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# *Escherichia coli* biofilm formation and control by phenolic compounds from *Salvia officinalis* L. extracts

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Biofilm is recognized as one of the virulence factors of uropathogenic *Escherichia coli*. It offers bacteria significantly increased tolerance to antibiotics and makes difficulties in infection eradication. In this study, the biofilm formation ability of clinical isolates of uropathogenic *E. coli* as well as the antibiofilm activity of the common Garden sage, *Salvia officinalis* L. extracts were investigated *in vitro*. The influence of growth conditions (nutrient composition and incubation period) on biofilm formation of *E. coli* strains was evaluated using crystal violet staining procedure. The tested strains better formed biofilms after a longer incubation period (48 h). In addition, biofilm formation depended on nutrient medium composition. The eight strains were slime producers (Congo red agar assay). The water, ethanol, acetone and diethyl ether *S. officinalis* extracts were screened for their ability to inhibit the formation and metabolic activity of *E. coli* biofilms using crystal violet and resazurin assay, respectively. The water extract was not active, while ethanol, acetone and diethyl ether extract exhibited antibiofilm activity (MBIC= 2.5-10 mg/mL). The microscopic visualization of treated *E. coli* LM1 biofilm has shown morphological and density changes. In addition, the concentrations of phenolic compounds were determined spectrophotometrically. The highest content of total phenolics was found in acetone extract while the flavonoids in water extract.

**Keywords**: Antimicrobials, Antibiofilm activity, Culinary sage, Garden sage

In most environments, bacteria form surface-adherent matrix-enclosed communities known as a biofilm. Biofilm represents a prominent mode of bacterial growth which enables adaptation and survival against different environmental conditions[1]. The cells in the biofilm produce an extracellular polymeric (EPS) matrix that gives the biofilm stability and helps it to adhere to a surface[1]. In comparison to planktonic (unattached, suspended) bacteria, biofilm is more tolerant to antibiotics, disinfectants and other stress factors[1,2]. In addition, biofilm is recognized as an additional virulence factor of pathogens because cells in matrix-enclosed aggregates are more tolerant toward host immune defenses what makes difficulties in control and eradication of bacterial infections[1].

The uropathogenic *Escherichia coli* (UPEC) (Fam. Enterobacteriaceae) is one of the most medically significant pathogenic bacteria. The UPEC is considered as the primary cause of urinary tract infections[3]. Its pathogenicity is due to the ability to adhere and colonize mucosal surfaces of epithelial host cells and to overcome host defense mechanisms. Moreover, the ability of UPEC to form biofilm contributes to the long-lasting persistence of bacterium in the genitourinary tract[4,5]. The UPEC virulence factors, type 1 FimH pili, curli fimbriae, antigen 43, flagella, polysaccharide capsule/slime, which enable the adhesion and colonization of epithelial host cells are also involved in biofilm development[4]. The biofilm development in UPEC occurs in several stages. The first stage comprises the reversible attachment of planktonic cells to surface. After this, the bacterium starts to produce adhesins, such as type 1 pili, curli fibers and antigen 43 which contribute to the irreversible attachment to surface. Once established, the cells grow and divide, start to produce the EPS matrix and form the three-dimensional structure. Finally, the last stage happens when cells release from the biofilm and change to a planktonic stage. This could lead to a new biofilm

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formation[5]. Except that UPEC strains form an intracellular bacterial community with biofilm-like properties, they are capable to develop biofilm on implanted medicinal devices and different abiotic surfaces[4].

Biofilm tolerance to antibiotics and difficulties in their control and eradication of bacterial infections have initiated the search for new antibiofilm agents. Plants have traditionally been provided as a source of novel bioactive compounds and their application as a new approach to control bacterial biofilms. In recent years, numerous studies have described the role of bioactive plant extracts and pure isolated compounds in inhibition of biofilm formation and development[6-8].

Garden sage, *Salvia officinalis* L. (Fam. Lamiaceae) is one of the oldest and still the most popular medicinal plants. A wide range of pharmacological activities has been reported including anticancer, anti-inflammatory, antinociceptive, antioxidant, antimutagenic, antidementia, hypoglycemic and hypolipidemic activity[9-11]. Besides these properties, *S. officinalis* antibacterial activity against bacteria testing in suspensions (planktonic form) has been extensively studied[12-15]. However, only little information is available on its potential use against bacterial biofilms, including *E. coli* biofilm. *Salvia officinalis* medicinal properties are attributed to production of different bioactive secondary metabolites, such as phenolic acids, flavonoids, phenolic diterpenoids and triterpenoids, tannins. The phenolic acids, such as rosmarinic, caffeic, salvianolic, protocatechuic, hydroxyl-cinnamic acids have been isolated from *S. officinalis* extracts[15]. Among flavonoids, apigenin, luteolin, and their glycosides are the dominant compounds[15]. In addition, the diterpenoids (carnosol, rosmanol) and triterpenoids (ursolic and oleanolic acids) are very common constituents in sage extracts[16].

A broad range of bioactivities and confirmation of its therapeutical properties in pharmacopeia of many countries have helped us to select *S. officinalis* as a potential agent in bacterial biofilm control. Therefore, this study was undertaken to investigate the ability of UPEC strains to form biofilm and, for the first time, the UPEC antibiofilm activity of different *Salvia officinalis* extracts *in vitro*. In this study, we tried to (i) check the ability of *E. coli* strains to form biofilm on abiotic surfaces under different growth conditions; (ii) detect slime production by tested strains, (iii) determine the content of phenolic compounds in *Salvia officinalis* extracts; (iv) evaluate the effects of *S. officinalis* extracts on the inhibition of *E. coli* biofilm formation and biofilm metabolic activity; and (v) visualize the effects of *S. officinalis* extracts on biofilm using fluorescence microscopy.

## Material and Methods

### *Escherichia coli* biofilm formation

*Bacterial strains*

A total of ten *Escherichia coli* strains (*E. coli* LM1 – LM10) from urine samples were selected for this study. The strains were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The *E. coli* ATCC 25922 (*E. coli* LM11), previously characterized as a biofilm producer[17], was used as a control. The strains were preserved in 20% glycerol/medium stock at −80°C. Working cultures were prepared by subculturing cells twice from the stock cultures onto Nutrient agar (Torlak, Serbia) and incubated at 37°C for 24 h.

*Biofilm formation assay on polystyrene*

Biofilm formation assay was performed in sterile 96-well tissue-culture polystyrene microtiter plates (Sarstedt, Germany) using crystal violet staining method[18]. Three nutrient media were tested: Mueller-Hinton broth (MHB), Biolife, Italy (beef extract 3 g/L, acid digest of casein 17.5 g/L and starch 1.5 g/L), Nutrient broth (NB), Torlak, Serbia (peptone 15 g/L, beef extract 3 g/L, NaCl 5 g/L and $K_2HPO_4$0.3 g/L) and Nutrient broth supplemented with 1% glucose (NB + 1% Glu). The 180 µL of a medium was inoculated with 20 µL of the bacterial suspension. The bacterial suspension was adjusted to a density of 2.0 McFarland units using a McFarland densitometer (BIOSAN, Latvia). To evaluate the impact of incubation period on biofilm formation, one series of inoculated microtiter plates were incubated at 37°C for 24 h, and the other series of inoculated plates for 48 h at 37°C, without shaking. After the incubation period, wells were washed with PBS (pH 7.2), three times; free (detached) cells were removed, and attached cells were fixed with 200 µL of 99% methanol. Afterward, the microtiter plates were air-dried and attached cells were stained with 0.1% crystal violet solution for 15 min. After washing, the crystal violet bound to the biofilm cells was solubilized with 200 µL of 96% ethanol and optical density (OD) was measured at 570 nm using a microplate reader (RT-2100C, Rayto, China). The experiment included the positive control, the *E. coli*

ATCC 25922 biofilm producing strain, and the negative control containing only the culture medium. The biofilm formation assay was performed in four replicate wells for each strain and results were presented as means ± standard deviations.

According to Stepanović[19] the strains were classified into four different categories as follows: OD < $OD_c$, no biofilm producer; $OD_c$ < OD < $2OD_c$, weak biofilm producer; $2OD_c$ < OD < $4OD_c$, moderate biofilm producer; and $4OD_c$ < OD, strong biofilm producer. The cut-off OD ($OD_c$) was defined as three standard deviations above the mean OD of the negative controls.

*Biofilm formation assay on glass*

The ability of *E. coli* strains to form biofilms on glass or ring formation on the air-liquid interphase was performed according to the method described by Hassan *et al*.[18] with certain modifications. Three types of media (MHB, NB, NB+1% Glu) and two incubation periods (24 and 48 h) were included in the experiment to test the effects of different growth conditions on biofilm formation. A total of 2700 µL of a medium was inoculated with 300 µL of bacterial suspension adjusted to a density of 2.0 McFarland units and further incubated at 37°C. After incubation, the contents of the tubes were carefully discarded, and the tubes were washed with PBS (pH 7.2) and air-dried. Afterward, the attached cells were stained with 0.25% safranin solution for 1 min, the dye was removed without a wash step and tubes were air-dried overnight. The test was considered positive when there was an adherent layer of stained material on the inner surface of the tube. The adherence was estimated as absent (/), weak (+), moderate (++) or strong (+++).

**Detection of slime production on Congo red agar**

Slime production by tested *E. coli* strains was checked on Congo red agar. Congo red agar plates [0.8 g of Congo red dye and 36 g of saccharose to 1L of Trypticase soy agar (Merck, Germany)] were inoculated and incubated at 37°C for 24 h. The results were interpreted according to colony colour change as follows: very black and black colonies indicated normal slime producer strains; dark, almost black color was referred to weak slime producers, while very red to bordeaux coloured colonies indicated strains unable to produce slime[20]. The experiment was done two times for each strain.

**Antibiofilm activity of *S. officinalis* extracts**

*Preparation of plant extracts*

The plant material (dried, ground leaves), obtained from a commercial source (Dr. Josif Pančić Institute, Serbia), was extracted by maceration with water, ethanol, acetone, and diethyl ether. In brief, 30 g of the plant material was soaked in 150 mL of the solvent. The plant material was macerated three times at 20°C using a fresh solvent every 24 h. After every 24 h, the samples were filtered, and the filtrates were collected and evaporated to dryness using a rotary evaporator (IKA, Germany) at 40°C. The obtained amounts of crude extracts of *S. officinalis* were: 3.5 g of water extract, 7.7 g of ethanol extract, 3.7 g of acetone extract, and 2.48 g of diethyl ether extract.

*Phytochemical analysis of plant extracts*

The quantitative estimation of phenolic compounds was performed spectrophotometrically. The total phenolic content was determined with Folin-Ciocalteu reagent[21] and the total flavonoid content with 2% aluminum chloride[22]. Results were expressed as gallic acid equivalent (mg of GAE/g of extract) and rutin equivalent (mg of RUE/g of extract), respectively. The total extractable tannin content was estimated indirectly by spectrophotometric measurement of the absorbance of the solution obtained after the precipitation of the tannins with polyvinyl polypyrrolidone as described by Makkar *et al*.[23] and expressed as gallic acid equivalent (mg GAE/g of extract). The proanthocyanidin content was measured by the butanol-HCl method with ferric ammonium sulfate as a catalyst as described by Porter *et al*.[24]. Cyanidin chloride was used as the standard and the proanthocyanidin content was expressed as cyanidin chloride equivalent (mg CChE/g of extract). All chemicals and reagents were purchased from Sigma-Aldrich, USA.

*Inhibition of biofilm formation assay*

The inhibition of biofilm formation was analyzed by crystal violet staining method, as previously described. Two-fold serial dilutions of plant extracts in MHB medium, concentrations from 0.078 mg/mL to 10 mg/mL were made in sterile 96-well tissue-culture microtiter plates. The final volumes of wells were 100 µL. Each well was inoculated with 10 µL of appropriated bacterial suspension ($10^7$ CFU/mL) and incubated at 37°C for 48 h. Optical densities (ODs) of stained adherent cells with crystal violet were recorded with a microplate reader at 570 nm. Each test included growth control (medium + bacteria),

negative control (medium + plant extract) and sterility control (only medium). Tetracycline, dissolved in the nutrient liquid medium, was used as a positive control, while 5% DMSO was used as the solvent control. The minimum concentration of plant extract required to prevent biofilm formation and reduce cell attachment was determined by comparing the OD value of the test sample ($OD_s$) with the OD value of the negative control ($OD_c$). The first extract concentration showed the same or lower OD value than OD value of the negative control ($OD_s \leq OD_c$) was considered as minimum biofilm inhibitory concentration (MBIC).

*Inhibition of biofilm metabolic activity assay*

The inhibition of biofilm metabolic activity by plant extracts was evaluated using the resazurin test. Resazurin is an indicator of metabolic activity of viable cells. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The test broth solutions containing variable concentrations of plant extracts, separately, (0.078, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 mg/mL), were inoculated with 10 µL of appropriated bacterial suspension ($10^7$ CFU/mL) in microtitre plates followed by incubation at 37°C for 48 h. After the incubation period, wells were washed with PBS (pH 7.2) and detached cells were removed. Thereafter, 100 µL of fresh MHB and 10 µL of 0.02% resazurin solution were added into each well. After 3 h of incubation, on the bases of indicator colour change, the results were determined. The concentration of plant extract that prevented resazurin colour change from blue to pink was defined as the lowest concentration which inhibited the metabolic activity of biofilm. Each test included growth control and sterility control.

**Fluorescence microscopy: Biofilm visualization**

Biofilm of *E. coli* LM1 strain treated with different concentrations (0.63, 1.25, 2.5, 5 and 10 mg/mL) of tested plant extracts was grown in a microtiter plate for 48 h. After the incubation period, wells were washed with PBS (pH 7.2) to remove the detached cells. After that, the formed biofilm was fixed with methanol for 2 min and stained with 0.1% acridine orange solution. The observation was done using an Olympus BX51 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan).

**Statistical analysis**

All results were expressed as means ± standard deviations where appropriate. Statistical analysis of data was performed using SPSS 20 software (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used for testing data normality distribution. Differences between groups were analyzed by Kruskal-Wallis 1-way ANOVA test, $P \leq 0.05$ was considered significant.

## Results

***Escherichia coli* biofilm formation ability**

The results of biofilm formation ability of tested *E. coli* strains on polystyrene (microtiter plates) in MHB, NB and NB + 1% Glu media are shown in Table 1. After 48 h of incubation, 63.6% (7/11), 72.7% (8/11) and 81.8% (9/11) of tested strains in MHB, NB + 1% Glu and NB media, formed biofilms, respectively. For comparison, after 24 h of incubation, only 20% of strains formed biofilms, depending on the growth medium. Regardless of the exposed growth

Table 1 — Biofilm formation ability of UPEC strains under different growth conditions in microtiter plates

| *E. coli* strains | Mueller – Hinton broth[c] | | Nutrient broth[c] | | Nutrient broth + 1% glucose[c] | |
|---|---|---|---|---|---|---|
| | 24 h[a] | 48 h[b] | 24 h[a] | 48 h[b] | 24 h[a] | 48 h[b] |
| LM1 | 0.135± 0.01 | 0.234± 0.03* | 0.125± 0.01 | 0.319± 0.06* | 0.121± 0.01 | 0.225± 0.03* |
| LM2 | 0.147± 0.02 | 0.256± 0.05* | 0.123± 0.01 | 0.256± 0.02* | 0.123± 0.01 | 0.266± 0.02* |
| LM3 | 0.134± 0.02 | 0.146± 0.02 | 0.120± 0.01 | 0.216± 0.03* | 0.128± 0.01 | 0.356± 0.02* |
| LM4 | 0.134± 0.01 | 0.151± 0.02 | 0.121± 0.01 | 0.190± 0.02* | 0.125± 0.01 | 0.182± 0.03* |
| LM5 | 0.194± 0.03* | 0.231± 0.02* | 0.132± 0.01 | 0.290± 0.03* | 0.129± 0.01 | 0.150± 0.01 |
| LM6 | 0.138± 0.01 | 0.197± 0.03* | 0.115± 0.01 | 0.161± 0.02 | 0.124± 0.01 | 0.164± 0.01 |
| LM7 | 0.172± 0.02 | 0.261± 0.03* | 0.126± 0.01 | 0.198± 0.01* | 0.132± 0.01 | 0.153± 0.01 |
| LM8 | 0.141± 0.01 | 0.177± 0.02 | 0.113± 0.01 | 0.166± 0.01* | 0.134± 0.01 | 0.238± 0.025* |
| LM9 | 0.128± 0.02 | 0.181± 0.07 | 0.122± 0.01 | 0.153± 0.01 | 0.156± 0.01* | 0.326± 0.01* |
| LM10 | 0.169± 0.01 | 0.227± 0.01* | 0.123± 0.01 | 0.167± 0.02* | 0.124± 0.01 | 0.203± 0.03* |
| LM11 | 0.261± 0.01* | 0.258± 0.05* | 0.139± 0.02 | 0.230± 0.01* | 0.142± 0.01 | 0.237± 0.01* |
| Negative control | 0.119± 0.02 | 0.134± 0.02 | 0.111± 0.02 | 0.131± 0.01 | 0.122± 0.01 | 0.137± 0.01 |
| $OD_c$ | 0.179 | 0.194 | 0.171 | 0.161 | 0.152 | 0.167 |

[Data are presented as a mean ± standard deviation of $OD_{570}$ nm of four wells for each strain. Means with different letters in the same row indicate significant ($P < 0.05$) differences. *biofilm producer strains]

conditions, tested *E. coli* strains produced weak biofilms.

The results for biofilm formation on glass were presented in Table 2. It was found that all 11 strains (100%), after 48 h, formed biofilms in NB medium, in MHB it was seven strains (63.6%) and only two (18.2%) in NB + 1% Glu. A relationship between a number of biofilm producer strains depending on nutrient media is presented as a venn diagram (Fig. 1).

**Detection of slime production**

Among the tested eleven *E. coli* strains, eight strains produce extracellular matrix and are classified into the group of slime producers and three strains are classified as no-slime producers (Table 2.). Regarding the gradient of colony colour produced on Congo agar, five strains formed black colonies (LM1, LM4, LM5, LM6, LM10, LM11), two strains formed almost black colonies (LM7, LM8) and three strains were with red colonies (*E. coli* LM2, LM3, LM9).

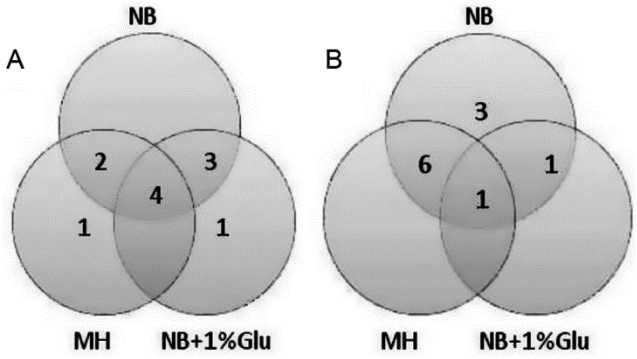


Fig. 1 — Relationship between number of biofilm producer strains depending on nutrient media after 48h incubation in (A) microtiter plates; and (B) glass tubes. [MH, Mueller-Hinton broth; NB, Nutrient broth; and NB+1%Glu, Nutrient broth + 1% glucose]

Table 2 — Biofilm formation ability of UPEC strains under different growth conditions in glass tubes and slime production

| *E. coli* strains | Mueller-Hinton broth | | Nutrient broth | | Nutrient broth + 1% glucose | | Slime production |
|---|---|---|---|---|---|---|---|
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h | |
| LM1 | - | - | + | + | - | - | + |
| LM2 | - | + | ++ | ++ | + | + | - |
| LM3 | + | + | + | ++ | - | - | - |
| LM4 | + | + | + | ++ | - | - | + |
| LM5 | - | - | + | + | - | - | + |
| LM6 | - | + | + | + | - | - | + |
| LM7 | + | + | + | + | - | - | + |
| LM8 | - | - | - | + | - | + | + |
| LM9 | - | - | + | + | - | - | - |
| LM10 | - | + | - | + | - | - | + |
| LM11 | - | + | - | + | - | - | + |

[(-) no biofilm or no slime production, (+) weak biofilm or slime production, (++) moderate biofilm and (+++) strong biofilm; „+" - slime producer]

**Antibiofilm activity of *S. officinalis* extracts**

*Phytochemical analysis*

The total phenolic content and the contents of various classes of polyphenols in *S. officinalis* extracts are presented in Table 3. The quantitative analysis of phenolic compounds showed differences in their total contents depending on solvent polarities used for the preparation of plant extracts. The highest content of total phenolics was measured in acetone extract followed by ethanol extract, diethyl ether extract and water extract. The total flavonoid content was from 35.05 ± 0.80 mg RUE/g to 54.91 ± 0.51 mg RUE/g. The water extract contained the highest concentration of flavonoids. The content of tannins was in similar quantities, except in diethyl ether extract. The concentrations of proanthocyanidins were the least, they were measured in ethanol, acetone and diethyl ether extract (1.33 ± 0.21, 1.89 ± 0.11, 1.59 ± 0.24 mg CChE/g, respectively).

*Effects of S. officinalis plant extracts on biofilm formation*

The inhibitory effects of water, ethanol, acetone and diethyl ether extract on biofilm formation were evaluated against seven *E. coli* strains capable to form biofilms in MHB medium. The results are presented in Fig. 2A. The water extract had no effect on biofilm formation (MBIC >10 mg/mL) while ethanol, acetone, and diethyl ether extracts were active at the concentration of 10 mg/mL, except acetone extract in relation to *E. coli* LM7 (5 mg/mL). The extracts showed similar activity against all tested strains. The antibiotic tetracycline was more active with inhibitory concentrations from 0.5 to 64 μg/mL.

Table 3 — Total phenolic, flavonoid, and condensed tannin contents

| Plant extract | Total phenolic content (mg GAE/g) | Total flavonoid content (mg RUE/g) | Total condensed tannin content | |
|---|---|---|---|---|
| | | | PVPP method (mg GAE/g) | Butanol-HCl method (mg CChE/g) |
| Water extract | 95.75 ± 0.89[a] | 54.91 ± 0.51[a] | 73.69 ± 1.24[a] | - |
| Ethanol extract | 134.16 ± 0.57[b] | 35.05 ± 0.80[b] | 74.24 ± 0.95[a] | 1.33 ± 0.21[a] |
| Acetone extract | 146.16 ± 0.50[c] | 46.32 ± 0.44[c] | 72.56 ± 1.41[a] | 1.89 ± 0.11[a] |
| Diethyl ether extract | 125.25 ± 0.69[d] | 39.75 ± 1.35[d] | 65.71 ± 0.39[b] | 1.59 ± 0.24[a] |

[Data are presented as a mean ± standard deviation. Means with different letters in the same column indicate significant ($P < 0.05$) differences. CChE/g cyanidin chloride equivalents; GAE/g gallic acid equivalents; RUE/g rutin equivalents; PVPP, polyvinylpolypyrrolidone; and -, not detected]

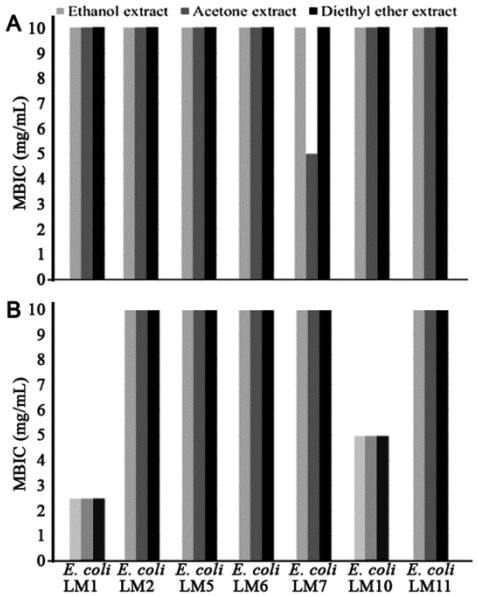


Fig. 2 — Inhibitory effects of water, ethanol, acetone, and diethyl ether extract on (A) biofilm formation; and (B) biofilm metabolic activity

*Effects of S. officinalis plant extracts on biofilm metabolic activity*

As indicated in Fig. 2B, the water extract had no activity, while the other tested extracts were active in the concentration range of 2.5-10 mg/mL. When comparing among all the strains, *E. coli* LM1 and *E. coli* LM10 were found to be most sensitive.

**Biofilm visualization**

The treated biofilm of *E. coli* LM1 strain displayed a significant reduction in the number of adherent cells and in the size of aggregates, which were reduced to small clusters or even single cells. The acetone, ethanol, and diethyl ether extracts completely prevented bacterial adherence at 1.25, 2.5, 5 and 10 mg/mL (Fig. 3B-D). On the other side, the low activity of water extract was confirmed (Fig. 3A).

## Discussion

Biofilm development is one of *Escherichia coli* (UPEC) virulence factors which enables survival and protection from host defensive mechanisms, antibiotics, and other environmental fluctuations.

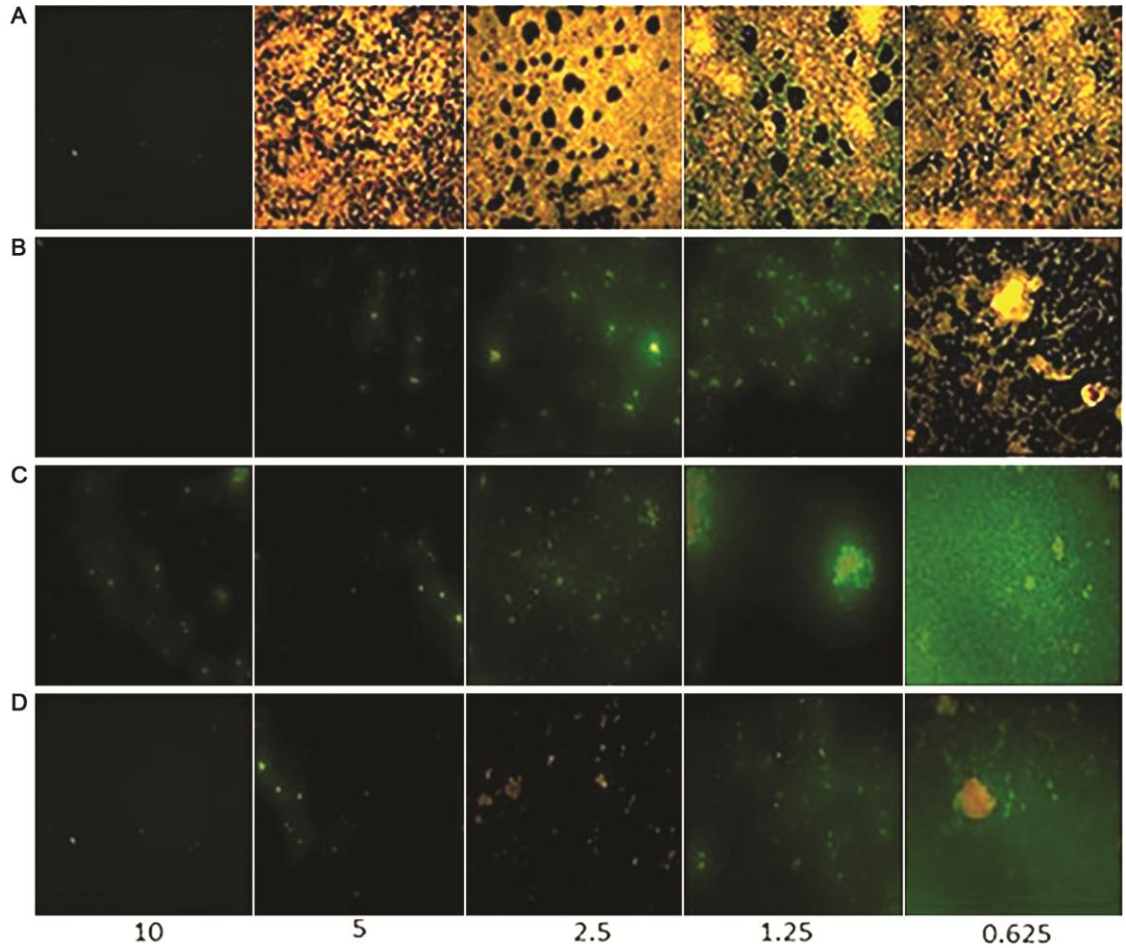


Fig. 3 — Microscopic images of *E. coli* LM1 biofilm treated with *Salvia officinalis* extracts. (A) water extract; (B) ethanol extract; (C) acetone extract; and (D) Diethyl ether extract. [Concentration 10-0.625 mg/mL]

In order to study the frequency of biofilm formation capacity of UPEC strains and to select biofilm producer strains for further antibiofilm studies, we analyzed the ability of 10 isolates to produce a biofilm on different abiotic surfaces and under different growth conditions.

The tested *E. coli* strains were capable to form biofilms on plastic surface (polystyrene) and on a glass when incubated in a rich medium. As expected, the process depended on the strain itself, culture medium, and incubation period. We observed that a longer incubation period better supported the biofilm formation ($P$ <0.05). In addition, biofilm formation, also, depended on nutrient medium composition but was not statistically significant ($P$ >0.05). We tested three nutrient media: MHB as standard antimicrobial susceptibility testing medium because of further antibiofilm testing, NB as standard, complex growth medium for bacteria and NB supplemented with 1% glucose to check the impact of monosaccharide (a carbon source) on biofilm formation. Our observation has indicated that the additional concentration of glucose did not enhance the attachment of bacterial cells and induce biofilm formation. These results agree with the results of the study by Dewanti & Wong[25] who noticed that increasing the concentration of glucose (0.04 to 0.1% and 1%) in the medium did not have any significant effect on the number of adherent bacteria. The tested *E. coli* isolates could form biofilm in nutritional rich media even though some literature sources described better biofilm formation in minimal media[26]. This could be partly attributed to the fact that higher levels of organic compounds (peptone, beef extract) lead to higher cell culture densities what facilitate the initial attachment of cells.

Furthermore, the EPS matrix possesses an important role in biofilm formation and maintenance of biofilm architecture. The UPEC strains produce an extracellular matrix composed of a protein fiber – curli and cellulose as major constituents[27]. Congo red dye was used to detect the curli formation in UPEC strains, because of the specificity of the Congo red dye to bind to the amyloid fibers and cellulose in the EPS matrix[20]. Our results showed that eight strains (73% of all strains) produce EPS matrix detected on Congo red agar. The EPS producing ability of tested strains correlates with the biofilm forming ability on microtiter plates and glass tubes.

According to the literature search, most of the studies found have mainly reported on the antibacterial activity of *S. officinalis* extracts against bacteria in suspension (planktonic form)[12-15]. To the best of our knowledge, this is the first report on antibiofilm effectiveness of *S. officinalis* extracts against UPEC strains. In this study, the antibiofilm activity was focused on cell attachment preventing and biofilm formation as well as inhibition of biofilm metabolic activity. The water extract was not active, while ethanol, acetone and diethyl ether extracts exhibited antibiofilm activity. The extracts' concentrations needed to inhibit the formation and metabolic activity of biofilms were similar. Microscopic observation clearly showed a gradual reduction in biofilm formation with the increased concentration of extracts indicating towards their ability to disrupt biofilm and its inhibition.

The antibiofilm activity of plant extracts is attributed to the quantity and composition of plant secondary metabolites. Phenolic compounds (simple phenolics, phenolic acids, flavonoids, coumarins, tannins and saponins) are an abundant group of secondary metabolites detected in plant extracts. The content of phenolic compounds in tested *S. officinalis* extracts was in correlation with their bioactivity. The quantity of total phenols, flavonoids, tannins in ethanol, acetone and diethyl ether extract contributed to the better activity of these extracts. Although the water extract was rich in flavonoids, it showed no activity at tested concentrations. Often, the activity of plant extracts depends on different groups of phenolic compounds and their synergistic acting.

Recently, several review articles have summarized the antibiofilm action of plant bioactive compounds[28,29]. Three levels of action were distinguished which target the different stages of biofilm development: inhibition of biofilm formation, reduction of formed biofilm, and inhibition of quorum sensing. Surface attachment is the first step for bacteria to establish a biofilm. Enterobacteriaceae produce an array of adhesive structures and proteins, enabling them to colonize their hosts or produce biofilms on abiotic surfaces. Such adhesion factors include curli fimbriae, flagella, cellulose, lipopolysaccharides, and several outer membrane proteins. It has been observed that plant active compounds act in the initial stages of biofilm formation by preventing adhesion and cell aggregation, next, preventing the secretion of adhesion proteins and other compounds, reducing the production of fimbriae, and inhibiting the motility of

bacteria. Physical mechanisms can also restrict biofilm formation in the initial stages by interfering with forces used by the bacterial cells for surface attachment[30]. Further, degradation of the EPS matrix under the influence of plant bioactive compounds contributes to the weakening and destruction of the mature biofilm. Through the pores of the EPS matrix, plant bioactive compounds can target mature biofilm by preventing the synthesis of EPS and degrading EPS[31]. In addition, inhibition of quorum sensing is crucial in biofilm control. Quorum sensing (QS) is the most well-characterized bacterial communication system utilized by bacterial communities to coordinate gene expression and consequently phenotypes including biofilm formation, in response to changes in population density. Different QS systems are used by various bacterial species, including the universal autoinducer-2 (AI-2) based system used by both Gram-negative and Gram-positive bacteria, acyl homoserine lactone (AHL) based systems found in Gram-negative bacteria, and autoinducing peptides (AIP) based systems found in Gram-positive bacteria[6]. The fundamental principle behind how the QS systems function is the release and detection of small, diffusible signaling molecules referred to as autoinducers. The two main proteins in QS systems are a synthase, which responds to population changes and environmental signals to make the autoinducer, and a receptor protein, which activates a response (change in gene expression) upon binding of the autoinducer over a threshold level[6]. Plant bioactive compounds act as active quorum quenchers because they disrupt these pathways by interfering with signal molecule synthesis, inactivating signals by destroying them, interfering with signal receptors in bacterial cells, and blocking target genes under QS controls[32,33].

Numerous medicinal plants used traditionally for urinary infection have been the focus of scientific studies. Cranberry (*Vaccinium macrocarpon*) is one of the most recommended plants for the prevention of urinary infection. Clinical and scientific studies support the use of cranberry, and it is found to be most effective against UPEC strains. The active phenolic compounds flavonols, anthocyanins, and proanthocyanidins inhibit bacterial adherence to uroepithelial cells[34]. In addition, Rodríguey-Pérez *et al*.[35] suggested that apart from proantho-cyanidins, flavonoids mainly, myricetin, quercitrin, dihydroferulic acid glucuronide, procyanidin A dimer, prodelphinidin B, also modify UPEC surface hydrophobicity *in vitro* and prevent *E. coli* biofilm formation. Besides direct interaction of the extract with outer membrane proteins of UPEC strains which preventing the adhesion of the bacteria to the host cells, also, inhibition of bacterial flagella expression and motility by cranberry extract have been described[36]. Proanthocyanidin-free cranberry extract exerted significant antiadhesive effects within *in vivo* studies in humans, which were due to an interaction with type 1 fimbriae of UPEC strains[36]. Wojnicz *et al*.[37] performed the study to determine the influence of *Betula pendula*, *Equisetum arvense*, *Herniaria glabra*, *Galium odoratum*, *Urtica dioica* and *Vaccinium vitis-idaea* water extracts on bacterial survival and virulence factors involved in tissue colonization and biofilm formation of UPEC strains. All the extracts exhibited antibiofilm activity. Hydroalcoholic extract from fruits of *Apium graveolens* (celery) exerted a dose-dependent antiadhesive quorum sensing inhibitory activities against UPEC strains. *In vivo* study showed a significant reduction of the bacterial load in bladder tissue treated with *A. graveolens* extract[38]. The published results showed that, primarily, medicinal plants used for urinary tract infection, possess antiadhesive activity. The mode of action has been described and indicated that plant bioactive compounds prevent biofilm formation by reducing the bacterial surface hydrophobicity and decreasing bacterial cell adhesion as well as motility.

In the present study, *Salvia officinalis* has been shown as a plant with promising antibiofilm activity, acting significantly on clinical isolates of UPEC. *Salvia officinalis* extracts control the development of *E. coli* biofilms in two ways, reduce initial biofilm formation and disturb the cell metabolism. Based on obtained results, *S. officinalis* could be included in a group of medicinal plants active against UPEC strains. With the effective action of *S. officinalis* proven *in vitro*, the next step is to design more complex research approaches, such as *in vivo* and clinical tests, to examine the role of *S. officinalis* as antibiofilm agents.

## Conclusion

The uropathogenic *Escherichia coli* (UPEC) is considered as the primary cause of urinary tract infections. Its pathogenicity, persistence in urinary tract and chronic infection, among other virulence

factors, are caused by biofilm development. The tested clinical isolates of UPEC were able to form biofilm *in vitro*. The process was affected by nutrient conditions and duration of incubation period. In addition, for the first time, *Salvia officinalis* extracts were found to be effective in inhibiting UPEC biofilm. The ethanol, acetone and diethyl ether extracts inhibit the formation of biofilms. The extracts act in two ways, reduce the cell attachment and biofilm formation as well as disturb the cell metabolism. The effectiveness of plant extracts, including *S. officinalis* extracts, in preventing biofilm formation is a promising tool for reducing microbial colonization and control of biofilms.

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**Conflict of Interest**

Authors declare no competing interests.

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