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SHORT COMMUNICATION

Evaluation of antibacterial effects of catechin and EDTA on planktonic and biofilm cells of *Pseudomonas aeruginosa*

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ABSTRACT

Aims: Today, bacterial biofilms contribute to 65% of nosocomial infections worldwide. One of the most common pathogens that can form biofilm is *Pseudomonas aeroginusa*. Therefore, the present study was aimed to evaluate the antimicrobial effects of catechin and ethylenediaminetetraacetic acid (EDTA) on planktonic and biofilm cells of *P. aeruginosa* standard strain.
Methodology and results: Standard strains of *P. aeruginosa* (ATCC 27853) were cultivated in nutrient agar medium for 24 h at 37 °C. The MICs values of EDTA, catechin and imipenem antibiotics on *P. aeruginosa* were determined with micro dilution test. Then, the biofilm of this bacterium was grown and finally the influences of these agents on biofilm inhibition were evaluated by colorimetric MTT and ATPase release assays. One way analyses of variance and then Fisher's least significant difference test were carried out to compare the different groups. The MIC values of catechin and EDTA on *P. aeruginosa* were 7.24 and 24.92 (µg/mL), respectively. Colorimetric assay with MTT showed that EDTA, and catechin inhibited biofilm formation significantly. ATPase assay indicated that the amount of released ATP from EDTA and catechin groups were significantly lower than the control group. Also, there was a significant difference between the EDTA and catechin groups with respect to the amount of the released ATP.
Conclusion, significance and impact of study: Our findings showed that EDTA and catechin can inhibit the growth of planktonic and biofilm cells of *P. aeruginosa*. From the results of the present study, we suggest using these agents to reduce or inhibit bacterial contamination of medical devices.

Keywords: *Pseudomonas aeruginosa*, bacterial biofilm, catechin, EDTA, antibacterial agent

INTRODUCTION

Nosocomial infections, also known as hospital-acquired infections (HAI), cause or contribute to a relatively high mortality rate worldwide. Sixty five percent of these infections are resulted from the formation of bacterial biofilms (Smith and Hunter, 2008). Biofilms are well-organized and complex aggregates of microorganisms, surrounded by a protective matrix of exopolysaccharides and can adhere to each other on various surfaces (Ehrlich *et al*., 2002). Bacterial biofilms are not homogenous structures. They are consisted of a wide variety of bacteria and microbial species. These complex substances are frequently found in medical implants and catheter (Costerton, 2001). *Pseudomonas aeruginosa*, one of the most important pathogenic agents and capable of forming biofilms, is generally found in skin and urinary tract infections, and in the lungs of patients suffering from cystic fibrosis disease (Costerton, 2001). This opportunistic and Gram negative bacterium causes virulent infections in immunocompromised patients (Costerton, 2001). Because of the ever-increasing prevalence of antibiotic resistance, it is necessary to find an antimicrobial substance fitted for reducing the pollution level in hospital settings and medical equipment.

Imipenem is broad-spectrum antibiotic and one of the most powerful antibiotics used for *Pseudomonas* infections (Hantson *et al*., 1999). As imipenem like other beta-lactams causes dysfunctions in central nervous system and leads to disorders like epilepsy (Chernish and Aaron, 2003), the need for safer alternatives with antimicrobial characteristics is obvious.

The application of EDTA, Ethylenediaminetetraacetic acid, for the treatment of biofilm infections has been

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investigated and the results are promising, however, the mechanism of its effect on biofilms is unknown *(*Banin *et al.*, 2006; Yakandawala *et al.*, 2007).

Catechin, one of the effective elements of green tea plays vital roles in various processes related to cell life including: signaling and cell cycle, arachidonic acid metabolism, cell division and apoptosis. It inhibits the growth of cancerous cells (Sun *et al.*, 2006; Caturla *et al.*, 2003). In addition to its antioxidant features, its antiviral function against tobacco mosaic virus was observed in a series of studies (Okada, 1971).

But to our knowledge there is not any study which investigated the antimicrobial properties of EDTA and catechin on planktonic and biofilm cells of *P. aeruginosa*. Therefore, this study aimed to comparing the antimicrobial properties of EDTA, catechin and Imipenem on Planktonic and Biofilm Cells of *P. aeruginosa*.

MATERIALS AND METHODS

Cultivation of bacteria

Standard *P. aeruginosa* strain ATCC 27853 was obtained from Al-Zahra University (Tehran, Iran) and cultivated in the nutrient agar medium. To obtain a single isolated colony of the sample, it was incubated at 35 °C for 18-24 h.

Preparation of different dilutions of ethylenediaminetetraacetic acid dipotassium salt (EDTA-K2)

Aqueous solutions of EDTA-K2 powder, ED2P-1KG (Sigma Co), were prepared and then filtered in different concentrations to perform the dilution test in the agar medium. The resulting dilutions were prepared in the following concentrations (g/mL): 0.1617, 0.1415, 0.1213, 0.1011, 0.0808, 0.0606, 0.0404, 0.0202, 0.00809, 0.00404, 0.00323, and 0.00243.

Preparation of different dilutions of catechin

Aqueous stock solution of the catechin powder, C0567-5MG (Sigma-Aldrich Co.), was prepared and then filtered in different concentrations to perform the dilution test in the agar medium. The resulting dilutions were prepared in the following concentrations (g/mL): 0.01, 0.02, and 0.03 of which the concentration of 0.02 g/mL was selected as the basic aqueous using a trial and error method.

Preparation of different dilutions of the broad-spectrum imipenem antibiotic

Since, imipenem antibiotic is a good soluble in sterile distilled water, its aqueous solution in different concentrations was used for different experiments of the study. The produced dilutions included 0.025, 0.016, 0.0125, 0.010 and 0.008 (g/mL).

Determining the minimum inhibitory concentrations (MICs) of EDTA, catechin and imipenem for the growth of *Pseudomonas aeruginosa* with dilution test

After the preparation of different dilutions of the above solutions, the dilution test was conducted according to the National Committee for Clinical Laboratory Standards (NCCLS). To perform the test, 10 µL of the diluted microbial suspension with 100 µL of the growth medium, Mueller-Hinton Broth, were added into each well of a 96-well microtiter plate. Then, different concentrations of the EDTA, catechin and imipenem were added to these wells and each test was repeated for three times. Following the incubation procedure, 10 µL of sample was withdrawn from each well and, was inoculated into the nutrient agar medium to determine and verify the number of colonies. Finally, the microplates were inserted into a 35 °C incubator for 24 h and the colonies were counted thereafter.

Evaluation of biofilm formation with MTT assay

Two hundred µL of the microbial suspension sample was added into each well of a 96-well polystyrene microtiter plate. The microtiter plate was heated at 37 °C for 24 h. Then, the contents of the wells were gently evacuated. To remove the weakly attached or unattached bacteria, at first, 200 µL of sterile PBS buffer was added into the wells and evacuated. Afterwards, EDTA, catechin and imipenem with a concentration of at least twice the MIC, predefined for each agent, were added into the wells separately. Then, 100 µL of the growth medium of TSB with 0.2% glucose was added into each well. This separation procedure was repeated three times for each antimicrobial agent. After the separation step, the above-mentioned microplate was incubated in a 37 °C incubator for 24 h. Following the incubation period, contents of the wells were completely withdrawn and then 200 µL of sterile PBS buffer was added into the wells and then evacuated. This procedure was repeated two times.

A 5 mg of MTT salt were dissolved into 1 mL of PBS buffer and sterilized with a 0.22 µm filter. Then 20 µL of the sterile MTT solution was added to each well of a 96-well microplate and heated at 37 °C for 5 h. Following this step, the microplates were removed and their contents were gently evacuated using a sampler. One hundred µL dimethyl sulfoxide (DMSO) 5% was added into the wells and heated at 37 °C for 15 min, then, the absorbance of each well at wavelength of 540/630 nm was measured using a standard ELISA-plate reader.

Evaluation of biofilm formation with ATPase assay

To prepare the suspension, the standard *P. aeruginosa* strain (ATCC 27853) was cultivated in the tryptic soy agar (TSA) medium enriched with 0.2% glucose at 37 °C for 24 h. The grown single colony in the cultivation medium was incubated in the tryptic soy broth (TSB) plus 0.2% glucose. To carry out light spectroscopy, contents of the

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test tubes were mixed and the light absorbance was set at 0.1 in wavelength of 650 nm for each sample.

Two hundred µL of the resultant cell suspension was added to each well of a 96-well microplate and heated at 37 °C for 24 h. Then, the content of each well was gently evacuated. To remove unattached or loosely attached bacteria, 200 µL of sterile phosphate buffered saline (PBS) buffer was added into the wells and then evacuated. In next step, EDTA, catechin and imipenem were added into the wells with a concentration equal to twice the respective MIC, which was defined previously for each of them. Then, 100 µL of the culture medium of TSA with 0.2% glucose was added into each well. This separation procedure was repeated three times. After the separation step, the above-mentioned microplate was heated in a 37 °C incubator for 24 h.

After the biofilm formation and incubation procedure, contents of the microplate wells in this test were not withdrawn. Instead, biofilm cells were removed from the microplate surface using tip of a sampler. Then, 100 µL of the content of each well, as well as 100 µL of a lysis buffer were transferred into the specialized plates of luminometer device and kept at room temperature for 10-20 s. Thereafter, 10 µL of luciferin and luciferase solutions were added into the each well. The amount of the released ATP was measured by the luminometer device.

Statistical analysis

The obtained data of the aforementioned assays were evaluated and compared with one way analyses of variance and Fisher's least significant difference tests. The data were analyzed using SPSS statistical package (Windows, version 16). A significance level of 0.05 was set for all statistical tests of the study.

RESULTS

For determining the minimum inhibitory concentrations of EDTA, catechin and imipenem, different concentrations (based on dilution preparation) of those were added into each well of a 96-well microtiter plate.

MIC results demonstrated that catechin and EDTA differ significantly from the control group regarding the derived inhibitory effects on the *Pseudomonas*. Catechin at the concentration of 7.24 µg/mL showed appropriate antibacterial property, which was much more effective than the EDTA (24.92) on the standard pseudomonas (p< 0.05), However the lowest concentration was belong to imipenem (0.43) (Table 1).

According to statistical analysis of the acquired data from the MTT technique (Figure 1), it was indicated that there was a significant difference between the wells containing two treatments groups (EDTA and catechin) and imipenem and control group, with relation to light absorbance values (p<0.01). Furthermore, results of this experiment showed a significant difference between the wells containing catechin and EDTA to those containing imipenem (p<0.01). And also there was a significant difference between EDTA and catechin (p<0.05).

Table 1: MIC values of various agents on standard *Pseudomonas aeruginosa* strain.

Agents	MIC (µg/mL)
Catechin	7.24
EDTA	24.92
Imipenem	0.43

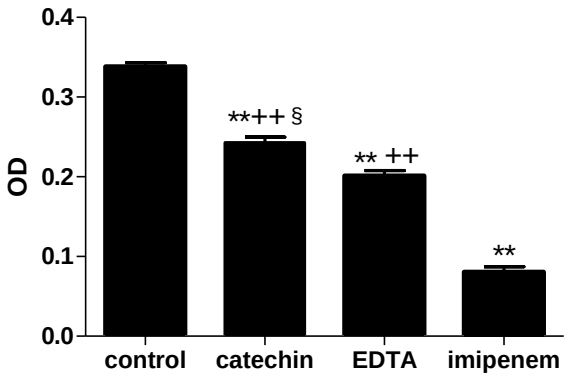

Figure 1: Results of the MTT assay for evaluation of antimicrobial effects of catechin, EDTA and imipenem on the *Pseudomonas* biofilm. ** denotes p<0.01, compared to the control group. ++ denotes p<0.01, compared to the imipenem. § denotes p<0.05, compared to the EDTA group. Values are presented as mean ± SEM.

Statistical analyses of the data obtained from the ATPase assay indicated that the amount of released ATP from imipenem, EDTA and catechin were significantly different from the value obtained from the control group (Figure 2) (p<0.01). However, there were no significant differences between the EDTA and catechin, and the positive control of imipenem groups with relation to the released ATP (p<0.05). However, there was a significant difference (p<0.05) between the EDTA and catechin groups with respect to the amount of the released ATP.

DISCUSSION

MIC analyses show that EDTA, catechin and imipenem are effective in inhibiting the growth of standard *P. aeruginosa* strain. Among treatment groups it was shown that catechin with the concentration equal to 1/3 of that of EDTA inhibited the growth of *P. aeruginosa* cells with the same inhibitory efficacy. Furthermore, catechin inhibited the growth of bacteria to a great extent and its effectiveness in both tests was not statistically different from that of the positive control group i.e. imipenem antibiotic. However, Imipenem group could inhibit the growth of bacteria with the least amount (0.43 µg/mL).

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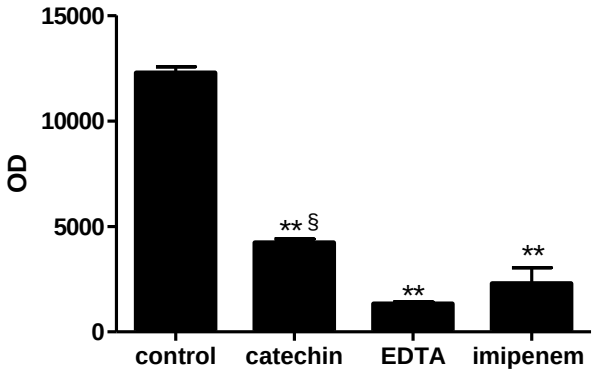

Figure 2: Results of the ATPase assay for evaluation of antimicrobial effects of catechin, EDTA and imipenem on the pseudomonas biofilm. ** denotes $p<0.01$ compared to the control group. § denotes $p<0.05$, compared to the EDTA group. Values are presented as mean ± SEM

ATPase assays demonstrated that treatment groups could effectively inhibit the growth of *pseudomonas aeruginosa* planktonic cells as compared to control group. However their effectiveness was not the same. Like the previous experiment, Imipenem suppress the growth of bacteria more vigorously in comparison to EDTA and catechin and also between the EDTA and catechin, EDTA exhibited more anti bacteria effect.

MTT assays verify the results of two previous experiments which two treatments group including EDTA and catechin inhibit the growth of bacteria in compare to control group but, with not the same powerfulness. As ATPase assays, MTT analyses also, showed that EDTA has more anti bacteria activity than catechin. Although in this experiment, as can be seen, EDTA exhibited more antibacterial effect than Imipenem but their difference was not significant.

Our research in regards of antibacterial effects of EDTA is in agreement to other research. For instance, Banin *et al.* (2006) indicated that EDTA chelates two-capacity cations which are necessary for the stability of biofilm matrix and causes the separation of cells from biofilm and subsequently death of biofilm cells. These researchers showed that addition of magnesium, calcium and iron ions protects pseudomonas bacterial biofilms against the effects of EDTA (Banin *et al.*, 2006). Furthermore, Raad *et al.* (2002), showed that a mixed compound of Minocycline and EDTA reduces the number of biofilm living cells significantly (Raad *et al.*, 2002). EDTA, a chelating agent for metals, affects the outer membrane permeability of microbial planktonic cells. This compound causes LPS to separate from cell surface by chelating two-capacity cations from outer membrane and increases outer membrane permeability (Banin *et al.*, 2006).

Plants generally have antimicrobial features because of the presence of phenolic, saponin and flavonoid metabolites in their structures. These agents typically affect cytoplasmic membrane and structural enzymes. In a study conducted by Sattari *et al.* (2006), they investigated the antibacterial effect of eucalyptus extract on *P. aeruginosa* and found that this extract inhibited the growth of bacteria Sattari *et al.* (2006). Tea plant leaves contain compounds such as catechin, thiamine (vitamin B1) and caffeine which can play an effective role on the antimicrobial property of the plant. The catechin in tea plant is classified under phenolic group. This compound comprises 30 percent of the dry tea weight (Graham, 1992). Catechin plays vital roles in various processes related to cell life including: signaling and cell cycle, arachidonic acid metabolism, cell division and apoptosis. (Okada, 1971).

Comparing the effectiveness of EDTA and catechin in recent study leads to interesting hint. Although in high concentration of EDTAD and catechin (used twice MIC) EDTA showed more antibacterial effects than catechin. But the concentration needs for completely inhibiting the growth of bacteria for catechin compare to EDTA was almost 1/3.

CONCLUSION

According to the data of this study which indicate the appropriate capability of EDTA and catechin in eliminating *Pseudomonas aeruginosa* biofilms, there is a hope for using naturally originated agents (like catechin), which in addition to their anti-tumor and antimicrobial features, to eliminate biofilms. Each agent with antimicrobial features and capable of eliminating biofilms is virtually helpful in preventing infections from spreading. The results of this study showed that catechin, as well as EDTA, can be considered as an eliminating agent of biofilms which exhibits an antimicrobial property and reduces the associated problems. This effectiveness can be widely used for the cleaning of medical equipment which is always exposed to pollution.

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