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The Effects of Capsicum annuum L. Extract on the Control of Single and Dual Biofilms of Common Pathogenic Strains Causing Urinary Tract Infection

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Abstract

***Background*:** Biofilms directly influence the virulence and pathogenicity of a pathogen, it is optimal to employ a strategy that effectively inhibits the formation of biofilm. In this study, the antibacterial and anti-biofilm activities of extract *Capsicum annuum* L were examined.
Materials and Methods: The eight strains were isolated from urine culture of hospitalized patients; growth and biofilm formation of strains were determined by microtiterplate method.
Results: The results revealed that the concentrations of 5 and 10 mg/mL are the most restrain in the biofilm formation of the isolated plates.
Conclusion: Results of this study suggest that the extract of *C. annuum* L may be useful alone to treat bacterial infections.

Introduction

Microorganisms are found in a wide range of ecosystems as highly structured multi-species communities, termed biofilms [1]. The downside of microbial biofilms is associated with their involvement in major problems related to industry, medicine and everyday life [2]. Biofilms constitute a protected mode of growth that allows microorganisms to survive in hostile environments. Antimicrobial agents were the main weapons used to control biofilms, acting either by interfering with microbial metabolism or by facilitating their detachment from the surface [3, 4]. The target of an antimicrobial strategy is to inactivate and reduce the number of microorganisms and to control the formation of biodeposits on surfaces [5].

Urinary tract infection (UTI) is the second most common type of infection in the body [6]. The most common cause of UTI is Gram negative bacteria that belong to the family of Enterobacteriaceae. Members of these families include *Escherichia coli*, Klebsiella, Enterobacter and Proteus. Also Gram positive Stapylococcus sp. plays a role in the infection [7]. Pepper (Capsicum), fruit and vegetables are important sources of bioactive compounds (such as phenolic compounds, terpenoids, steroids and alkaloids) known for their health-promoting effect against degenerative diseases [8]. In this study, the effects of extract *Capsicum annuum* L on the control of single and dual biofilms of common pathogenic strains resulting in urinary tract infection by microtiterplate method were studied.

There is a continuing quest for safe and effective antimicrobial agents. This need has been resolved recently by the emergence of many antimicrobial-resistant organisms such as *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *E. coli* and *Staphylococcus aureus*. Growth of bacterial strains as a biofilm mass occurs under a variety of environmental conditions.

Materials and Methods

Isolation of bacteria: This cross-sectional study was performed to evaluate 8 strains (*K. pneumonia*, *P. aeruginosa, E. coli* and *S. aureus*, two strains for each one) which were isolated from urine culture of hospitalized patients (Amir Al-Momenin hospital, Zabol, south-eastern Iran) who were suffering from urinary tract infections during the years 2010-2011. Isolated bacteria were identified by Gram's stain and standard biochemical tests [9] and the isolated Gram and catalase positive cocci were further tests for biochemical characterization, carbohydrates fermentation followed by urease, ONPG (ortho-Nitrophenyl-β-galactoside), vogues-proskauer, arginine ultilization, lysostaphin sensitivity, coagulase, clumping factor thermonuclease, haemolysin tests.

Antibiotics susceptibility: The susceptibility of all antibiotics was carried out using disc-diffusion method on Muller-Hinton agar as recommended by CLSI (Clinical and Laboratory Standard Institute). The procedure followed is briefly described here. The bacteria of isolates were grown overnight on nutrient agar and colony suspension was prepared using the sterile saline water equivalent to a 0.5 McFarland standard. Suspension (100 µL) was spread over the media plate and antibiotic disc was transferred aseptically on the surface of inoculated media plate. These plates were incubated afterward at 37ºC in antibiotic for 24 h. Zones of growth inhibition were recorded. Isolated plates were tested with different

antibiotics and their concentration shown in parenthesis viz. ampicillin (25 µg), trimethoprim-sulfamethoxazol (1.25+23.15 µg), ceftazidime (30 µg), gentamicin (10 µg), nalidixic acid (30 µg) and methicillin (30 µg) disks (Mast, UK) were placed on media in 20-30 mm with other disks [10].

Plant materials: The fruit *Capsicum annuum* L was collected in the region of Iran (Zahedan and Kerman, south-eastern, Iran) and were planted in Kerman Islamic Azad University herbarium received approval and to dry the plant: temperate room and dark place were used. Samples were crashed and transferred into glass container and preserved until extraction procedure was performed in the laboratory.

Preparation of extracts: Plants were properly dried and pulverized into a coarse powder as reported by Hanafy and Hatem [11]. Each of 20 g grinded powders was soaked in 60 mL ethanol 95%, separately for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered (Whatman No. 1 filter paper). Then the filtrates were evaporated using rotary evaporator. At last, 0.97 g of dried extracts were obtained and then stored at 4ºC in air tight screw-cap tube.

Minimum Inhibitory Concentration (MIC) of plant extracts: The broth microdilution method was used to determine MIC according to Yu et al. [12]. All tests were performed in Mueller Hinton Broth supplemented with Tween 80 at a final concentration of 0.5% (volume / volume). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 0.3 mg/mL to 10.00 mg/mL. To each well, 10 µL of indicator solution (prepared by dissolving a 10 mg extract in 2 mL of DMSO) and 10 µL of Mueller Hinton Broth were added. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) was added to each well to achieve the concentration of 10^4 CFU/mL. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37ºC for 18-24 h. The color change was then assessed visually. The lowest concentration at which the color change occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity.

Biofilm formation assay in presence of the biocides: After performing the procedure described above, the microplate was covered and incubated aerobically for 24 h at suitable temperature. At first, the OD (Optical Density) was measured (600 nm) by using an automated ELISA counter, then, the content of each well of the microplate was aspirated and each well was washed three times with 250 µL of sterile physiological saline.

The remaining attached bacteria were fixed with 200 µL of 99% methanol per well and after 15 min all of the wells were emptied and left to dry. Then, each well was stained for 5 minute with 0.2 mL of 2% crystal violet. Excess stain was rinsed off by washing the plate slowly with distilled water. After the plate was air dried, the dye bound to the adherent cells was resolubilized with 160 mL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 492 nm by using an automated ELISA counter.

Statistical analyses: The growth was compared at each experiment using analysis of variance (ANOVA) repeated measures (SPSS-16.0 for Windows). The level of statistical significance was set at $p< 0.01$.

Results

Eight clinical isolated plates included of *K. pneumonia* , *P. aeruginosa*, *E. coli* and *S. aureus* (two strains for each one) were obtained from the Amir al-Momenin hospital (Table 1) and the MIC *Capsicum annuum* L is shown in table 1. The changes of bacterial biofilms brought about treatment with various concentrations of extract plant are shown in table 2 and 3. Strains that were exposed to sub-MIC and MIC levels of extract exhibited a reduction in the OD_{492} reading compared to the control. These results showed that these strains exhibited an impaired ability to form a biofilm compared to the control. The achieved results showed that the bacteria resistant to cephalosporin compared to the sensitive ones are able to form more biofilms. Among under studies bacteria, *P. aeruginosa* antibiotic- resistant has made the most amount of biofilm during 2 h. The amount of formation has been increased by enhancing the duration. But as it can be seen in diagrams, different densities of extract have an inhibiting effect on the biofilm formation in comparison with the situation that there is no inhibitor factor in the environment.

Table 1. The isolates of bacterial for urinary tract infection and MIC extract of C. annum

Isolates of bacterial	Antibiotic-pattern						MIC extract plant
	Gm	CAZ	NA	AM	Me	SXT	(mg/mL)
E. coli1	I	S	R	R	S	S	5
E. coli2	S	R	R	I	S	S	10
Klebsella1	S	S	S	R	S	I	2.5
Klebsella2	R	R	S	R	S	R	5
P. aeruginosa1	S	S	S	R	S	S	1.25
P. aeruginosa2	S	R	S	S	S	S	5
S. aureus1	R	S	S	I	S	R	1.25
S. aureus2	S	s	S	S	R	R	1.25

Gm= gentamicin, CAZ=ceftazidime, NA=nalidixic acid, AM=ampicillin, SXT= sulfamethoxazol

Table 2. The effects of different concentrations of extract plant on the biofilm formation of bacterial and control (2 h)

Extract concentration (mg/mL) / Bacterial	0.62 mg/mL of extract	1.25 mg/mL of extract	2.5 mg/mL of extract	5 mg/mL of extract	10 mg/mL of extract	Control biofilm any extract
Ecoli 1	0.005	0.002	0.001	0.000	0.000	0.007
Ecoli2	0.003	0.003	0.003	0.002	0.001	0.011
Klebsella1	0.005	0.004	0.003	0.001	0.000	0.008
Klebsella2	0.009	0.007	0.004	0.003	0.003	0.010
Pseudomonas1	0.009	0.007	0.005	0.004	0.002	0.010
Pseudomonas2	0.012	0.007	0.007	0.006	0.005	0.016
S.aureus1	0.001	0.002	0.002	0.000	0.000	0.005
S.aureus2	0.003	0.003	0.005	0.000	0.000	0.008

Table 3. The effects of different concentrations of extract plant on the biofilm formation of bacterial and control (24 h)

Extract Concentration(mg/mL) / Bacterial	0.62 mg/mL of extract	1.25 mg/mL of extract	2.5 mg/mL of extract	5 mg/mL of extract	10mg/mL of extract	Control biofilm any extract
Ecoli 1	0.013	0.010	0.010	0.009	0.006	0.014
Ecoli2	0.014	0.011	0.010	0.009	0.008	0.016
Klebsella1	0.011	0.009	0.005	0.003	0.002	0.011
Klebsella2	0.014	0.012	0.010	0.008	0.005	0.015
Pseudomonas1	0.015	0.014	0.012	0.009	0.005	0.017
Pseudomonas2	0.017	0.015	0.010	0.007	0.005	0.023
S.aureus1	0.004	0.006	0.006	0.008	0.013	0.015
S.aureus2	0.007	0.009	0.012	0.012	0.015	0.018

Discussion

The result showed that concentrations of 5 and 10 mg/mL showed the most restrain in the biofilm formation of the isolated plates. Selected natural products that originate in plants can influence microbial biofilm formation. Neanderthals living 60,000 years ago in present location of Iraq used plants such as holly back, these plants are still widely used in ethnomedicine around the world. In this study, we report that the extract of *Capsicum annum* reduced the biofilm bacterial information. It also showed that the alcoholic extracts of *Capsicum annum* had potent antimicrobial activity against ESBL- product of *E. coli, K. pneumonia*, *P. aeruginosa* isolated plates (Table 1) and the result showed that concentrations of 5 and 10 mg/mL are the most restrain in the biofilm formation of the isolated plates. In the study of Careaga, the results showed that the minimum inhibitory extract concentration of *Capsicum annum* to prevent the growth of *Salmonella. typhimurium* in minced beef was 1.5 mL/100 g of meat; the addition of 1%, 2%, 3% and 4% w/w of sodium chloride did not have any additional inhibitory effect on Salmonella [13]. The result of de Marino et al. show that four new acyclic diterpene glycosides named capsianosides [1-4], together with 12 known compounds, were isolated from the fresh sweet pepper fruits of *Capsicum annuum* L., a plant used as a vegetable food, spice, and external medicine that known capsidiol [11] showed bacteriostatic properties in vitro against *Helicobacter pylori* with a minimum inhibitory concentration (MIC) of 200 µg/mL when compared with the commercial drug metronidazole (MIC, 250 µg/mL) [14]. The study of Koffi-Nevry et al., showed the effect of *Capsicum annuum* and *Capsicum frutescens* methanol and aqueous extracts on selected bacteria (*S. aureus, Salmonella typhimurium, Vibrio cholerae, P. aeruginosa, E. coli,* and *Shigella dysenteriae*) were investigated that result show both extracts were found to be effective against *Vibrio cholerae, S. aureus,* and *Salmonella typhimurium,* while methanol extracts showed the greatest effect [15]. The study of Keskin and Toroglu, *Capsicum annum* (red pepper) fruit extracts showed various antibacterial activities (7-20 mm 30Al-1 inhibition zone) to the microorganisms tested. The methanol extracts did not inhibited microorganisms tested except for *P. aeruginosa* [16]. Erturk was obtained antimicrobial activity of *C. annum* [17]. The extract of *Capsicum annum* could be an important factor that resulted in the reduction of biofilm formation. Also, it was suggested that essential oils and extract acted with the help of their lipophilic fraction reacting with the lipid parts of the cell membranes, investigation showed that damaging the microbial membrane structures could influence biofilm formation. It can be suggested that different climates plants grown and different extraction methods might be effective on the antimicrobial activity. Results of this study suggest that the extract of *Capsicum annum* may be useful either alone or when combined with antimicrobial agents, to treat bacterial infections. The antibacterial properties of *Capsicum annum* extract are mostly attributable to the *Capsicum annum* aldehyde.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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