RESEARCH ARTICLE

Evaluating the Biofilm Inhibitory Effect of Flavonoids Extracts Purified from Orange (*Citrus sinensis* L.) Peel

Sahar Mustafa[1], Wisam Al-Mayah[2], Mohammed K. A. Al-Maeeni[3], Khalood W. Abooud[4], Sura H. Al-Zubaidi[5], Hawra'a F. A. AL-Baghdady[6], Hayder A. Hammoodi[7]*

[1]*Medical Laboratory Technology, Ashur University College, Baghdad, Iraq*
[2]*Al-Manara College for Medical Sciences, Amarah, Iraq*
[3]*Al-Nisour University College, Baghdad, Iraq*
[4]*Medical Lab. Techniques Department, College of Medical Techology, Al-Farahidi University, Iraq*
[5]*Anesthesia Techniques Department, Al-Mustaqbal University College, Babylon, Iraq*
[6]*College of Dentistry, The Islamic University, Najaf, Iraq*
[7]*College of Pharmacy, Al-Ayen University, Thi-Qar, Iraq*

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ABSTRACT

Flavonoids were extracted from the peels of the Orange (*Citrus sinensis* L.) plant. The total flavonoids were detected with qualitative and quantitative assays using two solvents, n-hexane and methanol, then purified by silica gel chromatography. Varying bacterial genera from urinary tract infection (UTI) infections were tested for biofilm development. It was discovered that these isolates had different biofilm potential. The use of flavonoids led to reducing biofilm formation in a dose-dependent way so that *Pseudomonas aeruginosa* had the highest inhibitory activity at 70%, followed by *Klebsiella pneumoniae* at 68 % and *Candida albicans* at 65%. In contrast, *Escherichia coli* and *Staphylococcus aureus* showed lower inhibition rates of 33 and 34%, respectively. These findings suggest that flavonoids may effectively treat biofilm-associated bacterial infections and overcome multi-antibiotic resistance challenges.

Keywords: Biofilm formation, Flavonoids, Orange (*Citrus sinensis* L.).

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INTRODUCTION

Citrus flavonoids are a type of secondary metabolite that substantially impacts human health. These molecules can be found in various places, including citrus fruits.[1] Flavonoids are secondary metabolites found in plants that have a variety of roles in plant physiology and development, including oxidative stress protection and signaling, pigmentation, UV protection, defense, and plant-microbe signaling.[2] They have antioxidant, anti-inflammatory, antibacterial, antiviral, antiprotozoal, antifungal, anticancer, and antiallergic qualities that can help people stay healthy and avoid diseases.[3]

Bacterial biofilms are multicellular populations of bacteria encased in an extracellular polymeric substance (EPS) matrix.[4] Bacteria can colonize a variety of abiotic and biotic surfaces by creating biofilms. As a result, microorganisms must either defend their surfaces or at least regulate colonization, such as by interfering with the synthesis of bacterial EPS. Exopolysaccharides, secreted proteins (some of which can form amyloid fibers), extracellular DNA, and lipids make up matrix EPS.[5] Matrix components facilitate bacterial surface adherence, offer mechanical stability to biofilms, and protect bacteria from the harmful effects of chemical assaults and other environmental challenges by building a coherent polymer network.[4] Bacterial cells in biofilms are physiologically diverse, with spatially structured subpopulations coexisting in various stages of development, including many stress-resistant cells in biofilms' enormous stationary phase zones.[6,7]

Lung infections, ear and eye infections, dental illnesses, and urinary tract infections are only a few instances of pathogenic strains forming biofilms that cause major health problems. In industry, harmful biofilms are a problem because they induce corrosion in heat exchangers and pipes used to transport oil and service water. Biofilm cells persist, are

**Author for Correspondence: hayder.hammoodi@alayen.edu.iq*

antibiotic-resistant, and result in medical treatment failure.[8] As a result, an attempt was made in this work to extract and purify flavonoids from orange peel, as well as an assessment of Flavonoids' antibiofilm and antiadhesive properties against several biofilm-forming bacteria that cause UTI infections.

MATERIALS AND METHODS

Sample Preparation and Extraction

The orange peels were pulverized to a fine powder in a blender and dried in a drying oven at 70°C. N-Hexane (200 mL) was used to defat 100g of fine powder using a Soxhlet extractor. At a temperature of 65°C, the extraction took around six hours. The material was dried in a 50°C oven after extraction. Methanol was used to extract the extracted solution further (200 mL). The extract was then evaporated in a water bath to eliminate the solvent.

Primary Purification

The methanol extract was adjusted to 90% with water, then partitioned with a combination of t-Butylmethyl ether and n-hexane (9:1). The Methanol fraction split and evaporated after vigorous shaking.[9]

Quality Test for Flavonoid

The plant extracts (2 mL) were acidified with 1% HCl and dissolved in a 20% NaOH solution. A carnary yellow color represented flavonoid.[10]

Determination of Total Flavonoid Content

One mL of a diluted solution containing flavonoids, 0.7 mL of 5% (w/w) $NaNO_2$, and 10 ml of 30% (v/v) ethanol were combined and swirled for 5 minutes, after which 0.7 ml of 10% $AlCl_3$ (w/w) was added, and the mixture was stirred up.[11] A 5 mL of 1 M NaOH was added six minutes later. Before the measurement, the solution was diluted to 25 mL with 30% (v/v) ethanol. The absorption of the solution was measured at 500 nm with a spectrophotometer after 10 minutes of standing. By comparing flavonoids to a rutin standard curve, the levels of flavonoids were expressed in mg rutin per g, dry weight basis, and the yield of flavonoids was estimated using the following formula:

$Y = (6.404A + 0.2806)\ BV/W\ (mg/g)$

where: A: absorbance (500 nm) B: dilution factor W: dry weight (g), V: volume of the extracting agent (mL)

Secondary Purification of Flavonoids

The methanolic extract) was applied over the Silica gel column and eluted with a solvent mixture of (70:30:1.V/V) of CH_2Cl_2/ CH_3OH/H_2O, respectively. The fractions were further screened for flavonoids.[12]

Isolation and Identification of Bacteria from UTI Infection

Different bacterial species were collected from Baghdad hospitals*, including P. aeruginosa* and *Klebsiella pneumonia*, *E. coli*, *Proteus mirabilis,* and *S. aureus* and reidentified by VITEK 2 system using (GN-cards) and (GP-cards).

Screening of Biofilm Formation Ability

E. coli, Proteus merabolis, S. aureus and *C. albicans* well-isolated colonies grown overnight at 37°C on tryptic soy agar (TSA) were inoculated in tryptic soy broth (TSB) supplemented with 2% (w/w) glucose to examine the biofilm-forming potential. Culture supernatants from each isolate were diluted 1: 200 in TSB after being incubated at 37°C for 24 hours. A 96-well polystyrene microtiter plate with a flat bottom was used to transfer aliquots of bacterial suspension (200 µL, 1.5 10^8 CFU/mL, final concentration). The negative control was the medium without the bacterial suspension. The plates were incubated at 37°C for 24 hours, then decanted, and planktonic cells were removed by washing three times with phosphate-buffered saline (PBS; pH 7.4). Biofilms were washed with water before being dyed with 200 µL of gram's crystal violet for 1 minute. Biofilms that had been stained were rinsed with water and dried. The amount of crystal violet binding was determined by destaining the biofilms for 10 minutes with 200 µL of 33% acetic acid and measuring the absorbance of the crystal violet solution at 595 nm.[13] All of the assays were repeated three to five times with similar significant declines in absorbance values, and the results were averaged.

Biofilm and Adhesion Inhibition by Flavonoids

The purified flavonoids were tested to see if they might prevent biofilm development in the biofilm-producing bacteria stated before. When the cells were inoculated, the flavonoids were added to the growing medium, and the cells were allowed to develop a biofilm. Bacterial suspensions (200 µL 1.5 x 10^8 CFU/mL, final concentration) were mixed with various concentrations of pure flavonoids (50, 100, and 200 µg/mL) and water as a control before being placed on the plate. The plate was incubated for 24 hours at 37°C. Biofilms were washed with water before being dyed with 200 µL of Gram's crystal violet for 1 minute. Biofilms that had been stained were rinsed with water and dried. The amount of crystal violet binding was determined by destaining the biofilms for 10 minutes with 200 µL of 33% acetic acid and measuring the absorbance of the crystal violet solution at 595 nm. All assays were repeated three to five times, with similar dramatic reductions in absorbance values. For each harmful bacteria, the percentage of biofilm inhibition of pure flavonoids was calculated using the equation stated by[14]:

Percentage of biofilm inhibition (%) = [1- (A/Ao)] × 100

A refers to the absorbance of the well with the purified flavonoids and A_0 refers to the absorbance of the control well.

RESULTS AND DISCUSSION

Qualitative Screening of Flavonoids Formation

About 100 g of orange peel powder, when treated with n-Hexane, led to extract of flavonoids with a yield (+) using alkaline reagent for flavonoids with the production of bright yellow color. Then when it was extracted with methanol produced a yield (++) as reported in Table 1.

Quantitative Screening of Flavonoids Formation

Based on the standard curve for different concentrations of the standard rutin, the total quantity of flavonoids found in the

orange peel powder was 4.9 and 6.8 mg/mL total flavonoids when the extraction was done by n-Hexane and methanol, respectively (Table 1).

Secondary Purification of Flavonoids

The methanolic extract, when passed through a silica gel column and eluted with $CH_2Cl_2/CH_3OH/H_2O$ (70:30:1.v/v), led to appear three peaks and the total flavonoids **c**ontent was found in the second peak with a yield 12.16 mg/g as shown in Figure 1.

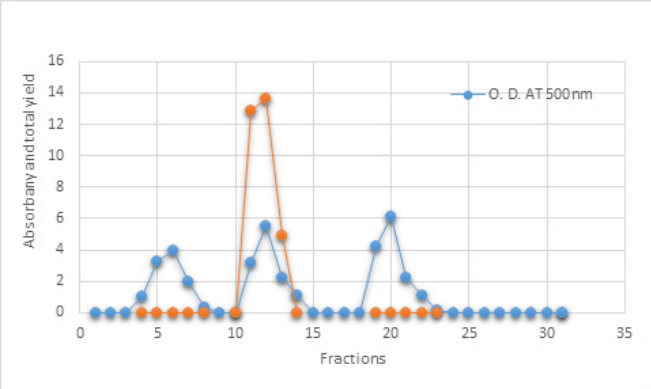

Figure 1: Purification of flavonoids by silica gel chromatography.

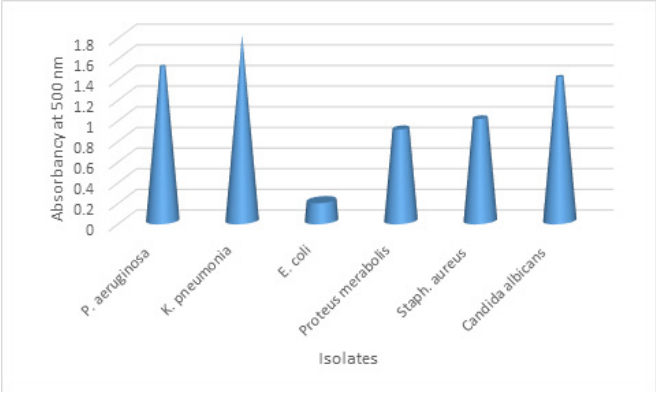

Figure 2: Screening biofilm formation by UTI infection isolates

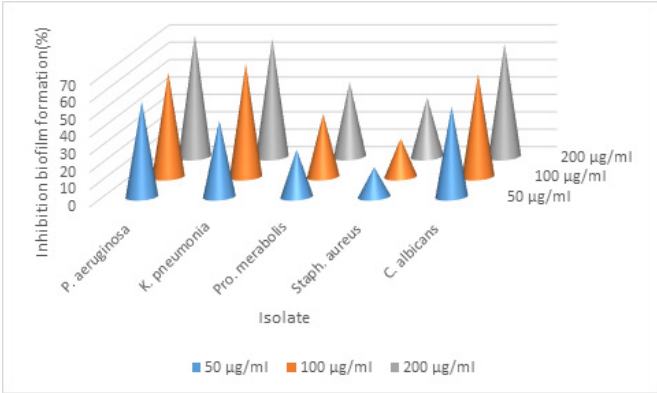

Figure 3: Inhibition of biofilm formation by purified flavonoids

Table 1: Qualitative and quantitative estimation of flavonoids

Solvent	*Qualitative estimation*	*Quantitative estimation (mg/g)*
n-Hexane	+	4.9
methanol	++	6.8

Screening of Biofilm Formation Ability

Varying bacterial genera from UTI infections were tested for biofilm development, and it was discovered that these isolates had different biofilm potential. *K. pneumoniae* developed the highest biofilm level, followed by *P. aeruginosa* and *C. albicans*, making those isolates good biofilm formers. *E. coli*, on the other hand, developed a lesser level of biofilm and is regarded as a poor biofilm producer. *S. aureus* and *P. merabolis*, on the other hand, developed a medium level of biofilm, as seen in Figure 2.

The ability to colonize polymeric surfaces, linked to cling to materials and form biofilm by producing multi-layered cell clusters embedded in extracellular material, is thought to be a key role in bacterial pathogenesis.[15] Each biofilm-forming bacterium produces different EPS components, which are one of the key constituents of the extracellular biofilm matrix. This EPS protects against the human innate immune system's attack.[16]

Biofilm and Adhesion Inhibition by Flavonoids

The findings revealed that all gram-negative and gram-positive biofilm-forming bacteria and fungi's ability to produce biofilms was dramatically reduced dose-dependent in the presence of flavonoids. According to the findings, *P. aeruginosa* had the highest inhibitory activity at 70%, followed by *K. pneumoniae* at 68% and *C. albicans* at 65%. In contrast, described in *Escherichia coli* and *S. aureus* showed lower inhibition rates of 33% and 34%, respectively (Figure 3).

These findings show flavonoids with sub-MICs suppress biofilm formation in S. aureus strains that overexpress efflux protein genes.[17] The presence of an O-glycosidic bond in a flavonoid glycone molecule is linked to a lack of affinity for the phospholipid bilayer or certain cell membrane receptors, resulting in decreased contact with target bacterial cells(18). Although a link between glycone and aglycone flavonoids' structure and antibacterial properties has been established, there are no studies of flavonoids' influence on biofilm formation inhibitory effects.

CONCLUSION

These findings suggest that flavonoids may effectively treat biofilm-associated bacterial infections and overcome multi-antibiotic resistance challenges.

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