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# The Study of Synergistic Effects of *n*.butanolic *Cyclamen coum* Extract and Ciprofloxacin on inhibition of *Pseudomonas aeruginosa* biofilm formation

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## Abstract

**Introduction**: Infections caused by *Pseudomonas aeruginosa* biofilm are the major causes of death in patients with cystic fibrosis (CF). Some studies revealed that biofilms are resistant to several antibiotics because of their impermeable structures. In order to re-sensitize bacteria to different antibiotics, biofilm formation should be inhibited. In this research, evaluation of antibiofilm activity of *n*-butanolic *Cyclamen coum* extract as a medicinal plant from Myrsinaceae family, in combination with ciprofloxacin was carried out.

**Materials and method**s: The biofilm formation ability by *P. aeruginosa* $PAO_1$ and one clinically isolated *P. aeruginosa* (PA214) was confirmed by microtiter plate method. Extraction of the tubers of *Cyclamen coum* was done by fractionation method. The antibiofilm and antibacterial properties of *n*-butanolic *C.* coum extract (which includes saponin compounds) alone and in combination with ciprofloxacin by using microdilution and crystal violet methods were examined. The cytotoxicity effect of the *n*-butanolic extract on HT-29 cells was assayed by MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide) test.

**Results**: The biofilm formation ability by *P. aeruginosa* strains was quantitatively confirmed. Saponin content of the *n*-butanolic *C.coum* extract was 156 µg/mL. The extract revealed antibacterial activity against the growth of planktonic *P. aeruginosa* strains. The combination of *n*-butanolic *C.coum* extract and ciprofloxacin significantly inhibited *P.aeruginosa* biofilm formation (ΣFBIC = 0.5). The *n*-butanolic *C.coum* extract showed insignificant cytotoxic effect against HT-29 human cancer cell line after 48 hours and 72 hours incubation.

**Discussion and conclusion:** It can be concluded that *n*-butanolic *C.coum* extract in combination with ciprofloxacin significantly revealed antibiofilm activity against *P. aeruginosa* biofilm however, further clinical investigations are required.

**Key words:** *Pseudomonas aeruginosa*, Biofilm, *Cyclamen coum,* Ciprofloxacin

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## Introduction

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is one of the most important agents involved in chronic lung infections in CF patients. The CF lung environment favours formation of biofilms by *P. aeruginosa* (1). Biofilms are microbial communities that are attached to a substratum and are embedded in a matrix of polysaccharide, proteins and DNA. Biofilm cells are up to 1000 times more resistant to antibiotics compared to motile cells allowing them to recolonize the host organs despite cycles of antibiotic therapy. Considering many biofilm related problems, it is clear that finding an effective and safe medicine with antimicrobial and antibiofilm properties is one of the interesting issues in clinical microbiology and infectious diseases. Thus, various treatments, including vaccines, interferon gamma, and even different plant extracts have been studied. Plant-based medicines not only have an effective role in the treatment of biofilm based diseases but also, have fewer side effects than synthetic medicines (2 and 3). *Cyclamn coum* as medicinal plant from *Myrsinaceae* family grows in the forests of Golestan province in Iran. According to Ahmadbeigi *et al.* study, tubers of *C. coum* contain large amounts of saponins and by using different solvents such as petroleum ether, ethanol 70% and *n*-buthanol high levels of saponins are extracted and are accumulated in butanolic phase. Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Saponins have several applications in pharmaceutical industry because of antibacterial, antifungal or antiviral properties (4).

The aim of this study was to evaluate the synergistic effects of n- butanolic *C. coum* extract in combination with ciprofloxacin on inhibition of biofilm formation of *P.aeruginosa*. The cytotoxicity effect of the extract against HT-29 cells was also studied.

## Materials and methods

### Bacterial strains

One sputum isolate, strain PA214, which has been used in previous studies (5 and 6) along with *P.aeruginosa* PAO1 were used in this study.

### Minimal Inhibitory Concentration (MIC) determination of Ciprofloxacin

The ciprofloxacin was dissolved in distilled water. Further dilutions were made according to CLSI document M100-S18. Ciprofloxacin was used in the concentrations range of 0.125 to 512 µg/mL. The adjusted bacterial inoculum ($1.6\times10^6$CFU/mL) were added to each well of sterile 96-well flat-bottomed microtiter plate containing the test concentrations of ciprofloxacin (100 µL/well). Two trials were performed for each concentration of ciprofloxacin. Two wells containing bacterial suspension with no drug (growth control) and two wells containing only media (background control) were included in this plate. Finally, optical densities were measured after 24 hours at 37°C using microplate reader (Bio-Tek-Synergy HT Microplate Reader, Bio-Tek Instruments and Winooski, Vt, USA).

**Biofilm formation assay**

A modified microtiter plate test was used to determine the ability of biofilm formation. An overnight culture of each strain was grown in Trypticase Soy agar plus 0.2% glucose (Merck, Germany) for 18 to 20 hours at 37°C. The bacterial suspensions were made in Trypticase Soy broth plus 0.2% glucose medium according to 0.5 McFarland turbidity standard as measured by absorbance (0.08 to 0.1 at 625 nm) in spectrophotometer (Schimadzu, model UV-120-01, Japan). Bacterial suspensions (200µL) were inoculated in three parallel wells of a 96-well plate.

After 24 hours incubation at 37°C, the content of each well was aspirated, and each well was washed with sterile physiological saline to remove all non-adherent cells. Attached bacteria were fixed with absolute methanol for 10 minutes. The plates were stained using crystal violet (1%W/V). Excess stain was washed off and the plates were rinsed with tap water. The bound dye was re-solubilized with 200 µL of glacial acetic acid (33%, v/v).The OD of each well was measured at 570 nm by using an ELISA reader. Un-inoculated wells containing media served as blanks. A three -grade scale was used to evaluate the ability of biofilm production: (-): ODs < 0.500; (+): ODs 0.500 to 1.500; (++): ODs >1.500 (6).

**Saponin extraction**

Tubers of *Cyclamen coum* were collected from Golestan Forest (Golestan Province in Iran). According to Ahmadbeigi *et al*. study, freshly collected tubers of *C. coum* were dried under shade, sliced into small pieces, pulverized using a mechanical grinder. Dried powder (100 g) was successively extracted using petroleum ether (300 mL) and ethanol 70% (300 mL) using Soxhlet apparatus. After 24 hours the extract was fractionated by *n*-buthanol to two phases. Lower phase (*n*-butanolic) which contained cyclamen saponins was dried by using a Rotavapor (Buchi Flawil, Switzerland). The solid precipitate was dissolved in dimethyl-sulphoxide (DMSO) taken in to account that the maximum concentration of DMSO in the test solution should not exceed one percent (4).

**Determination of total saponin content**

Total saponin content was determined by the vanillin-sulfuric acid method. *n*-butanolic *C.coum* extract was mixed with vanillin (8%, w/v) and sulfuric acid (72%, w/v). The mixture was incubated at 60 ˚C for 10 minutes followed by cooling in an ice water bath for 15 minutes. Absorbance was measured at 538 nm. All determinations were carried out in triplicates. A standard curve was obtained by using saponin (1mg/mL) (Merck, Germany) (7).

**Minimal Inhibitory Concentration (MIC) determination of *n*-butanolic *C.coum* extract**

Sterile 96-well microtiter plates were used for the assay. The *n*-butanolic *C.coum* extract was dissolved in a minimal amount of DMSO and was diluted to twice the desired initial test concentration with Mueller Hinton broth. All wells, except the first, were filled with Mueller Hinton broth (50µL). Test sample (100 µL) was added to the first well and serial two-fold dilutions were made down to the desired minimum concentration. One day old cultures of bacteria grown on Mueller Hinton agar

plates were suspended in Mueller Hinton broth until turbidity was equal to a 0.5 McFarland Standard. The plates were inoculated with the bacterial suspension ($1.6\times10^6$ CFU $mL^{-1}$) and incubated at 37°C overnight. The MIC values were taken as the lowest concentration of the extract in the well of the microtiter plate that showed no turbidity after incubation.

**Antibiofilm activity and synergy studies**

Serial dilutions of *n*-butanolic *C.coum* extract and ciprofloxacin were prepared in Tryptic Soy Broth (TSB) plus 0.2% glucose (antibiotic concentrations were from 0.125 µg $mL^{-1}$ to 64µg $mL^{1}$ and *n*-butanolic *C.coum* extract from 25 to 150 µg $mL^{-1}$ were prepared).

Checkerboard arrangements of *n*-butanoli *C. coum* extract and ciprofloxacin were made ready in 96-well microtiter plates as previously described (6). Each microtiter plate was contained 3 wells for sterility controls, 3 wells for growth controls, 9 wells for different concentrations of *n*-butanolic *C. coum* extract and ciprofloxacin alone and the combination of them. The turbidity of incubated bacterial suspension was adjusted to 0.5 McFarland standards to achieve $10^8$ CFU/mL and was added to each well. Thereafter, microtiter plates were incubated at 37ºC for 24 hours. A semiquantitative measure of biofilm formation was calculated by using crystal violet method (as mentioned above).

Fractional Biofilm Inhibitory Concentration (FBIC) of each agent was calculated from Minimum Biofilm Inhibitory Concentration (MBIC) amounts as follows.

$FBIC_{A} = MBIC_{A\ (C)} / MBIC_{A\ (A)}$ $FBIC_{B} = MBIC_{B\ (C)} / M\ BIC_{B\ (A)}$

$\sum FBIC = FBIC_A + FBIC_B$

Where subscripts A and B denote antimicrobial agents, subscripts in parentheses denote the activity measurements in combination and alone, respectively. The summation of both FBICs was used to array the combination of antimicrobial agents as synergistic ($\Sigma FBIC = 0.5$), partially synergistic ($0.5 < \Sigma FBIC = 1$), indifferent ($1 < \Sigma FBIC = 4$), or antagonistic ($\Sigma FBIC > 4$) (8).

**Cytotoxicity assay of *n*-butanolic *C. coum* extract**

Dried *n*-butanolic *C.coum* extract was dissolved in 1mL of DMSO to obtain a stock solution of the extract at 10mg/mL. Stock solution was further diluted in RPMI1640 (Sigma, MO, USA) to obtain final concentrations which were used in antibiofilm assay. In this study different concentrations of *n*-butanolic *C. coum* extract alone and in combination with ciprofloxacin were applied.

HT-29 human colon carcinoma cells were purchased from Pasteur Institute of Iran. In this study HT-29 cell line was used as a model for studying the epithelial transport of drugs. The cancer cells were grown at 37°C in a humidified $CO_2$ incubator with 5% CO2 in RPMI-1640 media supplemented with 10% fetal bovine serum (Invitrogen Corp., Auckland, New Zealand).

Cell suspension (5× $10^5$ cells/mL) was plated out into 96-well microtiter plate.

Thereafter, various concentrations of the *n*-butanolic *C.coum* extract alone and in combination with ciprofloxacin were plated out in triplicates. Each plate included untreated cell controls and a blank cell-free control. After 48hours and 72 hours of incubation, MTT (5 $\mu$g/mL) was added to each well and each microtiter plate was re-incubated for further 4 hours. The media were removed and isopropanol was added into each well to solubilize the formazan crystals. Finally, the absorbance was read at wavelength of 595 nm using a microtitre plate reader. Survival rate was calculated in the following manner:

$$\text{Survival rate } (\%) = \frac{A\ sample - A\ b}{Ac - Ab} \times 100$$

Where subscripts b and c denote blank and control, respectively (9).

**Statistical analysis**

Data analysis was performed by Microsoft Excel 2010 software. The results of the experiment were expressed as the mean ± standard deviations (SD) for three replicates in each test.

The significance of all the data was tested using Student's t-test (*P value*< 0.05) using Microsoft Excel 2010 software.

## Results

In this study *P. aeruginosa* strain PAO1 and one clinical isolate, *P. aeruginosa* strain PA 214, were assayed for biofilm formation using microtiter plate assay. This method is useful for quantitative assessment of biofilm formation (10). The results revealed that *P.aeruginosa* PAO1 was moderately positive (1.3 to 1.5 OD) and *P.aerugiosa* strain PA214 was strongly positive (1.7 to 1.8 OD) in biofilm formation. It seems that, these differences are due to the diversity of matrix materials. According to different studies, extracellular matrix of *P.aeruginosa* PAO1 is comprised mostly of DNA and different proteins but the matrix of *P.aerugiosa* strain PA214 is mostly composed of alginate.

Using petroleum ether, ethanol and *n*-butanol had significant effects on removing artifact compounds and increased extraction of the saponins from *cyclamen* tubers (4). According to the standard curve, the saponin content of the *n*-butanolic *C. coum* extract was 156µg/mL.

According to Table 1, the *n*-butanolic *C.coum* extract revealed good antibacterial activity against the growth of planktonic *P.aeruginosa*.

Table 1- Minimum inhibitory concentrations (MIC) of *n*-butanolic *C. coum* extract and ciprofloxacin using microtiter plate method according to CLSI protocols

| *P.aeruginosa* strains | *n*-butanolic *C.coum* extract | Ciprofloxacin | | |
|---|---|---|---|---|
| | MIC (µg/mL) | Inhibition zones Diameter (mm) | MIC (µg/mL) | MBC* (µg/mL) |
| PAO1 | 256 | 23 ± 0.1 | 0.25 | 0.5 |
| PA 214 | 254 | 17 ± 0.2 | 4 | 8 |

*Minimum Bactericidal Concentration

In recent years, the molecules that control bacterial biofilms have gained a lot of utility and attention. These molecules can act synergistically with conventional antibiotics to overcome infectious diseases. Some antibiofilm agents inhibit microbial biofilm formation, while the antibiotic will eliminate the bacterial population.

Over the years, the World Health Organization (WHO) advocated that countries should interact with traditional medicine with a view of identifying aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins (11 and 12).

Zeng *et al*. demonstrated the synergistic effect of a novel derivative of *Andrographis paniculata* (AL-1) and various antibiotics on the formation of *P.aeruginosa* biofilm. According to their study, AL-1 was identified as a potential inhibitor for the quorum sensing system (13).

Rogers *et al*. also reported that the combination of a natural anti-biofilm agent containing 2- aminoimidazole/triazole motif with antibiotics inhibits and disperses *P. aeruginosa*, *Acinetobacter baumannii*, *Bordetella bronchiseptica*, and *Staphylococcus aureus* biofilms. Furthermore, they have shown that it is possible to apply the 2-aminoimidazole/ triazole conjugate compound 1 as an adjuvant for re-sensitizing multidrug-resistant bacteria to the effects of several antibiotics (12).

According to the present study, the antibiotic alone (at 6xMIC) or *n*-butanolic *C. coum* extract (at 55± 0.3µg/mL) significantly disrupted *P. aeruginosa* biofilm formation but their combination inhibited more significantly biofilm formation (*P value*<0.05) (here antibiotic and *n*-butanolic *C. coum* extract concentrations reduced at 3xMIC and 38± 0.3µg/mL respectively) (Fig. 1).

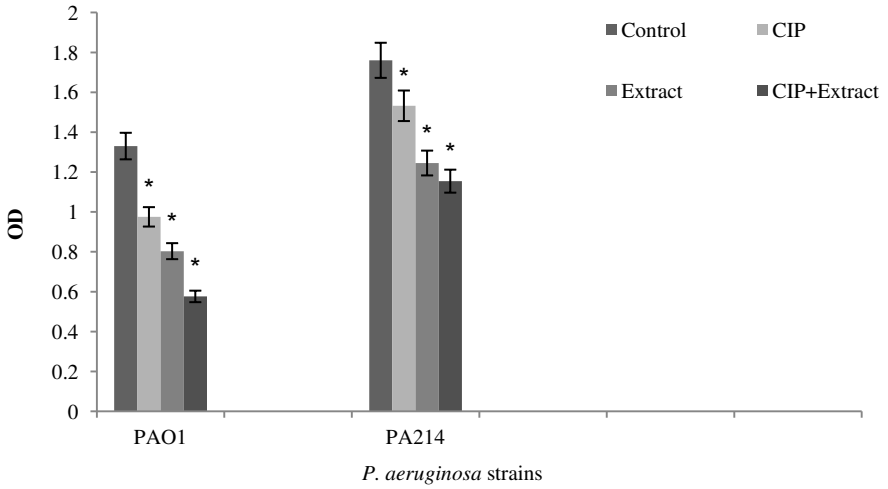


Fig. 1- The effect of ciprofloxacin (CIP), *n*-butanolic *C.coum* extract (Extract) and combined ciprofloxacin, *n*-butanolic *C.coum* extract (CIP+Extract) on biofilm formation of two *P.aeruginosa* strains. Bars represent the mean $OD_{570}$ values, and error bars represent the standard deviations (*P value* <0.05).

By checkerboard synergy technique, *n*-butanolic *C.coum* extract in combination with ciprofloxacin exhibited synergistic effect against *P. aeruginosa* biofilms (ΣFBIC = 0.496).

This observation may be due to the nature of biological active components of *n*-butanolic *C. coum* extract (saponins). Saponins are plant secondary metabolites which play a major role in plant defense mechanisms. It is reasonable to assume the extract may re-sensitize resistant bacteria to the antibiotics by inhibiting cell-to-cell communication (Quorum sensing) (14 and 15). In recent years, Saponins have been great applications in commercial use, but because of high toxicity effects they have limited medical applications. In this study cytotoxicity effect of *n*-butanolic *C. coum* extract was assayed.

A dose response curve for the percentages of viable cells (0 to 100%) was plotted against the concentrations used in this study. The *n*-butanolic *C. coum* extract alone and in combination with ciprofloxacin have not exhibited cytotoxic effects against HT-29 cancer cell lines when compared with the control RPMI1640 (survival rate was 100%).

## Discussion and conclusion

Results of this study offered a scientific basis for the traditional use of plant *Cyclamen coum* as a potential phytotherapeutic agent. The anti-biofilm activity could be enhanced if the active components are purified and used with conventional antibiotics, which is therefore employed in our further studies

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# بررسی اثرات سینرژیسمی عصاره بوتانلی گیاه *Cyclamen coum* و آنتی‌بیوتیک سیپروفلوکساسین بر مهار تشکیل بیوفیلم *Pseudomonas aeruginosa*

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## چکیده

**مقدمه:** عفونت‌های ناشی از *P. aeruginosa* مولد بیوفیلم یکی از عوامل مهم مرگ و میر در بیماران مبتلا به فیبروز سیستیک هستند. بر اساس نتایج بسیاری از پژوهش‌ها، بیوفیلم‌ها نسبت به بسیاری از آنتی‌بیوتیک‌ها به دلیل ساختار نفوذناپذیر مقاوم هستند. برای کاهش مقاومت باکتری‌ها به آنتی بیوتیک‌ها، باید تشکیل بیوفیلم مهار شود. در این مطالعه، اثرات ضدبیوفیلمی عصاره بوتانلی گیاه *C. coum* گیاه متعلق به خانواده Myrsinaceae در ترکیب با سیپروفلوکساسین علیه بیوفیلم *P. aeruginosa* بررسی شد.

**مواد و روش‌ها:** ابتدا توانایی تشکیل بیوفیلم، در سویه *P. aeruginosa* $PAO_1$ و سویه بالینی *P. aeruginosa* (PA214) توسط روش میکروتیترپلیت اثبات شد. عصاره‌گیری بخش توبر(ساقه زیرزمینی) گیاه C. coum توسط محلول‌های دی اتیل اتر، الکل اتانول و بوتانول انجام شد. فعالیت ضدمیکروبی و ضدبیوفیلمی عصاره بوتانلی گیاه *C. coum* (که دارای ترکیبات ساپونینی بود) به تنهایی و در ترکیب باآنتی‌بیوتیک سیپروفلوکساسین توسط روش‌های microdilution و کریستال ویوله بررسی شد. تاثیرات سایتوتوکسیسیته عصاره بوتانلی گیاه *C. coum* بر روی سلول‌های HT-29 توسط روش MTT بررسی شد.

**نتایج:** توانایی تشکیل بیوفیلم توسط سویه‌های *P. aeruginosa* به شکل کمی اثبات شد. میزان ساپونین عصاره بوتانلی گیاه *C. coum*، ۱۵۶ میکروگرم بر میلی‌لیتر بود. عصاره بوتانلی گیاه *C. coum* تاثیرات ضدمیکروبی علیه سویه‌های آزاد-زی *P. aeruginosa* نشان داد. ترکیب عصاره بوتانلی گیاه *C. coum* و آنتی بیوتیک سیپروفلوکساسین به‌طور معنی‌داری سبب مهار تشکیل بیوفیلم شد ($\Sigma FBIC \leq 0.5$). عصاره بوتانلی گیاه *C. coum* فاقد اثرات سایتوتوکسیک برروی سلول‌های HT-29 بود.

**بحث و نتیجه‌گیری:** ترکیب عصاره بوتانلی گیاه *C. coum* و آنتی بیوتیک سیپروفلوکساسین فعالیت ضدبیوفیلمی معنی‌داری علیه *P. aeruginosa* نشان داد، اما هنوز مستلزم تحقیقات بالینی بیشتری است.

**واژه‌های کلیدی:** *P. aeruginosa*، بیوفیلم، *Cyclamen coum*، سیپروفلوکساسین

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