metanolik ekstraktı ilave edilmiş diş macunlarının *Staphylococcus aureus* bakterisine karşı antibakteriyel etkinliğini incelemektir.

Yöntem: Çalışmada altı farklı diş macununun deney grubunu, florürsüz bir diş macununun pozitif kontrol grubunu ve distile suyun negatif kontrol grubunu oluşturduğu deney düzeneği kurulmuştur. *C. cassia* metanolik ekstraktının içeriği GC/MS ile belirlenmiştir. Diş macunlarının tek başlarına ve *C. cassia* metanol extraktı ile anti-*S. aureus* aktiviteleri agar disk difüzyon yöntemine göre ve n=3 tekrarlı olarak belirlenmiştir. İnhibisyon zon çapları dijital kumpas kullanılarak belirlenmiş ve tüm sonuçlar istatiksel olarak değerlendirilmiştir.

Bulgular: GC/MS ile belirlenen *C. cassia* metanolik ekstraktının ana maddelerinden olan cinnamaldehyde diğer bileşenlere göre daha yüksek oranda bulunmuştur. *C.*

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Geliş Tarihi / Received : 05.03.2020
Kabul Tarihi / Accepted : 02.07.2020

DOI ID : 10.5505/TurkHijyen.2021.25993

Tollu G, Erdoğan Eliuz EA. *In vitro* examination of toothpastes with *Cinnamomum cassia* methanolic extract.
Turk Hij Den Biyol Derg, 2021; 78(2): 197 - 204

The most effective toothpaste was found different after adding *C. cassia* methanol extract and the most effective toothpaste after adding *C. cassia* methanol extract (p <0.05).

Conclusion: With this study, it has been shown that toothpaste with low or no anti-*S. aureus* effect can be enriched with herbal extracts to increase their biological activities. Studies that evaluate the effectiveness of toothpaste-methanolic extract combinations proved by us to have antibacterial efficacy, such as remineralization, whitening, desensitization, which constitute oral and dental health, should also be evaluated. In further studies, it has been determined that various studies can be performed to use the essential oils used in the study in place of various antimicrobials in the content of toothpastes.

Key Words: Antibacterial, toothpaste, *in vitro*, *Staphylococcus aureus*

cassia metanol ekstraktı eklenmeden önce en etkili olan diş macunu ile *C. cassia* metanol ekstraktı eklendikten sonra en etkili diş macunu farklı bulunmuştur (p <0.05).

Sonuç: Bu çalışma ile anti-*S. aureus* etkisi düşük ya da hiç olmayan diş macunlarının bitkisel ekstraktlarla zenginleştirilerek biyolojik aktivitelerinin arttırılabileceği gösterilmiştir. Antibakteriyel etkinliği olduğu tarafımızdan kanıtlanan diş macunu-yağ kombinasyonlarının ağız ve diş sağlığını sağlayan remineralizasyonu, beyazlatma, hassasiyeti giderme gibi diğer bileşenleri için de etkinliklerinin değerlendirildiği çalışmalar yapılması gerekmektedir. Daha ileride yapılacak olan çalışmalarda, çalışmada kullanılan uçucu yağların, diş macunlarının içeriğinde çeşitli antimikrobiyallerin yerine kullanılması için çeşitli çalışmalar yapılabilineceği tespit edilmiştir.

Anahtar Kelimeler: Antibakteriyel, diş macunu, *in vitro*, *Staphylococcus aureus*

INTRODUCTION

Due to its anatomy, the oral cavity has a favorable environment for the settlement of different types of microorganisms. It is also susceptible to almost any microorganism known for its different temperatures, various nutrient contents, abundant humidity and different oxygen pressures. The microorganism placement in the mouth starts with birth and changes with factors such as feeding regimen, loss of teeth, use of prosthesis, oral hygiene and general health condition and formation of oral microflora occurs. Oral microbiota has a very rich microbial diversity and when this balance is disrupted, many diseases, especially dental caries and gum diseases, occur. This diversity arises from the fact that various assets meet each other's needs and from various nutrients (1). With antibacterial substances added to toothpastes, it aims to prevent various diseases and caries by controlling the pathogen formation in the bacterial plaque. Antibacterials are the most commonly used agents to affect the viability of bacteria in biofilm. However, if bacteria capable of producing biofilm develop resistance to antibiotics, pathologies will also progress (2). Therefore, researchers continue to search for an alternative product against synthetic chemicals (3). Phytochemicals found in traditional medicine and isolated from plants are seen as a good alternative to synthetic chemicals (4,5). Some studies have focused on reducing the microbial adhesion responsible for dental plaque formation by examining the effects of plant extracts and plant products on specific oral pathogens and their effects on inhibition of biofilm formation. (6,7).

The purpose of this study to compare the antibacterial activities of herbal ingredients after and before the addition of toothpaste. To determine the antibacterial activity on *S. aureus* bacteria after adding *C. cassia* content obtained by dissolving in methanol extract to toothpastes. Investigation of the effectiveness of herbal ingredients in preventing the activities of microorganisms in natural ways can be an example in preventing antibiotic resistance which is one of the biggest problems of today.

MATERIAL and METHOD

Microbiology studies were carried out in Mersin University Advanced Technology and Research Laboratory (MEITAM), between May 2019-August 2019 to investigate the anti-*S. aureus* activity of toothpastes with and without *C. cassia* methanolic extract.

Selecting toothpastes to be used

In this study, six fluoride toothpaste (C, I, Se, V, P and S) experiment groups, one fluoride-free toothpaste (R) positive control group and distilled water negative control group was formed. Since it is not ethically appropriate, the names of the manufacturers are not given, and the toothpastes are named with various letters.

Determination of the content of C. cassia methanolic extract

The qualitative and quantitative composition of *C. cassia* analysis were conducted at Giresun University central Research Laboratories Application and Research Center by GC-MS 7890A-(5975C inert MSD) instrument equipped with an Agilent 19091S-433 column (8). The chemical composition of *C. cassia* methanolic extract were determined by analyzing GC/MS in the scanning range of M+=50-550 m/z. 1L of the concentrated plant extract was filtered through 0.45 μL syringe filter and injected to GC-MS injection port (250 °C) in splitless mode. The extract was eluted using HP5-MS capillary column (30m x 0.25 mm x 0.25 μm) at helium gas (flow rate: 1.75 mL min-1) under fixed 21.21 psi of pressure. The study was performed by applying the following sample elution temperature system for a maximum of 70 minutes. The oven temperature was gradually increased after it was kept at 50 °C for 2 min. Then, it was increased to 100 °C at 5 °C min-1 and held for 5 min. Then, it was enhanced to 150 °C at 5 °C min-1 and performed for 8 min. Finally, increased to 250 °C at 5 °C min-1 and kept for 15 min. Characterization of *C. cassia* components was based on the library (Wiley and NIST) comparison with the mass spectra of the extract sample (8,9). The results of the GC / MS analysis contents are shown in Table 1.

Preparing of anti- S. aureus culture

The inoculum of *S. aureus* were prepared in 4 mL Triptic Soy Broth (Oxoid) for bacteria and incubated at 37°C, overnight. After 24 hours, the culture suspension were adjusted as $5.8x10^6$ cfu/mL and stored at +4 °C until further use.

Anti- S. aureus effects of toothpastes and toothpastes added to C. cassia methanolic extract

For anti- *S. aureus* activity of toothpaste samples and toothpastes combined with *C. cassia*, agar diffusion method used (10). Before the addition of *C. cassia* methanolic extract, toothpastes to be used in the study were formed the experimental groups, positive control group (fluoride-free toothpaste) and negative control group (distilled water).

For prepearing the toothpastes combined with *C. cassia*; *C. cassia* methanolic extract were added to newly opened toothpastes which had never been used before and thereon 5μL distilled water was added. This mixture was homogeneously dispersed.

According to experiment, 5 mm thick agar medium was poured into the 9 cm diameter sterile petri dishes and frozen. Then, 0.5 mL-culture of 1 night active liquid cultures of bacteria was spread onto the surface of the medium with sterile L baguette. After waiting for about one hour at 37 °C, 6 mm diameter

Table 1. Chemical composition of *C. cassia* methanolic extract

RT	C	%	RT	C	%
7.369	Oxime-, methoxy-phenyl-	3.32	35.343	Beta-Jonone	5.32
7.924	4-Penten-1-amine	3.61	35.778	Octadeamethyl-cyclononasiloxane	1.98
20.049	Cinnamaldehyde	21.41	36.814	Caryophyllenol	2.22
20.827	p-Cymen-3-ol	8.0	37.838	Methyl palmitate	8.99
21.141	Carvacrol	3.70	39.028	Cyclodecasiloxane, eicosamethyl-	1.62
21.920	Cyclohexasiloxane- dodecamethyl	5.01	41.746	Methyl stearate	6.11
26.806	Cycloheptasiloxane-tetradecamethy	4.59	41.906	Norepinephrine	3.67
27.195	2,4-di-tert-butylphenol	1.32	44.476	Ttetracosamethyl-cyclododecasiloxane	1.74
31.344	Isoquinoline	1.77	45.500	2-(trimethylsilyl)ethyl4-(diphenylphosphino) benzoate	1.71
31.864	Hexadecamethylcyclooctasiloxane	2.15	46.873	1H-Purin-6-amine, trihydrate	2.91
34.645	3-(2,5-dimethylphenyl)butan-2-one	4.99	50.175	Cheilanth-13(14)-enic Methyl Ester	3.83
Total					99.97

RT: Retention Time
C: Compounds name

wells were drilled in the middle of the petri dish and toothpastes alone and toothpastes combined with *C. cassia* were put into the wells (n = 3). An equal amount of samples were placed in the prepared wells without dilution. Petri dishes were incubated at 37 °C for 24 hours. Inhibition zones formed after incubation were measured with digital caliper. Statistical analysis the significances of antibacterial analyses were measured by ANOVA (least significant difference -LSD) test in one-way analysis of variance for inhibition zones (%) using SPSS 25. From the values obtained, the average value and standart deviation were evaluated in Table 2. While evaluating the study data, Kruskal Wallis test was used for comparing quantitative data between groups that did not show a normal distribution and Bonferroni Corrected Mann Whitney U test was used to determine the group that caused the difference. The experiments were repeated at least three times. Differences were considered as significant at $p \leq 0.05$.

RESULTS

According to GC/MS analysis, the main ingredients of *C. cassia* methanolic extract were cinnamaldehyde (21.41%), methyl palmitate (8.99%), p-Cymen-3-ol (8.0%), methyl stearate (6.11%) and beta-Jonone (5.32%). In this study, to determine the anti-*S. aureus* activity of toothpastes and control groups in pure form without the addition of *C. cassia* methanol extract, toothpastes having the highest anti-*S. aureus* activity among toothpastes were S toothpaste (19 mm ± 0.00 mm) and C toothpaste (15.12 mm

Table 2. Inhibition zones of toothpastes alone and with *C. cassia* (Cs) against *S. aureus* (mm)

Toothpaste alone on *S. aureus*							
S	C	V	Se	P	R	I	Co
19 [a] ± 0.0	15.12 [a] ± 0.07	11.26 [a] ± 0.02	8.0 [a] ± 0.2	0.0 [b] ± 0.0	12.0 [a] ± 0.0	7.33 [a] ± 0.3	0.0 [b] ± 0.0
Toothpaste with *C. cassia* on *S. aureus*							
S+Cs	C+Cs	V+Cs	Se+Cs	P+Cs	R+Cs	I+Cs	Co+Cs
16.4 [a] ± 0.8	19.8 [a] ± 0.4	14.5 [a] ± 0.5	13.3 [a] ± 1.53	22.0 [a] ± 0.1	12.0 [a] ± 0.2	8.60 [a] ± 0.3	0.0 [b] ± 0.0

a, b: There is a significant difference between those shown exponentially with different letters

± 0.07 mm). Inhibition zones were 11.26 mm for V toothpaste zone, 8 mm for Se toothpaste, 12 mm for Co toothpaste, 7.33 for I toothpaste. However, P toothpaste alone did not show any anti-*S. aureus* activity (0 mm ± 0.00 mm). The inhibition zone of P toothpaste were found to be statistically different from the other all toothpastes ($p \leq 0.05$). When the control groups were evaluated, positive control group R toothpaste (fluoride-free toothpaste) (12 mm ± 0.00 mm) was found to be S's toothpaste highest effective toothpaste. Distilled water (0 mm ± 0.00 mm), which is a negative control group, had no anti-S.aureus effect like P toothpaste. Also, after the addition of *C. cassia* methanol extract the highest anti-*S. aureus* activity among toothpastes was determined as P toothpaste (22.0 mm ± 0.1 mm) and C toothpaste (19.8 mm ± 0.4 mm). The inhibition zones were found as 16.4 mm for S toothpaste, 14.5 mm for V toothpaste zone, 13.26 mm for Se toothpaste and 8.60 mm for I toothpaste. When the control groups were evaluated, the positive control group had no fluoride-free toothpaste R toothpaste (12 mm ± 0.2 mm) and the negative control group distilled water (0 mm ± 0.00 mm) had no anti- *S. aureus* effect. According to the results of the study, it was seen that P toothpaste was the highest effective toothpaste in *C. cassia* methanol extract toothpaste mixture.

DISCUSSION

The seconder metabolites in plants exhibit different anti-*S. aureus* effects because they contain various components and differences in the mechanisms of action of each of these components (11). GC/MS analysis is essential for determining the composition of them (12). Since the pure forms of their do not use solvents, they are thought to be cytotoxic because their pure forms can be highly hemolyzed in plaques. The *Cinnamommum* species, which belongs to the *Lauraceae* family, contains more than 300 aromatic trees and shrubs that can remain green for four seasons (13). *C. cassia*, also known as *Chinese Cinnamon* used in this study, was purchased from a local market. In studies, investigating the antimicrobial activity of cinnamon oil, it has been reported to have an effect on different bacterial species and fungi. In a study evaluating the antimicrobial activity of volatile oil obtained from the bark of *Cinnamomum zeylanicum* on three bacteria and two yeast fungus that may cause tooth decay, the plant's ethanolic, methanolic and acetonic extracts showed an antibacterial effwct against *L. acidhoplilus* (14). According to the results of a previous study, *Cinnamommum* camphora essential oil showed the highest anti-*S. aureus* effect among the oils used (15). It is very important to increase the use of antimicrobial effective agents in the fight against Staphylococcus which is known to be a significant food poisoning and threatens food workers (16). In literature, the high antibacterial activity of cinnamon oil was attributed to cinemic aldehyde (17,18). In studies conducted with different cinnamon species, the rate of cinematic aldehyde contained in the essential oils was found to be between 50% and 88%. Different extraction methods, the use of different solvents and the yield of the plant from which the oil is obtained may affect the composition of the essential oil and the rate of the presence of the major component (19). Wang et al. (20) analyzed the essential oil obtained from *C. cassia* by steam distillation by GC/MS and found that it contains 50% cinematic aldehyde. In another study evaluating the antimicrobial activity of *C. cassia* essential oil obtained by hydrodistilation, it was reported that the cinemic aldehyde in the essential oil is 85% (18). Another Cinnamon species, C. zeylanicum from the study of the composition of the essential oil obtained by the method of hydrodistilation of 99.4% cinematic aldehyde was found in the oil obtained (21). According to GC/MS analyzes, the main component of *C. cassia* methanol extract used in this study was found to be cinemic aldehyde (21.41%). In this study, S toothpaste, one of the toothpastes used in the study, showed the highest anti-*S. aureus* activity. Bhattacharjee et al. (22) compared toothpastes with different properties and reported that herbal toothpastes showed higher anti-*S. aureus* effects than fluoride and triclosan toothpastes. All methanol extracts used in the study showed antibacterial activity against *S. aureus*. When *C. cassia* methanol extract was added to toothpastes, it was observed that the effectiveness of toothpastes increased. P toothpaste, which did not initially have any anti- *S. aureus* activity, showed the largest zone of inhibition, surprisingly gaining activity with the addition of methanol extract. When all groups were evaluated together, the group with the best anti-*S. aureus* effect was found to be P toothpaste-*C. cassia* methanol extract and the group with the weakest anti- *S. aureus* effect was S toothpaste. It should be considered that the differences in the zone diameters obtained for each solvent may be due to the different anti- *S. aureus* activities of the solvents and the interactions of the active ingredients in the solvents and toothpastes.

In conclusion, the toothpastes show antibacterial effect on *S. aureus* within the limits of our study in which the evaluation of anti- *S. aureus* activity of toothpastes and toothpastes with *C. cassia* methanol extract. R toothpaste from fluoride-free toothpastes showed lower inhibition zone diameter without addition of methanol extract, but higher after addition of methanol extract. R toothpaste also showed higher activity than some toothpastes containing fluoride. This may prove that fluoride-free toothpastes can be as effective as fluoride toothpastes and even show higher antimicrobial activity than some toothpastes. All toothpastes tested showed a higher inhibition zone diameter after addition of methanol extract. P toothpaste containing *C. cassia* extract and solvent showed the highest effect. P toothpaste showed no antimicrobial activity before the methanol extract was added. It is necessary to evaluate the efficacy of combinations of toothpaste methanol extract, which has been proven to have anti- *S. aureus* activity, for other components such as remineralization, whitening, desensitization, which form oral and dental health. In the future studies, it has been determined that methanol extracts used in the study can be used to replace various antimicrobials in the contents of toothpastes.

It is observed that toothpastes produced with different active substances examined in our study may show antimicrobial effects *in vitro*. However, taking into consideration the increasing variety of active substances in the market every day, *in vivo* studies are needed to examine the effects of toothpastes *in vitro* that will occur in the mouth environment. We all know that one of the biggest problems today is antibiotic resistance. It is seen that the efficiency on microorganisms increases by using plants with more natural content in the destruction of microorganisms. Plants and herbal ingredients are seen as the ideal alternative to prevent antibiotic resistance problem.

ACKNOWLEDGMENTS

Thanks to Giresun University and Mersin University Advanced Technology Research Centers to analysis supports.

ETHICS COMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

* This study was supported by Mersin University Scientific Research Projects Unit. Project no: 2018-2-AP3-2939.

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