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The *In Vitro* Antibacterial Activity of Some Tannin Extracts Against Pepper Spot Disease Caused by *Xanthomonas* spp.

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Abstract: Pepper is an industrially important cultivated plant. One of the disease agents that cause significant problems in pepper cultivation in our country is *Xanthomonas* spp. Tannins are compounds in the root, wood, crust, leaves and fruit of plants that have significant antimicrobial effects on molds, yeast, bacteria and certain viruses. In this study the antibacterial activity of tannins against different bacterial strains were determined at the *in vitro* conditions. Four different concentrations (0.5, 1, 2, and 3 mg ml^{-1}) of 3 different tannin extracts (Farmatan, Artutan and Artutan K) were tested against pepper bacterial spot disease causing 11 *Xanthomonas* spp. (strains from pepper fields of Kahramanmaras province). It has been identified that according to the diameter of the zone formed in the disk diffusion method, 3 tannins extract have anti-bacterial activity against the *Xanthomonas* spp. strains however, the effectiveness of Artutan K was found to be low. The most sensitive strains against three tannin extracts were identified as ZI364 and ZI370. It has been determined that strain ZI383 against the Farmatan and strains ZI94, ZI95, ZI107 and ZI110 against Artutan are resistant. It was also determined that the effect observed at the concentration of 1 mg ml^{-1} did not increase with the concentration increased. As a result, it is considered that when combined with appropriate concentration and sensitive strains, better efficacy than antibiotics can be achieved.

Keywords: Antibacterial activity, plant disease, plant extracts, tannin, *Xanthomonas*

In Vitro Ortamda Biber Leke Hastalık Etmeni *Xanthomonas* spp.'e Karşı Bazı Tanen Ekstraktlarının Antibakteriyel Aktivitesinin Değerlendirilmesi

Özet: Biber endüstriyel olarak önemli bir kültür bitkisidir. Ülkemizde biber yetiştiriciliğinde önemli sorunlara sebep olan hastalık etmenlerinden birisi; *Xanthomonas* spp. cinsi bakterilerdir. Tanenler, genellikle bitkilerin kök, odun, kabuk, yaprak ve meyvelerinde bulunan ve funguslar, mayalar, bakteriler ve bazı virüsler üzerine önemli düzeyde antimikrobiyal etkileri olan bileşiklerdir. Bu çalışmada *in vitro* ortamda 3 farklı tanen ekstraktının (Farmatan, Artutan ve Artutan K) 4 farklı konsantrasyonun (0.5, 1, 2, ve 3 mg ml^{-1}) biber bakteriyel leke hastalığına sebep olan ve Kahramanmaraş ili biber tarlalarından izole edilen 11 *Xanthomonas* spp. bakteri izolatlarına karşı antibakteriyel etkinliğini belirlemek ve etmenin mücadelesindeki önemini ortaya çıkarmak amaçlanmıştır. Disk difüzyon yönteminde oluşan zon çapına göre 3 tanen ekstraktından Farmatan ve Artutan'nın *Xanthomona s*spp. izolatlarına karşı antibakteriyel etkiye sahip olduğu ancak ArtutanK'nın etkinliğinin az olduğu belirlenmiştir. Her üç tanen ekstraktına karşı en duyarlı izolat ZI364 ve ZI370 olarak belirlenmiştir. Farmatan'a karşı ZI383 izolatı, Artutana'a karşı ise ZI94, ZI95, ZI107 ve ZI110 izolatları dayanıklı olduğu belirlenmiştir. Ayrıca 1 mg ml^{-1} konsantrasyon uygulamasında etkinin görüldüğü ve konsantrasyon arttıkça etkinin o oranda artmadığı belirlenmiştir. Sonuç olarak, uygun konsantrasyon ve duyarlı izolatların kombinasyon uygulamaları yapıldığında antibiyotiklere nazaran daha iyi etkinlik sağlanabileceği düşünülmektedir.

Anahtar Kelimeler: Antibakteriyel aktivite, bitki eksraktları, bitki hastalığı, *Xanthomonas*

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1. Introduction

Pepper (*Capsicum annum* L.) is a member of Solanaceae family and cultivated in field and greenhouse in the world and in our country, and it is an industrially important culture plant (Duman et al. 2002). Kahramanmaraş province is major production area of red hot pepper as spices in Turkey (Gençoğlan et al. 2006). One of the disease agents that causes important problems in pepper growing areas in our country is *Xanthomonas* spp. (Aysan and Şahin 2003). *Xanthomonas* spp. bacterial pathogens are gram-negative, moving and yellow pigment-forming (Mirik 2005). The leaves and fruit stalks of the paper plants are susceptible to bacterial spot disease. The first symptom starts with small water-soaked spots on the lower surface of the leaf, and then these stains become brown growing spots with 1-5 mm in diameter. As the disease progresses, it forms necrotic areas.

In fruit, small and irregular spots become browned and wart appearance and cause deformity. The disease agent, which is originated from seeds and soil, can sustain its existence on seeds and weeds for a long time, Maneb, Mancozeb applications and seed sterilization shown to be affective in management of the disease (Şahin and Miller 1997). Use of resistant varieties, certified disease free seeds, crop rotation and proper irrigation are known to be good for disease control. Alternative methods of control should be applied, however, because of the difficulty of management of bacterial the rapid spread of the agent in moisture and temperature, the excessive use of pesticides containing antibiotics should be prohibited in our country (Soylu et al., 2006). The presence of antibacterial substances in higher plants is well known (Srinivasan et al. 2001). Plant extracts and tannins have significant antimicrobial effects on molds, yeast, bacteria, fungi and some viruses (Bayar and Genç 2018; İnci et al., 2021). Tannins are polyphenolic and amorphous compounds in the form of a light yellow-brown powder, flake or spongy mass obtained from plants such as rape, pods, tea and sorghum. Tannins are generally found in the remains of wood, crust, leaves and scales (Scalbert 1991).

Tannins form a natural defensive mechanism against microbial infections that can occur in fruit before they are harvested. In this study, at the *in vitro* conditions it was aimed to determine the sensitivity of *Xanthomonas* spp., which causes pepper bacterial blight disease, to different tannin extracts and to reveal its significance to control to the agents.

2. Materyal ve Metot

2.1. Bacterial pathogens

In this study, 11 *Xanthomonas* spp. strain, previously isolated from Kahramanmaraş province pepper fields. In previous studies, these strain were determined as Xanthomonas according to morphological and biochemical tests. Strains with slimy yellow pigmentation were noted positive species of Xanthomonas on the medium. Positive Xanthomonas strains (yellow and mucoid colonies) were selected for research. These *Xanthomonas* spp. strain increased by shaking in an Nutrient Broth (Merck) environment at 25° C and 200 rpm for 24 hours on an orbital shaker. Firstly, 100 µl of the bacterial suspension from the solution was inoculated on Nutrient Agar(NA) medium. After 24 hours amount of bacteria colonies were taken and adjusted to a concentration of 10^6 cells ml^{-1} with glass tubes containing physiological water. 50 µl of the bacterial solution was taken and spread into the Nutrient Agar + Glucose nutrient media.

2.2. Preparation of tannin extracts

Commercial tannins including Artutan (65% ± 2 tannins; Valonia isolated from *Quercus ithaburensis* ssp. *macrolepis*), Artutan K ((63-65% tannin, isolated from scarlet, *Pinus brutia*) which belongs to the catechol tannin group) and Farmatan (soluble in water and isolated from chestnut tree, *Castanea sativa* Mill.) were used in the study. 1 g of each tannin was dissolved in 2 ml of 70% alcohol and vortexed by adding 8 ml of sterile distilled water.

2.3. Antimicrobial sensitivity test

The antimicrobial susceptibility was carried out according to the disc diffusion method of Cheesbrogh (1991). Filter paper discs (10 mm diameter) were placed on the bacteria contaminated NA + glucosemedium, with 4 disc each petri dishes. Each discs was separately diffused by pipetting at 0.5, 1, 2 and 3 mg ml^{-1}concentrations of tannin concentration. The experiment was repeated 3 times. The prepared petri dishes were incubated at 25 ± 1° C temperature for 48 hours.

2.4. Evaluation of data and statistical analysis

The inhibition zone diameters at the end of the study were measured with calipers and an average of 3 replicates with standard errors were formed. Subsequently, ANOVA test was performed and the differences of means were compared according to the TUKEY multiple comparison test. Data analysis was done using SPSS Ver. 20.0 (SPSS Inc., Chicago, IL).

3. Results

Antimicrobial activity against 11 strain of *Xanthomonas* spp. was determined at 4 different concentrations (0.5, 1, 2, and 3) of 3 different tannin extracts (Farmatan, Artutan and Artutan K). According to the zone diameter formed in

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the disk diffusion method, Farmatan and Artutan from extract of 3 tannins showed antibacterial activity against the *Xanthomonas* spp. strains (Figure 1).

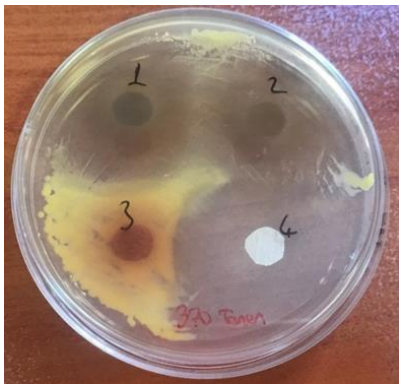

Figure 1. Antimicrobial activity of tannins on disk diffusion methods

As shown in Table 1, it was observed the antibacterial activity of Tannin extracts depending on the dose and type of tannin against eleven *Xanthomonas* spp. in different rates. Among tannins of Farmatan extract was determined as the most effective tannin. However, at the different dose of Farmatan did not show any antibacterial effect against Xanthomonas ZI383 isolate. Artutan tannin extract showed the most antibacterial effect against ZI364 isolate. This artutan exract, it either showed no antibacterial effect or showed certain rates against the other isolates. However, the extract of Artutan K was found to be least effective against *Xanthomonas* spp. In fact Artutan K showed no effect against most isolates. In briefly it was determined that the most sensitive strains, were found to be ZI364 and ZI370 strains in the extracts of Farmatan and Artutan tannins. Artutan K tannin extract showed only effect on ZI368 and ZI370 strains in all concentration, and on other strains in very high concentration; non antibacterial effect was detected in the others concentration. When the effects of tannin extracts were compared statistically, it was found that Farmatan application was statistically different with Artutan and Artutan K. But, ıt was found that Artutan and Artutan K application were not statistically different between ıt selves.

According to the variance analysis results in Table 2, a statistical difference was found between tannins and the most effective tannin was determined as Farmatan ($F_{2.132}$= 75.13; $p<0.01$). Ali-Shtayeh et al. (1998) reported in the studies they performed that the difference in antibacterial activity between tannin extracts is related to the variability of the components that they contain. The efficacy of tannins varies statistically according to bacterial isolates. When the results among the isolates were examined, the most susceptible isolates were ZI364 and ZI370, while the most resistant isolate was ZI383 ($F_{10.132}$= 178.06; $p<0.01$). When the efficacy between the strain of *Xanthomonas spp*. and four different concentrations of tannin extracts are examined, statistically significant difference was observed ($F_{3.132}$= 82.81; $p<0.01$). When the efficacy of each of the three tannins extracts at different doses between the isolates is examined, the doses were seen to be statistically different.

Table 1. Diffusion sensitivity test of different tannin extracts against strains of *Xanthomonas s*pp. at the *in vitro* conditions

		Strains											
Tannins	**Concentration (µL/ml)**	**ZI92**	**ZI94**	**ZI95**	**ZI99**	**ZI107**	**ZI109**	**ZI110**	**ZI364**	**ZI368**	**ZI370**	**ZI383**	**Value Means**
Farmatan	**0.5**	1.8±0.4	0.3±0.0	0.6± 0.0	0.6±0.0	NA	0.5±0.0	0.2±0.0	6.9± 0.2	5.0± 0.6	4.8± 1.3	NA	
	1	2.7± 0.5	3.1± 0.0	1.0± 0.1	2.1± 0.3	1.5±0.0	1.1±0.2	1.3± 0.4	9.2± 0.0	4.2± 2.3	9.6± 1.7	NA	1.61 ^{A}
	2	3.8±0.5	2.±0.5	3.6± 1.3	4.1± 0.0	2.3±1.8	2.3±0.1	1.6± 0.5	8.2± 2.3	4.7± 1.3	10.9±21	NA	
	3	5.4±1.5	3.1± 0.6	4.9± 0.9	4.3±0.4	3.3± 1.4	4.2±0.7	2.9± 0.2	11.6±22	5.0± 1.3	11.7±0.9	NA	
Artutan	**0.5**	2.1± 0.0	NA	NA	NA	0±0.00	NA	NA	8.7± 0.7	3.9± 0.2	4.7± 0.2	0.6± 0.3	
	1	2.7± 0.3	NA	NA	NA	NA	NA	NA	11.4±03	6.9± 0.8	8.4± 0.9	1.2± 0.0	1.16 ^{B}
	2	4.1± 0.1	NA	NA	1.3± 0.7	NA	0.2± 0.0	NA	11.0±07	4.8± 2.4	9.8± 1.0	2.5± 0.2	
	3	5.5± 0.3	NA	NA	1.2±0.0	NA	1.0± 0.0	0.9± 0.3	12.4±07	6.1± 1.5	11.4±0.2	3.4± 0.2	
Artutank	**0.5**	NA	NA	NA	NA	NA	NA	NA	NA	1.5± 0.3	0.5± 0.0	NA	
	1	NA	NA	NA	NA	NA	NA	NA	NA	2.6± 1.4	0.9± 0.0	NA	1.14 ^{B}
	2	NA	1.1±00	NA	NA	NA	NA	NA	NA	3.4± 1.2	1.1± 0.0	NA	
	3	NA	1.8± 1.2	NA	NA	NA	NA	NA	NA	4.4± 1.1	3.2± 0.0	NA	

NA- No Activity; Lower case indicate the differences lines and upper lines also the differences between columns (Mean±Std. Error); One-way ANOVA and TUKEY tests were applied to the data.

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Table 2. Variance analysis table of tannins by using disc diffusion methods

Source	Type III Sum of Squares	df	MeanSquare	Values F
Tannin	12.49	2.00	6.24	75.13**
Concentration	20.65	3.00	6.88	82.81**
Strain	147.99	10.00	14.80	178.06**
Tannin * Strain	55.17	20.00	2.76	33.19**
Tannin * Concent.	2.17	6.00	0.36	4.36**
Concent. * Strain	5.53	30.00	0.18	2.22**
Tannin* Concent. * Strain	7.43	60.00	0.12	1.49*
Error	10.97	132.00	0.08	
Total	710.83	264.00		
Corrected Total	262.40	263.00		

a. R Squared = .958 (Adjusted R Squared = .917)

4. Discussion and Conclusions

In this study, it was determined that 3 tannin extract showed variable antibacterial effect at four different concentrations against the *Xanthomonas* spp. strains causing the disease in pepper fields in Kahramanmaraş province. According to these results, the presence of antibacterial compounds in tannin extracts was determined. In current study, ZI370 and ZI368 strains were the most sensitive strains for each three tannin extracts and ZI383 strain was the most resistant strain among other strains. There was a difference between the activities of the different tannin extracts in the study. The Farmatan tannin extract was found to be more effective than the other tannins. This difference is thought to be due to the influence of the compounds contained in the tannin extracts. The antibacterial activity of tannic plant extract on food and animal pathogenic bacteria has been determined by several studies and there is no literature study on plant pathogenic bacteria (Doss et al., 2009; Salih et al., 2017). Bektas et al. (2020) demonstrated tannins can be used as an alternative to synthetic compounds in plant disease agents management. However, it has been determined that tannins may interact with bacteria membrane, cell walls and / or extracellular proteins or indirectly by rendering nutrients unusable on bacteria (Smith et al. 2005). For this reason, highly effective extracts can be obtained by making better purification. Firmansyah et al. (2015) based on the research result tannin is a exract that can inhibit the enzyme activities released by plant pathogen and thus is potential to be used as biocontrol agent against the plant disease agent. Canzoniere et al. (2021) demonstrated that condensed and tannins exhibited antimicrobial activity against *Pseudomonas syringae* p v. tomato(Pst), as shown by their in vitro effect to fully inhibit Pst growth 24 h after inoculation. Yılmaz et al. (2014) also stated that the content of extracts should be well known and the differences between the extracts should be compared so that plant extracts can be used as biopesticides. In addition, when the difference between the concentrations was examined, it was determined that the effect was observed at the concentration of 1 mg/ml and that the concentration did not increase at the same time as the concentration increased. Soylu et al. (2006) in their study, noted that plant extracts inhibit the growth of plant pathogens and these extracts have more inhibitory effect than some commonly used antibiotics, while varying according to microorganisms. When appropriate concentration and application of sensitive combination provided better activity than do strains to antibiotics. The fact that plant extracts are less risky to the environment and human health and the more effective against seed pathogens will provide an alternative to the control. All these reseach together with our reseach show that, tannins will support decrease of the use of copper salts in plant disease and pest control programmes, minimising possible effects of these materials on human and environmental health. Keles et al. (2001) stated that the microbial activities of plant extracts were limited and that the obtained extracts were not in pure state. For this reason, better extracts can be obtained by better purification. It has been determined that the Tannins are a hopeful alternative to reduce the harmful effects caused by use of synthetic pesticides. The results suggested that the extracts of tannins could be used as natural bactericides for the control of *Xanthomonas* spp. On the other hand, as antibacterial Activity tests have all been done *in vitro*, the next research is *in vivo* investigations to confirm if infection can be inhibited by the tannin extracts, demonstrating the safe use of these tannin extracts for the prevention of Xanthomonas spp. Future research may involve the identification of tannins secondary metabolites and the evaluation of their activity. Moreover, further research into manufacture of tannins, formularization and delivery may so assist and support the improving of tannins pesticides.

Author contributions

Mustafa Küsek: Conceptualization, Validation, Resource/Material/Instrument Supply, Supervision/ Observation/Advice, Project Administration; Zeynep İmecik: Methodology, Investigation; Ceyda Ceyhan Başaran: Formal Analysis, Data Curation, Original Draft Writing, Software; İdris Bektaş: Review and Editing; Visualization

Conflict of interest

As the authors of this study, we declare that we do not have any conflict of interest statement.

Ethics Committee Approval

As the authors of this study, we declare that we do not have any ethics committee approval.

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