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Evaluation of Pyocyanin induced systemic pathogenicity of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* (PA) is one of the most clinically significant nosocomial infectious agents. Clinical significance of this bacterium is intensified due to the phenomenon of its natural tendency for acquiring drug resistance mechanisms. PA produces pyocyanin (PCN), an important redox-active virulence factor. PCN has been detected in higher quantities in sputum samples of PA infected Cystic Fibrosis patients. PCN producing PA strains were isolated and characterized. Genomic 16s rRNA gene segment was amplified and sequenced (GenBank accession # jx280426). PCN was extracted and purified. *In silico* analysis yielded permeability and cytotoxic potential of PCN in modeled cell lines. PCN has high intestinal absorption, plasma protein binding potential, and permeability across biological membranes. Oral toxicity study in *in silico* rodent model classified PCN in class IV 'harmful if swallowed' (ld_{50} 0.3-2g/kg). Cytotoxicity was assessed by oxidative stress levels in different organs in balb/c mice induced by intra peritoneal PCN injection. Significant alterations in oxidative stress levels in different organs of balb/c mice were observed. Increased levels of oxidative stress were observed in lungs, and heart, lower in liver and spleen while muscle tissues showed no significant difference in comparison to control.

Keywords: *Pseudomonas aeruginosa*, pyocyanin, cytotoxicity, oxidative stress.

INTRODUCTION

Pseudomonas aeruginosa (PA) is a ubiquitous nosocomial infectious agent. Clinical significance of this bacterium is intensified due to its natural tendency for acquiring drug resistance mechanisms and as a result arising as Multi-Drug-Resistant (MRD) strains. Chronic nosocomial infections from MDR strains of PA in immune-compromised and transplant patients are becoming a very serious healthcare issue (Levy and Marshall, 2004, Zhe-Xian Tian, 2009). PA produces many virulence factors with multiple bioactivities. Prominent among all of them are pyocyanin (PCN), pyoverdine and prorubin. PCN is a redox active blue phenazine pigment. Bioactivities attributed to this pigment include antibacterial, antifungal, antiprotozoal, antiparasitic, antimalarial, immune-modulatory, pro-inflammatory, pro-apoptotic, enzyme inactivation and cytotoxicity.

All these bioactivities are one or the other way related to generation of Reactive Oxygen Species (ROS) and induction of oxidative stress (Bianchi *et al*., 2008, Britigan *et al*., 1999, Cheluvappa *et al*., 2008a, Denning *et al*., 1998, Sinha, 2008, Hashimoto *et al*., 2007, Hassan and Fridovich, 1980, Muller, 2006, Muller, 2002a, O'Malley *et al*., 2004, Price-Whelan *et al*., 2007, Ra, 2010). PCN alters the redox equilibrium inside a biological system. Cytotoxicity of PCN is considered to be its ability to generate ROS in particular H_2O_2 which induces oxidative stress in biological systems. Ranging from cardiac to chronic lung and liver diseases, oxidative stress is considered one of the important disease pathologies (Albano, 2006, Hashimoto *et al*., 2007, Lim *et al*., 2010, Muller, 2002a, O'Malley *et al*., 2004). ROS induce oxidative stress by interacting with most of the cellular macromolecules like proteins (structural alterations and functional impairment), lipids (peroxidation of phospholipids in plasma membranes) and DNA (Albano, 2006). PCN is vital for infection establishment and disease progression, this diffusing ability through biological membrane could pose a threat to other key body organs interconnected via the circulatory system.

This study was conducted to clarify the nature of the systemic effects of PCN on key body organs. We aimed to study PCN induced cytotoxicity in a bi-prong manner; (1) assessment of redox induced cytotoxicity using different computational tools and approaches and (2) evaluation of systemic cytopathology due to PCN's redox active nature via quantification of Glutathione S Transferase enzyme as a marker for induction of oxidative stress in animal models.

MATERIALS AND METHODS

Isolation, Characterization, and Antibiotic Susceptibility Profile of PA isolates

Environmental and clinical isolates were obtained from Islamabad. Ribotyping was performed using universal primers (Zahoor and Rehman, 2009) and the amplicon was sequenced. Overnight grown bacterial culture was harvested and cells were washed for Scanning Electron Microscopy sample preparations (Kumral *et al*., 2008). Scanning Electron Microscope (Analytical Scanning

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Electron Microscope, JSM 6490A, Jeol Japan), equipped with tungsten filament electron emitter and accelerating voltage of 30 kV was used for imaging. Antibiotic Susceptibility Profile was developed (Cheesbrough, 2006).

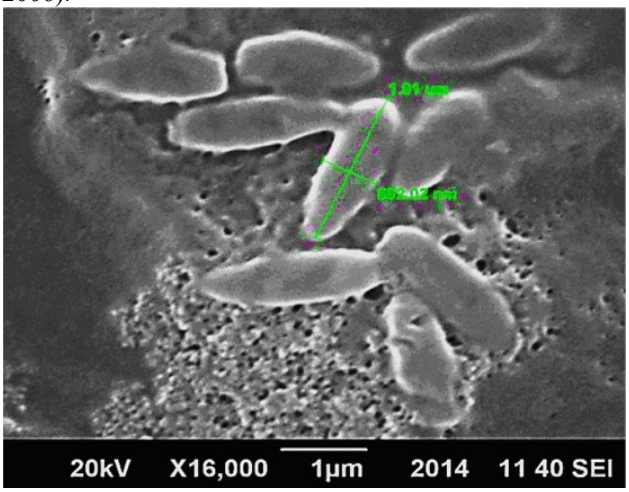

Fig. 1: Images of Scanning Electron Microscopy showing *P. aeruginosa isolate S-5.1.a* cellular morphology at 16000x magnifications

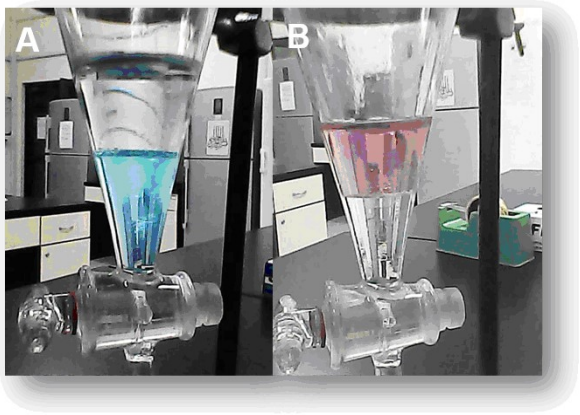

Fig. 2: PCN extraction and purification process.

PCN Purification and Quantification

PCN was extracted and quantified as mentioned (Essar *et al*., 1990, Cheluvappa *et al*., 2008b). For higher PCN production a MS, Modified Semi-synthetic media (composition: Tryptone 10g/L, $MgSO_4$ 1.5g/L, KCl 0.5g/L, Na_2HPO_4 2.5g/L, Glycerol 20mL/L, Lactose 10g/L, K_2HPO_4 0.25g/L& Yeast Extract 2.5g/L) was designed for batch fermentation experiments performed under conditions: incubation temperature 37°C, agitation at 250 *rpm*, pH 8 and in dark.

In silico PCN Cytotoxicity Studies

PROTOX server (http://tox.charite.de/tox/index.php?site=home) was used for calculation of PCN oral toxicity. PreADMET server (http://preadmet.bmdrc.kr/) provided estimations for Human intestinal absorption, MDCK (Mandin Darby Canine Kidney) cell permeability, skin permeability (transdermal delivery), Plasma Protein Binding, Caco2 (Human colorectal carcinoma) cell permeability and hERG channel inhibition values. Way2Drug server tool (http://www.way2drug.com/Cell-line/index.php) CLC-Pred predicted *in silico* cytotoxicity for tumor and non-tumor cell lines for PCN. eMolTox server (http://xundrug.cn/moltox) provides a thorough and profound range of *in silico* toxicity predictions. PCN was subjected to the server in order to study potential metabolic, cytotoxic and genotoxic implications. The predictions with confidence levels greater than 0.9 were considered as the probable impact of the prediction while prediction values lesser than confidence score 0.9 were discarded.

Study of redox bioactivity in the murine model

PCN was intra-peritonealy injected in adult BALB/c mice (n=3), to give a final systemic concentration of 10µM. Total blood volume was calculated for each mouse using the formula: blood volume (ml) = 0.06 x body weight (g) + 0.77(Lee and Blaufox). After 24 hours, mice were euthanized and liver, spleen, heart, lungs, and muscles were removed, homogenized in sterile PBS and oxidative stress estimation was done via GST assay (Cheluvappa *et al*., 2008c). Animal studies were approved by Institutional Review Board (IRB), Atta ur Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad. The study was conducted at Department of Industrial Biotechnology, Atta ur Rahman School of Applied Biosciences, National University of Sciences and Technology.

STATISTICAL ANALYSIS

GraphPad Prism (version 5) software was used to analyze results.

RESULTS

Isolation and Characterization

Individual green fluorescent colonies appeared after overnight incubation at 37°C. *Isolate* S-5.1.a with higher PCN production was selected and 16S rRNA gene segment was sequenced and submitted to GenBank (Ac#JX280426). BLAST analysis showed homology with Pseudomonas family entries. Cells were about 1.9µm long and 682.1nm wide (fig. 1). Antibiotic Susceptibility Profile yielded the resistance pattern in which *P. aeruginosa S-5.1.a* was highly susceptible to Topoisomerase inhibitors (Nucleic acid antibiotics) namely Ciprofloxacin and Levofloxacin while generally, translation inhibitors were more effective than the other two classes. Of total 21 antibiotics, *Isolate S-5.1.a* was totally resistant against 8 antibiotics (Silphamethoxazole Trimethoprin, Amoxicillin, Cephazolin, Trimethoprime, Bacitracin, Ampicillin, Linezolid, and Clindamycin), 8 antibiotics showed mild affectivity (Erythromycin,

Tetracycline, Streptomycin, Cefoperazone, Clarithromycin, Kanamycin and Ceftriaxone) while susceptible for 5 (Ciprofloxacin, Levofloxacin, Chloramphenicol, Gentamicin and Azithromycin). 50% (2/4) Nucleic Acid Inhibitors, 33.33% (2/4) of Cell Envelope Antibiotics and 72% (8/11) of Translation Inhibitor antibiotics were effective against *P. aeruginosa S-5.1.a*.

Pyocyanin production and purification

Optimization experiments showed maximum yield at pH 8, incubation temperature 37°C, and agitation at 250 rpm. Batch fermentation using MS (Modified Semi-synthetic) media yielded 3x fold increase in PCN yield (~ 6µg/mL) as indicated by experimental data depicting PCN yields of different media formulated for improved production (fig. 2).

In silico PCN Cytotoxicity Studies

Rodent oral toxicity server classified PCN in class IV (harmful if ingested, LD_{50} 0.3-2g/kg). Percent Human intestinal absorption was 95.08%, *in vitro* cell permeability rates for MDCK and Caco2 were 216.14 and 24.466 nMol/sec respectively. Percent plasma protein binding was 76.22%. The transdermal delivery rate for PCN was -3.75 logKp, cm/hour which was slower than the average of the dataset tested (table 1). For hERG channel inhibition the server yielded qualitative results suggesting medium degree threat for channel inhibition by PCN. Cell line toxicity prediction revealed PCN cytotoxicity effectively in MRC5; Embryonic lung fibroblast cells, BJ; Foreskin fibroblast cells, WIL2-NS; Lymphoblastoid cells, WI-38; Embryonic lung fibroblast cells while non-significant cytotoxic effects were estimated for TERT-RPE1; Retinal pigmented epithelial cells and WRL68; Embryonic hepatoma cells (table 2).

The eMolTox server predicted 13 different high confidence predictions for PCN, indicating the serious implications of its role during infection against the host (table 3). These effects ranged from interfering with signaling pathways to inhibition of transporter proteins to mutagenicity. Liver was predicted to be the prime target for PCN as it interfered with four signaling pathways as activator for the aryl hydrocarbon receptor (AhR) and human pregnane X receptor (PXR) signaling pathways, agonist for the constitutive androstane receptor (CAR) and antioxidant response element (ARE) signaling pathways, inhibitor for CYP1A2 activity and blocker for OATP1B3 transporter protein. Endocrine, Central Nervous System (CNS) and immune systems were predicted to be affected by PCN due to its role as an agonist of the estrogen receptor alpha (ER-alpha) and androgen receptor (AR) signaling pathways while antagonist of the glucocorticoid receptor (GR) signaling pathway. Kidney, Heart, and immune components were affected due to PCN's computed role as the agonist of the peroxisome proliferator-activated receptor gamma (PPARg) signaling pathway. Another key implication was the role of PCN in DNA damage as an agonist of the p53 signaling pathway while in two other assessments PCN was predicted to be genotoxic as well as mutagenic.

Systemic redox bioactivity

Redox bioactivity was assayed in mice (*n=3*) pre-injected with PCN (24 hrs) and euthanized. Test organs/tissues i.e. brain, heart, liver, spleen, lungs, and muscles were obtained and homogenized. Total proteins were estimated and a standard curve was developed. Enzymatic activity of Glutathione S-Transferase (GST) was calculated in relation to the total protein content of the experimental (PCN injected) and test organs (fig. 3). Results suggested lower levels of oxidative stress in liver and spleen, higher in lungs and heart while no significant difference in muscles. Levels of oxidative stress in tissues were calculated to the activity of GST enzymes over time in tissues in relation to total proteins of organ homogenate.

DISCUSSION

In this study, we evaluated PA's prominent virulence factor PCN's potential to inflict damage to the host body. PCN is an important redox active virulence factor produced by PA, which is one of topmost critical nosocomial infectious threats (Polisetti *et al*., 2017, Pathak *et al*., 2017). It infects almost every tissue of immune-compromised patients like those of AIDS, CF, transplant recipients and burn wound patients (Hewer and Smyth, 2017, Pollini *et al*., 2018, Berger and Badell, 2017). Antibiotic resistance profile showed that the pattern of drug resistance; PA isolate *S-5.1.a* was highly susceptible to Fluoroquinolones: topoisomerase inhibitors, while overall translation inhibitors antibiotics were more effective against the bacterium. But the resistance against Fluoroquinolones is increasing as evidenced in the Annual report of the European Antimicrobial Resistance Surveillance Network (Yang *et al*., 2018, Bryan *et al*., 1976, Denys *et al*., 2005, 2008, Geli *et al*., 2012, Levy and Marshall, 2004, Llanes *et al*., 2004, Moellering, 1998, Noschese and Zeitlin, 2007, Tsurusako and Fukushima, 1999, Wasaznik *et al*., 2009).

The predicted cell line cytotoxicity is in accordance with the reported sites of infections by PA. As it causes chronic pulmonary infections in lungs, urinary tract infections, eye infections and also induces apoptosis in neutrophils via PCN. The implications of eMolTox server predictions have painted a serious picture. Being an activator for the aryl hydrocarbon receptor (AhR) and as an antagonist of the glucocorticoid receptor (GR) signaling pathways, PCN has the ability to modulate expression profiles in various tissue types, as well as already established immunomodulatory role (Esser, 2012, Cole *et al*., 2018, Denning *et al*., 2003, Baschant and Tuckermann, 2010).

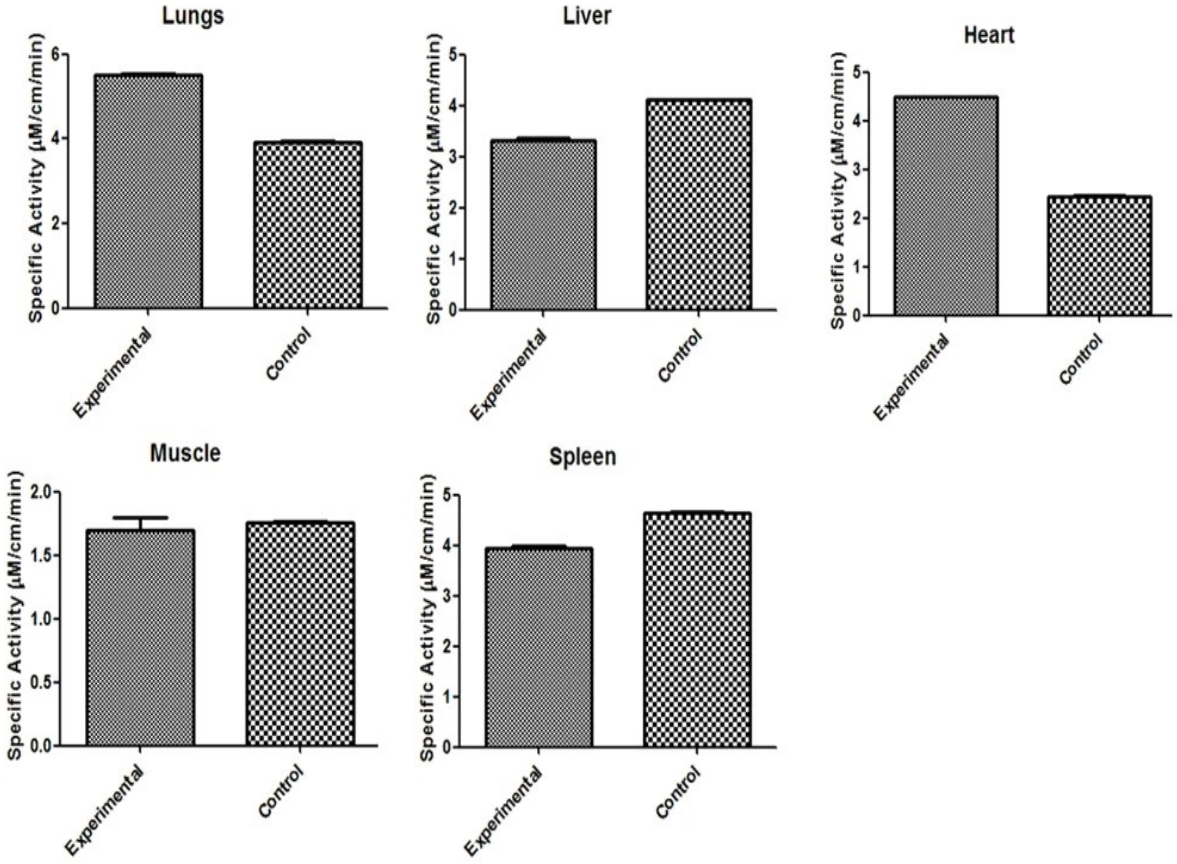

Fig. 3: GST enzymatic activity of experimental (PCN injected) and control mice. Experimental values superscripted with * are significantly different from control values ($p<0.0001$). One Way ANOVA was performed using Graphpad Prism v5.

Table 1: PCN *In silico* analyses by PreADMET server.

ID	Description	Value
HIA %	Human intestinal absorption	95.082379
MDCK	in vitro MDCK cell permeability (Mandin Darby Canine Kidney) (nm/sec)	216.14
Skin_Permeability	in vitro skin permeability (transdermal delivery) (logKp, cm/hour)	-3.7595
PPB%	Plasma Protein Binding	76.22525
Caco2	in vitro Caco2 cell permeability (Human colorectal carcinoma) (nm/sec)	24.466
hERG_inhibition	in vitro Human ether-a-go-go related gene channel inhibition	medium_risk

Table 2: List of Cell lines tested for PCN toxicity potential evaluated by CLC-Pred tool.

Activity potential (Pa)	Inactivity potential (Pi)	Cell-line	Cell-line full name	Tissue
0.301	0.041	MRC5	Embryonic lung fibroblast cells	Lung
0.241	0.149	BJ	Foreskin fibroblast cells	Foreskin
0.085	0.021	WIL2-NS	Lymphoblastoid cells	Haematopoietic and lymphoid tissue
0.115	0.065	WI-38	Embryonic lung fibroblast cells	Lung
0.036	0.014	TERT-RPE1	Retinal pigmented epithelial cells	Retina
0.048	0.034	WRL68	Embryonic hepatoma cells	Liver

Table 3: List of cytotoxicities computationally predicted for PCN via *in silico* analysis of PCN chemical structure by eMolTox server.

Action	Injury	Outcome	Confidence	Similar Positive Molecule (SMILES)
Agonist of the p53 signaling pathway	DNA damage	Positive	0.965	CCCCNC(=O)n1c(NC(=O)OC)nc2ccccc12
Agonist of the estrogen receptor alpha (ER-alpha) signaling pathway	Endocrine	Positive	0.919	Cc1ccc(O)c(c1)-n1nc2ccccc2n1
Agonist of the androgen receptor (AR) signaling pathway	Endocrine, Central nervous system	Positive	0.926	CCn1c2ccccc2c2cc(ccc12)[N+]([O-])=O
Antagonist of the glucocorticoid receptor (GR) signaling pathway	Endocrine, immune, Nervous system	Positive	0.912	CN1c2ccc(Cl)cc2C(=NCC1=O)c1ccccc1
Induce genotoxicity in human embryonic kidney cells	Genotoxicity	Positive	0.958	O=C1C(C(=O)c2ccccc12)c1ccc2ccccc2n1
Agonist of the peroxisome proliferator-activated receptor gamma (PPARg) signaling pathway	Kidney, Heart, immune	Positive	0.915	Clc1nc2ccccc2nc1Cl
Activator the aryl hydrocarbon receptor (AhR) signaling pathway	Liver	Positive	0.97	CCCCNC(=O)n1c(NC(=O)OC)nc2ccccc12
Inhibit CYP1A2 Activity	Liver	Positive	0.963	Cc1c2Cn3c(cccc3=O)-c2nc2ccccc12
Agonist of the constitutive androstane receptor (CAR) signaling pathway	Liver	Positive	0.958	Cc1nc2ccccc2n1Cc1ccc(Cl)cc1
Block OATP1B3 transporter	Liver	Positive	0.932	CC[C@@]1(O)C(=O)OCC2=C1C=C3N (Cc4cc5ccccc5nc34)C2=O
Agonist of the antioxidant response element (ARE) signaling pathway	Liver	Positive	0.925	Cc1nc2ccccc2n1Cc1ccc(Cl)cc1
Activators of the human pregnane X receptor (PXR) signaling pathway	Liver	Positive	0.901	CNC(=O)N(C)c1nc2ccccc2s1
Mutagenicity	Mutagenicity	Positive	0.927	Cn1c(N)nc2ccccc12

Being a potential agonist of the peroxisome proliferator-activated receptor gamma (PPARg) signaling pathway also contribute to its immunomodulatory role (Ricote *et al*., 1998). As the agonist of the constitutive androstane receptor (CAR) and an activator of human pregnane X receptor (PXR) signaling pathways PCN might be able to regulate CYP3A4 and CYP2B6 gene expression (Faucette *et al*., 2007). It was interesting to observed PCN predicted as the agonist of the antioxidant response element (ARE) signaling pathway, considering its redox active nature (Chen *et al*., 2000, Muller, 2002b). Potential inhibition of CYP1A2 activity may have affect drug metabolism (Landi *et al*., 1999). Blocking of OATP1B3 transporter has serious effects on drug elimination and drug pharmacokinetics (Smith *et al*., 2005). Thus any potential interference with these two proteins might result in the accumulation of xenobiotics in the host body. PCN interaction with the estrogen receptor alpha (ER-alpha) signaling pathways can be explained as the anti-PCN activity of Raloxifene (Selective Estrogen Receptor Modulator) as it has been demonstrated to inhibit PCN production in a dose-dependent manner (Jayaseelan *et al*., 2014, Sui *et al*., 2012). Interacting with both estrogen receptor alpha (ER-alpha) and androgen receptor (AR) may potentially pave the way for modulation of endocrine systems as these two are key endocrine hormones (Davey and Grossmann, 2016). The server not only predicted for PCN to be an agonist of the p53 signaling pathway but it also predicted PCN to induce genotoxicity in human embryonic kidney cells and have mutagenic potential. So far there has been no study on genotoxic potential of PCN yet a single study reported the parent molecule PCN is derived from Phenazine to be cytotoxic as well as genotoxic in two human cell lines [Human hepatocarcinoma (HepG2; HB-8065) and human bladder carcinoma (T24; HTB-4)] (McGuigan and Li, 2014). Despite that fact that oxidative stress as reactive oxygen species has been attributed towards genotoxicity there has been no such investigation to our knowledge that focused on PCN and its genotoxic potential (Shukla *et al*., 2011, Cao *et al*., 2008, Chen *et al*., 2009).

Study of redox bioactivity in the murine model suggested that intraperitoneal injection of PCN significantly altered levels of GST enzyme activity in five (Heart, lungs, liver, and spleen) out of five test organs/tissues. Redox bioactivity was measured to be lower in the liver which is in accordance with previous studies conducted on liver cell lines (Cheluvappa *et al*., 2008a, Cheluvappa *et al*., 2007, Hashimoto *et al*., 2007). One possible reason for these reduced levels could be the presence of multiple antioxidant mechanisms are expressed in the liver (Di

Mascio *et al*., 1991). Higher levels of oxidative stress in lungs, the primary site of PA infection in CF are in accordance with previous studies which have concluded that PCN is vital for the establishment of lung infections on the basis of studies conducted in murine models (Armstrong *et al*., 1971, Greenhagen *et al*., 2008, Lau *et al*., 2004). Role of PCN has not been investigated well in a systemic manner probably because of the lack of specified site of infection beyond pulmonary and UTI's. Also, higher levels of oxidative stress in cardiac tissues have provided an indication of the possibility of PCN's ability to pass through the biological barriers via diffusion. However further experiments and /or clinical evidence will be required.

Virulence factors and drug resistance mechanisms cover ultimate research interest in PA, the factors and mechanisms by which this bacterium damages the human body and how does it manage to escape therapeutic agents. With the highest frequency of nosocomial infections, PA needs to be studied in detail for obtaining knowledge and information for application in medical and pharmaceutical sciences.

CONCLUSION

In this study, we have covered the important public health concerns regarding *P. aeruginosa*'s virulence mediated via its primary virulence factor PCN. Computational predictions estimated PCN to be diffusible across biological membranes as evident from *in vitro* cell permeability rates for MDCK and Caco2 cell lines. PCN showed good intestinal absorption but poor permeability across the skin. Although if swallowed PCN is predicted to be mildly toxic (LD_{50}: 300-2000mg/kg). Considering the conventional infection sites, overall pathogen count during infection and effect of host-pathogen interaction on microbial metabolism this quantity is rarely produced under normal conditions. The predicted interactions and interferences with signaling pathways indicated the liver to be the primary target for PCN, followed by Kidney, Heart, Endocrine, immune and Nervous system. We report that PCN is capable of inducing oxidative stress in lungs and cardiac tissues while computational analysis yielded the potential risk for hERG channel inhibition, genotoxicity, and mutagenicity.

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