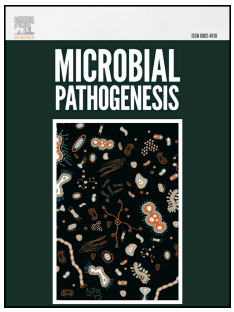
# Accepted Manuscript




Effect of human placental extract in the management of biofilm mediated drug resistance – A focus on wound management

Sutapa Goswami, Ratul Sarkar, Pritam Saha, Amit Maity, Tridib Sarkar, Debmalya Das, Piyali Datta Chakraborty, Subhasri Bandyopadhyay, Chandan Kumar Ghosh, Sanmoy Karmakar, Tuhinadri Sen

PII: S0882-4010(17)30139-0

DOI: 10.1016/j.micpath.2017.08.041

Reference: YMPAT 2431

To appear in: *Microbial Pathogenesis*

Received Date: 14 February 2017

Revised Date: 23 August 2017

Accepted Date: 30 August 2017

Please cite this article as: Goswami S, Sarkar R, Saha P, Maity A, Sarkar T, Das D, Chakraborty PD, Bandyopadhyay S, Ghosh CK, Karmakar S, Sen T, Effect of human placental extract in the management of biofilm mediated drug resistance – A focus on wound management, *Microbial Pathogenesis* (2017), doi: 10.1016/j.micpath.2017.08.041.

# Effect of human placental extract in the management of biofilm mediated drug resistance – A focus on wound management

**Sutapa Goswami[a,b*], Ratul Sarkar[a], Pritam Saha[a], Amit Maity[a], Tridib Sarkar[a], Debmalya Das[c], Piyali Datta Chakraborty[b], Subhasri Bandyopadhyay[b], Chandan Kumar Ghosh[c], Sanmoy Karmakar[a,d], Tuhinadri Sen[a]**

[a]Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India

[b]Albert David Ltd., Kolkata 700001. India

[c]School of Material Science and Nanotechnology, Jadavpur University, Kolkata 700032, India

[d]Bioequivalence Study Center, Jadavpur University ,Kolkata 700032, India

*Corresponding Author
Sutapa Goswami,
Department of Pharmaceutical Technology,
Jadavpur University,
Ph no- 033-24572056
Email. sutapa44goswami@gmail.com

•This paper is dedicated to the memory of our revered mentor Prof. Tuhinadri Sen. He stimulated our scientific curiosity and nurtured our development as researcher and we still admire and respect him as our teacher, scientist and great scholar.

**Abstract**

Management of infectious wounds, particularly chronic wounds and burn injuries, is a matter of global concern. Worldwide estimates reveal that, billions of dollars are being spent annually for the management of such chronic ailments. Evidently, bacterial biofilms pose a greater problem in the effective management of infection in chronic wounds, since most of the currently available antibiotics are unable to act on the microorganisms residing inside the protected environment of the biofilms. Accordingly, in the present study, we have attempted to evaluate the anti-biofilm properties of human placental extract (PLX) and also other virulence factors that are mediated via the quorum sensing (QS) signalling system. PLX is well known for its anti inflammatory action and it has been shown earlier some anti microbial and enzymatic activity also. PLX was found to produce significant inhibition of biofilm formation and also decreased the levels of pyoverdin and pyocyanin. The microscopic analysis (both light microscopy and atomic force microscopy) of biofilms was also used for substantiating the findings from spectrophotometric (crystal violet estimation) and fluorescence analysis (resazurin uptake). PLX pre-treatment decreased the hydrophobicity of gram-positive and gram negative cells, indicating the effect of placental extract on adherence property of planktonic cell, serving as an indicator for its antibiofilm effect on microorganisms. The reduced extracellular DNA (eDNA) content in biofilm matrix following treatment with PLX also indicates the effectiveness of placenta extract on bacterial adherence, which in turn serves as evidence substantiating the antibiofilm effects of the PLX. Furthermore, PLX was also found to be significantly effective in the *in vitro* wound biofilm model. Thus the present study, the first of its kind with PLX, establishes the therapeutic benefit of the same particularly in infected wounds, opening up newer avenue for further exploration.

**Key words:** Human Placenta Extract, Wound, Quorum Sensing, Biofilm, Motility, eDNA

**Abbreviations**

Pelvic inflammatory diseases: PID; Human placental extract: PLX; Nutrient Agar: NA; Mueller Hinton broth: MHB; colony-forming unit: CFU; Microgram: µg; Milligram: mg; Milliliter: ml; Microliter: µl; Hours: hrs; Nanometer: nm; Molar: M; millimolar: mM; Second: Sec; Ethylenediaminetetraacetic acid: EDTA; Phosphate buffer saline: PBS; Atomic force Microscopy: AFM; Optical Density: OD; weight/weight: w/w; Relative Fluorescence Unit: RFU; Quorum sensing: QS; extracellular genomic DNA: eDNA; *Staphylococcus aureus: S. aureus; Pseudomonas aeruginosa: P. aeruginosa;* Cipro : Ciprofloxacin

## 1. Introduction

Management of infected wounds remains a major cause of concern, and as per US reports, several billion dollars are being spent annually towards treatment cost [1]. It has been found that the moisture content and a steady supply of nutrients facilitates bacterial colonization of wounds and in a majority of wounds (about 60-80%) the colonizing organism are known to produce biofilms, thereby hindering the healing process to a great extent [2]. Bacterial biofilms have been found to be associated with chronic lung infections (cystic fibrosis patients), otitis media, dental caries, pelvic inflammatory disorder (PID), skin infection and many other disorders. Presence of polymicrobial biofilms have been reported in foot ulcers, pressure ulcers, venous leg ulcers and also in burn wounds [1].

A wide spectrum of microorganisms, during adverse environmental conditions, are known to form biofilms (as a protective measure), composed of self-secreting matrix of extra cellular polysaccharides and peptides. A biofilm also contains non cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed. Interestingly, such extracellular polysaccharide matrix is known to impart resistance to a wide array of antibiotics. Polymicrobial antibiotic resistant biofilms have also been found to be associated with infections arising from the use of catheters, medical implants or intrauterine devices [3]. A vast majority of chronic wounds have been found to be colonized with *Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Candida albicans* [1]. According to reports, chronic infection associated with biofilm are resistant to conventional antibiotic treatments, where the minimal bactericidal concentration for eradication of biofilm (containing altered phenotype of the infecting organisms) was found to be several fold higher (10-1000) when compared to their planktonic counterparts [4,5]. Therefore, in the management of biofilm mediated chronic infections removal of foreign bodies, changes of prosthesis or implants, draining of abscesses, debridement of wounds combined with suitable antimicrobial therapy may be utilized to attenuate the bacterial biofilm infections [3].

Placenta has been known to serve as a depot of many biologically active components with significant healing attributes [6]. In many countries, placental extract is commercially available for medicinal use [7, 8]. Studies have revealed the clinical efficacy of an aqueous extract of human placenta in the management of wounds including chronic non-healing wounds [9]. Interestingly, a number of reports published in Indian medical journals, indicate

the usefulness of human placenta extract in the management of PID (that has been commonly associated with the use of intrauterine devices) [10] as well as for the treatment of infected wounds [11, 12, 13].

Characterizations of placental extract reveal the presence of some bioactive components like peptides, NADPH, polydeoxyribonucletides (PDRNs), amino acids etc. Peptide of around 7.4 kDa has been identified and partial amino acid sequence from mass spectrometric analysis showed its homology with 10th type-III fibronectin peptide. This peptide contains the 'RGD' (Arg-Gly-Asp) signature sequence endowed with cell adhesion properties [14]. The importance of fibronectin in cutaneous wound healing is well documented [15]. Affinity chromatography, reversed-phase HPLC followed by mass spectrometry have shown the presence of free and bound NADPH in the extract [16]**.** Placental extract has been found to stimulate nitric oxide (NO) production in mouse peritoneal macrophages and also induces NO mediated wound healing in the presence of NADPH [17]. PDRNs identified by fluorescence spectroscopy as well as HPLC in the extract are capable of suppressing the chemical mediators of inflammation and thereby exerting an anti-inflammatory action [18, 19]. The placental extract was also found to contain ubiquitin like proteins possessing distinct proteolytic activity [20]**.** Investigation also revealed that the peptide fraction of placenta extract stabilizes serine proteases against their auto-digestion by reversibly inactivating them, which enhances the efficiency of proteolytic enzymes thereby facilitates wound healing [21]. Proteases regulate the balance between tissue degradation and regeneration during wound healing. Preliminary studies with the extract reveal anti-microbial property against a number of pathological microorganisms, particularly those associated with infected wound and burns [22].

Considering the increasing importance of biofilm mediated chronic infections and antibiotic resistance, an effort has been made in the present study to evaluate the effect of placental extract on bioflims with a special focus on the management of infected wounds.

## 2. Materials and Method

### *2.1. Preparation of Human Placenta extract*

Human placental extract (PLX) and Placenta gel, containing 10% fresh human placenta extract (received as a gift from Albert David Lid., Kolkata 700001, India) were utilized in the present investigation. PLX contains 100µg/ml protein (Bradford assay method)

[23]. Overall manufacturing procedure holding confidentiality of the proprietary terms has been described earlier [16].

An aqueous extract of rat stomach tissue (containing 250 µg/ml protein; Bradford assay method) has been prepared by using the same method as that of PLX [16], served as biological control to check any nonspecific effect (Institutional Ethical Committee approval number is 1237/PO/ReBi/S/2008/CPCSEA). The rat stomach tissue extract was used for experiments after proper dilution with respect to the protein concentration of PLX.

*2.2. Bacterial strains*

Bacterial Strains used for the assays**:** Gram positive: *S. aureus* (ATCC25923) Gram negative: *P. aeruginosa* (ATCC278531). The strains were maintained on Nutrient Agar (NA) (Himedia) plate and stored at 4°C. A single colony was transferred to Mueller Hinton broth (MHB) (Himedia), and incubated at 37°C. Density of the broth (containing the suspended organisms) was adjusted to 0.5 McFarland standards [24].

*2.2.1. Growth curve analysis*

*P. aeruginosa*, cultivated in presence and absence of PLX was used for studying the effect of PLX on bacterial growth. Briefly, standardized test inoculums (50 µl of $10^5$ CFU/ml suspension) were added to 1ml MHB in test tubes, with and without PLX (10 µg/ml protein). The culture were incubated at 37°C and the OD were recorded at 590 nm (Spectramax M5; Molecular Devices) at 0, ½, 1, 2, 4, 6, 8, 12, 18 and 24 hrs intervals [25].

*2.2.2. Effect of PLX on biofilm formation*

The effect of PLX on biofilm formation by *P. aeruginosa* was studied according to the method of Pompilio *et al.*, 2011 [25]. Briefly, 5µl of the cell suspension ($10^5$ CFU/ml) was used for inoculating MHB, containing either PLX (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml protein) or the standard antibiotic (ciprofloxacin;0.0781 µg/ml) and thereafter the suspension were inoculated for 24 hrs. After removal of the non-adherent cells, the biofilm were stained with either 0.1% crystal violet or resazurin (in PBS; pH 7.4; 0.15 mg/ml). In case of crystal violet stained biofilm, the dye were extracted with glacial acetic acid and then the concentration of the same was determined spectrophotometrically at 492 nm (Spectramax M5; Molecular Devices).

In another case, biofilms were stained with resazurin and the fluorescence produced by living cell were measured using Spectramax M5; Molecular Devices (Excitation at 560 nm; Emission at 590 nm) [26].

*2.2.2.1. Microscopic Analysis of Biofilm*

Microscopic analysis of biofilm of *P. aeruginosa* was performed on small glass pieces (cover slip; 10 mm×10 mm). The glass pieces were placed in 12-well polystyrene plates, and the bacterial culture ($10^5$ CFU/ml; in MHB media) were grown in presence or absence of the test samples [10 µg/ml protein] or standard drug [0.0781 µg/ml]) for 24 hrs (37°C). The cover slips were carefully removed, washed with (PBS; pH 7.4) fixed at 60°C, stained with 0.1% crystal violet. The stained biofilm (biomass) were observed under light microscope [27] (EVOS XL, Life Technologies).

Atomic force Microscopy (AFM) was also used for studying the surface topography of the biofilms of *P. aeruginosa*. In this analysis, the biofilm grown on glass slides (5mm×5mm) were air dried and then analyzed by AFM (NMDT nano-K$^{TM}$ VIBRATION ISOLATION by minus k TECHNOLOGY). AFM was performed in the tapping mode and the height was determined by using the NOVA software [28].

*2.2.3. The effect of PLX on preformed biofilm*

Biofilms were formed by incubating 5µl of the *P. aeruginosa* cell suspension ($10^5$ CFU/ml) in 100µl of MHB. After 24hrs incubation at 37°C, planktonic cells were gently removed by washing three times with PBS (pH 7.4). Matured biofilms were then treated with PLX (30µg/ml, 40µg/ml, 50µg/ml protein). Ciprofloxacin was used as positive control (2.5µg/ml). Accordingly the plates were again incubated at 37°C for 24 hrs, remaining biofilms were then scrapped and the isolated cells were subsequently mixed with 100µl of 0.25% trypsin-EDTA. The viability of the cell was determined by CFU count after diluting the samples [27].

*2.2.4. Effect of PLX on QS mediated virulence factors*

*2.2.4.1. Pyoverdin assay*

Bacterial pyoverdin competes with eukaryotic transferrin for iron uptake in the prokaryotic organism stimulating bacterial growth and pathogenicity. *P. aeruginosa* cells were inoculated for 16 hrs (37°C) with different concentration of the PLX (10 µg/ml, 5 µg/ml,

2.5 µg/ml protein) and Salicylic acid (50 µg/ml). Thereafter, the cells were centrifuged and the fluorescence (Excitation- 400 nm; Emission- 460 nm) of the supernatant was used measured (Spectramax M5; Molecular Devices) for determination of pyoverdin content [29]. The activity was expressed in Relative fluorescence unit (RFU).

*2.2.4.2. Pyocyanin Assay*

Pyocyanin, a cytotoxic secondary metabolite of *P. aeruginosa* is often used as a marker of QS induced virulence. Cultures (in MHB); $10^5$ CFU/ml) of *P. aeruginosa* were exposed to different concentration of either PLX (10 µg/ml, 5 µg/ml, 2.5 µg/ml protein) or the standard drug (Salicylic acid; 50µg/ml) for 24 hrs at 37°C (shaking incubation;125 rpm). At the end of the incubation period, liquid culture were extracted with chloroform then mixed with 0.2 M HCl. The absorption of the organic layer was determined spectrophotometrically (Spectramax M5; Molecular Devices) at 520 nm [30].

*2.2.4.3. Extracellular DNA (e DNA) assay*

Standardized inoculums (250 µl of a 1–5×$10^5$ CFU/ml suspension) of *P. aeruginosa* was inoculated with 2 ml of fresh MHB in absence (control) and presence (treated) of PLX for 24 hrs at 37°C. The cell free supernatant was carefully removed and 1ml of Tris-EDTA (10 mM Tris; 1 mM EDTA; pH8) was added to it after centrifugation. The adhered cells were gently vortexed and then centrifuged at (13000g for 30 Sec). The supernatant was removed and the cell pellet was re-suspended in Tris-EDTA buffer and then again centrifuged. eDNA concentration of the supernatant was determined using standard curve, prepared as per manufacturer (Cubit® 2.0 Fluorormeter; Life technologies) guidelines [31].

*2.2.5. Effect on Bacterial motility*

*P. aeruginosa* $10^5$CFU/ml cell suspension was utilized for the investigation.

*2.2.5.1. Swarming motility*

Agar plates (1% peptone, 0.5% NaCl, 0.5% agar and 0.5% D glucose; Himedia, India) containing different concentration of PLX and the standard drug (salicylic acid; 50µg/ml) were prepared for the swarming motility assay. Plates were inoculated (point inoculation) at the center and thereafter incubated for 24 hrs at 37°C. The swarming zone (Diameter; cm) was determined for each plate [29].

*2.2.5.2. Twitching motility*

Agar plates (1% agar) containing different concentration of PLX or the standard (salicylic acid; 50µg/ml) were inoculated (stab inoculum) and the plates were incubated for 24 hrs (37°C). The presence of a somewhat cloudy zone was considered as the sign of twitching motility and the diameter (cm) of the same was determined [29].

*2.2.6. Cell surface hydrophobicity*

Cell surface hydrophobicity was determined by microbial adhesion to hydrocarbon (MATH) assay with slight modification. Overnight grown *P. aeruginosa* and *S. aureus* culture (5%; $10^5$CFU/ml) were inoculated with 3 ml of MHB, supplemented with and without (control), PLX (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25µg/ml protein) respectively. After 24 hrs (37°C) of incubation, 500 µl of toluene was added. The mixture was vortexed vigorously for at least 3 min and allowed to stand at room temperature for the separation of two phases. Absorbance of the aqueous layer was measured at 600 nm [31].

% hydrophobicity = [1-(OD 600 nm after addition of toluene /OD 600 nm before addition of toluene)] ×100.

*2.2.7. In vitro wound biofilm model for assessing effectiveness of placenta gel contains 10% fresh human placenta extract.*

To evaluate the effectiveness of PLX (gel, containing 10% fresh human placenta extract) in the prevention of the development of bacterial biofilm, untreated gauze (control) or gauze containing PLX (gel, containing 10% fresh human placenta extract; 400 mg/ gauge) or standard 2% w/w Mupirocin (400 mg/ gauge), 1% w/w Framycetin (400 mg/ gauge) were placed over the sterile 6 mm disks immediately after inoculation with the strain $10^3$ CFU of *P. aeruginosa*. Direct contact between the antibiotic and the inoculated disks was ascertained by placing light-weight bars on each gauze and an adhesive tape attached to the sides of the plate to restrict the movement. Twenty-four hours after incubation (37°C), the gauze was removed and the disks were rinsed gently in PBS (pH: 7.4) to remove loosely attached cells. Each disk was then transferred to a microcentrifuge tube containing 1 ml of PBS and vortexed to disrupt the biofilms and detach the cells from the disks. Suspended cells were then suitably diluted and plated on NA plates. CFU were counted after 16 hrs of incubation at 37°C [32].

*2.3. Statistical analysis*

All the reported values represent the average of six independent experiments. Statistical analysis was performed with one-way analysis of variance followed by post-hoc

Dunnett's test. Unless otherwise mentioned, 'P' values less than 0.05 was considered to be statistically significant.

## 3. Results

### *3.1. Growth curve analysis*

The analysis of the growth curve of *P. aeruginosa* did not display any significant difference in the growth, when PLX (10µg/ml protein) treated culture was compared to the untreated one (Fig. 1).

### *3.2. Effect of PLX on biofilm formation*

Effect of PLX on biofilm formation was evaluated by crystal violet and resazurin assay. In case of crystal violet assay, a decrease in biofilm formation was observed following PLX pre-treatment. As evident from Fig. 2, biofilm formation was found to be reduced by PLX, in a concentration dependent manner in case both *P. aeruginosa* (10 µg/ml, 68± 2.50% ; 5 µg1/ml, 61± 1.00%; 2.5 µg/ml, 49± 1.50%; 1.25 µg/ml, 37± 4.00%; 0.625 µg/ml, 31± 4.00%; 0.312 µg/ml, 18± 2.50% ) and *S. aureus* (10 µg/ml, 54± 3.21%; 5 µg/ml, 44± 2.03%; 2.5 µg/ml, 34± 3.38%; 1.25 µg/ml, 21± 2.65%; 0.625 µg/ml, 07± 1.53%). The resazurin assay also displayed the effectiveness of the PLX (decrease of RFU) indicating the reduction of biofilm formation and the effect of PLX were also found to be concentration dependent (Fig. 3). As evident from crystal violet (Fig. 2) and resazurin assays, PLX was more effective against the gram negative organism as compared to the gram positive. The images obtained from Light microscope (Fig. 4) further support the observation from crystal violet assay, where pre-incubation with PLX were found to produce significant reduction of biofilm formation of *P. aeruginosa*. Rat stomach tissue extract was used as (negative) control (Fig. 2) in each of the experiment and exhibited no considerable inhibitory effects.

As evident from AFM analysis (50 µm × 50 µm), the average thickness of the untreated sample was found to be 450 nm as compared to 200 nm (PLX; 10 µg/ml protein) and 100 nm for the Ciprofloxacin (0.0781 µg/ml) (Fig. 5).

### *3.3. Effect of PLX on Preformed biofilm*

The viability of cells within the matured biofilms of *P. aeruginosa* were found to be reduced after treating with PLX (30µg/ml, 75± 3.20%; 40µg/ml, 48± 2.20%; 50µg/ml, 32±

2.50%) (Fig.6).These concentrations were higher as compared to the concentration (10 µg/ml) used to inhibit bacterial biofilm formation.

*3.4. Pyoverdin assay*

Pyoverdin production is a QS regulated process and it is known to compete with mammalian transferrin, thereby affecting iron uptake in the eukaryotic cells. In our present investigation, pretreatment of *P. aeruginosa* with PLX (10, 5, 2.5 µg/ml protein equivalent) produced significant inhibition of pyoverdin production (71.2%, 46.1%, and 28.6%) in a dose-dependent manner (Table-1). Salicylic acid was used as the standard drug which produced 54.4% inhibition.

*3.5. Pyocyanin Assay*

Pyocyanin (phenazine pigment) is a QS regulated virulence factor produced by the Gram-negative bacteria. In *P. aeruginosa*, pyocyanin (green coloured pigment) functions as a redox active toxin, which is known to release extracellular DNA (eDNA) from entrapped cells. Pretreatment of *P. aeruginosa* with PLX (10, 5, 2.5 µg/ml protein equivalent) produced significant inhibition of pyocyanin production (41.4%, 33.8%, and 25.1%) and the effect was found to be dose-dependent (Table-1).

*3.6. Extracellular DNA (e DNA) assay:*

eDNA present in biofilm matrix plays an important role in the cellular attachment and subsequent biofilm formation [33]. PLX pre-treatment (10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml of protein) was found to reduce eDNA formation in *P. aeruginosa* (82%, 62%, 48%, 30%) biofilms and the effect were found to be concentration dependent (Fig. 7).

*3.7. Swarming and twitching motility*

PLX significantly decreased the swarming zone, used as an index of flagellum-mediated swarming motility responsible for colonization (Table 2). Furthermore the PLX also produced significant reduction of twitching movement in *P. aeruginosa*, (Table 2), thus indicating the effect of the extract on bacterial movement.

*3.8. Cell surface hydrophobicity*

Initial adherence of bacterial cells to a solid surface (like glass or polystyrene plates) may favour biofilm formation. Such adherence is known to be governed by cell surface hydrophobicity. In the present context, PLX treatment was found the decrease in cell surface hydrophobicity both in *P. aeruginosa* and *S. aureus* (Fig. 8). This, reduced hydrophobicity may be correlated to poor attachment capacity, and slackened microcolony formation on polystyrene plates, which may have ultimately lead to a reduction of biofilm forming ability of the PLX treated cells.

*3.9. In vitro wound biofilm model for assessing effectiveness of placenta gel*

A freshly debrided wound was simulated in a disc, containing $10^2$ - $10^3$ CFU (the microbial load was similar to a fresh wound) of *P. aeruginosa*, notorious for triggering biofilm formation [32]. As evident from our present investigation PLX (containing 10% PLX; 400mg/gauge), produced significant reduction 99% reduction of CFU count of *P. aeruginosa*. Whereas, 1% Framycetin (400mg/gauge) or 2% mupirocin ointment (400mg/gauge), used as the standard drugs, produced 85.41% and 97.5% inhibition respectively (Table 3).

## 4. Discussion

During tissue injury, the defensive functions are perturbed, along with reduction in different acidic secretions like lactic acids from the sweat and sebaceous glands leading to loss of normal protective mechanism of the body [34]. Utilizing the impairment of protective systems microorganisms like *S. aureus, E. coli, P. aeruginosa*, *Klebsiella*, *Serratia* etc. invade and colonize the wounds [35, 36, 37, 32]. According to the recent reports, delay or failure of wound healing has been associated with biofilm formation [1]. Microorganism, infecting wounds, release diffusible signal molecules like acyl homoserine lactones, auto inducer peptides, furanosyl borate diesters. When these autoinducers attain critical concentrations with time cellular communication takes place between same or different species, a phenomenon known as QS [38, 39]. QS helps the organisms in regulating different physiological activities including biofilm formation. Considering the ineffectiveness of a vast majority of antimicrobials to penetrate the biofilm matrix, a number of studies are being conducted for evolving novel strategies that may be utilized to disrupt or prevent the bioflim formation.

In the present study PLX displayed significant reduction of biofilm forming ability of *P. aeruginosa* and *S. aureus* (organism often associated with nosocomial and community infectious). However bacterial growth curve analysis shows that the planktonic cell growth is unaltered (Fig 1). The concentration chosen for PLX (10 µg/ml of protein) is much below the MIC [22], to avoid selective pressure on the microorganisms, shows significant biofilm inhibitory property against both gram-positive and gram-negative organisms (Fig 2 & Fig. 3). The rat stomach tissue extract (as negative control) did not display any effect on biofilm inhibition (Fig 2 & Fig. 3). Reduction of colony formation of *P. aeruginosa* with reduced dispersion of micro-colonies was confirmed by microscopic investigation of PLX treated sample (Fig. 4 & 5). PLX at higher concentration (50 µg/ml of protein) reduced the viable cells entrapped in matured biofilm (Fig 6). Thus, the requirement of anti biofilm substance for inhibition of bacterial growth in matured biofilm may be higher than the concentration needed for inhibiting biofilm formation [40, 41].

Bacterial motility is QS mediated phenomenon and is a major determinant influencing biofilm development [29, 42]. In our present investigation pretreatment with PLX inhibited bacterial motility (swarming and twitching) in significant dose dependent manner (Table 2). Similar report with a synthetic cationic peptide 1037 was found to inhibit bacterial biofilm formation by reducing bacterial motility [42]. QS system is also shown to regulate the productions of other virulence factors like pyoverdin and pyocyanin, which are involved in host cell damage [30, 42, 43]. Analysis of the cell supernatants (from *P. aeruginosa* liquid culture), treated with PLX, showed significant reduction in concentration of both pyocyanin and pyoverdin, in dose dependent manner (Table 1).

Microorganism releases eDNA, an important component of biofilm (extracellular matrix), which promotes adhesion of the biofilm to abiotic surfaces through acid–base interactions [44]. Certain enzymes (DNase I, trypsin, proteinase K) have been found to make eDNA more soluble as well as susceptible to chemical agents [45]. Therefore, enzymatic treatment may improve the accessibility of conventional antimicrobials, in biofilm related chronic infections [46, 47]. Pretreatment with PLX caused significant reduction of eDNA content of *P. aeruginosa* biofilm which was dose dependent (Fig 7).

Increased cell surface hydrophobicity has been found to enhance the adherence of planktonic cell [31], a property often correlated to biofilm formation [48]. Pretreatment of *P. aeruginosa* and *S. aureus* with the PLX not only reduced biofilm development but also resulted in a decrease of surface hydrophobicity (Fig 8).

The effectiveness of PLX was also evaluated utilizing the *in vitro* wound biofilm model (a biofilm prevention model), that was utilized for mimicking an infected wound environment. From our observations, it was evident that the anti-biofilm activity of PLX gel was found to be more effective than Framycetin cream and mupirocin ointment (Table 3).

In recent studies application of various proteases to bacterial cultures are often shown to result in the reduction of biofilm formation and in the dispersal of established biofilms [49]. In our earlier report it has been shown that the peptide fraction of PLX containing ubiquitin has distinct proteolytic activity [20]. Thus, peptides of PLX and the enzymatic activity may be responsible for the anti-biofilm property and the future endeavor will be to identify the component/s responsible. Therefore any substance that can reduce formation or break the biofilm shall definitely take care of the above problems. PLX therapy has shown significant reduction in signs and symptoms of PID along with wound healing [50]. The healing time, recurrences of the disease was considerably brought down compared to antibiotics alone. Administering PLX along with antibiotics improves the patient compliance and may decrease the chance of antibiotic resistance [50, 51].

## 5. Conclusion

In the present study it has been observed for the first time that PLX significantly inhibits QS dependent bioflim formation in both gram-positive and gram-negative organisms. It also attenuated pyoverdin and pyocyanin formation and reduced eDNA concentration. In vitro wound biofilm assay model further demonstrated effectiveness of PLX. This property of PLX to combat bacterial biofilms could open up new avenues in the management of infected wounds and may be useful in limiting burn induced sepsis like situations, associated with serious burn injuries. Expedited healing has been observed in clinical studies with PLX and antibiotics with considerable shortening in recovery time improved MAGS score, neoangiogenesis and healthy collagen formation [52]. However, further characterization of PLX and purification of active components would be able to highlight the role of individual or combination of different components on anti-biofilm properties.

## Conflict of Interest

The authors declare that they have no conflict of interests.

## Acknowledgements

Authors are thankful to Albert David Ltd., Kolkata (for extending financial assistance for this project) and the University Grants Commission, New Delhi (DSA Phase-III and UPE-II programme of UGC) for the instrumental facilities developed under the aforesaid programmes. The authors would like to thank Dr. Joydeep Mukherjee (School of Environmental Studies, Jadavpur University) for providing the bacterial strains.

**Reference**

[1] G. Brackman, L.De Meyer, H.J. Nelis, T. Coenye, Biofilm inhibitory and eradication activity of wound care products against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm in an *in vitro* chronic wound model, J Appl Microbiol. 114 (2013) 1833-1842.

[2] G.A. James, E. Swogger, R. Wolcott, E. Pulcini, P. Secor, J. Sestrich, W.J Costerton, P.S Stewart, Biofilms in chronic wounds, Wound Repair Regen. 16 (2008) 37-44.

[3] H. Wu , C. Moser, H.Z. Wang, N. Høiby, Z.J. Song, Strategies for combating bacterial biofilm infections, Int J Oral Sci. 7 (2015) 1-7.

[4] N. Høiby, O. Ciofu , H.K. Johansen, Z.J. Song, C. Moser, P.Ø. Jensen, S. Molin, M. Givskov, T.Tolker-Nielsen, T. Bjarnsholt, The clinical impact of bacterial biofilms, Int J Oral Sci. 3 (2011) 55-65.

[5] W. Hengzhuang, H. Wu, O. Ciofu Z. Song, N. Høiby, Pharmacokinetic/pharmacodynamics of colistin and imipenem on mucoid and nonmucoid *Pseudomonas aeruginosa* biofilm, Antimicrob Agents Chemother. 55 (2011) 4469-4474.

[6] G. Tonello, M. Daglio, N. Zaccarelli, E. Sottofattori, M. Mazzei, A. Balbi, Characterization and quantitation of the active polynucleotide fraction (PDRN) from human placenta, a tissue repair-stimulating agent, J. Pharm Biomed Anal. 14 (1996) 1555-1560.

[7] P.D. Chakraborty**,** D. De, S. Bandyopadhyay, D. Bhattacharyya, Human aqueous placental extract as a wound healer, J Wound Care. 18 (2009) 462 – 467.

[8] J.W. Hong, W.J. Lee, S.B. Hahn, B.J. Kim, D.H. Lew, The effect of human placenta extract in a wound healing model, Ann Plast Surg. 65 (2010) 96-100.

[9] C.H. Wu , G.Y. Chang, W.C. Chang, C.T. Hsu, R.S. Chen, Wound healing effects of porcine placental extracts on rats with thermal injury, Br J Dermatol. 148 (2003) 236 – 245.

[10] N. Agarwal, V. Kulshrestha, A. Kriplan, Clinical efficacy of Placentrex injection in pelvic inflammatory disease, J Indian Med Assoc. 108 (2010) 117-118.

[11] A. Chandanwale, D. Langade, V. Mohod, S. Sinha, A. Ramteke, G. D. Bakhshi, M. Motwani, Comparative evaluation of human placental extract for its healing potential in surgical wounds after orthopaedic surgery: an open, randomised, comparative study, J Indian Med Assoc. 106 (2008) 405-408.

[12] Prameela, K.D. Sharma, Clinical Efficacy of Placentrex Injection in Pelvic Inflammatory Disease, IJOGR. 3 (2016) 65-67.

[13] V.M. Shinde, A.R. Paradakar, K.R. Mahadik, Human Placenta: potential source of bioactive compounds, Pharmacologyonline. 1 (2009) 40-51.

[14] P.D. Chakraborty, D. Bhattacharyya, Isolation of fibronectin Type III like peptide from human placental extract used as wound healer, J Chromatogr B Analyt Technol Biomed Life Sci. 818 (2005) 67-73.

[15] S. Nath, D. Bhattacharyya, Cell adhesion by aqueous extract of human placenta used as wound healer, Indian J Exp Biol. 45 (2007) 732-738.

[16] P. Datta, D. Bhattacharyya, Spectroscopic and chromatographic evidences of NADPH in human placental extract used as wound healer, J Pharm Biomed Anal. 34 (2004) 1091 -1098.

[17] P.D. Chakraborty, D. Bhattacharyya, S. Pal, N. Ali, *In vitro* nitric oxide induction by mouse peritoneal macrophages treated with human placental extract, Int Immunopharmacol. 6 (2006) 100-107.

[18] P. Bianchini, N. Tellini, A.M. Morani, M.G. Folloni, Pharmacological Data on Polydeoxiribonucleotide of Human Placenta, Int J Tiss Reac. 3 (1981) 151- 154.

[19] P.D. Chakraborty, S. Goswami, S. Bera, I. Mukhopadhyay, Quantitation of Polydeoxyribonucletides (PDRNs) in Human Placental Extract by Fluorescence Spectroscopy Using Ethidium Bromide, AJAC. 5 (2014) 784-795.

[20] D. De, P.D. Chakraborty, J. Mitra, K. Sharma, S. Mandal, S. Das, S. Chakrabarti, D. Bhattacharyya, Ubiquitin-like Protein from Human Placental Extract has Collagenase Activity, PLoS ONE.8 (2013) e59585.

[21] D. De, P.D. Chakraborty, D. Bhattacharyya, Regulation of trypsin activity by peptide fraction of an aqueous extract of human placenta used as wound healer, J Cell Physiol. 226 (2011) 2033-2040.

[22] P.D. Chakraborty, D. Bhattacharyya, In Vitro growth inhibition of microbes by human placental extract, Current Science. 88 (2005) 782-786.

[23] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem. 72 (1976) 248–254.

[24] J.M. Andrews, Determination minimum inhibitory concentrations, J Antimicrob Chemother. 48 (2001) 5–16.

[25] A. Pompilio, M. Scocchi, S. Pomponio, F. Guida, A. Di Primio, E. Fiscarelli, R. Gennaro, G. Di Bonaventura, Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients, Peptides. 32 (2011) 1807-1814.

[26] E. Peeters, H.J. Nelis, T. Coenye, Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates, J Microbiol Methods. 72 (2008) 157-165.

[27] S. Gowrishankar, N.D. Mosioma, S. K. Pandian, Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and–susceptible *Staphylococcus aureus* biofilms, Evid Based Complement Alternat Med. 2012 (2012) 862374.

[28] S. Chatterjee, N. Biswas, A. Datta, R. Dey, P. Maiti, Atomic force microscopy in biofilm study, Microscopy. 63 (2014) 269-278.

[29] E. Déziel, Y. Comeau, R. Villemur, Initiation of biofilm formation of *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly

adherent phenotypic variants deficient in swimming, swarming, and twitching motilities, J Bacteriol. 183 (2001) 1195-1204.

[30] C.Y. Chang, T. Krishnan, H. Wang, Y. Chen, W. F. Yin, Y.M. Chong, L.Y. Tan, T.M. Chong, K.G. Chan, Non-antibiotic quorum sensing inhibitors acting against N-acyl homoserine lactone synthase as druggable target, Sci Rep. 4 (2014) 7245.

[31] S. Gowrishankar, M. Sivaranjani, A. Kamaladevi, V.A. Ravi, K. Balamurugan, K.A. Pandian, Cyclic dipeptide cyclo(L-leucyl-L-prolyl)from marine *Bacillus amyloliquefaciens* mitigates biofilm formation and virulence in *Listeria monocytogenes*, Pathog Dis. 74 (2016) 017.

[32] A.A. Hammond, K.G. Miller, C.J. Kruczek, J. Dertien, H. Colmer, J.A. Griswold, A.R. Horswill, A.N. Hamood, An *in vitro* biofilm model to examine the effect of antibiotic ointments on biofilms produced by burn wound bacterial isolates, Burns. 37 (2011) 312-321.

[33] A. Ghafoor, I. D. Hay, B.H.A. Rehm. Role of Exopolysaccharides in *Pseudomonas aeruginosa* Biofilm Formation and Architecture, Appl Environ Microbiol.77 (2011) 5238–5246.

[34] P. Kennedy, S. Brammah, E. Wills. Burns, biofilm and a new appraisal of burn wound sepsis, Burns. 36 (2010) 49-56.

[35] M. Kooistra-Smid, M. Nieuwenhuis, V. A. Belkum, H. Verbrugh, The role of nasal carriage in *Staphylococcus aureus* burn wound colonization, FEMS Immunol Med Microbiol. 57 (2009) 1–13.

[36] J.P. Barret, D. N. Herndon, Effects of burn wound excision on bacterial colonization and invasion, Plast Reconstr Surg. 111 (2003) 744–752.

[37] B.A. Pruitt, A.T. McManus, S.H. Kim, C.W. Goodwin, Burn wound infections: current status. World J Surg. 22 (1998) 135– 145.

[38] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, Clin Microbiol Rev. 15 (2002) 167–193.

[39] Y.H. Li, X. Tian, Quorum Sensing and Bacterial Social Interactions in Biofilms, Sensors (Basel), 12 (2012) 2519–2538.

[40] R. Sarkar, K.S. Chaudhary, A. Sharma, K.K. Yadav, K.N. Nema, M. Sekhoacha, S. Karmakar, C.F. Braga, G. M. Matsabisa, P.K. Mukherjee, T. Sen , Anti-biofilm activity of Marula - a study with the standardized bark extract, J Ethnopharmacol. 154 (2014) 170-175.

[41] A. Nostro, A. S. Roccaro, G. Bisignano, A. Marino, M.A. Cannatelli, F.C. Pizzimenti, P.L Cioni, F. Procopio, A.R. Blanco, Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms, J Med Microbiol.56 (2007) 519–523.

[42] C. de la Fuente-Núñez, V. Korolik, M. Bains, U. Nguyen, E.B. Breidenstein, S. Horsman, S. Lewenza, L. Burrows, R.E. Hancock, Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide, Antimicrob Agents Chemother. 56 (2012) 2696-2704.

[43] A. Adonizio, K.F. Kong, K. Mathee, Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by South Florida plant extracts, Antimicrob Agents Chemother. 52 (2008) 198-203.

[44] M. Okshevsky, R.L. Meyer, The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms, Crit Rev Microbiol.41 (2015) 341-352.

[45] M. Chen, Q. Yu, H. Sun, Novel Strategies for the Prevention and Treatment of Biofilm Related Infections Int, J. Mol. Sci. 14 (2013) 18488-18501.

[46] L. Montanaro, A. Poggi, L. Visai, S. Ravaioli, D. Campoccia, P. Speziale, C.R. Arciola, Extracellular DNA in biofilms, Int J Artif Organs. 34 (2011) 824-831.

[47] K. Sharma, C. Mukherjee, S. Roy, D. De, D. Bhattacharyya, Human placental extract mediated inhibition of proteinase K: implications of heparin and glycoproteins in wound physiology, J Cell Physiol. 229 (2014) 1212-1223.

[48] B. Kouidhi, T. Zmantar, H. Hentati, A. Bakhrouf, Cell surface hydrophobicitry, biofilm formation adhesives properties and molecular detection of adhesions genes in *Staphylococcus aureus* associated to dental caries, Microb Pathog. 49 (2010) 14-22.

[49] R. Mukherji, A. Patil, A. Prabhune, Role of Extracellular Proteases in Biofilm Disruption of Gram Positive Bacteria with Special Emphasis on *Staphylococcus aureus* Biofilms, Enz Eng. 4 (2015) 126-132.

[50] P. Dahiya, A. Paul, A randomised study to evaluate the efficacy and safety of placentrex injection in patients suffering from pelvic inflammatory disease, J Indian Med Assoc. 111 (2013) 352-353.

[51] A. Chandanwale, D. Langade, V. Mehod, S. Sinha, Comparative evaluation of human placental extract for its healing potential in surgical wounds after orthopaedic surgery: an open, randomised, comparative study, J Indian Med Assoc. 106 (2008) 405-408.

[52] S.K. Tiwary, D. Shukla, A.K. Tripathi, S. Agrawal, M.K. Singh, V.K. Shukla, Effect of placental-extract gel and cream on non-healing wounds, J Wound Care. 15 (2006) 325-328.

**Figure legends**

**Fig. 1.** Influence of PLX on growth of *P. aeruginosa* (incubated at 37 °C for 24 hrs). The data represent the mean values of growth curve experiment in triplicate.

**Fig. 2.** Biofilm inhibition assay for PLX (Protein content) and ciprofloxacin (Cipro) at different concentrations. The biofilm inhibition with *P. aeruginosa* and *S. aureus* were assessed colorimetrically, using crystal violet. The results are expressed as percentage of biofilm inhibition with respect to the untreated one. Rat stomach tissue extract used as a control (negative). All values are expressed as mean ±SEM (n=6).

**Fig. 3.** The effect of PLX (Protein content) and ciprofloxacin (Cipro) on biofilm formation by *P. aeruginosa* and *S. aureus* were assessed with resazurin, a dye which is taken up by living cells and the fluroscense emission (RFU) was recorded (Excitation at 560 nm; Emission at 590 nm). Rat stomach tissue extract used as a control (negative). All values are expressed as mean ±SEM (n=6). *$P < 0.05$ (vs control).

**Fig. 4.** Light Microscopic images (400×) of *P. aeruginosa* biofilm grown in absence [A] or presence [B] of PLX. Ciprofloxacin was used as the standard drug [C].

**Fig. 5.** Effect of vehicle (A), PLX (B) or Ciprofloxacin (C) on *P. aeruginosa* biofilm formation, visualized by Atomic force microscopy (AFM). The biofilm topography (50µm×50µm scan) in three dimensions determined in the tapping mode.

**Fig. 6.** Effect of PLX (Protein content) at different concentrations against *P. aeruginosa* on preformed biofilm. The results are expressed as a percentage of the biofilm viability, assessed by colony counting, with respect to untreated control (taken as 100%). The data represent the mean values of experiment ±SEM (n=6).

**Fig. 7.** Effect of PLX (in terms of Protein) on eDNA content of *P. aeruginosa* biofilms. All values are expressed as mean ±SEM (n=6).

**Fig. 8.** Alteration of cell surface hydrophobicity of *P. aeruginosa* and *S. aureus*, following pretreatment with PLX (Protein content). The hydrophobicity was calculated from the absorbance (measured at 600nm) of the aqueous layer (before toluene addition and after partitioning with toluene). All values are expressed as mean ±SEM (n=6).

## TABLES

### Table 1

Pyoverdin and Pyocyanin production in *P. aeruginosa*, grown in presence of PLX or salicylic acid (standard).

| Treatment | Protein Concentration (µg/ml) | Pyoverdin production | | Pyocyanin production | |
|---|---|---|---|---|---|
| | | RFU (Excitation 405nm; Emission 465 nm) | % of Inhibition | Absorbance at 520 nm | % of Inhibition |
| Control | | 9581.666±131 | 0.0 | 0.408±0.006 | 0.0 |
| PLX | 2.5 | 6838.333±245* | 28.6 | 0.306±0.008* | 25.1 |
| | 5 | 5166.333±91* | 46.1 | 0.270±0.008* | 33.8 |
| | 10 | 2760.666±157* | 71.2 | 0.239±0.01* | 41.4 |
| Salicylic acid (50 µg/ml) | | 4365.666±132* | 54.4 | 0.265±0.007* | 35.1 |

RFU: Relative fluorescence Unit; The fluorescence emission spectrum was recorded at 465 nm by exciting at 405 nm
Values are expressed as mean ± SEM.; (n = 6); *P < 0.05 (vs control).

### Table 2

Swarming and twitching motility of *P. aeruginosa* cells, evaluated on semi-solid agar in the presence of either PLX or salicylic acid (standard).

| Treatment | Protein Concentration (µg/ml) | Swarming motility Distance of migration (cm) | Twitching motility Distance of migration (cm) |
|---|---|---|---|
| Control | | 2.16±0.12 | 1.6±0.07 |
| PLX | 2.5 | 0.97±0.04* | 0.83±0.03* |
| | 5 | 0.54±0.02* | 0.63±0.03* |
| | 10 | 0.37±0.01* | 0.24±0.02* |
| Salicylic acid (50 µg/ml) | | 0.23±0.01* | 0.9±0.04* |

Values are expressed as mean ± SEM.; (n = 6); *P < 0.05 (vs control).

**Table 3**

Effect of the PLX (in a gel containing 10% human placenta extract) or 2% w/w Mupirocin or 1% w/w Framycetin on biofilm formation, studied in *P. aeruginosa*. A representative wound biofilm model (*in vitro*).

| | Blank | PLX | Mupirocin | Framycetin |
|---|---|---|---|---|
| CFU/Disk | $4.8\times10^{7}$ | $1\times10^{3}$* | $1.2\times10^{6}$* | $7\times10^{6}$* |

Disks were inoculated with 103 CFU of P. aeruginosa. The inoculated disk were covered with untreated gauze, or treated with 10% human placenta extract, 2% mupirocin ointment, 1% Framycetin cream and incubated for 24hrs. Numbers of CFU were determined by plating after suitable dilution on NA plate. Values are expressed as mean CFU; (n = 6).*P < 0.05 (vs control).