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Quercetin and Cinnamaldehyde Show Antipathogenic Activity Against *Proteus mirabilis* Isolates: Inhibition of Swarming Motility and Urease Activity

Kersetin ve Sinnamaldehitin *Proteus mirabilis* İzolatlarına Karşı Antipatojenik Etkileri: Swarming Hareketi ve Üreaz Aktivitesinin İnhibisyonu

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ABSTRACT

Introduction: *Antipathogenic molecules target not the vital structures but the virulence factors of pathogenic microorganisms to prevent the development of an infection. Proteus mirabilis is a right candidate for testing antipathogenic molecules due to its distinctive features as an uropathogen such as spreading on urinary catheter surfaces by swarming motility, forming crystalline biofilm by intense urease activity and causing kidney stones.*

Materials and Methods: *In this study, the potential antipathogenic effects of quercetin and cinnamaldehyde against swarming motility and urease activity of P. mirabilis isolates from various services and clinics of Balcali Hospital in Cukurova University were investigated. The effect on swarming motility was investigated by measuring the swarm diameter on the agar surface of the isolates exposed to the respective compounds. The effect on urease activity was determined by the analysis of colorimetric changes caused by isolates in the media containing urea and phenol red. In addition, a selected isolate exposed to the related compounds was examined by electron microscopy (SEM) to investigate the morphological changes caused by the compounds.*

Results: *We observed that quercetin inhibited swarming motility for all isolates in a dose-dependent manner. Besides, the cells were found to be in short form and deformed for the quercetin-exposed isolate examined by SEM. Cinnamaldehyde exhibited moderate inhibitory activity against urease despite not being effective against the swarming motility.*

Conclusion: *These molecules, which are commonly found in plants consumed as food or used in phytotherapy, are known to have low toxicity. It is therefore contemplated that these compounds can be used as a prophylactic in urinary tract infections and as a coating agent in catheters. Such potentials should be explored by further in vivo studies.*

Key Words: *Proteus mirabilis; Quercetin; Cinnamaldehyde; Swarming motility; Urease activity*

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ÖZ

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Giriş: *Antipatojenik moleküller, patojenik mikroorganizmaların hayati yapılarını değil virülans faktörlerini hedef alır. Proteus mirabilis, swarm hareketi ile üriner kateterlerin yüzeyinde yayılması, yoğun üreaz aktivitesi ile kristal içerikli biyofilm oluşturması ve böbrek taşlarına neden olması gibi karakteristik özellikleri nedeniyle, antipatojenik moleküllerin test edilmesi için ideal bir adaydır.*

Materyal ve Metod: *Bu çalışmada, Çukurova Üniversitesi Balcalı Hastanesindeki çeşitli servis ve kliniklerden izole edilen P. mirabilis izolatlarının swarm hareketi ve üreaz aktivitesine karşı, kersetin ve sinnamaldehitin potansiyel antipatojenik etkileri incelenmiştir. Swarm hareketine etki, ilgili bileşiklere maruz kalan izolatların agar yüzeyindeki swarm çapının ölçülmesiyle araştırılmıştır. Üreaz aktivitesine etki ise, üre ve fenol kırmızısı içeren besiyerlerinde izolatların neden olduğu kolorimetrik değişikliklerin analiziyle ortaya konmuştur. Ayrıca, ilgili bileşiklere maruz kalan belirli bir izolat elektron mikroskobuyla (SEM) incelenerek, bileşiklerin neden olduğu morfolojik değişiklikler araştırılmıştır.*

Bulgular: *Kersetinin tüm izolatlarda swarm hareketini doza bağlı bir şekilde inhibe ettiği görülmüştür. Ayrıca, SEM incelemeleri neticesinde, kersetine maruz kalan hücrelerin kısa formda ve deforme olduğu tespit edilmiştir. Sinnamaldehit üreaz aktivitesine karşı orta derecede inhibitör etkili bulunmuş, fakat swarm hareketine karşı etkili olmamıştır.*

Sonuç: *Gıda olarak tüketilen ve fitoterapide sıklıkla kullanılan bitkilerde yaygın olarak bulunan bu moleküllerin düşük toksisiteye sahip olduğu bilinmektedir. Dolayısıyla ilgili bileşiklerden idrar yolu infeksiyonlarında profilaktik olarak ve kateterlerde kaplama ajanı olarak yararlanılabileceği öngörülmektedir. Daha ileri düzeyde in vivo çalışmalarla bu tür potansiyeller araştırılmalıdır.*

Anahtar Kelimeler: *Proteus mirabilis; Kersetin; Sinnamaldehit; Swarm hareketi; Üreaz aktivitesi*

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infections. They are seen in all age groups and both sexes. Members of the colon flora are usually transmitted from the perianal region to the urethral opening and travel through the urethra (ascending) to the bladder and kidneys[1,2]. On the other hand, the infection may develop as a result of urinary catheterization. The unique swarming motility of *Proteus mirabilis* plays a primary role in UTI pathogenesis by spreading through the urinary tract epithelium and the catheter surface[3,4].

P. mirabilis is an urease-positive bacterium. Like the swarming motility, *P. mirabilis* urease also plays a vital role in UTI pathogenesis. Urease acts as a catalyst for the release of carbon dioxide and ammonia as a result of hydrolysis of urea. The formed ammonia makes the urine alkaline. Thus, various ions dissolved in the urine precipitate. These precipitates (struvite and apatite crystals) cause the formation of urinary tract stones and accumulate in the biofilm on the catheter surface. This crystalline biofilm clogs the catheter lumen. Due to obstruction of urine flow, urinary incontinence and vesicoureteral reflux emerge. These disorders are predisposing factors for UTIs[5-7].

It is known that many biologically active phytochemicals can exhibit antimicrobial activity through various mechanisms. Phenolic phytochemicals such as cinnamaldehyde, curcumin, eugenol, quercetin, resveratrol show antibacterial and antipathogenic activity by oxidation of hydroxyl groups to free oxygen radicals[8-12]. These radicals interact with functional groups of nucleophilic amino acids, leading to the inhibition of vital enzymes. In addition, phenols, whose lipophilic character is dominant, disrupt the integrity of the cell membrane. They also make complexes

with dissolved proteins and bacterial cell wall. Thus, they inhibit bacterial adhesins, enzymes, and carrier proteins in the cell membrane[13,14].

In our previous studies, potential antipathogenic effects of quercetin and cinnamaldehyde on *P. mirabilis* HI4320 (Mobley Research Laboratory, the University of Michigan Medical School, Ann Arbor, USA) were examined from different angles and this report builds on our prior study by examining detected antipathogenic effects of related phytochemicals on multiple clinical isolates from UTI infections[15,16]. In this context, the effect of the related molecules on viability, the swarming motility, urease activity, and general cell morphology of *P. mirabilis* isolates was investigated by phenotypic tests.

MATERIALS and METHODS

Test Molecules and Media

Quercetin and cinnamaldehyde used in the experiments were purchased commercially (Sigma-Aldrich, USA). Sufficient amount of sterile stock solutions of the molecules in dimethyl sulfoxide (DMSO) at 100 mM concentration were prepared. For the sterilization of solutions, DMSO-compatible regenerated cellulose membrane filters (EMD Millipore, Germany) with a diameter of 0.45 µm were used. Stock solutions were diluted at the required ratios and used in experiments. Lysogeny Broth (LB), LB agar, Mueller Hinton Broth (MHB) and Brain-Heart Infusion Broth (BHIB) (Neogen, UK) were used where appropriate as a medium.

Proteus mirabilis Isolates

The studies were carried out with 10 *P. mirabilis* strains isolated from various services and clinics of Balcali Hospital in Cukurova University (Adana, Turkey) (Table 1). All isolates were stocked at -80°C in BHIB containing 15% glycerin. Studies were performed with fresh cultures grown on LB agar with an overnight incubation at 37°C.

MIC Determination

Minimum inhibitory concentrations (MICs) of the test molecules were determined for all isolates before investigating their antipathogenic effects. By the standards used in MIC determination, test molecules were diluted with LB broth in 96-well U-base microplates at the required concentrations[17]. Then the inoculums, 100-fold dilutions of suspensions of the isolates at 0.5 McFarland density, were added to the wells in the same volume. After one night of incubation at 36 ± 1°C, the microplates were evaluated visually, and the lowest concentration without growth was determined as MIC. Dilutions containing only DMSO were also included in the experiments for control.

Swarming Motility Experiments

A previous method was used to determine the effect of quercetin and cinnamaldehyde on swarming motility[18]. LB agar plates (1.5%) containing the molecule to be tested at the required concentrations (0.1 to 0.4 mM) were prepared

Table 1. Characteristics of the *Proteus mirabilis* isolates studied

Number	Sample type	Origin of isolation
1	Midstream urine	Pediatric nephrology
2	Midstream urine	Orthopedics and traumatology service
3	Midstream urine	Urology service
4	Midstream urine	Pediatric nephrology
5	Midstream urine	Pediatric surgery
6	Urinary catheter	Pediatric intensive care service
7	Urinary catheter	Neurosurgery service
8	Midstream urine	Oncology service
9	Suprapubic	Dermatology service
10	Midstream urine	Hematology service

for both quercetin and cinnamaldehyde. After preparation, the plates were allowed to dry for 24 hours at 37°C. The plates containing DMSO was also included in the experiments as the control. The inoculums to be used in the experiments were obtained by inoculating fresh culture into LB broth medium and culturing for several hours. By taking 1 mL of the LB broth culture into the microcentrifuge tube, the cells were washed a few times with centrifugation (Thermo Scientific, USA) (10.000 rpm, 1 min) and resuspension cycles in serum physiologic (SF). After washing, final suspension was adjusted to 0.5 McFarland density by a densitometer (Grant Instruments, UK). Onto the previously dried plates, 5 µL was added from the prepared inoculum and incubated at 37°C for 12 hours. At the 12th hour, the swarming diameter was measured in millimeters and recorded. For each concentration, the mean of the swarm diameter detected in three plates was taken, and the results were analyzed by comparing with the control group.

Urease Activity Experiments

In order to determine the effect of quercetin and cinnamaldehyde on urease activity, isolates that were cultured in LB agar plates (0.1% urea) containing quercetin or cinnamaldehyde at 0.3 mM concentration were used[19,20]. DMSO containing control plates were also included again in the experiment. After 12 hours of incubation, suspensions of the isolates at 3 McFarland density were prepared. By taking 1 mL of the prepared suspension into the microcentrifuge tubes for each isolate, the cells were washed a few times with centrifugation (10.000 rpm, 1 min) and resuspension cycles in serum physiologic. After washing, the supernatant was discarded, and the remaining pellet was suspended by the addition of 1% sodium dodecyl sulfate (SDS) and incubated for 2 min. After one last centrifugation (10.000 rpm, 1 min), the urease activity of the soluble protein in the supernatant was determined spectrophotometrically. For this purpose, 75 µL of supernatants were added to the flat bottom microplate containing the same volume of indicator solution (7 µL/mL phenol red, 120 mM urea). The absorbance value of the wells was determined reading at OD550 nm by a microplate reader (Thermo Scientific, USA) and evaluated according to the formula given below[21].

Urease Inhibition Rate (%) = (A-B)/A x 100

(A: Absorbance value for the control group; B: Absorbance value for the molecules)

Examining the Effects of the Molecules on *P. mirabilis* Morphology by SEM

The effect of the swarming inhibitory molecule(s) (here only quercetin) on *P. mirabilis* morphology was examined by observing the changes in the surface morphology of the isolates exposed to the inhibitory molecule by FEI Quanta 650 Field Emission (Thermo Scientific, USA). For sample preparation, LB agar plates with quercetin at 0.2 mM concentration were prepared[22]. Then plates were inoculated according to 'swarming motility experiments' by taking from a fresh culture of the selected isolate (no. 2). At the end of 12-hour incubation, a small amount of the bacterium spread on the surface of the plates was resuspended in 2.5% glutaraldehyde and incubated for 5 min. Subsequently, the cells collected by centrifugation (10.000 rpm, 1 min) were resuspended in SF. With a single-use disposable plastic loop, 10 µL suspension was taken up, spread on a clean slide, and allowed to dry overnight. After proper gold coating and grounding procedures, the prepared slide was examined[23].

RESULTS

According to the microdilution method applied to the isolates, MIC values of cinnamaldehyde for all isolates were 256 µg/mL. Quercetin did not show inhibitory activity against any isolates at the tested concentrations (> 1024 µg/mL).

The effect of quercetin and cinnamaldehyde on swarming motility of the isolates is represented in the graph in Figure 1. When the swarming diameter was measured at the 12th hour, significant swarming inhibition was detected for all isolates grown on the quercetin containing plates (except isolate no. 3 at 0.1 mM concentration) compared with the control plate containing only DMSO ($p< 0.01$). It was observed that the inhibitory activity increased in direct proportion to the concentration. Also, cinnamaldehyde caused dose-dependent inhibition in isolates no. 4,8,9

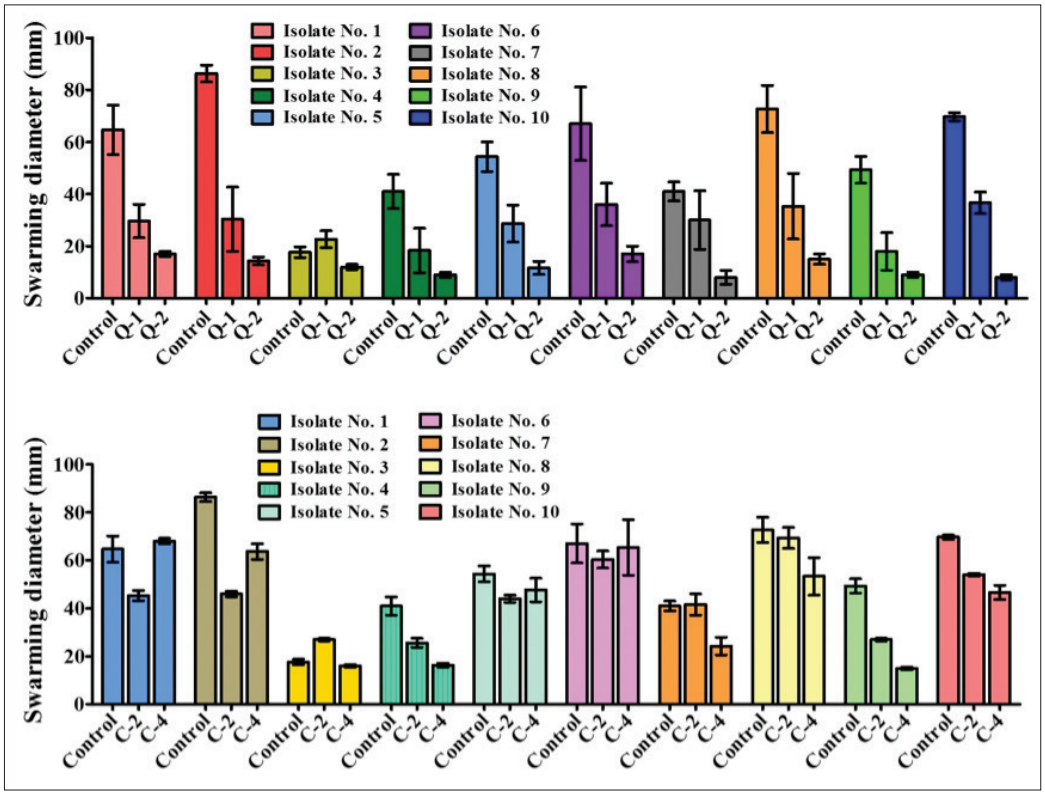

Figure 1. Effect of quercetin and cinnamaldehyde on the swarming diameter for various concentrations. The control groups contain only DMSO, which is used as a solvent. The bars in the columns represent means and standard deviations for the data obtained from three separate plates. Q-1: Quercetin (at 0.1 mM concentration); Q-2: Quercetin (at 0.2 mM concentration); C-2: Cinnamaldehyde (at 0.2 mM concentration); C-4: Cinnamaldehyde (at 0.4 mM concentration).

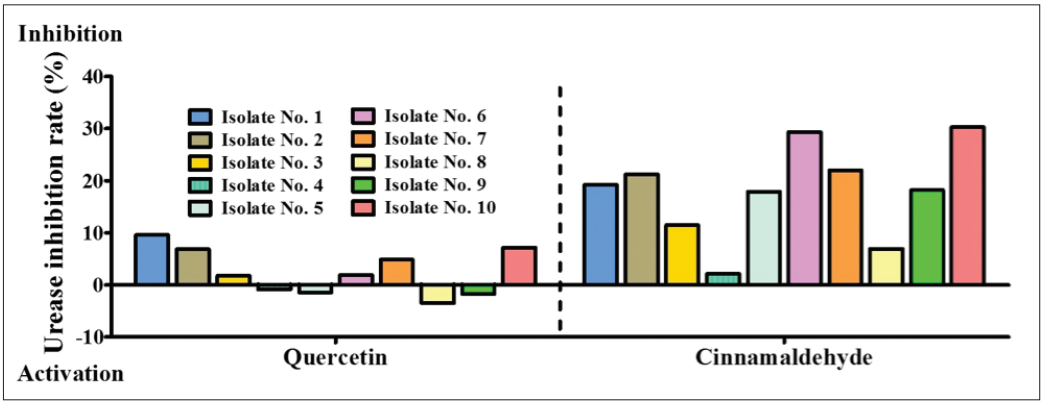

Figure 2. Effect of quercetin (0.3 mM) and cinnamaldehyde (0.3 mM) on urease activity (%). Control groups are omitted for clarity of the graph, and relative results were given for quercetin and cinnamaldehyde.

and 10. On the other hand, for the various concentrations of cinnamaldehyde, dose-independent inhibition was observed in isolates 1, 2, 3, 5, 6 and 7.

The effect of quercetin and cinnamaldehyde at 0.3 mM concentration (an average inhibitory dose) on the urease activity of isolates was represented in the graph in Figure 2. At this concentration, in the spectrophotometric measurements after 12

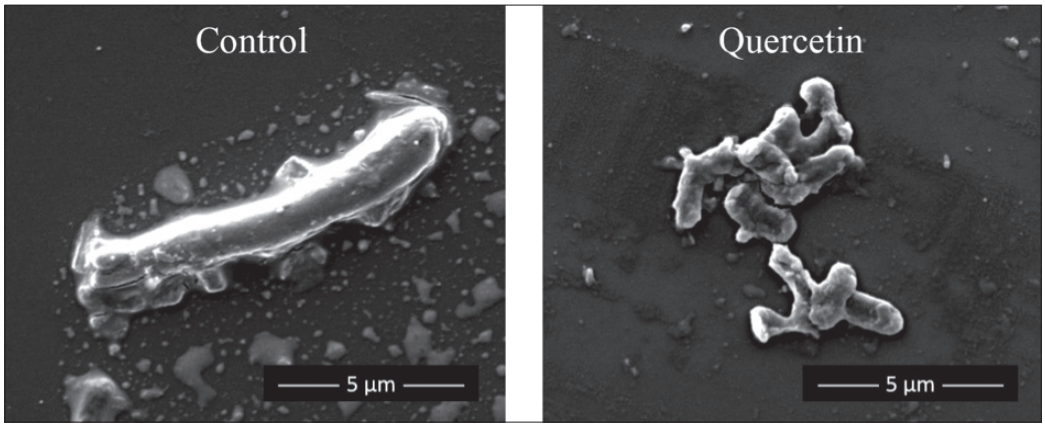

Figure 3. SEM images of preparations from isolate no. 2 grown on plates containing quercetin (0.2 mM) or only DMSO as a control.

hours incubation, a significant urease inhibition was observed in isolates that were cultured in cinnamaldehyde containing plates (except isolates no. 4 and 8) when compared to control groups containing only DMSO. Over 10% inhibition rate was detected for 8 isolates by cinnamaldehyde and 4 of them were higher than 20%. No significant inhibition of urease activity was detected for any isolates grown on quercetin containing plates.

The effect of quercetin on the morphology of selected isolate no. 2 was demonstrated by direct observation with SEM (Figure 3). Typical, quite long bacilli were observed in the control group, while the cells observed in quercetin containing plates were found to be in short form and deformed. On the other hand, cinnamaldehyde did not cause any visible changes in cell morphology.

DISCUSSION

By the determined objectives at the beginning of our study, quercetin and cinnamaldehyde were found to be effective inhibitors of *P. mirabilis* swarming motility and urease activity. Such approaches targeting virulence factors that play a role in infection pathogenesis aim to neutralize pathogenic bacteria without killing. Thus, molecules which are both active and less likely to develop resistance due to selective stress can be developed. In general, the higher the bactericidal effect of a molecule, the higher the probability of resistance. Therefore, molecules such as quercetin and cinnamaldehyde, which have no known toxic effects for bacteria at concentrations where they are effective as virulence inhibitors, may be an alternative option to solve the resistance problem.

Phytochemicals such as resveratrol, geraniol, citral, tannic acid, curcumin have been reported to inhibit swarming motility of various bacteria including *P. mirabilis*[9,12,24-26]. In our study, quercetin inhibited the swarming similarly or more effectively compared to these studies. As can be clearly understood from the SEM image of the selected isolate, quercetin-exposed cells cannot perform swarm differentiation and remain in short form. On the other hand, no significant inhibition of swarming was detected with cinnamaldehyde. The inhibition of swarming was evident for all isolates with increasing doses of quercetin, whereas for only 3 isolates a similar situation was observed with cinnamaldehyde. When urease inhibition was evaluated, cinnamaldehyde was found to be moderately active, and no significant inhibition was observed with quercetin.

For both swarming inhibition by quercetin and urease inhibition by cinnamaldehyde, the results of our previous studies performed with *P. mirabilis* HI4320 are similar with the data obtained from clinical isolates in this study, except that cinnamaldehyde inhibits not only urease but also swarming of *P. mirabilis* HI4320. In addition, cinnamaldehyde inhibited urease at different rates in different isolates. It is thought that these variations are due to differences between the urease activity of the isolates, which requires investigating this issue at the molecular level. Besides, the ineffectiveness of quercetin in this study may also be related to the method followed. Because

quercetin is a yellow compound, it can lead to misleading results in spectrophotometric methods. Therefore, it is planned to investigate the effect of urease expression by molecular methods with future studies.

In the prevention and treatment of infectious diseases caused by urease-positive bacteria, the potential benefits of urease inhibitors lack the attention they deserve. It is possible to find alternatives to licensed but highly toxic molecules such as acetohydroxamic acid among many phytochemicals with low toxicity and readily available[27]. The urease inhibitory qualification of cinnamaldehyde, which is shown in our study, should be evaluated in this respect. This compound, which is frequently used in the food and cosmetic industry and traditional folk medicine, has no known toxic effects at concentrations which shows inhibitory activity. It has been shown in various studies that cinnamaldehyde also has other therapeutic properties that can be used in many conditions. Similarly, quercetin is also a commonly known and widely searched molecule in the medical literature. The effectiveness of these molecules in UTI prophylaxis and catheter coating should be examined in detail with further studies.

As a result of this research, cinnemaldehyde and quercetin had antibacterial and antipathogenic potential against *P. mirabilis* isolates. It can be envisaged that these compounds may be utilized against *P. mirabilis* and similar pathogens which may develop significant resistance to antibiotics used in urinary tract infections such as cotrimoxazole and beta-lactams[28].

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CONFLICT of INTEREST

Authors have no competing interests to disclose.

AUTHORSHIP CONTRIBUTIONS

Concept/Design: AA, PÇ

Analysis/Interpretation: AA, FK, PÇ

Data Acquisition: AA

Writting: AA

Critical Revision: AA, FK, PÇ

Final Approval: AA, FK, PÇ

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