Turkiye Parazitol Derg 2024;48(2):72-6
DOI: 10.4274/tpd.galenos.2024.91855

Investigation of the Antitrichomonal Activity of Cinnamaldehyde, Carvacrol and Thymol and Synergy with Metronidazole

Sinnamaldehit, Karvakrol ve Timolün Antitrichomonal Aktivitesi ve Metronidazol ile Sinerjisinin Araştırılması

Yener Özel[1], İbrahim Çavuş[2], Gülhan Ünlü[1], Mehmet Ünlü[1], Ahmet Özbilgin[2]

[1]Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
[2]Manisa Celal Bayar University Faculty of Medicine, Department of Medical Parasitology, Manisa, Türkiye

Cite this article as: Özel Y, Çavuş İ, Ünlü G, Ünlü M, Özbilgin A. Investigation of the Antitrichomonal Activity of Cinnamaldehyde, Carvacrol and Thymol and Synergy with Metronidazole. Turkiye Parazitol Derg. 2024;48(2):72-6.

ABSTRACT

Objective: *Trichomonas vaginalis* is a sexually transmitted protozoan parasite that usually causes infections in women. Metronidazole is used as the first choice in the treatment of this parasitic disease, but there is a need for new drugs since 1980's with increasing numbers of reported resistance. In this study, it was aimed to determine the antitrichomonal activity of the major components of *Cinnamomum zeylanicum* (cinnamon) and *Thymus vulgaris* (thyme) essential oils, cinnamaldehyde, carvacrol and thymol against metronidazole resistant and susceptible *T. vaginalis* strains, and to determine their interaction with metronidazole by checkerboard method.

Methods: Cinnamaldehyde, carvacrol, thymol and metronidazole were obtained commercially. Two clinical isolates and one metronidazole resistant *T. vaginalis* reference strain were used in the study. MIC_{50} and MLC values of essential oil components and metronidazole were determined by broth microdilution method. The combinations of essential oil components with metronidazole were determined by the checkerboard method.

Results: According to *in vitro* activity tests, cinnamaldehyde was determined to be most effective essential oil component. Clinical isolates were susceptible to metronidazole. In combination study, metronidazole showed synergy with cinnamaldehyde and carvacrol, and partial synergy with thymol.

Conclusion: It was determined that cinnamaldehyde, carvacrol and thymol, which are known to have high antimicrobial activity, also have strong activity against *T. vaginalis* isolates and show a synergistic interaction with metronidazole. The use of metronidazole at lower doses in the synergistic interaction may contribute to the literature in terms of reducing drug side effects, creating a versatile antimicrobial target, and reducing the rate of resistance development.

Keywords: Checkerboard, cinnamaldehyde, carvacrol, metronidazole, synergy, thymol, *T. vaginalis*

ÖZ

Amaç: *Trichomonas vaginalis* genellikle kadınlarda enfeksiyona neden olan ve cinsel yolla bulaşan bir protozoon parazittir. Parazitin neden olduğu hastalığın tedavisinde ilk tercih olarak metronidazol kullanılmaktadır. Ancak 1980 yılından sonra artan sayılarda direnç gelişiminin rapor edilmesi ile yeni ilaç arayışlarına ihtiyaç duyulmuştur. Bu çalışmada, *Cinnamomum zeylanicum* (tarçın) ve *Thymus vulgaris* (kekik) uçucu yağlarının majör bileşenleri olan sinnamaldehit, karvakrol ve timolün metronidazole dirençli ve duyarlı *T. vaginalis* izolatlarına karşı anti-trichomonal etkinliğinin belirlenmesi ve metronidazol ile etkileşiminin checkerboard (dama tahtası) yöntemi ile gösterilmesi amaçlandı.

Yöntemler: Çalışmada kullanılan sinnamaldehit, karvakrol, timol ve metronidazolün saf formları ticari olarak temin edildi. Çalışmada, iki klinik izolat ve bir adet metronidazole dirençli *T. vaginalis* standart (ATCC 50143) suşu kullanıldı. Uçucu yağ bileşenlerinin ve metronidazolün MIK_{50} ve MLK (minimum letal konsantrasyonu) değerleri sıvı mikrodilüsyon yöntemi, metronidazol ile kombinasyonu ise checkerboard (dama tahtası) yöntemi ile saptandı.

Bulgular: *İn vitro* etkinlik testlerine göre, en etkili uçucu yağ bileşeninin sinnamaldehit olduğu belirlendi. Klinik izolatların metronidazole duyarlı olduğu saptandı. Checkerboard yöntemi ile yapılan kombinasyon çalışması değerlendirildiğinde, sinnamaldehit ve karvakrolün metronidazol ile kombinasyonunda sinerji, timolün metronidazol ile kombinasyonunda ise kısmi sinerji görüldü.

Received/Geliş Tarihi: 10.04.2023 **Accepted/Kabul Tarihi:** 25.05.2024

Address for Correspondence/Yazar Adresi: Yener Özel, Balıkesir University Faculty of Medicine, Department of Medical Microbiology, Balıkesir, Türkiye
Phone/Tel: +90 266 612 10 10 **E-mail/E-Posta:** yener_ozel@hotmail.com **ORCID ID:** orcid.org/0000-0001-6618-8251

Türkiye PARAZİTOLOJİ Dergisi

Sonuç: Yüksek antimikrobiyal aktiviteye sahip olduğu bilinen sinnamaldehit, karvakrol ve timol'ün *T. vaginalis* izolatlarına karşı güçlü aktiviteye sahip olduğu ve metronidazol ile sinerjistik etkileşim gösterdiği belirlendi. Sinerjik etkileşimde metronidazolün daha düşük dozlarda kullanılması ilaç yan etkilerinin azaltılması, çok yönlü bir antimikrobiyal hedef oluşturulması ve direnç gelişme hızının düşürülmesi açısından literatüre katkı sağlayabilir.
Anahtar Kelimeler: Checkerboard, sinnamaldehit, karvakrol, metronidazol, sinerji, timol, *T. vaginalis*

INTRODUCTION

Trichomoniasis is a non-viral and sexually transmitted urogenital infection that affects both men and women. According to the World Health Organization, it is estimated that more than 156 million new cases of trichomoniasis will occur in 2020 (1). However, prevalence data vary according to the population studied and the method used for diagnosis. Higher prevalence values have been reported when compared with nucleic acid amplification tests, direct microscopic examination and vaginal pH tests (2,3). *Trichomonas vaginalis* is a protozoan parasite and is usually associated with clinical symptoms including yellow-green vaginal discharge, vulvovaginal irritation and dysuria. Although the infection is usually asymptomatic in men, symptoms such as urethral irritation, discharge, burning after urination or ejaculation and swelling of the prostate gland may be observed (4). The parasite's soluble and membrane-associated enzymes with phospholipase A activity cause microulcerations and microscopic haemorrhages in the vaginal wall and exocervix by causing the breakdown of nucleated cells. Although humoral and cell-mediated immune responses develop against infection, the development of repeated infections indicates non-protective immunity against the parasite (5). Metronidazole is used as the first choice in the treatment of the disease caused by the parasite, yet, after 1980, an increasing number of reports of resistance development led to the need to search for new drugs. In recent years, natural active ingredients derived from plants, called phytochemicals, have become the focus of interest with the discovery of their strong antimicrobial effects (6,7). This study aimed to determine the antitrichomonal activity of cinnamaldehyde, carvacrol and thymol, the major components of *Cinnamomum zeylanicum* (cinnamon) and *Thymus vulgaris* (thyme) essential oils against metronidazole-resistant and susceptible *T. vaginalis* isolates and their synergistic interactions with metronidazole.

METHODS

Ethically Approval

No clinical material or data were used in this study. Therefore, ethics committee approval is not required.

Study Design

Minimum inhibition concentration (MIC_{50}) and (minimum lethal concentration (MLC) values of phytochemicals and metronidazole were determined *in vitro* by broth microdilution method and the combination of each phytochemical with metronidazole was investigated by checkerboard method (Figure 1).

Essential Oil Components and Parasite Strains

Pure forms of cinnamaldehyde, carvacrol, thymol and metronidazole used in this research were obtained commercially (Sigma, the USA). Two clinical isolates and one metronidazole-resistant *T. vaginalis* reference strain (ATCC 50143) were used in this study. Clinical isolates were produced from vaginal swabs sent to our laboratory by sowing them on TYM (trypticase yeast-extract maltose) medium and subcultured until they entered the logarithmic phase (8). Metronidazole-resistant *T. vaginalis* reference strain (ATCC 50143) was obtained from Manisa Celal Bayar University Faculty of Medicine Parasitology Bank.

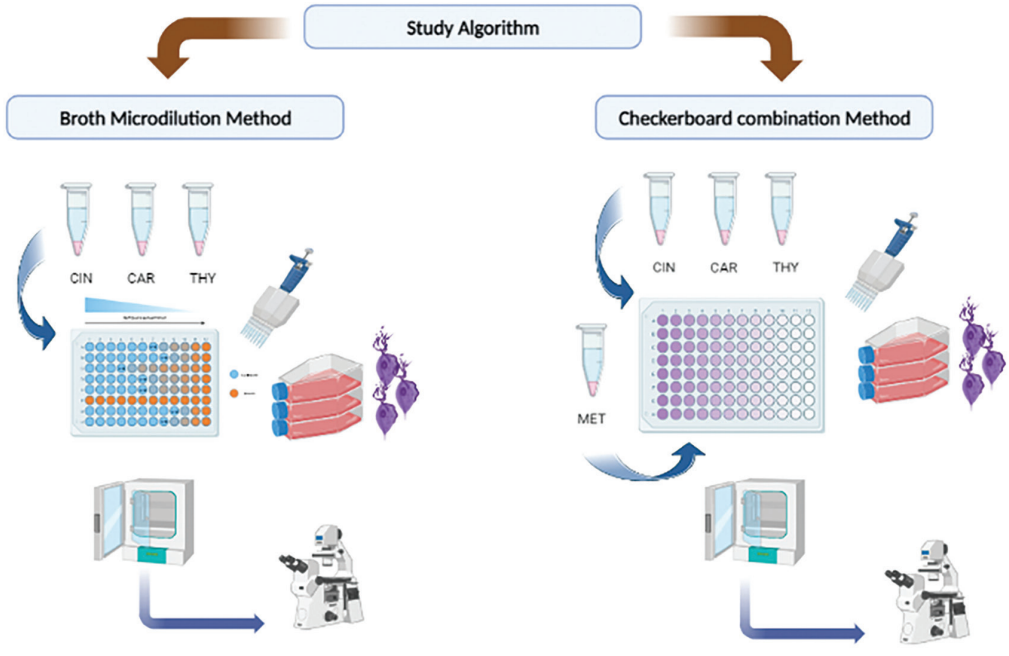

Figure 1. Study workflow

In vitro Drug Screening Test

For all isolates, MIC_{50} and MLC values of phytochemicals and metronidazole were determined *in vitro* by broth microdilution method in 96-well microplates (9). The dilution range for phytochemicals was set to be 1600-0.9 µg/mL and 12500-3 µM for metronidazole. $5x10^3$ parasites/mL *T. vaginalis* trophozoites were added to each well except for negative control. Microplates were incubated at 37 °C for 48 hours. At the end of incubation, the viability and motility of *T. vaginalis* trophozoites were evaluated under a light microscope using a counting chamber. All studies were repeated 3 times on independent days.

Checkerboard Combination Test

The combination of each phytochemical with metronidazole was investigated against a metronidazole-resistant *T. vaginalis* reference (ATCC 50143) strain by checkerboard method (10). Two 96-well microplates were used to determine the combination of phytochemicals with metronidazole. Serial dilutions of phytochemicals were made from top to bottom in the first microplate and metronidazole from right to left in the second microplate. Wells containing all combinations of both substances were obtained by transferring serial dilutions in the second microplate to the corresponding wells in the first microplate. $5x10^3$ parasites/mL *T. vaginalis* trophozoites were added to each well except for negative control. The plates were incubated at 37 °C for 48 hours and the viability and motility of *T. vaginalis* trophozoites were evaluated under a light microscope and FICI (fractional inhibitory concentration index) values were calculated. Interactions were interpreted as synergy if the FICI value was <0.5, partial synergy if between 0.5-0.75, additive if between 0.75-1, indifferent if between 1-4, and antagonism if >4 (11). All studies were repeated 3 times on independent days.

Statistical Analysis

In this study, statistical analysis was not required.

RESULTS

In vitro Drug Screening Tests

MIC_{50} and MLC values of cinnamaldehyde, carvacrol, thymol and metronidazole were respectively 3.9/31.25 µg/mL, 15.6/125 µg/mL, 62.5/500 µg/mL and 6/24 µM for the first clinical isolate; 1.8/7.81 µg/mL, 31.25/250 µg/mL, 15.6/125 µg/mL and 6/24 µM for the second clinical isolate; 3.9/15.6 µg/mL, 62.5/250 µg/mL, 125/500 µg/mL and 48/390 µM for metronidazole resistant isolate (Table 1).

Checkerboard Combination Test

The FICI values for cinnamaldehyde/metronidazole, carvacrol/metronidazole and thymol/metronidazole combinations were 0.374, 0.187 and 0.750, respectively (Table 2). According to these values, the combination of cinnamaldehyde and carvacrol with metronidazole was found to be synergistic and thymol partially synergistic.

DISCUSSION

Although trichomoniasis is thought to be a simple parasitic disease, it is a serious parasitic infection that can cause premature birth and rupture of membranes in pregnant women, and outside of pregnancy, it is associated with the post-partum syndrome with fever and foul-smelling discharge and can predispose to many infections. Many studies have reported that cervical cancer, atypical pelvic inflammatory disease and infertility are more

Table 1. Antitrichomonal activity values of essential oil components and metronidazole

Isolates	Phytochemicals (µg/mL)	MIC_{50}	MLC
T. vaginalis BAUN-TV1	Cinnamaldehyde	3.9	31.25
	Carvacrol	15.62	125
	Thymol	62.5	500
	Metronidazole (µM)	6	24
T. vaginalis BAUN-TV2	Cinnamaldehyde	1.8	7.81
	Carvacrol	31.25	250
	Thymol	15.62	125
	Metronidazole (µM)	6	24
T. vaginalis ATCC 50143	Cinnamaldehyde	3.9	15.62
	Carvacrol	62.5	250
	Thymol	125	500
	Metronidazole (µM)	48	390

MIC_{50}: Minimum inhibition concentration, MLC: Minimum lethal concentration

Table 2. Results of checkerboard combination of phytochemicals and metronidazole

Isolate	Combination	ΣFICI	Interaction
T. vaginalis ATCC 50143	Cinnamaldehyde/metronidazole	0.374	Synergy
	Carvacrol/metronidazole	0.187	Synergy
	Thymol/metronidazole	0.75	Partial synergy

ΣFICI: Fractional inhibition concentration index

common in patients infected with *T. vaginalis*. In addition, in women infected with *T. vaginalis*, the collection of HIV-infected cells such as lymphocytes and macrophages in the vagina and cervix facilitates the transmission of HIV (12,13). In a study conducted by Gram et al. (14) on 43016 Norwegian women, *T. vaginalis* infection was shown to increase the risk of cervical neoplasia caused by human papillomavirus (HPV). Studies conducted in Finland (15) and India (16) also indicated a similar correlation between trichomoniasis and cervical cancer caused by HPV. Another study demonstrated that *T. vaginalis* infection increased HPV infection 6.5 times (17).

The 5-nitroimidazole group of drugs is widely used in the treatment of trichomoniasis. Among these drugs, only metronidazole and tinidazole are authorized by the Food and Drug Administration in the United States for the treatment of trichomoniasis. Metronidazole is a relatively inexpensive, effective, and generally well-tolerated drug as well as generally mild gastrointestinal side effects. Occasionally, hematologic and neurotoxic side effects have also been reported. Recently, however, metronidazole-induced side effects have become a real problem in resistant and recurrent cases of trichomoniasis. Treatment of such infections requires a long-term treatment protocol with high doses of metronidazole. Increasing the drug dose leads to an increase in side effects and treatment fails with discontinuation of prophylaxis (18). New drug alternatives are needed today due to the emergence of resistant strains due to incomplete treatment processes and serious side effects of existing drugs at high doses (19).

In traditional medicine, the use of medicinal plants for the treatment of various diseases dates back thousands of years, according to records from ancient Babylon, Egypt, China and India. Despite the wide variety of chemically synthesized molecules in the modern pharmaceutical industry, natural components play a key role in drug development (20). Currently, around 35% of approved medicines are derived from natural ingredients or semi-synthetic derivatives, while 30% are synthetic molecules inspired by natural products. Remarkably, 65% of the 15 antiparasitic drugs approved by health authorities between January 1981 and June 2006 were natural ingredients or derivatives (21). Therefore, interest in medicinal plants has greatly increased in recent years. Given the need for new alternatives in the treatment of trichomoniasis, research focusing on the efficacy of natural ingredients against *T. vaginalis* has also increased.

Cinnamaldehyde, carvacrol and thymol, whose anti-trichomonal activity we investigated in our research, are the major natural components found in Cinnamomum zeylanicum and Thymus vulgaris essential oil. The antimicrobial activity of these components has been indicated by many researchers (22,23). Nevertheless, there are no studies demonstrating the efficacy of cinnamaldehyde, carvacrol and thymol against *T. vaginalis*. In our study, these components were found to reveal potent antitrichomonal activity. In particular, the fact that cinnamaldehyde is effective at very low concentrations of 0.9 μg/mL and has no cellular cytotoxicity at these doses (24) makes this essential oil component an important drug alternative. There is no clear information in the literature on the antimicrobial mechanisms of action of cinnamaldehyde, carvacrol and thymol, yet the general view is that these compounds bind to cell membranes due to their lipophilic character, increase membrane permeability, cause physical damage by accumulating in the membrane, inhibit the production of various enzymes and kill the microorganism by negatively affecting energy metabolism (25,26). At the MLCs determined for all three essential oil components, it was detected that the cell integrity of *T. vaginalis* trophozoites was preserved but they were immobile and lifeless. This suggests that these components may have an effect on the energy metabolism of the parasite.

In persistent infections caused by drug-resistant strains, increasing the drug dose often leads to treatment failure due to serious side effects. In such cases, combined drug use is often preferred as an alternative method. Phytochemicals offer promising adjuvants for antimicrobial drugs and the synergistic interaction of these metabolites with antimicrobials has been identified by researchers (6). The most common method used to determine the synergistic interaction between antimicrobials is the checkerboard method. In our study, a synergistic interaction was found between cinnamaldehyde, carvacrol and thymol in combination with metronidazole against metronidazole-resistant *T. vaginalis* strains. The observation of synergistic interaction between essential oil components and metronidazole may contribute to the literature in terms of using metronidazole at lower doses, reducing drug side effects, creating a versatile antimicrobial target and reducing the rate of resistance development.

CONCLUSION

Cinnamaldehyde, carvacrol and thymol, which have been shown to have antimicrobial activity against many microorganisms, were also found to have strong activity against *T. vaginalis* isolates. In addition, synergy was detected in combinations of essential oil components with metronidazole against metronidazole-resistant *T. vaginalis* isolate. The synergistic interaction in combinations provides advantages in treatment in terms of using the drug at lower doses, reducing drug side effects and preventing the development of resistance. Investigating both the individual efficacy of phytochemicals and their combinations with existing drugs can contribute to efforts to combat drug resistance.

* Ethics

Ethics Committee Approval: No clinical material or data were used in this study. Therefore, ethics committee approval is not required.

Informed Consent: Not required.

* Authorship Contributions

Concept: Y.Ö., İ.Ç., Design: Y.Ö., İ.Ç., G.Ü., M.Ü., A.Ö., Data Collection or Processing: Y.Ö., İ.Ç., G.Ü., M.Ü., A.Ö., Analysis or Interpretation: Y.Ö., İ.Ç., G.Ü., M.Ü., A.Ö., Literature Search: Y.Ö., İ.Ç., G.Ü., M.Ü., A.Ö., Writing: Y.Ö., İ.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Fact Sheet - Sexually Transmitted Infections (STIs). World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis) (Accessed on March 10, 2023).

2. Schwebke JR, Gaydos CA, Nyirjesy P, Paradis S, Kodsi S, Cooper CK. Diagnostic Performance of a Molecular Test versus Clinician Assessment of Vaginitis. J Clin Microbiol. 2018; 56: e00252-18.
3. Danby CS, Althouse AD, Hillier SL, Wiesenfeld HC. Nucleic Acid Amplification Testing Compared With Cultures, Gram Stain, and Microscopy in the Diagnosis of Vaginitis. J Low Genit Tract Dis. 2021; 25: 76-80.
4. Rein MF. Trichomoniasis. Hunter's Tropical Medicine and Emerging Infectious Disease, Elsevier. 2020; 731-3.
5. Meites,E, Workowski KA. (2018). In: Trichomonas vaginalis. S.S. Long, C.G., Prober, M. Fischer (Eds.), Principles and Practice of Pediatric Infectious Diseases, Elsevier, pp. 1364-1366.
6. Hemaiswarya S, Kruthiventi AK, Doble M. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine. 2008; 15: 639-52.
7. Langeveld WT, Veldhuizen EJ, Burt SA. Synergy between essential oil components and antibiotics: a review. Crit Rev Microbiol. 2014; 40: 76-94.
8. Mcmillan A. Laboratory Diagnostic Methods and Cryopreservation of Trichomonads. In: Trichomonads Parasitic in Humans (Ed). BM Honigsberg. Spinger, New York. 1989, pp: 299-310.
9. Clinical and Laboratory Standards Institute 2014. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement M100-S24, Vol. 34, No. 1, 950 West Valley Road, Suite 2500 Wayne, PA 19087 USA.
10. Eliopoulos G, Moellering RC. Antimicrobial Combinations. In: Lorian V, (Ed), Antibiotics in Laboratory Medicine, 4th ed, Williams & Wilkins Co., Baltimore, 1996, pp: 330-396.
11. Li YJ, Pan CZ, Zhao ZW, Zhao ZX, Chen HL, Lu WB. Effects of a combination of amlodipine and imipenem on 42 clinical isolates of Acinetobacter baumannii obtained from a teaching hospital in Guangzhou, China. BMC Infect Dis. 2013; 13: 548.
12. Laga M, Manoka A, Kivuvu M, Malele B, Tuliza M, Nzila N, et al. Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS. 1993; 95-102.
13. Petrin D, Delgaty K, Bhatt R, Garber G. Clinical and microbiological aspects of Trichomonas vaginalis. Clin Microbiol Rev. 1998; 11: 300-17.
14. Gram IT, Macaluso M, Churchill J, Stalsberg H. Trichomonas vaginalis (TV) and human papillomavirus (HPV) infection and the incidence of cervical intraepithelial neoplasia (CIN) grade III. Cancer Causes Control. 1992; 3: 231-6.
15. Viikki M, Pukkala E, Nieminen P, Hakama M. Gynaecological infections as risk determinants of subsequent cervical neoplasia. Acta Oncol. 2000; 39: 71-5.
16. Ghosh I, Muwonge R, Mittal S, Banerjee D, Kundu P, Mandal R, et al. Association between high-risk human papillomavirus infection and co-infection with *Candida* spp. and Trichomonas vaginalis in women with cervical premalignant and malignant lesions. J Clin Virol. 2017; 87: 43-8.
17. Lazenby GB, Taylor PT, Badman BS, McHaki E, Korte JE, Soper DE, Young Pierce J. An association between Trichomonas vaginalis and high-risk human papillomavirus in rural Tanzanian women undergoing cervical cancer screening. Clin Ther. 2014; 36: 38-45.
18. Howe K, Kissinger PJ. Single-Dose Compared with Multidose Metronidazole for the Treatment of Trichomoniasis in Women: A Meta-Analysis. Sex Transm Dis. 2017; 44: 29-34.
19. Hirt RP, Sherrard J. Trichomonas vaginalis origins, molecular pathobiology and clinical considerations. Curr Opin Infect Dis. 2015; 28: 72-9.
20. Ngo LT, Okogun JI, Folk WR. 21st century natural product research and drug development and traditional medicines. Nat Prod Rep. 2013; 30: 584-92.
21. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010 J Nat Prod. 2012; 75: 311-35.
22. Doyle AA, Stephens JC. A review of cinnamaldehyde and its derivatives as antibacterial agents. Fitoterapia. 2019; 139: 104405.
23. Özel Y, Yılmaz U, Ünlü M, Vardar Ünlü G. Çeşitli Uçucu Yağ Bileşenleri ile Antibiyotiklerin Antibakteriyel Etkinliği ve Sinerjik Etkileşimi [Antibacterial Activity and Synergistic Interaction of Various Essential Oil Components and Antibiotics]. Mikrobiyol Bul. 2022; 56: 95-102.
24. García-Salinas S, Elizondo-Castillo H, Arruebo M, Mendoza G, Irusta S. Evaluation of the Antimicrobial Activity and Cytotoxicity of Different Components of Natural Origin Present in Essential Oils. Molecules. 2018; 23: 1399.
25. Lv F, Liang H, Yuan QP, Li CF. *In vitro* antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. Food Research International. 2011; 44: 3057-64.
26. Xu J, Zhou F, Ji BP, Pei RS, Xu N. The antibacterial mechanism of carvacrol and thymol against Escherichia coli. Lett Appl Microbiol. 2008; 47: 174-9.